

Regulation of the Growth of Multinucleated Muscle Cells by an NFATC2-dependent Pathway

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Abstract. The nuclear factor of activated T cells (NFAT) family of transcription factors regulates the development and differentiation of several tissue types. Here, we examine the role of NFATC2 in skeletal muscle by analyzing adult NFATC2^{-/-} mice. These mice exhibit reduced muscle size due to a decrease in myofiber cross-sectional area, suggesting that growth is blunted. Muscle growth was examined during regeneration after injury, wherein NFATC2-null myofibers form normally but display impaired growth. The growth defect is intrinsic to muscle cells, since the lack of NFATC2 in primary muscle cultures results in reduced cell size and myonuclear number in myotubes. Retroviral-mediated expression of

NFATC2 in the mutant cells rescues this cellular phenotype. Myonuclear number is similarly decreased in NFATC2^{-/-} mice. Taken together, these results implicate a novel role for NFATC2 in skeletal muscle growth. We demonstrate that during growth of multinucleated muscle cells, myoblasts initially fuse to form myotubes with a limited number of nuclei and that subsequent nuclear addition and increases in myotube size are controlled by a molecular pathway regulated by NFATC2.

Key words: NFATC2 • myotube • muscle growth • nuclear number • fusion

Introduction

Calcium is an important regulator of skeletal muscle physiology. Increases in intracellular calcium can activate the calmodulin-regulated phosphatase calcineurin. Calcineurin-dependent pathways have been implicated in expression of muscle-specific genes (Friday and Pavlath, 2001), myoblast differentiation (Delling et al., 2000; Friday et al., 2000), skeletal muscle hypertrophy (Musaro et al., 1999; Semsarian et al., 1999; Dunn et al., 2000), and regulation of slow myofiber-specific gene expression (Chin et al., 1998; Bigard et al., 2000; Delling et al., 2000; Naya et al., 2000).

One of the downstream targets of calcineurin is the nuclear factor of activated T cells (NFAT)¹ family of transcription factors (Rao et al., 1997). This family consists of four calcium-sensitive members, NFATC1–4. Under basal conditions, NFAT proteins are phosphorylated and localized to the cytoplasm. In response to increases in intracellular calcium, calcineurin becomes activated and dephosphorylates NFAT proteins, thereby allowing their nuclear translocation. Once

in the nucleus, NFAT proteins, in association with other transcription factors, bind to a consensus DNA sequence and activate gene transcription. Rephosphorylation of NFAT proteins by several kinases results in nuclear export.

Although originally described in T cells, recent evidence suggests that this family of transcription factors regulates development of several tissue types (de la Pompa et al., 1998; Ho et al., 1998; Ranger et al., 1998a,b, 2000; Kegley et al., 2001). Different NFAT proteins have distinct functions in both developing and postnatal tissues as revealed by the study of mice with targeted mutations in specific NFAT genes. Other studies indicate that multiple types of NFAT proteins can be expressed in a tissue, but only during specific stages of development do individual NFAT proteins undergo nuclear translocation in response to increases in intracellular calcium. Such developmental specificity in nuclear translocation is observed in T cells (Adachi et al., 2000) and skeletal muscle cells (Abbott et al., 1998; Delling et al., 2000). These results imply that different NFAT proteins may regulate specific sets of genes required for distinct phases of tissue development.

During myogenesis *in vitro*, mononucleated myoblasts are induced to withdraw from the cell cycle by a decrease in growth factors, initiate muscle specific gene expression, and subsequently fuse with one another to form multinucleated myotubes. Myotubes undergo further maturation

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¹Abbreviations used in this paper: CSA, cross-sectional area; DM, differentiation medium; EMyHC, embryonic MyHC; MyHC, myosin heavy chain; NFAT, nuclear factor of activated T cells; RT, reverse transcription; TA, tibialis anterior.

as evidenced by increases in size and changes in expression of contractile proteins. The various stages of myogenesis, myoblast, nascent myotube, and mature myotube, are not associated with differences in the amounts or types of NFAT proteins expressed (Abbott et al., 1998). However, treatment of muscle cells at different stages of myogenesis with the calcium ionophore ionomycin resulted in nuclear translocation of NFATC3 only in myoblasts, and NFATC1 and NFATC2 only in myotubes (Abbott et al., 1998). The nuclear translocation of NFATC2 was restricted to newly formed myotubes. This limited activation of NFATC2 suggested that NFATC2 might play a role in regulating the growth of multinucleated muscle cells.

During development of skeletal muscle tissue, myoblasts differentiate and fuse together to form multinucleated myofibers, which are analogous to myotubes *in vitro*. After myofiber formation during embryogenesis, the size of individual myofibers greatly increases during the immediate postnatal period. Satellite cells are a population of quiescent muscle precursor cells that exist within postnatal muscle tissue. During periods of growth, satellite cells re-enter the cell cycle and fuse with adjacent myofibers, thereby increasing the number of myonuclei within the myofiber (Moss and Leblond, 1971). Myonuclei exist within a common cytoplasm and are thought to regulate the expression of individual proteins for a defined amount of cytoplasm. This concept of a "myonuclear domain" suggests that the number of myonuclei limits the size of a myofiber (Allen et al., 1999). Supporting this theory, the fusion of satellite cells with myofibers is required for mammalian postnatal growth (Darr and Schultz, 1989; Rosenblatt and Parry, 1993; Mozdziaik et al., 2000) and hypertrophy (Rosenblatt et al., 1994; Phelan and Gonyea, 1997; Barton-Davis et al., 1999). However, the molecular mechanisms that regulate the addition of myonuclei during muscle growth are not well defined.

The differential regulation of NFATC2 during myogenesis led us to examine its role in the skeletal muscle of NFATC2^{-/-} mice. These mice have demonstrated that NFATC2 regulates development and differentiation of several tissue types. T cells from NFATC2^{-/-} mice (Hodge et al., 1996; Xanthoudakis et al., 1996) hyperproliferate and have abnormal cytokine production. In addition, chondrogenesis is induced in extraarticular tissues of older mice lacking NFATC2, implicating NFATC2 as a repressor of cartilage cell growth and differentiation (Ranger et al., 2000). In this report, we demonstrate a role for NFATC2 in skeletal muscle growth. We demonstrate that during growth of multinucleated muscle cells, myoblasts initially fuse to form myotubes with a limited number of nuclei and that subsequent nuclear addition and increases in myotube size are controlled by a molecular pathway regulated by NFATC2.

Materials and Methods

Animals

NFATC2^{-/-} mice were generated by heterozygous or homozygous matings of mice carrying a targeted disruption in the NFATC2 gene leading to a loss of function mutation (Hodge et al., 1996). NFATC3^{-/-} mice were generated by heterozygous matings of mice carrying a targeted disruption in the NFATC3 gene (Oukka et al., 1998). Wild-type mice controls for each of the mutant mice were generated from heterozygous matings. Mice

were genotyped by PCR analyses of tail DNA (Hodge et al., 1996; Oukka et al., 1998). Male mice between 8 and 12 wk of age were used for these studies. All animals were handled in accordance with the institutional guidelines of Emory University.

