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DNA binding sites target nuclear NFATc1 to heterochromatin regions in adult skeletal muscle fibers

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Abstract

We have previously demonstrated that Ca^{2+} /calcineurin-dependent dephosphorylation of the transcription factor nuclear factor of activated T cells subtype 1 (NFATc1) during repetitive skeletal muscle activity causes NFAT nuclear translocation and concentration in subnuclear NFAT foci. We now show that NFAT nuclear foci colocalize with heterochromatin regions of intense staining by DAPI or TO-PRO-3 that are present in the nucleus prior to NFATc1 nuclear entry. Nuclear NFATc1 also colocalizes with the heterochromatin markers trimethyl-histone H3 (Lys9) and heterochromatin protein 1 α . Mutation of the NFATc1 DNA binding sites prevents entry and localization of NFATc1 in heterochromatin regions. However, fluorescence in situ hybridization shows that the NFAT-regulated genes for slow and fast myosin heavy chains are not localized within the heterochromatin regions. Fluorescence recovery after photobleaching shows that within a given nucleus, NFATc1 redistributes relatively rapidly ($t_{1/2} < 1$ min) between NFAT foci. Nuclear export of an NFATc1 mutant not concentrated in NFAT foci is accelerated following nuclear entry during fiber activity, indicating buffering of free nuclear NFATc1 by NFATc1 within the NFAT foci. Taken together, our results suggest that NFAT foci serve as nuclear storage sites for NFATc1, allowing it to rapidly mobilize to other nuclear regions as required.

Keywords

NFAT; Nuclear foci; Heterochromatin; Skeletal muscle

Introduction

The transcription factor nuclear factor of activated T cells (NFAT) is a multigene family containing five members: NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5. Four NFAT family members (c1–c4) are regulated by the calcium-activated protein phosphatase calcineurin (Hogan et al. 2003). The fifth member, NFAT5, is activated in response to osmotic stress (Miyakawa et al. 1999). Each of the four Ca^{2+} sensitive NFAT family members possess nuclear localization sequences (NLSs) and nuclear export signals (NESs) that promote nuclear transport receptor-mediated entry into and CRM1-mediated exit from the nucleus, respectively (Beals et al. 1997; Klemm et al. 1997). When phosphorylated, these NFAT family members reside in the cytoplasm. During periods of elevated cytosolic $[\text{Ca}^{2+}]$, calcineurin is activated, leading to NFAT dephosphorylation, exposure of NFAT nuclear localization signals, and NFAT translocation into the nucleus (Beals et al. 1997; Hogan et al. 2003). Recent work has revealed that NFAT plays regulatory roles in various tissues such as skeletal muscle, heart, and the central neuron system, etc. (Crabtree and Olson 2002; Hogan et al. 2003). NFATc1–c4 are all expressed in skeletal muscle (Hoey et al. 1995; Parsons et al. 2003; Calabria et al. 2009), and NFATc1 is a specific determinant of slow muscle gene expression (Calabria et al. 2009). Calcineurin-mediated signal transduction via NFATc1 has been implicated in fast-twitch to slow-twitch skeletal muscle fiber type transformation (Chin et al. 1998; Kubis et al. 2003; McCullagh et al. 2004; Bassel-Duby and Olson 2006; Mu et al. 2007).

Three isoforms of NFATc1, NFATc1/A-C, are produced from alternative splicing of the primary transcript (Rao et al. 1997) and the NFATc1/A variant is the one examined in this study. All splice variants of NFATc1 contain an N-terminal regulatory domain and a C-terminal DNA binding domain (DBD) (Rao et al. 1997). In resting muscle fibers or myotubes, NFATc1A (henceforth referred to here simply as NFATc1) is located in the cytoplasm, but can translocate into the nucleus in response to an elevation of intracellular calcium concentration induced by slow fiber type electrical stimulation of skeletal muscle fibers (Liu et al. 2001) or by exposure myotubes to the calcium ionophore A23187 (Kubis et al. 2003). Our previous studies have shown that within a skeletal muscle nucleus, NFATc1 displays both a diffuse nuclear distribution and concentration into subnuclear foci (Liu et al. 2001; Shen et al. 2006), which could correspond to subnuclear domains of specialized function (Misteli 2005; Matera et al. 2009). The intranuclear NFAT foci exhibit a range of diameters, from less than 0.5 μm to greater than 2 μm , and vary between 4 and 9 per nucleus (Liu et al. 2001). Similar intranuclear NFAT foci have been described in living mouse skeletal muscles (Tothova et al. 2006) as well as in HEK 293T cells with exogenously expressed NFATc1–GFP (Sheridan et al. 2002). Thus, nuclear NFAT foci appear to be present both in vivo and in vitro, and in both muscle and non-muscle cell types. However, the nature of the NFAT foci has not been determined. We previously found that both the transcription factor MEF2, which together with NFATc1 activates slow muscle gene expression, and the splicing factor SC35 are excluded from the nuclear NFAT foci in muscle fibers, suggesting that the NFAT foci might not be locations of active genes or RNA processing (Liu et al. 2001).

In the present study we examine the molecular basis and functional effects of NFATc1 localization in NFAT foci in skeletal muscle fiber nuclei. Our results show that nuclear NFAT foci correspond to pre-existing heterochromatin regions where NFATc1 co-localizes

with heterochromatin marker proteins trimethyl-histone H3 (Lys9; H3K9m3) and heterochromatin protein 1 α (HP1 α) in nuclei of muscle fibers. The targeting of NFATc1 to nuclear heterochromatin regions requires NFATc1 DNA binding sites, but neither the slow nor the fast myosin heavy chain genes are co-localized with NFAT foci in nuclei of cultured muscle fibers. Finally, nuclear NFATc1 is highly dynamic and that it redistributes relatively rapidly between NFAT foci within a given muscle fiber nucleus, buffering the non-localized nuclear NFATc1 and thereby retarding its nuclear efflux after entry during previous muscle activity. Our results suggest that NFAT foci might serve as reservoirs of activated nuclear NFATc1.

