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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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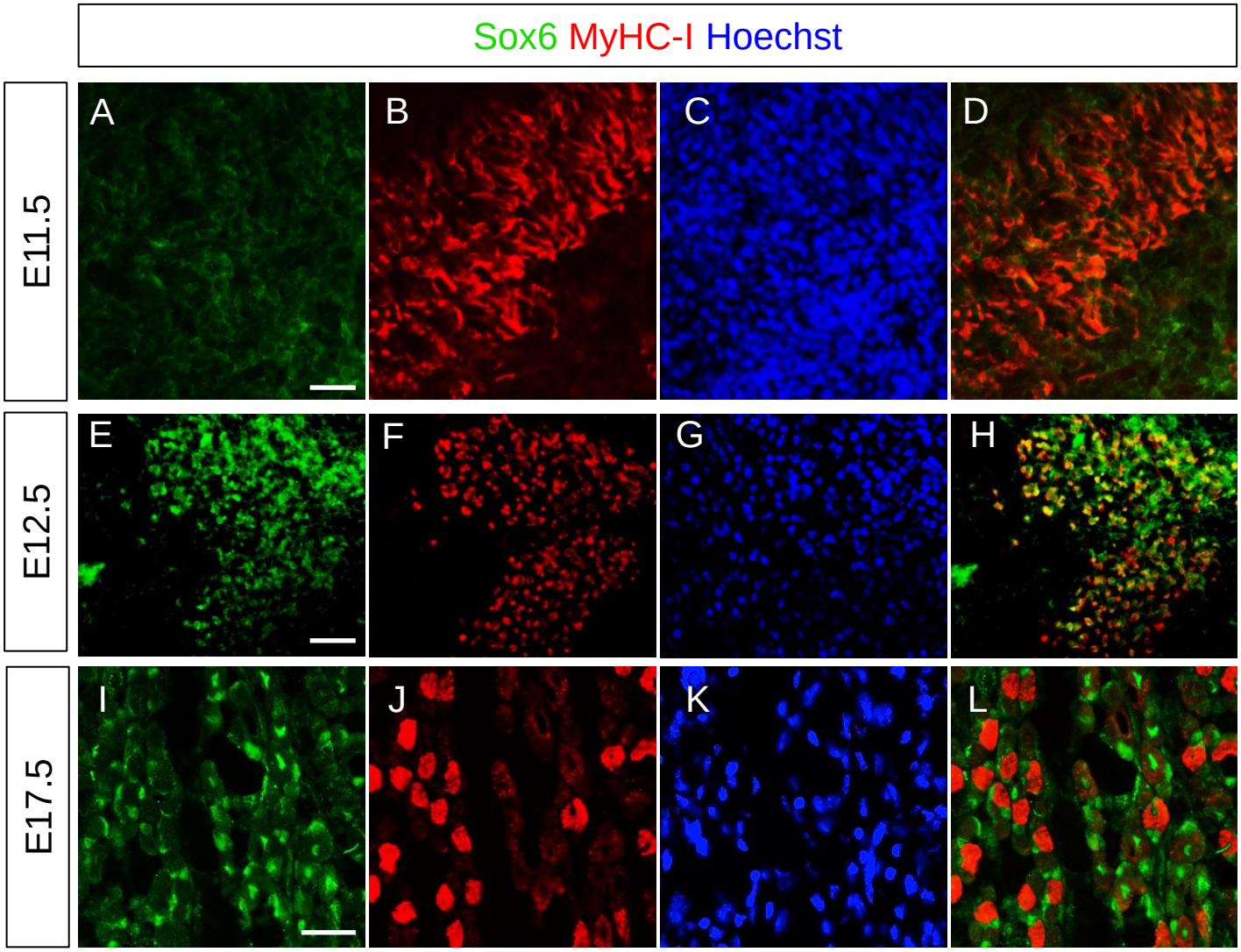
