A combinatorial role for NFAT5 in both myoblast migration and differentiation during skeletal muscle myogenesis

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Summary

Skeletal muscle regeneration depends on myoblast migration, differentiation and myofiber formation. Isoforms of the nuclear factor of activated T cells (NFAT) family of transcription factors display nonredundant roles in skeletal muscle. NFAT5, a new isoform of NFAT, displays many differences from NFATc1-c4. Here, we examine the role of NFAT5 in myogenesis. NFAT5$^{+/-}$ mice displayed a defect in muscle regeneration with fewer myofibers formed at early times after injury. NFAT5 has a muscle-intrinsic function because inhibition of NFAT5 transcriptional activity caused both a migratory and differentiation defect in cultured myoblasts. We identified Cyr61 as a target of NFAT5 signaling in skeletal muscle cells. Addition of Cyr61 to cells expressing inhibitory forms of NFAT5 rescued the migratory phenotype. These results demonstrate a role for NFAT5 in skeletal muscle cell migration and differentiation. Furthermore, as cell-cell interactions are crucial for myoblast differentiation, these data suggest that myoblast migration and differentiation are coupled and that NFAT5 is a key regulator.

Key words: Skeletal muscle, NFAT5, TonEBP, Myogenesis, Migration, Differentiation

Introduction

Skeletal muscle is composed of bundles of myofibers containing multiple nuclei in a continuous cytoplasm. Accounting for 50-60% of lean body mass (Prior et al., 2001) skeletal muscle is frequently damaged by direct trauma, invasive surgery and contractile activity. Muscle injury can induce sarcolemmal disruption, cytoskeletal destruction and myofiber degradation. As myofibers are terminally differentiated and myonuclei are postmitotic, de novo myofiber formation during regeneration is dependent upon satellite cells, a population of stem cells that exist beneath the basal lamina that surrounds each myofiber (Charge and Rudnicki, 2004). In response to injury, satellite cells proliferate and their progeny myoblasts migrate to sites of damage. Eventually myoblasts withdraw from the cell cycle and undergo differentiation leading to their fusion either with each other to form nascent myofibers or with existing myofibers, and muscle architecture is restored.

The nuclear factor of activated T cells (NFAT) proteins are a family of Ca^{2+}-activated transcription factors that function in numerous cell types (Horsley and Pavlath, 2002). NFATc1-c4 isoforms display a high degree of similarity in their DNA-binding and calcineurin-interaction domains (Hogan et al., 2003; Jain et al., 1995; Rao et al., 1997). We have shown that skeletal muscle expresses NFATc1-c3 but not NFATc4 and that these isoforms are differentially regulated in skeletal muscle depending on its state of differentiation (Abbott et al., 1998). NFATc2 and c3 have non-redundant functions in controlling muscle size (Horsley et al., 2001; Kegley et al., 2001). NFATc2 regulates the growth of nascent myotubes (Horsley et al., 2001) through an IL-4-dependent mechanism (Horsley et al., 2003), which is required for myoblast-myotube fusion. By contrast, NFATc3 directs primary myofiber formation during embryonic development through unknown mechanisms (Kegley et al., 2001).

NFAT5 is the newest member of the NFAT family of transcription factors based upon homology of its DNA-binding domain. The DNA-binding domain of NFAT5 exhibits approximately 40% homology to the NFATc1-c4 family members (Lopez-Rodriguez et al., 1999a). A number of key differences exist between NFAT5 and the NFATc1-c4 isoforms in terms of their activation and DNA binding. (1) NFATc1-c4 exist in the cytoplasm in an inactive phosphorylated state. Upon increases in intracellular Ca^{2+}, the phosphatase calcineurin is activated, which dephosphorylates NFAT, allowing nuclear translocation (Hogan et al., 2003). By contrast, NFAT5 is constitutively nuclear in many cell types (Lopez-Rodriguez et al., 1999b) regardless of whether calcineurin is active. (2) NFATc1-c4 bind to DNA in conjunction with a binding partner such as AP-1, which is activated by PKC signaling. By contrast, NFAT5 binds to DNA as a dimer (Lee et al., 2002; Lopez-Rodriguez et al., 2001; Lopez-Rodriguez et al., 1999b; Stroud et al., 2002). (3) The DNA binding sequences recognized by NFATc1-c4 versus NFAT5, although similar in a core GGAA recognition sequence, otherwise differ in the complete consensus sequence necessary for optimal binding (Lopez-Rodriguez et al., 1999b). (4) NFAT5 is activated in response to hypertonicity. It is

expressed in virtually all tissues, including many never normally exposed to extremes of hypertonicity (Dalski et al., 2000; Lopez-Rodriguez et al., 1999b; Miyakawa et al., 1999; Trama et al., 2000; Zhang et al., 2003). In isotonic environments, NFAT5 can be activated by amino acid depletion (Franchi-Gazzola et al., 2001; Trama et al., 2002) as well as engagement of extracellular receptors such as the T-cell receptor (Trama et al., 2000) and α6β4 integrin (Jauliac et al., 2002). By contrast, NFATc1-c4 are not known to be activated by hypertonicity. (5) In response to hypertonicity, NFAT5 activity is regulated at multiple stages: nuclear translocation (Cha et al., 2001; Dahl et al., 2001; Miyakawa et al., 1999; Woo et al., 2000a; Woo et al., 2000b), increase of its transcriptional activity (Ferraris et al., 2002) and increased expression (Miyakawa et al., 1999; Trama et al., 2000; Woo et al., 2002). The primary factor regulating the activity of NFATc1-c4 is nuclear translocation. Thus, NFAT5-dependent transcription could be modulated in multiple ways depending on cell type.

In the kidney, NFAT5 stimulates transcription of genes necessary for intracellular accumulation of osmolytes that act to control cellular ionic strength. These genes include transporters (Ito et al., 2004; Na et al., 2003), enzymes (Miyakawa et al., 1999) and molecular chaperones (Woo et al., 2002). In addition, NFAT5 regulates the expression of cytokines such as TNF and LTβ in osmotically stressed T cells (Esensten et al., 2005; Lopez-Rodriguez et al., 2001) suggesting roles for NFAT5 besides controlling cellular ionic strength. The widespread expression and activity of NFAT5 in cells never exposed normally to extreme hypertonicity raises the question of its role in non-renal tissues.

Genetically engineered mice mutant for NFAT5 have shed some light on the role of NFAT5 in non-renal tissues. Mice expressing either a dominant-negative NFAT5 (Trama et al., 2002) or containing a targeted deletion of its DNA-binding domain (Go et al., 2004) demonstrate a key role for NFAT5 in supporting an adaptive immune response in vivo. Mice with partial loss of NFAT5 function exhibit lymphoid hypocellularity (Go et al., 2004; Trama et al., 2002) and an impaired antigen-specific antibody response (Go et al., 2004; Trama et al., 2002). T cells isolated from these mice exhibit significantly impaired proliferative responses under conditions of hyperosmotic stress (Go et al., 2004; Trama et al., 2002). Transgenic mice expressing dominant-negative NFAT5 in the lens have defects in lens development (Wang et al., 2005). Furthermore, dominant-negative NFAT5 blocks cancer cell invasion and metastasis (Jauliac et al., 2002). The genes regulated by NFAT5 under isotonic conditions or in non-renal/non-lymphocytic cells are unknown.

In this study we hypothesized a physiological role for NFAT5 in skeletal muscle because several other NFAT isoforms have distinct roles during myogenesis. We demonstrate that NFAT5 is transcriptionally active in both primary muscle cells in vitro as well as in skeletal muscle tissue in vivo. Importantly, NFAT5 regulates myofiber formation during muscle regeneration in vivo. A muscle-intrinsic role for NFAT5 probably contributes to the defects in muscle regeneration because myoblasts expressing dominant-negative NFAT5 exhibit defects in migration and differentiation in vitro. Finally, we identified the secreted cysteine-rich CCN (connective tissue growth factor) matrix protein Cyr61 as a target gene of NFAT5 signaling that regulates myoblast migration. These results demonstrate a novel role for NFAT5 in muscle physiology and extend our knowledge of the role of various NFAT isoforms in myogenesis.

