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Supplemental Information

Nfix Regulates Temporal Progression of Muscle Regeneration through Modulation of Myostatin Expression

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Supplemental Information

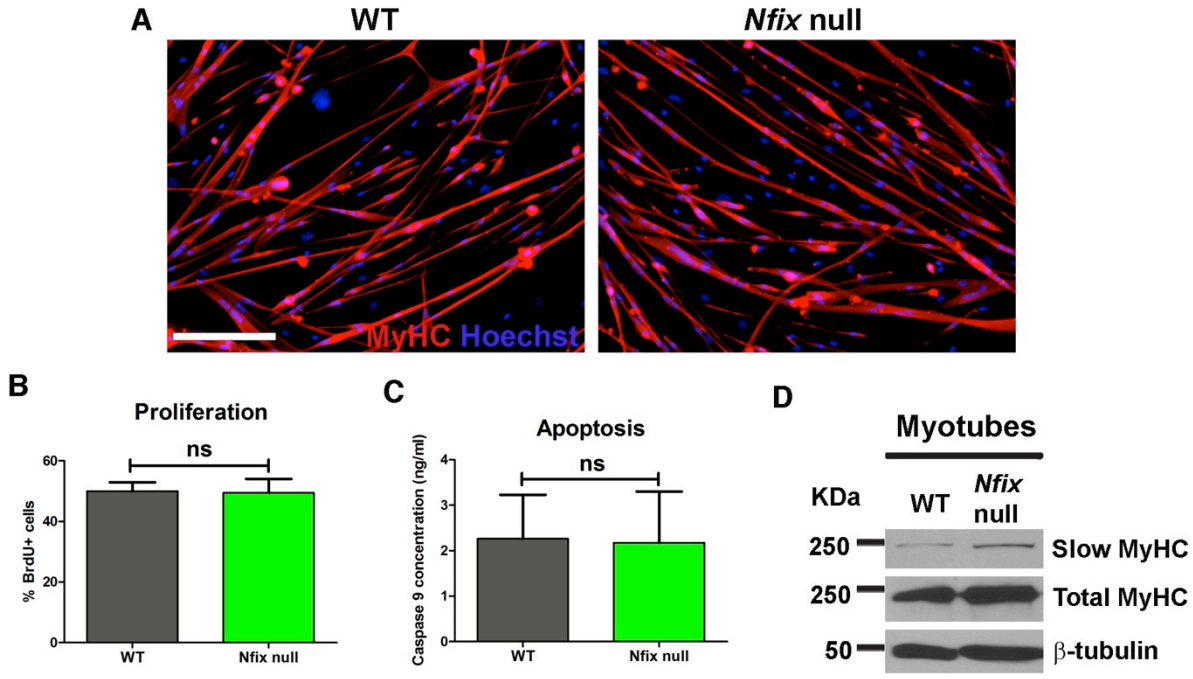

Fig.S1. Isolated satellite cells in culture correctly differentiate in absence of Nfix, while expressing higher levels of slow MyHC, Related to Fig.1.

(A) Immunofluorescence analysis of total MyHC expression (red) on wild-type and *Nfix* null satellite cell-derived myotubes after 48 h in differentiation medium. Hoechst was used to stain nuclei. n=3 independent myoblast preparations. Scale bar represents 50μm.

(B) Quantification of the percentage of wild-type and *Nfix* null BrdU incorporating myoblasts after 1h of exposure to 50μM BrdU. The experiment was performed in duplicate (n=2 independent myoblast preparations) in proliferation conditions. Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test.

(C) ELISA assay measuring caspase 9 concentration as a marker of apoptosis. The test was performed on protein extracts from wild-type and *Nfix* null proliferating myoblasts. Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test. n=2 assays performed on independent myoblast preparations.

(D) Western blot analysis of slow and total MyHC expression on wild-type and *Nfix* null satellite cell-derived myotubes after 48 h in differentiation medium. β-tubulin was used to normalize the amount of loaded proteins.

