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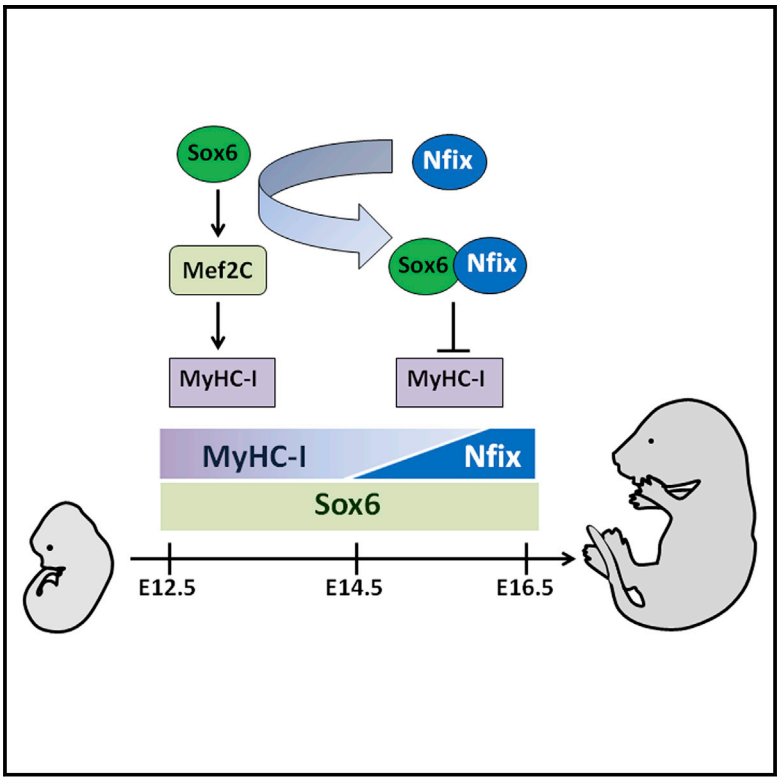
Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

Graphical Abstract



Authors

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In Brief

Taglietti et al. reveal molecular mechanisms defining muscle fiber specification, driven by the key transcription factors Nfix and Sox6, during mouse and zebrafish development. They show that Nfix reverses Sox6 function between embryonic and fetal phases of myogenesis, allowing the proper expression of slow MyHC.

Highlights

- Sox6 has opposite roles in MyHC-I regulation during embryonic and fetal myogenesis
- In embryonic muscle, Sox6 enhances MyHC-I expression via regulation of Mef2C
- In fetal muscle, Nfix is required for Sox6-mediated repression of MyHC-I
- The Sox6 and Nfixa orthologs cooperate in repressing *smyhc1* in zebrafish

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Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

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SUMMARY

Sox6 belongs to the Sox gene family and plays a pivotal role in fiber type differentiation, suppressing transcription of slow-fiber-specific genes during fetal development. Here, we show that Sox6 plays opposite roles in MyHC-I regulation, acting as a positive and negative regulator of MyHC-I expression during embryonic and fetal myogenesis, respectively. During embryonic myogenesis, Sox6 positively regulates MyHC-I via transcriptional activation of Mef2C, whereas during fetal myogenesis, Sox6 requires and cooperates with the transcription factor Nfix in repressing MyHC-I expression. Mechanistically, Nfix is necessary for Sox6 binding to the MyHC-I promoter and thus for Sox6 repressive function, revealing a key role for Nfix in driving Sox6 activity. This feature is evolutionarily conserved, since the orthologs Nfixa and Sox6 contribute to repression of the slow-twitch phenotype in zebrafish embryos. These data demonstrate functional cooperation between Sox6 and Nfix in regulating MyHC-I expression during prenatal muscle development.

INTRODUCTION

In vertebrates, the process of skeletal muscle development occurs in subsequent steps that involve distinct populations of myogenic progenitors, the myoblasts, which arise from the dermomyotomal domain of somitic mesoderm (Christ and Ordahl, 1995). The process of myogenic differentiation is initiated in mesodermal cells by a family of basic-helix-loop-helix (bHLH) transcription factors, named muscle regulatory factors (MRFs), that are able to activate transcription of muscle-specific markers such as the myosin heavy chain (MyHC) isoforms (Pinney et al., 1988; Cao et al., 2010). Embryonic myoblasts sustain a first wave of myogenesis between embryonic day 10 (E10) and E12 in the mouse and give rise to primary myofibers that establish the primitive shape of muscle and express high levels of the slow MyHC isoform (MyHC-I, encoded by the *Myh7* gene) and of the embryonic MyHC isoform (MyHC-emb, encoded by *Myh3*) (Schiaffino et al., 1986; Stockdale, 1992). A second wave of muscle differentiation takes place between E15 and E18, driven by fetal myoblasts that form secondary fibers, characterized by low levels of MyHC-I and high levels of neonatal MyHC (MyHC-neo, encoded by *Myh8*) (Eusebi et al., 1986; Lyons et al., 1990; Daou et al., 2013). Eventually, primary fibers conserve the slow-twitch phenotype typical of embryonic muscle, while secondary fibers lose the expression of several embryonic-specific markers such as MyHC-I and acquire expression of fast-twitch markers (Ferrari et al., 1997; Biressi et al., 2007). Embryonic and fetal myoblasts, once isolated from the embryo, are committed to a specific fiber type, suggesting the involvement of intrinsic factors rather than nerve activity in the establishment of fiber phenotype (Page et al., 1992). These observations suggest that the proper transition of skeletal muscle from the embryonic to the fetal/post-natal phenotype requires a switch in the transcriptional status of differentiating myoblasts.

In our previous study, we have shown that the transcription factor Nfix, a member of the nuclear factor I (Nfi) family, has a key role in the establishment of fetal muscle phenotype and in the downregulation of slow MyHC both in fetal and adult muscles (Messina et al., 2010; Rossi et al., 2016). We have shown that Nfix is strongly expressed in fetal myoblasts and indirectly represses MyHC-I expression via the transcription factor Nfatc4, a positive regulator of *MyHC-I* in skeletal muscle (Calabria et al., 2009; Messina et al., 2010). We have also reported that the zebrafish (*Danio rerio*) Nfix ortholog Nfixa has an evolutionarily conserved role in the transition from slow-twitch to fast-twitch myogenesis (Pistocchi et al., 2013). In the past few years, it has been shown that Sox6, a member of the Sry-related HMG box (Sox) factor family, which is highly conserved in vertebrates, plays a critical role in fetal fiber specification through direct repression of *MyHC-I* by binding to the 5′-upstream region in two different binding sites. The first is located −200 bp from the transcription start site (TSS) in the proximal promoter and is sufficient for Sox6-dependent *MyHC-I* repression in fetal myotubes (Hagiwara et al., 2007; An et al., 2011), and the second

is located −2,900 bp from the TSS in a distal muscle enhancer that is required for full promoter activity (Giger et al., 2000; Blow et al., 2010). As a consequence, *Sox6*-null mouse muscle displays increased levels of MyHC-I and a general switch toward a slower phenotype (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011). Of note, studies in zebrafish have shown that Sox6 is restricted to fast-twitch fibers during embryonic muscle development and that ectopic Sox6 expression in adaxial cells (the slow muscle progenitors in zebrafish) leads to the silencing of slow-twitch genes (such as the slow MyHC isoform *smyhc1* and the transcription factor *prox1a*) (von Hofsten et al., 2008; Wang et al., 2011; Jackson et al., 2015).

Here, we observed that Sox6, at variance with Nfix, is expressed at comparable levels in embryonic and fetal myoblasts, despite its role of inhibitor of slow twitching program. Intriguingly, we demonstrated that Sox6 has opposite roles in regulating MyHC-I expression between embryonic and fetal myogenesis in mouse. Specifically, during embryonic myogenesis, Sox6 indirectly promotes *MyHC-I* expression via transcriptional activation of Mef2C. As a consequence, Sox6 deficiency in embryonic muscle leads to a strong downregulation of *MyHC-I*. On the contrary, during fetal myogenesis, Sox6 cooperates with Nfix to repress *MyHC-I* in a complex in which Nfix is necessary for the proper binding of Sox6 to the *MyHC-I* promoter in fetal myotubes. Finally, we show that Nfixa and Sox6 together regulate sMyHC in zebrafish embryos, revealing an evolutionarily conserved mechanism that is required for the acquisition of normal muscle phenotype.

RESULTS

Sox6 Transcriptionally Promotes MyHC-I Expression during Embryonic Myogenesis

Sox6 has been intensively studied as an inhibitor of slow muscle phenotype during the fetal period. However, we observed that Sox6 is also expressed during embryonic myogenesis, which is mainly characterized by the expression of typical slow genes such as the slow MyHC isoform MyHC-I. *Myf5*$^{GFP\text{-}P/+}$ embryos and fetuses were collected at E12.5 or E16.5, and GFP-positive myoblasts were isolated via fluorescence-activated cell sorting (FACS) as previously described (Messina et al., 2010). Using quantitative real-time PCR and western blot, we found that *Sox6* levels do not significantly change, whereas *Nfix*, as known, is drastically upregulated in fetal progenitors (Figures 1A–1C). Importantly, these data show that Sox6 and Nfix proteins are co-expressed only during fetal myogenesis. We also performed extensive immunofluorescence analysis on frozen mouse embryo sections from E10.5 to E18.5 in order to follow Sox6 protein expression throughout development. Notably, Sox6 is first expressed between E11.5 and E12.5 in primary fibers that express high levels of MyHC-I (Figures S1A–S1H), whereas at E17.5, the localization of Sox6 is almost completely associated with secondary fibers that are negative for MyHC-I (Figures S1I–S1L), as previously described (An et al., 2011; Richard et al., 2011). We therefore decided to investigate the possible function of Sox6 during embryonic myogenesis, a function not apparently linked to repression of MyHC-I. To this aim, we performed immunofluorescence analysis on E12.5 muscle sections from homozygous mice carrying the *Sox6*^{lacZ} allele (hence referred to as *Sox6*-null mice) (Smits et al., 2001). Surprisingly, the staining for MyHC-I is strongly decreased in *Sox6* null in comparison to wild-type (WT) muscle (Figures 1D and 1E). Importantly, no differences in total MyHC content were assessed in embryonic muscle groups of *Sox6*-null embryos (Figures 1G and 1H), implying that the decrease in MyHC-I expression is not due to delayed or aberrant muscle differentiation. We also performed western blot and quantitative real-time PCR on embryonic muscle lysates to confirm the immunofluorescence data, and our results showed a decrease of MyHC-I protein in *Sox6*-null samples, without major changes in total MyHC content (Figures 1J and 1K). Interestingly, the phenotype of *Sox6*-null embryonic muscle is reminiscent of the Tg:Mlc1f-*Nfix2* gain-of-function embryo (Figures 1F and 1I), in which the Nfix2 splice variant is ectopically expressed in muscle cells from E11.5, leading to a fetal-like muscle phenotype (Kelly et al., 1997; Messina et al., 2010). Importantly, Nfix expression in embryonic skeletal muscle is not altered in the absence of Sox6 at both the protein and mRNA levels (Figures S2A–S2D). Taken together, these results show that Sox6 is expressed at equal levels in skeletal muscle during embryonic and fetal myogenesis and that deletion of *Sox6* during the embryonic period unexpectedly leads to downregulation of *MyHC-I*.

