Supplemental Information:

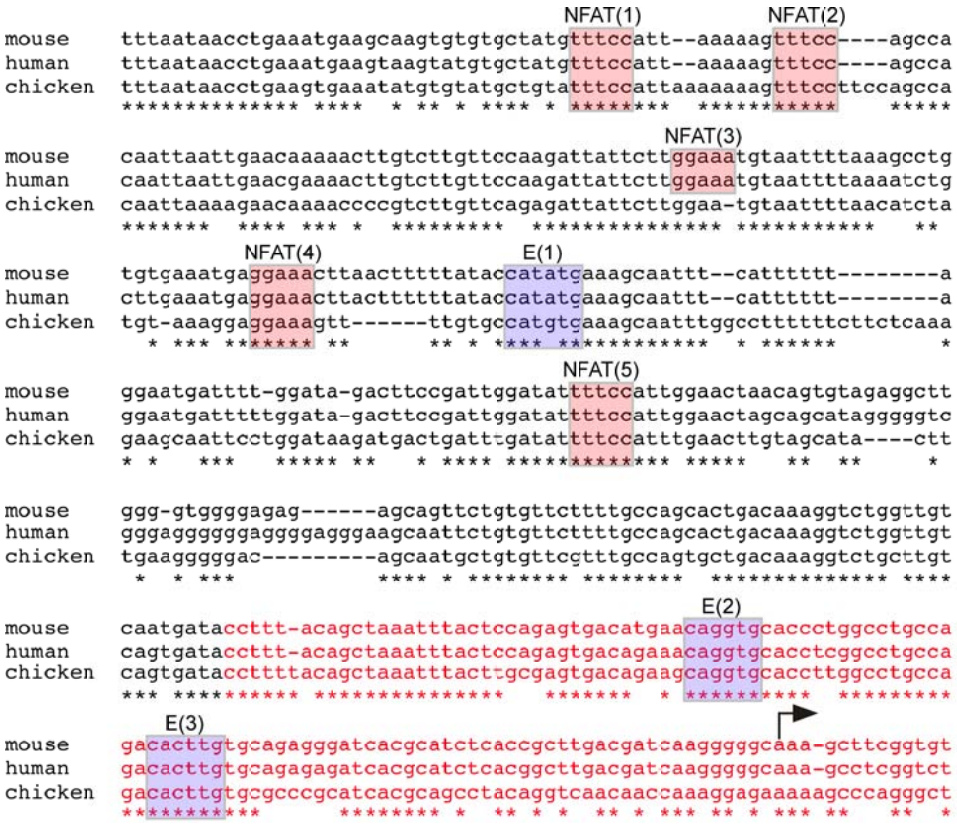


Figure S1, related to Figure 1. The *Mef2c* skeletal muscle promoter contains multiple NFAT sites

The evolutionarily conserved skeletal muscle promoter resides upstream of exon 1 in mouse *Mef2c* (Dodou et al. 2003) (Genbank accession #AY321453). Sequence alignment of the mouse, human, and chicken *Mef2c* loci shows an evolutionarily conserved 429bp region upstream of the first transcribed exon. Nucleotides indicated with an asterisk are conserved. Red boxes indicate the evolutionarily conserved, candidate cis-acting NFAT consensus sites and blue boxes indicate conserved E box elements. The nucleotides shown in red text correspond to the sequence of the truncated *Mef2c* reporter lacking NFAT sites. The arrow indicates the transcriptional start site obtained from Genbank (accession #AK009139).