Materials and methods

Plasmid constructs

The full-length human NFATc1 cDNA (NFAT2 isoform A, amino acids 1–716; Rao et al. 1997) and the cDNA encoding NFATc1 with serine-rich region (SRR) mutations (NFATc1–mSRR), in which all serine residues in the SRR are mutated to the non-phosphorylatable alanine, were gifts from Dr. Gerald R. Crabtree (Beals et al. 1997; Howard Hughes Medical Institute, Stanford, CA). The expression constructs of NFATc1–GFP, NFATc1–YFP, NFATc1–mSRR–GFP and their recombinant adenoviruses were as described previously (Liu et al. 2001). Expression plasmids carrying NFATc1(1–417) which contains the N-terminal regulatory region with deletion of the C-terminal amino acids 418–716, and NFATc1(1–591), which contains the N-terminal regulatory region and the whole DNA binding domain of NFATc1, were fused to YFP to form the fusion protein NFATc1(1–417)–YFP or NFATc1(1–591)–YFP, respectively. In vitro mutagenesis (Quickchange lightning site-directed mutagenesis kit, Stratagene, La Jolla, CA) was used to change residues R439 and Y442, two DNA binding sites of NFATc1, to alanine to construct GFP or YFP fusion expression plasmid NFATc1(R439A/Y442A)–GFP or NFATc1(R439A/Y442A)–YFP. The oligonucleotides used for the mutagenesis were: 5'-CAA GTC CCA CCA CGC AGC CCA CGC CGA GAC GGA GGG C-3' for sense and 3'-GTT CAG GGT GGT GCG TCG GGT GCG GCT CTG CCT CCC G-5' for antisense. All constructs were confirmed by sequencing, and also expressed in cultured adult FDB muscle fibers using available adenoviruses [NFATc1 (1–716)–YFP, NFATc1(1–417)–YFP, and NFATc1 (R439A/Y442A)–YFP] or expressed in 293T cells [NFATc1(1–591)–YFP] by plasmid transfection for western blot analysis. Blots were probed with the Living Colors A.v. Peptide antibody (1 μ g/ml) which reacts with YFP and YFP fusion proteins (BD Biosciences Clontech, CA).

Construct expression by intramuscular DNA injection and electroporation

Experiments were carried out on 4- to 6-week-old CD-1 mice. Experimental protocols were approved by the University of Maryland Institutional Animal Care and Use Committee. The intramuscular injection of various NFATc1 expression DNA plasmids is done, with minor modifications, according to the method of DiFranco et al. (2006). Briefly, one footpad of an anesthetized mouse is injected subcutaneously with 5 μ l of 2 mg/ml hyaluronidase through a 33-gauge needle. Two hours later, the mouse is again anesthetized and 40 μ g of plasmid DNA is injected into the footpad. Ten minutes later, two gold plated stainless steel electrodes are placed subcutaneously close to the proximal and distal tendons of the flexor digitorum brevis (FDB) muscle and 20 pulses of 100 V/cm, 20 ms in duration, are applied at 1 Hz (Pulse master A300, World Precision Instruments) driving a custom high current capacity output stage. One week later, single muscle fibers are enzymatically dissociated from the injected FDB muscles and cultured as previously described (Liu et al. 1997). Muscle fibers were imaged 20 h after plated in culture.

Adenoviral infection of FDB skeletal muscle fibers

For in vitro adenovirus infection, individual muscle fibers were enzymatically dissociated from un-injected mouse FDB muscles and cultured as previously described (Liu et al. 1997). Infection of muscle fiber cultures by recombinant adenoviruses was carried out about 20 h after the fibers were plated in culture dishes (Liu et al. 2001). Infected muscle fibers were continuously cultured for 2 days before imaging.

Microscopy, image acquisition, and muscle fiber treatment

The culture medium of the isolated single muscle fibers was changed to Ringer's solution (135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, and 1.8 mM CaCl₂, pH 7.4). The culture chamber was then mounted on an Olympus IX70 inverted microscope equipped with an Olympus FLUOVIEW 500 laser scanning confocal imaging system, utilizing an excitation wavelength of 488 nm (for GFP) or 514 nm (for YFP). Fibers were viewed with an Olympus 60X/1.2 NA water-immersion objective and scanned at 3.0× zoom (for Fig. 1a at 1.0× zoom) using constant laser power and gain. The confocal aperture was set to the optimal setting for each wavelength (Shen et al. 2006). For electrical field stimulation, two platinum electrodes connected to a stimulator were placed into the fiber culture chamber to give field stimulation, and FDB muscle fibers expressing either NFATc1-GFP or NFATc1-YFP via adenoviral transfection were stimulated with a slow fiber type pattern of activity (10 Hz trains: a 5 s train of 10 Hz stimuli once every 50 s, Liu et al. 2001) for 60 min to induce the NFATc1 nuclear translocation and concentration into nuclear foci. Fibers were exposed to DAPI during stimulation and imaged before and immediately after electrical stimulation. DAPI was added at a concentration of 10 µg/ml to Ringer's solution for 1 h before imaging. Alternatively, fibers were fixed after stimulation and then stained with TO-PRO-3 (1:1,000 in PBS) or used for fluorescence immunocytochemistry (see below). For calcium ionophore A23187 treatment, living isolated single muscle fibers expressing either NFATc1-YFP or its deletion or site mutants, transfected by intramuscular DNA injection, were incubated in Ringer's solution with 0.75 µM of A23187 for 2 h. Fibers were imaged before and immediately after A23187 treatment.

Fluorescence immunocytochemistry

FDB muscle fibers (with or without expressed NFATc1-GFP) were fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilized with 0.2% Triton-X-100 in PBS for 20 min. Nonspecific binding sites were blocked by incubation with 5% normal serum. Immunostaining was performed using the antibodies as indicated, i.e., antibody, respectively, against heterochromatin protein 1 α , acetylated histone H3, p300, or small ubiquitin-like modifier 1 (Santa Cruz Biotechnology, Santa Cruz, CA), or antibody against trimethyl-histone H3 (Lys9, Upstate, Lake Placid, NY). Fibers were incubated with the specific primary antibody against the protein of interest for 48 h at 4°C followed by overnight incubation with the fluorescent protein conjugated secondary antibody. The stained fibers were visualized using the confocal laser scanning imaging system described above.