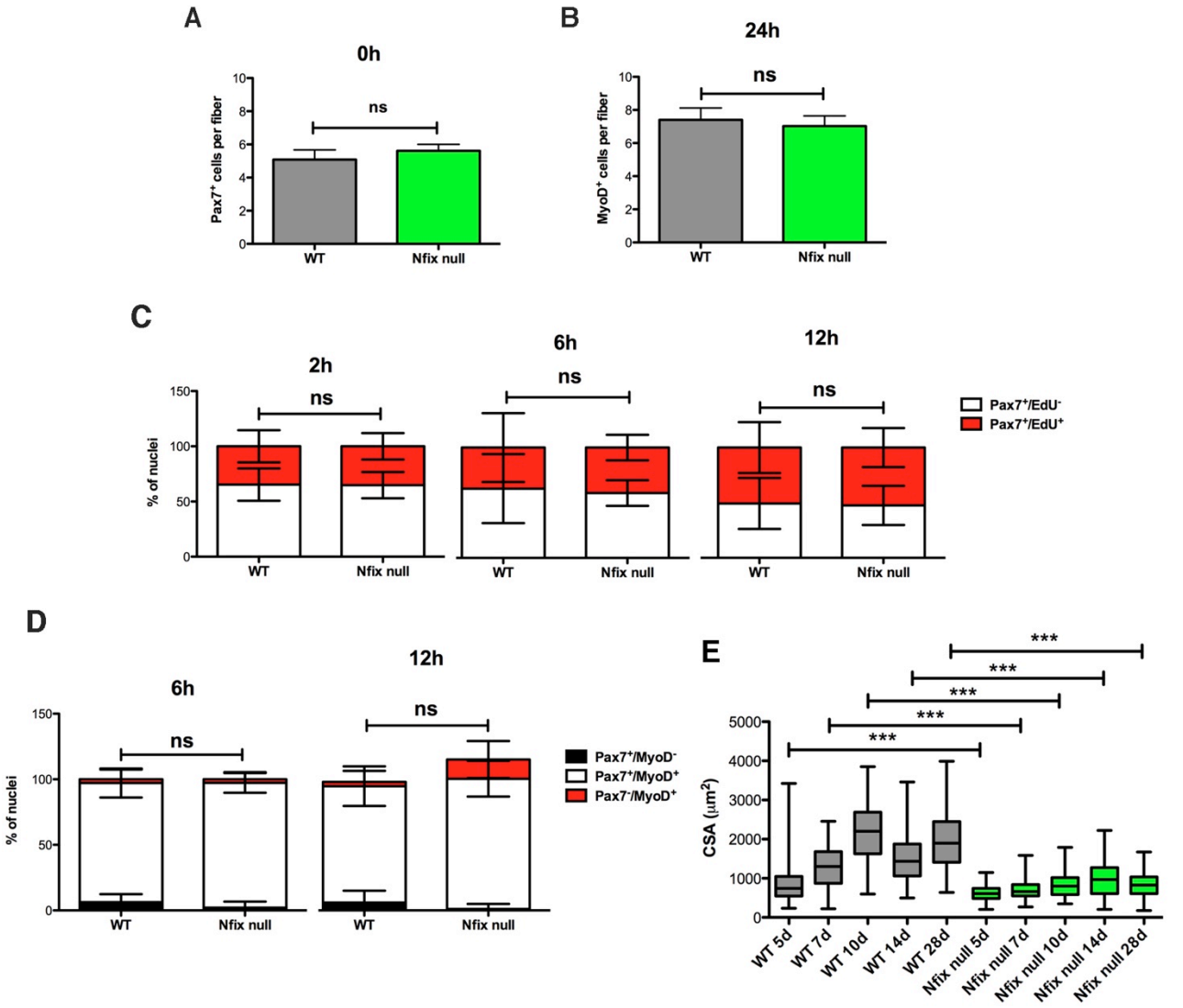

Fig.S2. Number and proliferation of satellite cells associated to myofibers does not change in the absence of Nfix, Related to Figs.2 and 3.

(A) Quantification of Pax7$^+$ satellite cells associated to freshly isolated wild-type and *Nfix* null myofibers. (n=35 WT and 41 *Nfix* null myofibers). Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test.

(B) Quantification of MyoD$^+$ satellite cells associated to wild-type and *Nfix* null myofibers after 24 hours in culture. (n=32 WT and 34 *Nfix* null myofibers). Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test.

(C) Quantification of the percentage of proliferating (EdU$^+$) wild-type and *Nfix* null satellite cells after 2 (n=18 WT and 14 *Nfix* null myofibers), 6 (n=7 WT and 15 *Nfix* null myofibers), and 12 hours (n=8 WT and 16 *Nfix* null myofibers) (h) in culture. Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test.

(D) Quantification of the percentage of satellite cells expressing MyoD and Pax7 after 6 (n=9 WT and 22 *Nfix* null myofibers) and 12 hours (n=10 WT and 24 *Nfix* null myofibers) in culture. Data are presented as mean ± SD. ns: not significant, two-tailed unpaired *t* Test.