Sox6 Positively Regulates the Slow-Twitch Phenotype of Embryonic Myoblasts by Binding to *Mef2C* Promoter

In order to define a possible mechanism by which Sox6 regulates the transcription of MyHC-I during embryonic myogenesis, we performed chromatin immunoprecipitation (ChIP) for Sox6 in differentiated embryonic myoblasts. We found that Sox6 does not significantly bind either to the proximal or to the distal regulative regions upstream of *MyHC-I* (Figure 2A), thus suggesting that Sox6 is not able to directly regulate *MyHC-I* transcription in embryonic muscle. To confirm this hypothesis, we performed luciferase assays on WT and *Sox6*-null embryonic differentiated myoblasts with vectors containing the 3,500-bp *MyHC-I* full 5′-upstream region (MyHC-I 3500), the 408-bp *MyHC-I* proximal promoter sequence (MyHC-I 408), or the mutated forms of the distal and proximal canonical Sox6 binding sites, MyHC-I 3500 m and MyHC-I 408 m (Figure S3A) (Hagiwara et al., 2007; An et al., 2011). As expected, in the absence of Sox6, we found a significant reduction of firefly luciferase activity in all the conditions with the only notable exception of the 408 WT construct (Figure 2B), suggesting that Sox6 is promoting MyHC-I expression in embryonic myocytes without direct binding to its canonical binding sites. In order to identify a possible indirect mechanism by which Sox6 enhances MyHC-I expression in embryonic muscle, we focused on the transcription factor Mef2C, a known positive regulator of the slow phenotype (Wu et al., 2000; Potthoff et al., 2007; Anderson et al., 2015). To verify the interaction between Mef2C and the *MyHC-I* promoter in embryonic myoblasts, we performed a ChIP assay, which showed direct binding of Mef2C on the proximal *MyHC-I* promoter (Figure S3B). Interestingly, *Mef2C* mRNA is downregulated in E12.5 *Sox6*-null muscle, at variance with the closely related *Mef2A* (Figure 2C). By ChIP on embryonic differentiated myoblasts, we found that Sox6 directly binds to a region located in

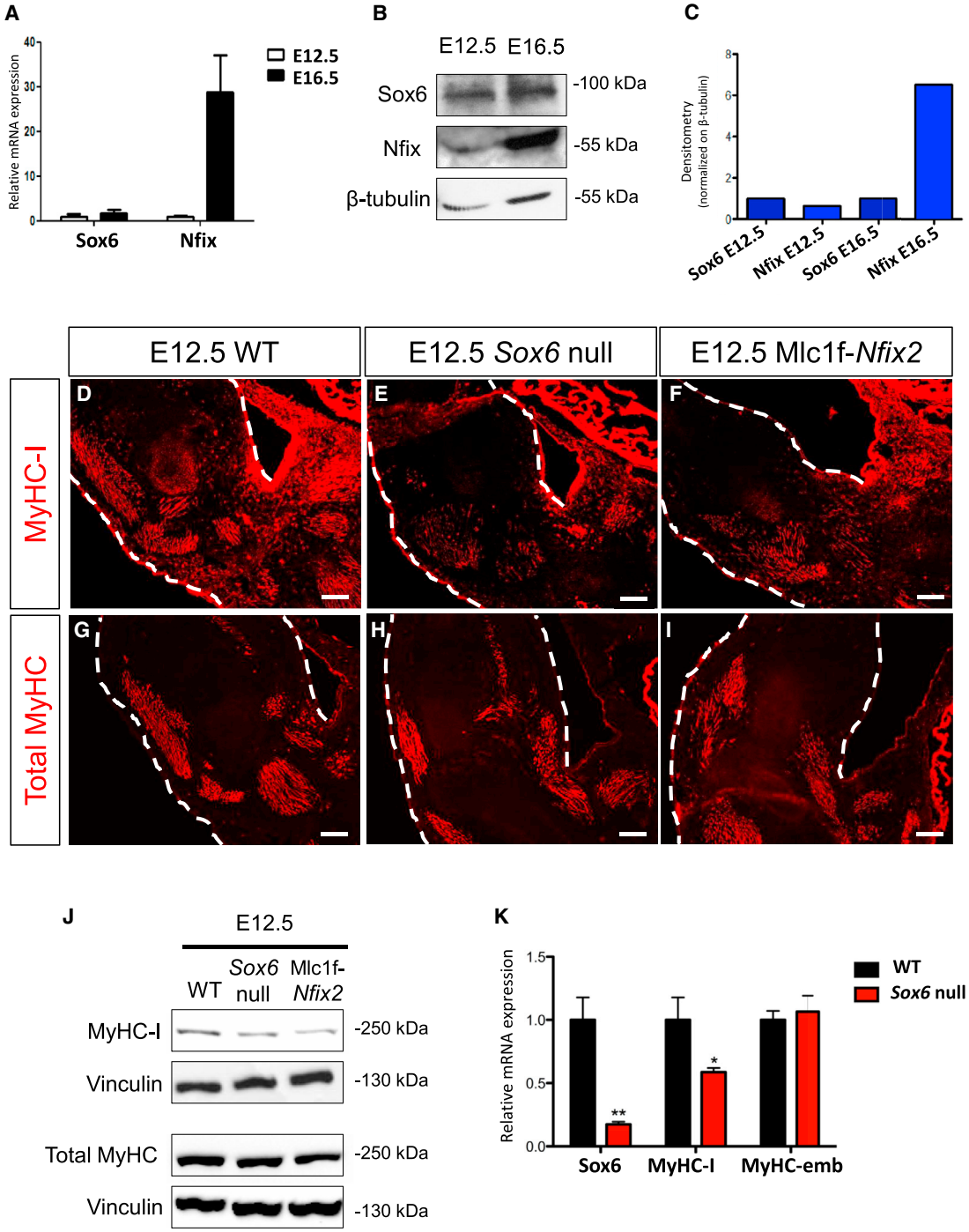


Figure 1. Sox6 Acts a Positive Regulator of the Slow-Twitch Phenotype during Embryonic Myogenesis

(A) Quantitative real-time PCR analysis on freshly isolated *Myf5*$^{GFP-P/+}$ embryonic (E12.5) and fetal (E16.5) myoblasts showing relative expression of *Sox6* and *Nfix* transcripts in the two populations.

(B) Western blot on lysates from freshly isolated *Myf5*$^{GFP-P/+}$ embryonic and fetal myoblasts. β-Tubulin was used to normalize the amount of protein loaded.

(C) Quantitative densitometry of the protein expression levels of Sox6 and Nfix at E12.5 and E16.5.

(D–I) Immunofluorescence on E12.5 muscle sections from WT (D and G), *Sox6* null (E and H), and Tg:Mlc1f-*Nfix2* (F and I) mice stained with anti-MyHC-I (D–F) or anti-MyHCs (MF20) antibodies (G-I). Dashed lines highlight the forelimb anlagen contour. Scale bars, 100 μm.

(J) Western blot on E12.5 muscle samples from WT, *Sox6* null, and Tg:Mlc1f-*Nfix2* mice. Vinculin was used to normalize the amount of loaded proteins.

(K) Quantitative real-time PCR on E12.5 muscle tissue from WT and *Sox6*-null mice (*$p < 0.05$; **$p < 0.01$; $n = 3$).

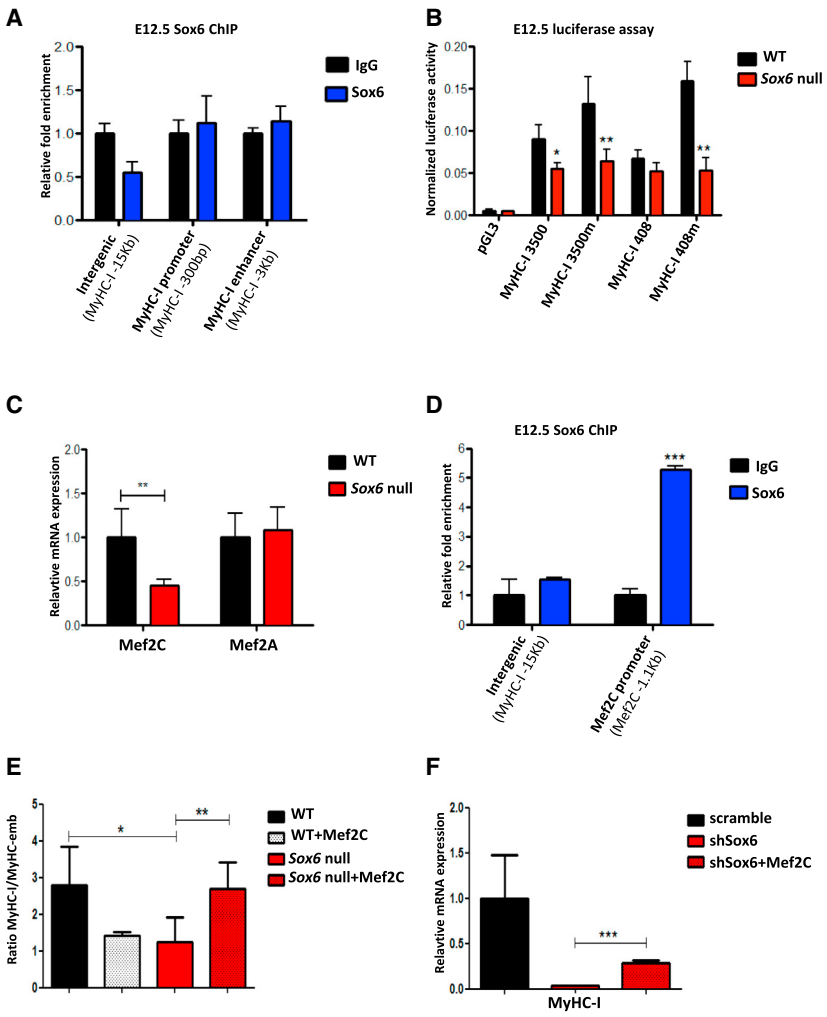


Figure 2. Sox6 Indirectly Activates MyHC-I Expression in Embryonic Myoblasts via a Mef2C-Dependent Mechanism

(A) ChIP assay with anti-Sox6 antibody on E12.5 differentiated myoblasts. Three different chromatin regions were tested: a negative control region (intergenic) located 15 kb upstream of *MyHC-I* TSS, the *MyHC-I* proximal promoter (−375 bp; MyHC-I promoter), and the *MyHC-I* distal enhancer (−2,900 bp; MyHC-I enhancer).

(B) Luciferase report assay on WT and *Sox6*-null E12.5 differentiated myoblasts transfected with control pGL3-basic, MyHC-I 408, MyHC-I 408 m, MyHC-I 3,500, and MyHC-I 3,500 m constructs (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(C) Quantitative real-time PCR for *Mef2C* and *Mef2A* on E12.5 muscle tissue from WT and *Sox6*-null mice (**$p < 0.01$; $n = 2$).

(D) ChIP assay with anti-Sox6 antibody on E12.5 differentiated primary myoblasts showing binding of Sox6 to a region located −1.1 kb upstream of *Mef2C* TSS (***$p < 0.001$; $n = 3$). As negative control, we used the *MyHC-I* −15 kb intergenic region.

(E) Quantitative real-time PCR for *MyHC-I* and MyHC-emb (MyHC-I/MyHC-emb ratio) on WT and *Sox6*-null differentiated embryonic myoblasts transfected with a Mef2C-overexpressing vector (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(F) Quantitative real-time PCR for MyHC-I on embryonic myoblasts purified from $Myf5^{GFP-P/+}$ mice, infected with scramble or shSox6 virus. $Myf5^{GFP-P/+}$ purified embryonic myoblasts infected by lentiviruses expressing the shRNA for Sox6 were transfected with Mef2C-overespressing vector (***$p < 0.001$; $n = 2$).

the *Mef2C* promoter (Figure 2D), indicating that Sox6 is a direct activator of Mef2C during embryonic myogenesis. In order to validate a possible Mef2C-mediated mechanism of MyHC-I regulation, we transfected WT and *Sox6*-null unpurified embryonic myoblasts with a vector overexpressing Mef2C. Mef2C overexpression in Sox6-deficient cells (Figure S3C) leads to an increase of *MyHC-I* expression when normalized on the levels of *MyHC-emb* to account for the total number of myogenic cells (Figure 2E). To validate these data, we also transduced purified embryonic myoblasts with a lentiviral vector expressing small hairpin RNA (shRNA) against Sox6 to achieve in vitro Sox6 knockdown (Figure S3D). We then transfected cells with Mef2C-overexpression vector (Figure S3D) and assessed the levels of *MyHC-I* mRNA by quantitative real-time PCR (Figure 2F). Strikingly, overexpression of Mef2C was able to partially rescue MyHC-I expression in shSox6 cells (up to 30% of the levels of scramble-transduced cells). These results show that during the embryonic period, Sox6 acts indirectly as a positive regulator of MyHC-I via a Mef2C-dependent mechanism, in sharp contrast with its well-known and characterized function during fetal myogenesis (An et al., 2011; Quiat et al., 2011; see below).