Fluorescence in situ hybridization

Bacterial artificial chromosomes (BACs) used in this study including RP24-317F19 (slow fiber gene of beta myosin heavy chain; β -MHC) and RP23-294E23 (fast myosin heavy chain IId/x; MHCIIId/x) were supplied by BAC Resources (Children's Hospital Oakland Research Institute). The fluorescence in situ hybridization (FISH) is done according to the method of Meaburn and Misteli (2008). Briefly, the DNA probes were labeled by nick translations with biotin-16-dUTP (Roche, Indianapolis, IN). For hybridization to DNA, isolated muscle fibers (resting or after electrical stimulation by 10 Hz trains for 60 min)

were denatured in 50% formamide and 2× SSC at 90°C for 10 min followed by hybridization to labeled probes at 37°C for 20 h. Hybridization probes were detected with Alexa Fluor 546 conjugated streptavidin. The slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and imaged under confocal microscope. The FISH images in Fig. 8 were acquired on a Zeiss LSM 5 LIVE system, based on an Axiovert 200M inverted microscope using a UPlan Apo 63× NA 1.20 water-immersion objective. Excitation was provided by a 405-nm laser for DAPI and a 532-nm laser for Alexa-546, with emissions detected with 420–480 nm band-pass filter or 560 nm long-pass filter, respectively. The acquired images were contrast enhanced in order to visualize the gene locations (Meaburn et al. 2009). No other images presented or analyzed in this paper were contrast enhanced.

Nuclear fluorescence photobleaching analysis

Muscle fibers were infected with adenovirus encoding the constitutively nuclear targeted NFATc1–mSRR–GFP (Beals et al. 1997). Nuclear or nuclear foci of NFAT fluorescence recovery after photobleaching (FRAP) analysis was carried out using the Olympus Fluoview 500 confocal microscope. After recording a pre-bleach image, a rectangular region, slightly larger than a region of interest (either a whole nucleus or a single NFAT focus) was photobleached by a 488-nm laser line for GFP at the maximum laser power. Subsequently, the recovery of the fluorescence signal in the bleached area was recorded by sequential imaging at lower laser power. The average pixel fluorescence within user-specified areas of interest (AOI) of either the nucleus or nuclear foci in each image was quantified by software custom-written in the IDL programming language (Research Systems, Boulder, CO). Results are expressed as the mean ± SEM.

Results

Localization of NFATc1 in pre-existing subnuclear heterochromatin regions

Previous studies in our laboratory have shown that exogenously expressed NFATc1–GFP fusion protein, produced after adenovirus infection, is predominantly located in the cytoplasm of cultured resting adult mouse FDB skeletal muscle fibers (Liu et al. 2001). Electrical stimulation of FDB muscle fibers with a repetitive slow fiber type activity (a 5 s train of 10 Hz stimuli once every 50 s; denoted as “10 Hz trains”; Hennig and Lomo 1985) caused nuclear translocation of NFATc1–GFP. Inside the muscle fiber nuclei, NFATc1–GFP displayed both a diffuse distribution and concentration into subnuclear NFAT foci (Liu et al. 2001). In this study, we further characterize the subnuclear NFAT foci in cultured adult skeletal muscle fibers, determine the signals for targeting NFATc1 to the nuclear NFAT foci, and analyze the functional effects of NFAT foci on NFATc1 movements within and out of muscle fiber nuclei.

Cultured adult mouse skeletal muscle fibers were infected with adenovirus containing the cDNA of the fusion protein NFATc1–YFP. Two days after virus infection, muscle fibers were electrically stimulated for 60 min with 10 Hz trains to induce NFATc1–YFP translocation from cytoplasm to nucleus (Liu et al. 2001), and exposed to DAPI during stimulation. Alternatively, fibers were fixed after stimulation and then stained with TO-PRO-3. DAPI preferentially intercalates into the AT-rich DNA present in the heterochromatin of mouse cells (Schnedl et al. 1977). TO-PRO-3 has also been used to stain mouse heterochromatin (Matamalas et al. 2009). Figure 1a shows lower magnification (zoom 1×) images of a muscle fiber expressing NFATc1–YFP before (left), after (middle) electrical stimulation as well as the image of the same fiber under transmitted light (right). After electrical stimulation, NFATc1–YFP translocated from cytoplasm into nuclei of muscle fibers and formed nuclear NFAT foci [Fig. 1a, arrows denote 3 nuclei, with each

nucleus exhibiting a similar change from no nuclear fluorescence before stimulation to the appearance of several discrete foci on NFATc1–YFP fluorescence after stimulation as previously described (Liu et al. 2001; Shen et al. 2006)]. We now find that the NFAT foci (Fig. 1b, c, left, green arrows) corresponded to heterochromatin regions intensely stained by either DAPI (Fig. 1b, middle, red arrows) or TO-PRO-3 (Fig. 1c, middle, red arrows). When applied simultaneously to fixed fibers, DAPI and TO-PRO-3 stain the same heterochromatin regions in nuclei of cultured FDB muscle fibers (data not shown). Other muscle fibers expressing NFATc1–GFP were electrically stimulated for 60 min with the same pattern as above, fixed and stained with TO-PRO-3 and then imaged at successive focal planes separated by 2 μ m. The resulting images show that all NFAT foci within a given muscle fiber nucleus correspond to regions of TO-PRO-3 heterochromatin staining (Fig. 2).

We next investigated whether the same heterochromatin regions exist before the entry of NFATc1–YFP into the nucleus. Living muscle fibers expressing NFATc1–YFP were first stained with DAPI and then electrically stimulated with 10 Hz trains for 60 min. Note that only DAPI, but not TO-PRO-3, can be used to stain heterochromatin in living cells. Before stimulation, muscle fibers showed negligible NFATc1–YFP in the nucleus (Fig. 3a), but did exhibit clear DAPI-stained regions (Fig. 3b, red arrows). After electrical stimulation, NFATc1–YFP moved from cytoplasm into the nucleus where the nuclear NFATc1–YFP (Fig. 3d, green arrows) co-localized with DAPI (Fig. 3e, red arrows). Electrical stimulation did not change the pattern of DAPI staining (compare Fig. 3b and 3e, red arrows). Taken together, these results demonstrate that NFATc1–YFP accumulates in pre-existing heterochromatin regions of muscle fiber nuclei after slow fiber type electrical stimulation-induced nuclear translocation. The nuclear regions in which NFATc1–YFP localizes are thus not artifacts of NFATc1–YFP within the nucleus, but were present prior to NFATc1–YFP entry. Note that all images in Fig. 3 are of the same muscle fiber nucleus, either before (Fig. 3, control) or after (Fig. 3, stimulation) 60 min of slow fiber type electrical stimulation. This experiment also shows that DAPI does not seem to affect the recruitment of NFATc1 to the heterochromatin regions, and thus may not affect chromatin structure and DNA–protein interaction.