(E) Measurement of the myofiber cross sectional area distribution in WT and *Nfix* null regenerating muscles at different time points from injury. n=225 myofibers. Data are presented as mean±whiskers from min to max. ***P < 0.001; two-tailed unpaired t Test.

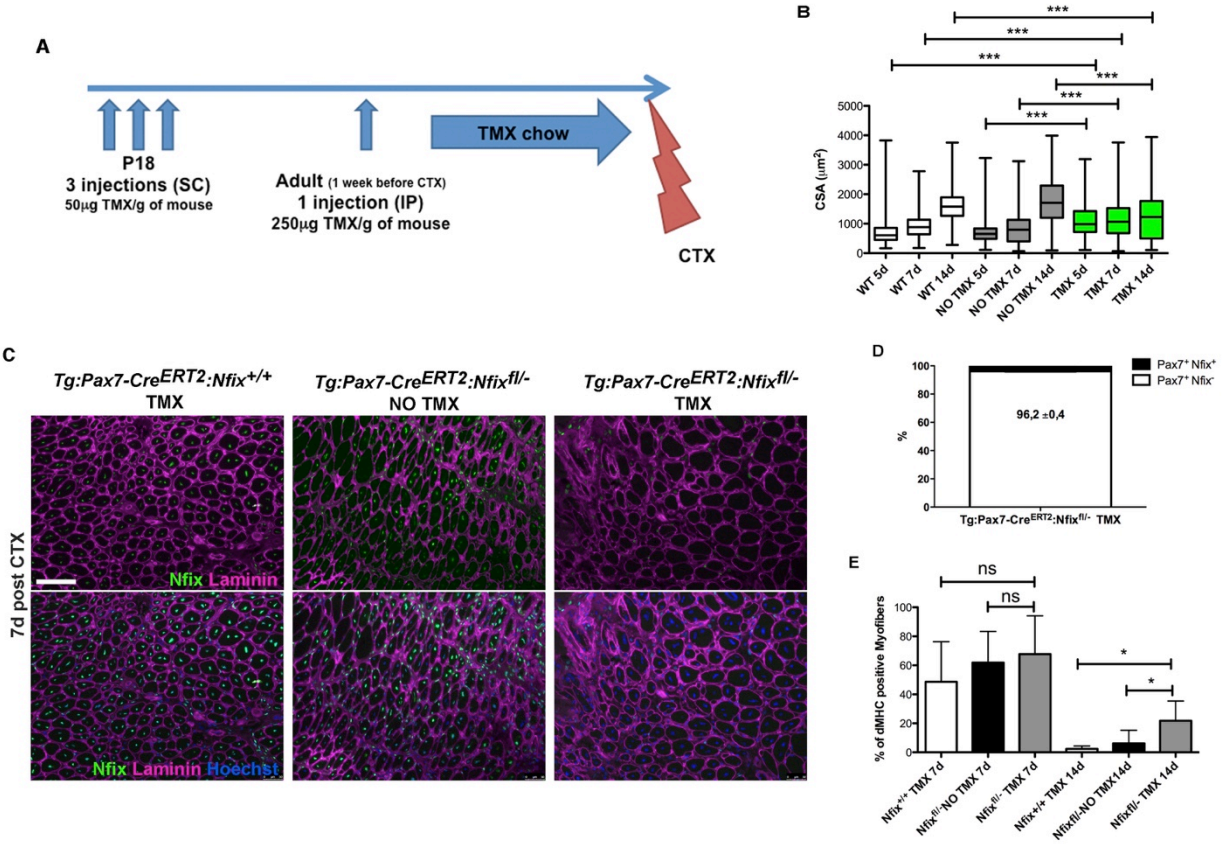

Fig.S3. Tamoxifen treatment of *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* mice determines Nfix excision in satellite cells, Related to Fig.4.

(A) Scheme representing the protocol used for tamoxifen treatment (TMX) and cardiotoxin (CTX) injection. SC, subcutaneous. IP, intraperitoneal.

(B) Measurement of the myofiber cross sectional area distribution in regenerating muscles of *Tg:Pax7-Cre^{ERT2}:Nfix$^{+/+}$* (indicated as WT) and *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* mice with (TMX) or without (NO TMX) tamoxifen at different time points from injury. n=750 myofibers. Data are presented as mean±whiskers from min to max. ***P < 0.001; two-tailed unpaired *t* Test.