Sox6 Is Necessary for Nfix Binding to the *MyHC-I* Promoter during the Fetal Period

Since Sox6 has opposite roles in MyHC-I regulation in embryonic and fetal muscle, and since Nfix is only expressed during fetal myogenesis (Messina et al., 2010), we decided to investigate the possible cooperation between Nfix and Sox6 during fetal myogenesis. We first investigated Nfix expression and function in *Sox6*-null fetuses (E16.5). As previously described in other *Sox6*-null mouse models (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011), fetal fiber specification is completely disrupted in the absence of Sox6. Indeed, in contrast to WT, *Sox6*-null fetal muscle displays very high levels of MyHC-I by immunofluorescence, quantitative real-time PCR, and western blot (Figures S4A–S4D). We also looked at Nfix on sections from the *Sox6*-null mice and found that despite the dramatic increase in MyHC-I expression, Nfix is correctly expressed in the nuclei of muscle fibers in the absence of Sox6 (Figures 3A–3D), and western blot analysis did not reveal differences in Nfix protein content (Figure S4C). These data suggest that Nfix is normally expressed in fetal muscles lacking Sox6 but unable to properly repress *MyHC-I.* To verify whether Sox6 is required for Nfix function, we performed ChIP for Nfix in WT fetal myotubes, which revealed binding of Nfix to *MyHC-I* promoter in the same region of the proximal Sox6 binding site (−200 bp) (Figure 3E). Interestingly, we

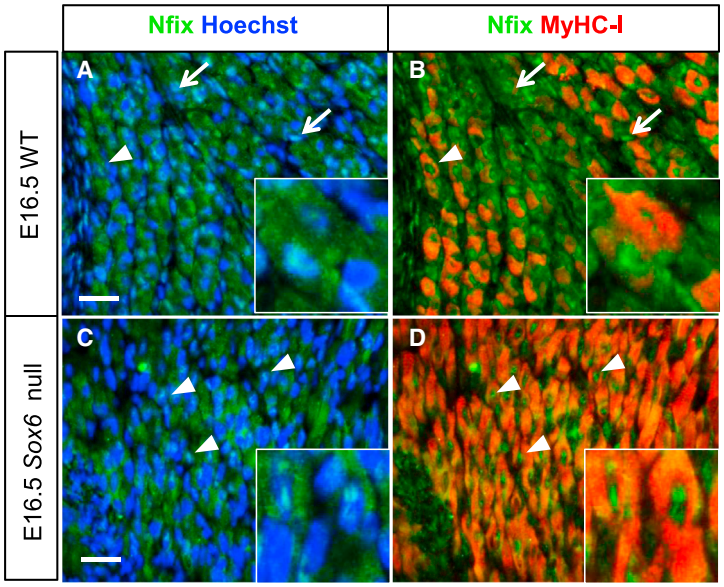
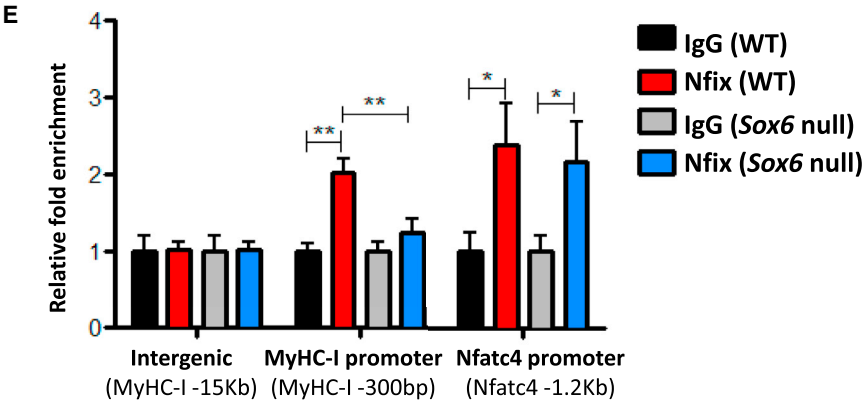


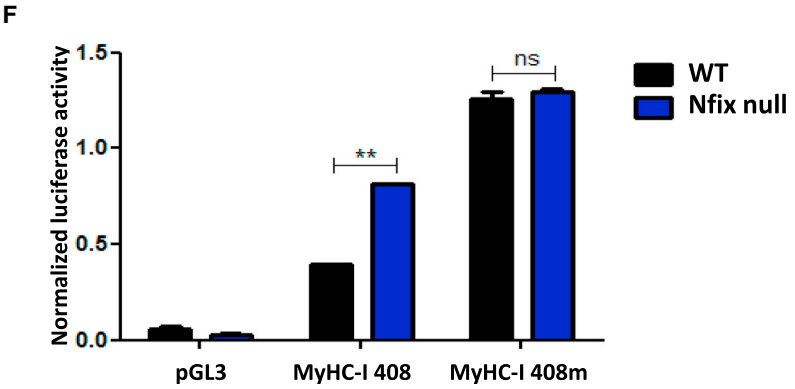
Figure 3. Sox6 Is Required for Normal Nfix Function in Fetal Muscle

(A–D) Immunofluorescence with anti-Nfix (green) and anti-MyHC-I (red) antibodies on E16.5 muscle sections from WT (A and B) or *Sox6*-null (C and D) mice. Arrows indicate Nfix-positive nuclei in secondary (MyHC-I negative) fibers. Arrowheads indicate Nfix-positive nuclei in MyHC-I-positive fibers. Nuclei are counterstained with Hoechst. Scale bars, 25 μm. Higher magnifications of Nfix-positive fibers are shown in the insets.

(E) ChIP assay with anti-Nfix antibody on WT and *Sox6*-null E16.5 myotubes on negative control region (intergenic) located 15 kb upstream of *MyHC-I* TSS, the *MyHC-I* proximal promoter (−375 bp; MyHC-I promoter), and the *Nfatc4* promoter region (−1.2 kb upstream of the Nfatc4 TSS) (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(F) Luciferase report assay on WT and *Nfix*-null differentiated fetal myoblasts (E16.5) transfected with control pGL3-basic, MyHC-I 408, and MyHC-I 408 m vectors (**$p < 0.01$; $n = 2$).





observed that in *Sox6*-null fetal myotubes, the binding of Nfix to the *MyHC-I* promoter is significantly reduced. Importantly, Nfix binding to *Nfatc4* promoter is not impaired in the absence of Sox6 (Figure 3E), suggesting that Nfix is still able to bind to other transcriptional targets in the absence of Sox6. We further validated the repressive role of Nfix on the *MyHC-I* proximal promoter by performing a luciferase assay with MyHC-I 408 and MyHC-I 408 m vectors in WT and *Nfix*-null fetal myotubes (Figure 3F). Our results showed increased luciferase expression in the absence of Nfix with the vector carrying the WT sequence, suggesting that indeed Nfix represses MyHC-I by acting on the proximal promoter regulative region, in spite of the absence of any Nfix consensus sequences (data not shown). Importantly, Nfix-dependent negative regulation of MyHC-I is lost when the proximal Sox6 binding sequence is mutated, demonstrating that Sox6 is required for Nfix binding to the proximal MyHC-I promoter (Figure 3F). Overall, these results suggest a possible crosstalk between Nfix and Sox6 in regulating MyHC-I expression at the proximal promoter region.

Nfix Is Necessary and Sufficient for Sox6 Regulation of MyHC-I Expression

To better investigate a possible reciprocal interplay between Nfix and Sox6, we performed immunofluorescence for Sox6 and total MyHC on frozen muscle sections from E16.5 WT and *Nfix*-null mice (Campbell et al., 2008). We found that Sox6 is expressed in both WT and *Nfix*-null muscles (Figures S4E and S4F), implying that Nfix is dispensable for normal Sox6 expression in fetal muscle. Interestingly, immunofluorescence for Sox6 and MyHC-I on sections from the same mice revealed that in the absence of Nfix, there is a marked increase in the number of Sox6-positive fibers that co-express MyHC-I, in contrast to WT muscles (Figures 4A–4D). We further

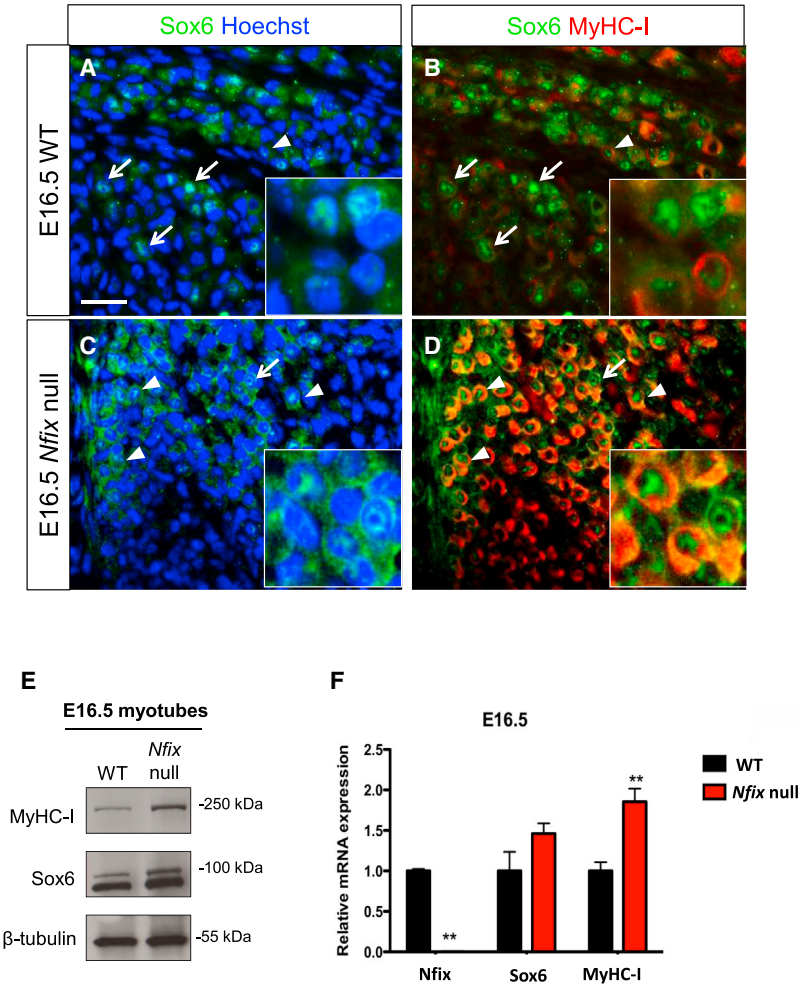


Figure 4. Nfix Is Necessary for the Correct Function of Sox6 in Fetal Muscle

(A–D) Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on fetal (E16.5) muscle sections from WT (A and B) and *Nfix*-null (C and D) mice. Arrows indicate secondary fibers, which present nuclear Sox6 expression and low or absent staining for MyHC-I. Arrowheads indicate fibers co-expressing nuclear Sox6 and MyHC-I. Nuclei are counterstained with Hoechst. Scale bars, 25 μm. Higher magnifications are shown in the insets.

(E) Western blot on lysates from WT and *Nfix*-null E16.5 myotubes. β-Tubulin was used to normalize the amount of proteins loaded.

(F) Quantitative real-time PCR on WT and *Nfix*-null E16.5 myotubes (**$p < 0.01$; $n = 3$).

validated these data by western blot and quantitative real-time PCR on differentiated fetal myotubes and observed that *Nfix*-null myotubes express higher levels of MyHC-I than WT cells (Figures 4E and 4F). These data show that in the absence of Nfix, the repressive activity of Sox6 on MyHC-I is partially impaired, even if the normal expression pattern of Sox6 is maintained. The results obtained in both *Sox6* and *Nfix*-null fetuses led us to hypothesize a functional cooperation between Nfix and Sox6 in MyHC-I regulation. Therefore, we tested the ability of Nfix and Sox6 to bind to each other in a multi-protein complex. To this aim, we used fetal myotubes transiently transfected with an Nfix2 hemagglutinin (HA) vector (see Experimental Procedures) and performed a co-immunoprecipitation (coIP) assay from nuclear extracts for HA and Sox6. CoIP revealed the presence of a complex containing both Sox6 and Nfix2-HA, as shown in Figure 5A. Therefore, we wondered whether, as observed for Nfix fetuses in absence of Sox6, Nfix might be required for the binding of Sox6 to the *MyHC-I* promoter as well. Hence, we performed ChIP for Sox6 in WT and *Nfix*-null fetal myotubes. Our results showed a decrease in Sox6 binding to the two different sites in the *MyHC-I* 5′-upstream region (Figure 5B). Notably, the binding with the proximal promoter was completely lost in the absence of Nfix, whereas the binding with the distal enhancer was reduced by 50%. Interestingly, ChIP for Sox6 on the *Mef2C* promoter reveals that in WT fetal myotubes, Sox6 is not able to bind to the *Mef2C* promoter, at variance with what happens during the embryonic stage. On the contrary, Sox6 binding on the *Mef2C* promoter still occurs in *Nfix*-null fetal myotubes. These data indicate that Nfix is required for the proper binding of Sox6 to the *MyHC-I* promoter during fetal myogenesis. Finally, we performed ChIP for Sox6 on WT and Tg:Mlc1f-*Nfix2* embryonic myoblasts (Figure 5C). Strikingly, Nfix overexpression leads to a switch in the binding properties of Sox6; indeed, we found that Sox6 is bound to the *MyHC-I* promoter, but not to the *Mef2C* promoter, in Tg:Mlc1f-*Nfix2* embryonic myoblasts. These data demonstrate that Nfix is necessary and sufficient for the binding of Sox6 to the MyHC-I proximal promoter and therefore for Sox6 repressive activity on MyHC-I.