Results

NFAT5 is expressed and transcriptionally active in skeletal muscle in vivo

Expression of NFAT5 has been reported previously in myoblasts of the C2C12 myogenic cell line by immunocytochemistry (Lopez-Rodriguez et al., 1999b). To determine whether NFAT5 is expressed in skeletal muscle in vivo, sections from both control and regenerating tibialis anterior (TA) muscles were immunostained with an antibody against NFAT5. Whereas control sections displayed diffuse NFAT5 localization throughout the entire muscle (Fig. 1A), in regenerating muscles NFAT5 was prominently present in both mononucleated cells as well as regenerating myofibers (Fig. 1B). In fact, NFAT5 was present both in the nuclei and cytoplasm of regenerating myofibers. NFAT5 transcriptional activity in vivo was measured by electroporating either an NFAT5 reporter or a control reporter containing mutated NFAT5-binding sites into TA muscles. In our hands, plasmid injection and electroporation resulted in myofiber degeneration in part of the muscle (Fig. 1D), therefore, these experiments measure NFAT5 transcriptional activity in regenerating muscle. Consistent with the nuclear localization observed in regenerating muscles, NFAT5 was transcriptionally active in regenerating muscle as measured by luciferase assays (Fig. 1C). These data suggest that NFAT5 regulates some aspect of myogenesis during regeneration.

NFAT5 is transcriptionally active throughout myogenesis

To examine whether NFAT5 is differentially expressed during myogenesis, primary myoblasts were differentiated for 24 hours (nascent myotubes) and 48 hours (mature myotubes). Immunoblot analyses demonstrated NFAT5 was increased 2.8±0.4-fold and 2.9±0.8-fold in nascent and mature myotubes, respectively, compared with proliferating myoblasts (Fig. 2A). Together, these data suggest roles for NFAT5 in both early and late stages of myogenesis.

Immunocytochemical analyses revealed that NFAT5 was constitutively nuclear in myoblasts and nascent myotubes (Fig. 2B). However, in large multinucleated myotubes, NFAT5 displayed variable localization with a subset of nuclei containing less NFAT5 (arrows). As NFAT5 localization is tonicity-sensitive in immortalized cell lines (Woo et al., 2000a; Zhang et al., 2003) we measured the osmolarity of the culture media. Both growth medium (308 mOsm/l) and differentiation medium (327 mOsm/l) were hyperosmotic. The nuclear distribution of NFAT5 during myogenesis did not change in iso-osmotic media (290 mOsm/l) (data not shown) indicating that the nuclear distribution of NFAT5 is independent of media osmolarity.

Consistent with its nuclear localization, we confirmed that NFAT5 is transcriptionally active during myogenesis using a series of reporter assays. As $NFAT5^{-/-}$ mice are perinatal lethal (Go et al., 2004), myoblasts were isolated from wild-type and $NFAT5^{+/-}$ mice. NFAT5 transcriptional activity following treatment with mannitol, a membrane-impermeant osmotic stimulus (Gosmanov et al., 2003), was measured using a

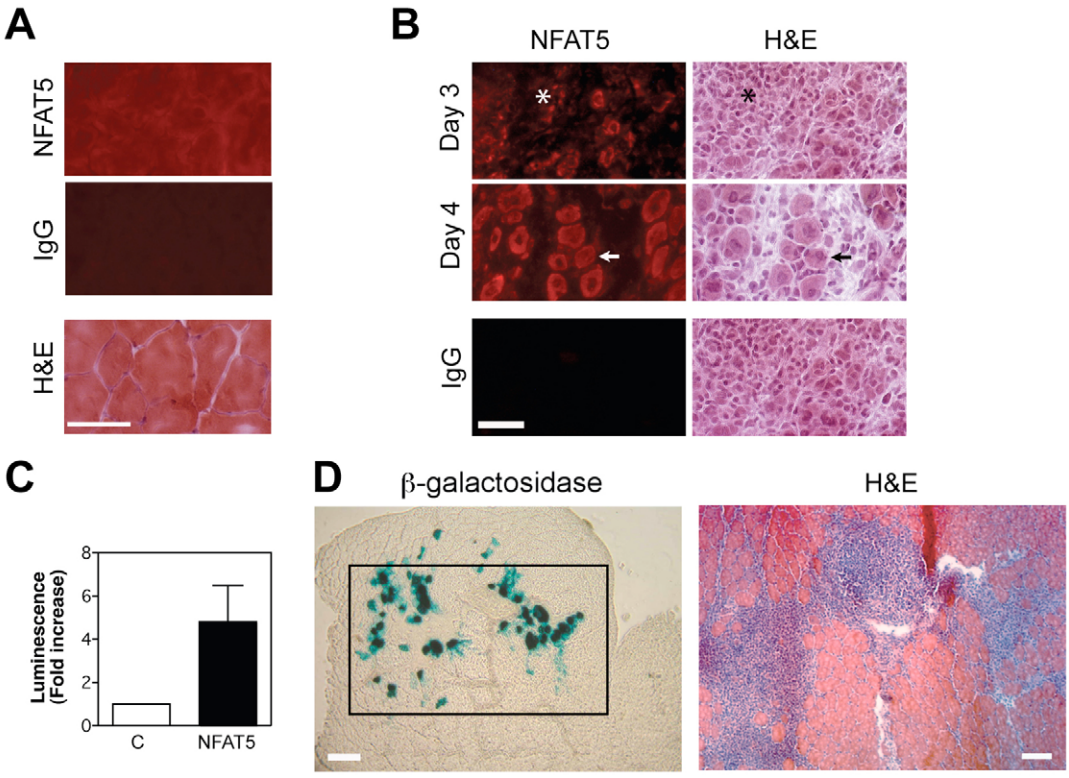

Fig. 1. NFAT5 expression and activity during myogenesis in vivo. (A) Immunostaining of TA sections from uninjured mice with an anti-NFAT5 antibody revealed a diffuse low level of staining throughout the section. No immunostaining was observed in sections following incubation with control IgG. Serial sections were stained with hematoxylin and eosin (H&E). Bar, 50 μm. (B) Immunostaining of TA sections 3 and 4 days following injury with an antibody against NFAT5 indicated bright staining of both mononucleated cells (*) and regenerating myofibers. NFAT5 appeared both nuclear (arrows) and cytoplasmic in newly formed myofibers (day 4). Arrow indicates the same myofiber in both sections. Bar, 50 μm. (C) TA muscles were electroporated with either an NFAT5 or control (C) reporter. Muscles were homogenized after 6 days and luciferase activity was determined. NFAT5 was transcriptionally active in vivo. Data are expressed as fold increase in luminescence relative to the control reporter containing mutated NFAT5 binding sites. Data are mean ± s.e.m. from four mice. (D) TA muscles were electroporated with a construct encoding β-gal and collected 6 days later. β-gal activity was confined to regenerating areas. The H&E stained section is a higher magnification view of the area in the indicated rectangle on the β-gal stained section. Bar, 100 μm.

luciferase-based NFAT5 reporter. A 52% decrease in NFAT5 transcriptional activity was observed in NFAT5$^{+/-}$ muscle cells in the presence of 100 mM mannitol (Fig. 3A). Lower concentrations of mannitol were also effective in inducing NFAT5 transcriptional activity in wild-type muscle cells (Fig. 3B). In addition, glucose, an alternate hyperosmotic agent commonly used in dialysis (Nette et al., 2002) and cell culture models of osmotic stress (Ohnishi et al., 2006), also induced NFAT5 transcriptional activity (Fig. 3C). Together, these data demonstrate NFAT5 is tonicity sensitive in skeletal muscle cells. The activity of the NFAT5 reporter was independent of the calcineurin-dependent NFATc1-c4 isoforms because VIVIT, a peptide inhibitor of NFATc1-c4 activation (Aramburu et al., 1998), had no effect on luciferase activity (Fig. 3C). By contrast, VIVIT caused a 60% inhibition of the pKA7 reporter, which measures luciferase activity mediated by NFATc1-c4 (Fig. 3D). Subsequently, we assessed native NFAT5 activity during myogenesis. NFAT5 was equally active in myoblasts, nascent myotubes and mature myotubes (Fig. 3E) despite the increased protein levels in myotubes. However, induction of NFAT5 activity following treatment with mannitol was threefold greater in myotubes than myoblasts (Fig. 3F) consistent with the threefold higher levels of NFAT5 in myotubes. These data suggest that NFAT5 integrates signals present in both proliferating and differentiated muscle cells resulting in NFAT5-mediated gene transcription.

