NFATc1 nucleocytoplasmic shuttling is controlled by nerve activity in skeletal muscle

Jana Tothova[1,2,*], Bert Blaauw[1,2,3,*], Giorgia Pallafacchina[1,2,*], Rüdiger Rudolf[1,2], Carla Argentini[1,2], Carlo Reggiani[3,4] and Stefano Schiaffino[1,2,4,‡]

[1]Department of Biomedical Sciences, University of Padova, Padova, Italy
[2]Venetian Institute of Molecular Medicine (VIMM), Padova, Italy
[3]Department of Anatomy and Physiology, University of Padova, Padova, Italy
[4]CNR Institute of Neurosciences, University of Padova, Padova, Italy

*These authors contributed equally to this work
‡Author for correspondence (e-mail: stefano.schiaffino@unipd.it)

Accepted 4 January 2006
Journal of Cell Science 119, 1604-1611 Published by The Company of Biologists 2006
doi:10.1242/jcs.02875

Summary

Calcineurin-NFAT signaling has been shown to control activity-dependent muscle gene regulation and induce a program of gene expression typical of slow oxidative muscle fibers. Following Ca^{2+}-calmodulin stimulation, calcineurin dephosphorylates NFAT proteins and induces their translocation into the nucleus. However, NFAT nuclear translocation has never been investigated in skeletal muscle in vivo. To determine whether NFATc1 nucleocytoplasmic shuttling depends on muscle activity, we transfected fast and slow mouse muscles with plasmids coding for an NFATc1-GFP fusion protein. We found that NFATc1-GFP has a predominantly cytoplasmic localization in the fast tibialis anterior muscle but a predominantly nuclear localization in the slow soleus muscle, with a characteristic focal intranuclear distribution. Two hours of complete inactivity, induced by denervation or anaesthesia, cause NFATc1 export out of the nucleus in soleus muscle fibers, whereas electrostimulation of tibialis anterior with a low-frequency tonic impulse pattern, mimicking the firing pattern of slow motor neurons, causes NFATc1 nuclear translocation. The activity-dependent nuclear import and export of NFATc1 is a rapid event, as visualized directly in vivo by two-photon microscopy. The calcineurin inhibitor cain/cabin1 causes nuclear export of NFATc1 both in normal soleus and stimulated tibialis anterior muscle. These findings support the notion that in skeletal muscle NFATc1 is a calcineurin-dependent nerve activity sensor.

Key words: Fast and slow skeletal muscles, NFAT, Nuclear translocation, Calcineurin, Nerve activity, Electrostimulation

Introduction

The transcription factors of the nuclear factor of activated T cells (NFAT) gene family comprise four isoforms, NFATc1-c4, also known as NFAT1-4, whose activity is controlled by Ca^{2+}-calcineurin (Cn) signaling (Crabtree and Olson, 2002; Hogan et al., 2003). NFAT nucleocytoplasmic shuttling is a key event in Cn-NFAT signaling and is dependent on the phosphorylation/dephosphorylation status of two types of serine-rich motifs, the SRR-1 and SP motifs, which are conserved within the NFAT family. Following dephosphorylation of the SRR-1 and SP motifs by calcineurin, NFATs translocate to the nucleus and activate or repress target genes, acting often in combination with other transcription factors or co-activators. NFAT nuclear export depends on the activity of different kinases, including CK1, which phosphorylates the SRR-1 motif (the primary region required for NFAT nuclear import) and GSK3, which targets SP-2 (one of the SP motifs) and synergizes with CK1 to regulate NFAT nuclear export (Okamura et al., 2004).

Several lines of evidence indicate that Cn-NFAT signaling is involved in fiber-type specification induced by nerve activity in skeletal muscle. Chin et al. first suggested that slow motor neuron activity leads to activation of the Cn-NFAT pathway promoting the transcriptional activation of slow oxidative muscle genes (Chin et al., 1998). This interpretation has been supported in subsequent studies, although contradictory results have also been reported (see Schiaffino and Serrano, 2002). Using an in vivo transfection approach in regenerating and adult rat muscles, we have shown that Cn is involved in the induction and maintenance of the slow-muscle gene program by nerve activity (Serrano et al., 2001). More recently, using the same approach, we have shown that, (1) NFAT activity, monitored with NFAT-dependent reporters, is selectively stimulated by electrostimulation of denervated muscles with impulse patterns mimicking the firing pattern of slow motor neurons, (2) the activation of the slow-muscle gene program by slow motor neuron activity in regenerating slow muscles is blocked by VIVIT, a specific peptide inhibitor of the Cn-mediated NFAT activation, and (3) a constitutively active NFATc1 mutant is able to activate the slow gene program in regenerating muscle (McCullagh et al., 2004).

We have now used NFATc1 fused to green fluorescent protein (GFP; NFATc1-GFP) to monitor NFATc1 nucleocytoplasmic shuttling in muscle fibers in vivo. Here, we show that NFATc1 translocates rapidly to the nucleus in response to 'slow-type' stimulation patterns, supporting the notion that NFAT is a nerve activity sensor in skeletal muscle fibers.

Results

Localization of NFATc1-GFP in fast and slow mouse muscles

Mouse skeletal muscles were transfected with plasmids coding for an NFATc1-GFP fusion protein by intramuscular DNA injection followed by electroporation. Seven days after transfection the muscles were either directly removed for analysis or subjected to denervation or electrostimulation through the common peroneal nerve. We first determined the cellular localization of NFATc1-GFP in the normal slow soleus and fast tibialis anterior (TA) muscles. As shown in Fig. 1A-D, NFATc1-GFP has a predominantly nuclear localization in soleus muscle fibers (panels A and C) but a predominantly cytoplasmic localization in TA fibers (panels B and D). By contrast, a flag-tagged constitutively active NFATc1 mutant showed an exclusively nuclear localization in both muscles (Fig. 1E,F). Soleus muscles transfected with NFATc1-GFP contain both fibers with exclusively nuclear localization and fibers with higher nuclear than cytoplasmic fluorescence (see Fig. 1C). By contrast, TA muscles transfected with NFATc1-GFP contain a majority of fibers with a lower or identical level of fluorescence in the nucleus compared with the cytoplasm (Fig. 1D, inset). It should be stressed that in this study we did not quantify the relative proportion of nuclear and cytoplasmic NFAT and, therefore, we cannot establish the precise fraction of NFAT activated. As shown in Fig. 2, about 66% of soleus muscle fibers display predominantly nuclear localization of NFATc1-GFP, whereas only about 14% of TA fibers show predominantly nuclear localization of NFAT. The nuclei of transfected soleus muscle fibers showed a typical focal distribution of fluorescence (see inset to Fig. 1D), similar to that previously described in isolated muscle fibers (Liu et al., 2001).

