IKK/NF-κB regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis

Nadine Bakkar,[1,2] Jingxin Wang,[1] Katherine J. Ladner,[1] Huating Wang,[1] Jason M. Dahlman,[1] Micheal Carathers,[1] Swarnali Acharyya,[1] Michael A. Rudnicki,[4] Andrew D. Hollenbach,[5] and Denis C. Guttridge[1,2,3]

[1]Human Cancer Genetics Program, Department of Molecular Virology, Immunology, and Medical Genetics, [2]Molecular, Cellular, and Developmental Biology Graduate Program, and [3]Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210
[4]Molecular Medicine Program, Ottawa Health Research Institute, Ottawa K1Y 4E9, Ontario, Canada
[5]Department of Genetics, Louisiana State University, New Orleans, LA 70112

Nuclear factor κB (NF-κB) is involved in multiple skeletal muscle disorders, but how it functions in differentiation remains elusive given that both anti- and promyogenic activities have been described. In this study, we resolve this by showing that myogenesis is controlled by opposing NF-κB signaling pathways. We find that myogenesis is enhanced in MyoD-expressing fibroblasts deficient in classical pathway components RelA/p65, inhibitor of κB kinase β (IKKβ), or IKKγ. Similar increases occur in myoblasts lacking RelA/p65 or IKKβ, and muscles from RelA/p65 or IKKβ mutant mice also contain higher fiber numbers. Moreover, we show that during differentiation, classical NF-κB signaling decreases, whereas the induction of alternative members IKKα, RelB, and p52 occurs late in myogenesis. Myotube formation does not require alternative signaling, but it is important for myotube maintenance in response to metabolic stress. Furthermore, overexpression or knockdown of IKKα regulates mitochondrial content and function, suggesting that alternative signaling stimulates mitochondrial biogenesis. Together, these data reveal a unique IKK/NF-κB signaling switch that functions to both inhibit differentiation and promote myotube homeostasis.

Introduction

Nuclear factor κB (NF-κB) is a ubiquitously expressed transcription factor and, in mammals, consists of five family members: RelA/p65, c-Rel, RelB, p50 (the processed form of p105), and p52 (the processed form of p100; Hayden and Ghosh, 2004). These subunits contain a DNA-binding protein dimerization domain and nuclear localization signal, but only RelA/p65 (hereafter referred to as p65), c-Rel, and RelB possess transactivation domains (TAs). NF-κB forms homo- and heterodimers, with the p50–p65 complex being the most common. In most cells, NF-κB is bound to IκB inhibitor proteins that mask its nuclear signal and sequester it in the cytoplasm (Huxford et al., 1998).

Correspondence to Denis C. Guttridge: denis.guttridge@osumc.edu

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; DM, differentiation medium; GM, growth medium; IKK, inhibitor of κB kinase; MEF, mouse embryonic fibroblast; MSCV, murine stem cell virus; MyHC, myosin heavy chain; NF-κB, nuclear factor κB; NIK, NF-κB–inducing kinase; Rb, retinoblastoma; SR, superrepressor; TA, transactivation domain; Tn, troponin.

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NF-κB is regulated by a variety of factors, such as inflammatory cytokines that direct NF-κB by what is now referred to as the classical pathway (Ghosh and Karin, 2002). This occurs through stimulation of the inhibitor of κB kinase (IKK) complex consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NF-κB essential modulator/IKKAP1 (Karin, 1999). Once activated, IKKβ phosphorylates IκB proteins, targeting them for ubiquitination and proteasomal degradation. This releases p50–p65 or p50–c-Rel dimers to translocate to the nucleus and bind DNA, where they induce gene expression. Mice null for IKKβ, IKKγ, or p65 are embryonic lethal as a result of massive liver apoptosis, and cells derived from these embryos are unresponsive to classical NF-κB inducers (Beg et al., 1995b; Tanaka et al., 1999; Rudolph et al., 2000), demonstrating a signaling link between p65, IKKβ, and IKKγ subunits.