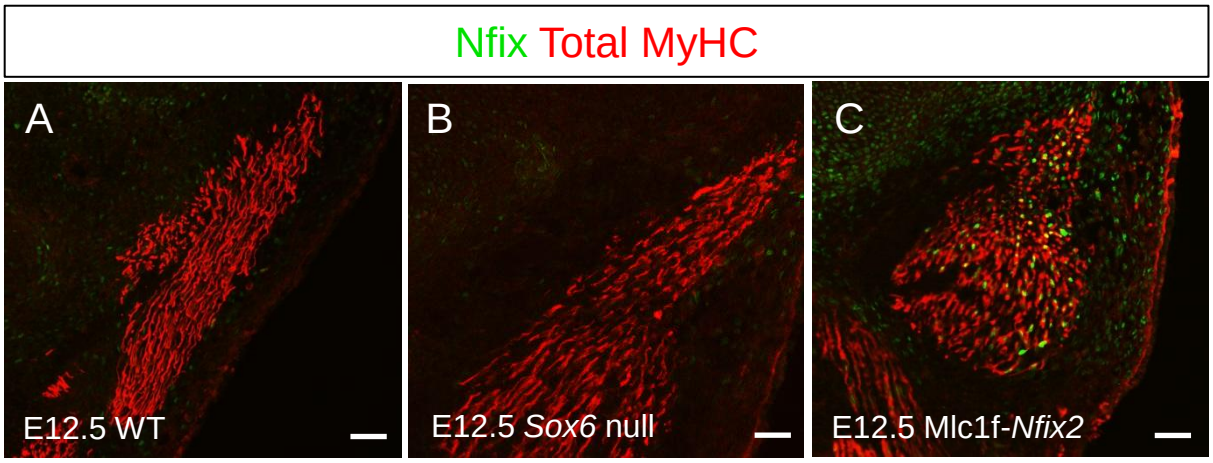
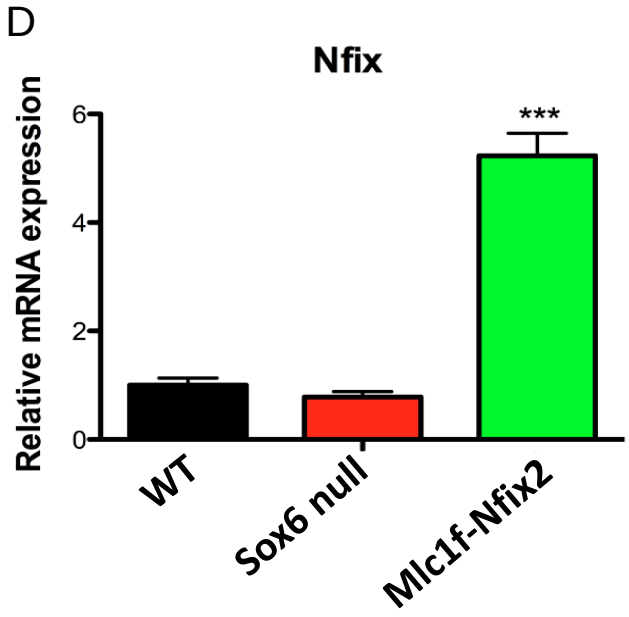


Supplemental Figure S1. Related to Figure 1. Sox6 is expressed in both embryonic and fetal muscle cells.