Nuclear export of NFATc1-GFP is induced by muscle inactivity in the soleus muscle

To determine whether the nuclear localization of NFAT depends on muscle activity, we first examined the effect of complete inactivity induced by anesthesia or denervation in mice with transfected soleus muscles. A 2-hour period of anesthesia or denervation caused nuclear export of NFATc1-GFP in most soleus muscle fibers (Fig. 3A,B), reducing the amount of fibers with positive nuclei to 9.9% or 7.5% upon anesthesia or denervation, respectively (Fig. 2). Interestingly, the proportion of fibers with nuclear localization of NFATc1-GFP was increased to almost 90% in the soleus muscle, which was overloaded due to denervation of the contralateral leg from the same animals (Fig. 2).

Nuclear import of NFATc1-GFP is induced by low-frequency but not high-frequency electrostimulation in the TA muscle

We next asked whether specific activity patterns induced by electrostimulation affect the nuclear localization of NFAT. To select the optimal paradigms for stimulation of slow- and fast-muscle-types, we characterized the contractile response of TA to stimulation via the common peroneal nerve at increasing stimulation frequency. A force-frequency curve, from single twitches (frequency 1 Hz) to completely fused tetanus (frequency 150 Hz) is shown in Fig. 4. Based on these data, we then selected two frequency values at the lower and upper ends of the steep part of the force-frequency curve. Whereas one pattern consisted of long trains at low frequency (20 Hz, for 10 seconds every 30 seconds), the other comprised short trains at high frequency (100 Hz, for 0.6 seconds every 60 seconds). These impulse patterns resemble the discharge pattern of slow and fast motor units, respectively (Hennig and Lomo, 1985), and have been shown to lead to marked changes in the myosin-

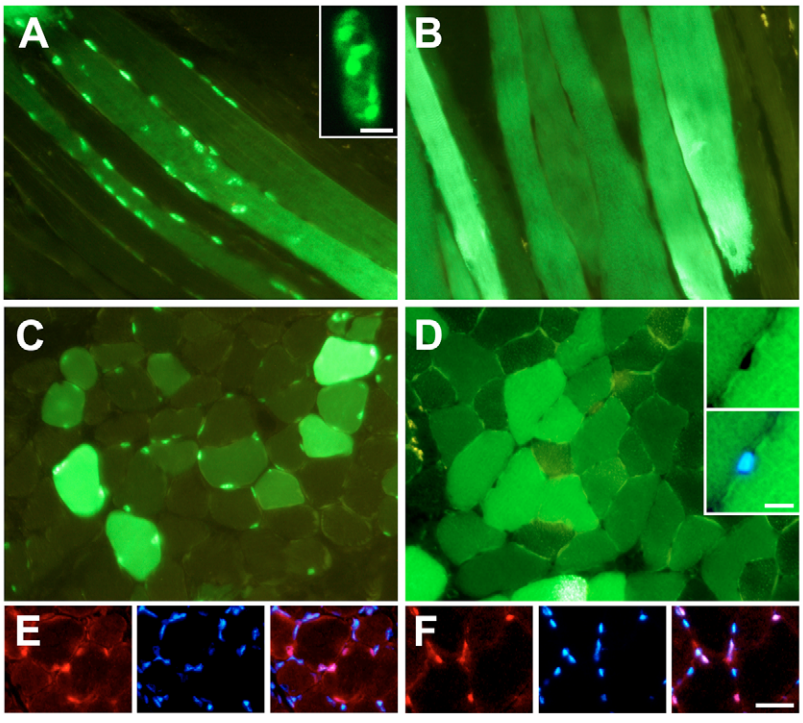

Fig. 1. NFATc1-GFP has a predominantly nuclear localization in most slow muscle fibers and a predominantly cytoplasmic localization in most fast fibers. (A-D) Longitudinal (A,B) and transversal (C,D) sections of mouse soleus (A,C) and tibialis anterior (TA) muscles (B,D) transfected with plasmids coding for NFATc1-GFP fusion protein. Notice the nuclear localization of NFATc1-GFP in soleus (A,C) and the presence of multiple foci of GFP fluorescence when nuclei are examined at higher magnification (A, inset). By contrast, NFATc1-GFP is either homogeneously distributed in the nuclei and cytoplasm of transfected TA muscle fibers (B,D) or some nuclear profiles are GFP-negative in this muscle, as shown by DAPI staining (D, inset). (E-F) Transverse sections of soleus (E) and TA (F) muscles transfected with plasmids coding for a constitutively active flag-tagged mutant of NFATc1 (caNFATc1) and analyzed with anti-flag antibodies (left panels) and nuclear DAPI staining (central panels). Images were merged to demonstrate colocalization (right panels). Bars, 30 μm (A-F); 5 μm (inset in A); 10 μm (inset in D).

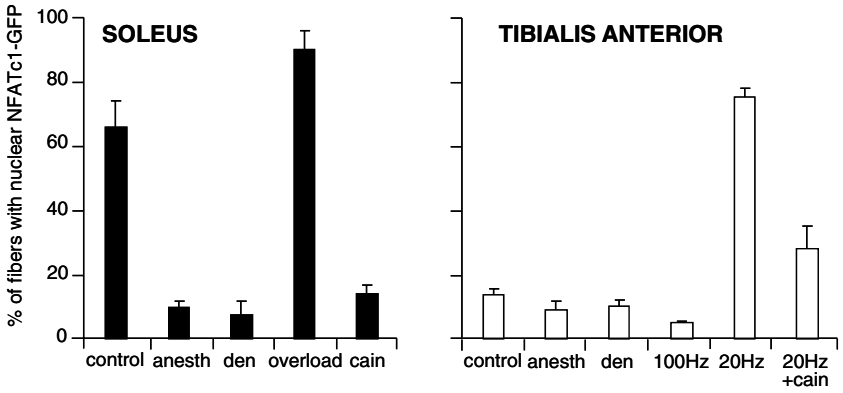

Fig. 2. Proportion of muscle fibers showing predominantly nuclear localization of NFATc1-GFP in normal mouse soleus and TA muscles, and in muscles subjected to various experimental conditions. Values are expressed as mean ± s.e.m. Muscles were transfected with plasmid coding for NFATc1-GFP and 7 days later were either immediately removed for analysis (control) or subjected to various experimental conditions: anesth, 2 hours anaesthesia; den, 2 hours denervation; overload, soleus muscle from contralateral leg; cain, soleus muscle co-transfected with the calcineurin inhibitor cain; 100 Hz or 20 Hz, TA muscles stimulated for 2 hours at 100 Hz or 20 Hz, respectively; 20 Hz + cain, TA muscles co-transfected with cain and stimulated for 2 hours at 20 Hz.

