Supporting Information

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SI Materials and Methods

The Calcineurin (Cn)-Nuclear Factor of Activated T Cell (NFAT) Pathway in Skeletal Muscle: Role of Different NFAT Isoforms. Several lines of evidence indicate Cn as an important regulator of muscle differentiation, from early stages of myogenesis to fiber type diversification in adult muscle. However, the function of NFAT in these processes has not been conclusively established, because (*i*) not all NFAT isoforms were analyzed, and (*ii*) different lines of research reached substantially diverging conclusions. A detailed overview of these studies can be found in the paragraphs below. We have subdivided previous reports on Cn-NFAT according to the experimental system used. Indeed, it should be kept in mind that the induction of myogenic programs in myoblasts in culture is largely different at the molecular level from the induction and maintenance of muscle specific transcriptional programs in muscle fibers in vivo. Active Cn was shown to induce both slow and fast muscle-specific promoters in C2C12 myoblasts, but not in whole muscles in vivo (1). Also, when Cn was inhibited from early stages of mouse myogenesis by transgenic overexpression of the Cn inhibitor modulatory Cn-interacting protein (MCIP)-1, muscle development and muscle fiber type patterning occurred normally during embryogenesis, but maintenance of slow fibers was compromised in postnatal life (2).

Contribution of the Present Work. We have analyzed the role of the 4 NFAT isoforms in parallel in skeletal muscle in vivo, an original experimental setup, considering also that expression of NFATc3 and c4 in skeletal muscle was a matter of controversy (1, 3, 4). Also, unlike many previous works that are based on overexpression of chimeric NFAT-GFP or mutant constructs, our experiments are based on the analysis of endogenous NFATs. We have chosen RNAi of the 4 individual NFAT isoforms in vivo as a loss of function approach to explore their specific role in activity-dependent muscle gene regulation in adult muscles. We demonstrate that NFAT transcription factors are involved not only in slow, but also in fast fiber type specification in adult muscle, and that different NFAT isoforms have different roles in this respect. Several lines of evidence support this view. First, RNAi experiments show that NFATc2, c3, and c4, but not c1, control the activation of myosin heavy chain (MyHC)-2A and -2X promoters, whereas NFATc4, but not the other isoforms, control the MyHC-2B promoter (Fig. 4). Second, electrical stimulation experiments show that, although NFATc1 undergoes nuclear translocation on slow-, but not fast-like stimulation, NFATc3 undergoes nuclear translocation also after fast-like stimulation (Fig. 5; Fig. S5). Third, the finding that NFATc4 is constitutively nuclear even in unstimulated muscles, whereas NFATc1 is predominantly nuclear in slow (soleus) and cytoplasmic in fast (EDL) muscle, and NFATc2 and c3 show an intermediate pattern of distribution, supports our hypothesis of a differential role of NFAT isoforms in relaying nerve activity to gene regulation.

Previous Studies at the Basis of Our Work. ***Fiber type diversification in vivo.*** The role of Cn in intact skeletal muscle in vivo was explored by using either pharmacological inhibition with CsA and FK506 or overexpression of activated Cn. Rats treated with CsA displayed a slow-to-fast fiber transition, with a net increase in fast MyHC isoforms in soleus muscle, suggesting the importance of Cn signaling in the maintenance of fiber type diversification (5). Both contractile and metabolic genes were shown to undergo a slow-to-fast fiber type shift under these conditions (6), in contrast with the results of Oh et al. (2), showing that Cn inhibition affects MyHC-slow expression, but not the oxidative capacity and mitochondrial content of muscle fibers. Other studies with CsA also reported that the Cn pathway controls fiber type specification in conditions of increased activity, such as muscle overload, but not under normal weightbearing conditions and that NFATc1 is activated downstream of Cn in this process (7, 8). However, transgenic mice overexpressing activated Cn under the control of the muscle creatine kinase (MCK) promoter displayed an increase in slow fibers; because the fast-to-slow transformation was incomplete, the authors suggest that Cn-independent pathways must be implicated in fiber type specification (8). A major impulse to the study of NFAT in fiber type diversification in vivo was given by the in vivo manipulation of adult skeletal muscle through transient transfection of inhibitors, active mutants, or sensors of the Cn-NFAT pathway. Transient overexpression of the general NFAT inhibitor peptide VIVIT blocked the expression of MyHC-slow; likewise, overexpression of constitutively active NFATc1 stimulated the expression of MyHC-slow while repressing the fast MyHC-2X and -2B in rat muscle in vivo (9). NFATc1 nucleocytoplasmic shuttling in mouse muscle depends on slow, but not fast nerve activity, indicating that NFATc1 is a specific slow activity sensor in vivo (10).