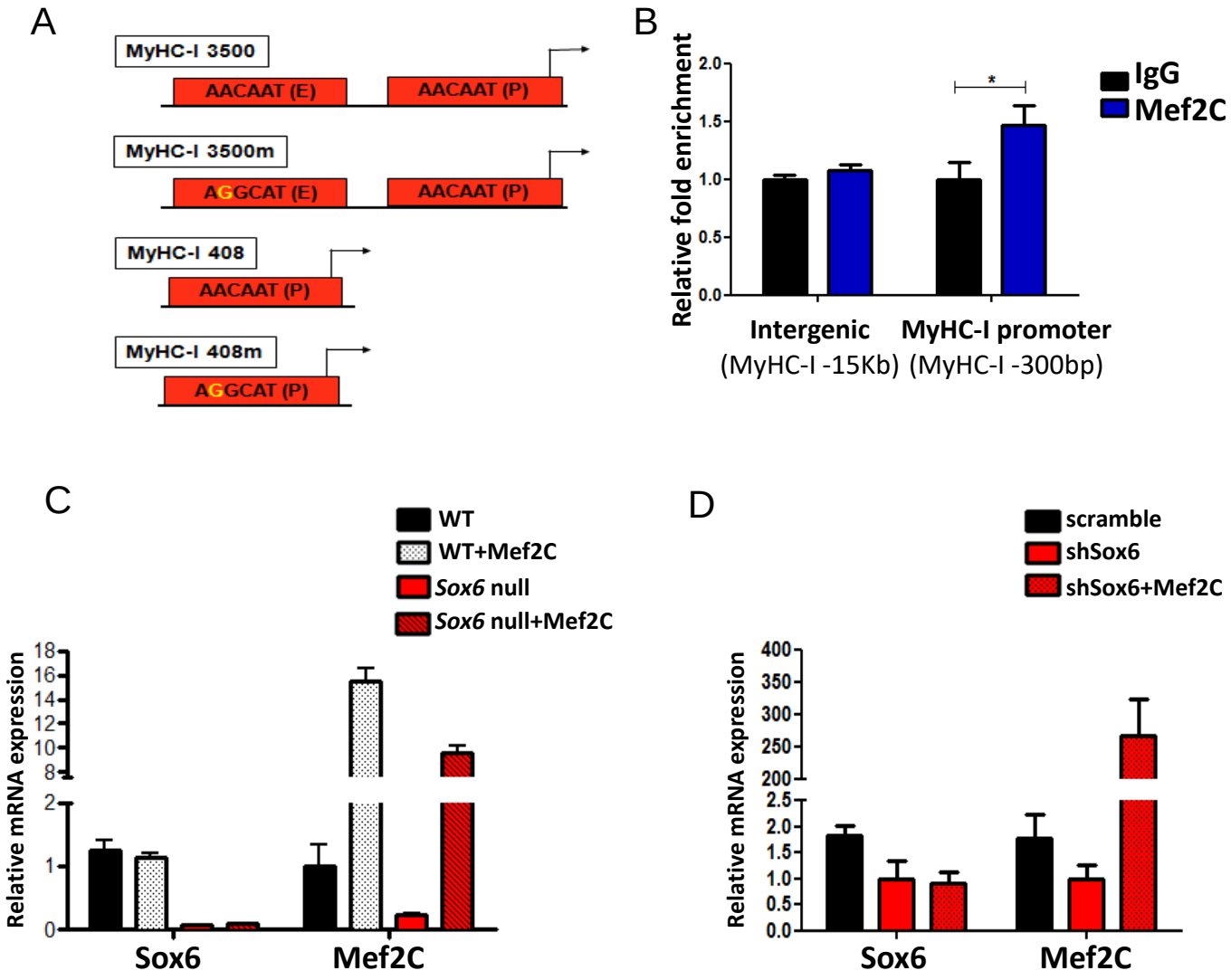
A-L) Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on E11.5 (**A-D**), E12.5 (**E-H**) and E17.5 **(I-L)** muscle sections. Nuclei are counterstained with Hoechst. Scale bars: 25 μm.