isoform expression pattern after chronic stimulation in rat skeletal muscles (Ausoni et al., 1990). TA muscles were stimulated for 2 hours via the common peroneal nerve with either the 20 Hz or the 100 Hz pattern, while the mouse was kept under anaesthesia. As shown in Figs 2 and 5, low frequency stimulation caused nuclear translocation of NFATc1-GFP in about 75% of the fibers, whereas the high-frequency trains did not affect NFATc1-GFP localization and, indeed, only about 5% of the fibers in high-frequency stimulated muscles showed nuclear NFAT. Thus, the nerve stimulation experiments not only confirmed that activity is responsible for NFATc1-GFP localization, but also demonstrated that specific patterns of neural discharge on the muscle fibers are required to shift NFATc1-GFP to the nucleus. The tonic low-frequency repetitive stimulation was able to reproduce in the fast fibers of TA a localization pattern similar to that present in the slow soleus muscle fibers, whereas phasic high-frequency stimulation did not. The intranuclear focal distribution of fluorescence was also very similar to that observed in soleus fibers (Fig. 5B, inset).

NFATc1-GFP nucleocytoplasmic shuttling can be visualized in the living animal by two-photon microscopy

Detection of NFATc1-GFP localization in cryosections of muscles dissected in various experimental conditions could clearly demonstrate how the stimulation pattern applied to the muscle is crucial for the nuclear translocation of NFATc1-GFP, but left unanswered questions as to when a first obvious translocation would eventually come up, how this translocation occurs in individual nuclei, and whether all nuclei are filled-up with the same time course. We, therefore, undertook an approach that permitted us to visualize NFATc1-GFP nuclear import and export in real-time in the living animal. To this aim, TA muscles transiently expressing NFATc1-GFP were directly observed in situ by means of two-photon microscopy during and after the low-frequency (20 Hz trains) stimulation applied via the common peroneal nerve, which was found to induce nuclear translocation of NFATc1-GFP (see above). As shown in Fig. 6, both transport of NFATc1-GFP into and out of the nucleus during stimulation (A) and after the end of the stimulation (B), respectively, could be followed under these conditions. The earliest fill-up of some nuclei was detectable already after 10 minutes of stimulation, and 30 minutes after the onset of stimulation most the nuclei in transfected fibers show a marked increase of NFATc1-GFP fluorescence (Fig. 6A). Both nuclear import and export of NFATc1 were apparently continuous during the analyzed time periods (Fig. 6C). In most cases, we observed that the focal localization of NFATc1-GFP inside the nuclei, such as shown in Fig. 6D, was present from the earliest time-points, and subsequent fill-up consisted in both – enlargement of existing and addition of new foci (not shown). At 60 minutes after stimulation the average number of intranuclear foci per nucleus was 12.3 and their average size was 1.3 μm in diameter, ranging from 0.4 to 3.4 μm.

Nuclear import of NFATc1-GFP depends on calcineurin activity

To determine whether the nuclear translocation of NFATc1-GFP is due to calcineurin activity, normal soleus or TA muscles were co-transfected with plasmids coding for NFATc1-GFP and plasmids coding for cain/cabin1, a peptide with a specific inhibitory action on calcineurin. Most soleus muscle fibers expressing cain/cabin1 do not show nuclear localization of

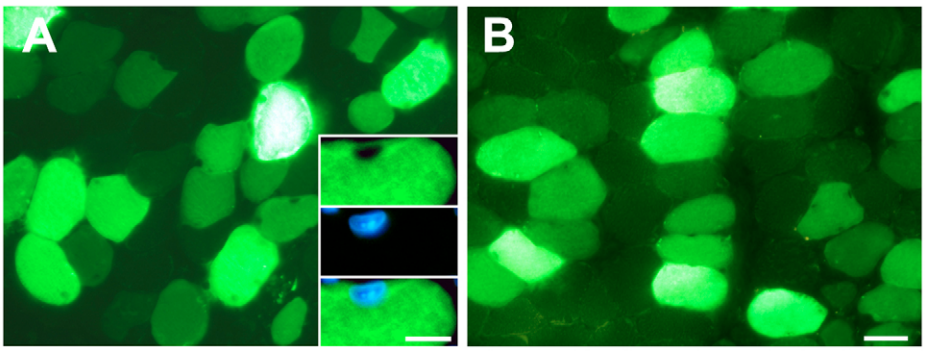

Fig. 3. Nucleus-to-cytoplasm translocation of NFATc1-GFP is rapidly induced by inactivity in slow muscle fibers. Transversal sections of soleus muscles transfected with NFATc1-GFP and examined 2 hours after section of the sciatic nerve (A) or after 2 hours anaesthesia (B). Notice cytoplasmic distribution of NFATc1-GFP (A,B) with no GFP fluorescence in DAPI-stained nuclei (A, inset). Bars, 30 μm (A,B); 10 μm (inset in A).