Collection of Muscles and Morphometric Measurements

Tibialis anterior (TA), soleus, and masseter muscles were collected using standardized dissection methods, embedded in TBS tissue freezing medium (Fisher Scientific), and frozen in isopentane cooled in liquid nitrogen. For histological analyses, 10–12 serial 14- μ m sections were collected along the entire length of the muscle at 400–500- μ m intervals and stained with hematoxylin and eosin. All analyses and photography were performed on an Axioplan (ZEISS) microscope equipped with a video camera and Scion Image and Adobe Photoshop[®] software.

The cross-sectional area (CSA) of myofibers was determined in the soleus, TA, and masseter muscles of wild-type and NFATC2^{-/-} mice. For the soleus, the CSA of sections throughout the soleus muscles was measured using computer-assisted morphometric measurements, and the region spanning the belly of the soleus was subsequently used for all further analyses described below. For the masseter and TA, anatomical markers of each muscle were used to find the same region in different samples, and these sections were subsequently used for analysis. The CSA of individual myofibers in the masseter and TA was determined by capturing an image in the center of each section and analyzing 100–250 myofibers within this 307,200- μ m² field.

TA muscles were subject to a standardized local freeze damage as described previously (Pavlati et al., 1998). In experiments analyzing regenerating TA muscles, sections containing the largest area of damage were selected for measurements. The core of the damaged area, as defined by the region that was the least regenerated, was visualized, and the image was captured to a computer screen. The number of centrally nucleated myofibers in this 307,200- μ m² field was determined on day 7 after injury. In addition, the CSA of individual regenerating myofibers was also measured at various time points after damage. Myofibers with CSA smaller than 100 μ m² were not included in the analysis so as not to mistake regenerating myofibers for mononucleated cells in the damaged area.

Immunohistochemistry

To analyze particular fiber types, cross sections of soleus muscles were stained with antibodies against type I (BA-D5) (Schiaffino et al., 1998) and type II (My32) (Sigma-Aldrich) myosin heavy chain (MyHC) as described (Kegley et al., 2001).

To quantify the number of myonuclei in wild-type and NFATC2^{-/-} soleus muscles, duplicate sections were incubated in antibody against dystrophin (MANDYS8, 1:400) (Sigma-Aldrich) for 1 h at room temperature or 4°C overnight. After washing in TNT (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20), the sections were incubated in Texas red-conjugated goat anti-mouse IgG (1:50) (Cappel). After further washing in TNT, the sections were fixed in 2% formaldehyde for 10 min at room temperature. The sections were then washed in TNT and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). Nuclei within the dystrophin-positive sarcolemma were counted for 100–150 myofibers, and the number of myonuclei was expressed per 100 myofibers.

Cell Culture

Primary cultures were derived from TA muscles of adult wild-type or NFATC2^{-/-} mice 2 d after induced muscle damage (Pavlati et al., 1998), and myoblasts were purified to >99% in selective medium (Rando and Blau, 1994). Myoblasts were grown in growth medium (Ham's F10, 20% FBS, 5 ng/ml bFGF, 200 U/ml penicillin G, 200 μ g/ml streptomycin) on collagen or entactin-collagen-laminin-coated (Upstate Biotechnology) dishes in a humidified 5% CO₂ atmosphere at 37°C. Differentiation was induced by switching confluent myoblast cultures grown on E-C-L-coated dishes to low serum, low mitogen differentiation medium (DM: DME, 2% horse serum, 200 U/ml penicillin G, 200 μ g/ml streptomycin) for 48–72 h.

In Vitro Nuclear Number Assays

Primary myoblasts were plated at 3×10^5 cells per well of 6-well dishes. After 2 h, cells were placed in DM and allowed to differentiate for 48–72 h before fixation in 3.7% formaldehyde. To identify multinucleated myotubes, cells were washed in PBS and incubated with blocking buffer containing 5% goat serum, 0.5% BSA, and 0.25% Triton X-100 in PBS for 1 h at room temperature, followed by incubation with an antibody against em-

myonic MyHC (EMyHC) (F1.652, neat hybridoma supernatant; Developmental Studies Hybridoma Bank) for 1 h at room temperature. Cells were washed in PBS/Tween and then incubated in biotinylated goat anti-mouse IgG (1:200) (Jackson ImmunoResearch Laboratories). Antibody binding was detected using Vectastain Elite ABC reagent (Vector Laboratories) and diaminobenzidine. The number of nuclei within individual myotubes (≥ 2 nuclei) was counted for 50–200 myotubes. Myotubes were grouped into two categories, those with two to four nuclei and those with five or more nuclei. The percentage of myotubes in each category was calculated.

Retroviral Plasmids, Retroviral Production, and Infection

A retroviral vector expressing full-length human NFATC2 was created by PCR amplification of NFATC2 cDNA from the vector pREP4-NFATC2 to create a 2.8-kb product (Ranger et al., 2000). The forward primer consisted of an SrfI site followed by bases 224–241 of the human NFATC2 mRNA (sequence data available from GenBank/EMBL/DDBJ under accession number U43342), whereas the reverse primer contained an Sall site and bases 2978–2998. PCR was performed using the Expand Long Template PCR system (Roche), and the PCR product was digested with SrfI and Sall. The amplified NFATC2 cDNA was then cloned into a basic retroviral vector in which expression of NFATC2 is driven by the retroviral 5' long terminal repeat. Production of infectious retrovirus and infection of primary myoblasts were performed as described previously (Abbott et al., 1998). The efficiency of one round of retroviral infection is estimated at >60 and $>90\%$ after two rounds based on visualization of cells infected with vectors containing green fluorescent protein.

Reporter Assays

Myoblasts in 24-well plates were infected by two rounds of infection with a combination of either control or NFATC2 retroviruses and an NFAT-responsive reporter retrovirus (Abbott et al., 1998). 24 h after the last infection, the medium was replaced with DM, and the cells were allowed to differentiate for 48 h. Cells were collected and assayed for luciferase as described previously (Abbott et al., 1998).

Immunoblotting

Cells were lysed with RIPA-2 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (Mini Complete; Roche). Equal amounts of protein (10 $\mu\text{g}/\text{lane}$) (Bradford, 1976) were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore). Immunoblots were performed as described (Friday et al., 2000), and antibody binding was detected using enhanced chemiluminescence.

Northern Blotting

RNA was prepared from TA muscles using Trizol Reagent (Life Technologies) following the manufacturer's protocol. RNA (10 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes. Membranes were hybridized with random-primed cDNA probes (Rediprime II; Amersham Pharmacia Biotech) labeled with [α - ^{32}P]dCTP in Rapid-hyb buffer (Amersham Pharmacia Biotech). After high stringency washing, membranes were visualized by autoradiography.