NFAT5 regulates myofiber formation during muscle regeneration

We used a mouse model of muscle regeneration in wild-type and NFAT5$^{+/-}$ mice to assess the role of NFAT5 in regulating muscle growth in vivo. However, NFAT5 is expressed in immune cells (Lopez-Rodriguez et al., 1999b; Trama et al., 2002; Trama et al., 2000) and efficient muscle regeneration is highly dependent on neutrophil and macrophage infiltration soon after injury (Lescaudron et al., 1999; Robertson et al., 1992). Therefore, infiltration of Mac-1^{+} cells (neutrophils and macrophages) was measured in regenerating wild-type and NFAT5$^{+/-}$ TA muscles injured by exposure to $BaCl_2$ along the length of the muscles. Three days after $BaCl_2$ injection, flow cytometric analyses demonstrated similar percentages of Mac-1^{+} cells in regenerating muscle of both genotypes (Fig. 4A). The extent of damage as assessed by the percentage of the muscle showing degenerative changes was not different between the two genotypes (data not shown). Although the cross-sectional area (XSA) of the entire TA muscle in the belly

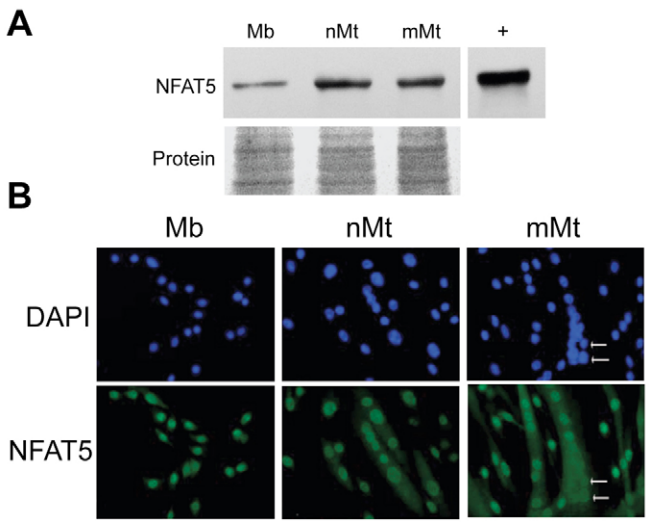

Fig. 2. NFAT5 expression and localization during myogenesis in vitro. (A) Lysates were prepared from primary myoblasts (Mb), nascent myotubes (nMt) and mature myotubes (mMt) as well as from murine kidney (+). Representative immunoblot analyses demonstrated NFAT5 protein was increased in myotubes compared with myoblasts. (B) NFAT5 localization during myogenesis was detected by immunostaining with an antibody against NFAT5. Nuclei were counterstained with the fluorescent dye DAPI. NFAT5 appeared predominantly nuclear in myoblasts, nascent myotubes (nMt) and mature myotubes (mMt). A subset of nuclei in mMT contained less NFAT5 (arrows) than others. No immunostaining was observed with an IgG control (data not shown).

region was similar in both wild-type and NFAT5$^{+/-}$ regenerating muscle (wild type: 132±15 mm^2; NFAT5$^{+/-}$: 144±15 mm^2), the actual number of myofibers appeared to be decreased in NFAT5$^{+/-}$ muscles 5 days after injury (Fig. 4B), therefore, we quantified the number of regenerating myofibers per field. As seen in Fig. 4C, myofiber number was significantly decreased by 25% in NFAT5$^{+/-}$ TA muscles at day 5 after injury, but not day 14. By contrast, the mean XSA of regenerating myofibers did not differ between these two genotypes at day 5 after injury but NFAT5$^{+/-}$ myofibers were 28% smaller at day 14 (Fig. 4D). The smaller mean XSA at day 14 probably reflects new myofiber formation between days 5 and 14 in NFAT5$^{+/-}$ muscles. To determine whether the effects of NFAT5 are specific to regenerating muscle, myofiber number and XSA were quantified in uninjured soleus muscles from wild-type and NFAT5$^{+/-}$ mice. No differences were observed either in myofiber number (Fig. 4E) or XSA (data not shown) in uninjured muscles. Taken together, these data suggest that NFAT5 regulates myofiber formation during regeneration.

NFAT5 regulates myoblast differentiation

The observed defects in myofiber formation during regeneration suggest that early stages of myogenesis such as myoblast proliferation and differentiation may be regulated by NFAT5. To determine a muscle-intrinsic role for NFAT5, myoblast proliferation and differentiation were analyzed in vitro. As NFAT5$^{+/-}$ muscle cells retain 50% transcriptional activity, we generated a retroviral vector expressing dominant-negative NFAT5 (NFAT5-DN). Following retroviral infection of wild-type myoblasts with NFAT5-DN, NFAT5

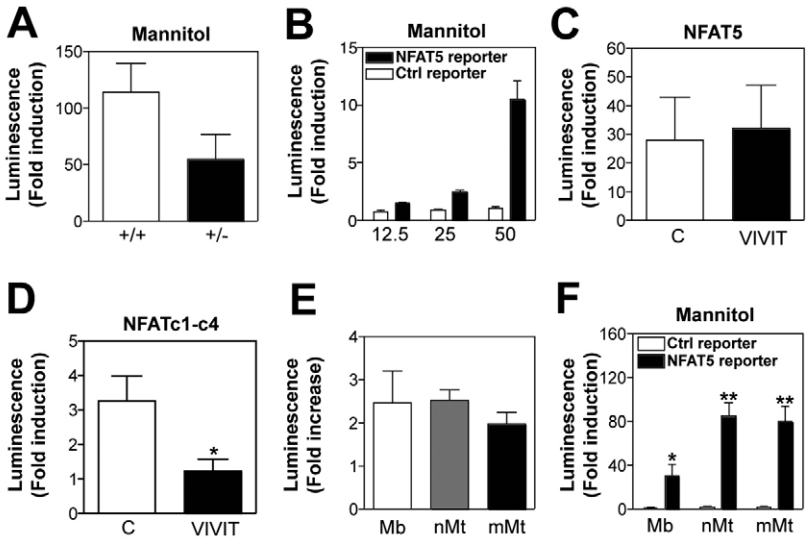

Fig. 3. NFAT5 is transcriptionally active in skeletal muscle cells in vitro. (A) Wild-type (+/+) and NFAT5$^{+/-}$ myoblasts containing an NFAT5 reporter plasmid were differentiated for 24 hours and subsequently treated with 100 mM mannitol for 5 hours. Luciferase activity was diminished by 52% in mannitol-treated NFAT5$^{+/-}$ muscle cells compared to the wild type. Data are fold induction over basal levels of luciferase activity in unstimulated cells. (B) Wild-type myoblasts containing either control or NFAT5 reporters were differentiated for 40 hours and subsequently treated with lower concentrations of mannitol (mM) for 5 hours. Data are fold induction over basal levels of luciferase activity in unstimulated cells. (C) Wild-type myoblasts containing an NFAT5 reporter plasmid and VIVIT, a peptide inhibitor of NFATc1-c4 activation, were differentiated for 48 hours and treated with 100 mM glucose. Luciferase activity was similar in the presence or absence of VIVIT. Data are fold induction over basal levels of luciferase activity in unstimulated cells. (D) In parallel experiments, co-transfection of an NFATc1-c4 reporter plasmid and VIVIT led to decreased luciferase activity in cells treated with PMA and ionomycin (*P<0.05). Data are fold induction over basal levels of luciferase activity in unstimulated cells. (E) Wild-type myoblasts containing either control or NFAT5 reporters were differentiated for 24 hours (nMt) or 48 hours (mMt) and analyzed by luciferase assay. NFAT5 was transcriptionally active at all stages of myogenesis. Data are fold increase over levels of luciferase activity in cells containing a reporter with mutated NFAT5 binding sites. (F) NFAT5 transcriptional activity was analyzed as in E, but cells were treated with 100 mM mannitol to induce NFAT5 activity. Maximal NFAT5 activity was observed in myotubes (*P<0.05 for Mb relative to ctrl; **P<0.05 for Mt relative to Mb). Data are fold induction over basal levels of luciferase activity in unstimulated cells. All data in this figure are mean ± s.e.m. from three to four independent experiments.

transcriptional activity was decreased by 74% in the presence of mannitol (Fig. 5C). To analyze the effects on cell proliferation, myoblasts infected with either control or NFAT5-DN retroviruses were labeled with BrdU. Immunocytochemical analyses demonstrated an equivalent percentage of $BrdU^+$ cells in control and NFAT5-DN myoblasts (Fig. 5A,B). Thus, NFAT5 does not appear to play a significant role in regulating myoblast proliferation.