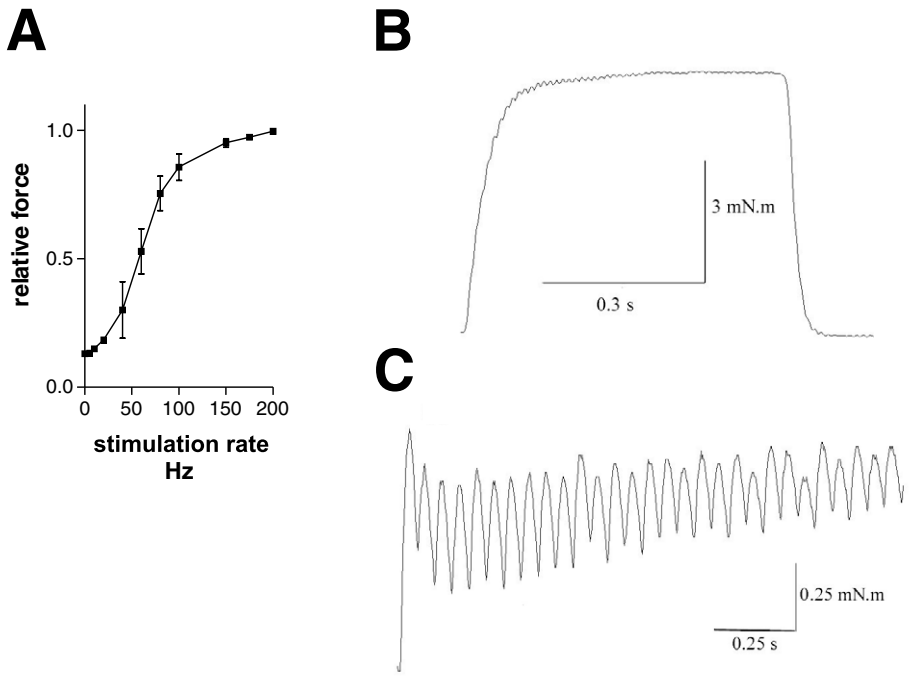

Fig. 4. (A) Force-frequency relation. (B,C) Representative mechanical responses at (B) high stimulation frequency (100 Hz) and (C) low stimulation frequency (20 Hz). Tibialis anterior was stimulated via common peroneal nerve and force was recorded with a force transducer (FT03E, Grass, Warwick, USA) connected to the mouse foot.

NFATc1-GFP (Fig. 2, Fig. 7A,B). Similar results were observed in TA muscles stimulated with the 20-Hz pattern (Fig. 2, Fig. 7C,D). Interestingly, nuclear translocation was clearly evident in the occasional fibers positive for NFATc1-GFP but negative for cain/cabin (Fig. 7E-H). These results indicate that the nuclear translocation of NFAT in skeletal muscle fibers depends on calcineurin activity.

Discussion

Skeletal muscle fibers can adapt to changing functional demands by changing their size, metabolism and contractile properties. Activity patterns, such as the low-frequency tonic pattern of impulses induced by slow-motor-neuron firing, are well known to trigger a transformation towards the oxidative and slow phenotype (Lomo, 2003; Pette and Vrbova, 1992). Previous studies have shown that the calcineurin pathway is involved in this fast-to-slow transformation and that NFAT transcription factors are major targets of calcineurin phosphatase activity in the transcriptional regulation of muscle genes (for reviews see Bassel-Duby and Olson, 2003; Horsley and Pavlath, 2002; Schiaffino and Serrano, 2002). Studies in vitro on cultured muscle cells have shown that NFAT undergoes nuclear translocation following electrical stimulation with a low-frequency tonic pattern of impulses (Kubis et al., 2002; Liu et al., 2001) and is able to activate slow-fiber-specific gene transcription (Chin et al., 1998; Torgan and Daniels, 2001). We have recently provided direct evidence for a major role of NFATc1 in vivo to establish the slow phenotype during regeneration and also to maintain the slow-phenotype in adult muscles (McCullagh et al., 2004). NFATc1 is able to sense slow patterns of electrical nerve activity and to mediate transcriptional responses. In this process NFAT dephosphorylation operated by calcineurin and subsequent nuclear translocation are two essential steps. In the present study, we provide the first demonstration in vivo that nuclear translocation of NFAT is activity-dependent and that it is induced only by specific patterns of activity.

Compared with the in vitro experiments (Kubis et al., 2002; Liu et al., 2001), the study in vivo allowed us to demonstrate that in the slow soleus muscle NFAT nuclear localization strictly depends on contractile activity. In soleus fibers, the nuclear translocation of NFATc1-GFP can be quickly reversed by denervation or by transient suppression of contractile activity during deep anesthesia. Most interestingly, nerve stimulation can induce NFAT translocation also in fast muscles, but only in response to appropriate stimulation patterns. Although the issue of the stimulation pattern was also taken up in the in vitro studies, only in vivo experiments, like those

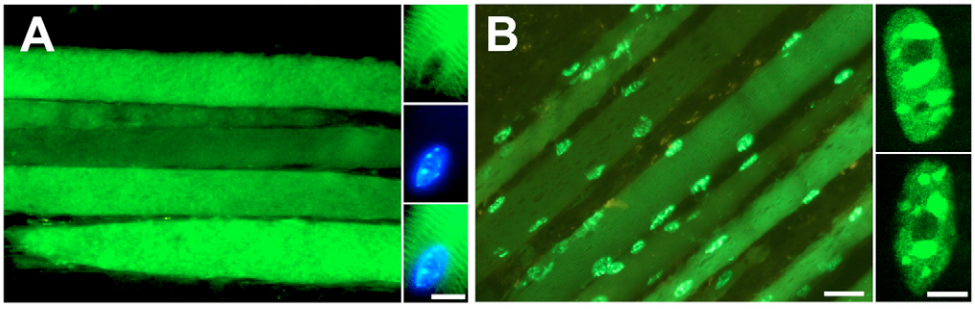

Fig. 5. Nuclear translocation of NFATc1-GFP is induced in fast muscle fibers by electrostimulation with a tonic low-frequency pattern of impulses but not by a phasic high-frequency pattern. TA muscles transfected with NFATc1-GFP were electrostimulated for 2 hours via the common peroneal nerve with two distinct impulse patterns: (A) a phasic high-frequency (100 Hz) pattern, which resembles the firing pattern of fast motor neurons or (B) a tonic low-frequency (20 Hz) pattern, which resembles the firing pattern of slow motor neurons. Notice that NFATc1-GFP maintains a cytoplasmic localization after stimulation with the 'fast' pattern (A), with negative nuclear profiles (see merge of GFP and DAPI staining in A, insets), but shows a nuclear translocation after stimulation with the 'slow' pattern (B), with multiple intranuclear foci of fluorescence, similar to those seen in soleus muscle fibers (B, inset, compare with Fig. 1A). Bars, 30 μm (A-B); 10 μm (inset in A); 5 μm (inset in B).

presented here, allow the evaluation of the real conditions under which muscle contraction occurs. Previous experiments in vitro could not take into account the mechanical characteristics of the contractile response, the degree of fusion and the possible occurrence of fatigue.