Nucleocytoplasmic distribution of NFAT in cultured adult muscle fibers. Fibers dissociated from adult muscles and maintained in culture have been extensively used to analyze activity-dependent nuclear translocation of NFAT. Electrical stimulation with a pattern of impulses mimicking slow-twitch fiber activity has been shown to induce nuclear translocation of NFATc1-GFP and of NFATc3-GFP, whereas fast-like impulses had no effect on NFATc1-GFP; NFATc3-GFP was not analyzed under the latter conditions (11, 12). The nucleocytoplasmic translocation and the functional role of the other NFAT isoforms in fiber type specification was never addressed in this experimental system.

Myoblasts differentiation in culture. There is a general consensus on the important role had by Cn in the early stages of muscle cell differentiation. It has been shown that myoblast differentiation and fusion in culture is controlled by Cn, because treatment with a Cn inhibitor, the immunosuppressant drug CsA, inhibits the expression of differentiation markers such as creatine kinase and embryonic MyHC, as well as the formation of multinucleated myotubes.

Downstream of Cn, 3 NFAT isoforms were shown to be expressed in primary cultures of mouse and human muscle cells, and to translocate to the nucleus in human muscle cells stimulated with thapsigargin at different stages of myogenesis (3). In particular, NFATc1 and c2 were found to be nuclear and active in response to thapsigargin in multinucleated myotubes, but not in myoblasts, whereas NFATc3 was nuclear in myoblasts, but not in myotubes.

However, the role of NFAT as a downstream effector in myogenesis, at least in the cell culture system, is controversial. Friday and coworkers (13) showed that both CsA and infection of myoblasts with adenovirus expressing the Cn inhibitor protein Cain, suppressed the induction of myogenin and differentiation. Interestingly, because myogenin induction occurred also in the presence of the NFAT inhibitor peptide VIVIT, the authors conclude that NFAT is not an essential target of Cn during early stages of myogenesis. The evidence that Cn promotes myoblasts differentiation is also present in the work of Delling et al. (14),

showing that Cn is activated at early stages of differentiation, and that overexpression of activated Cn promotes myoblast fusion dependent on NFATc3, but not on other isoforms.

Expression of fiber-specific genes in culture. Cultured myoblasts largely express the embryonic-neonatal isoforms of contractile proteins, and differentiation induces the expression of some adult slow and/or fast-specific proteins. In C2C12 myoblasts, forced overexpression of activated Cn has been shown to upregulate slow fiber-specific genes, such as TnI-slow and myoglobin, which required intact NFAT binding sites (5). Delling et al. (14) also report a role of Cn in fiber type specification in cultured muscle cells. Cn was shown to promote MyHC-slow expression, whereas the upstream kinase of p38, MKK6, promoted fast MyHC expression. This effect was enhanced by cotransfection with NFATc3, but not with NFATc1 and c2, neither WT nor constitutively active.

It should be emphasized that regulated expression of differentiation markers, such as those found in mature fibers in vivo, is never fully achieved in in vitro conditions. Therefore, the conclusions on Cn-NFAT arising from studies in cultured muscle cells cannot be directly extended to adult muscle.