Functional Cooperation of Sox6 and Nfixa Is Evolutionarily Conserved in Zebrafish

It was previously demonstrated that both Sox6 and Nfixa have an evolutionarily conserved role in the repression of slow-twitch genes in zebrafish (von Hofsten et al., 2008; Pistocchi et al., 2013; Jackson et al., 2015). We thus wondered whether a functional interplay between Nfixa and Sox6 is conserved in zebrafish myogenesis. As a preliminary analysis, we performed quantitative real-time PCR on trunk and tail regions isolated from zebrafish embryos at 1, 2, and 3 days post-fertilization (dpf) and found that the *sox6* transcript is expressed at high levels at 1 dpf and is steadily downregulated up to 3 dpf (Figure S5A), whereas the *nfixa* transcript peaks at 2 dpf (Figure S5B), as shown previously (Pistocchi et al., 2013). Additionally, we performed immunofluorescence for Sox6 and total MyHC (MF20 antibody) or the slow MyHC isoform sMyHC (F59

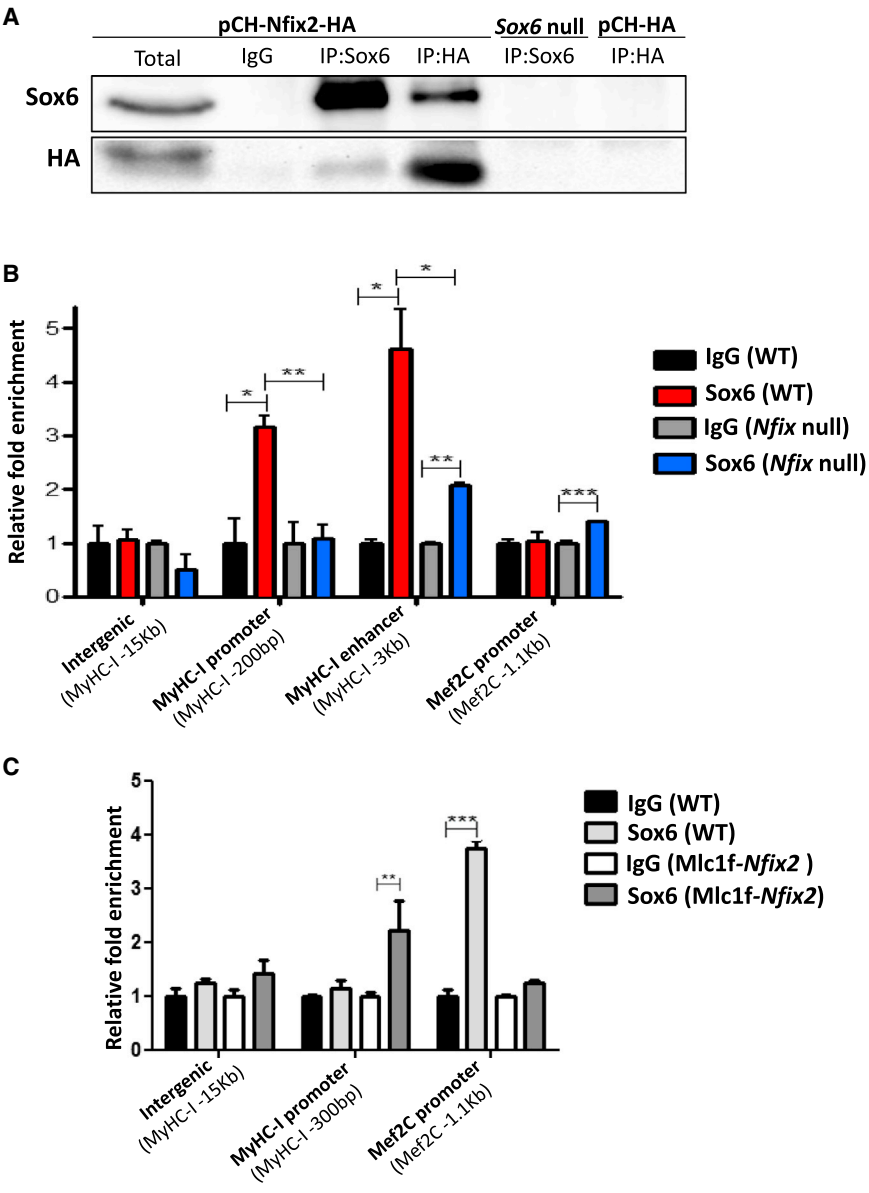


Figure 5. Nfix Is Required for Fetal-Specific Binding of Sox6 to *MyHC-I* Regulative Regions

(A) Immunoprecipitation assay, from nuclear extracts, on fetal myoblasts transfected with Nfix2-HA vector, showing the immunoprecipitation of Sox6 (IP Sox6) and HA (IP HA). T, total lysate; IgG, negative control; IP, immunoprecipitated. The coIPs for Sox6 on *Sox6*-null myotubes and for HA on fetal myoblasts transfected with the HA-only expressing vector (pCH-HA) were used as negative controls.

(B) ChIP assay with anti-Sox6 antibody on WT and *Nfix*-null E16.5 myotubes on the same chromatin regions described in Figures 2A and 2D (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 2$). As a negative control, we used only the *MyHC-I* −15 kb region.

(C) ChIP assay on unpurified embryonic myoblasts from WT or Tg:Mlc1f-*Nfix2* to test Sox6 binding on MyHC-I and the Mef2C promoter. The intergenic region was used as a negative control and IgG as an unrelated antibody (**$p < 0.01$; ***$p < 0.001$; $n = 2$).

antibody) on 2 dpf embryos and found that Sox6 protein is specifically expressed in the nuclei of fast muscle fibers that are negative for sMyHC (Figures 6A and 6B), whereas the outer superficial fibers positive for sMyHC are mostly negative for Sox6 (data not shown). Interestingly, we observed that a minority of slow superficial fibers show cytoplasmic staining for Sox6 (Figures 6C–6J), suggesting that Sox6 subcellular localization might be spatially regulated in the different muscle domains. In order to elucidate the role of Sox6 in slow-twitch genes regulation, we performed morpholino (MO)-mediated knockdown of *sox6* (von Hofsten et al., 2008). By quantitative real-time PCR analysis (Figures S5C and S5D), we found that the expression of *smyhc1* is markedly increased in *sox6* morphants at 2 dpf, whereas the fast-twitch gene *mylpfa* (fast myosin light chain isoform) is expressed at equal levels. Moreover, the *nfixa* transcript is drastically upregulated in *sox6* morphants, suggesting that Sox6 might negatively regulate *nfixa* (Figure S5E). We conclude that Sox6 is a critical repressor of the slow-twitch phenotype in zebrafish and that Nfixa is not able to compensate for *sox6* knockdown. To verify a possible cooperation between Nfixa and Sox6, we performed co-injections of morpholinos against *sox6* and *nfixa* at lower doses with respect to those previously described (von Hofsten et al., 2008; Pistocchi et al., 2013) in order to minimize their effect when injected separately (see Experimental Procedures). Co-injection of these doses of MOs resulted in synergistic defects in motility in touch-response assays (Figure S6). Control embryos and the vast majority of *sox6* or *nfixa* morphants readily swam away when touch-stimulated. On the contrary, 66% of double partial morphants were either shivering or bending their tails before eventually moving away but within a shorter distance (Figures S6A and S6C; Movie S1). The synergistic effect is more evident when lowering the doses (see Figures S6B and S6D). Strikingly, quantitative real-time PCR results show that *smyhc1* is significantly upregulated only in the double morphants at 48 hpf, whereas the level of *myl*1, an early marker for differentiating fast muscle cells (Burguière et al., 2011), does not change (Figure 6K). Moreover, we validated the increased expression of sMyHC (F59 antibody) in *sox6*/*nfixa* double morphants by western blot (Figure 6L). We thus conclude that functional cooperation of Sox6 and Nfix is required for proper skeletal muscle development and that this cooperation is evolutionarily conserved in mouse and zebrafish.

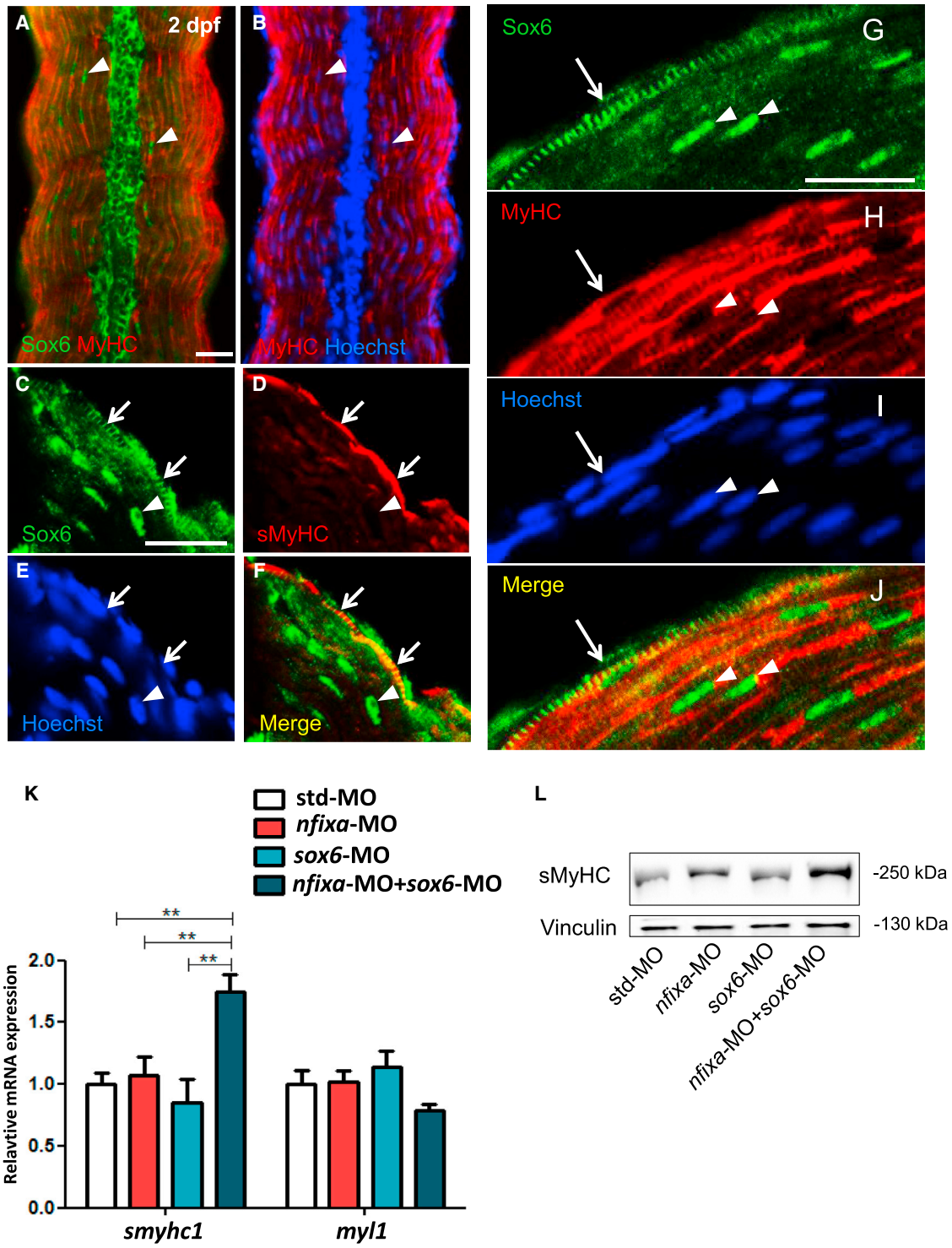


Figure 6. Functional Cooperation of Sox6 and Nfixa Is Conserved in Zebrafish

(A–J) Immunofluorescence with anti-Sox6 antibody (green) and anti-MyHC antibody (red) (A, B, and G–J) or with anti-Sox6 (green) and anti-sMyHC (F59, red) (C–F) on 2 dpf zebrafish muscle longitudinal sections. Arrowheads indicate Sox-positive nuclei in fast-twitch muscle fibers. Arrows indicate Sox6 staining in the cytoplasm of superficial slow fibers. Approximately one-fifth of the sMyHC-positive superficial cells displayed cytoplasmic Sox6 staining, whereas fast fibers negative for sMyHC only displayed nuclear Sox6 staining. Nuclei are counterstained with Hoechst. Scale bars, 25 μm.