As shown by the determination of the force-frequency curve, the two selected stimulation patterns are located at the lower (20 Hz) and upper (100 Hz) end of the steep part of the force-frequency curve. In the former condition (20 Hz), fusion between the contractile responses is present but only to a minor degree, so that virtually no summation and increase of tension above the twitch level occur. Contractile responses follow closely to each other and no rest interval is left between the end of a response and the beginning of the next one, so that muscle fibers are engaged in low-intensity contractile activity for 1/3 of their time. The condition obtained with the high-frequency pattern (100 Hz) is the opposite because muscle fibers are activated in quasi-maximal contractions for short periods (1/100 of their time). In either case, no fatigue or loss of excitability occurred during the two hours of stimulation and the amplitude of the contractile response remained virtually constant. However, the total amount of stimuli administered was different: 400 stimuli were delivered every minute with the low-frequency pattern, whereas only 60 stimuli per minute were delivered with the high-frequency pattern. In vitro studies support the view that the pattern of stimulation is more important than the total number of stimuli (Liu et al., 2001). Further tests in vivo might contribute to set this point. Previous studies (Hennig and Lomo, 1985) show that slow and fast motor units differ for discharge frequency as well as for the total number of stimuli delivered and the fraction of time they are active during the day: from 5-8 hours a day for the slow motor units to few minutes a day for the fast 2B motor units. Stimulation patterns that mimick these patterns of motor neuron firing and are similar to those used in the present study were shown to be effective in inducing fiber-type switching in denervated rat muscles (Ausoni et al., 1990). No direct information is presently available on patterns of motor neuron discharge in the adult mouse, however the motor unit discharge rate in the soleus of 14- to 15-day-old mice is similar to that recorded in the adult rat (Personius and Balice-Gordon, 2001).

In vitro studies on isolated mouse muscle fibers (Liu et al., 2001) and in cultured rabbit myotubes (Kubis et al., 2002) have previously shown that NFAT nuclear translocation depends upon specific stimulation patterns. The stimulation pattern reported by Liu et al. (Liu et al., 2001) to induce NFAT nuclear translocation (10 Hz for 5 seconds every 50 seconds), is comparable with the pattern used in this study (20 Hz, for 10 seconds every 30 seconds), if we take into account that intracellular Ca^{2+} kinetics is probably slower in fibers in culture at room temperature than in a mouse at body temperature. The frequencies adopted by Kubis et al. (Kubis et al., 2002) to induce translocation in vitro were lower (1-10 Hz); however, those experiments were carried out on rabbit myotubes, i.e. immature muscle cells and not on fully differentiated fibers.

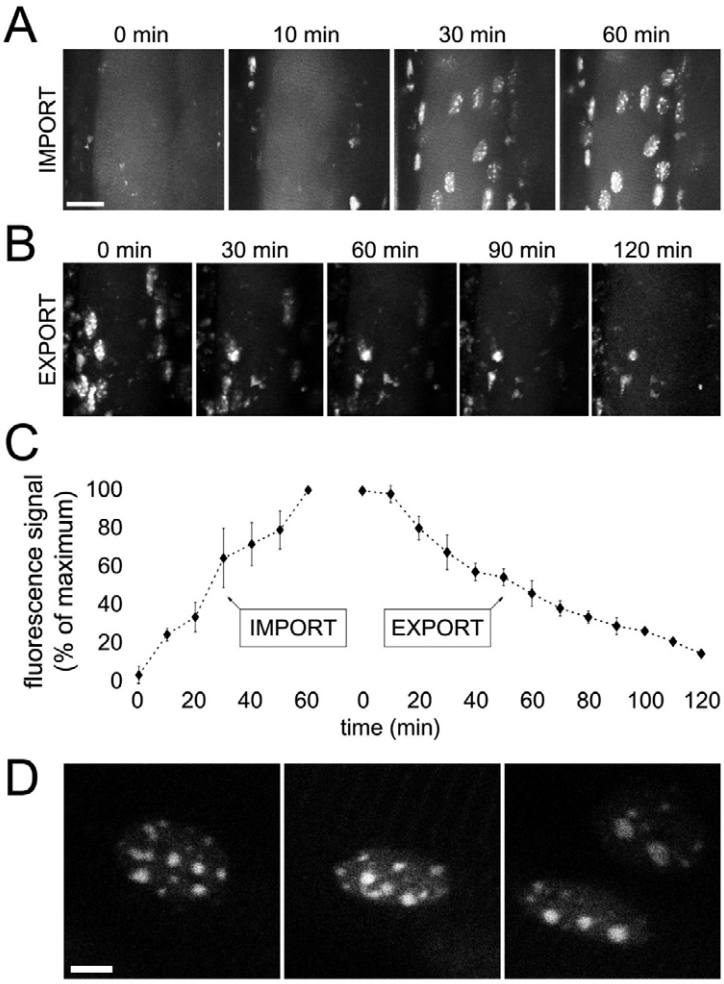

Fig. 6. Nucleocytoplasmic shuttling of NFATc1-GFP visualized in muscles of living mice. TA muscle transiently expressing NFATc1-GFP was observed in situ by using two-photon microscopy as described in Materials and Methods, either during application or after suspension of low-frequency stimulation. (A,B) Micrographs depicting maximum-intensity projections of either 15 confocal sections of individual fibers taken at the indicated time points after start (A) or suspension of low-frequency stimulation (B); bar, 25 μm. (C) Graphs show the mean fluorescence intensity of 30 (import, *n*=3 fibers) or 49 nuclei (export, *n*=3 fibers). Data (mean ± s.e.m.) were obtained from background-corrected sum plots and were either normalized to the 60-minute values (import, left panel) or 0-minute values (export, right panel). (D) High-resolution confocal micrographs of individual nuclei showing the concentration of NFATc1-GFP in punctuate structures. Bar, 5 μm.

In agreement with the results of the studies in vitro, nuclear translocation is detectable after short periods of activity and disappears after short periods of reduced or suppressed activity (denervation, anaesthesia). Two-photon microscopy, showing for the first time the translocation of NFATc1 in live muscle in situ, revealed that, (1) already few minutes of repetitive low-frequency stimulation are sufficient to drive this transcription factor into nuclei of fast-muscle fibers, (2) the dynamics of its import and export may vary between different nuclei and, (3) nuclear NFATc1-GFP shows a characteristic punctuate pattern of fluorescent foci.

Although a detailed study on kinetics falls outside of the

scope of this study, the demonstration that contractile activity in vivo can produce nuclear translocation in a short time is interesting, particularly taking into account that the first transcriptional effects of low-frequency stimulation are detectable only after 24-30 hours (Barton-Davis et al., 1996; Kirschbaum et al., 1990; Kirschbaum et al., 1989). It should be stressed that NFAT nuclear translocation is a necessary but not sufficient condition for NFAT transcriptional activity. Indeed, in preliminary experiments using an NFAT-dependent reporter, we found no detectable activation of the reporter even after 10 hours of low-frequency stimulation in anaesthetized mice (our unpublished observations). We have recently shown that constitutively active NFAT can upregulate slow-fiber-specific genes in the slow muscle soleus but not in the fast muscle EDL (McCullagh et al., 2004). The present experiments show that the nuclear localization of NFAT occurs both in soleus and in tibialis anterior, provided that a suitable stimulation pattern is applied. Thus, the signaling pathway initiated by contractile activity appears to be effective in the same way in fast and slow muscles, however, transcriptional activity is probably affected by other factors. We have previously suggested that the response of fiber-specific genes is modulated by chromatin remodeling during fiber maturation (McCullagh et al., 2004).