To further test the subnuclear distribution of NFAT foci relative to heterochromatin, we compared the spatial distribution of NFAT foci with the antibody staining patterns for heterochromatin protein 1 α (HP1 α) and trimethyl-histone H3 (Lys9, H3K9m3), two hallmarks of heterochromatin. Consistent with NFAT concentration in DAPI-stained heterochromatin described above, the antibody staining for H3K9m3 (Fig. 4a, red arrow) or HP1 α (Fig. 4b, red arrows) co-localized with NFAT foci (Fig. 4a, b, respectively, green arrows) in FDB fiber nuclei. As expected, staining of acetylated histone 3 (ac H3) or p300, two proteins associated with nuclear euchromatin, was very low (Fig. 4c, d; white arrows point to low levels of ac H3 or p300 at the location of NFATc1 foci) within the NFAT foci (Fig. 4c or d, green arrows). Only the diffuse nuclear NFATc1–GFP overlapped with regions containing proteins associated with euchromatin (Fig. 4c, d). From these results, we conclude that nuclear NFATc1 of muscle fibers is enriched in heterochromatin regions.

Co-localization of NFATc1 nuclear foci with small ubiquitin-like modifier 1

Sumoylation can modify the subnuclear localization of targeted proteins (Verger et al. 2003; Terui et al. 2004). It has been shown that sumoylation of the transcription factor CCAAT/enhancer-binding protein β (C/EBP β) results in its relocation to heterochromatin (Berberich-Siebelt et al. 2006). In the present study, we fixed cultured adult skeletal muscle fibers after 60 min of 10 Hz train stimulation and then we used antibody staining to detect the small ubiquitin-like modifier 1 (SUMO-1) and co-stained for heterochromatin with TO-PRO-3. This electrical stimulation causes NFATc1–YFP nuclear translocation and nuclear foci formation (Fig. 1). Our results show that staining of SUMO-1 was very low (Fig. 5, left; the

white arrow points to low levels of SUMO-1 at location of the heterochromatin) within the TO-PRO-3-rich heterochromatin regions (Fig. 5, middle, red arrow). Since TO-PRO-3-stained heterochromatin co-localizes with nuclear NFAT foci (Fig. 1c), our results indicate that nuclear NFAT foci are regions of relatively low concentration of SUMO-1, suggesting that sumoylation by SUMO-1 may not be related to heterochromatin targeting of NFATc1. In agreement with our results, it was recently reported that NFATc1/A (the isoform used in the present study) has only one extremely weak sumoylation site and is not co-localized with SUMO-1 in human lymphoid A3.01 cells (Nayak et al. 2009).

The NFATc1 DNA binding domain contains the structural motif that determines localization of NFATc1 into nuclear heterochromatin

In order to analyze the molecular basis underlying the formation of nuclear NFAT foci, we generated a series of YFP-tagged deletion mutants covering the full-length NFATc1/A protein sequence (Fig. 6a). The wild-type and mutant NFATc1 proteins were expressed and showed their expected molecular weights as demonstrated by western blot analysis (Fig. 6b). NFAT proteins from each of these constructs were expressed by intramuscular plasmid injection into FDB muscle followed by electroporation in the live mouse and subsequent muscle and muscle fiber isolation and fiber culture 1 week later. Deletion of the C-terminal amino acids 418–716 from NFATc1, leaving only the regulatory domain [NFATc1(1–417), Fig. 6a, second panel], did not affect NFATc1 nuclear translocation during application of the calcium ionophore A23187. However, intranuclear NFATc1 (1–417)–YFP was not concentrated in discrete nuclear foci but was instead diffusely distributed in the fiber nuclei (Fig. 6c, middle panels) and thus lacked the characteristic feature of full-length NFATc1–YFP [NFATc1(1–716), Fig. 6a, first panel] to be localized in nuclear foci (Fig. 6c, left, white arrows). Thus, at least some part(s) of the sequence within amino acids 418–716 of the C-terminal part of wild-type NFATc1 is (are) required for the targeting of nuclear NFATc1 to the nuclear heterochromatin regions.

The C-terminal part of wild-type NFATc1 consists mainly of a Rel homology region containing the DNA binding domain (DBD; amino acids 416–591; Fig. 6a, first panel; Wolfe et al. 1997) and a C-terminal domain containing an NLS. Thus, we constructed a mutant NFATc1(1–591) which contains both the N-terminal regulatory region and only the DNA binding domain within the Rel homology region of NFATc1 and fused it to YFP to form the fusion protein NFATc1(1–591)–YFP (Fig. 6a, third panel). This fusion protein NFATc1(1–591)–YFP entered the nucleus during application of calcium ionophore A23187 and clearly exhibited targeting to the intranuclear foci (Fig. 6c, right, white arrows). These intranuclear NFAT foci of NFATc1(1–591)–YFP were co-localized with DAPI-stained heterochromatin (data not shown). These results indicate that the DNA binding domain of NFATc1 is necessary for nuclear NFAT foci formation in skeletal muscle fibers.