The effects of NFAT5 on myoblast differentiation were also assessed in cells expressing NFAT5-DN. Differentiating myoblasts were immunostained for eMyHC, a marker of differentiated muscle cells (arrows, Fig. 5D). At 10 hours following the induction of differentiation, the number of $eMyHC^+$ cells was decreased 39% in NFAT5-DN cultures (Fig. 5E). However, this defect was transient as the differentiation index was similar in both control and NFAT5-DN cells by 24 hours (Fig. 5F). These data demonstrate a role for NFAT5 during the early stages of differentiation.

NFAT5 regulates myoblast motility

As myoblast migration is integral to myogenesis both in vitro (Bonavaud et al., 1997; Dedieu et al., 2004) and in vivo (Neuhaus et al., 2003; Watt et al., 1994), wild-type and $NFAT5^{+/-}$ myoblasts were analyzed by time-lapse microscopy every 6 minutes for 36 minutes. The paths of individual cells were tracked revealing that wild-type myoblasts migrated further than $NFAT5^{+/-}$ myoblasts (Fig. 6A). In addition, the average velocity of $NFAT5^{+/-}$ myoblasts was decreased by 18% compared to the wild type (Fig. 6B). Analysis of velocity as a frequency distribution revealed a population of wild-type myoblasts (10%) migrating at velocities of 85-125 μm/hour that were absent in $NFAT5^{+/-}$ myoblasts.

Cyr61, an NFAT5 target gene, enhances myoblast motility

Cyr61 is an extracellular-matrix-associated protein regulating cell adhesion, migration, chemotaxis and differentiation in fibroblasts and endothelial cells (Chen et al., 2000; Leu et al., 2002). Transacting factors mediating transcription of Cyr61 are unknown. However, as induction of Cyr61 expression is tonicity sensitive in the kidney (Nahm et al., 2002) and Cyr61 contains three putative NFAT5-binding sites in its proximal promoter region (data not shown) we hypothesized that NFAT5 regulates Cyr61 gene transcription in skeletal muscle. RT-PCR analyses demonstrated that Cyr61 was expressed at all stages of myogenesis in vitro (Fig. 7A). Cyr61 was also expressed in skeletal muscle in vivo (Fig. 7B). Cyr61 expression was observed in uninjured muscle as well as throughout muscle regeneration during the formation of new myofibers. Retroviral infection of myoblasts with NFAT5-DN decreased Cyr61 protein expression suggesting Cyr61 transcription is either directly or indirectly regulated by NFAT5 (Fig. 7C). Similar results were obtained with NFAT5 siRNA (data not shown). Suprisingly, a decrease in Cyr61 expression was not observed in regenerating $NFAT5^{+/-}$ muscle (data not shown). However, multiple cell types known to express Cyr61 [endothelial cells (Glienke et al., 2000) and fibroblasts (O'Brien et al., 1990)] are also present in regenerating muscle tissue. Possibly, Cyr61 induction may occur in non-muscle cells in an NFAT5-independent manner during regeneration.

Subsequently, cell migration was analyzed in NFAT5-DN cells using time-lapse microscopy. Similarly to $NFAT5^{+/-}$ cells, the average velocity of NFAT5-DN cells was decreased by 17% compared with control cells (Fig. 7D). Although the NFAT5-DN construct demonstrated greater efficacy with regard to NFAT5 transcriptional inhibition following mannitol stimulation (Fig. 3A and Fig. 5C), transcriptional repression in the absence of external stimulation was similar between NFAT5-DN and $NFAT5^{+/-}$ cells (22% vs 35%, respectively).

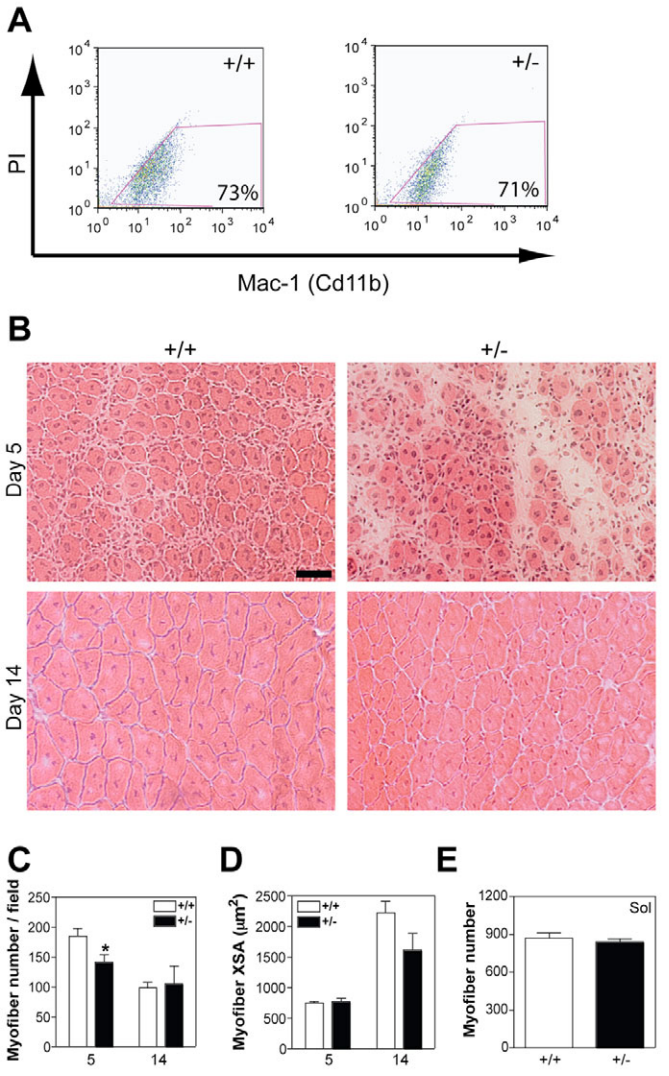

Fig. 4. NFAT5 regulates myofiber formation during muscle regeneration in vivo. (A) Cells were isolated from the TA muscles of wild-type (+/+) and $NFAT5^{+/-}$ mice 3 days following injury and analyzed for Mac-1 expression by flow cytometry. Similar percentages of $Mac\text{-}1^+$ macrophages were observed in regenerating muscle from wild-type (+/+) and $NFAT5^{+/-}$ mice. (B) TA sections obtained from wild-type(+/+) and $NFAT5^{+/-}$ mice were stained with hematoxylin and eosin 5 and 14 days following injury. Bar, 50 μm. (C) The number of regenerating TA myofibers per field was reduced in $NFAT5^{+/-}$ mice 5 days (*P<0.05) but not 14 days following injury. (D) The XSA of regenerating TA myofibers was similar in both wild-type (+/+) and $NFAT5^{+/-}$ mice 5 days following injury but decreased in $NFAT5^{+/-}$ mice 14 days following injury. (E) Myofiber number was similar in uninjured soleus muscles of both wild-type (+/+) and $NFAT5^{+/-}$ mice. All data are mean ± s.e.m., n=4-8 for each genotype.