It is of interest that the changes in muscle activity we have examined in this study neither seem to be sufficient to induce nuclear localization in all fibers efficiently transfected with NFATc1-GFP nor to cancel any presence of fluorescent nuclei. Thus, physiologically active soleus muscles contain a minority of fibers in which NFATc1-GFP does not localize in the nucleus. A plausible explanation for this could be that not all motor units are continuously recruited in the mouse soleus, as shown in the rat soleus (see Hennig and Lomo, 1985). The alternative explanation that the mouse soleus contains also motor units of the fast fatigue-resistant type (corresponding to type 2A fibers) seems unlikely because staining with specific anti-myosin antibodies revealed nuclear localization of fluorescence in both slow and fast 2A fibers (data not shown). The suppression of activity for 2 hours induced by anesthesia or denervation is not sufficient to cause nuclear export of NFATc1-GFP from all the nuclei, some nuclear fluorescence still being present in less than 10% of the fibers.

In the tibialis anterior muscle, the large majority of fibers exhibited a predominantly cytoplasmic localization of NFATc1-GFP before tonic stimulation. After stimulation, the proportion of the fibers with nuclear localization of fluorescence was very high but variable, ranging from 60 to 90%. It is likely that the low voltages applied were not sufficient to stimulate all axons in the peroneus nerve and, therefore, a corresponding population of muscle fibers did not participate in contractile activity and did not display nuclear translocation.

In agreement with previous observations in isolated muscle fibers (Liu et al., 2001), NFAT appears grouped in blocks inside the nucleus. The discrete distribution of the NFATc1-GFP fusion protein encoded by a plasmid vector was present both in the soleus nuclei in response to spontaneous contractile activity, and in the tibialis anterior nuclei after adequate nerve stimulation. This confirms that the discrete distribution was not an artifact due to the adenovirus vector used by Liu et al. (Liu et al., 2001). The same focal distribution of NFAT was observed by using tagged NFAT instead of the NFATc1-GFP fusion protein and, therefore, is not due to GFP (data not shown). The structure of the NFATc1-GFP foci and the lack of overlay with staining for the transcription factor MEF-2 and the splicing factor SC-35 (Liu et al., 2001) are in contrast with the punctuate pattern of NFAT intranuclear distribution and the partial overlap with MEF-2 pattern demonstrated by Kubis et al. by immunostaining with specific antibodies (Kubis et al., 2002). Our data are in support of the pattern observed by (Liu et al., 2001). Again, it should be stressed that Kubis and co-workers used cultured myotubes (Kubis et al., 2002), and not mature muscle fibers either isolated in vitro (Liu et al., 2001) or in situ (present study).

In conclusion, the present study demonstrates that a suitable pattern of contractile activity induces nuclear localization of NFATc1 and that this is the result of the phosphatase activity of calcineurin. Taken together with the previous evidence on activation of the calcineurin-NFAT pathway as a function of neuromuscular activity in transgenic mice (Wu et al., 2001; Rosenberg et al., 2004) and in transfected muscles of adult rats (Serrano et al., 2001; McCullagh et al., 2004), these results identify a pathway going from contractile activity to transcriptional regulation

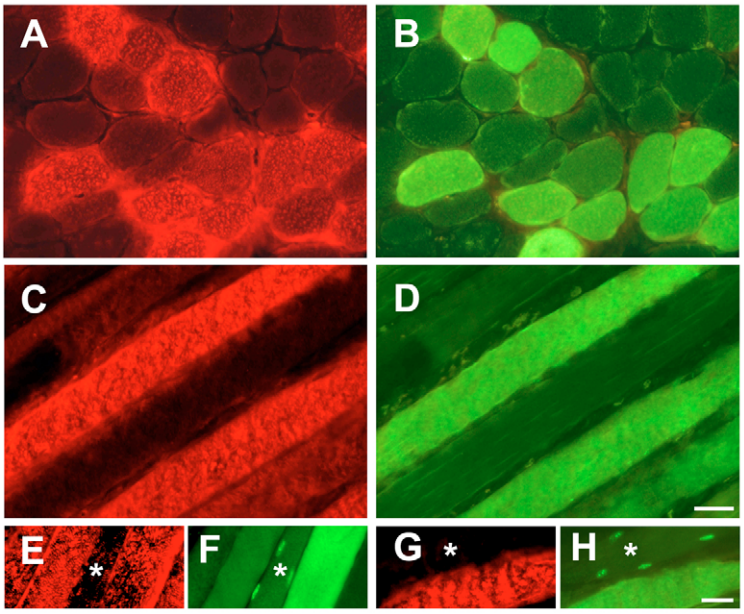

Fig. 7. The calcineurin inhibitor cain/cabin1 blocks the nuclear translocation of NFATc1-GFP. (A,B) Soleus muscles co-transfected with plasmids coding for NFATc1-GFP and myc-tagged cain. (C,D) TA muscles co-transfected with plasmids coding for NFATc1-GFP and myc-tagged cain and electrostimulated for 2 hours with a 20 Hz impulse pattern. Serial sections were either stained with anti-myc (A,C,E,G) or examined for GFP fluorescence (B,D,F,H). (E-H) Occasional fibers in electrostimulated TA muscles that do not express cain (asterisks) maintain a nuclear localization of NFATc1, whereas neighboring fibers that do express cain show a cytoplasmic localization of NFATc1-GFP. Bar, 30 μm.

that might be responsible for the determination of the slow-phenotype following increased contractile activity.

A major open issue concerns the response of the other NFAT isoforms (NFATc2, NFATc3 and NFATc4) to contractile activity and their role in nerve-activity-dependent specification of muscle-fiber types.