Relative Reverse Transcription PCR

RNA was isolated using Trizol Reagent (Life Technologies) from wild-type and NFATC2 $^{-/-}$ myoblasts in growth medium. Reverse transcription (RT)-PCR was performed in duplicate for each sample using primers specific for NFATC1 (sense, 5'-TCCGGCGCATGCGAGCCGTCATC-GACTGTGCTGGGATCCTGA-3'; antisense, 5'-GGACCCGGGTCA-ATTGGCAGGAAGGTACGTGAAACG-3'), NFATC4 (sense, 5'-GAGGAGCCCTACCGGACTC-3'; antisense, 5'-AAGGGG-CGGGAAGGAAGG-3'), and NFATC3 (sense, 5'-CCCAAGAA-GAATCAGAAGACTGTG-3'; antisense, 5'-GCTCTAAAGATGGC-TCCGTGCTTAAGG-3'). All RT reactions were performed using 2.5 μg of total RNA, and all PCR products were amplified in their linear range. To quantify the amplicons, the PCR reactions were spiked with [α - ^{32}P]dCTP (specific activity 3,000 Ci/mmol) (ICN Biomedicals), and 18S rRNA was used as an internal control in each sample using QuantumRNA 18S primers (Ambion). The products were resolved using 6% nondenaturing PAGE, and individual band intensities in the dried gel were determined using a PhosphorImager (Molecular Dynamics).

The density of the amplicons for each NFAT isoform was normalized to the density of the 18S rRNA amplicon and expressed as arbitrary units.

Results

Myofiber CSA Is Decreased in NFATC2 $^{-/-}$ Mice

Our previous studies in cultured muscle cells indicated that individual NFAT isoforms are specifically induced to undergo nuclear translocation in response to changes in intracellular calcium at different stages in myogenesis (Abbott et al., 1998). Such activation of NFATC2 occurred only in small nascent myotubes, suggesting that NFATC2 may play a role in regulating growth of differentiated muscle cells. To test this hypothesis in vivo, we analyzed the soleus muscles from adult wild-type and NFATC2 $^{-/-}$ mice. Soleus muscles were examined because in BALB/c mice this muscle is composed of approximately equal amounts of slow and fast myofibers (Kegley et al., 2001), allowing us to determine if NFATC2 has similar roles in different fiber types. As seen in the top of Fig. 1 A, the CSA of soleus muscles from NFATC2 $^{-/-}$ mice is smaller than wild-type muscles. This size difference correlates with a decrease in the CSA of individual myofibers (Fig. 1 A, bottom). To determine if type I or type II myofibers are differentially affected by the absence of NFATC2, the CSA of myofibers expressing type I and type II MyHC was measured. The CSA of both type I and type II myofibers is decreased significantly in NFATC2 $^{-/-}$ mice (Fig. 1 B) with a 44 and 33% decrease, respectively. These results suggest that NFATC2 plays a role in regulating myofiber size.

The CSA of an entire muscle is affected not only by the size of individual myofibers but also by the number of myofibers. We have shown previously that muscles of adult NFATC3 $^{-/-}$ mice are smaller than wild-type muscles due to a decrease in the number but not the CSA of myofibers (Kegley et al., 2001). This decrease in myofiber number is a result from a defect in myofiber formation during embryonic development. Thus, we analyzed the total number of myofibers in soleus muscles from adult wild-type and NFATC2 $^{-/-}$ mice to determine if NFATC2 also plays a role in regulating myofiber number. No difference in the number of myofibers is observed between wild-type and NFATC2 $^{-/-}$ mice (Fig. 1 C). Thus, in direct contrast to NFATC3 $^{-/-}$ mice, the smaller size of NFATC2 $^{-/-}$ soleus muscles is due to a decrease in the CSA and not in the number of myofibers. These data suggest that myofiber formation during embryogenesis is normal in NFATC2 $^{-/-}$ mice but that prenatal and postnatal growth of myofibers is altered.

Since calcineurin-dependent signaling pathways have been proposed to regulate MyHC isoform expression (Chin et al., 1998; Bigard et al., 2000; Delling et al., 2000; Naya et al., 2000), we also examined whether fiber-type proportions are altered in NFATC2 $^{-/-}$ mice. Serial cross sections of soleus muscles were stained with antibodies recognizing type I or type II MyHC (Fig. 1 D, left). The percentage of myofibers expressing type I MyHC is slightly but significantly decreased by 5% in NFATC2 $^{-/-}$ muscles compared with wild-type (Fig. 1 D, right). No difference was noted in the percentage of myofibers expressing type II MyHC (Fig. 1 D, right). Changes in one MyHC isoform do not necessarily correlate with changes in another MyHC isoform, since myofibers can coexpress different

