

# IL-4 Acts as a Myoblast Recruitment Factor during Mammalian Muscle Growth

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## Summary

Skeletal muscle formation and growth require the fusion of myoblasts to form multinucleated myofibers or myotubes, but few molecules are known to regulate myoblast fusion in mammals. The transcription factor NFATc2 controls myoblast fusion at a specific stage of myogenesis after the initial formation of a myotube and is necessary for further cell growth. By examining genes regulated by NFATc2 in muscle, this study identifies the cytokine IL-4 as a molecular signal that controls myoblast fusion with myotubes. Muscle cells lacking IL-4 or the IL-4 $\alpha$  receptor subunit form normally but are reduced in size and myonuclear number. IL-4 is expressed by a subset of muscle cells in fusing muscle cultures and requires the IL-4 $\alpha$  receptor subunit on myoblasts to promote fusion and growth. These data demonstrate that following myotube formation, myotubes recruit myoblast fusion by secretion of IL-4, leading to muscle growth.

## Introduction

Skeletal muscle fibers are syncytia that form from the fusion of mononucleated myoblasts. Myoblast fusion is also required for muscle growth (Darr and Schultz, 1989; Rosenblatt and Parry, 1992; Phelan and Gonyea, 1997; Horsley et al., 2001; Mitchell and Pavlath, 2001), as it increases the number of nuclei within the myofiber cytoplasm, allowing each nucleus to regulate more cytoplasm (Allen et al., 1999), and causes the muscle cell to increase in size. While the regulation of myoblast fusion is important during embryonic development as well as in maintenance and repair of adult muscle, the mechanisms by which myoblast fusion is controlled during myogenesis are largely unknown.

Calcium-dependent pathways regulate many aspects of skeletal myogenesis that contribute to skeletal muscle growth (Delling et al., 2000), including muscle cell fusion (Barnoy et al., 1996; Constantin et al., 1996). One signaling pathway activated by calcium involves the nuclear factor of activated T cells (NFAT) family of transcription factors (Crabtree and Olson, 2002). Activation and nuclear translocation of NFAT proteins is controlled by calcineurin, a calcium-sensitive phosphatase. Several NFAT isoforms are expressed in skeletal muscle, but each undergoes activation and nuclear translocation only at specific stages of myogenesis (Abbott et al.,

1998). For instance, the NFATc2 isoform is activated only in newly formed or nascent myotubes (Abbott et al., 1998) where it plays a crucial role in the accretion of nuclei and myotube growth (Horsley et al., 2001), suggesting that NFATc2 is important for the fusion of cells with the growing myotube. However, the genes regulated by NFATc2 that control muscle growth are unknown.

NFAT proteins regulate the expression of many secreted cytokines including IL-4 and IL-13 (Rao et al., 1997). IL-4 and IL-13 can induce macrophage fusion (McInnes and Rennick, 1988; DeFife et al., 1997). Both of these cytokines are produced primarily by Th2 lymphocytes and many of the functions of these cytokines are redundant due to a shared receptor subunit, IL-4R $\alpha$ , which is expressed in a wide range of tissues (Nelms et al., 1999). We hypothesized that the regulation of cell fusion may be conserved between macrophages and skeletal muscle and asked whether NFATc2 regulates a secreted cytokine to control muscle cell fusion. We demonstrate that NFATc2 regulates IL-4 in a subset of fusing muscle cells and induces muscle growth through the IL-4 receptor. Our data suggest that following the initial fusion of myoblasts to form myotubes, IL-4 acts as a myoblast recruitment factor, leading to further nuclear addition and increases in myotube size.

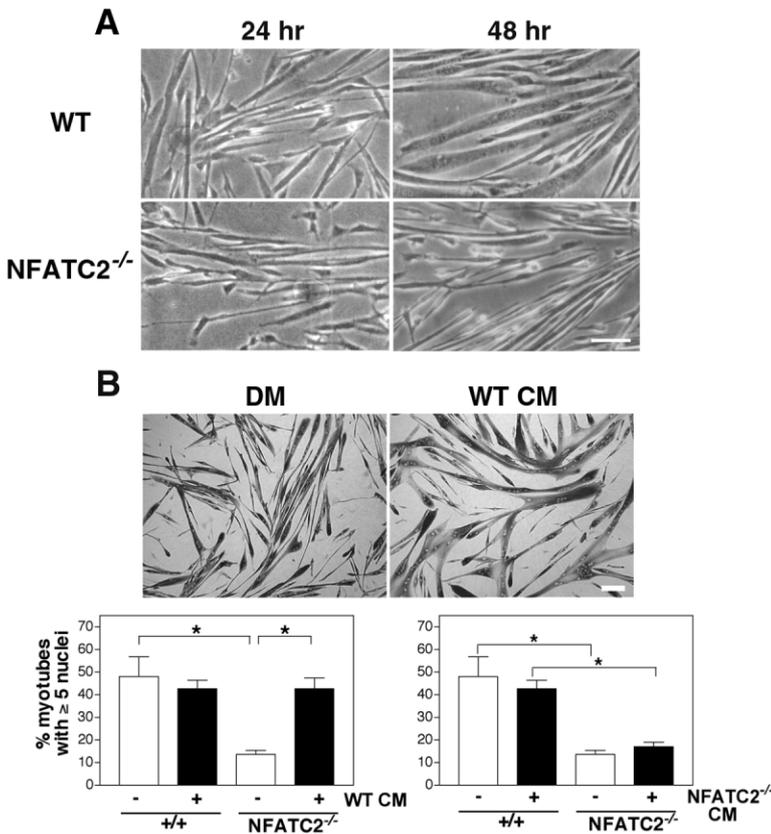
## Results

### A Protein Secreted by Wild-Type Muscle Cells Induces Growth of NFATc2<sup>-/-</sup> Myotubes

Previously, we described a role for NFATc2 in muscle growth following differentiation (Horsley et al., 2001). After 24 hr in differentiation media (DM), both wild-type and NFATc2<sup>-/-</sup> myoblasts have begun myotube formation and are indistinguishable in size (Figure 1A). However, after 48 hr in DM, wild-type myotubes are large, while NFATc2<sup>-/-</sup> myotubes remain small. This defect in cell size also correlates with a reduction in myonuclear number (Horsley et al., 2001). Thus, NFATc2<sup>-/-</sup> myotubes do not have a defect in the initial formation of a multinucleated myotube but rather in the further fusion of cells with myotubes.

To regulate cell fusion with the nascent myotube, NFATc2 may regulate the expression of two types of molecules: a cell surface molecule that mediates cell interaction and/or a secreted factor that recruits cell fusion. To test the hypothesis that NFATc2 regulates a secreted factor, the effect of media conditioned (CM) by wild-type myotubes on the growth of NFATc2<sup>-/-</sup> myotubes was analyzed. When NFATc2<sup>-/-</sup> muscle cells are induced to differentiate in wild-type CM, these cells form large myotubes similar to wild-type cells in DM (Figure 1B). The increased size of NFATc2<sup>-/-</sup> myotubes in CM is correlated with increases in myonuclear number to wild-type levels (Figure 1B, bottom left). To rule out that NFATc2<sup>-/-</sup> cells do not release a factor that inhibits muscle growth, the effect of NFATc2<sup>-/-</sup> CM was tested on wild-type myotubes. NFATc2<sup>-/-</sup> CM has no effect

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**Figure 1. Wild-Type CM Contains a Secreted Factor Involved in Myotube Growth**

(A) Bright field images of wild-type and NFATc2<sup>-/-</sup> myotubes after 24 and 48 hr in differentiation media (DM). Bar: 30  $\mu$ m.