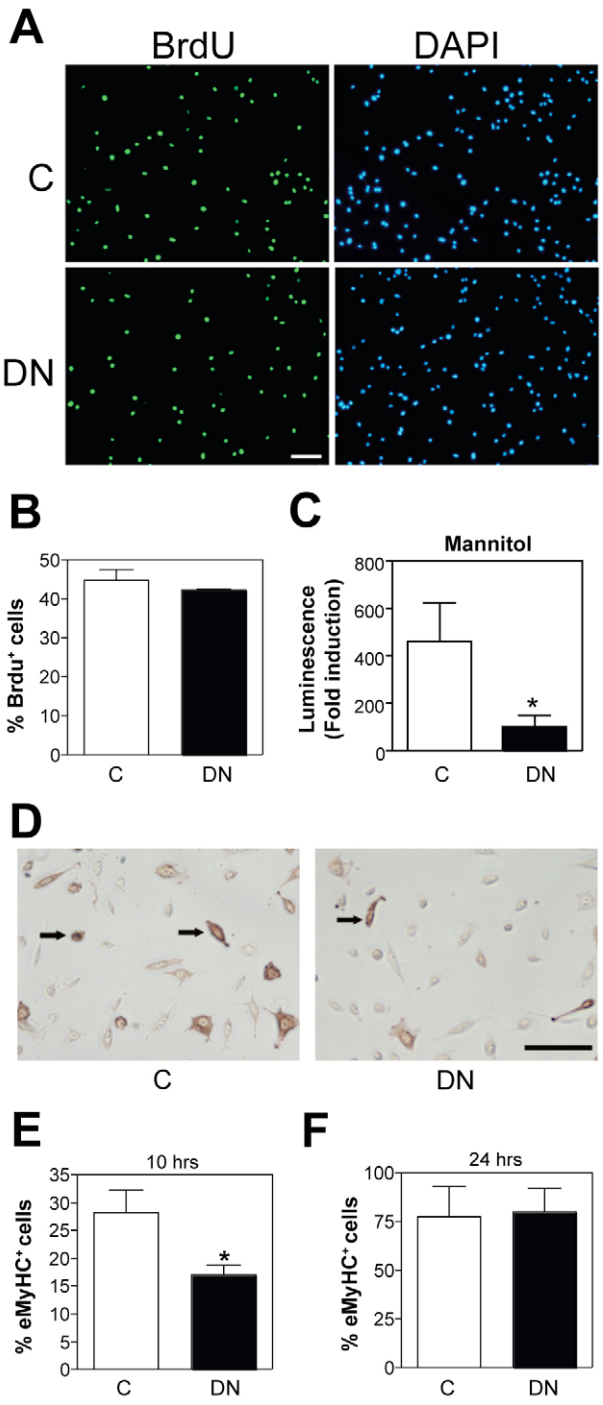

Fig. 5. Myoblast differentiation is transiently decreased in muscle cells containing dominant-negative NFAT5. (A) Primary myoblasts containing either control (C) or dominant-negative NFAT5 (DN) plasmids were labeled with BrdU and subsequently immunostained. Nuclei were counterstained with DAPI. Bar, 100 μm. (B) The percentage of $BrdU^+$ cells was similar in both control and DN cultures. (C) Nascent myotubes containing the NFAT5 reporter and either C or DN plasmids were treated with 100 mM mannitol for 5 hours. Luciferase activity was reduced by 74% in DN cells (*P<0.05). Data are fold induction over basal levels of luciferase activity in unstimulated cells. (D) eMyHC immunostaining (arrows) in primary myoblasts containing either C or DN retroviruses. Bar, 100 μm. (E) Ten hours following the induction of differentiation, the percentage of $eMyHC^+$ cells in DN cultures was decreased (*P<0.05). (F) After 24 hours of differentiation, the percentage of $eMyHC^+$ cells in DN cultures was similar to levels in the control. All data are mean ± s.e.m. from three to four independent experiments.

Thus, the defects in cell motility in cells expressing NFAT5-DN are comparable with $NFAT5^{+/-}$ cells. The addition of 5 μg/ml recombinant Cyr61 significantly enhanced motility of NFAT5-DN cells such that the mean velocity of NFAT5-DN cells returned to control values (Fig. 7D). Complementing these data, frequency distribution analyses also demonstrated increased velocities in NFAT5-DN cells treated with Cyr61 (Fig. 7E). However, recombinant Cyr61 had no effect on the velocity of wild-type myoblasts (data not shown), suggesting sufficient levels of Cyr61 are present in the extracellular matrix milieu and that Cyr61 growth-factor-mediated signaling is tightly regulated. Together, these data identify Cyr61 as a target of NFAT5 signaling in skeletal muscle cells and suggest Cyr61 is important for regulating myoblast motility.

Discussion

Our data identify a novel role for NFAT5 in cell physiology. We demonstrate that NFAT5 regulates myofiber formation during regeneration. Muscle-intrinsic defects probably contribute to this impaired regeneration because myoblasts with impaired NFAT5 signaling migrated shorter distances and at lower velocities than normal. Addition of the NFAT5-target gene Cyr61 rescued the migratory defect. Defects in cell migration may underlie the transient delay in myoblast differentiation we observed, because cell-cell contact is crucial for the initiation of myoblast differentiation (Kang et al., 2004; Krauss et al., 2005). These data demonstrate a role for NFAT5 during the early stages of myogenesis and suggest myoblast migration and differentiation are closely linked. The muscle defects observed in the presence of impaired NFAT5 signaling are different from other NFAT isoforms studied to date. Previous studies from our lab indicated NFATc3 was crucial for myofiber formation during embryogenesis (Kegley et al., 2001). However, no defects were observed in NFATc3-null mice during post-natal myogenesis (Horsley et al., 2001). By contrast, developmental myogenesis was normal in mice lacking NFATc2 but post-natal muscle growth and regeneration after injury were impaired (Horsley et al., 2001). Although the number of newly formed myofibers was similar in regenerating muscle of wild-type and $NFATc2^{-/-}$ mice a muscle-intrinsic defect in myoblast fusion prevented myonuclear addition and subsequent increases in myofiber XSA. In the current study, impaired myofiber formation was seen after injury in $NFAT5^{+/-}$ muscles (Fig. 4B,C). Thus, the defect in $NFAT5^{+/-}$ muscle is distinct from the phenotypes in $NFATc3^{-/-}$ and $NFATc2^{-/-}$ mice. The effects of NFAT5 are specific to regenerating muscle because the number of myofibers and myofiber XSA in uninjured muscle was identical in wild-type and $NFAT5^{+/-}$ mice indicating that NFAT5 does not influence embryonic muscle development. Embryonic, fetal and adult myoblasts have been extensively characterized (Miller et al., 1993; Stockdale, 1992). These cells differ with respect to signaling pathways and gene expression. Such disparity may underlie the differential effects of NFAT isoforms on embryonic muscle development compared with post-natal regeneration. Our

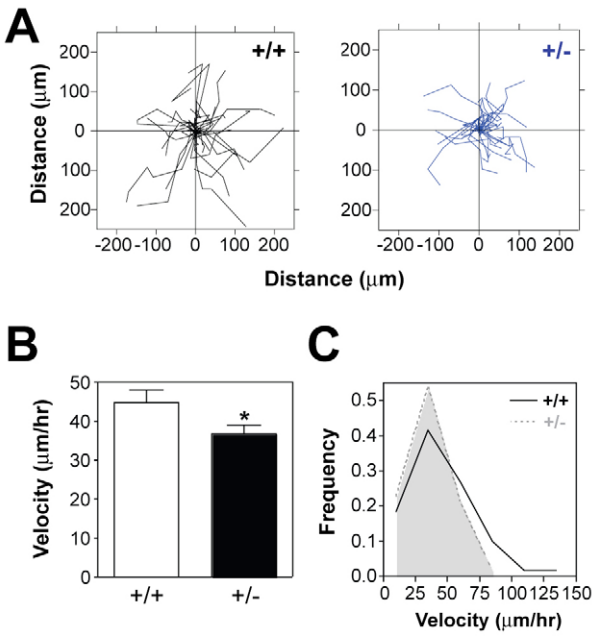

Fig. 6. NFAT5$^{+/-}$ muscle cells are less motile. (A) Migration of wild-type (+/+) and NFAT5$^{+/-}$ myoblasts was analyzed using time-lapse microscopy. Individual cell traces from 30 cells of each genotype are shown. NFAT5$^{+/-}$ myoblasts migrated shorter distances than the wild type. Three independent isolates for each genotype were analyzed. (B) Mean velocity was decreased in NFAT5$^{+/-}$ myoblasts (*P<0.05). Data are mean ± s.e.m. of 60 cells (20 cells from each of three independent isolates). (C) Frequency histogram illustrating the distribution of cell velocities for wild-type and NFAT5$^{+/-}$ myoblasts. A population of rapidly moving cells was absent in NFAT5$^{+/-}$ myoblasts. Data are mean ± s.e.m. of 60 cells (20 cells from each of three independent isolates).

results provide further evidence that NFAT isoforms perform non-redundant functions in skeletal muscle.

A muscle-intrinsic role for NFAT5 exists in myoblasts and probably contributes to the defects in myofiber formation during regeneration. Migratory defects were observed in NFAT5$^{+/-}$ myoblasts in vitro (Fig. 6). Further migration analyses confirmed similar migratory defects in myoblasts expressing dominant-negative NFAT5 (Fig. 7D,E). Following muscle injury, a release of diffusible factors stimulates myoblast migration to the site of damage, which is important for myofiber formation (Watt et al., 1994). Our studies support a general role for NFAT5 in cell migration. A previous study demonstrated that activation of NFAT5 by integrin engagement promoted cancer cell invasion and migration through Matrigel-coated filters in Boyden chambers (Jauliac et al., 2002). Thus, NFAT5 appears to be important for both physiological and pathological cell migration.