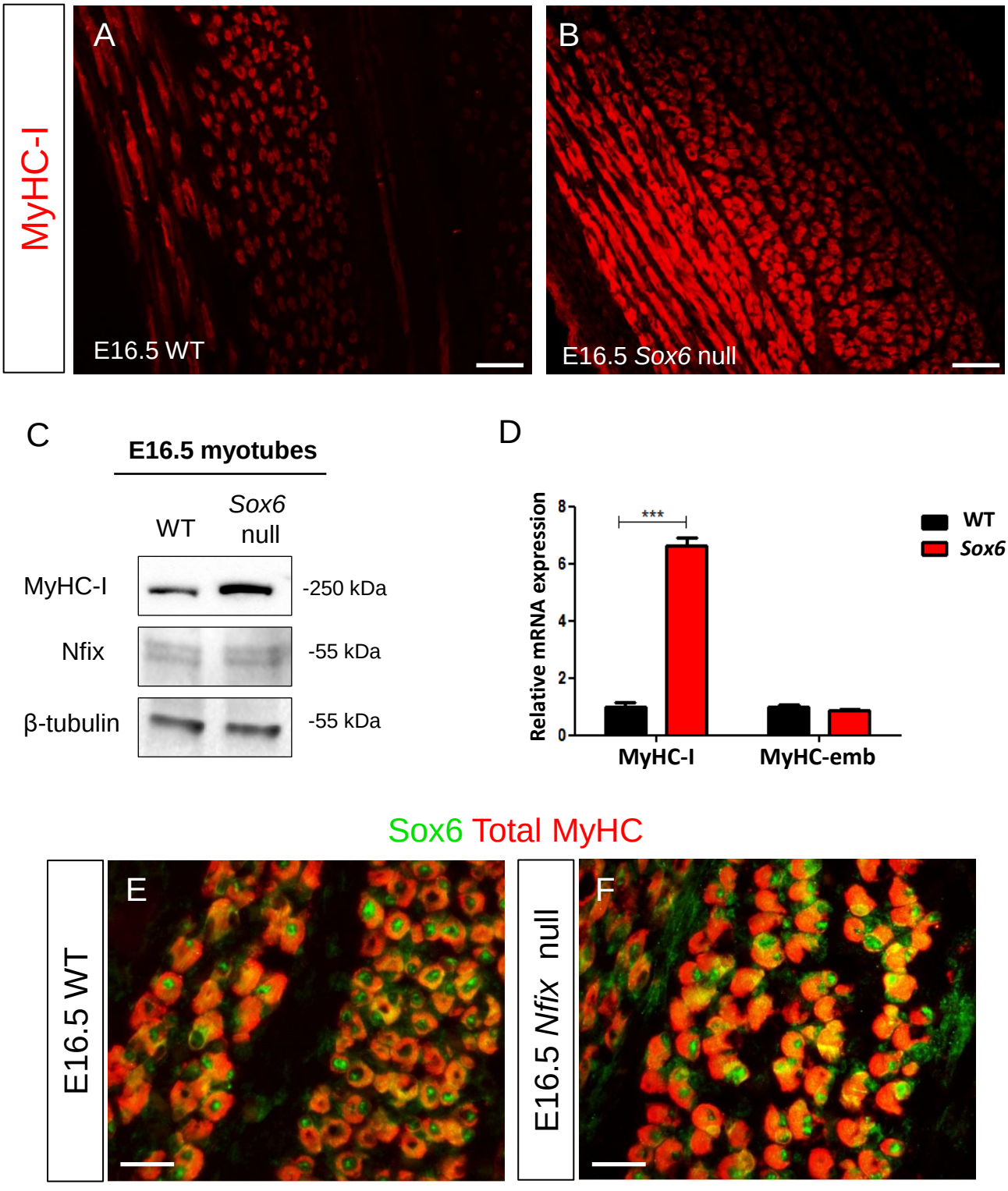
Supplemental Figure S2. Related to Figure 1. Nfix is not up-regulated in *Sox6*-null embryonic muscle.

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).