(B) NFATc2<sup>-/-</sup> myoblasts were induced to differentiate in DM or in wild-type conditioned media (CM) for 48 hr. Bar: 100  $\mu$ m. The number of nuclei in individual wild-type and NFATc2<sup>-/-</sup> myotubes was analyzed after incubation in DM or wild-type CM for 48 hr (bottom left). Wild-type and NFATc2<sup>-/-</sup> myotubes were treated with NFATc2<sup>-/-</sup> CM for 48 hr and analyzed as described above (bottom right). Data are mean  $\pm$  standard error of three independent cell isolates. (\*significantly different,  $p < 0.05$ ).

either on the size (data not shown) or the nuclear number of wild-type myotubes (Figure 1B, bottom right), suggesting that the reduced size of NFATc2<sup>-/-</sup> myotubes is not due to production of a growth inhibitory factor by these mutant cells. Together, these data suggest that wild-type CM contains a secreted factor that is not produced by NFATc2<sup>-/-</sup> muscle cells during myotube growth. Further studies demonstrated that NFATc2 regulates the expression of a secreted protein(s) between 5 and 30 kDa that is necessary for nuclear addition and muscle growth (data not shown).

#### IL-4 Is the Factor in CM that Induces Growth of NFATc2<sup>-/-</sup> Myotubes

Since IL-4 is known to induce the fusion of macrophages (McInnes and Rennick, 1988), we hypothesized that IL-4 may be the bioactive factor in CM. Initially, the effect of exogenous IL-4 on NFATc2<sup>-/-</sup> myotubes was determined. Addition of IL-4 induces NFATc2<sup>-/-</sup> myoblasts to form large myotubes with a dose-dependent increase in the percentage of myotubes with  $\geq 5$  nuclei (Figure 2A). Concentrations of IL-4  $\geq 3$  ng/ml result in restoration of myonuclear number to wild-type levels. Since the IL-4 receptor (IL-4R) shares a common subunit, the IL-4R $\alpha$  chain, with the IL-13 receptor (Nelms et al., 1999), the ability of IL-13 to induce growth of NFATc2<sup>-/-</sup> myotubes was also tested (Figure 2A). Compared to IL-4, higher concentrations of IL-13 ( $\geq 10$  ng/ml) are required to induce the increases in myonuclear number of NFATc2<sup>-/-</sup> myotubes to wild-type levels. This difference is consistent with signaling through the IL-4R since IL-13 binds

to the IL-4R with lower affinity than IL-4 (Murata et al., 1998).

IL-4 may lead to increases in myonuclear number by enhancing cell proliferation, survival, differentiation, or fusion. To determine if IL-4 affects cell proliferation and/or survival, the DNA content of cultures treated with vehicle or IL-4 was determined and no significant difference is observed (Figure 2B). In addition, no difference is observed in the number of differentiated cells after 24 hr of IL-4 treatment in DM (data not shown). To test if IL-4 enhances the overall number of muscle cells that fuse, the fusion index was determined. The percentage of total nuclei in myotubes is not different between vehicle or IL-4-treated cells (Figure 2C). These data indicate that IL-4 is not a general fusion-promoting factor. Rather, the increases in myonuclear content observed with IL-4 (Figure 2A) suggest that IL-4 augments the fusion of cells with myotubes to increase muscle cell size.

Subsequently, to examine whether IL-4 or IL-13 is responsible for the growth-promoting bioactivity in CM, CM was treated with neutralizing antibodies against either IL-4 or IL-13. No differences are observed between untreated CM and CM treated with control IgG antibodies or IL-13 antibodies (Figure 2D). In contrast, treatment with IL-4 antibodies results in a dose-dependent loss of the bioactivity. To further test that IL-4 is responsible for the bioactivity in the CM, CM from IL-4<sup>-/-</sup> myotubes was also tested in the myonuclear number assay. NFATc2<sup>-/-</sup> myotubes treated with IL-4<sup>-/-</sup> CM are similar to NFATc2<sup>-/-</sup> myotubes in DM (Figure 2E), indicating

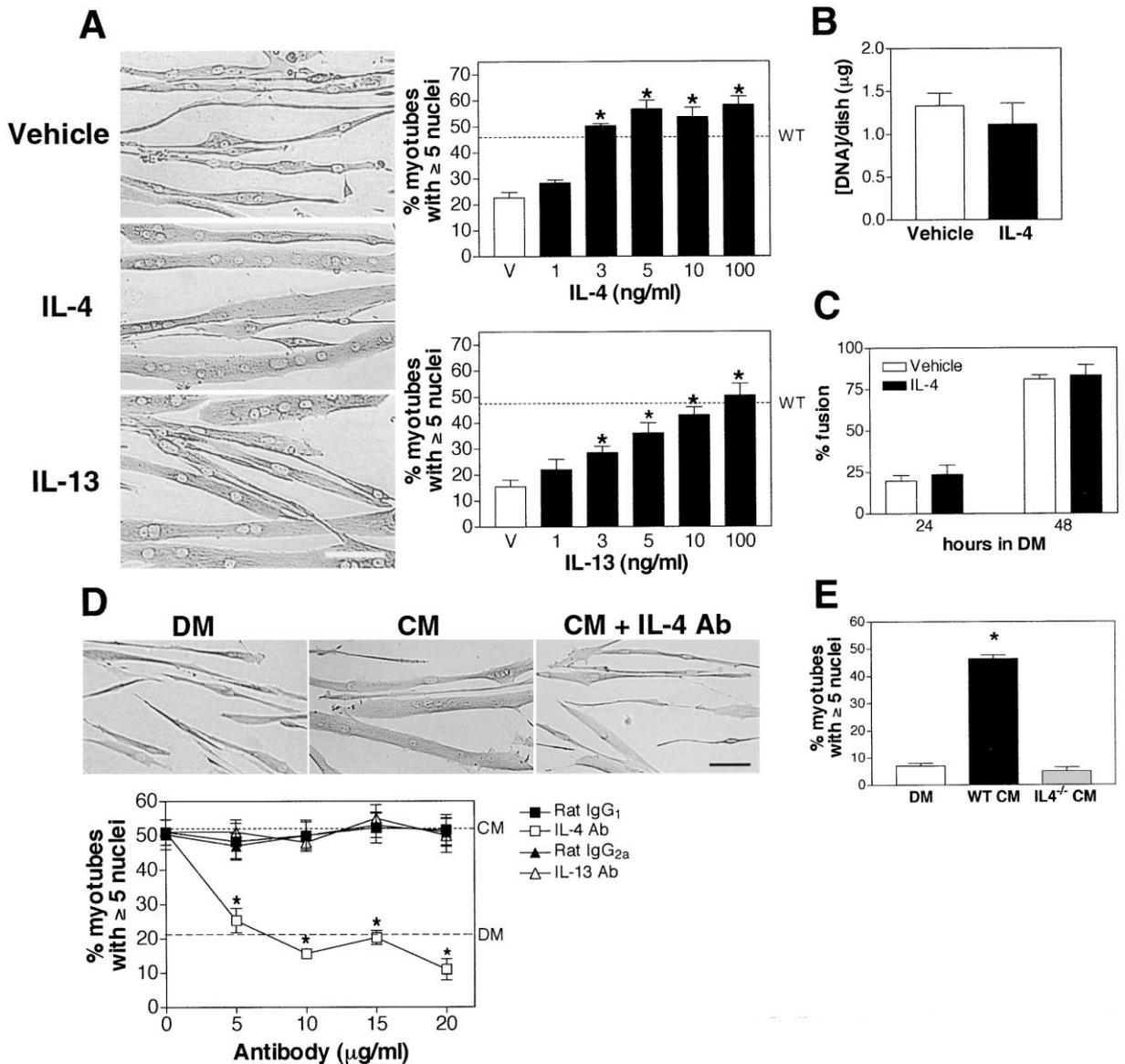


Figure 2. IL-4 Is the Component of Wild-Type CM that Enhances Myotube Growth

(A) NFATc2<sup>-/-</sup> myoblasts were treated with vehicle, 5 ng/ml IL-4, or 10 ng/ml IL-13 for 48 hr in differentiation media (DM). Bar: 60 µm. Cells were treated with the indicated doses of IL-4 or IL-13 for 48 hr, and the myotubes were analyzed as in Figure 1B. (B) The DNA content of NFATc2<sup>-/-</sup> cells treated with vehicle or 5 ng/ml IL-4 was quantified after 48 hr in DM. (C) The percentage of nuclei within myotubes was calculated in NFATc2<sup>-/-</sup> cultures following treatment with vehicle or 5 ng/ml IL-4 at indicated times. (D) NFATc2<sup>-/-</sup> myoblasts were incubated in DM, wild-type conditioned media (CM), or wild-type CM treated with indicated doses of control IgG, IL-4, or IL-13 antibodies for 48 hr and were analyzed as in Figure 1B. Bar: 60 µm. (E) NFATc2<sup>-/-</sup> myoblasts were treated with DM, wild-type CM, or IL-4<sup>-/-</sup> CM for 48 hr and analyzed as in Figure 1B. Data are mean ± standard error of three independent cell isolates (\*significantly different from DM, p < 0.05).

that wild-type CM increases the size and nuclear number of NFATc2<sup>-/-</sup> myotubes due to the presence of IL-4.