In addition to myoblast migration, NFAT5 was also important for the early stages of myoblast differentiation (Fig. 5D,E). The number of differentiated myoblasts as assessed by eMyHC immunostaining was transiently decreased during early differentiation in dominant-negative NFAT5 cells. Other signaling pathways might compensate for impaired NFAT5 signaling and allow normal differentiation to occur at later times in differentiation. Cell-cell contact between myoblasts needs to occur for activation of intracellular signaling leading to myogenic differentiation. The "community effect" describes the process of contact-dependent myogenic differentiation (Gurdon, 1988; Krauss et al., 2005). Initially identified in *Xenopus* myogenic explants (Gurdon et al., 1993) and later complemented by studies in murine myogenesis (Cossu et al., 1995), a threshold number of myoblasts is required for muscle differentiation. An optimal density of myoblasts promotes cell-cell contact leading to the generation of adhesion-dependent signals that activate myogenic gene transcription. For example, the formation of a cell-surface complex involving Ig superfamily members (CDO, BOC), cadherins and neogenin induces bHLH transcription factor activation (Cole et al., 2004; Kang et al., 2002; Kang et al., 2004; Knudsen et al., 1990). The migratory defects present in NFAT5$^{+/-}$ myoblasts might limit cell-cell contact and subsequent activation of myogenic gene transcription. Furthermore, secreted proteins regulating myogenic gene transcription in a paracrine and autocrine manner might also be diluted, with increased distances between cells. Muscle is not the only cell type in which migration and differentiation are coupled. For example, in response to chemokines, adipose precursor cells migrate into clusters where sequential gene induction and repression results in differentiation (Omatsu-Kanbe et al., 2006). Similarly, platelet-derived chemoattractants regulate trophoblast migration and subsequent differentiation along the vessel wall of maternal arteries (Sato et al., 2005). During vascular development, endothelial cell migration and cell-cell contact are required for differentiation and formation of arteries and veins (Fuller et al., 2003). Furthermore, axonal migration in the dorsal cerebral cortex is coupled to neuronal differentiation and the formation of functionally competent GABAergic neurons (Bellion et al., 2003). Thus, coupled cell migration and differentiation are vital for the formation of several tissue types.

The identity of genes regulated by NFAT5 in muscle cells is clearly of interest for understanding the phenotype of NFAT5$^{+/-}$ and NFAT5 dominant-negative cells. We identified Cyr61 as a target gene of NFAT5 signaling in muscle cells because dominant-negative NFAT5 decreased its expression at both the RNA (data not shown) and protein level (Fig. 7C). These results do not distinguish between indirect and direct regulation of Cyr61 gene expression by NFAT5. Cyr61 (CCN1) is one member of the six-member CCN family of genes (Perbal, 2004). CCN proteins are secreted cysteine-rich proteins that are found on the cell surface as well as in the extracellular matrix. All family members have a conserved multi-modular structure consisting of four domains sharing identity with insulin-like growth factor (IGF) binding proteins, Von Willebrand factor, thrombospondin and cysteine-knot-containing growth regulators. Cyr61 has been associated with proliferation, adhesion, migration and differentiation in fibroblasts and endothelial cells (Babic et al., 1998; Kireeva et al., 1996). These activities are thought to occur in part through the ability of Cyr61 to bind and activate cell-surface integrins (Grzeszkiewicz et al., 2001; Kireeva et al., 1998; Leu et al., 2002; Schober et al., 2002) as well as heparan sulfate proteoglycans (Chen et al., 2004).

Cyr61 was expressed in regenerating skeletal muscle (Fig. 7B) consistent with a role for NFAT5 in regulating myofiber formation after injury. Cyr61 was also present in muscle tissue

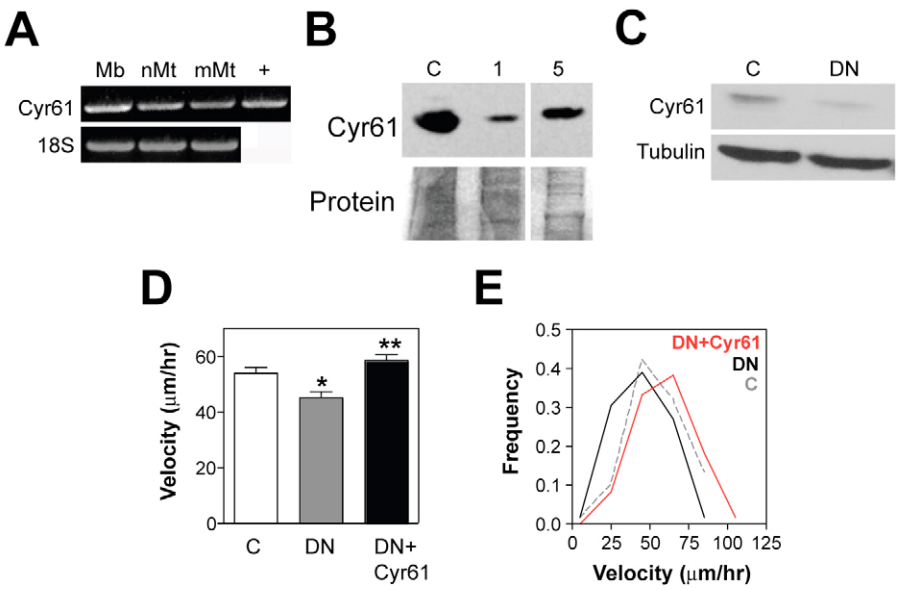

Fig. 7. Cyr61, an NFAT5 target gene in skeletal muscle, regulates myoblast motility. (A) RNA was isolated from primary myoblasts (Mb), nascent myotubes (nMt) and mature myotubes (mMt) as well as from murine kidney (+). RT-PCR analyses demonstrated that Cyr61 was expressed in skeletal muscle cells at all stages of myogenesis. Equivalent input of cDNA was confirmed by amplification of the ribosomal gene 18S. (B) Immunoblot analyses demonstrated that Cyr61 protein was expressed in both uninjured (C) and regenerating (n=3 animals at each time point except n=2 for day 5) TA muscles at days 1 and 5 after injury. Equivalent protein loading was confirmed by Coomassie Blue staining. (C) Lysates from myoblasts containing either control (C) or dominant-negative NFAT5 (DN) plasmids were immunoblotted for Cyr61. Cyr61 was decreased in DN cells. Tubulin was detected as a loading control. (D) The average velocity of myoblasts expressing DN was decreased relative to the control. (*P<0.05). Average velocity returned to control levels following the addition of 5 μg/ml Cyr61 to these cells (**P<0.05 relative to DN). Data are mean ± s.e.m. of 60 cells (20 cells from each of three independent isolates). (E) Frequency histogram illustrating the distribution of cell velocities for control, DN and DN+Cyr61 cells. The DN curve was shifted to the left relative to control. The addition of 5 μg/ml Cyr61 to DN cells caused a rightward shift in the population. Data are mean ± s.e.m. of 60 cells (20 cells from each of three independent isolates).

before injury. Cyr61 might be important for both muscle and non-muscle cells during normal muscle maintenance. Cyr61 might regulate myoblasts in several ways. First, Cyr61 may be tethered to the extracellular matrix through its heparin-sulfate-binding domain and act as a ligand for integrin receptors. Subsequent activation of integrin signaling could effect actin polymerization and focal adhesion formation with the ECM (Lo, 2006) allowing myoblasts to migrate to sites of damage. In addition, Cyr61 might also induce matrix metalloproteinase production (Nguyen et al., 2006) cleaving ECM proteins that act as a barrier to migration of myoblasts in injured muscle tissue. Finally, Cyr61 may play a recruitment function in the extracellular matrix, assembling multiple factors including IGF-1Ea, IGF-1Eb and mechano growth factor through its IGF binding protein domain providing a dense pool of factors regulating myoblast function.

What activates NFAT5 signaling during myogenesis? NFAT5 was originally characterized in kidney cells as a tonicity-sensitive transcription factor (Miyakawa et al., 1999). In response to hyperosmotic solutions, NFAT5 activation leads to increased gene transcription of a number of volume regulatory transporters (Miyakawa et al., 1998; Rim et al., 1998), which draw fluid back into the cell. Subsequently, NFAT5-mediated osmocompensatory gene transcription was observed in T lymphocytes (Trama et al., 2000) and neurons (Loyher et al., 2004). NFAT5 is also tonicity sensitive in skeletal muscle, because NFAT5 activity was enhanced following stimulation with the osmotic inducers glucose and mannitol (Fig. 3). Despite NFAT5 activation following hypertonic stimulation, NFAT5 also remained constitutively nuclear in primary muscle cells at isotonic osmolarities (290 mOsm/l). Therefore, other extracellular signals besides hypertonicity might activate NFAT5 nuclear translocation and signaling during myogenesis in vitro. In isotonic environments NFAT5 can be activated by amino acid depletion (Franchi-Gazzola et al., 2001; Trama et al., 2002) as well as engagement of extracellular receptors such as the T-cell receptor (Trama et al., 2000) and α6β4 integrin (Jauliac et al., 2002).