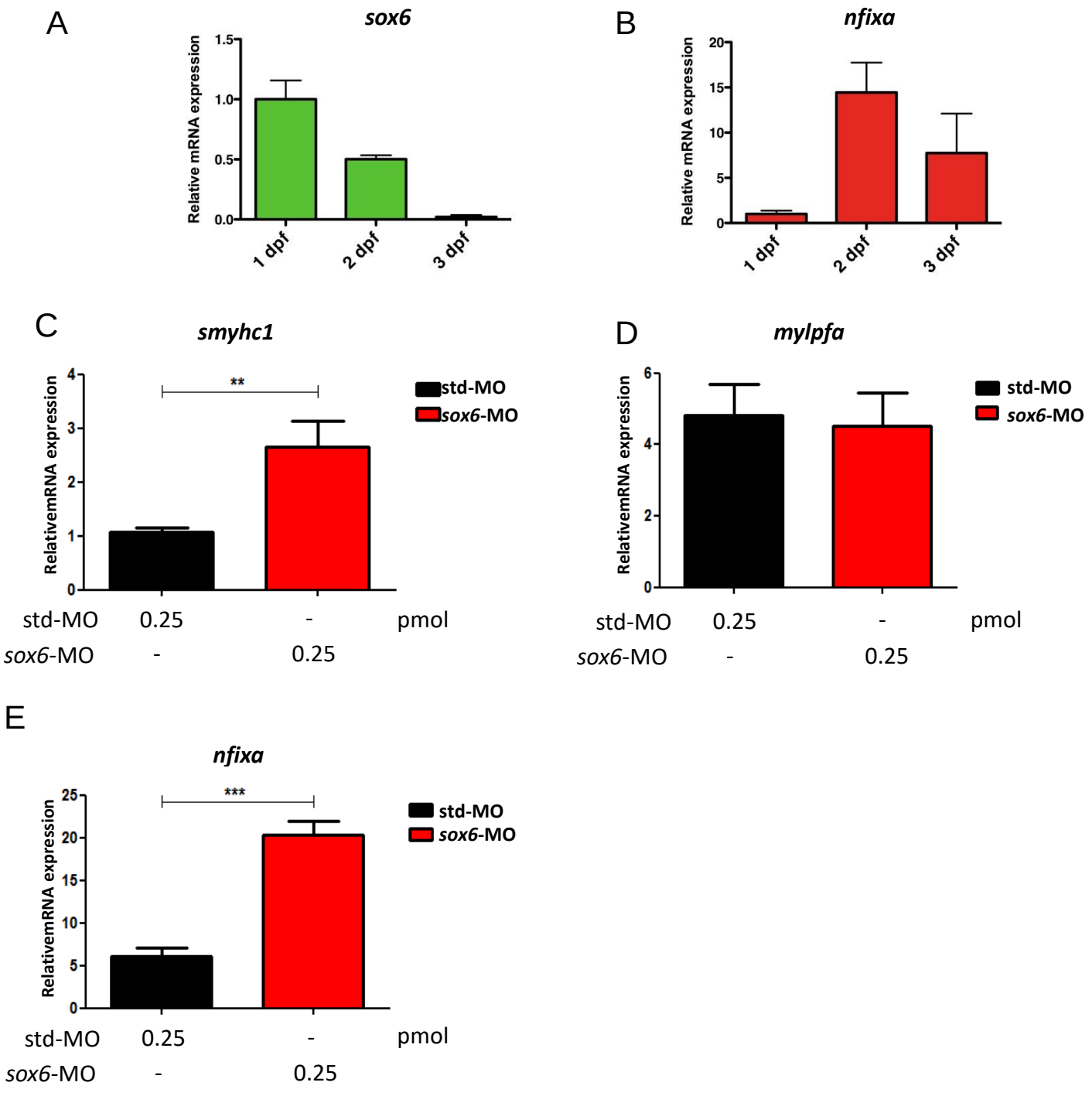
Supplemental Figure S3. Related to Figure 2. Sox6, through Mef2C, positively regulates MyHC-I in embryonic myotubes.

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.