(C) Immunofluorescence analysis of Nfix expression (green) in *Tg:Pax7-Cre^{ERT2}:Nfix$^{+/+}$* and *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* mice with (TMX) or without (NO TMX) tamoxifen treatment 7 days (d) after cardiotoxin injection. Laminin, purple; Hoechst, nuclei. n=3 mice for each group. Scale bar represents 100µm.

(D) Graphical representation of the percentage of Nfix excision in tamoxifen treated *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* mice. Quantification was obtained counting the percentage of cells expressing Pax7 and Nfix in muscle sections. n=2 mice. Data are presented as mean ± SD.

(E) Quantification of the percentage of dMHC positive myofibers in *Tg:Pax7-Cre^{ERT2}:Nfix$^{+/+}$* and *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* mice with (TMX) or without (NO TMX) tamoxifen treatment 7 and 14 days following cardiotoxin injection. For time point 7, n=5 *Tg:Pax7-Cre^{ERT2}:Nfix$^{+/+}$*, n=8 *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* NO TMX mice, n=6 *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* TMX mice. For time point 14, n=5 *Tg:Pax7-Cre^{ERT2}:Nfix$^{+/+}$*, n=6 *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* NO TMX mice, n=6 *Tg:Pax7-Cre^{ERT2}:Nfix$^{fl/-}$* TMX mice. Data are presented as mean ± SD. ns: not significant, *P <0,05, two-tailed unpaired *t* Test.

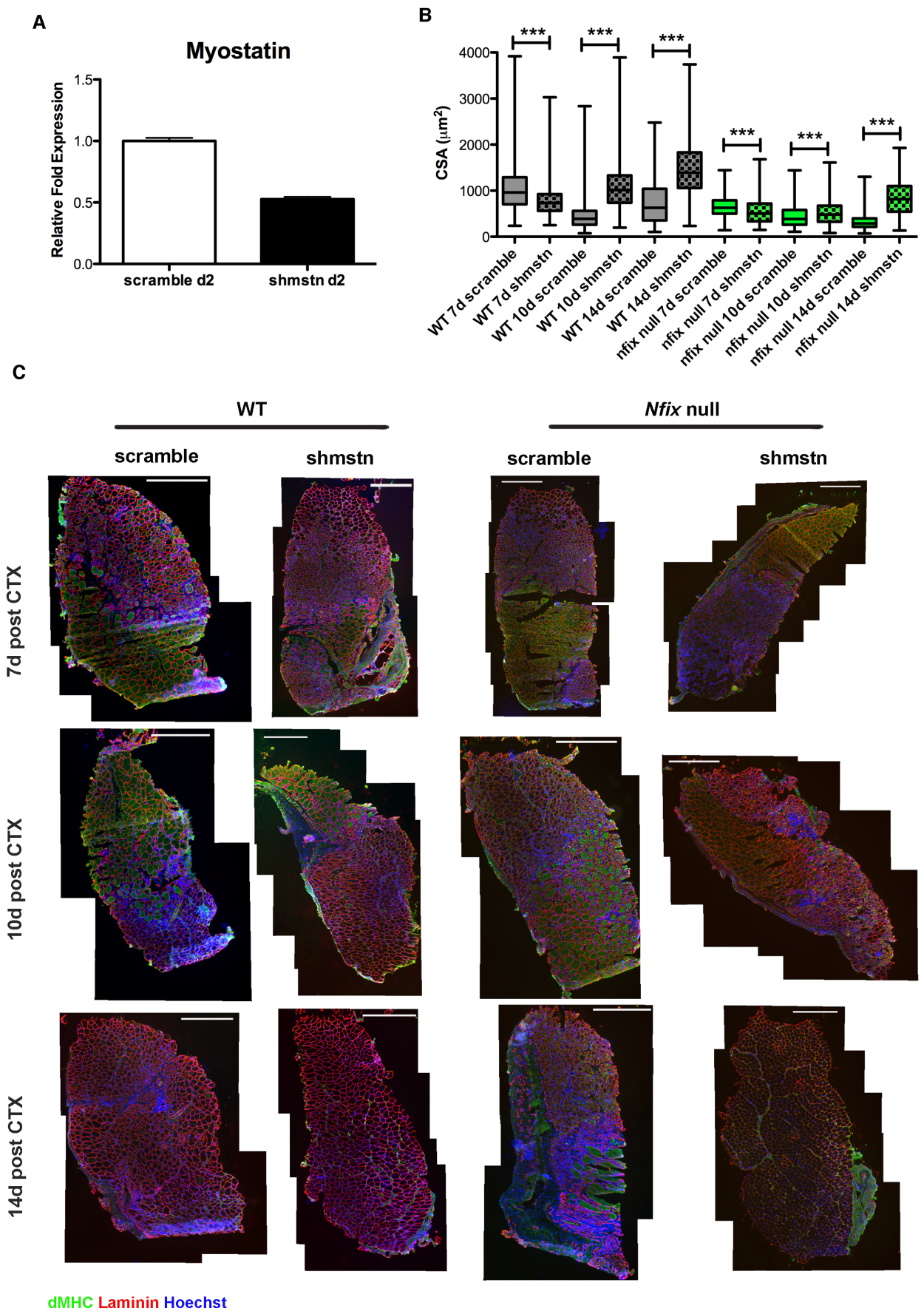
A
Myostatin
Relative Fold Expression
1.5
1.0
0.5
0.0
scramble d2
shmstn d2
B
CSA (μm²)
4000
3000
2000
1000
0
***
WT 7d scramble
WT 7d shmstn
WT 10d scramble
WT 10d shmstn
WT 14d scramble
WT 14d shmstn
nfix null 7d scramble
nfix null 7d shmstn
nfix null 10d scramble
nfix null 10d shmstn
nfix null 14d scramble
nfix null 14d shmstn
C
WT
Nfix null
scramble
shmstn
scramble
shmstn
7d post CTX
10d post CTX
14d post CTX
dMHC Laminin Hoechst