#### NFATc2 Regulates the Expression of IL-4 in Skeletal Muscle

To confirm that IL-4 is expressed by muscle cells and regulated by NFATc2, both RT-PCR and ELISA analyses were utilized. Both IL-4 mRNA and protein are expressed by wild-type muscle cells, but not NFATc2<sup>-/-</sup> cells (Fig-

ures 3A and 3B). Retroviral-mediated expression of NFATc2 in NFATc2<sup>-/-</sup> myoblasts induces expression of IL-4 mRNA. These data demonstrate NFATc2 regulates IL-4 expression in skeletal muscle cells.

#### IL-4 Is Expressed by a Subset of Muscle Cells during Muscle Growth

Next, IL-4 and IL-4R $\alpha$  expression were examined during myogenesis by immunostaining. No IL-4 expression is

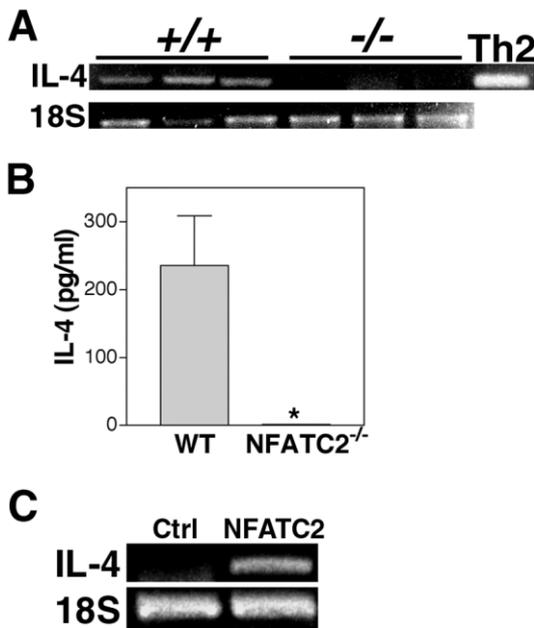


Figure 3. NFATc2 Regulates IL-4 Expression in Skeletal Muscle Cells

(A) IL-4 mRNA expression was examined by RT-PCR in wild-type and NFATc2<sup>-/-</sup> muscle cells after 24 hr in differentiated media (DM). Wild-type Th2 cells were included as a control. Representative ethidium bromide staining of agarose gel with three independent muscle cell isolates of each genotype is shown with 18S rRNA as an internal control.

(B) The expression of IL-4 protein was analyzed in wild-type and NFATc2<sup>-/-</sup> CM by ELISA. Data are mean  $\pm$  standard error of three independent cell isolates (\*significantly different,  $p < 0.05$ ).

(C) NFATc2<sup>-/-</sup> myoblasts were infected either with control retrovirus (Ctrl) or with a retrovirus expressing recombinant NFATc2. IL-4 mRNA expression was analyzed by RT-PCR after 24 hr in DM. Representative ethidium bromide staining of agarose gel is shown with 18S rRNA as an internal control. Data are indicative of results from three independent experiments.

observed in muscle cells before differentiation (0 hr in DM) (Figure 4A). Cells begin expressing IL-4 after 24 hr in DM, a time when most of the cells are differentiated and beginning to form nascent myotubes. After 36 hr in DM when myotube growth peaks, approximately 35% of the cells are positive for IL-4. However, once mature myotubes have formed and growth is reduced (48 hr), the number of positive cells decreases. With exception of a few mononucleated cells at 24 hr, IL-4 staining is primarily observed in small myotubes. No staining is detected in NFATc2<sup>-/-</sup> cells incubated with IL-4 antibodies or in wild-type cells incubated with secondary antibodies alone (Figure 4B). Thus, IL-4 is expressed by a subset of muscle cells during myogenesis. In contrast, IL-4R $\alpha$  is expressed on all cells (Figure 4C).

The expression of IL-4 during muscle regeneration was examined also (Figure 4D). At days 8 and 14 after injury, new myofibers have formed in the injured area, and the majority of muscle growth has not occurred. In these areas of regeneration, IL-4 is located primarily outside small myofibers (Figure 4D). No staining is observed in sections incubated with secondary antibodies alone or in regenerating muscles of IL-4<sup>-/-</sup> mice incu-

bated with antibodies against IL-4 (Figure 4D). Together, these data suggest that IL-4 expression is limited to specific stages of muscle growth.

The receptor for IL-4 consists of the IL-4R $\alpha$  subunit and the  $\gamma$ c subunit in immune cells or IL-13R $\alpha$ 1 in non-lymphoid tissues (Nelms et al., 1999). In addition, a distinct IL-13 receptor chain, IL-13R $\alpha$ 2, does not bind IL-4 (Donaldson et al., 1998) but can inhibit IL-4-dependent signaling (Rahaman et al., 2002). To characterize the receptor subunits that are expressed in skeletal muscle, RT-PCR was performed on muscle cells, and mRNA expression was detected for IL-4R $\alpha$  and IL-13R $\alpha$ 1, but not  $\gamma$ c or IL-13R $\alpha$ 2 (Figure 4E). These data suggest that the IL-4 receptor in skeletal muscle is comprised of IL-4R $\alpha$ 1 and IL-13R $\alpha$ 1 subunits.

#### IL-4 and the IL-4R $\alpha$ Are Required for Muscle Growth

To analyze the function of IL-4 and the IL-4R in skeletal muscle *in vivo*, myofiber cross-sectional area in IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice was analyzed and compared to wild-type. The cross-sectional area of myofibers in TA muscles is significantly reduced in the mutant mice (Figure 5A). In addition, the size distribution of myofibers in the TA muscles was examined. IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> TA muscles are characterized by an increase in small myofibers (<2400  $\mu$ m<sup>2</sup>) and a reduction in large myofibers (>3700  $\mu$ m<sup>2</sup>) (Figure 5B). Similarly, the soleus muscles of IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice exhibit an increase in small myofibers with a cross-sectional area <1200  $\mu$ m<sup>2</sup> and a decrease in large myofibers (>1700  $\mu$ m<sup>2</sup>). Myonuclear number was assayed in soleus muscles (Horsley et al., 2001; Mitchell and Pavlath, 2001) to determine if cell fusion is also defective in these mice. The number of DAPI-stained nuclei was determined inside of the dystrophin-stained sarcolemma (Figure 5C). Myonuclear number is decreased in IL-4<sup>-/-</sup> soleus muscles as compared to wild-type (Figure 5C), suggesting that the reduced cross-sectional area of myofibers in IL-4<sup>-/-</sup> mice results from defects in the ability of muscle cells to fuse with the myofiber *in vivo*. These analyses support a role for IL-4 in the developmental growth of myofibers *in vivo*.

To analyze the role of IL-4 and the IL-4R $\alpha$  in muscle growth during regeneration, the cross-sectional area of regenerating myofibers in the TA of IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice was measured at different times after injury. At days 8 and 14 after injury, the cross-sectional area of regenerating myofibers is not significantly different among wild-type, IL-4<sup>-/-</sup>, or IL-4R $\alpha$ <sup>-/-</sup> mice (Figure 5D). However, as further growth of the regenerating myofibers occurs, the mean cross-sectional area of both mutants is decreased compared to wild-type at day 25 after injury. Thus, IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> myofibers form normally during regeneration but have a subsequent defect in myofiber growth and cannot attain the size of wild-type myofibers. These data suggest that IL-4 is important for the growth of myofibers after their initial formation.