The NFATc1 DNA binding sites determine the localization of NFATc1 in nuclear heterochromatin regions

Previous studies have shown that residues R439 and Y442 in the DNA binding domain of NFATc1 are critical for DNA binding (Jain et al. 1995; Wolfe et al. 1997). To test whether these residues are required for NFAT foci formation, we mutated both R439 and Y442 in the full-length NFATc1 to alanine and fused it to YFP to form the mutant fusion protein NFATc1(R439A/Y442A)–YFP (Fig. 7a). Alanine substitution was used to minimize the possibility of causing global alterations in protein structure (Jain et al. 1995). The wild-type and the mutant NFATc1 proteins were expressed and showed their expected molecular weights as demonstrated by western blot analysis (Fig. 7b). In resting muscle fibers, NFATc1(R439A/Y442A)–YFP is located only in the cytoplasm (data not shown). Exposure of muscle fibers to calcium ionophore A23187 caused nuclear translocation of

NFATc1(R439A/Y442A)–YFP (Fig. 7c, d, left). However, intranuclear NFATc1(R439A/Y442A)–YFP was not concentrated in discrete nuclear foci but was instead diffusely distributed in the fiber nuclei (Fig. 7c, d, left). Co-staining with either DAPI or TO-PRO-3 clearly shows that after mutation of the DNA binding sites R439 and Y442, nuclear NFATc1(R439A/Y442A)–YFP (Fig. 7c, d, left) no longer entered the DAPI or TO-PRO-3-rich heterochromatin regions in muscle fibers (Fig. 7c, d, middle, red arrows), indicating that the NFATc1 DNA binding sites are required for localization of NFATc1 in nuclear heterochromatin. Interestingly, not only is the mutant construct NFATc1 (R439A/Y442A)–YFP not concentrated in heterochromatin regions, but also actually excluded from these regions (Fig. 7c, d, middle, red arrows). Thus, the mutated residues are needed for NFATc1 to access the heterochromatin regions.

Localization of muscle fiber genes relative to nuclear NFAT foci

In order for NFATc1 within the heterochromatin to promote transcriptional activity, an obvious requirement is that the corresponding NFATc1 regulated genes also would have to be located within the heterochromatin. To test whether NFATc1 within the heterochromatin is directly related to activation of its target genes, we used fluorescence in situ hybridization (FISH) to localize two genes regulated by NFATc1, slow fiber β -MHC and fast fiber MHCIIx/d, within individual FDB fiber nuclei counterstained with DAPI. NFATc1 has been shown previously to stimulate β -MHC expression, and to inhibit the expression of MHCIIx/d (McCullagh et al. 2004; Meissner et al. 2007; Calabria et al. 2009). We find that both β -MHC (Fig. 8a, left, without stimulation; c, left, after 60 min of 10 Hz train electrical stimulation, 2 genes per nucleus, green arrowheads) and MHC-IIId/x genes (Fig. 8b, left, without stimulation; d, left, after 60 min of 10 Hz train electrical stimulation, left, 2 genes per nucleus, green arrowheads) are located outside the DAPI-stained heterochromatin regions (Fig. 8a–d, middle, red arrows). As shown in Fig. 1, NFAT foci co-localized with DAPI-stained heterochromatin, thus, our FISH results indicate that the function of NFATc1 within the heterochromatin is unlikely to be direct regulation of muscle fiber gene transcription. However, concentration of NFAT in the heterochromatin regions does not preclude its activity at lower concentration elsewhere in the nucleus.

NFATc1 equilibrates relatively rapidly between intranuclear NFAT foci

We next analyzed the mobility of NFATc1–GFP proteins among NFAT foci within a given muscle fiber nucleus. For this purpose, we carried out fluorescence recovery after photobleaching (FRAP) analysis. For our initial FRAP studies, muscle fibers were infected with adenovirus encoding NFATc1–mSRR–GFP (Fig. 9a–c), a NFATc1 mutant GFP fusion construct constitutively targeted to the nucleus (Beals et al. 1997). This mutant is exclusively nuclear, so cannot move into or out of the nucleus during the FRAP measurements. In fibers infected with this construct, NFATc1–mSRR–GFP fluorescence was concentrated inside nuclei and in nuclear foci, with negligible cytoplasmic fluorescence (Fig. 9a–c). In our FRAP experiment, a defined area of either a single NFAT focus (Fig. 9a, white rectangle) or a whole nucleus (Fig. 9b, white rectangle) was bleached using maximum laser power, followed by sequential imaging scans to record the recovery of the fluorescence signal in the bleached area and the corresponding fluorescence loss in the unbleached area of the same nucleus due to NFATc1–mSRR–GFP movement out of non-bleached foci into other regions of the nucleus. Our results show that the bleached NFAT foci (Fig. 9a, arrows) recovered 52% of their original fluorescence 2 min after photobleaching, with the $t_{1/2}$ less than 1 min (Fig. 9d, filled triangle; 5 nuclear foci from 5 different fibers). The failure to recover to 100% of the initial fluorescence is due to the fact that the bleached focus constitutes a major fraction of the total nuclear NFATc1–mSRR–GFP. This is consistent with the finding that the fluorescence of a neighboring NFAT focus within the same nucleus (Fig. 9a, arrowheads) decreased to 62% of its original level in 2 min (Fig. 9d, open triangles;

5 nuclear foci from the same nuclei as the bleached nuclear foci). Furthermore, both the post-bleach recovery of fluorescence in the bleached foci and the post-bleach decline of fluorescence in the unbleached foci are approaching the same steady state, corresponding to about 55% of their pre-bleach value, indicating that about 45% of the total nuclear fluorescence was bleached. The decrease of fluorescence level in the neighboring NFAT foci is not caused by the sequential imaging scans, as the repeated imaging scans did not significantly change the fluorescence level of the control NFAT foci in non-bleached nuclei (Fig. 9c). The repeated imaging scans decreased the fluorescence level of the control NFAT foci by 7% (Fig. 9d, open circles; 6 nuclear foci from 6 different fibers), indicating that there is only a small, limited photobleaching during imaging. When the whole nucleus was photobleached (Fig. 9b), there was no recovery of its fluorescence level within 2 min (Fig. 9d, filled circles; 6 nuclei from 6 different fibers), suggesting no detectable movement of NFATc1-mSRR-GFP into the nucleus from cytoplasm within the 2-min time period of the recording. Therefore, the partial recovery of the fluorescence level of the bleached NFAT foci cannot be due to nuclear entry of NFATc1-mSRR-GFP from the cytoplasm. It must be due to the redistribution of NFATc1-mSRR-GFP protein within the nucleus, including that from the neighboring NFAT foci, which consequently decline in fluorescence. Together, our results demonstrate that NFATc1 exhibits a relatively high degree of mobility among NFAT foci in muscle fibers.