Myoblasts are not the only site of NFAT5 signaling during myogenesis. NFAT5 is constitutively nuclear and transcriptionally active in myotubes (Fig. 2B and Fig. 3E) as well as expressed and transcriptionally active in regenerating muscle in vivo (Fig. 1B,D) suggesting further roles in differentiated muscle cells. In large myotubes however, a subset of nuclei contained less NFAT5, revealing that NFAT5 is spatially regulated in skeletal muscle cells. NFAT5 might remain nuclear during early stages of myogenesis because NFAT5-mediated transcription is necessary for the migration of myoblasts and their subsequent differentiation. Thus, in mature myotubes that have completed their myogenic program, nuclear export may commence. Further work is required to define the role of NFAT5 in myotubes.

In summary, NFAT5 signaling contributes to myoblast migration and differentiation, both of which are required for myofiber formation during muscle regeneration. Cyr61, a target gene of NFAT5 in muscle cells, is required for optimal myoblast migration and probably contributes to an adequate density of myoblasts at sites of injury. Thus, activators of NFAT5 signaling may be useful in enhancing muscle repair. Finally, these studies suggest that targets of NFAT5 signaling such as Cyr61 may be important in regulating cell migration in a number of cell types.

Materials and Methods

Animals and cell culture

NFAT5$^{+/-}$ mice, backcrossed for eight generations onto a C57BL/6 background, and their non-transgenic littermates were used at 8-12 weeks of age. Genotyping was performed by PCR analyses of tail tissue DNA as described (Go et al., 2004). Mice were housed under a 12 hour dark/light cycle and received food and water ad

libitum. All procedures were approved by Emory University's Institutional Animal Care and Use Committee.

Primary myoblasts (>90% purity) were isolated from the hindlimb muscles of wild-type, NFAT5$^{+/-}$ and Balb/c mice as described previously (Mitchell and Pavlath, 2004) excluding differential centrifugation with Percoll. Primary myoblasts were grown and differentiated as described previously (Horsley et al., 2001; Mitchell and Pavlath, 2004). C2C12 myoblasts (Blau et al., 1985) were cultured in medium comprising DMEM, 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin.

Muscle injury and immunohistochemical analyses

Male wild-type and NFAT5$^{+/-}$ mice were anesthetized by intraperitoneal injection of a mixture of xylazine (15 mg/kg) and ketamine (100 mg/kg). Muscle injury was induced by injection of 50 μl of 1.2% $BaCl_2$ in PBS using a Hamilton syringe and a 27 G needle from the origin to the insertion of the left TA muscle, whereas the contralateral muscle served as a control. Mice were euthanized 5 and 14 days following injury (4-8 mice per experimental group). Injured and control TA muscles as well as control soleus muscles were collected, frozen and serial 14 μm cross-sections obtained as described previously (Pavlath et al., 1998). Cryosections from the belly of the TA muscle were analyzed by quantifying myofiber number and XSA for 100-250 myofibers in a 307,200 μm^2 field in the core of the regenerating muscle (Horsley et al., 2003). In addition, the XSA of the entire TA muscle in the belly region was visualized using a 4× objective and subsequently quantified. For control TA muscles, myofiber XSA was also quantified in the belly of the muscle. Myofiber number and XSA of the soleus were visualized and quantified in similar anatomical regions. All analyses were performed blinded.

NFAT5 immunostaining was performed in both uninjured muscle and during muscle regeneration 3 and 4 days after injury to the TA by $BaCl_2$ as described above. Muscle sections were treated with blocking buffer containing 10% donkey serum and 0.25% Triton X-100 in PBS for 1 hour and incubated overnight at 4°C with NFAT5 antiserum or purified rabbit IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking buffer. Following successive washes in TNT (0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% Tween-20), sections were incubated with biotin-conjugated donkey anti-rabbit F(ab′)$_2$ fragments diluted 1:250 in PBS containing 2% donkey serum for 1 hour. Following repeated washes in a solution of TNT, the sections were incubated in Texas-Red-conjugated streptavidin diluted 1:1000 in PBS for 30 minutes. Serial sections were stained with hematoxylin and eosin.

RT-PCR analyses

Total RNA was isolated from cells using TRIzol reagent (Life Technologies) in accordance with the manufacturer's instructions. The reverse transcriptase reaction was performed using 2.5 μg of total RNA/sample. cDNA (1 μl/sample) was amplified using 2.5 μM of each primer and the Expand High Fidelity PCR system (Roche) in a final volume of 50 μl. Cyr61 primers (sense: 5′-GACGGCTG-CGGCTGCTGTAAGGT-3′ and antisense: 5′-ACTCGCGGTTCGGTGCCAAA-GAC-3′) were designed from GenBank accession number NM_010516. Cyr61 primers spanned intron-exon boundaries to control for genomic contamination and were specific for Cyr61 by Blast search.

Amplification was performed in the linear phase and consisted of 30 cycles of 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds, and a final termination step at 72°C for 5 minutes generating an amplicon of 503 bp. As an internal control 18S cDNA was amplified with QuantumRNA 18S primers (Ambion). PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. Duplicate independent experiments were analyzed to confirm the pattern of Cyr61 gene expression during myogenesis.

Immunoblotting

Cells were harvested in RIPA-2 (Horsley et al., 2001) containing protease inhibitors (Mini Complete; Roche) and immunoblots were performed as described (Friday and Pavlath, 2001) using 30 μg protein/lane, a 1:10,000 dilution of rabbit NFAT5 antiserum (Woo et al., 2002) and a 1:10,000 dilution of HRP-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). To demonstrate equal protein loading, the membrane was stained with Coomassie Blue (Bio-Rad). Triplicate independent experiments were analyzed to confirm the pattern of NFAT5 expression during myogenesis.

Expression of Cyr61 protein in vitro was determined following retroviral infection of C2C12 myoblasts with either control or dominant-negative NFAT5 vectors. Immunoblotting was performed using 80 μg/lane, a 1:500 dilution of rabbit Cyr61 antiserum (Abcam) and a 1:5000 dilution of an HRP-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). Equivalent protein loading per lane was confirmed using a 1:500 dilution of mouse monoclonal antibody to tubulin (Sigma) and a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch).

Cyr61 protein expression during skeletal muscle regeneration was examined in C57BL/6 mice injured by $BaCl_2$ injection as described above. TA muscles (n=2-3 at each time point) were collected 1 and 5 days following injury. Muscles were homogenized with RIPA-2 containing protease inhibitors (Mini Complete; Roche) and subsequently centrifuged at 1100 ***g*** for 30 minutes at 4°C. Immunoblotting was performed with Cyr61 antiserum as described above. Equal protein loading was confirmed by staining the membrane with Coomassie Blue.

Immunocytochemistry

NFAT5 immunostaining was performed using a TSA Green Tyramide Signal Amplification kit (PerkinElmer). Primary myoblasts were maintained in growth media or differentiated for 24 and 48 hours. Cells were fixed with –20°C methanol for 10 minutes. Following successive washes with PBS, the cells were treated with blocking buffer containing 5% donkey serum, 0.5% BSA, 0.25% Triton X-100 in PBS for 1 hour. The cells were incubated overnight at 4°C with either the NFAT5 antiserum or purified rabbit IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking buffer. All subsequent steps were performed at room temperature. The cells were rinsed with PBS containing 0.2% Tween 20 (PBS-T) and incubated with biotin-conjugated donkey anti-rabbit F(ab′)$_2$ fragments (Jackson-ImmunoResearch) diluted 1:500 in PBS-T for 1 hour. Following successive washes in PBS-T, further incubations were performed with HRP-conjugated streptavidin diluted 1:200 in TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% blocking reagent) for 30 minutes followed by TSA Green, diluted 1:200 in amplification diluent for 5 minutes. Nuclei were counterstained with 25 μM 4′,6-diamidino-2-phenylindole (DAPI) in PBS-T. Triplicate independent experiments were performed to confirm NFAT5 localization during myogenesis.

Osmolarity measurements

The osmolarity of culture media was measured using the freezing point depression method by osmometry (Micro Osmometer-Precision Systems Inc.). Instrument calibration was confirmed both before and after culture media measurements using osmolarity standards (290 mOsm/l, Wescor). Triplicate aliquots of both growth and differentiation media were analyzed.