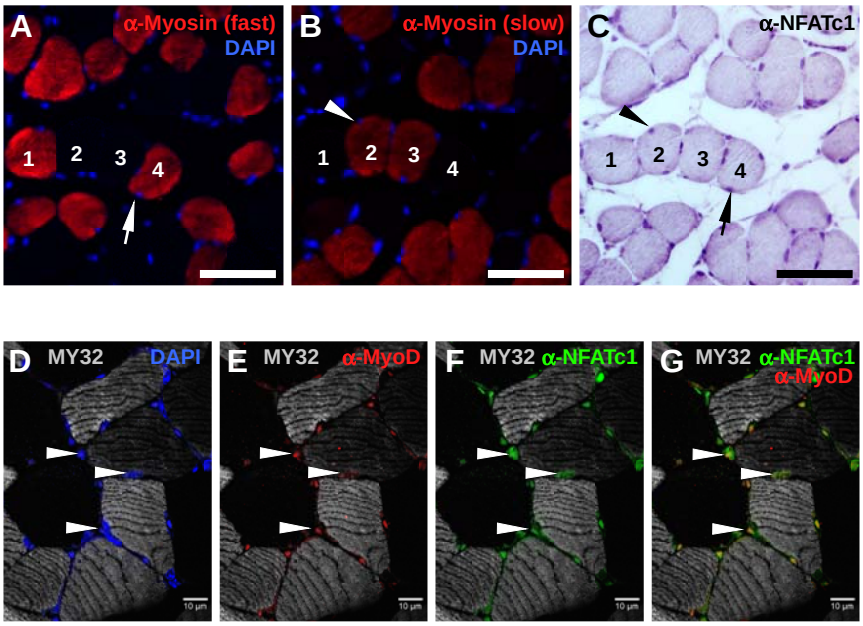


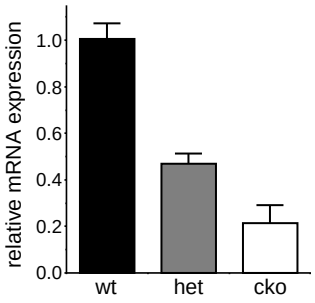
Figure S2, related to Figure 2. NFATc1 is expressed in slow and fast myofibers and colocalizes with MyoD

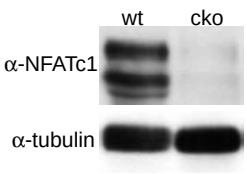
(A-C) Immunohistochemistry for NFATc1 (C) shows NFATc1 positive nuclei are present in both fast (A, fibers labeled 1 and 4) and slow (B, fibers labeled 2 and 3) muscle fibers as shown by immunofluorescence labeling of fast and slow myosin. Experiments were done using serial sections of soleus muscle from adult male mice. Scale bar, 100μm.

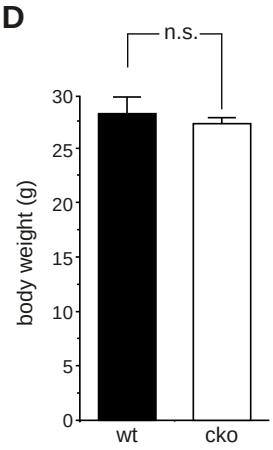
(D-G) Co-immunofluorescence for fast myosin heavy chain (MY32, gray fluorescence in all panels) and DAPI to stain nuclei (D), MyoD (E), NFATc1 (F) and NFATc1 + MyoD (G) on a representative section from adult soleus muscle from male mice. Nuclei showing colocalization of MyoD and NFATc1 are shown with arrowheads. Fibers that do not show MY32 staining are slow fibers. Scale bar, 10μm.

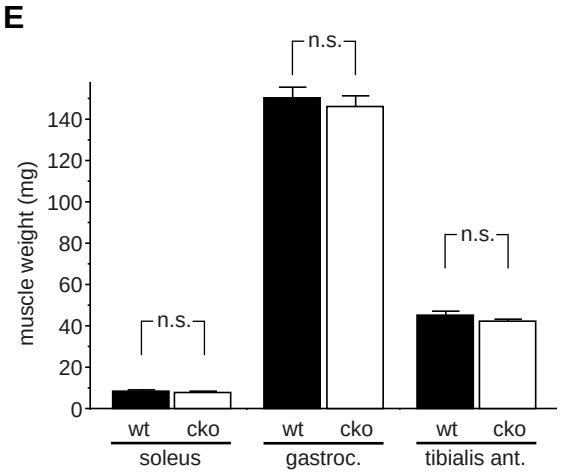
A

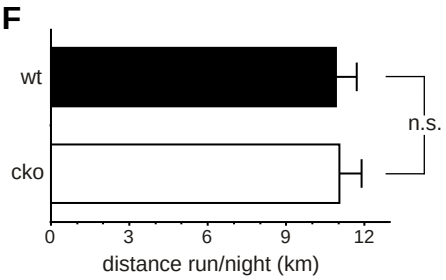
Genotype	Expected	Observed
nfatc1$^{flox/+}$ (wt)	43.5	48
nfatc1$^{flox/+}$;*mef2c-73k-Cre*$^{Tg/0}$	43.5	49
nfatc1$^{flox/\Delta}$ (het)	43.5	33
nfatc1$^{flox/\Delta}$;*mef2c-73k-Cre*$^{Tg/0}$ (cko)	43.5	44











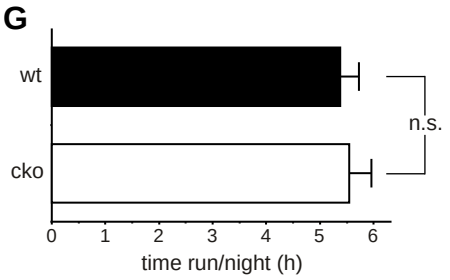


Figure S3, related to Figure 2. *nfatc1*^{SkMKO} mice are born at normal Mendelian frequency and do not display deficits in growth or exercise capabilities

(A) Expected and observed offspring of each genotype from crosses to generate *nfatc1*^{SkMKO} mice. All genotypes were born at expected Mendelian ratios and survived to adulthood (χ^2 = 3.701, 3 df; p = 0.2956, n.s.).