NFATc1 concentration in intranuclear foci retards NFATc1 nuclear efflux

A predicted functional implication of NFAT binding in nuclear foci is that the NFAT within the nuclear foci is not directly available for efflux via the CRM1 efflux carrier. This would tend to slow the overall efflux of nuclear NFAT from the nucleus. We tested this prediction by activating NFATc1-GFP entry into nuclei during 60 min of repetitive stimulation, and then by stopping stimulation and comparing the decline of nuclear NFATc1 outside the nuclear foci for wild-type NFATc1-GFP and for the DNA binding site mutant NFATc1(R439A/Y442A)-GFP that does not target nuclear foci. Here we used the same protocol as used previously by our laboratory to characterize the control of NFATc1-GFP nuclear efflux by specific kinases (Shen et al. 2007), but now to compare efflux for constructs that do or do not concentrate in the NFAT nuclear foci. As anticipated, the decline of non-nuclear foci NFATc1 [NFATc1(R439A/Y442A)-GFP] during export after cessation of electrical stimulation was slightly faster [Fig. 10c, average of 10 nuclei from 10 different fibers for NFATc1-GFP and average of 7 nuclei from 6 different fibers for NFATc1(R439A/Y442A)-GFP]. A similar difference in nuclear efflux of wild-type NFATc1-GFP was previously shown to be produced by partial inhibition of casein kinase, one of the kinases responsible for rephosphorylation of nuclear NFATc1 prior to nuclear efflux (Shen et al. 2007). Thus, NFATc1 concentration in NFAT foci has the functional consequence of slowing NFAT nuclear efflux after NFAT entry into the nucleus.

Discussion

In the present study we have characterized the intranuclear distribution, the molecular basis, and the functional effects of NFATc1 localization in NFAT foci in cultured adult skeletal muscle fibers. Our results clearly show that nuclear NFAT foci are within heterochromatin regions that are present prior to NFATc1 nuclear entry and that the DNA binding sites in NFATc1 are required for targeting to NFATc1 the nuclear heterochromatin. The nuclear NFAT foci are not the sites of gene localization for either slow or fast myosin heavy chain, suggesting that nuclear NFAT foci are not locations of either active or suppressed muscle gene transcription. Furthermore, NFATc1 exhibits a relatively high degree of mobility among the nuclear foci within a given muscle fiber nucleus. Nuclear export of an NFATc1 mutant not concentrated in NFAT foci is accelerated following nuclear entry during fiber

activity, indicating buffering of free nuclear NFATc1 by NFATc1 in NFAT foci. We propose that nuclear NFAT foci might serve as storage sites for nuclear NFATc1, allowing rapid mobilization to other nuclear sites when its functions at those sites are required.

Figure 11 presents a cartoon summary of a model for the movements of NFATc1 into the nucleus and into the subnuclear heterochromatin regions based on the present work and on previous studies from this and other laboratories. Dephosphorylated NFAT (bottom left) has an exposed nuclear localization sequence (Beals et al. 1997), and consequently moves unidirectionally from the cytoplasm into the nucleus via a nuclear import system. Inside the nucleus, dephosphorylated NFATc1 can bind directly to NFAT-regulated genes (green; bottom center) or can enter regions of dense heterochromatin (NFAT foci, grey). Within the foci, unbound NFATc1 would be present at the same concentration in the foci water as the concentration in the non-foci water outside the foci. However, NFATc1 is concentrated in the foci by binding to an as yet undetermined site or sites in the foci (X) via the DNA binding domain (DBD) of NFATc1. Due to the higher density of non-aqueous material in the heterochromatin regions, if NFATc1 is prevented from binding to sites X in the foci by mutation of the NFATc1 DBD, the concentration of NFATc1 per unit foci volume then becomes less than that in the general nucleoplasmic space, where non-aqueous material is presumably less densely packed than in the foci. Whether the high concentration of NFAT in heterochromatin simply correlates with the high concentration of DNA in these regions remains to be determined. As shown by our FRAP studies, the movement of NFATc1 into and out of the foci is relatively rapid, consistent with the absence of a membrane barrier surrounding the foci. Phosphorylation of NFATc1 by nuclear GSK3 β and casein kinase (Shen et al. 2007) unmasks the nuclear export signal (NES) on NFATc1-P, allowing NFATc1-P to be transported out of the nucleus, and obscures the NLS on NFATc1-P (Beals et al. 1997). The binding of NFATc1 within the foci buffers the total nuclear content of NFATc1, and thus retards the NFATc1 nuclear efflux after nuclear accumulation of NFATc1 following muscle activity. During muscle activity, the Ca²⁺ dependent phosphatase calcineurin is activated, causing dephosphorylation of NFATc1-P, restarting the cycle of NFATc1 nuclear entry.

The nucleus contains distinct nuclear chromatin domains that are morphologically defined as euchromatin and heterochromatin. The euchromatin consists of less-condensed genome regions and is generally considered to be transcriptionally active, whereas heterochromatin is characterized as regions of chromatin in which DNA is tightly coiled and densely packed around histones that are both deacetylated (by the enzymatic activity of histone deacetylases, HDACs) and methylated. Heterochromatin is often thought of enriched in inactive and silenced genome regions. Consistent with NFAT concentration in heterochromatin, our antibody staining results show that the heterochromatin marker proteins HP1 α and H3K9m3 co-localize with NFAT foci in FDB fibers. H3K9m3 has been recognized as a key feature in maintaining pericentric heterochromatin and in silencing of euchromatic genes (Lachner et al. 2003).

In the present study, we clearly establish that the DNA binding site, and in particular amino acid residues R439 and Y442 of NFATc1, is the molecular basis by which NFATc1 is targeted to heterochromatin regions. However, our findings cannot distinguish NFATc1 binding via this region to either DNA or protein. Other transcription factors such as Ikaros and C/EBP α may accumulate in heterochromatin by direct DNA binding (Cobb et al. 2000; Liu et al. 2007). Ikaros has been found to co-localize with γ -satellite DNA (Cobb et al. 2000), and C/EBP α has been found to directly bind to the α -satellite DNA in the pericentromeric heterochromatin (Liu et al. 2007). Satellite DNA is an abundant repetitive element found at the centromeres of all murine chromosomes (Vissel and Choo 1989). It will be interesting in the future experiments to test whether NFATc1 binds directly to

satellite DNA repeat elements or to protein partners such as HP1a, H3K9m3, or others in the heterochromatin.