In response to a second set of factors that include CD40L, B cell–activating factor, and lymphotoxin β, NF-κB is activated through an alternative pathway independent of IKKγ (Pomerantz and Baltimore, 2002). Instead, activation proceeds through the

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NF-κB–inducing kinase (NIK) that phosphorylates and activates IKKα homodimers and, in turn, phosphorylates p100 in complex with RelB. This leads to ubiquitin-dependent processing of p100 to p52 and translocation of p52–RelB to the nucleus (Senftleben et al., 2001; Xiao et al., 2001). B cell–activating factor, NIK, and p100–p52 knockout mice have similar phenotypes (Gerondakis et al., 1999), confirming that these molecules are also part of the same linear nonclassical signaling cascade. In addition, the classical and alternative pathways are thought to regulate distinct genes in response to their various activators (Dejardin et al., 2002; Bonizzi et al., 2004).

Aside from its more commonly accepted role as a regulator of innate immunity and cell survival, NF-κB is also prominent in regulating cellular differentiation. In hematopoietic cells, c-Rel and RelB are essential for B cell lymphopoiesis and T cell maturation (Weih et al., 1996; Gerondakis et al., 1999). NF-κB is also required for osteoclastogenesis, and mice lacking p50 and p52 display severe osteopetrosis (Iotsova et al., 1997). Furthermore, IKKα is important for skin differentiation as well as skeletal and craniofacial morphogenesis (Takeda et al., 1999; Hu et al., 2001; Sil et al., 2004), a function thought to be independent of its kinase activity.

Over the past years, an increasing number of studies have also implicated NF-κB in skeletal muscle differentiation, a process regulated by transcription factors MyoD, Myf5, myogenin, MRF4/Myf6/herculin, and MEF2A-D (Naya and Olson, 1999; Sabourin and Rudnicki, 2000; Pownall et al., 2002). These factors regulate myoblasts to undergo growth arrest and fuse into multinucleated myotubes. However, in contrast to hematopoiesis, the function of NF-κB in myogenesis is less defined, and results have conflicted as to whether NF-κB promotes or inhibits this differentiation process. On the one hand, studies demonstrate that NF-κB DNA binding and transcriptional activities decrease during differentiation (Lehtinen et al., 1996; Guttridge et al., 1999) and that inhibition of NF-κB via expression of the IκBα superrepressor (SR) mutant accelerates myogenesis (Guttridge et al., 1999). In addition, activators of NF-κB such as TNFα, IL-1β, or the receptor-interacting protein (RIP) homologue RIP2 act as potent inhibitors of differentiation (Guttridge et al., 2000; Langen et al., 2001; Munz et al., 2002), which together support the notion that NF-κB functions as an inhibitor of myogenesis. NF-κB mediates this regulation through the induction of cyclin D1 (Guttridge et al., 1999) or by suppressing MyoD synthesis through a destabilization element in the MyoD transcript (Guttridge et al., 2000; Sitcheran et al., 2003). More recent data suggest that NF-κB can also inhibit myogenesis by stimulating expression of the Polycomb group protein YY1 (Wang et al., 2007).

In contrast, similarly performed studies have reported that NF-κB activity increases during myogenesis in response to insulin-like growth factor (Kaliman et al., 1999; Conejo et al., 2002). Insulin-like growth factor activation is mediated, in part, through the classical pathway, causing IκBα degradation and p65 nuclear translocation, although the alternative pathway also appears to be involved because the overexpression of IKKα or NIK was seen to enhance myogenesis (Canicio et al., 2001). In addition, the expression of IκBα-SR in L6 rat myoblasts was found to inhibit terminal differentiation markers, and recently it was also determined that p38 MAPK-induced myogenesis functions through IL-6 synthesis in an NF-κB–dependent manner (Baeza-Raja and Munoz-Canoves, 2004).