Fig.S4. *In vivo* silencing of Myostatin rescues the regeneration defects of *Nfix* null muscles, Related to Fig.6.

(A) Real-Time qPCR showing Myostatin expression in *Tibialis anterior* muscles at 2 days (d) after electroporation with control (scramble) or shMyostatin (shmstn) plasmids. Data are presented as mean ± SD.

(B) Measurement of the myofiber cross sectional area distribution in WT and *Nfix* null regenerating muscles electroporated with scramble or shmstn plasmids. n=475 myofibers. Data are presented as mean±whiskers from min to max.***P < 0.001; two-tailed unpaired *t* Test.

(C) Immunofluorescence analysis of developmental MyHC expression (dMHC, green) on regenerating wild-type and *Nfix* null *Tibialis Anterior* m uscle sections after muscle electroporation with a control plasmid (scramble) or with a plasmid carrying an shRNA targeting myostatin (shmstn). Muscle sections were collected and stained 7, 10 and 14 days (d) after cardiotoxin (CTX) injection. Muscles were electroporated 4 days (d) after cardiotoxin injection. Laminin, red; Hoechst, nuclei. Images represent photomerge reconstruction of the entire muscle section shown in Fig.6. Scale bar represents 500μm.

Table S1. Related to Experimental Procedures. List of primers used for mouse genotyping

Primer Name	Sequence
Nfix I1F5	ATGGACATGTCATGGGTGCGACAG
Nfix I2R2-CEC	AAGCCCCTCAGCTCTAGCACAGAG
Nfix I1R1	AACCAGAGGCACGAGAGCTTGTC
***Pax7-Cre*^{ERT2} for**	CCACACCTCCCCCTGAACCTGAAACATAAA
***Pax7-Cre*^{ERT2} rev**	GAATTCCCCGGGGAGTCGCATCCGCGG

Table S2. Related to Experimental Procedures. List of primers used for qRT-PCR

Primer Name	Sequence
Myostatin for	AAGATGACGATTATGACGCTACC
Myostatin rev	CCGCTTGCATTAGAAAGTCAGA
GAPDH for	AGGTCGGTGTGAACGGATTTG
GAPDH rev	TGTAGACCATGTAGTTGAGGTCA

Table S3. Related to Experimental Procedures. List of primers used for ChiP

Primer Name	Sequence
Myostatin promoter (415-539)for	TTGTGGAGCAGGAGCCAATC
Myostatin promoter (415-539)rev	GTACCGTCCGAGAGACAACC
Myostatin promoter (116-369)for	GTAACAAAACAGCACTCCAAGTC
Myostatin promoter (116-369)rev	CCCTGTCTGTCACAAGTCACC
Nfatc4 promoter for	GGCGCTTAACCCTTTAGGTG
Nfatc4 promoter rev	CAAGACAGGGGAGCAGTCAC
Intergenic region for	GACCTGCCTGTTCCTTCTTG
Intergenic region rev	GTTACCCAGCACTGCAAAGG