Materials and Methods

Animals and in vivo transfection

Experiments were carried out on 4- to 6-weeks-old male CD1 mice. Experimental protocols were reviewed and approved by the local Animal Care Committee of the University of Padova. Before surgery or muscle transfection, mice were anesthetized by intraperitoneal injection of a mixture of Zoletil 100® (a combination of Zolazapam and Tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Rompun® (Xilazine 2%, 0.06 ml/kg, Bayer). Leg skin was opened in anaesthetized mice, tibialis anterior (TA) 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 a ECM830 BTX porator (Genetronics, San Diego, CA) with the following settings: 5 pulses of 20 milliseconds each and 200 milliseconds interval, the voltage was adjusted according to the thickness of the muscle (5 V/mm).

Plasmids

Most experiments were carried out with a pEGFP–N1 (Clontech) plasmid containing a full-length wild-type NFATc1 linked to E-GFP (NFATc1-GFP) (Chin et al., 1998). Plasmids coding for a flag-tagged constitutively active NFATc1 mutant (Neal and Clipstone, 2001) were used in some experiments. To inhibit calcineurin activity, we used a plasmid coding for a myc-tagged calcineurin inhibitory domain from cain (Lai et al., 2000), that was previously shown to efficiently block NFAT-dependent reporters and calcineurin-NFAT-dependent induction of slow-myosin heavy chain in regenerating rat soleus muscle (McCullagh et al., 2004; Serrano et al., 2001).

Denervation and electrical stimulation

Seven days after transfection, the cytoplasmic or nuclear localization of NFATc1-GFP was examined in TA and soleus muscles. Mice were anesthetized as described above and muscles were dissected either immediately after anesthesia induction (control group), or after two hours of anesthesia (anesthesia group), after two hours of denervation (denervation group), or after two hours of electrostimulation of TA at 20 Hz or 100 Hz (stimulation 20 Hz and 100 Hz groups). Denervation was performed by cutting the sciatic nerve high in the thigh. To perform stimulation of the TA muscle, the common peroneal nerve was exposed proximally to the knee on the lateral side of the thigh. Teflon-covered stainless steel electrodes (Cooner wire, Chatsworth, CA) were implanted near to the branch of the peroneal nerve as it emerges distally from the popliteal fossa. The two thin electrodes were sewed on both side of the nerve and the skin above was sutured. The electrodes were connected to an AMP Master-8 stimulator (AMP Instruments, Jerusalem, Israel). The appropriate voltage was set to obtain nerve stimulation and avoid direct muscle stimulation. The movement (foot dorsal flexion) was checked as indication of the correct stimulation. Electrical stimulation was carried out for two hours while the mouse was kept under anesthesia. Two main patterns of stimulation were tested. (1) Slow pattern: trains of 10-second duration and 20-Hz frequency given every 30 seconds. (2) Fast pattern: trains of 0.6-second duration and 100-Hz frequency given every 60 seconds. Immediately after dissection the muscles were frozen in isopentane, cooled in liquid nitrogen and stored at –80°C till subsequent analysis.

Force measurement

To measure the force development during the electrical stimulation of the TA muscle via the common peroneal nerve, the foot was connected to a Grass force transducer (FT03E, Grass, Warwick, USA). The signals from the force transducer were displayed on the screen of a storage oscilloscope (mod 5113, Tektronix, Beaverton, Oregon, USA) and processed for A/D conversion (interface CED 1401 plus, Cambridge, UK). For data storage, recall and analysis the software Spike 2 (CED, Cambridge, UK) was used.

NFAT detection

Longitudinal or transversal cryosections, about 8-10 μm thick, were prepared from muscle samples, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at 4°C, rinsed three times in PBS and mounted in Elvanol. Nuclei were stained with DAPI (Sigma-Aldrich). The localization of fluorescent NFATc1-GFP was detected on an Olympus BX51 fluorescent microscope, digital images were acquired using an Olympus DP50 CCD camera and Twain imaging software. Confocal images were acquired using a BioRad Laboratories confocal microscope (Radiance 2100 MP equipped with an argon laser for 488 nm excitation of GFP fluorescence, a Nikon 60x/1.4 Plan Apo objective, a 500 DCLPXR beamsplitter and HQ515/30 emission filter; all filters were from Chroma Technology Corp.) using Lasersharp 2000 software (BioRad). NFAT nuclear translocation was quantified by counting the number of fibers showing nuclear NFATc1-GFP localization over the total GFP-positive fibers on digital images of transverse sections from transfected muscles. We scored, as fibers with a predominantly nuclear NFATc1-GFP, those with a higher level of fluorescence in the nucleus compared with the cytoplasm. For each experimental group, at least five muscles were analyzed and at least 350 fibers per TA and 200 fibers per soleus were counted.

Sections from muscles transfected with flag-tagged constitutively active NFATc1 and myc-tagged cain were stained with rabbit anti-flag (Sigma-Aldrich) and goat anti-myc (Santa Cruz Biotechnologies) primary polyclonal antibodies, respectively. Sheep anti-rabbit Cy3 (Sigma-Aldrich) and donkey anti-goat Alexa Fluor 568 (Molecular Probes) were used as secondary antibodies.

Two-photon microscopy

In vivo two-photon microscopy of tibialis anterior muscle transiently expressing NFATc1-GFP was performed as recently described (Rudolf et al., 2004), with minor modifications. In brief, animals were used for analysis 7-8 days after transfection and mounted onto the microscope stage, exposing the muscle for observation. Innervation and circulation were left intact. NFATc1-GFP fluorescence was measured using a Bio-Rad Life Science (Hertfordshire, UK) Radiance 2100 MP multi-photon system, equipped with a Tsunami mode-locked, tunable, femto-second-pulsed Ti:sapphire laser, optically pumped by a Millennia VsS 5 W green laser (both from Spectraphysics Lasers, Mountain View, CA). For excitation, infrared light of 830 nm was used and emission was collected with a BioRad Direct Detection System equipped with a 500LP DC dichroic mirror and a HQ535/50 emission filter (Chroma Technology, Rockingham, VT). The LaserSharp 2000 software package (Bio-Rad) was used for data acquisition and images were taken at 1024×1024 pixels with a scan rate of 166 lines per second. For each time point, a series of 15 different focal planes was taken. During acquisition, stimulation was intermitted and the time points indicated in Fig. 6A,C (left panel) represent effective duration of stimulation.

The kinetics of NFATc1-GFP translocation were obtained for all nuclei visible in the microscopic field at the end of the stimulation period using the slow stimulation pattern. Therefore, with ImageJ 1.32j software (freeware, available at http://rsb.info.nih.gov/ij/) sum plots of all confocal layers were produced for each time point. Then, the total fluorescence intensity of each nucleus was determined for all tested time points by the following term:

$$I_\tau = (N_\tau – BG_\tau) \times A_{max} ,$$

where I is the total nuclear fluorescence intensity, N the mean nuclear fluorescence intensity and BG the mean background fluorescence intensity, all measured at the time point τ. A_{max} represents the nuclear area at the end of the stimulation period. All values are shown as percentage of I_{max} (total nuclear fluorescence intensity at the end of the stimulation period). For presentation purposes maximum-intensity projections, prepared with ImageJ and arranged with Adobe Photoshop v.6.0, have been used.