Myofiber growth is dependent on both nonmuscle and muscle cell types. To test the hypothesis that the *in vivo* defects in growth are muscle-cell intrinsic, we examined primary IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> muscle cells *in vitro*. After 48 hr in DM, both mutants form myotubes, but these

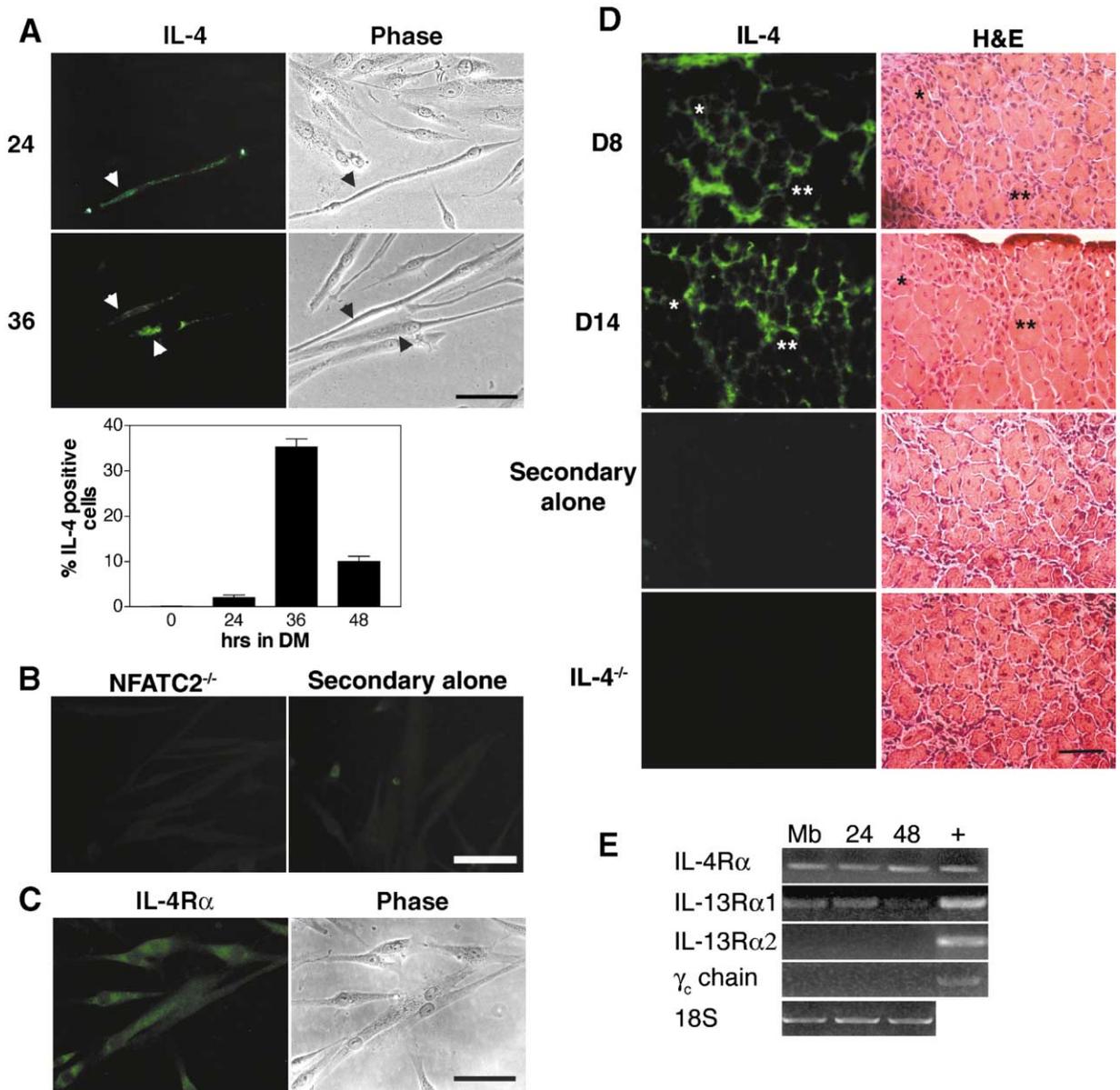


Figure 4. IL-4 Is Expressed by a Subset of Myotubes during Muscle Growth

(A) Representative images of fusing cultures immunostained with an antibody against IL-4 after 24 or 36 hr in differentiation media (DM). Arrowheads indicate the same cell in both fluorescent and phase images. Bar: 60  $\mu$ m. The percentage of IL-4 positive cells was determined at the indicated times in three independent experiments.

(B) Images of NFATc2<sup>-/-</sup> cells incubated with antibodies against IL-4 and wild-type cells incubated with secondary antibodies alone after 24 hr in differentiation media (DM).

(C) After 24 hr in DM, wild-type cells were immunostained with an antibody against IL-4R $\alpha$  and representative images are shown. Bar: 60  $\mu$ m.

(D) At days 8 and 14 after injury, wild-type regenerating muscle sections were immunostained with an antibody against IL-4. Asterisks indicate the same myofibers in both fluorescent and haematoxylin and eosin (H&E) stained images. Regenerating muscle sections at day 8 after injury from wild-type mice incubated with secondary antibodies alone and from IL-4<sup>-/-</sup> mice incubated with IL-4 antibodies are shown. Bar: 60  $\mu$ m.

(E) mRNA expression for IL-4R $\alpha$ , IL-13R $\alpha$ 1, IL-13R $\alpha$ 2, and  $\gamma_c$  was examined by RT-PCR in cells after 0, 24, and 48 hr in DM. Macrophage mRNA was included as a control for IL-4R $\alpha$ , IL-13R $\alpha$ 1, and  $\gamma_c$ , and mRNA from the glioblastoma cell line U251 was included as a control for IL-13R $\alpha$ 2. Representative ethidium bromide staining of agarose gel is shown with 18S rRNA as an internal control. Data are indicative of results from three independent experiments.

cells are small and thin as compared to wild-type myotubes (Figure 5E). The vast majority of IL-4<sup>-/-</sup> (84%) and IL-4R $\alpha$ <sup>-/-</sup> (91%) myotubes contain two to four nuclei, whereas wild-type cultures contain an equal proportion of myotubes with two to four nuclei and those with  $\geq$ 5

nuclei. Thus, the reduced size of the mutant myotubes correlates with a decrease in the number of nuclei within the cells similar to that observed in vivo. This phenotype of IL-4<sup>-/-</sup> muscle cells can be rescued by retroviral expression of IL-4 (Figure 5F). In addition, retroviral overex-