(legend continued on next page)

DISCUSSION

Adult skeletal muscle is composed of two major fiber types presenting a wide range of physiological and biochemical differences. Slow-twitch type I fibers use oxidative metabolism and express the slow MyHC isoform MyHC-I; in contrast, fast-twitch type II fibers present glycolytic or mixed metabolism and express three fast MyHC isoforms (MyHC-IIa, IIx/d, and IIb) (Peter et al., 1972; Schiaffino et al., 1988; Chakkalakal et al., 2012). The phenotype of post-natal muscle fibers is strictly regulated by extrinsic signals such as muscle activity and hormones (Butler-Browne et al., 1982; Gambke et al., 1983; Russell et al., 1988). Additionally, different factors controlling adult muscle plasticity have been identified, including the Nfatc (Calabria et al., 2009) and Mef2 (Wu et al., 2000; Potthoff et al., 2007; Anderson et al., 2015) transcription factor families and PGC-1α (Li et al., 2002). On the contrary, the molecular mechanisms by which muscle fiber diversity is achieved during pre-natal development are still poorly understood. It was shown that the intrinsic transcriptional properties of embryonic and fetal myogenic progenitors are important to set the fiber type in the absence of nerve activity (Cho et al., 1993; Cusella-De Angelis et al., 1994). Moreover, in recent years, several transcription factors contributing to developmental muscle fiber specification have been identified, including Sox6 (Hagiwara et al., 2007; An et al., 2011), Nfix (Messina et al., 2010), Six1/Six4 (Richard et al., 2011), and Nfatc2 (Daou et al., 2013). However, the network of transcription factors controlling fiber specification during embryogenesis is still far from being fully characterized, and until now, functional interactions among the different regulators were completely unknown.

In this work, we provided evidence for functional interplay between Nfix and Sox6 in controlling expression of the slow MyHC isoform during mouse pre-natal muscle development.

In contrast to Nfix, which is a specific marker of fetal myogenesis (Messina et al., 2010; Mourikis et al., 2012), Sox6 is expressed in both embryonic and fetal purified myoblasts at the mRNA and protein levels. Consistently, we found that Sox6 protein is expressed in skeletal muscle in vivo starting between E11.5 and E12.5. This was unexpected, since Sox6 is known to be a repressor of MyHC-I, which along with MyHC-emb is expressed in all embryonic fibers (Hagiwara et al., 2007; Hutcheson et al., 2009). It is known that *Sox6* transcript is absent in mouse primary myotome between E9.5 and E10.75 (Vincent et al., 2012), suggesting that Sox6 is quickly activated in embryonic myoblasts at the beginning of primary myogenesis. Unexpectedly, Sox6 deficiency during primary myogenesis leads to a transient faster muscle phenotype with low levels of MyHC-I. This is followed in *Sox6*-null fetuses by dramatic upregulation of MyHC-I at the transcription level, consistently with previous characterizations of the *Sox6*-null phenotype (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011). Our results demonstrate that Sox6 plays opposite roles in MyHC-I expression during development. Importantly, in embryonic myogenic cells, Sox6 does not bind to the two canonical *MyHC-I* binding sites, which are both dispensable for Sox6-dependent embryonic regulation of MyHC-I, at least in our culturing conditions. These data strongly suggest an indirect mechanism of regulation. We found that Sox6 directly binds to the promoter of *Mef2C* and activates its expression in primary myofibers. Mef2C is part of a transcription factor family that is constitutively expressed in muscle cells since early embryogenesis (Edmondson et al., 1994) and plays an important role in the specification and maintenance of type I fibers (Chin et al., 1998; Wu et al., 2000; Anderson et al., 2015). Importantly, conditional deletion of *Mef2C* in skeletal muscle leads to a drastic reduction in slow-twitch fibers (Potthoff et al., 2007). Our rescue experiment, although partial, clearly shows that one of the Sox6-dependent effects on MyHC-I transcription in embryonic myofibers is mediated by Mef2C, which is directly targeted by Sox6 in embryonic, but not fetal, muscle. This function led us to hypothesize that the binding ability of Sox6 is differentially regulated in discrete myogenic progenitor populations or at different times during development according to different co-factors, which contribute to the high versatility of Sox6 functions. It is known that SoxD factors, lacking *trans*-acting functional domains, have a critical requirement for co-factors in order to regulate transcription of target genes (reviewed in Hagiwara, 2011). Therefore, it is likely that the reversal in Sox6 function is due to different factors that are progressively recruited and activated during muscle development. Indeed, we found that during fetal myogenesis, Nfix acts as a fundamental co-factor of Sox6 and is able to form a complex with Sox6, which is no longer able to bind to the *Mef2C* promoter but can bind to *MyHC-I* regulative regions, in particular the proximal promoter that was shown to be critical for Sox6-dependent fetal repression of MyHC-I (Hagiwara et al., 2007). In our previous study, we showed that Nfix negatively regulates MyHC-I by repressing Nfatc4, a positive regulator of MyHC-I (Messina et al., 2010). Interestingly, we have now found that during fetal myogenesis, Nfix is also present at the *MyHC-I* proximal promoter along with Sox6, suggesting that a physical association between these two proteins may be required for proper MyHC-I downregulation. Since Nfix presents both a *trans*-repression domain and a *trans*-activation domain, we hypothesized that formation of a complex with Sox6 might provide the basis for the transcriptional repression at the *MyHC-I* locus (Figure 7). Indeed, Nfix and Sox6 were found to be part of the same complex in fetal myotubes and are both present at the *MyHC-I* proximal promoter. Moreover, our study on *Nfix*-null and *Sox6*-null fetuses clearly shows that Sox6 and Nfix are independently expressed during secondary myogenesis and that neither Sox6 nor Nfix is able to correctly downregulate MyHC-I expression when the other one is not present. A scheme of the scenario in which Sox6 and Nfix behave

(K) Quantitative real-time PCR analysis on trunk and tail regions at 48 hpf from embryos injected with std-MO or suboptimal doses of *nfixa*-MO (0.25 pmol), *sox6*-MO (0.1 pmol), or *nfixa*-MO + *sox6*-MO (**$p < 0.01$; $N = 2$).

(L) Western blot for sMyHC at 52 hpf on trunk and tail regions of embryos injected with std-MO or suboptimal doses of *nfixa*-MO, *sox6*-MO, or *nfixa*-MO + *sox6*-MO. Vinculin was used to normalize the amount of loaded proteins.

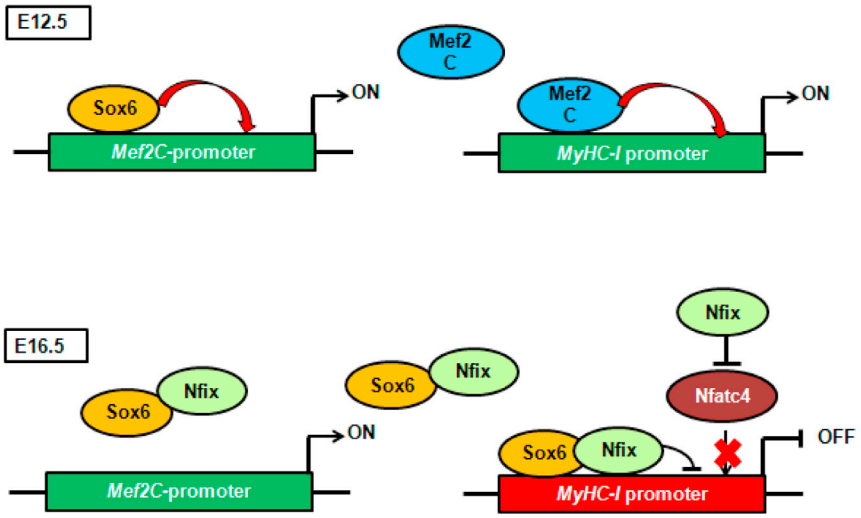


Figure 7. Model of Sox6 and Nfix Interplay during Pre-natal Myogenesis

Scheme illustrating the functions of Sox6 and Nfix during embryonic (E12.5) and fetal (E16.5) myogenesis. Transcription factors are represented by circles, whereas the regulative region of *MyHC-I* is indicated by rectangles that are green when transcription is activated and red when transcription is repressed.

and cooperate during pre-natal muscle development is shown in Figure 7.

In this work, we have also provided evidence for a conserved transcriptional cooperation of Sox6 and Nfixa in zebrafish. We have shown that Sox6 is crucial for the regulation of *smyhc1*, in line with the findings of Jackson et al. (2015). It was shown that Sox6 expression is silenced in zebrafish slow fibers by the combined effects of Prdm1a and miR-499 (von Hofsten et al., 2008; Wang et al., 2011). We found that a minority of sMyHC-positive cells display cytoplasmic staining for Sox6, suggesting that Sox6 might also be regulated through subcellular localization in different fiber types. Notably, our double partial knockdown performed with suboptimal doses of *sox6*-MO (von Hofsten et al., 2008) and *nfixa*-MO (Pistocchi et al., 2013) caused a severe impairment in the touch-evoked escape response, suggesting that Sox6 and Nfixa cooperate in proper muscle function. Molecularly, our data suggest that the two transcription factors can act together to repress *smyhc1* expression in zebrafish embryos, whereas the expression of a typical fast-twitch gene, such as *myl1* (Burguière et al., 2011), was unaffected in double partial morphants.

In conclusion, we have presented a complex model of regulation of embryonic to fetal MyHC-I regulation that involves a functional interplay between Nfix and Sox6 that is conserved in mammals and teleosts.

EXPERIMENTAL PROCEDURES

Mouse Strains and Fish Lines

The following murine lines were used: $Myf5^{GFP\text{-}P/+}$ (Kassar-Duchossoy et al., 2004), *Nfix* null (Campbell et al., 2008), $Sox6^{lacZ/+}$ (Smits et al., 2001), and Tg:Mlc1f-*Nfix2* (Messina et al., 2010). For each of these lines, the genotyping strategy has been described in the references. CD1 WT mice (Jackson Laboratory) were used as well. Mice were kept in pathogen-free and controlled conditions, and all procedures conformed to Italian law (D. Lgs n° 2014/26, implementation of the 2010/63/UE) and were approved by the Animal Welfare Body of the University of Milan and the Italian Minister of Health (1212/2015PR). Zebrafish were raised and maintained according to established techniques. The following line was used: AB (from Carole Wilson, UCL, London, UK).

Immunofluorescence on Sections

Mouse or zebrafish embryos were fixed in 4% paraformaldehyde, extensively washed in PBS, and incubated overnight in PBS containing 15% sucrose. Samples were then frozen in nitrogen-chilled isopentane and kept at −80°C until use. Cryostat sections (8 μm thick) were permeabilized in 1% BSA, 0.2% Triton X-100 in PBS for 30 min at room temperature and then incubated for 1 hr in blocking solution (10% goat serum in PBS) and overnight with the primary antibody or with mock PBS. After incubation for 45 min with the fluorescent-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), sections were washed in PBS 0.2% Triton X-100 and mounted, and fluorescent immunolabeling was recorded with a DM6000 Leica microscope. The following primary antibodies were used: rabbit anti-Sox6 (Abcam, 1:300), rabbit anti-Nfix (Novus Biological, 1:200), mouse anti-MyHC-I (Sigma, 1:200), BAD5 (monoclonal, 1:2), MF20 (monoclonal, 1:2), and F59 (monoclonal, 1:10). Nuclei were stained with Hoechst (1:1,000).

Cell Sorting and Culturing

Dissected $Myf5^{GFP\text{-}P/+}$ embryonic or fetal muscles were digested by 0.15 mg/mL Collagenase (Sigma), 1.5 mg/mL Dispase (GIBCO), and 0.1 mg/ml Dnase I (Sigma) for 30 min at 37°C in agitation as described in Biressi et al. (2007). After centrifugation and filtration, cells were collected in DMEM, 20% fetal bovine serum (FBS), 2 mM EDTA, and 20 mM HEPES. For cell sorting, we used the Sorter BD FACSAria. GFP-positive cells were collected for mRNA and protein extraction. For the preparation of unpurified fetal myoblasts after digestion of tissue, cells were pre-plated for 30 min on a plastic dish to lose fibroblasts, which normally adhere to plastic. Unpurified cells were kept in incubation at 37°C in 20% HS (20% horse serum in DMEM) and 24 hr later allowed to differentiate in DM (2% horse serum in DMEM) for 48 hr.

Lentivirus Production, Transduction, and Transfection

Preparation of viral particles were performed by co-transfecting pLKO.1-shSox6 vectors (Thermo Fisher Scientific) or non-targeting shRNA vectors (30 μg), together with the packaging plasmids pMDLg/p (16.25 μg), pCMV-VSVG (9 μg), and pRSV-REV (6.25 μg), in HEK293T cells. Transfection was performed using the calcium phosphate transfection method. Viral particles were collected 40 hr after transfection and subjected to ultracentrifugation at 20,000 rpm for 2 hr at 20°C (Beckman Coulter, Optima L-100 XP). The concentrated viral particles were re-suspended in PBS and stored in aliquots at −80°C until further use. Embryonic myoblasts were transduced by overnight incubation with viral preparation. These preparations were used to transduce embryonic myoblasts at an MOI of 10. The day after transduction, embryonic myoblasts were transfected with Mef2C (pCDNAI/A-Mef2C) or the empty vector as a negative control using Lipofectamine LTX (Invitrogen).