Additional Materials and Methods. ***Plasmids used.*** NFATc1-GFP (gift of R. S. Williams), NFATc2-HA (gift of A. Rao), NFATc3-GFP (gift of S. Miyatake), NFATc4 (gift of C. W. Chow), MyHC-slow 1.1 Kb (gift of R. N. Kitsis), MyHC-2A 0.8 Kb and MyHC-2 × 1 Kb (gift of L. Leinwand), MyHC-2B 2.8 Kb (gift of S. J. Swoap), NFAT sensor-LUC (gift of J. D. Molkentin), SNAP (synaptosome-associated protein of 25 KD)-GFP (gift of T. Pozzan), MyHC-slow −348 WT and ΔNFAT (gift of I. K. Farrance), pSuper (Oligoengine), and pRenilla-TK (Promega).

In vivo transfection and electrostimulation. For the transfection of adult muscles, Wistar rats were anesthetized by i.p. injection of a mixture of Zoletil 100 (Zolazapam and Tiletamine, 1:1, 10 mg/kg) and Xilor (Xilazine 2%, 0.06 mL/kg), or by using an isoflurane vaporizer maintained at 2% isoflurane, 1 L/m oxygen. EDL and soleus muscles were exposed and injected with plasmid DNA (20 μg in saline). Injection was followed by electroporation with stainless steel electrodes connected to an ECM830 BTX porator (Genetronics) with the following settings: 5 pulses of 20 ms each and 200-ms interval, voltage adjusted to the thickness of the leg (220 V/cm). For the transfection of regenerating muscles, plasmid DNA (20 μg in 20% sucrose) was directly injected into the muscles at day 3 after bupivacaine treatment. Muscles were removed 7 days after transfection, frozen in isopentane cooled in liquid nitrogen, and stored at −80 °C.

For electrostimulation experiments of EDL, 7 days after transfection the common peroneal nerve was exposed proximally to the knee on the lateral side of the thigh. Teflon-covered stainless steel electrodes (Cooner wire) were implanted near the branch of the peroneal nerve, and the stimulation was carried out for 2 h by using an AMP Master-8 stimulator (AMP Instruments).

RNAi-mediated gene silencing. The sequences of each NFAT gene were retrieved and analyzed. The target sequences were selected from regions common to all splice variants of each gene. Target oligos were designed by using the criteria defined by Reynolds et al. (15). We selected at least 4 oligos per gene on the basis of a specificity screening performed with BLAST analysis. The selected oligos were cloned into the pSUPER vector. We cotransfected an epitope-tagged cDNA together with each pSUPER. As a control, we used pSUPER constructs targeting either LacZ or GFP. To exclude that the observed down-regulation of NFATs could be due to off-target effects, we selected at least 2 sequences with high silencing efficiency for each gene, and performed every experiment with both of them. We used at least 2 sequences to target each NFAT isoforms, indicated below with the corresponding position on the targeted NFAT sequence.

NFATc1: 1423 TGAACCTCTCACGCTACAG 1441 and 928 CACCACCCAGTATACCAGC 946 [National Center for Biotechnology Information (NCBI) Reference Sequence XM_001058445].

NFATc2: 307 CCCTATCGAAGAAGAACCG 325 and 320 GAACCGATCGCACATAAGG 338 (NCBI Reference Sequence XM_001107805).

NFATc3: 1886 ACTCCGCAATTCAGATATA 1904 and 2064 ATTGAGAAGTACAGTATCA 2082 (NCBI Reference Sequence NM_001108447).

NFATc4: 1832 ATTCAGACATTGAGCTACG 1850 and 2307 GGAGGAGCCCCTACCAGAC 2325 (NCBI Reference Sequence NM_001107264).