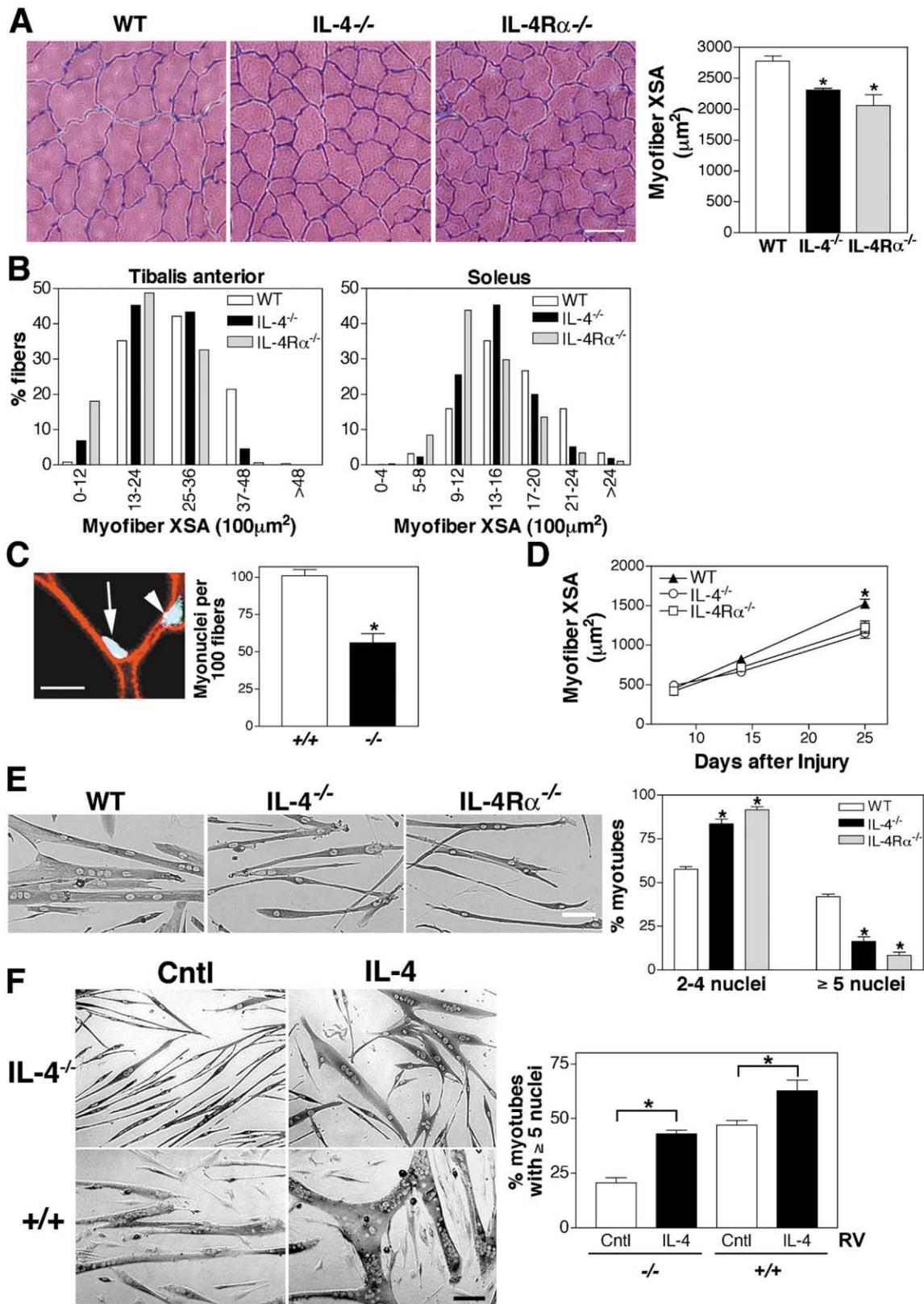


Figure 5. Reduced Myofiber Size in IL-4<sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> TA and Soleus Muscles Is Muscle Cell Intrinsic

(A) Representative sections of wild-type, IL-4<sup>-/-</sup>, and IL-4R $\alpha$ <sup>-/-</sup> TA muscles are shown. Bar: 60  $\mu\text{m}$ . Data for myofiber cross-sectional areas (XSA) are mean  $\pm$  standard error; n = 3 for wild-type, n = 7 for IL-4<sup>-/-</sup>, and n = 4 for IL-4R $\alpha$ <sup>-/-</sup>.

(B) Frequency histograms showing the distribution of myofiber XSA in wild-type (n = 256), IL-4<sup>-/-</sup> (n = 731), and IL-4R $\alpha$ <sup>-/-</sup> (n = 482) Tibialis anterior (TA) muscles (left) and wild-type (n = 447), IL-4<sup>-/-</sup> (n = 1060), and IL-4R $\alpha$ <sup>-/-</sup> (n = 726) soleus muscles (right).

pression of IL-4 in wild-type cells augments myotube size and myonuclear number (Figure 5F). Therefore, these data further support a role for IL-4 in the myonuclear accretion that occurs in nascent myotubes.

#### IL-4 Acts as a Recruitment Factor for Muscle-Cell Fusion during Growth

Our data suggest that IL-4 is secreted by myotubes and recruits further cell fusion during muscle growth. However, the cellular target of IL-4 (unfused mononucleated cells versus myotube) is not known. If the unfused cells are the target of IL-4, IL-4R $\alpha^{-/-}$  mononucleated cells should not be recruited to fuse with wild-type nascent myotubes. To test this hypothesis, wild-type nascent myotubes were cocultured with IL-4R $\alpha^{-/-}$  mononucleated cells. To identify each cell population, cells were stained with CellTracker fluorescent dyes that have been used previously for cell-fusion studies (Wunschmann and Stapleton, 2000; Huerta et al., 2002). Coculture of wild-type nascent myotubes with mononucleated wild-type cells results in 89% of myotubes with both fluorescent labels (Figure 6), demonstrating efficient cell mixing and cell fusion in the assay. In contrast, coculture of wild-type myotubes with IL-4R $\alpha^{-/-}$  mononucleated cells results in only 14% of the myotubes with dual label, suggesting that IL-4R $\alpha^{-/-}$  cells have a defect in their ability to fuse with wild-type nascent myotubes. To determine if IL-4 also acts on myotubes, IL-4R $\alpha^{-/-}$  nascent myotubes were cocultured with wild-type mononucleated cells. The majority of IL-4R $\alpha^{-/-}$  myotubes contain both labels, indicating that wild-type mononucleated cells can fuse with IL-4R $\alpha^{-/-}$  myotubes. Interestingly, these chimeric myotubes are larger than IL-4R $\alpha^{-/-}$  myotubes. We also cocultured IL-4R $\alpha^{-/-}$  nascent myotubes with IL-4R $\alpha^{-/-}$  mononucleated cells to confirm the defect in the recruitment of IL-4R $\alpha^{-/-}$  muscle cells. Consistent with the phenotype of IL-4R $\alpha^{-/-}$  myotubes, few IL-4R $\alpha^{-/-}$  myotubes contain dual label when cultured with IL-4R $\alpha^{-/-}$  mononucleated cells, further suggesting that IL-4R $\alpha^{-/-}$  muscle cells are unable to fuse with nascent myotubes. Together, these data indicate that mononucleated cells are the target of IL-4, leading to their recruitment and fusion with nascent myotubes.

#### Discussion

Myoblast fusion is required for skeletal myogenesis as it allows the formation and growth of multinucleated myotubes. This process requires multiple steps involving cell migration, alignment, recognition, adhesion, and

membrane fusion (Wakelam, 1985). Of the limited number of molecules known to regulate muscle cell fusion in mammals, the majority of these molecules mediate the myoblast-myoblast fusion that forms a myotube (Wakelam, 1985; Rosen et al., 1992; Yagami-Hiromasa et al., 1995; Barnoy et al., 1996; Gorza and Vitadello, 2000). Previous studies of NFATc2 $^{-/-}$  muscle cells suggested that the initial fusion of myoblasts that forms small nascent myotubes is distinct from the subsequent fusion of differentiated muscle cells with myotubes during muscle growth (Horsley et al., 2001). This model is also supported by recent data from *Drosophila* that identified rolling pebbles as a molecule that is required for fusion of myoblasts with the growing myofiber (Rau et al., 2001). Here, we demonstrate that the second phase of myoblast fusion that occurs with myotubes is dependent on IL-4 and the IL-4R $\alpha$  subunit of the IL-4 receptor (Figure 7). Data in support of this model are discussed below. Together, these data further demonstrate that mechanisms that control myoblast-myoblast fusion are distinct from mechanisms that regulate myoblast fusion with myotubes in mammals.

To our knowledge, our data are the first description of IL-4 expression by a nonimmune cell and suggest that regulation of IL-4 expression by NFATc2 also occurs in nonlymphoid cells. The regulation of IL-4 expression in T cells is complex as multiple NFAT isoforms, NFATc1–NFATc3, seem to control IL-4 expression (Hodge et al., 1996; Monticelli and Rao, 2002; Rengarajan et al., 2002). Skeletal muscle expresses NFATc1–NFATc3 in both myoblasts and myotubes (Abbott et al., 1998), and the expression of NFATc1 and NFATc3 is unaffected in NFATc2 $^{-/-}$  muscle cells (Horsley et al., 2001). Here, we demonstrate that NFATc2 $^{-/-}$  muscle cells do not express IL-4 (Figures 3A and 3B), and the expression of recombinant NFATc2 restores expression of IL-4 (Figure 3C). The timing of IL-4 expression (Figure 4A) also correlates with NFATc2 activation during myogenesis (Abbott et al., 1998). Together, these data indicate that NFATc2 is the primary NFAT isoform responsible for IL-4 expression in skeletal muscle. Whether other NFAT isoforms regulate IL-4 expression in concert with NFATc2 in muscle is unknown.