The biological significance of NFATc1 targeting to the heterochromatin is currently unknown. Our novel finding that NFATc1 is enriched in intranuclear heterochromatin regions suggests that NFAT foci might not be needed for ongoing transcriptional activity. In support of this hypothesis, we now show that both p300, a transcriptional coactivator which is normally found near the regions where active transcriptional reactions occur, and acetylated H3, a hallmark of active transcription sites, are excluded from nuclear NFAT foci. NFATc1 has previously been shown to activate slow myosin and inhibit fast myosin heavy chain genes. Activation of NFATc1 stimulates the promoter of β -MHC (McCullagh et al. 2004; Meissner et al. 2007) and induce β -MHC expression (McCullagh et al. 2004), but inhibits the promoter of MHCIIx/d (Meissner et al. 2007). Selective knock down of NFATc1 by RNAi significantly decreased the promoter activity of β -MHC and the expression level of β -MHC in regenerating muscle (Calabria et al. 2009). Previously results have also shown that inhibition of CaN-mediated NFAT activation by the specific peptide inhibitor VIVIT leads to decreased expression of β -MHC and up-regulation of MHCIIx/d in adult rat soleus muscle (McCullagh et al. 2004). However, our results of gene localization by FISH show that nuclear NFAT foci are not the sites of gene localization for either β -MHC or MHCIIx/d, suggesting that NFATc1 protein located in the heterochromatin regions may be indirectly related to the regulation of gene expression for either β -MHC or MHCIIx/d. The present results are in agreement with our previous findings that the splicing factor SC35 and the transcription factor MEF2, which together with NFATc1 activates slow muscle fiber type gene expression, are excluded from the nuclear NFAT foci (Liu et al. 2001). Our finding of muscle gene location outside of the heterochromatin is analogous to a previous report that the myogenin gene, another muscle-specific gene, does not exhibit a specific relationship to heterochromatin in either undifferentiated or differentiated cells (Moen et al. 2004). Furthermore, using RNA FISH analysis it was reported that the genes for human β -cardiac myosin heavy chain and myogenin were localized to the periphery of an SC-35 domain in terminally differentiated muscle nuclei (Smith et al. 1999; Moen et al. 2004).

The sequestration of NFAT in nuclear foci is sufficient to retard the overall nuclear efflux of NFATc1 after nuclear entry, thus prolonging the transcriptional effect of NFATc1 that entered the nucleus during prior muscle activity. FRAP analysis revealed that bleached molecules of NFATc1-GFP within a nuclear foci are rapidly replaced by unbleached NFATc1-GFP from the same nucleus. Our observations that nuclear NFATc1 proteins which reside in nuclear foci are rapidly exchanged among the foci indicate they are in continuous flux and suggest that nuclear NFAT foci might serve as storage sites for NFATc1, allowing rapid mobilization to other nuclear sites containing euchromatin when its functions at those sites are required.

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Fig. 1.

Localization of NFATc1 in nuclear heterochromatin regions. **a** Images of lower (zoom 1×) magnification of a muscle fiber expressing NFATc1–YFP before (*left*) and after (*middle*) electrical stimulation with 10 Hz trains for 60 min to induce NFATc1–YFP nuclear translocation. The image on the *right* was taken for the same fiber under transmitted light. *Arrows* denote nuclei. Note that the transmitted image has a very large depth of focus, so non-muscle cells on the coverslip are seen in transmitted light image (*right, arrowhead*), but are not seen in the confocal YFP fluorescence image (*middle, arrowhead*) with focus at the center of the fiber, even though the non-muscle cells were highly fluorescent when the confocal plane is set at the level of the coverslip (not shown). *Scale bar* 20 μm. **b, c** Muscle fibers expressing NFATc1–YFP were stimulated electrically with 10 Hz trains for 60 min and then either counterstained with DAPI in living fiber (**b**) or TO-PRO-3 in fixed fiber (**c**). *Scale bar* 5 μm. Here, and in other figures with overlays, the *green arrows* in the left and overlay (*right*) panels, and the *red arrows* in the center and overlay panels are located in the identical positions. Representative images are shown. *n* = 4 (**a**), *n* = 14 (**b**), *n* = 8 (**c**)

Fig. 2.

All NFAT foci within a given muscle fiber nucleus co-localize with heterochromatin. **a–c** A muscle fiber expressing NFATc1–GFP was stimulated electrically with 10 Hz trains for 60 min to induce NFATc1–GFP nuclear translocation, fixed, counterstained with TO-PRO-3, and then imaged at successive focal planes separated by 2 μ m for NFATc1–GFP (*left*) and TO-PRO-3 (*right*). The corresponding *arrows* in each pair of panels in **a–c** are positioned in the identical locations for NFATc1–GFP (*left*) and TO-PRO-3 (*right*). *Scale bar* 5 μ m. Representative images are shown ($n = 4$)

Fig. 3.

NFATc1–YFP accumulates in pre-existing heterochromatin regions of muscle nuclei. A living muscle fiber expressing NFATc1–YFP in **a** was first stained with DAPI in **b** and then stimulated electrically with 10 Hz trains for 60 min to induce NFATc1–YFP nuclear translocation in **d**. The *green arrow* in **d** indicates that the NFATc1–YFP nuclear focus formed after muscle activity coincides with pre-existing DAPI-stained heterochromatin region in **e** (*red arrow*). Electrical stimulation does not change the pattern of DAPI staining (**b**, **e** *red arrows*). Scale bar 5 μ m. Representative images are shown ($n = 9$)

Fig. 4.

Co-localization of NFATc1–GFP with heterochromatin marker proteins and exclusion of euchromatin marker proteins from NFATc1 nuclear foci. Muscle fibers expressing NFATc1–GFP were first stimulated electrically with 10 Hz trains for 60 min to induce NFATc1–GFP nuclear translocation, then fixed and subjected to indirect immunofluorescence using antibody to either heterochromatin proteins H3K9m3 (**a**) or HP1 α (**b**), or to euchromatic proteins ac H3 (**c**), or p300 (**d**), respectively. The *white arrows* are located in the identical positions in the center and right (*overlay*) panels in **c** and **d**. Scale bar 5 μ m. Representative images are shown. *n* = 6 (**a**), *n* = 6 (**b**), *n* = 8 (**c**), *n* = 6 (**d**)

Fig. 5.