Immunohistochemistry. Muscle cryosections, 10 μm thick, were analyzed for GFP fluorescence and/or processed for immunostaining. For GFP and MyHC staining, sections were fixed with 2% PFA at room temperature for 5 min. Cy2- and Cy3-labeled (115-225-146 and 115-165-146, respectively) second antibodies were purchased from Jackson Laboratories. For NFATc1 and c4 staining, cryosections were fixed with methanol (10 min at −20 °C in precooled methanol) or with 4% PFA (10 min at room temperature) and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. For NFATc2 and c3 staining, cryosections were denatured in citrate buffer at pH 6 for 10 min in a microwave oven (750 W) before staining. Endogenous peroxidase activity was blocked incubating sections with 0.3% H_2O_2 in methanol at room temperature for 30 min. Sections were then incubated with an Avidin/Biotin blocking kit (SP-2100; Vector Laboratories), to block nonspecific binding of Biotin/Avidin system reagents, and incubated with the various antibodies as indicated. Bound primary antibodies were detected by using biotinylated secondary antibodies (BA-1000 and BA-2100; Vector Laboratories) that were visualized by using a streptavidin-horseradish peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride (D5905; Sigma-Aldrich) or Vector VIP substrate kit (SK-4600; Vector Laboratories) as supplied with the Vectastain Elite ABC-peroxidase kit (PK-6100; Vector Laboratories). Controls were performed without primary antibodies. Where indicated, controls were also performed by preincubating the primary antibody with the specific immunizing peptide at room temperature for 2 h, or overnight at 4 °C, under gentle shaking. A mock incubation was performed in parallel. The antibody was then used to process cryosections under standard conditions. For NFAT antibodies used, see Table S1.

Primers for quantitative real-time PCR. The primers (except CyclophilinA) (16) were designed and analyzed with Primer3 (freeware) and Vector NTI (freeware; Invitrogen). Identity of the amplicons was confirmed by their dissociation profiles and gel analysis.

Quantitative PCR standard curves were constructed by using serial dilutions of pooled cDNAs of the analyzed samples, using at least 4 dilution points and the efficiency of all primer sets was between 94 and 105%. The data were normalized with Cyclophilin A.

Primer sequences, designed to amplify all known splice variants, were as follows:

NFAT-C1, 5′ ACC AGC TCT GCT ATT GTG 3′ and 5′ GAG ACT TGA TAG GGA CAC C 3′;

NFAT-C2, 5′ TCT GCT GTT CTC ATG GAT GC 3′ and 5′ ACGGGA GTT GGA TCA GGA C 3′;

NFAT-C3, 5′ CTG TGC AAA CCC CAC CTC 3′ and 5′ GCC CAG AAA TCG GTG AAC 3′;

NFAT-C4, 5′ TAC AGC AAC AAG CGG GTG TC 3′ and 5′ CGG AGA GAT GAG TCT GGT AGG G 3′.

Lysate preparation and Western blotting (WB). HEK293 cells were lysed in RIPA buffer containing "complete" protease inhibitor mixture (1 tablet per 50 mL; Roche), 1 mM sodium orthovanadate, 50 mM sodium fluoride and sonicated for 5 s. For total

muscle lysates, muscles were pulverized in liquid nitrogen and homogenized in ice-cold lysis buffer containing 50 mM Tris·HCl, pH 7.5/1 mM EGTA/1 mM EDTA/1% Triton X-100/1 mM sodium orthovanadate/50 mM sodium fluoride/5 mM sodium pyrophosphate/0.27 mol/L sucrose. Homogenates were centrifuged at 13,000 × *g* for 30 min at 4 °C, supernatants were removed, and aliquots were snap frozen in liquid nitrogen.

Muscle or cell lysates (25–80 μg) were heated in SDS sample buffer and subjected to SDS/PAGE and electrotransfer to nitrocellulose membranes. Membranes were then blocked in 50 mmol/L Tris·HCl, pH 7.5/0.15 mol/L NaCl/0.05% Tween (TBST) containing 5% skimmed milk and probed for 16 h at 4 °C in TBST, 5% skimmed milk, and the antibody concentrations indicated in Table S1. Detection of proteins was performed by using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and the enhanced chemiluminescence reagent.

1. Swoap SJ, et al. (2000) The Cn-NFAT pathway and muscle fiber-type gene expression. *Am J Physiol Cell Physiol* 279:C915–C924.
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15. Delling U, et al. (2000) A Cn-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. *Mol Cell Biol* 20:6600–6611.
16. Reynolds, et al. (2004) Rational siRNA design for RNA interference. *Nat Biotech* 22:326–330.
17. Sanchez, et al. (2006) Quantification by real-time PCR of developmental and adult myosin mRNA in rat muscles. *Biochem Biophys Res Commun* 340:165–174.