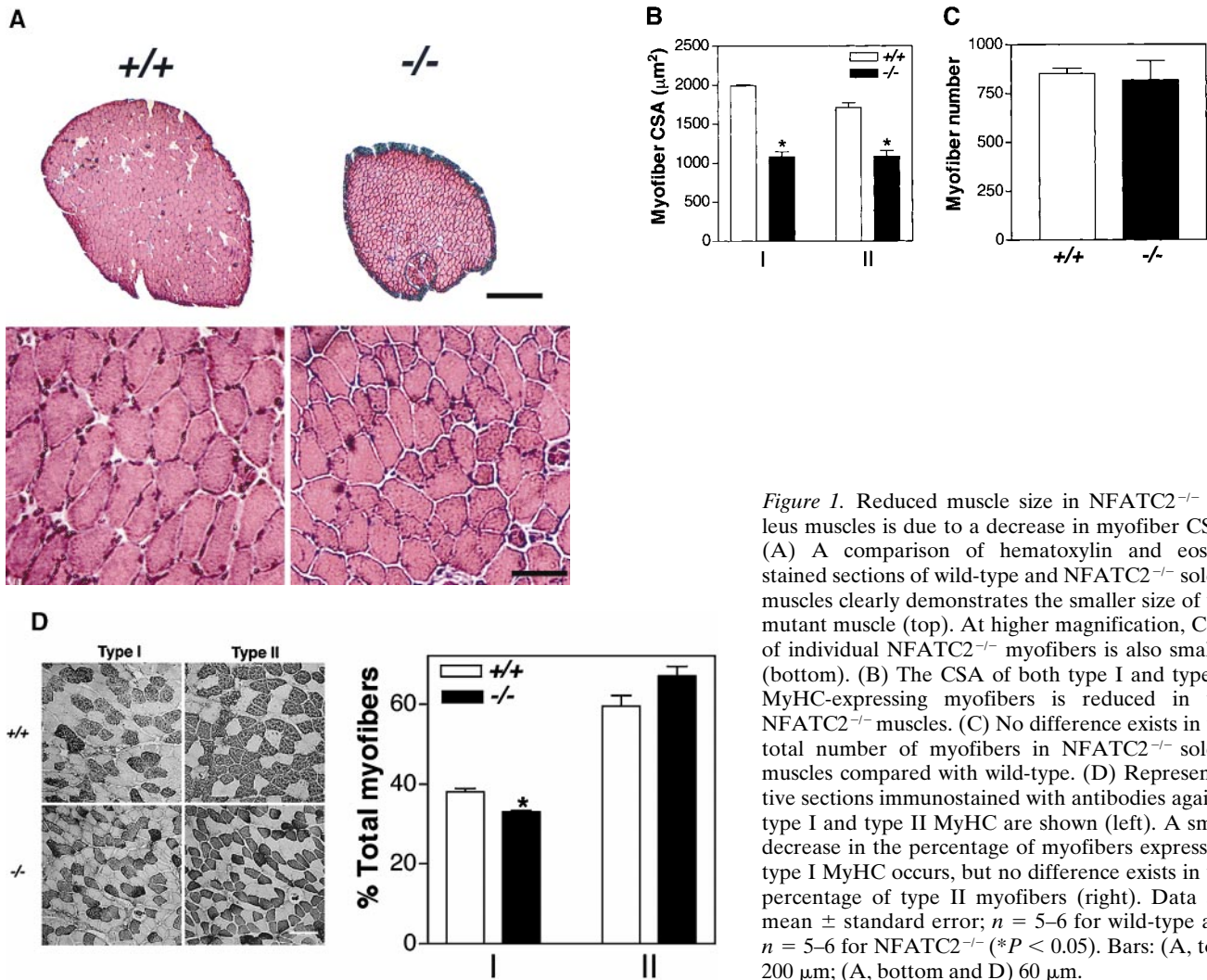


Figure 1. Reduced muscle size in *NFATC2*^{-/-} soleus muscles is due to a decrease in myofiber CSA. (A) A comparison of hematoxylin and eosin-stained sections of wild-type and *NFATC2*^{-/-} soleus muscles clearly demonstrates the smaller size of the mutant muscle (top). At higher magnification, CSA of individual *NFATC2*^{-/-} myofibers is also smaller (bottom). (B) The CSA of both type I and type II MyHC-expressing myofibers is reduced in the *NFATC2*^{-/-} muscles. (C) No difference exists in the total number of myofibers in *NFATC2*^{-/-} soleus muscles compared with wild-type. (D) Representative sections immunostained with antibodies against type I and type II MyHC are shown (left). A small decrease in the percentage of myofibers expressing type I MyHC occurs, but no difference exists in the percentage of type II myofibers (right). Data are mean \pm standard error; $n = 5-6$ for wild-type and $n = 5-6$ for *NFATC2*^{-/-} (* $P < 0.05$). Bars: (A, top) 200 μm ; (A, bottom and D) 60 μm .

MyHC isoforms. Thus, *NFATC2* does not appear to play a major role in regulating the expression of MyHC isoforms.

To determine if the reduced myofiber CSA in *NFATC2*^{-/-} mice is specific to the soleus muscle or a generalized muscle phenomenon, we analyzed myofiber CSA of TA and masseter muscles. These muscles were chosen for analysis because they differ from the soleus in several properties. In contrast with the soleus muscle, which is composed mainly of type I and type IIA MyHC isoforms, the TA contains primarily type IIB, and the masseter contains predominantly type IIX MyHC (Eason et al., 2000). In addition, the masseter exhibits differences in myoblast populations compared with limb muscles (Pavlath et al., 1998). The myofiber CSA of the TA is decreased 25% in *NFATC2*^{-/-} mice compared with wild-type (Fig. 2). A similar decrease is observed in the masseter (Fig. 2). These data suggest that *NFATC2* has a general role in regulating myofiber growth.

Myofiber Growth Is Defective in NFATC2^{-/-} Mice during Regeneration

During postnatal development and during regeneration after injury, myofibers grow in size, a process involving addition of nuclei to the myofiber. Since myonuclei are post-mitotic, myofiber growth involves the fusion of muscle precursor cells with myofibers. These muscle precursor

cells, called satellite cells, lie underneath the basal lamina surrounding each myofiber and are in close juxtaposition to the myofiber itself. Satellite cells are normally quiescent but become activated and start proliferating in response to growth factors. Since satellite cells are required for skeletal muscle regeneration (Robertson et al., 1992; Quinlan et al., 1997) and muscle growth (Darr and Schultz, 1989; Rosenblatt and Parry, 1993; Mozdziaik et al., 2000), we used regenerating muscle as a model to examine satellite cell function in *NFATC2*^{-/-} mice. Northern analysis was performed on regenerating TA muscles of wild-type and *NFATC2*^{-/-} mice on day 4 after injury using myoD and myogenin as markers of activation/proliferation and differentiation, respectively. At this time point, satellite cells have become activated and are proliferating and beginning to differentiate. No differences are observed in the levels of either MyoD or myogenin mRNA (Fig. 3 A). In addition, the number of myoblasts isolated from wild-type and *NFATC2*^{-/-} muscles 2 d after regeneration does not differ (data not shown), further suggesting that *NFATC2*^{-/-} satellite cells are able to activate and proliferate normally. We next analyzed the ability of satellite cells to fuse and form new myofibers. When myofibers form after injury, their nuclei are centrally localized, facilitating identification of regenerating myofibers. The number of cen-

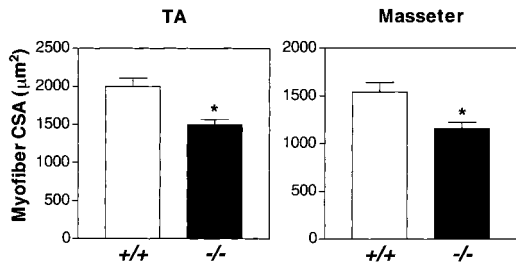


Figure 2. Myofiber CSA in other muscles is also reduced in NFATC2^{-/-} mice. The CSA of individual myofibers was measured in TA and masseter muscles of wild-type and NFATC2^{-/-} mice. Data are mean \pm standard error; $n = 5-8$ for wild-type and $n = 3$ for NFATC2^{-/-} (* $P < 0.05$).

trally nucleated myofibers in the core of the injury 7 d after injury was counted as described in Materials and Methods. At this time point, the area of injury is completely filled with these regenerating myofibers. As seen in Fig. 3 B, no significant difference exists in the number of regenerated myofibers between wild-type and NFATC2^{-/-} mice. Therefore, satellite cell function appears normal in the early stages of muscle regeneration in the absence of NFATC2.

To determine if NFATC2 regulates further growth of regenerating myofibers, the CSA of regenerating myofibers was measured at different times after injury. At day 7 after injury, the CSA of regenerating myofibers is the same for both wild-type and NFATC2^{-/-}. As further growth of the regenerating myofibers occurs, the CSA of myofibers in the mutant at each time point is significantly decreased compared with wild-type (Fig. 3 C). At 50 d after injury, both wild-type and NFATC2^{-/-} myofibers are almost completely recovered to the normal size for each genotype (Fig. 2 A). However, the CSA of regenerating wild-type and NFATC3^{-/-} myofibers is not different at various time points after damage, indicating the specificity of NFATC2 in regulating muscle growth (Fig. 3 C, bottom inset). Taken together, these data demonstrate that myofiber growth but not formation is impaired during regeneration of NFATC2^{-/-} mice. Thus, the function of NFATC2 is required for growth of myofibers as suggested by our previous *in vitro* studies (Abbott et al., 1998).