Localization of SUMO-1 in stimulated muscle fibers. A muscle fiber stimulated electrically with 10 Hz trains for 60 min was then subjected to indirect immunofluorescence using antibody specific to SUMO-1 and then counterstained with TO-PRO-3. The *white arrow* is in the identical location in the *right* and *left* panels. *Scale bar* 5 μ m. Representative images are shown ($n = 7$)

Fig. 6.

The NFATc1 DNA binding domain contains the structural motif that determines the localization of NFATc1 in nuclear heterochromatin. **a** Schematic representation of YFP-tagged wild type (1–716) or different deletion mutants of NFATc1. Representative domains are indicated: *NLS* nuclear localization signal, *DBD* DNA binding domain. **b** Western blot of total protein extracts (NuPAGE 4–12% Bis–Tris gel) using the Living Colors A.v. Peptide antibody for protein detection. Positions of marker proteins (in kDa) are indicated. **c** Intracellular distribution of various exogenously expressed NFATc1 expression vectors as indicated in skeletal muscle fibers before (*control*) or after calcium ionophore A23187 treatments (A23187, 0.75 μ M for 2 h, *bottom*). Arrows indicate NFAT foci. Scale bar 5 μ m. Representative images are shown. $n = 8$ [NFATc1(1–716)–YFP], $n = 18$ [NFATc1(1–417)–YFP], $n = 10$ [NFATc1(1–591)–YFP]

Fig. 7.

The NFATc1 DNA binding sites are required for localization of NFATc1 in nuclear heterochromatin. **a** Schematic representation of YFP-tagged DNA binding sites mutant of NFATc1. **b** Western blot of total protein extracts (NuPAGE 4–12% Bis–Tris gel) using the Living Colors A.v. Peptide antibody for protein detection. Positions of marker proteins (in kDa) are indicated. **c**, **d** muscle fibers expressing NFATc1(R439A/Y442A)–YFP treated with 2 h of A23187 were counterstained either with DAPI (**c**) or with TO-PRO-3 (**d**) after fixation. Scale bar 5 μ m. Representative images are shown. $n = 6$ (**c**), $n = 6$ (**d**)

Fig. 8.

Localization of muscle fiber genes relative to nuclear heterochromatin. Genes for beta myosin heavy chain (β -MHC; **a** and **c**, *green arrowheads*) with (**c**) or without (**a**) 10 Hz trains electrical stimulation for 60 min or for myosin heavy chain IIId/x (MHC-IIId/x; **b** and **d**, *green arrowheads*) with (**d**) or without (**b**) 10 Hz trains electrical stimulation for 60 min in cultured FDB muscle fibers were detected using fluorescence in situ hybridization and counterstained with DAPI (*red arrows*). Images were contrast enhanced to visualize the genes. Both genes are located outside the DAPI-stained heterochromatin. No other images in this paper were contrast enhanced. *Scale bar* 5 μ m. Representative images are shown. $n = 13$ (**a**), $n = 8$ (**b** and **d**), $n = 7$ (**c**)

Fig. 9. NFATc1 equilibrates in intra nuclear foci. **a–c** Muscle fibers expressing NFATc1–mSRR–GFP were imaged before (*control*) and after bleaching of either an NFATc1 nuclear focus (indicated by arrow in **a**, bleached for 10 s) or a whole nucleus (**b**, bleached for 30 s). The area of the bleach is indicated with a *rectangle*. The NFAT focus in **c** (indicated by *arrow*) functions as a control for photobleaching. *Scale bar* 5 μ m. **d** Kinetics of the recovery after photobleaching. Bleached whole nucleus (*filled circles*) average of 6 nuclei from 6 different fibers bleached as in **b**; control nuclear foci (*open circles* no bleaching): average of 6 nuclear foci as the one in **c** (indicated by *arrow*) from 6 different fibers; bleached nuclear foci (*filled triangles*): average of 5 nuclear foci from 5 different fibers bleached as the one in **a** (indicated by *arrow*); neighboring non-bleached nuclear foci (open triangles): average of 5 nuclear foci as the one in **a** (indicated by *arrowhead*) from the same nuclei as the bleached nuclear foci. Note that error bars are about the same size as the symbols

Fig. 10.

NFATc1 concentration in intra nuclear foci slows NFATc1 nuclear efflux. **a, b** images of two different FDB fibers expressing either NFATc1–GFP (**a**) or NFATc1(R439A/Y442A)–GFP (**b**) after 60 min of 10 Hz train electrical stimulation, demonstrating how the cytoplasmic AOI (**c**) and nuclear (outside of the nuclear foci) AOI (**n**) are defined. *Scale bar* 5 μ m. **c** Time courses of nuclear NFATc1 outside the nuclear foci for wild-type NFATc1–GFP (10 nuclei from 10 fibers) or NFATc1(R439A/Y442A)–GFP (7 nuclei from 6 fibers) showing nuclear translocation during electrical stimulation with 10 Hz trains for 60 min and nuclear export during 90 min (recovery) after cessation of electrical stimulation. For nucleus, the mean pixel fluorescence values for the AOI at each time point were divided (normalized) by the mean pixel fluorescence values in the cytoplasmic AOI at the corresponding time point in the same fiber. This method of normalization by cytoplasmic fluorescence was used to avoid variations caused by the difference in expression level. Results are expressed as the mean $\pm$ SEM and normalized to the mean value in each group at the end of the 60 min stimulation period (larger, *half-filled symbol*). Note that *error bars* are about the same size as the *symbols*

Fig. 11.

Cartoon representation of model for NFAT movement between cytoplasm, nucleus and subnuclear heterochromatin regions. Dephosphorylated NFAT (*lower left*) has exposed nuclear localization sequence (NLS) and is carried into the nucleus (*green arrow*) where it can bind to NFAT-regulated genes (*bottom center*) via its DNA binding domain (DBD), or enter the subnuclear heterochromatin regions (foci, *grey*) by simple diffusion, where it is concentrated due to binding to an as yet unspecified site or sites (X?), also via its DBD. Phosphorylation (P) of NFAT masks the NLS and exposes the nuclear export signal (NES), causing NFAT nuclear efflux (*red arrow*). The *two upper pairs of vertical arrows* represent enzymatic phosphorylation/dephosphorylation of NFAT, whereas the *two lower pairs of vertical arrows* represent simple binding/unbinding without any covalent modification