Plasmid production and retroviral infection

Oligonucleotides containing two NFAT5 response elements (TGGAAAATTACCG) or mutated response elements (TttAAAATTACCG) were cloned into the retroviral luciferase plasmid pKA9 (Abbott et al., 1998) to generate an NFAT5 retroviral reporter and a control reporter, respectively. The identity of the inserted fragments was confirmed by diagnostic digest and sequencing (Iowa State University Sequencing Facility).

To generate a dominant-negative NFAT5 retroviral vector, a cDNA fragment encoding the NFAT5 DNA-binding domain was subcloned from the pJT501 construct (Trama et al., 2002) into the retroviral vector pTJ84 (Abbott et al., 2000). pTJ84 was subsequently used as the control retroviral vector in appropriate experiments. Retroviral production and infection were performed as described previously (Abbott et al., 1998). Cells were grown for 48 hours to ensure optimal gene expression before use in experiments. The efficiency of retroviral-mediated gene transfer was >95%, based on cell survival in the presence of drug selection following two rounds of retroviral infection.

Luciferase reporter assays

The TA muscles of Balb/c mice (n=4) were electroporated with the NFAT5 retroviral reporter plasmid and the contralateral leg received the control plasmid containing mutated NFAT5 binding sites described above. Intramuscular injection of 50 μg DNA (2 μg/μl in PBS) was accompanied by several square wave pulses (200 V/cm) generated by a Grass S8800 stimulator. A pulse duration of 50 milliseconds and pulse interval of 270 milliseconds were used. The electrodes were a two-needle array fixed 5 mm apart (BTX) and inserted into the TA muscles. After 6 days, TA muscles were collected and homogenized in 1 ml lysis reagent (Promega). The homogenates were centrifuged for 10 minutes at 4°C and the supernatants were collected and analyzed by luciferase assay. In vivo electroporation of a lacZ plasmid was analyzed by histochemical analyses to assess the extent of muscle injury from the electroporation protocol and determine the efficiency of gene delivery. X-gal staining was performed as described (Rando et al., 1995).

NFAT5 transcriptional activity in wild-type and NFAT5$^{+/-}$ muscle cells was measured following retroviral infection of primary myoblasts with the NFAT5 reporter construct. Cells were differentiated for 24 hours and NFAT5 transcriptional activity was determined by luciferase assay following treatment with 100 mM mannitol (Sigma) for 5 hours and compared with the luminescence of untreated cells. Quadruplicate independent experiments were performed. In addition, wild-type cells were differentiated for 40 hours and transcriptional activity was determined by luciferase assay following stimulation with 12.5, 25 and 50 mM mannitol for 5 hours. Triplicate independent experiments were performed.

To verify that the NFAT5 reporter measures transcriptional activity independent of the calcineurin-dependent NFATc1-c4 isoforms, primary myoblasts were co-transfected with the NFAT5 reporter construct and a construct encoding VIVIT, an inhibitor of NFATc1-c4 activation described previously (Aramburu et al., 1999) at a ratio of 1:3, respectively. Alternatively, myoblasts were co-transfected with the NFATc1-c4 reporter pKA7 (Abbott et al., 1998) and VIVIT at a ratio of 1:3, respectively. Cells were differentiated for 43 hours and myotubes were treated with 100 mM glucose (NFAT5 reporter) or 10^{-8} M PMA and 10^{-6} M ionomycin (NFATc1-c4 reporter) for an additional 5 hours and luciferase assays were

performed. Quadruplicate independent experiments were performed. To control for potential variations in gene transduction with plasmid transfections, in parallel experiments cells were transfected with an eGFP plasmid construct. The percentage of cells expressing eGFP was subsequently quantified and did not vary significantly between experiments.

Native NFAT5 transcriptional activity during myogenesis was measured following retroviral infection of primary myoblasts with either the NFAT5 or control reporter constructs. Cells were harvested as myoblasts or differentiated for 24 hours (nascent myotubes) or 48 hours (mature myotubes) and assayed for luciferase activity. By contrast, induced NFAT5 transcriptional activity was determined by luciferase assay following stimulation of myoblasts, nascent and mature myotubes with 100 mM mannitol for 5 hours. Triplicate independent experiments were performed. To confirm that NFAT5 activity decreases following expression of dominant-negative NFAT5, primary muscle cells were retrovirally infected with the NFAT5 reporter construct and either the dominant-negative NFAT5 construct or a control vector, pTJ84 at a ratio of 1:9, respectively. Cells were differentiated for 24 hours, treated with 100 mM mannitol for an additional 5 hours and luciferase assays were performed. Triplicate independent experiments were performed.

Flow cytometry

The TA muscles of wild-type and NFAT5$^{+/-}$ mice (n=4 per genotype) were injured by 1.2% $BaCl_2$ as described above. Three days following injury, the muscles were dissected and enzymatically dissociated as described previously (Mitchell and Pavlath, 2004). Following differential centrifugation through an isotonic Percoll gradient (Yablonka-Reuveni et al., 1987), cells were isolated, quantified and resuspended in cold PBS containing 0.25% BSA. Cells were incubated with either FITC-conjugated anti-CD11b or isotype control IgG2bκ (BD Pharmingen) at a concentration of 2.35 μg/10^6 cells for 20 minutes. After a series of washes, the cells were resuspended in PBS, 0.25% BSA and 0.5 μg/ml propidium iodide (Sigma) for flow cytometric analyses. All flow cytometry was performed on a FACSVantage (Becton-Dickinson) and analyzed using FlowJo software (version 6.2.1, Tree Star).

Cell proliferation and differentiation assays

Primary myoblasts were infected with either control or dominant-negative NFAT5 retroviral vectors. For proliferation assays, cells were grown for 23 hours and pulsed with 25 μM bromodeoxyuridine (BrdU) (Sigma) for 1 hour. Cells were immunostained for BrdU as described previously (Mitchell and Pavlath, 2004) and the percentage of BrdU$^+$ nuclei was quantified. Triplicate independent experiments were performed. For differentiation assays, cells were differentiated for 10 and 24 hours and immunostained for embryonic myosin heavy chain (eMyHC) as described previously (Horsley et al., 2003). To analyze differentiation, the number of nuclei in cells expressing eMyHC was counted and expressed as a percentage of the total nuclei analyzed. Quadruplicate independent experiments were performed.

Cell migration assays

Primary wild-type and NFAT5$^{+/-}$ myoblasts were allowed to adhere to tissue culture dishes coated with entactin-collagen IV-laminin (E-C-L) (Upstate Cell Signaling Solutions) for 1 hour. Subsequently, the medium was buffered with 25 mM HEPES and the dish transferred to a 37°C heated microscope stage. Cell movements were visualized using a Zeiss Axiovert 200M microscope with a 0.3 NA 10× Zeiss Plan-Neofluar objective and images recorded (QImaging camera and OpenLab version 3.1.4 software, Improvision) every 6 minutes for 36 minutes. Migratory paths were digitized by tracking the paths of individual cells using ImageJ software (version 1.36) with manual tracking plug ins. The initial point of each migratory path was mathematically set at zero and subsequent displacement and velocity were calculated for each cell using Excel software (version 5.0). Cell migration was quantified from triplicate independent experiments on pooled isolates (n=3 per genotype). Approximately 20 cells per genotype were analyzed per experiment.

In some experiments, primary myoblasts infected with either control or dominant-negative NFAT5 retroviral vectors were analyzed. Some wells received 5 μg/ml Cyr61 (Abnova) immediately before the start of time-lapse photography. Migration analyses were performed as described above for three independent experiments.

Image preparation and statistical analyses

Images were obtained using a Zeiss Axioplan microscope with a 0.3 NA 10× Zeiss Plan-Neofluar objective unless otherwise stated. Images were captured using Scion Image (version 1.63) and globally processed for brightness and contrast using Adobe Photoshop (version 7.0).

To determine statistical significance, the mean data obtained from two groups were analyzed using unpaired Student's t-tests. Data from multiple groups with a single independent variable were analyzed by one-way ANOVA with Neuman-Keuls post test comparisons using GraphPad Prism version 4.0a (GraphPad Software). Data from multiple groups with two independent variables were analyzed by two-way ANOVA with Neuman-Keuls post-test comparisons using SigmaStat Software version 2.03 (SPSS Science Inc.). For all tests, the significance of the difference between means was accepted at the 0.05 level.

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