Defects in Myofiber Growth in NFATC2^{-/-} Mice Are Muscle Cell Intrinsic

Myofiber growth is dependent on both nonmuscle and muscle cells. To determine if the defects observed in myofiber growth in NFATC2^{-/-} mice are intrinsic to muscle cells, myoblasts were isolated from wild-type and NFATC2^{-/-} mice and studied *in vitro*. To determine if NFATC2^{-/-} myoblasts can differentiate normally, immunoblot analyses were performed on muscle cells in DM using antibodies against myogenin and EMyHC, markers of myogenic commitment and differentiation, respectively (Merlie and Gros, 1976; Andres and Walsh, 1996). Myogenin levels increase at 24 h and are downregulated at 48 h in both wild-type and NFATC2^{-/-} cells (Fig. 4 A). A small amount of myogenin is detected in both types of myoblasts, presumably due to spontaneous differentiation in the cultures. EMyHC is expressed at similar levels in both

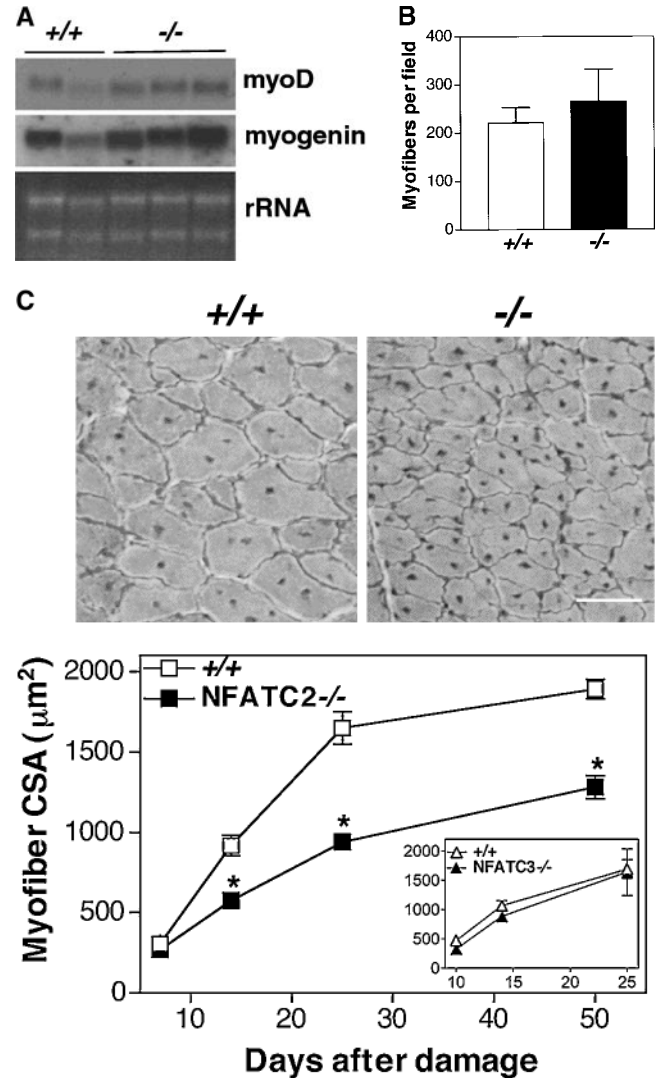


Figure 3. Regenerating myofibers form, but their growth is impaired in NFATC2^{-/-} mice. (A) Northern blots of TA muscles from wild-type and NFATC2^{-/-} mice 4 d after injury are shown. A portion of an ethidium bromide-stained gel containing 28S and 18S rRNAs demonstrates relative RNA loading. (B) 7 d after injury, TA muscles were collected from wild-type and NFATC2^{-/-} mice, and the number of regenerated myofibers was counted in sections from the central region of the lesion. (C) A comparison of hematoxylin and eosin-stained sections of regenerating TA muscles at day 25 after injury demonstrates the smaller size of the mutant myofibers (top). The CSA of regenerated myofibers in the central region of the lesion was determined for various time points after injury (bottom). Data are mean \pm standard error; $n = 3-7$ for each genotype (* $P < 0.05$). Bar, 60 μm .

cell types after 24 and 48 h in DM. Thus, the expression patterns of myogenin and EMyHC in wild-type and NFATC2^{-/-} muscle cells are similar, indicating that the early stages of muscle differentiation are not regulated by NFATC2. Consistent with these results, after 48 h in DM wild-type and NFATC2^{-/-} cultures appear morphologically differentiated, containing many multinucleated cells with an elongated appearance. However, NFATC2^{-/-} cells form myotubes that are smaller than wild-type myotubes (Fig. 4 B), indicating a muscle-intrinsic role for NFATC2 in regulating myotube size.

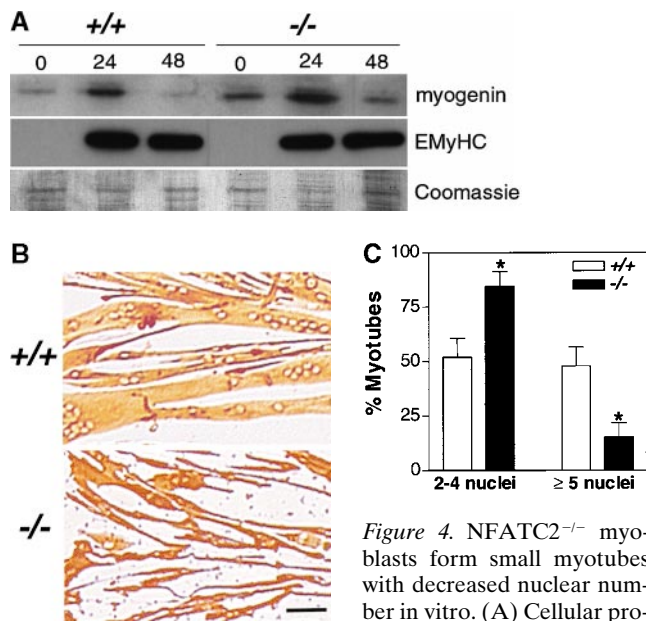


Figure 4. NFATC2^{-/-} myoblasts form small myotubes with decreased nuclear number in vitro. (A) Cellular proteins from wild-type and

NFATC2^{-/-} myoblasts were collected at various time points during differentiation and analyzed by immunoblotting for myogenin and EMyHC. The expression patterns of myogenin and EMyHC are similar between differentiating wild-type and NFATC2^{-/-} muscle cells. A portion of a Coomassie-stained gel demonstrates relative protein loading. (B) Wild-type and NFATC2^{-/-} myoblasts were induced to differentiate in DM for 48 h and then immunostained for EMyHC. (C) The number of nuclei within individual myotubes (at least two nuclei) was counted. Myotubes were grouped into two categories, and the percentage of myotubes in each category was determined. Each bar represents mean \pm standard error of three independent cell isolates each performed in duplicate (* $P < 0.05$). Bar, 60 μ m.