Protein Extraction and Western Blot

Cultured cells were washed twice in ice-cold PBS and then lysed (30 min in ice) with RIPA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton-X, 0.1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl in deionized water) plus protease inhibitors (1 mM PMSF). For zebrafish, only trunk and tail regions were used, cutting out the head, and protein was extracted in Laemmli buffer

(3 μL per embryo). Protein was harvested after centrifugation, quantified by absorbance reading at 750 nm, and stored at −80°C. 30 μg protein was resolved on 8%–12% polyacrylamide gels or on MiniProtean TDX Gels (Bio-Rad) after denaturation at 95°C for 5 min with SDS-PAGE Loading sample buffer 2X (100 mM Tris [pH 6.8], 4% SDS, 0.2% Bromophenol blue, 20% glycerol, and 10 mM dithiothreitol). For western blot analysis, proteins were transferred onto nitrocellulose with the iBlot Dry Blotting System (Invitrogen). Following transfer, membranes were blocked in 5% milk in Tris-buffered saline (TBS)-T (TBS plus 0.02% Tween20) for 1 hr at room temperature. The primary antibodies used were rabbit anti-Sox6 (Abcam; 1:1,000), mouse anti-MyHC-I (Sigma; 1:5,000), mouse anti-β-tubulin (Santa Cruz Biotechnology; 1:5,000), rabbit anti-Nfix (Novus Biologicals; 1:500), mouse anti-HA (Covance; 1:500), mouse anti-vinculin (Sigma; 1:1,000), and mouse anti-sMyHC (F59, Hybridoma Bank) anti-mouse sarcomeric MyHC (MF20, Hybridoma Bank). Horseradish peroxidase (HRP)-conjugated antibodies (Bio-Rad) were used as secondary antibodies, and the signal was revealed with the ChemiDoc MP System (Bio-Rad).

Transfection and Co-immunoprecipitation Assays

For transfection of fetal myoblasts, WT or Sox6-null cells were plated on 90-mm dishes and allowed to reach 80% confluence in proliferating conditions. Cells were transfected with pCH-Nfix2-HA or pCH-HA plasmids with Lipofectamine LTX (Invitrogen) overnight at 37°C. Cells were kept in DM for 36 hr after transfection, and then nuclear extracts were prepared by collecting 5×10^6 cells in 400 μL ice-cold hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, protease inhibitors, 1 mM PMSF, and phosphatase inhibitors) for 15 min in ice. Then, NP40 was added to a final concentration of 0.625%, and cells were centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was discarded as cytoplasmatic extract, while the pelleted nuclei were resuspended in 100 μL immunoprecipitation lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% NP40, 1 mM EDTA, 100 mM NaCl, and protease and phosphatase inhibitors. Immunoprecipitation was performed using the immunoprecipitation kit Dynabeads Protein A (Life Technologies). Dynabeads Protein A (50 μL) was incubated for 2 hr with gentle rotation at room temperature with 5 μg rabbit anti-Sox6 antibody (Abcam), rabbit anti-HA antibody (Santa Cruz), or non-related rabbit immunoglobulin G (IgG) (Santa Cruz). Then, the bead-antibody complex was incubated with gentle rotation for 2 hr at 4°C with 1.5 mg total protein lysates per condition. The eluted proteins were denatured with non-reducing SDS-PAGE loading sample buffer (100 mM Tris [pH 6.8], 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and loaded onto a gel.

Chromatin Immunoprecipitation and Reporter Assay

ChIP for Sox6 and luciferase assays were performed as previously published (An et al., 2011). For the detailed protocol, see Supplemental Experimental Procedures.

RNA Extraction and Analysis

RNA from homogenized mouse or zebrafish embryos and isolated cells was extracted with NucleoSpin RNA kits (Macherey-Nagel) following the manufacturer's instructions. Eluted RNA was checked on agarose gels, quantified with a Nanodrop spectrophotomer, and stored at −80°C. Approximately 0.5 μg RNA was used with the ImProm-II Reverse Transcriptase kit (Promega). Real-time PCR was performed on cDNA using SYBR Green Supermix (Bio-Rad) and the CFX Connect Real Time System (Bio-Rad). After amplification, relative mRNA expression levels were calculated using standard curves from cDNA dilutions and normalized on the *Gapdh* expression levels. For quantitative real-time PCR in zebrafish, we used *rpl8* to normalize the mRNA levels. The primers used are listed in Tables S1 and S2.

MO Microinjections

The antisense MOs (Gene Tools) used in this study, *sox6*-MO1 (von Hofsten et al., 2008) and *nfixa*-MO (Pistocchi et al., 2013), were previously described. MOs, diluted in Danieau buffer (Nasevicius and Ekker, 2000), were injected at the one- to two-cell stage. Escalating doses of each MO were tested for phenotypic effects; as control for unspecific effects, each experiment was performed in parallel with an std-MO (standard control oligo) with no target in zebrafish embryos. We usually injected 0.25 pmol *sox6*-MO per embryo. For combined knockdown experiments, we injected *sox6*-MO and *nfixa*-MO at 0.1 and 0.25 or 0.08 and 0.125 pmol per embryo, respectively.

Statistical Analysis

Values were expressed as means ± SD. Statistical significance was assessed by unpaired Student's t test with Prism 5 software. Statistical significance with a probability of less than 5%, 1%, or 0.1% is indicated in each graph with a single, double, or triple asterisk, respectively, followed by the number of independent experiments (n).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.082.

AUTHOR CONTRIBUTIONS

Conceptualization, G. Maroli, V.T., and G. Messina; Methodology, S.C., A.F., and S.M.; Investigation, V.T., G. Maroli, S.M., S.C., A.F., and G.R.; Writing – Original Draft, G. Maroli and V.T.; Writing – Review & Editing, V.T., S.C., G.R., G.C., M.B., and G. Messina; Supervision, M.B. and G. Messina; Funding Acquisition, G. Messina.

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Supplemental Information

Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

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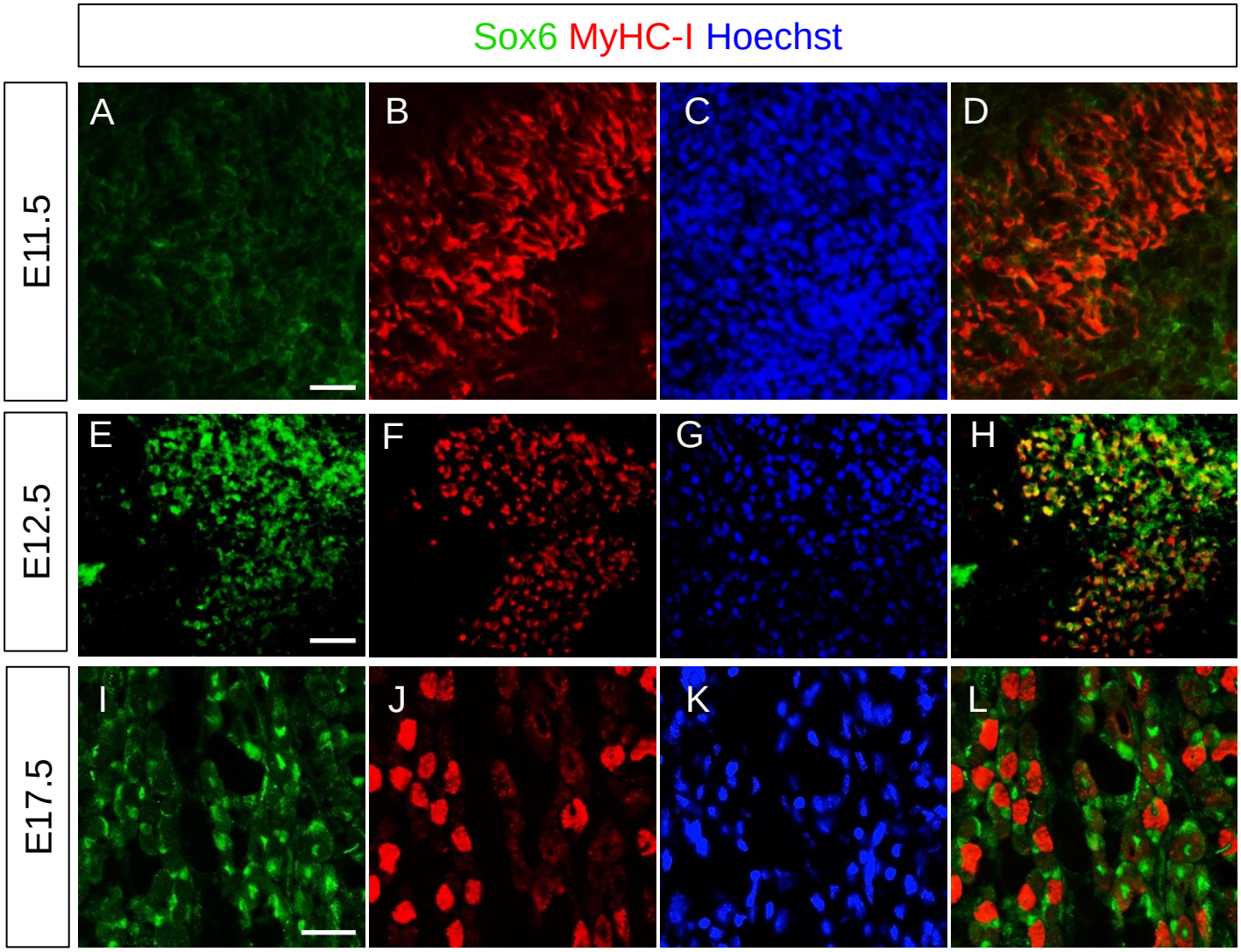
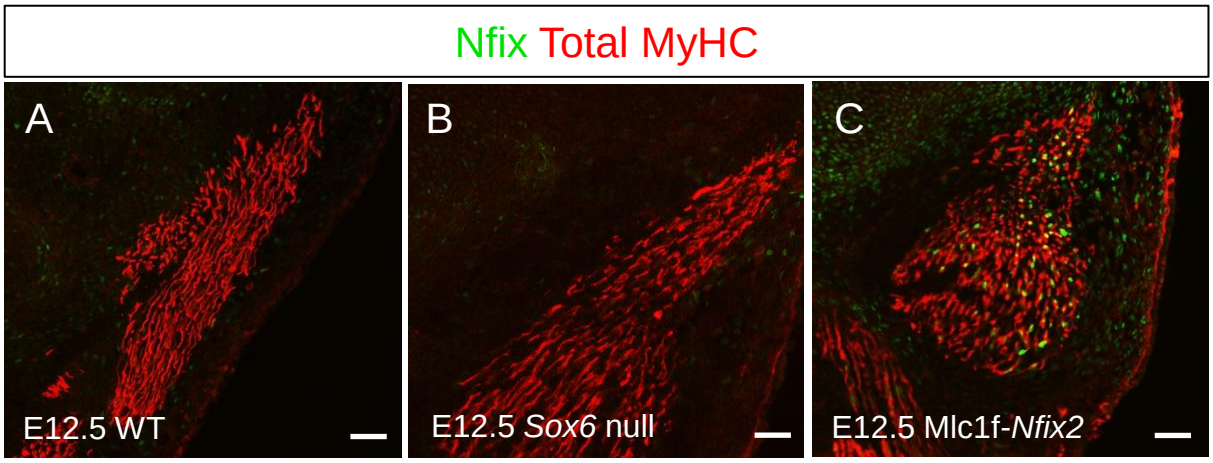
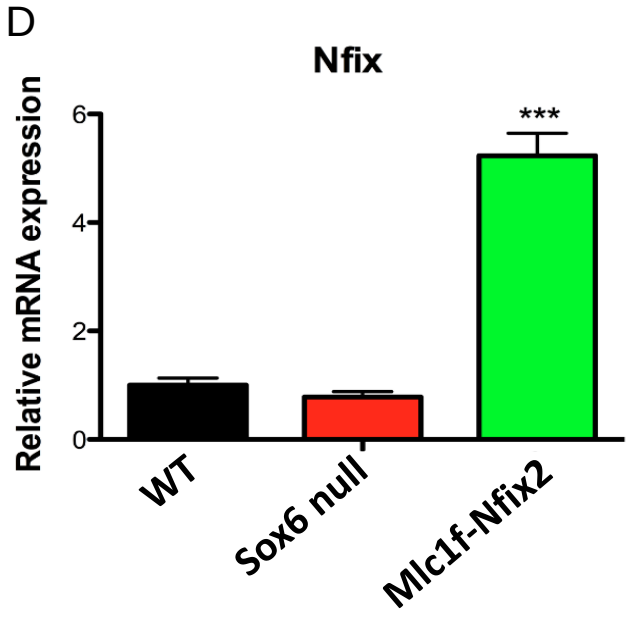


Supplemental Figure S1. Related to Figure 1. Sox6 is expressed in both embryonic and fetal muscle cells.