(B) qPCR on RNA isolated from the soleus muscles of 6 week old adult male mice. *nfatc1* expression was reduced by greater than 80% in *nfatc1*^{SkMKO} (cko) muscle compared to wild type (wt) control mice (*nfatc1*$^{flox/+}$). Results are expressed as the relative mean mRNA expression plus SEM from 3 mice of each genotype.

(C) Western blot for NFATc1 protein showed a marked reduction in NFATc1 protein expression in the gastrocnemius muscles from adult *nfatc1*^{SkMKO} (cko) compared to wild type control mice. α-tubulin was examined as a loading control. Highly similar results were obtained in 3 independent experiments.

(D) No significant difference in mean body weight of 49-day old male mice between *nfatc1*^{SkMKO} mice (cko, white bars) and *nfatc1*$^{flox/+}$ control mice (wt, black bars).

(E) No significant differences (n.s.) were observed in the mean weight of the soleus, gastrocnemius, or tibialis anterior muscles of 49-day old control (wt, black bars) and *nfatc1*^{SkMKO} mice (cko, white bars) male mice.

(F, G) Adult male mice were subjected to voluntary wheel running. Measurement of distance run/night (F) and time run/night (G) were taken each day for 7 days and then averaged. No significant differences (n.s.) between wild type mice (black bars, n =10) and *nfatc1*^{SkMKO} mice (n = 12) were observed for distance run/night (F) or time run/night (G). Error bars represent SEM.