Since myofiber size correlates with myonuclear number (Allen et al., 1999), the formation of smaller myotubes could result from defects in either myoblast–myotube fusion or myotube–myotube fusion. Thus, assays were performed on myotube cultures after 48 h in DM to compare the number of nuclei within wild-type and NFATC2^{-/-} myotubes. To clearly define the nuclei within myotubes, the myotubes were immunostained for EMyHC and the number of nuclei within individual myotubes (at least two nuclei) was counted. Myotubes were grouped into two categories, those with two to four nuclei and those with five or more nuclei. The percentage of the myotubes in each category was calculated. The majority of NFATC2^{-/-} myotubes contain two to four nuclei (Fig. 4 C), whereas wild-type myotubes are equally distributed between the two nuclear groupings. In addition, an increased number of myotubes occurs in the mutant cultures (data not shown). These results suggest that NFATC2^{-/-} muscle cells can differentiate and fuse to form the initial multinucleated cell but are unable to recruit the fusion of myoblasts or myotubes efficiently and thus remain small compared with wild-type myotubes. The increased number of myotubes in the NFATC2^{-/-} cultures suggests that if myoblasts are not recruited into growing myotubes, they differentiate and fuse with other myoblasts, thus forming many small myotubes. Alternatively, many small myotubes could result from an inability of NFATC2^{-/-} myotubes to fuse with one another.

Retroviral Expression of NFATC2 Rescues the Defects in Myotube Size and Nuclear Number

To confirm that the absence of NFATC2 is responsible for the observed defects in myotube size and nuclear number in vitro, two types of experiments were performed. First, the expression levels of other NFAT isoforms in the mutant cells were analyzed to rule out compensatory changes that could contribute to defects in myotube size. We analyzed the expression of the three other known calcium-responsive NFAT isoforms, NFATC1, NFATC3, and NFATC4, in wild-type and NFATC2^{-/-} cells. RT-PCR was performed on RNA isolated from high-density wild-type and NFATC2^{-/-} myoblasts using gene-specific primers and normalized to the expression of 18S RNA. NFATC4 was not detected in either genotype (data not shown). The expression of NFATC1 and NFATC3 is not significantly different between the two types of muscle cells (Fig. 5 A). Thus, the absence of NFATC2 does not alter the expression of NFATC1 or NFATC3 in skeletal muscle cells. These results are consistent with the results of Ranger et al. (2000), indicating that no compensatory changes occur in the expression of individual NFAT isoforms in chondrocytes lacking NFATC2.

In the second line of experiments, NFATC2^{-/-} myoblasts were infected with a retrovirus expressing NFATC2 to determine if expression of NFATC2 could rescue the reduced size and nuclear number of NFATC2^{-/-} myotubes. Initially, the activity of the recombinant NFATC2 was demonstrated in wild-type cells containing an NFAT-responsive reporter construct (Fig. 5 B). Myoblasts infected with either control or NFATC2 retroviruses were induced to differentiate, and luciferase activity was determined. Luciferase activity is increased in NFATC2-infected cells by approximately sevenfold, confirming the transcriptional activity of the recombinant NFATC2 in skeletal muscle cells. Subsequently, nuclear number assays were performed on myotubes in wild-type and NFATC2^{-/-} cultures infected with either control or NFATC2 retrovirus. No significant difference is observed in nuclear number of wild-type cells infected with either control or NFATC2 retrovirus (data not shown), suggesting that overexpressing NFATC2 in wild-type muscle cells does not affect size or nuclear number of myotubes. However, NFATC2^{-/-} muscle cells infected with the NFATC2 retrovirus form myotubes similar in size to wild-type myotubes (Fig. 5 C) and with nuclear number proportions similar to wild-type (Fig. 5 D). Together, these results show that the absence of NFATC2 is responsible for the defects in myotube size and nuclear number in NFATC2^{-/-} cells.

Myonuclear Number Is also Decreased in NFATC2^{-/-} Soleus Muscles

The reduced number of nuclei in NFATC2^{-/-} myotubes suggests that NFATC2^{-/-} myofibers in vivo may also have reduced myonuclei. To determine if myonuclear number is reduced in NFATC2^{-/-} myofibers, cross sections of soleus muscles were immunostained with dystrophin to outline the myofiber sarcolemma and stained with DAPI to label nuclei (Fig. 6 A). Nuclei inside the outline of dystrophin were counted in wild-type and NFATC2^{-/-} soleus muscles. A 50% decrease in myonuclei is observed in NFATC2^{-/-} soleus muscles compared with wild-type (Fig.

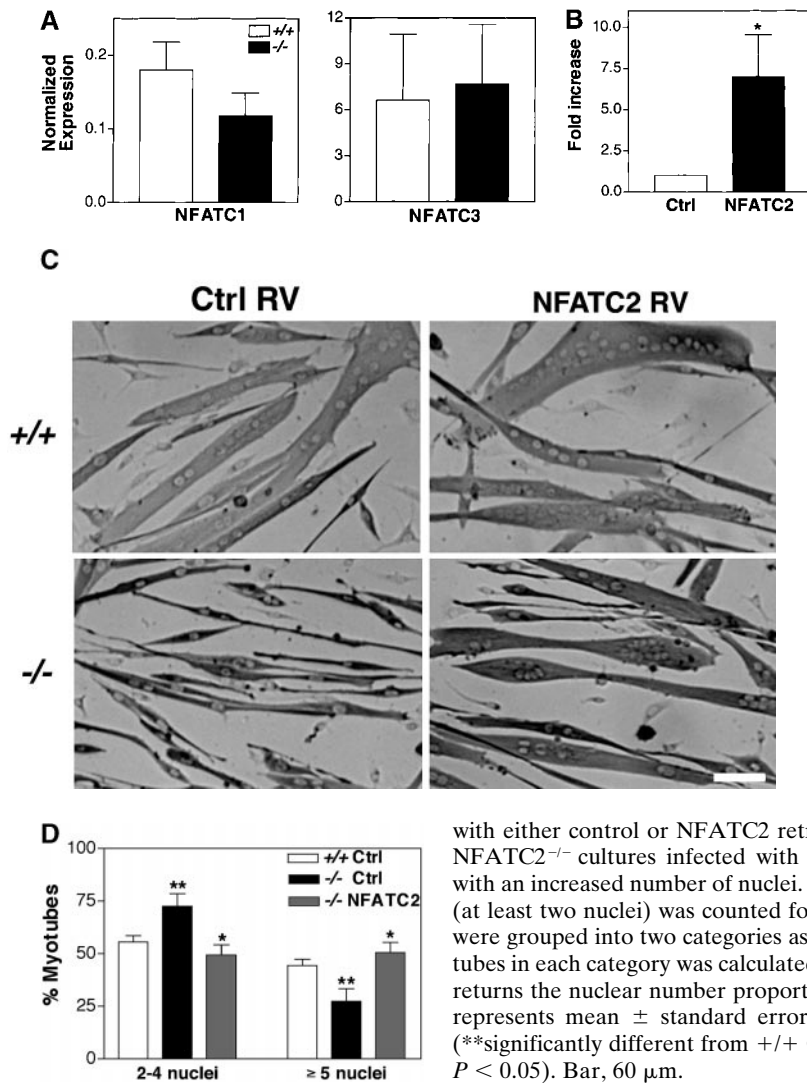


Figure 5. The lack of NFATC2 in mutant myotubes is responsible for the cellular phenotype. (A) RT-PCR of RNA from wild-type and NFATC2^{-/-} myoblasts was performed for various NFAT isoforms and normalized to the expression of 18S rRNA. NFATC4 is not expressed in myoblasts of either genotype (data not shown). Data are mean \pm standard error; $n = 3$ for each genotype. (B) Primary myoblasts were infected with an NFAT-responsive reporter and either control (Ctrl) or NFATC2 retroviruses. Cells were induced to differentiate, and luciferase assays were subsequently performed. Data are reported as fold increase in luciferase activity over control cells. Each bar represents the mean \pm standard error of four independent experiments each performed in triplicate ($*P < 0.05$). (C) Wild-type and NFATC2^{-/-} myoblasts were infected