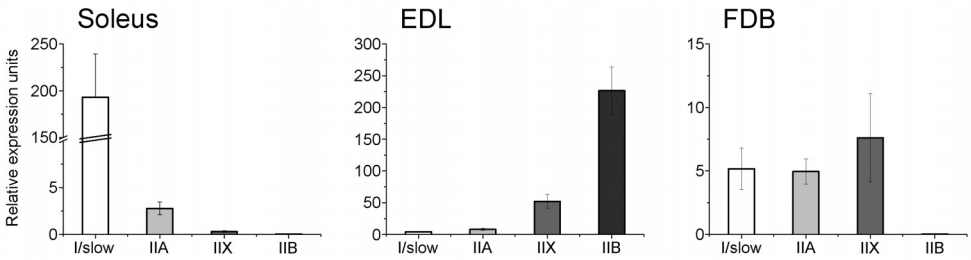

Fig. S1. Quantification of MyHC isoform expression in soleus, EDL, and FDB muscles. RNA from total muscles was processed as described and analyzed by qPCR with primers specific for the 4 adult isoforms of MyHC. Note the interruption in the I/slow bar in soleus. The lower values in FDB reflect underrepresentation of muscle transcripts due to the presence of abundant connective tissue in this muscle.

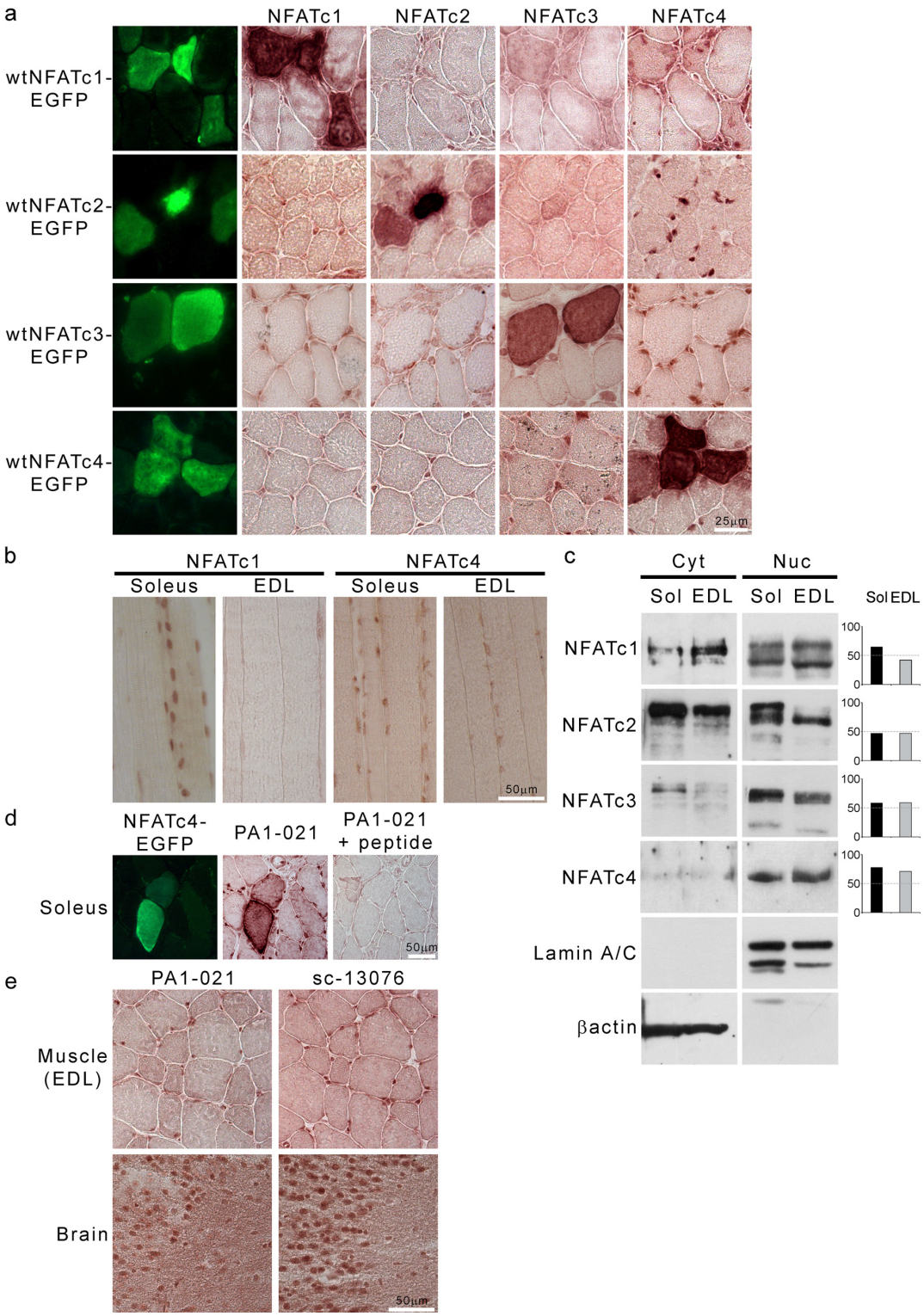

Fig. S2. Specificity of anti-NFAT antibodies. (a) Serial sections of EDL were stained with all 4 isoform-specific anti-NFAT antibodies. The left column shows for comparison the GFP signal of the transfected NFAT-GFP chimera. (*b*) Longitudinal sections of rat soleus and EDL muscles stained with Abs specific for NFATc1 (*Left*) and NFATc4 (*Right*), respectively. (*c*) Representative WB of nuclear and cytosolic extracts analyzed with NFAT isoform-specific antibodies. Lamin A/C and actin show nuclear and cytosolic fraction purity, respectively. The values in the right column show the ratio of nuclear/total NFAT