The induction of IL-4 expression by NFATc2 promotes cell fusion, leading to muscle growth. Previously, we demonstrated that NFATc2 is required for the nuclear addition or cell fusion following myoblast differentiation (Horsley et al., 2001) and initial myotube formation (Figure 1A). Here, a target of NFATc2 in skeletal muscle, IL-4, also regulates cell fusion. Media from wild-type myotube cultures induces nuclear addition in NFATc2 $^{-/-}$

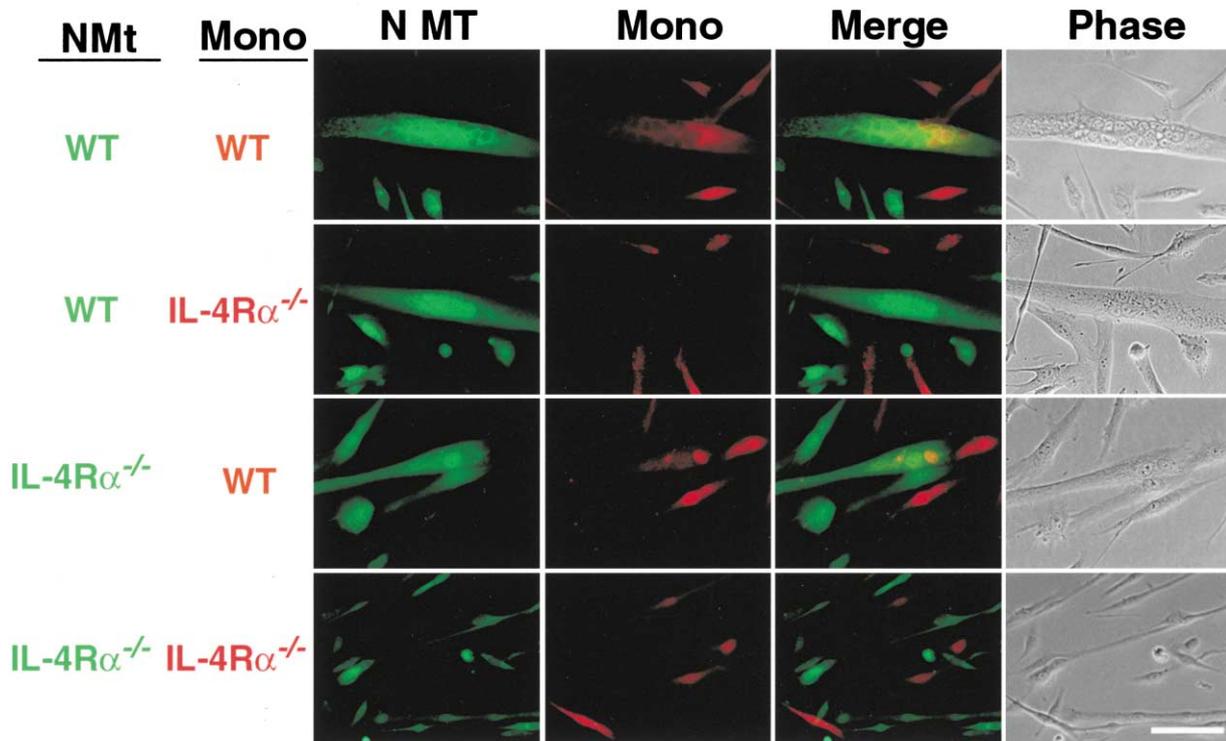
(C) A representative wild-type myofiber immunostained with an antibody against dystrophin (red) and stained with DAPI (blue) illustrates the myonuclear number assay. The number of DAPI-stained nuclei within the dystrophin positive sarcolemma (arrow) were counted in soleus muscles from wild-type and IL-4 $^{-/-}$  mice and expressed per 100 myofibers. Arrowhead indicates a nucleus outside the myofiber. Bar: 20  $\mu$ m. Data are mean  $\pm$  standard error; n = 3 for each genotype.

(D) The XSA of regenerating TA myofibers was determined at various time points after injury. Data are mean  $\pm$  standard error; n = 4–7 for each genotype.

(E) Wild-type, IL-4 $^{-/-}$ , and IL-4R $\alpha^{-/-}$  myoblasts were induced to differentiate in differentiation media (DM) for 48 hr. Bar: 60  $\mu$ m. Myonuclear content was examined as in Figure 1B. Data are mean  $\pm$  standard error of three independent cell isolates.

(F) Wild-type or IL-4 $^{-/-}$  myoblasts were infected with either a control retrovirus (Cntl) or a retrovirus expressing IL-4 (IL-4) and induced to differentiate for 48 hr. Bar: 30  $\mu$ m. Myotube cultures were analyzed as in Figure 1B. Data are mean  $\pm$  standard error of three independent cell isolates (\*indicates significantly different, p < 0.05).

**A**



**B**

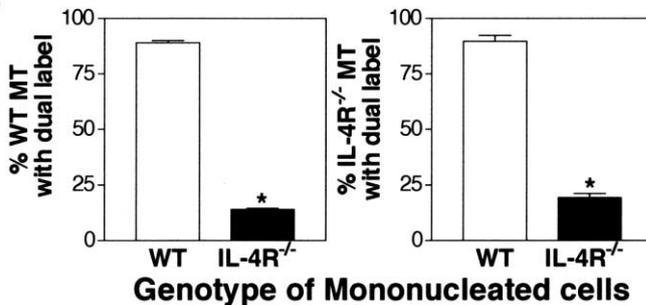


Figure 6. IL-4 Acts on Myoblasts to Induce Myonuclear Accretion in Myotubes

(A) Wild-type nascent myotubes (NMt) were cocultured for 24 hr in differentiation media (DM) with either wild-type or IL-4R $\alpha^{-/-}$  differentiated, mononucleated muscle cells (Mono). In addition, IL-4R $\alpha^{-/-}$  NMt were cocultured for 24 hr in DM with either wild-type or IL-4R $\alpha^{-/-}$  differentiated, mononucleated cells. Label colors represent the particular CellTracker dye used to stain each cell type. Bar: 60  $\mu$ m.

(B) The percentage of myotubes containing dual label was determined and expressed as a percentage of the total myotubes analyzed (100). Data are the mean  $\pm$  standard error of three independent cell isolates (\*indicates significantly different,  $p < 0.05$ ).

myotubes (Figure 1B), which is blocked with the addition of antibodies against IL-4 (Figure 2D). Likewise, addition of exogenous IL-4 rescues defects in myonuclear number (Figure 2A). Furthermore, the phenotype of IL-4 $^{-/-}$  muscle cells also supports a role for IL-4 in the regulation of cell fusion during muscle growth in vivo and in vitro. IL-4 $^{-/-}$  myotubes and myofibers form but are reduced in size likely due to a decreased number of myonuclei (Figure 5). Expression of recombinant IL-4 induces nuclear accretion in wild-type and IL-4 $^{-/-}$  myotubes (Figure 5F). Interestingly, these myotubes expressing recombinant IL-4 demonstrate a branched appearance, suggesting that overexpression of IL-4 leads to cell fusion at multiple locations along the myotube. Clearly, IL-4

expression is required for cell fusion with nascent myotubes during development (Figures 5A–5C) and regeneration (Figure 5D), but whether IL-4 regulates nuclear accretion that occurs with hypertrophy (Rosenblatt and Parry, 1992) or recovery from atrophy (Mitchell and Pavlath, 2001) is unknown.