with either control or NFATC2 retroviruses (RV) and induced to differentiate for 48 h. NFATC2^{-/-} cultures infected with NFATC2 retrovirus display increased myotube size with an increased number of nuclei. (D) The number of nuclei within individual myotubes (at least two nuclei) was counted for each of the retrovirally infected cultures. Myotubes were grouped into two categories as in the legend to Fig. 4 C, and the percentage of myotubes in each category was calculated. The expression of NFATC2 in NFATC2^{-/-} cultures returns the nuclear number proportions back to that of wild-type distributions. Each bar represents mean \pm standard error of three experiments each performed in duplicate (**significantly different from +/+ Ctrl $P < 0.05$; *significantly different from -/- Ctrl $P < 0.05$). Bar, 60 μ m.

6 B). These data correlate with the *in vitro* results, showing that NFATC2^{-/-} myotubes have fewer nuclei than wild-type myotubes. Since fusion of myoblasts and thus addition of myonuclei is required for growth of mammalian myofibers (Darr and Schultz, 1989; Rosenblatt and Parry, 1993; Mozdziaik et al., 2000), these data also suggest that the reduced CSA of myofibers in NFATC2^{-/-} mice results from defects in the ability of muscle cells to fuse with the myofiber and to add nuclei to the myofiber.

Discussion

We have shown previously that individual NFAT proteins translocate to the nucleus of muscle cells at specific stages of myogenesis (Abbott et al., 1998). Since NFATC2 translocates to the nucleus only in newly formed myotubes, we examined whether NFATC2 has a role in muscle growth using NFATC2^{-/-} mice. Histological analyses of soleus muscles of adult NFATC2^{-/-} mice revealed that the mutant mice have smaller soleus muscles due to a decrease in the CSA of myofibers. Other muscles with divergent functions, MyHC composition, and embryonic origins, such as the TA and masseter, also exhibit decreases in myofiber CSA in NFATC2^{-/-} mice. To determine if this defect is

due to impaired myofiber growth, we examined regenerating skeletal muscle as a model for myofiber growth. Injured muscles in NFATC2^{-/-} mice can form new myofibers normally, but subsequent myofiber growth is impaired. Defects in myofiber growth are intrinsic to muscle cells since cultured muscle cells lacking NFATC2 form small myotubes with few nuclei. Retroviral expression of recombinant NFATC2 in the mutant muscle cells rescues the defects in myotube size and nuclear number, indicating that the lack of NFATC2 is responsible for the observed cellular phenotype of NFATC2^{-/-} cells. The reduction in the number of myonuclei *in vitro* is also found *in vivo* in myofibers of NFATC2^{-/-} mice. Taken together, these data implicate a novel role for NFATC2 in skeletal muscle growth.

Muscle growth results from an ordered sequence of events. Withdrawal of mitogens *in vitro* causes myoblasts to exit the cell cycle and activate the expression of differentiation specific genes. Subsequently, myoblasts migrate toward one another, elongate, align, and through cell-cell interactions fuse together to form a multinucleated cell. Multiple proteins are required for migration and fusion of myoblasts such as integrins and other cell adhesion molecules, metalloproteases, and phospholipases (Knudsen, 1992). In addition, increases in intracellular calcium (Con-

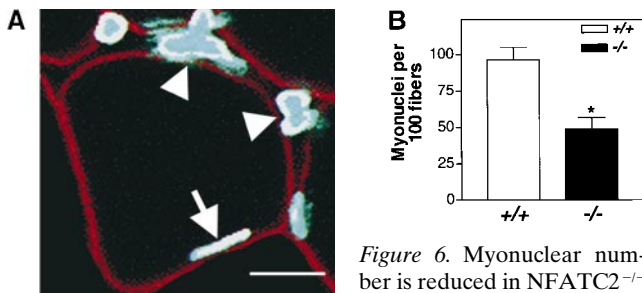


Figure 6. Myonuclear number is reduced in NFATC2^{-/-} myofibers. (A) A representative wild-type myofiber immunostained with an antibody against dystrophin (red) and stained with DAPI (blue) illustrates the myonuclear number assay. Arrow indicates a myonucleus within the dystrophin border, and arrowheads indicate nuclei outside the myofiber. (B) DAPI-stained nuclei within the dystrophin-positive sarcolemma were counted in soleus muscles from wild-type and NFATC2^{-/-} mice, and the number of myonuclei was expressed per 100 myofibers. Data are mean \pm standard error; $n = 6$ for each genotype ($*P < 0.05$). Bar, 20 μ m.

stantin et al., 1996) and several calcium-dependent molecules (Barnoy et al., 1996; Gorza and Vitadello, 2000) are essential for myoblast fusion, including calmodulin (Kim et al., 1992), which can regulate calcineurin. Once myotubes form, additional myoblasts fuse with the myotube, and the myotube grows in size. Postnatal muscle growth in vivo is also characterized by myoblast fusion with myofibers, leading to an increase in myonuclear number. Numerous studies indicate that myoblasts are critical for muscle growth in vivo. When the proliferative capacity of myoblasts is attenuated, increases in myonuclear number and myofiber size are blocked in growing rats (Darr and Schultz, 1989; Rosenblatt and Parry, 1993; Mozdziaik et al., 2000). Additionally, the lack of myoblast growth factors such as leukemia inhibitory factor (Kurek et al., 1997) leads to reductions in the size of myofibers after regeneration. Thus, numerous molecules contribute to proper muscle cell size.

Our data indicate that NFATC2 is involved in muscle growth. Myoblast proliferation and differentiation occur normally in NFATC2^{-/-} muscle. However, the reduced number of nuclei in NFATC2^{-/-} myotubes and myofibers suggests that a later stage involving the fusion of differentiated muscle cells with the multinucleated muscle cell is impaired. The decrease in the number of nuclei is correlated with a decrease in myotube size, implicating an NFATC2-dependent pathway in the control of myotube

size. Since a few NFATC2^{-/-} myotubes do form with large number of nuclei, NFATC2-independent pathways for muscle growth also must exist. However, the majority of NFATC2^{-/-} myotubes do not have increased nuclear number and are small in size. Thus, the downstream targets of NFATC2 allow the fusion of differentiated muscle cells with newly formed myotubes and the subsequent growth of the myotube. In addition, our data suggest that fusion of differentiated muscle cells with myotubes/myofibers is distinct from the initial fusion of myoblasts to form a multinucleated muscle cell.