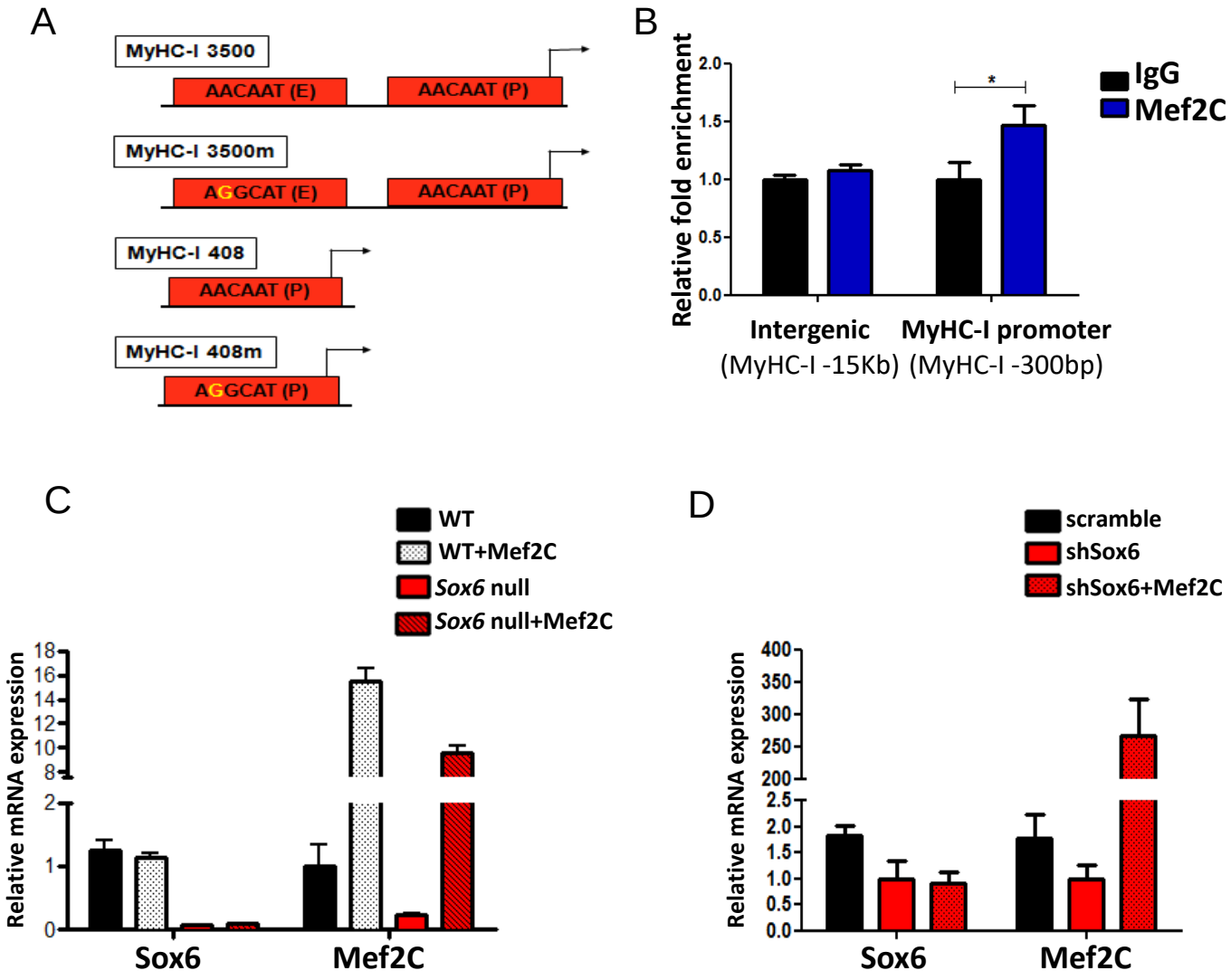
A-L) Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on E11.5 (**A-D**), E12.5 (**E-H**) and E17.5 **(I-L)** muscle sections. Nuclei are counterstained with Hoechst. Scale bars: 25 μm.





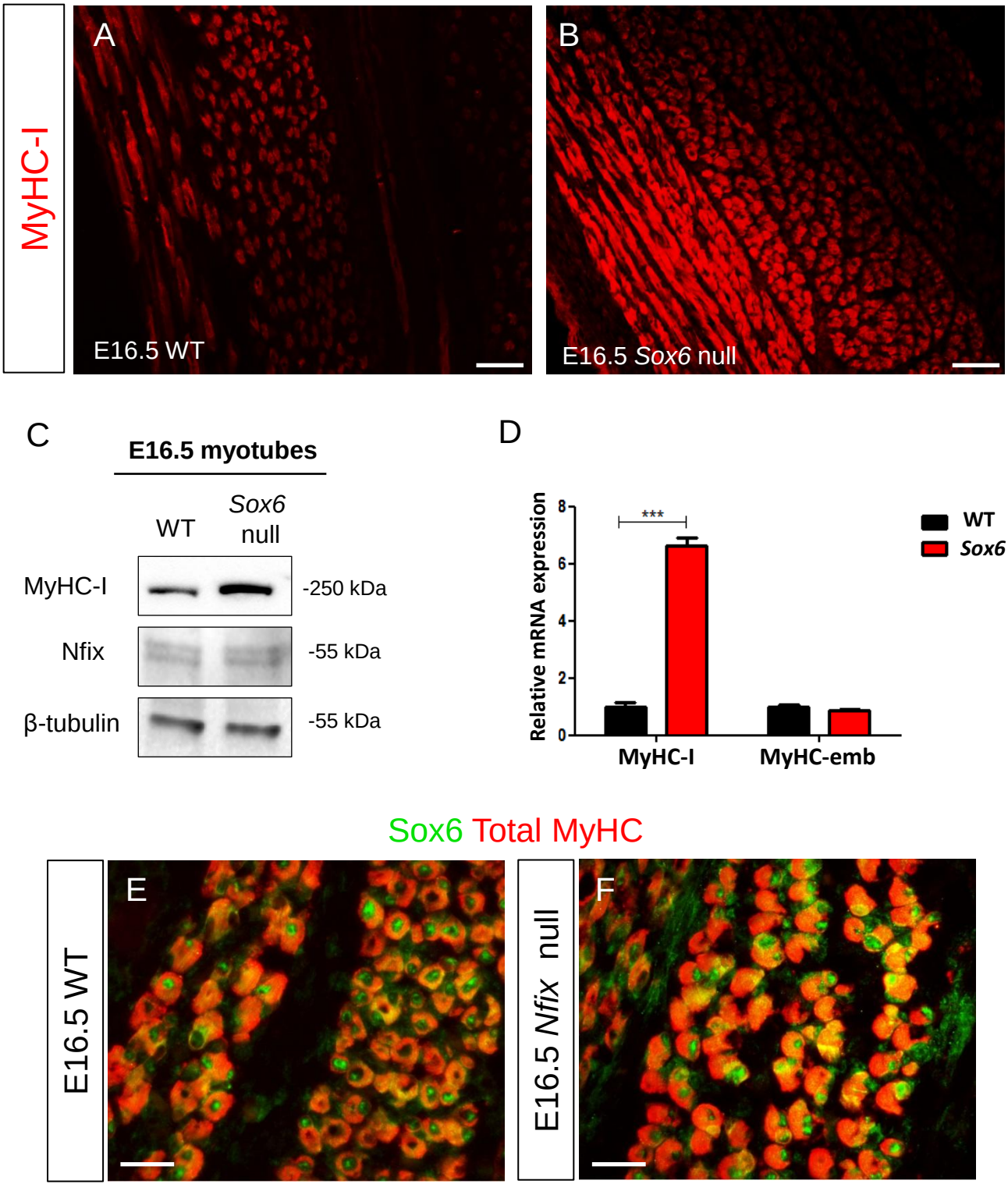
Supplemental Figure S2. Related to Figure 1. Nfix is not up-regulated in *Sox6*-null embryonic muscle.

A-C) Immunofluorescence with anti-Nfix antibody (green) and MF20 (red) on E12.5 muscle sections from WT (**A**), *Sox6* null (**B**) and Tg:Mlc1f-*Nfix2* (**C**) embryos. Scale bar: 50 μm. **D)** qRT-PCR for *Nfix* on WT, *Sox6* null and Tg:Mlc1f-*Nfix2* E12.5 muscle tissue (***p<0.001; N=3).



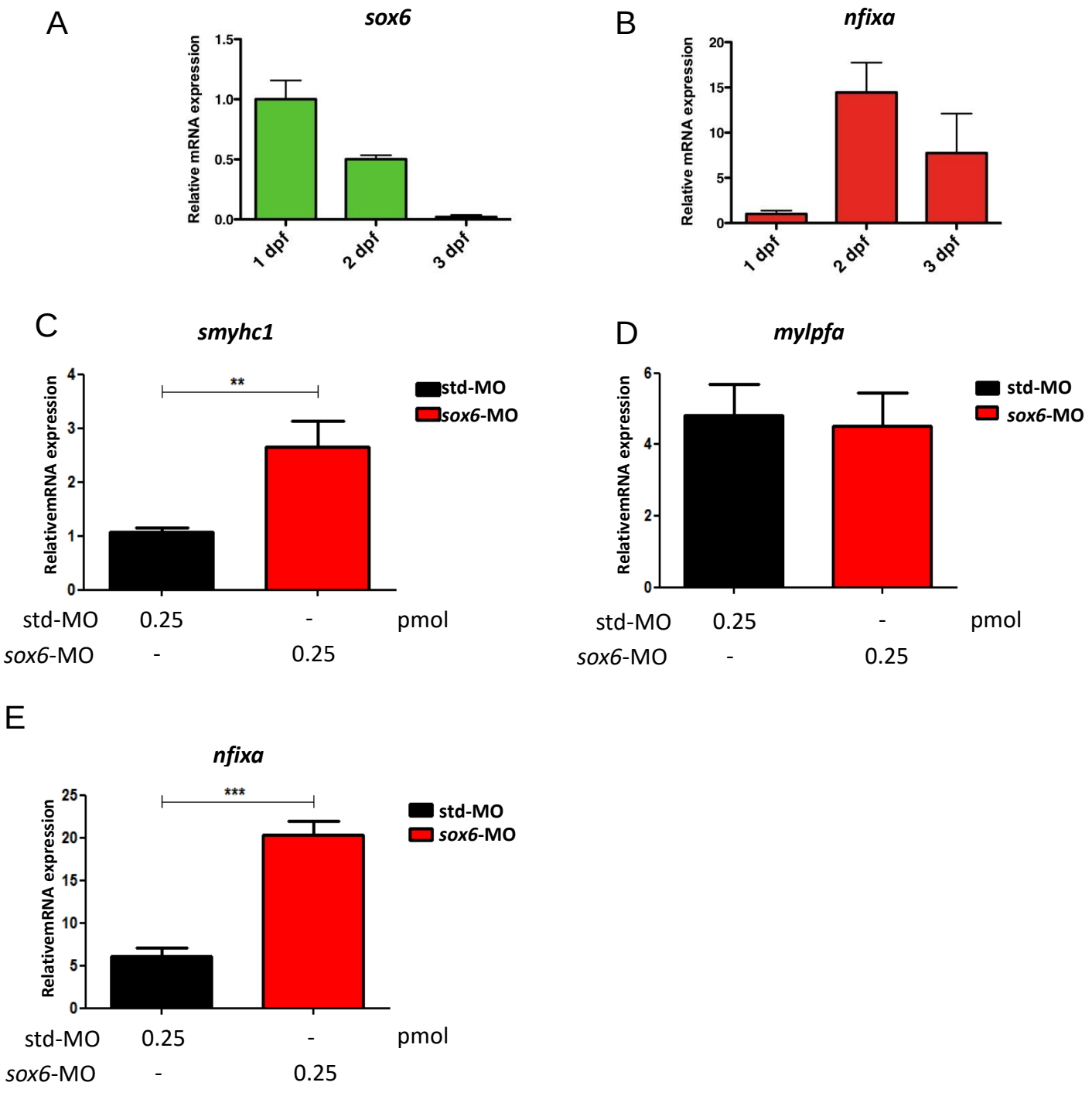
Supplemental Figure S3. Related to Figure 2. Sox6, through Mef2C, positively regulates MyHC-I in embryonic myotubes.

A) Scheme of the luciferase vectors used in Fig. 2B and Fig. 3F. See also Hagiwara et al. 2007 and An et al. 2011 for a complete description of the vectors. **B)** Chromatin immunoprecipitation (ChIP) for Mef2C on an intergenic region located at -15 Kb upstream of the MyHC-I TSS and on the MyHC-I proximal promoter. (*$p<0.05$; N=2). **C)** qRT-PCR for Sox6 and Mef2C on WT and *Sox6* null differentiated embryonic myoblasts transfected with a Mef2C-overexpressing vector. Related to Fig. 2E. **D)** qRT-PCR for Sox6 and Mef2C on purified embryonic myoblasts treated with control shRNA lentivirus (scramble), with anti-Sox6 shRNA lentivirus (shSox6) and with both shSox6 lentivirus and Mef2C overexpressing vectors (shSox6+Mef2C). Related to Fig. 2F.