with a value of 50 meaning equal nuclear-cytosolic distribution. Data were obtained analyzing densitometric values of the bands; $n = 3$. (*d*) Staining with anti-NFATc4 is specific as shown by the total removal of the signal after preincubation with the specific peptide. (*e*) Two different anti-NFATc4 antibodies used (PA1-021 and sc-13076) show the same staining pattern in muscle and in brain, where they have a much stronger cytosolic signal than in muscle.

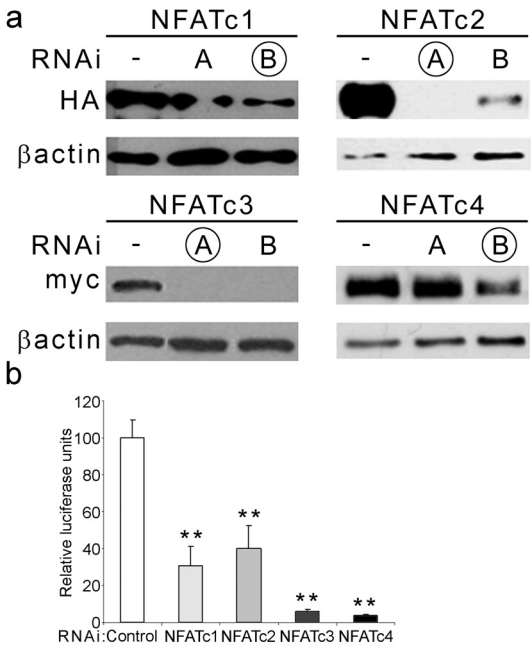

Fig. S3. Specificity of siRNAs. (*a*) WB of NFAT isoforms cotransfected in HEK 293 cells with either an empty pSuper vector (−) or with 2 different (A, B) siRNAs specific for the indicated isoforms. We refer to them as siRNAs for simplicity, although they are shRNAs. Circles indicate siRNA sequences used as first choice. (*b*) RNAi of NFAT isoforms detected measuring the expression in vivo of an NFAT sensor composed of 9 consensus NFAT binding sites linked to luciferase. The reporter was cotransfected with siRNAs specific for either GFP as a control or for the indicated NFAT isoforms.