Collectively, these studies show that NF-κB function in skeletal muscle differentiation remains at best enigmatic. Resolving this will not only provide insight into the involvement of NF-κB during muscle development and repair but may also increase our understanding of its participation in a growing list of muscle-wasting disorders, including cachexia (Guttridge et al., 2000; Cai et al., 2004; Mourkioti et al., 2006), disuse atrophy (Hunter and Kandarian, 2004), muscular dystrophies (Baghdiguian et al., 1999; Kumar and Boriek, 2003; Acharyya et al., 2007), and inflammatory myopathies (Monici et al., 2003). To this end, we used a genetic approach to decipher the role of NF-κB/IKK subunits during myogenic differentiation. Our results provide an explanation for the previously reported anti- and promyogenic activities of NF-κB by revealing that myogenesis involves both classical and alternative NF-κB pathways. Although constitutive activation of the classical pathway functions in myoblasts to inhibit differentiation, NF-κB signaling switches to the alternative pathway late in the myogenic program to promote mitochondrial biogenesis and myotube homeostasis.

Results

Myogenic activity is enhanced in p65$^{-/-}$ MEFs expressing MyoD

To extend our understanding of NF-κB in skeletal myogenesis, we used established mouse embryonic fibroblasts (MEFs) that were wild type or null for individual NF-κB subunits converted to skeletal muscle by exogenous expression of MyoD (Davis et al., 1987). We initiated this analysis with p65 because this subunit is constitutively active in myoblast nuclei (Guttridge et al., 1999). Results showed that myogenic activity derived from a troponin (Tn) I enhancer reporter plasmid (TnI-luc) was significantly enhanced in *p65*$^{-/-}$ MEFs compared with wild-type cells (Fig. 1 A). Similar findings were obtained with plasmids containing the acetylcholine receptor promoter (AchR-luc) or multimerized muscle regulatory factor binding sites (4RTK-luc), arguing that this effect was not reporter specific. To verify the specificity of p65 regulation, reporter assays were repeated in early passaged primary fibroblasts prepared from embryonic day (E) 13.5 *p65*$^{+/+}$, *p65*$^{+/-}$, and *p65*$^{-/-}$ embryos. Myogenic activity was again elevated in MEFs lacking p65, which occurred in a gene dosage–dependent manner (Fig. 1 B). This confirmed that p65 regulation of myogenesis was not a consequence of cell immortalization. As a control, myogenesis was also assessed in primary MEFs that were wild type or null for the retinoblastoma (Rb) protein, a cell cycle checkpoint required for skeletal muscle differentiation (Novitch et al., 1999). As predicted, reporter activity was significantly impaired in *Rb*$^{-/-}$ MEFs (Fig. 1 B), thus supporting the relevance of our findings with p65. To further address p65 specificity, myogenic assays were extended to MEFs lacking other NF-κB subunits. Results showed that activity from *cRel*$^{-/-}$ or *p50*$^{-/-}$ MEFs was considerably lower than that for *p65*$^{-/-}$ cells (Fig. 1 C). In addition, we used MEFs deficient in IκBα that contain constitutive levels of nuclear p65