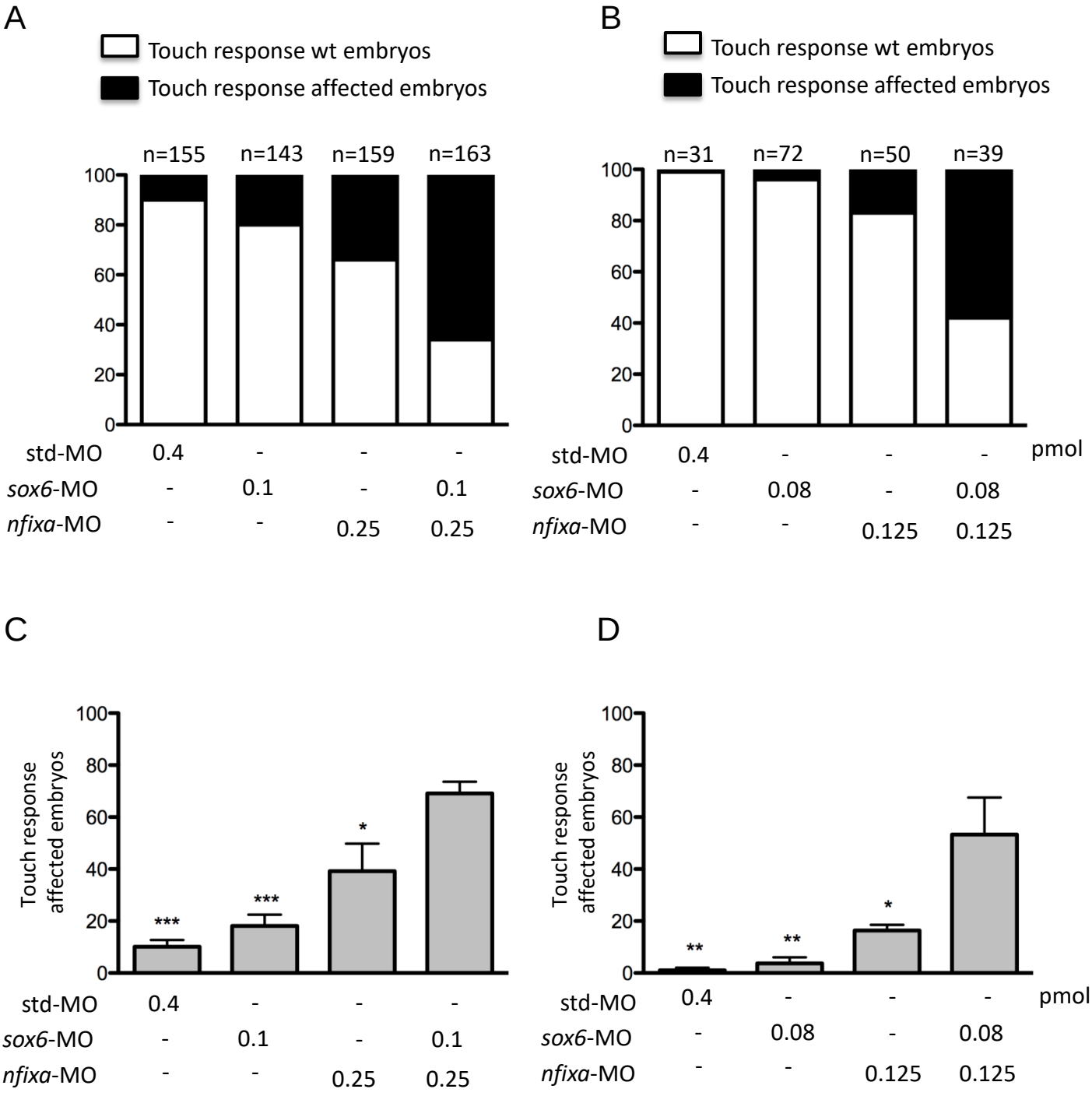
Supplemental Figure S4. Related to Figure 3 and Figure 4. Sox6 and Nfix are independently expressed in fetal muscle.

A, B) Immunofluorescence with anti-MyHC-I on E16.5 muscle sections from WT (**A**) and *Sox6* null **(B)** mice. Scale bar: 50 µm. **C)** Western blot on E16.5 WT and *Sox6* null myotubes. β-tubulin was used to normalize the amount of proteins loaded. **D)** qRT-PCR analysis on E16.5 WT and *Sox6* null muscle tissue (***p<0.001; N=3). **E, F)** Immunofluorescence with anti-Sox6 antibody (green) and anti-MyHC antibody (red) on fetal (E16.5) muscle sections from WT and *Nfix* null mice. Scale bars: 25 µm.



Supplemental Figure S5. Related to Figure 6. *sox6* and *nfixa* expression levels during wt embryo development and gene expression analysis in *sox6* morphants.

A, B) qRT-PCR for *sox6* **(A)** and *nfixa* **(B)** on trunk/tail regions collected from 1, 2 and 3 dpf zebrafish embryos. **C-E)** qRT-PCR for *smyhc1*, *mylpfa, and nfixa* on trunk/tail regions at 2 dpf collected from embryos injected with std-MO or *sox6*-MO (**p<0.01; ***p<0.001; N=2).



Supplemental Figure S6. Related to Figure 6. Touch-evoked escape response assay on std-MO, *sox6*-MO, *nfixa*-MO and *nfixa*-MO+*sox6*-MO injected embryos.

A, B) Touch-evoked escape response assay was performed on *sox6* and *nfixa* single morphants and on the combined double partial morphants, injected using slightly higher **(A)** or lower **(B)** doses. Graphs show the percentage of embryos with or without defects (white and black bars, respectively) in touch-evoked escape response assay. The co-injection of both lower and higher doses of MOs resulted in synergic defects in motility in touch-response assays. **C, D)** Statistical analysis of touch-evoked escape response assay shown in A and B, respectively (*p<0.05; **p<0.01; ***p<0.001).

Supplemental Movie S1. Related to Figure 6. Impaired escape response in double partial *sox6/nfixa* morphants.

The movie shows, sequentially, touch-evoked escape responses of 2 pdf control embryos (std-MO 0.4 pmol), single partial *sox6* or *nfixa* knocked down embryos (*sox6*-MO or *nfixa*-MO, 0.1 pmol and 0.25 pmol, respectively), and double partial morphants (*sox6*-MO+*nfixa*-MO). The most representative embryos are shown for each condition (see Supplemental Fig. S6A). In particular, the *sox6/nfixa* double partial morphant corresponds to the most drastic and representative phenotype, within the range of escape-response impairments observed.

SUPPLEMENTAL TABLES

Supplemental Table S1. Primers used for mRNA expression analysis with quantitative Real Time PCR (*Mus musculus*)

Gene	Primers sequence	Reference
Sox6	F: 5'-AATGCACAACAAACCTCACTCT-3' R: 5'-AGGTAGACGTATTTCGGAAGGA-3'	
MyHC-I (Myh7)	F: 5'-AGGGCGACCTCAACGAGAT-3' R: 5'-CAGCAGACTCTGGAGGCTCTT-3'	Mathew et al. 2011
MyHC-emb (Myh3)	F: 5'-GCAAAGACCCGTGACTTCACCTCTAG-3' R: 5'-GCATGTGGAAAAGTGATACGTGG-3'	Mathew et al. 2011
Nfix	F: 5'-CACTGGGGCGACTTGTAGAG-3' R: 5'-AGGCTGACAAGGTGTGGC-3'	Mourikis et al. 2012
Mef2c	F: 5'-AGTACACCGAGTACAACGAGC-3' R: 5'-GCCTGTGTTACCTGCACTTGG-3'	
Mef2a	F: 5'-TTGATGGGGGAATGACAACT-3' R: 5'-TAGTCCTGTGGGGAATGGAT-3'	
Gapdh	F: 5'-GGCATGGACTGTGGTCATGA-3' R: 5'-TTCACCACCATGGAGAAGGC-3'	

Supplemental Table S2. Primers used for mRNA expression analysis with quantitative Real Time PCR (*Danio rerio*)

Gene	Primers sequence
nfixa	F: 5'-ACCGAAGAAACCGACTGGTG-3' R: 5'-TCTGTGGCCATTGTAGTTCAGG-3'
sox6	F: 5'-GCGCATGGAATCGGACAG-3' R: 5'-GGCTTGTGTGGAGAGGTAGAG -3'
smyhc1	F: 5'-GCTAACAGGCAGGCATCAGA-3' R: 5'-GTTGCATTTGGGAATCCTTGACA-3'
mylpfa	F: 5'-GCGGCTTCAGACTTCTCTTCTTG-3' R: 5'- CTTCTTGGGTGCCATGTCGAG-3'
myl1	F: 5'-GGAACAGTGATGGGTGCTGA-3' R: 5'-CGTTTTCATCCTCCTGGCCT-3'
rpl8	F: 5'-GCAAACAGAGCCGTTGTTG-3' R: 5'-CCTTCAGGATGGGTTTGTCA-3'

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reporter assay

Luciferase expression vectors driven by the *MyHC-I* promoter were kindly provided by Dr. K.M. Baldwin (University of California, Irvine) and Dr. N. Hagiwara (University of California, Davis). The MyHC-I 408 and MyHC-I 3500 constructs contain 408 bp and 3500 bp of the 5'-upstream sequence of the rat *MyHC-I* gene, respectively (Fig. S2A; Huey et al. 2002). The MyHC-I 408m and MyHC-I 3500m constructs were generated by introducing ACA to CAG substitutions to disrupt the proximal and the distal Sox6 binding sites, respectively (Fig. S2A; Hagiwara et al. 2007; An et al. 2011). These lucifearse vectors were co-transfected with the *Renilla* luciferase vector (pcDNA-Rluc) into embryonic or fetal primary myoblasts using Lipofectamine 2000 (Invitrogen). The day after transfection, cells were washed in PBS and allowed differentiation in DM. After 2 more days, firefly and *Renilla* luciferase activities were measured using Dual-Glo Luciferase Assay System (Promega) and a luminometer according to the manufacturer's instructions. pGL3-basic vector was used as a negative control.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously published (An et al. 2011). Briefly, primary myoblasts were crosslinked for 10 min at room temperature in 1% formaldehyde (Sigma) in PBS, then the crosslinking was quenched with 0,25M glycine in PBS (10 min) and the cells were pelleted and lysed in Sonication Buffer containing 10mM Tris-HCl pH 8.0, 1mM EDTA, 1% SDS in deionized water. Sonication was performed in a Bioruptor Diagenode with three pulses of 5 min at maximum intensity. Chromatin was de-crosslinked and checked for 200-500bp DNA fragments enrichment. Immunoprecipitation was performed with 3-5 µg of the following antibodies: rabbit anti-Sox6 (Abcam), rabbit anti-Nfix (Novus Biologicals), rabbit anti-Mef2C (Cell Signaling) and normal goat IgG (Santa Cruz). After elution, de-crosslinking, DNA extraction and precipitation, the samples were analyzed by qRT-PCR.

The following primers were used for amplification of the *MyHC-I* proximal promoter region (promoter): 5'-CCCCACCCCCTGGAACT-3' (fw), 5'-CCAGCTAGGAAACAATTGGAAGTG-3' (rev); for the *MyHC-I* distal promoter region (enhancer): 5'-ACACCGCCCACTCAATACAC-3' (fw), 5'-GCCCTCTCCAAACACTCTTG-3' (rev); for a negative control region located 15 Kb upstream of

the *MyHC-I* gene (intergenic): 5'-TCGGACCGGAGTGTTAGGAA-3' (fw), 5'-ACCCTGGAGTCTCAGCATCG-3' (rev) (An et al. 2011); *Nfatc4* promoter (-1.2Kb): 5'–GGCGCTTAACCCTTTAGGTG-3' (fw). 5'-CAAGACAGGGGAGCAGTCAC-3' (rev). We also used the following primers for the *Mef2c* promoter (-1.1Kb): 5´-AACCTAAGGGTTTTGTTATGACGC-3' (fw), 5´-ACGGGTGGGACTTTTTAGGAG-3´(rev). The fold enrichment of each sample was calculated as percentage of input for internal control and then normalized on the IgG value.

Touch-evoked escape response assay

The screening for embryonic motility was performed as follows: embryos at 2 dpf were subjected to a tactile stimulus. Using a needle, a gentle stimulus was applied to the tail of the larvae and their reaction observed. Wild-type embryos at this stage of development have a normal activity. Upon application of the tactile stimulus they swim away from the source of the stimulus (Granato et al. 1996).

Movies were recorded with a Leica MZFLIII stereomicroscope equipped with a DFC 480-R2 digital camera and the LAS imaging software. Movies were edited using the Adobe Premiere program.

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