To control cell fusion, IL-4 acts through the IL-4R on myoblasts not myotubes (Figure 7). Both myoblasts and myotubes express the IL-4R $\alpha$  subunit of the IL-4R (Figures 4C and 4E), and IL-4R $\alpha$  is required for muscle growth (Figure 5) and likely paired with the IL-13R $\alpha$ 1 subunit (Figure 4E). Myoblasts that lack IL-4R $\alpha$  cannot be recruited by nascent myotubes that secrete IL-4 (Figure 6). However, IL-4R $\alpha^{-/-}$  nascent myotubes can recruit

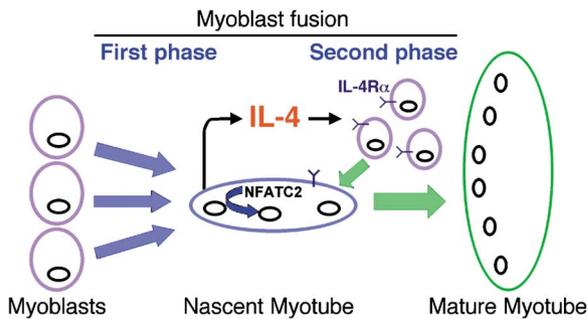


Figure 7. Model for the Role of IL-4 in the Recruitment of Myoblast Fusion during Muscle Growth

Myoblast fusion occurs in two stages. In the first phase, a subset of differentiated myoblasts fuse together to form a nascent myotube with a limited number of nuclei. A second phase of myoblast fusion occurs with nascent myotubes. Under the control of NFATc2, IL-4 is secreted by nascent myotubes and induces this second phase of fusion through IL-4R $\alpha$  on myoblasts. This action allows the accretion of nuclei within nascent myotubes and, along with protein accumulation, the formation of a large, mature myotube.

cell fusion of wild-type myoblasts. Together, these data indicate that signaling via the IL-4R in myoblasts is required for cell fusion with nascent myotubes. While the signaling pathways downstream of the IL-4R in skeletal muscle are unknown, several targets have been identified in other cell types, including Janus kinases, insulin receptor substrate-1/2, phosphoinositide-3-kinase [PI(3)K], MAP kinase, and Stat-6 (Nelms et al., 1999). The activation of PI(3)K is of particular interest given recent data implicating this pathway in muscle growth downstream of IGF-1 (Rommel et al., 2001).

The expression of IL-4 is restricted to a subpopulation of multinucleated myotubes (Figure 4). Heterogeneity in muscle cells is likely a common mechanism to control cell fusion. In *Drosophila*, unique gene expression patterns in myoblasts form two populations, founder myoblasts and fusion competent myoblasts, resulting in asymmetric fusion of these myoblast populations (Taylor, 2002). Our data suggest that heterogeneity during cell fusion also exists in mammalian muscle cells and may allow IL-4 to act as either a general or a specific signal for fusion. As a general signal, IL-4 may lead to enhanced fusion competency of muscle cells, regulating fusion with both IL-4 expressing and nonexpressing myotubes. To direct specific fusion, IL-4 may recruit the fusion of myoblasts with the distinct subset of myotubes that secrete IL-4, allowing other myotubes to grow through alternative signals.

Our data support a role for multinucleated muscle cells in the promotion of cell fusion and growth (Figure 7). Other studies also demonstrate that multinucleated muscle cells can control fusion. During embryogenesis, subpopulations of myoblasts fuse with specific myotubes in the development of primary and secondary myofibers (Harris et al., 1989; Zhang and McLennan, 1995). In addition, unidentified secreted proteins produced by myotubes can control cell fusion (Konigsberg, 1971; Doering and Fischman, 1977; Yeoh and Holtzer, 1977). To our knowledge, our data are the first to identify a secreted protein produced by myotubes that promotes

the fusion of myoblasts with myotubes. In addition, in *Drosophila*, nascent myotubes express a cytoplasmic protein, rolling pebbles, that is required for fusion of myoblasts with growing muscle cells (Rau et al., 2001). Since homologs of NFATc2 are not expressed in *Drosophila*, the control of cell fusion by the NFATc2 pathway may have evolved in higher eukaryotes to regulate the plasticity of muscle growth, allowing maintenance of muscle mass over an extended lifespan and the growth of muscles containing multiple myofibers.

The involvement of IL-4 in regulating cell fusion in both macrophages and muscle cells suggests that fusion mechanisms are conserved among these cell types. To control macrophage fusion, IL-4 induces expression of the integrins  $\beta_1$  and  $\beta_2$  (McNally and Anderson, 2002).  $\beta_3$  integrin is also regulated by IL-4 in macrophages (Kitazawa et al., 1995). Thus, IL-4 may mediate the fusion of myoblasts by increasing the expression of cell adhesion molecules. In fact, IL-4 can induce the expression of intracellular adhesion molecule-1 (ICAM-1) on myoblasts (Marino et al., 2001). Intriguingly, the expression of VCAM-1, which is specifically expressed on myoblasts and is required for myotube formation in vitro (Rosen et al., 1992), is controlled by IL-4 in vascular endothelial cells (Schleimer et al., 1992) and smooth muscle cells (Barks et al., 1997). In addition to the induction of adhesion molecules, IL-4 stimulates chemotaxis of osteoblasts (Lind et al., 1995), suggesting that IL-4 may also regulate myoblast chemotaxis during myotube growth. The mechanisms by which IL-4 induces cell fusion are currently under investigation.

As a molecular signal that promotes fusion of myoblasts with myotubes, IL-4 treatment may be useful clinically in muscle disorders. Induction of endogenous myoblast fusion may ameliorate muscle diseases or speed recovery of muscle atrophy and/or injury. Also, inducing the fusion of exogenous myoblasts (Blau and Springer, 1995) or stem cells may improve gene therapy protocols utilizing transplanted cells to treat muscle disorders. Further work determining the molecular pathways by which IL-4 induces myoblast fusion will aid in the development of possible clinical therapies.

#### Experimental Procedures

##### Animals

Wild-type Balb/c controls and NFATc2 $^{-/-}$  mice were generated by heterozygous mating of mice carrying a targeted disruption in the NFATc2 gene (Hodge et al., 1996). Mice were genotyped by PCR analyses of tail DNA (Hodge et al., 1996). Wild-type Balb/cJ, IL-4 $^{-/-}$  and IL-4R $\alpha^{-/-}$  were purchased from Jackson Laboratories. Adult male mice between 8–12 weeks of age were used for all studies. All animals were handled in accordance with the institutional guidelines of Emory University.

##### Primary Muscle Cell Culture and Cytokine Treatment

Primary cultures were derived from tibialis anterior (TA) muscles of wild-type, NFATc2 $^{-/-}$ , IL-4 $^{-/-}$ , and IL-4R $\alpha^{-/-}$  mice 2 days after induced muscle damage (Paviath et al., 1998), and myoblasts were purified to >99% in selective media (Rando and Blau, 1994). All experiments were performed with independent cell isolates from three animals for each genotype. Myoblasts were grown in growth media (GM: Ham's F10, 20% fetal bovine serum, 5 ng/ml bFGF, 100 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin) on collagen-coated dishes in a humidified 5% CO $_2$  atmosphere at 37°C. Differentiation was induced by plating 2  $\times$  10 $^5$  myoblasts per well of E-C-L (Upstate

Biotechnology) coated 6-well dishes and after 2 hr, switching the media to a low serum, low-mitogen differentiation media (DM: DMEM, 100 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin) containing either 2% horse serum or Insulin-Transferrin-Selenium-A supplement (Gibco) for 48 hr. In experiments using exogenous cytokines, vehicle (0.1% bovine serum albumin in PBS), IL-4, or IL-13 (R&D Systems) were added to DM and replenished at 24 hr for a total treatment of 48 hr.

#### Conditioned Media Experiments

Conditioned media (CM) was collected from muscle cells after 24 and 48 hr in DM. In all assays, myoblasts were placed in CM, and CM was replenished at 24 hr. All experiments were analyzed after 48 hr of treatment. In control experiments, no differences were observed in the formation of wild-type myotubes in wild-type CM compared to DM. Neutralization of CM was performed by treating CM with rat monoclonal antibodies against either IL-4, IL-13, or their corresponding control rat IgG antibodies (R&D systems, Sigma). In control experiments, addition of IL-4 together with antibodies against IL-4 abolished the ability of IL-4 to induce growth of NFATc2<sup>-/-</sup> myotubes.

#### Fusion Assays

After 48 hr in DM or CM, cells were fixed and immunostained with antibodies against embryonic myosin heavy chain (F1.652, Developmental Studies Hybridoma Bank, Iowa City, Iowa) as previously described (Horsley et al., 2001). The fusion index was determined by dividing the number of nuclei in myotubes ( $\geq 2$  nuclei) by the total number of nuclei analyzed (100–250). Fusion was also analyzed by performing nuclear number assays (Horsley et al., 2001). The number of nuclei in individual myotubes was counted for 50–100 myotubes. Myotubes were grouped into two categories: those with two to four nuclei and those with five or more nuclei. The percentage of myotubes in each category was calculated.