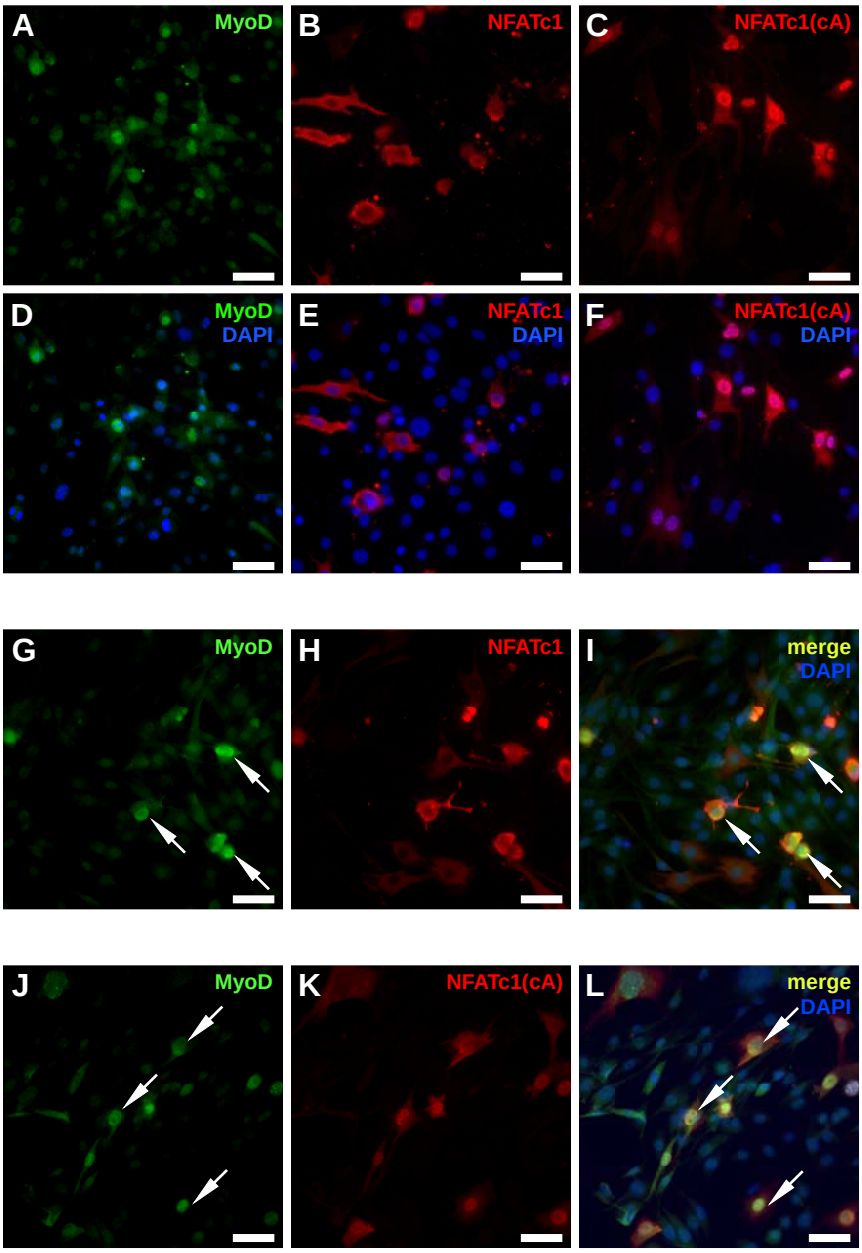

A
MyoD
B
NFATc1
C
NFATc1(cA)
D
MyoD
DAPI
E
NFATc1
DAPI
F
NFATc1(cA)
DAPI
G
MyoD
H
NFATc1
I
merge
DAPI
J
MyoD
K
NFATc1(cA)
L
merge
DAPI

Figure S4, related to Figure 4. NFATc1 does not affect MyoD nuclear localization

(A-C) Immunofluorescence labeling of MyoD (panel A, green fluorescence), NFATc1 (panel B, red fluorescence), and constitutively active (cA) NFATc1 (panel C, red fluorescence) transfected C3H10T1/2 cells shows nuclear localization of MyoD (A), cytoplasmic and nuclear localization of full length NFATc1 (B), and nuclear localization of NFATc1(cA) after 24 h in low serum differentiation media.

(D-F).Merged images of MyoD, NFATc1, and NFATc1(cA) immunofluorescence and DAPI (blue) to highlight nuclei in each panel.

(G-I) Immunofluorescence of C3H10T1/2 cells transfected with MyoD (panel G, green fluorescence) and NFATc1 (panel H, red fluorescence) and the merged image (panel I) after 24 hours in low serum differentiation media shows MyoD expression remains restricted to nuclei in the presence of NFATc1 (white arrows). The merged image of NFATc1 and MyoD (panel I) shows colocalization in numerous nuclei (white arrows).

(J-L) Immunofluorescence of C3H10T1/2 cells transfected with MyoD (panel J, green fluorescence) and NFATc1(cA) (panel K, red fluorescence) and the merged image (panel L) after 24 hours in low serum differentiation media shows MyoD expression remains restricted to nuclei in the presence of NFATc1 (white arrows). The merged image shows colocalization in numerous nuclei (white arrows). Scale bars, 100μm in all panels.

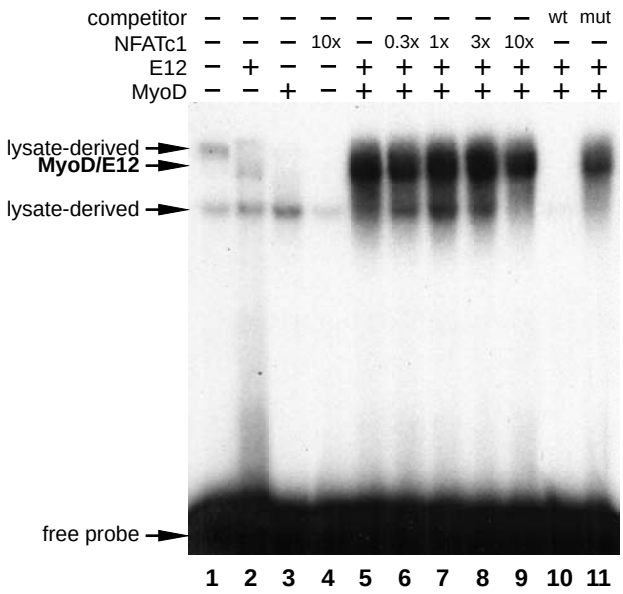


Figure S5, related to Figure 4. NFATc1 does not inhibit MyoD dimerization with E proteins

Recombinant MyoD, E12, and NFATc1 proteins were transcribed and translated *in vitro* and used in EMSA with a radiolabeled, double-stranded oligonucleotide probe corresponding to the E2 E-box from the *Mef2c* skeletal muscle promoter. Neither E12 nor MyoD bound individually to the E-box (lanes 2 and 3), but when both were present, a robust shift was observed (lane 5). NFATc1 did not bind directly to the E box (lane 4). The addition of NFATc1 in increasing amounts had a negligible effect on MyoD/E12 heterodimer formation and subsequent binding to the E-box binding site (lanes 6-9). MyoD/E12 binding was specific, as it was efficiently competed by wild type unlabeled competitor oligonucleotide (lane 10), but not by a mutant version of the *Mef2c* E box (lane 11). Unprogrammed reticulocyte lysate control is shown (lane 1). Lysate-derived bands, free (unbound) probe, and the MyoD/E12 bound probe art indicated.