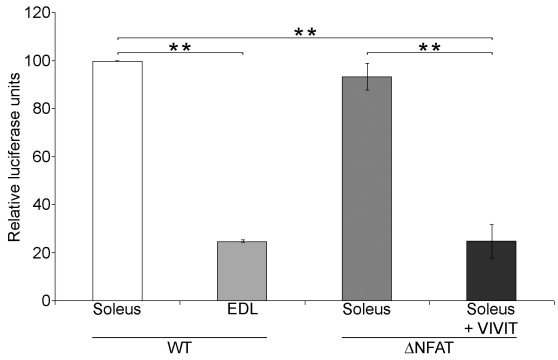

Fig. S4. Expression of WT and ΔNFAT −348 MyHC promoter. The short MyHC-slow construct retains the fiber type specificity of the longer promoter, as indicated by the higher luciferase activity in soleus than in EDL. The luciferase activity is essentially identical for the WT and ΔNFAT construct. Sensitivity to the NFAT inhibitor peptide VIVIT is maintained in the mutated construct, indicating that NFAT controls the expression of MyHC-slow also indirectly (see *Discussion*).

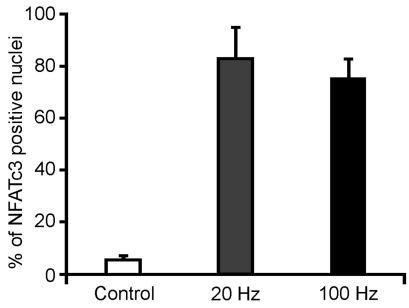

Fig. S5. Electrical stimulation at both 20 and 100 Hz causes nuclear translocation of NFATc3-GFP. Mouse tibialis anterior muscles were transfected with NFATc3-GFP and electrically stimulated for 2 h as indicated. The percentage of fibers with nuclear staining was quantified by microscopical analysis of muscle sections.

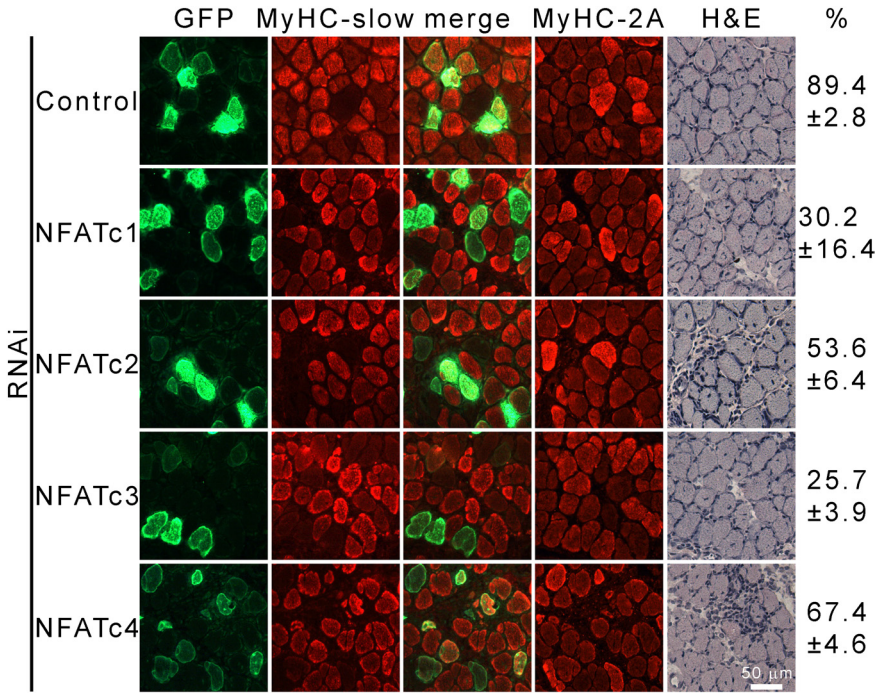

Fig. S6. All NFAT isoforms are necessary for the expression of MyHC-slow in regenerating muscle. Regenerating soleus was cotransfected with NFAT isoform-specific siRNAs and SNAP-GFP at day 3 after myotoxic damage induced with bupivacaine. Analyses were performed 1 week later. Serial cross sections were stained with antibodies specific for GFP, MyHC-slow (and the images were merged), MyHC-2A, and with hematoxylin/eosin (H&E). The right column shows the percentage of MyHC-slow-positive transfected (i.e., GFP-positive) fibers.