This work was supported by grants from the European Union (Network of Excellence MYORES, contract LSHG-CT-2004-511978 to S.S., Integrated Project EXGENESIS, contract LSHM-CT-2004-005272 to S.S.), from the Italian Ministry of Education, University and Research (PRIN 2004 and FIRB 2001 RBNE015AX4 to S.S., PRIN 2004 and FIRB 2001 RBNE01AY9E_002 to C.R.) and from Telethon (grant number GGP04227). We gratefully acknowledge the gifts of plasmids from R. S. Williams and R. Bassel-Duby, N. Clipstone, and S. Snyder.

References

Ausoni, S., Gorza, L., Schiaffino, S., Gundersen, K. and Lomo, T. (1990). Expression of myosin heavy chain isoforms in stimulated fast and slow rat muscles. *J. Neurosci.* **10**, 153-160.

Barton-Davis, E. R., LaFramboise, W. A. and Kushmerick, M. J. (1996). Activity-dependent induction of slow myosin gene expression in isolated fast-twitch mouse muscle. *Am. J. Physiol.* **271**, C1409-C1414.

Bassel-Duby, R. and Olson, E. N. (2003). Role of calcineurin in striated muscle: development, adaptation, and disease. *Biochem. Biophys. Res. Commun.* **311**, 1133-1141.

Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R. and Williams, R. S. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev.* **12**, 2499-2509.

Crabtree, G. R. and Olson, E. N. (2002). NFAT signaling. Choreographing the social lives of cells. *Cell* **109**, S67-S79.

Hennig, R. and Lomo, T. (1985). Firing patterns of motor units in normal rats. *Nature* **314**, 164-166.

Hogan, P. G., Chen, L., Nardone, J. and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205-2232.

Horsley, V. and Pavlath, G. K. (2002). Nfat: ubiquitous regulator of cell differentiation and adaptation. *J. Cell Biol.* **156**, 771-774.

Kirschbaum, B. J., Simoneau, J. A., Bar, A., Barton, P. J., Buckingham, M. E. and Pette, D. (1989). Chronic stimulation-induced changes of myosin light chains at the mRNA and protein levels in rat fast-twitch muscle. *Eur. J. Biochem.* **179**, 23-29.

Kirschbaum, B. J., Schneider, S., Izumo, S., Mahdavi, V., Nadal-Ginard, B. and Pette, D. (1990). Rapid and reversible changes in myosin heavy chain expression in response to increased neuromuscular activity of rat fast-twitch muscle. *FEBS Lett.* **268**, 75-78.

Kubis, H. P., Scheibe, R. J., Meissner, J. D., Hornung, G. and Gros, G. (2002). Fast-to-slow transformation and nuclear import/export kinetics of the transcription factor NFATc1 during electrostimulation of rabbit muscle cells in culture. *J. Physiol.* **541**, 835-847.

Lai, M. M., Luo, H. R., Burnett, P. E., Hong, J. J. and Snyder, S. H. (2000). The calcineurin-binding protein cain is a negative regulator of synaptic vesicle endocytosis. *J. Biol. Chem.* **275**, 34017-34020.

Liu, Y., Cseresnyes, Z., Randall, W. R. and Schneider, M. F. (2001). Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. *J. Cell Biol.* **155**, 27-39.

Lomo, T. (2003). Nerve-muscle interactions. In *Clinical Neurophysiology of Disorders of Muscle and the Neuromuscular Junction in Adults and Children: IFSCN Handbook of Clinical Neurophysiology, Vol. 4* (ed. E. Stålberg), pp. 47-65. Amsterdam: Elsevier.

McCullagh, K. J., Calabria, E., Pallafacchina, G., Ciciliot, S., Serrano, A. L., Argentini, C., Kalhovde, J. M., Lomo, T. and Schiaffino, S. (2004). NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proc. Natl. Acad. Sci. USA* **101**, 10590-10595.

Neal, J. W. and Clipstone, N. A. (2001). Glycogen synthase kinase-3 inhibits the DNA binding activity of NFATc. *J. Biol. Chem.* **276**, 3666-3673.

Okamura, H., Garcia-Rodriguez, C., Martinson, H., Qin, J., Virshup, D. M. and Rao, A. (2004). A conserved docking motif for CK1 binding controls the nuclear localization of NFAT1. *Mol. Cell. Biol.* **24**, 4184-4195.

Personius, K. E. and Balice-Gordon, R. J. (2001). Loss of correlated motor neuron activity during synaptic competition at developing neuromuscular synapses. *Neuron* **31**, 395-408.

Pette, D. and Vrbova, G. (1992). Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. *Rev. Physiol. Biochem. Pharmacol.* **120**, 115-202.

Rosenberg, P., Hawkins, A., Stiber, J., Shelton, J. M., Hutcheson, K., Bassel-Duby, R., Shin, D. M., Yan, Z. and Williams, R. S. (2004). TRPC3 channels confer cellular memory of recent neuromuscular activity. *Proc. Natl. Acad. Sci. USA* **101**, 9387-9392.

Rudolf, R., Mongillo, M., Magalhaes, P. J. and Pozzan, T. (2004). In vivo monitoring of $Ca(2^+)$ uptake into mitochondria of mouse skeletal muscle during contraction. *J. Cell Biol.* **166**, 527-536.

Schiaffino, S. and Serrano, A. (2002). Calcineurin signaling and neural control of skeletal muscle fiber type and size. *Trends Pharmacol. Sci.* **23**, 569-575.

Serrano, A. L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T. and Schiaffino, S. (2001). Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc. Natl. Acad. Sci. USA* **98**, 13108-13113.

Torgan, C. E. and Daniels, M. P. (2001). Regulation of myosin heavy chain expression during rat skeletal muscle development in vitro. *Mol. Biol. Cell* **12**, 1499-1508.

Wu, H., Rothermel, B., Kanatous, S., Rosenberg, P., Naya, F. J., Shelton, J. M., Hutcheson, K. A., DiMaio, J. M., Olson, E. N., Bassel-Duby, R. et al. (2001). Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *EMBO J.* **20**, 6414-6423.