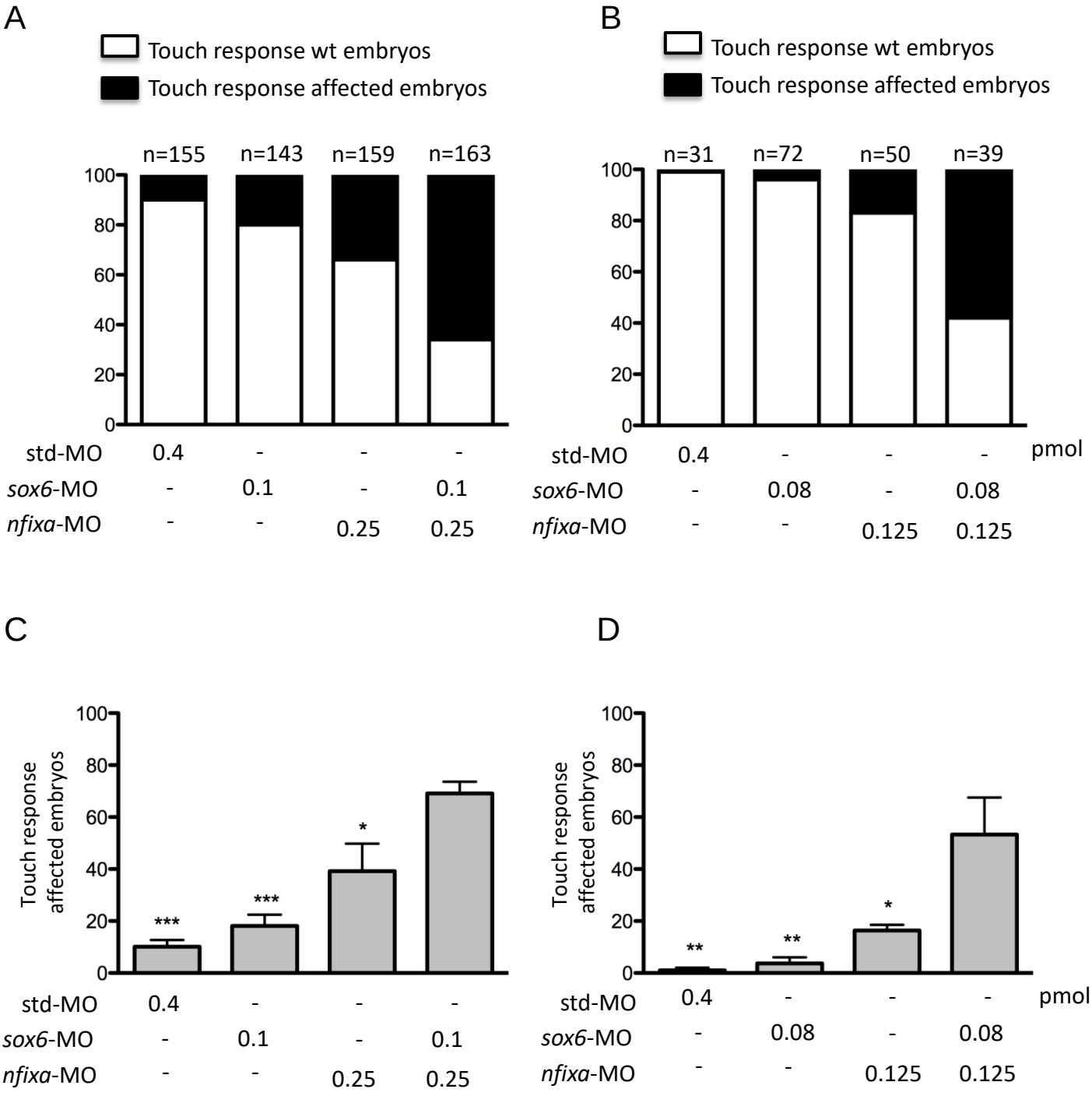
Supplemental Figure S4. Related to Figure 3 and Figure 4. Sox6 and Nfix are independently expressed in fetal muscle.

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.

SUPPLEMENTAL REFERENCES

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