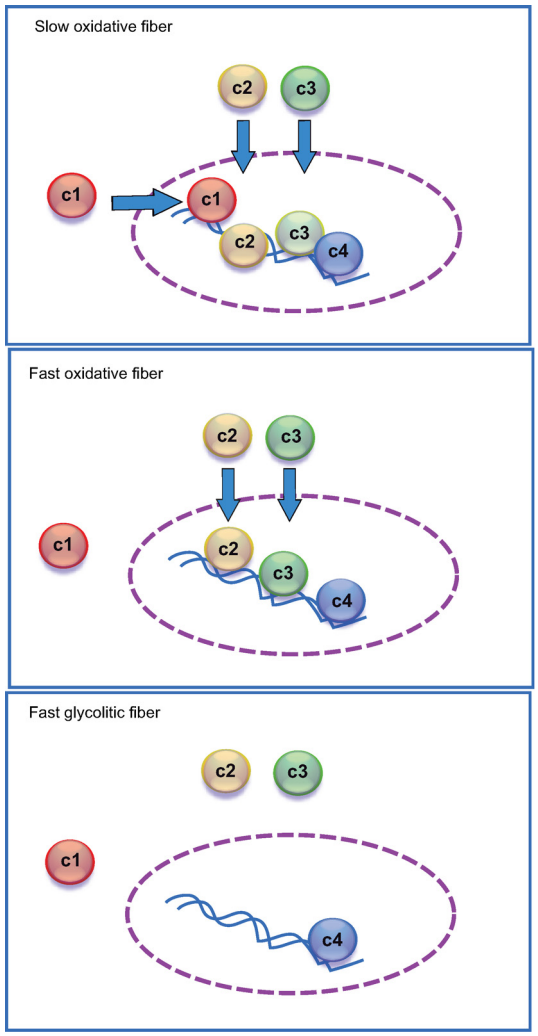

Fig. S7. A graded nuclear recruitment of NFAT transcription factors in response to activity modulates the expression of MyHCs. The pattern of activity that characterizes slow fibers induces the nuclear translocation and activation of all 4 NFAT isoforms, which are necessary for the transcription of MyHC-slow. NFATc1, in particular, only translocates to the nucleus in response to slow-type activity (10, 12) and switches the specificity of the transcriptional machinery toward slow MyHC; by inhibiting fast MyHC expression (9), it also ensures that the fast and slow gene programs are mutually exclusive. In fast-oxidative (2A/2X) fibers, NFATc1 is mostly cytosolic and NFATc2 and c3 are transcriptionally active; thus, contributing to the transcriptional activation of MyHC-2A and -2X. We hypothesize that in fast-glycolytic fibers, where MyHC-2B is the only transcribed isoform, only NFATc4 is nuclear and transcriptionally active (Fig. 4).

Table S1. Anti-NFAT antibodies used in this study

	Antibody	IF/IHC	Concentration	WB	Concentration
NFATc1	SCBT 7A6 mouse monoclonal (sc-7294 X)	Very good	1:500	Very good	1:1,000
NFATc2	SCBT 4G6-G5 (sc-7256)	Does not work	—	Very good	1:500
	Abcam Ab2722	Denaturing conditions	1:500	—	—
	G1G9 Crabtree	Denaturing conditions	1:500	Does not work	—
NFATc3	SCBT F1 mouse monoclonal (sc-8405)	Does not work	—	Good	1:500
	H75 rabbit polyclonal (sc-8321)	Denaturing conditions	—	Good	1:1,000
NFATc4	SCBT H74 rabbit polyclonal (sc-13036)	Good	1:1,000	Weak	1:1,000
	ABR rabbit polyclonal(PA1–021)	Good	1:500	Weak	1:2,000

IF/IHC, immunofluorescence/immunohistochemistry; WB, Western blot; —, not tested.