#### DNA Quantification

After 48 hr in DM, cells were collected by scraping and centrifuged at 10,000  $\times$  g at 4°C. Cells were resuspended in 250  $\mu$ l of saline-phosphate buffer (0.05 M NaPO<sub>4</sub> and 2 M NaCl [pH 7.4]) and frozen. Samples were thawed, sonicated for 15 s, and a 50  $\mu$ l aliquot of each sample was added to the buffer containing 0.5  $\mu$ g/ml Hoechst 33258 (Molecular Probes). DNA concentration was determined as previously described (Mitchell and Pavlath, 2001).

#### Retroviral Plasmids, Production, and Infection

The NFATc2 construct has been previously described (Horsley et al., 2001). A retroviral vector expressing full-length mouse IL-4 was created by excision of IL-4 cDNA from a vector containing the full-length IL-4 cDNA (gift from Dr. M. Brown) by BamHI and SalI to create a 0.6 kb product. The IL-4 cDNA was then cloned into a basic retroviral vector in which expression of IL-4 is driven by the retroviral 5'-LTR. Production of infectious retrovirus and infection of primary myoblasts were performed as previously described (Abbott et al., 1998). Cells were subject to two rounds of infection with an efficiency of gene transfer of >90% based on visualization of green fluorescent protein.

#### RT-PCR and Protein Analyses

RNA was isolated using Trizol Reagent (Life Technologies) from muscle cells. RT-PCR was performed for each sample using primers specific for IL-4 (accession number: NM\_021283) (*sense*, 5'-AACC CCAGCTAGTTGTCATCCTG-3'; *antisense*, 5'-CATCGAAAAGCCC GAAAGAGTCTC-3'), IL-4R $\alpha$  (accession number: NM\_010557) (*sense*, 5'-TG TAGCCCGGCCCAAATCC-3'; *antisense*, 5'-GGCGGCACCC TGCTTCACTG-3'), IL-13R $\alpha$ 1 (accession number: S80963) (*sense*, 5'-CCGCGGCACAGAAGTTCAGC-3'; *antisense*, 5'-ACAGCGGACTC AGGATCACCTTCA-3'), IL-13R $\alpha$ 2 (accession number: NM\_008356) (*sense*, 5'-GAGCACACCTGGAGGCCATTCC-3'; *antisense*, 5'-GGC CCTGTGTAACCTTCCCAACATTC-3'), and  $\gamma$ c (accession number: NM\_013563) (*sense*, 5'-GAGCCGCTATAACCAATCTGTGG-3'; *antisense*, 5'-GGGAGGCCAGTAAGGGCTATGC-3'). All primer pairs span intron/exon boundaries to control for DNA contamination in RNA samples. All RT reactions were performed using 2.5  $\mu$ g of

total RNA. 18S rRNA was used as control for each sample using QuantumRNA 18S primers (Ambion). IL-4 cDNA was amplified using Expand High Fidelity PCR system (Roche) by incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and termination at 72°C for 5 min. Similar amplification cycles were performed for the IL-4R $\alpha$ , IL-13R $\alpha$ 1, IL-13R $\alpha$ 2, and  $\gamma$ c receptor subunits with annealing temperatures of 58°C for IL-13R $\alpha$ 1, IL-13R $\alpha$ 2, and  $\gamma$ c, and 56°C for IL-4R $\alpha$ . For IL-4, 5  $\mu$ l of the initial PCR reaction was removed, and PCR reagents were replenished for a second round of amplification. The amplicons were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

A 10-fold concentration of wild-type and NFATc2<sup>-/-</sup> CM was analyzed for IL-4 levels using Quantikine M-ELISA (R&D Systems) with a lower detection limit of 2 pg/ml.

#### Collection of Muscles and Morphometric Measurements

Collection and analysis of undamaged and regenerating muscles were performed as previously described (Horsley et al., 2001). Anatomical markers were used to find the same region in different samples, and these sections were subsequently used for analyses of myofiber cross-sectional area. To examine the growth of regenerating muscles, TA muscles were subject to a standardized local freeze damage (Pavlath et al., 1998) and the cross-sectional area of regenerating myofibers was measured. The cross-sectional area of undamaged and regenerating myofibers in the TA was expressed as a mean of the total fibers analyzed (75–225). The cross-sectional area was also analyzed in the undamaged TA and soleus as a distribution and expressed as a percentage of the total number of myofibers analyzed. All photography was performed on an Axioplan microscope (Zeiss) equipped with a video camera and Adobe Photoshop and Scion Image software. All cross-sectional area measurements were performed using Scion Image software.

#### In Vivo Nuclear Number Assay

Myonuclei were quantified as described previously (Horsley et al., 2001). Briefly, soleus muscles were incubated in antibody against dystrophin (MANDYS8, Sigma; 1:400) for 1 hr at room temperature. After washing in TNT (0.1 M Tris-HCl [pH 7.5], 0.15M NaCl, and 0.05% Tween 20), the sections were incubated in Texas red conjugated goat anti-mouse IgG (Cappel, Durham, NC; 1:50). After further washing in TNT, the sections were mounted in Vectashield mounting media containing DAPI (Vector Labs). Nuclei within the dystrophin-positive sarcolemma were counted for 100–150 myofibers, and the number of myonuclei was expressed per 100 myofibers.

#### Cellular Analyses of IL-4 and IL-4R $\alpha$ Expression

At 12 hr intervals in DM, muscle cells were fixed with ice-cold methanol for 10 min at room temperature. After washing in PBS, nonspecific binding was blocked with TNB and cells were incubated with a rat monoclonal antibody against IL-4 (500ng/ml) or IL-4R $\alpha$  (500ng/ml) (R&D Systems) at 4°C overnight. After washing in TNT, the cells were incubated in biotin conjugated goat anti-rat IgG (Jackson Laboratories; 1:250) for 1 hr at room temperature, washed in TNT, and incubated in HRP-streptavidin (1:50) (NEN Life Sciences) for 30 min at room temperature. After further washes in TNT, the cells were incubated in the TSA green reagent (NEN Life Sciences) diluted 1:50 in amplification buffer for 5 min at room temperature, followed by a final wash in TNT. The number of IL-4-positive cells was quantified and expressed as a percentage of the total number of cells analyzed (100–200).

Sections of regenerating TA muscles were fixed in 3.7% formaldehyde, treated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS, washed in PBS, blocked with TNB, and incubated with primary and secondary antibodies as described above. Sections were mounted in Vectashield mounting media containing DAPI (Vector Labs). No staining was observed in control sections incubated with secondary antibodies alone or in IL-4<sup>-/-</sup> muscles.

#### Cell Mixing Experiments

Cells were plated at  $2 \times 10^5$  cells per well of a 6-well plate to produce nascent myotubes, and cells were plated at  $1 \times 10^5$  cells per well to produce differentiated, mononucleated cells. After 24 hr in DM,

nascent myotubes were stained with 0.5  $\mu$ M CellTracker green CMFDA (5-chloromethyl-7-hydroxycoumarin) (Molecular Probes) and mononucleated cells were stained with 3  $\mu$ M CellTracker orange CMTMR (5-(and-6)-((4-chloromethyl) benzoyl) amino) tetramethylrhodamine) (Molecular Probes) for 10 min at 37°C. Cells were washed twice with PBS, trypsinized, and plated at equal cell number with a final cell number of  $2 \times 10^5$  in each well of a 6-well plate. After 24 hr, cocultures were fixed with 3.7% formaldehyde. The presence of dual label was analyzed in 50–100 myotubes with  $\geq$  three to four nuclei and was only observed in cells with multiple nuclei. In control experiments, wild-type cells showed 100% staining efficiency with no deleterious effects on cell fusion.

#### Statistics

To determine significance between two groups, comparisons were made using Student's *t* tests. Analyses of multiple groups were performed using one-way ANOVA with Bonferroni's posttest using GraphPad Prism version 3.0a for Macintosh (GraphPad Software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

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