Based on our results, we present the model outlined in Fig. 7 for the NFATC2-mediated signaling pathway that leads to muscle growth. Increases in intracellular calcium lead to the activation of calcineurin. Calcineurin dephosphorylates NFATC2 in newly formed myotubes, permitting nuclear translocation of NFATC2. Once in the nucleus, NFATC2 along with other transcription factors influences gene transcription. NFATC2 may either directly or indirectly regulate gene transcription of a cell surface protein (pathway 1) that mediates cell-cell interaction or cell fusion between mononucleated muscle cells and newly formed myotubes. Evidence exists for cell surface proteins that mediate the fusion of myoblasts with myotubes. The integrin very late antigen 4 on multinucleated muscle cells and its counterreceptor vascular cell adhesion molecule 1 on myoblasts are thought to mediate myoblast fusion with myofibers during development (Rosen et al., 1992). In addition, glycoproteins may mediate myoblast-myotube interactions to allow fusion and muscle growth, since wheat germ agglutinin can block myoblast fusion with myotubes and decrease myotube size (Muroya et al., 1994). Alternatively, NFATC2 could either directly or indirectly regulate gene transcription of a secreted protein (pathway 2) that recruits differentiated mononucleated myoblasts to fuse with adjacent myotubes. Secreted factors apparently can regulate the fusion of cells with myotubes. Fibroblasts cocultured with young or old myotubes can acquire myogenic characteristics and fuse with myotubes (Breton et al., 1995), suggesting that a secreted factor from the myotube recruits fibroblast fusion. Though not shown, both pathway 1 and 2 could also mediate myotube-myotube fusion. Specific molecules downstream from NFATC2 that contribute to muscle growth, though not examined in this study, are currently being investigated.

Our data suggest that multinucleated muscle cells may regulate the fusion of myoblasts/myotubes. Myotube-

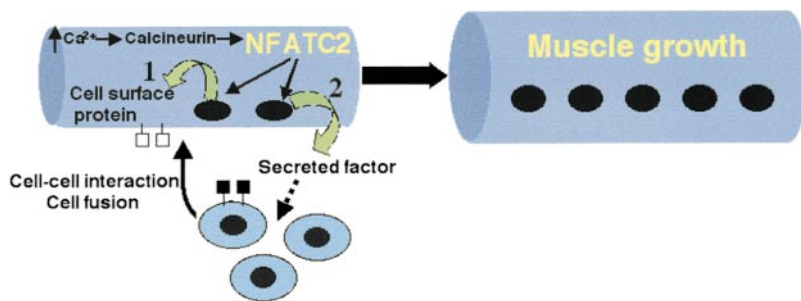


Figure 7. Model for the role of NFATC2 in the growth of myotubes. Increases in intracellular calcium activate the phosphatase calcineurin, which dephosphorylates NFATC2 in nascent myofibers causing nuclear translocation of NFATC2. Myofibers lacking NFATC2 have reduced nuclear number and size. Thus, the downstream targets of NFATC2 recruit fusion of myoblasts/myotubes with multinucleated muscle cells and contribute to increases in muscle size. Only myoblast fusion is represented in

the model. NFATC2 may regulate a cell surface protein (pathway 1) that mediates cell-cell interaction or cell fusion between differentiated muscle cells and newly formed myofibers. Alternatively, NFATC2 could regulate gene transcription of a secreted protein (pathway 2) that recruits differentiated muscle cells to fuse with adjacent myofibers.

myotube fusion has been suggested to occur during regeneration (Robertson et al., 1990), but nothing is known about its regulation. Several lines of evidence implicate multinucleated cells as controlling the site and extent of myoblast fusion. During the development of mammalian skeletal muscle, primary myofibers form initially and are followed by the formation of secondary myofibers. Primary myofibers control the site of secondary myofiber assembly, since secondary myofibers form only at the site of innervation on the primary myofiber, independently of the nerve (Duxson et al., 1989; Duxson and Sheard, 1995). In addition, the primary myofiber seems to restrict the fusion of secondary myoblasts, whereas the secondary myofiber seems to recruit fusion, since secondary myoblasts fuse primarily with the forming secondary myofiber (Harris et al., 1989; Zhang and McLennan, 1995). This specificity of fusion shares analogy with *Drosophila* muscle development in which founder myoblasts express dumbfounded, an attractant for myoblast fusion, and recruit fusion-competent myoblasts to fuse with founder myoblasts and not with other myoblasts (Ruiz-Gomez et al., 2000). The mechanisms used by founder myoblasts may share similarities with how a newly formed myotube or myofiber recruits myoblasts/myotubes to fuse. Further suggesting that myofibers can control the location of myoblast fusion, myofibers elongate by fusion of myoblasts at their ends during mammalian postnatal growth (Williams and Goldspink, 1971; Zhang and McLennan, 1995). The mechanisms by which myofibers regulate the fusion of myoblasts are unknown but may involve expression of proteins such as those mentioned above. This control of fusion by multinucleated muscle cells is likely one mechanism by which the size of muscle cells is regulated.

Since increased intracellular calcium activates NFATC2 only in newly formed myotubes (Abbott et al., 1998) and calcineurin is the only known phosphatase that can control nuclear translocation of NFAT proteins, NFATC2 is likely regulated by calcineurin to control myotube/myofiber growth. Calcineurin has been shown to be involved in skeletal muscle hypertrophy (Musaro et al., 1999; Semsarian et al., 1999; Dunn et al., 2000). The inability of NFATC2 overexpression to induce an increase in myotube size and nuclear number in wild-type myotubes suggests that NFATC2 is not involved in skeletal muscle hypertrophy. Several possibilities exist to explain this lack of effect on growth of wild-type myotubes. (a) Calcineurin may not activate the recombinant NFATC2 once myotubes reach a certain state of maturity, after the addition of further myonuclei. This hypothesis is supported by the fact that NFATC2 cannot be activated by a calcium ionophore in mature myotubes (Abbott et al., 1998). (b) Other convergent pathways may be necessary for NFATC2-mediated effects on cell growth. These pathways could involve the activation of additional transcription factors necessary for forming a transcriptional complex with NFATC2. (c) Changes may occur in unfused myoblasts or myotubes with time such that they are either no longer responsive to a putative secreted factor regulated by NFATC2 or they downregulate a cell surface molecule necessary for fusion with myotubes. (d) Limits may exist on the size of primary myotubes in culture. Thus, wild-type myotubes already may be at their maximal cell size and no further increases are possible even in the presence of the recombinant

NFATC2. Further work is needed to define the relation of NFATC2-dependent muscle growth with hypertrophy.

In summary, we demonstrate that the calcium-regulated transcription factor NFATC2 regulates muscle growth. These data implicate an NFAT-dependent pathway in newly formed myotubes that controls further cell fusion in a mechanism that is distinct from the initial formation of multinucleated muscle cells. Myoblast fusion with multinucleated cells is central to proper muscle development but is also clinically relevant. Enhancing myoblast fusion with multinucleated cells has potential therapeutic value by improving the fusion of endogenous myoblasts in the treatment of muscle injuries or muscle atrophy or by enhancing the fusion of exogenous transplanted myoblasts in gene therapy protocols (Blau and Springer, 1995). Both myotonic dystrophy (Farkas-Bargeton et al., 1988) and centronuclear myopathy (Wockel et al., 1998) are characterized by reduced myofiber size, suggesting that myoblast fusion may be defective in these muscle disorders. Further studies will be directed towards identifying genes regulated by NFATC2 and their regulation of the fusion of differentiated muscle cells with multinucleated muscle cells.

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