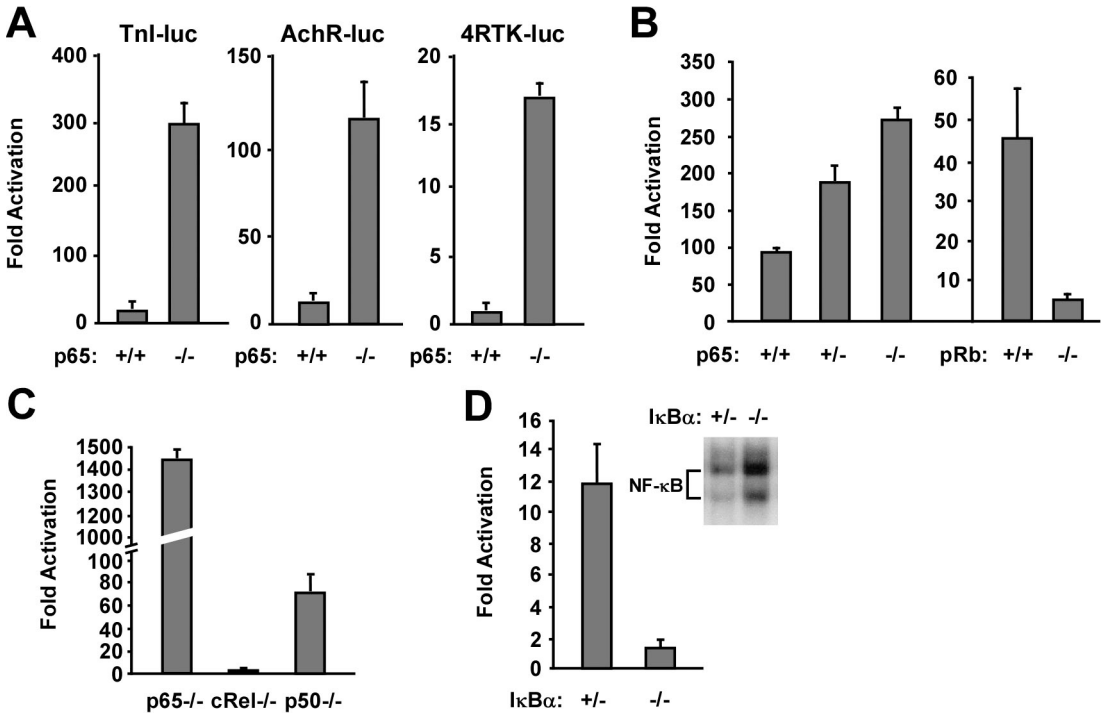


Figure 1. **Loss of p65 enhances myogenic activity in MEFs.** (A) $p65^{+/+}$ and $p65^{-/-}$ MEFs were cotransfected with cytomegalovirus-MyoD and either of the following reporter constructs: TnI-luc, AchR-luc, or 4RTK-luc. The next day, cells were switched to differentiation medium (DM), and, after 48 h, lysates were prepared and assayed for luciferase activity. (B) $p65^{+/+}$, $p65^{+/-}$, $p65^{-/-}$ and $pRb^{+/+}$, and $pRb^{-/-}$ primary MEFs were transfected with MyoD and TnI-luc. Cells were differentiated as in A, and luciferase assays were performed. (C) $p65^{-/-}$, $cRel^{-/-}$, and $p50^{-/-}$ MEFs were transfected with MyoD and TnI-luc, differentiated, and monitored for luciferase activity. (D) Myogenic assays similar to those described in A–C were performed in $I\kappa B\alpha^{+/-}$ and $I\kappa B\alpha^{-/-}$ cells. (inset) Electrophoretic mobility shift assay analysis of $I\kappa B\alpha^{+/-}$ and $I\kappa B\alpha^{-/-}$ MEFs. Error bars represent SEM.

(Beg et al., 1995a). Electrophoretic mobility shift assay confirmed that NF-κB binding was higher in $I\kappa B\alpha^{-/-}$ MEFs, which correlated with lower myogenic activity (Fig. 1 D). Together, these genetic data indicated that p65 functions as a negative regulator of MyoD-induced myogenesis.

MEFs null for p65 are accelerated in their myogenic program

To examine how the absence of p65 exerts its effects on the myogenic program, MyoD was stably expressed in $p65^{+/+}$ and $p65^{-/-}$ MEFs using a murine stem cell virus (MSCV)–MyoD-IRES-GFP retrovirus. After selection, cells were sorted by flow cytometry for GFP to ensure equal levels of MyoD (Fig. 2 A). Cells were then differentiated, and myogenic markers were analyzed over a 4-d period. This analysis revealed that both the induction and overall expression of markers muscle creatine kinase, Tn, myosin heavy chain (MyHC), and tropomyosin were greater in cells lacking p65 (Fig. 2 B). Myotube formation was also strikingly higher in $p65^{-/-}$ cells (Fig. 2 C), which together supported the aforementioned reporter data that p65 functions as an inhibitor of myogenesis.

The transcriptional activity of p65 derives from three TAs located in its C terminus (Schmitz et al., 1994). To determine whether the regulation of myogenesis was dependent on p65 transcriptional activity, reporter assays were repeated in $p65^{-/-}$ MEFs reconstituted with either p65 wild type (1–551 amino acids) or mutants truncated in TA1 (1–521) or all three (1–313) TA domains. Compared with vector, the addition of wild-type p65 (1–551) or TA mutant (1–521) strongly repressed myogenesis, whereas the expression of mutant (1–313) was effective in partially rescuing this regulation (Fig. 2 D). To verify these results, MyoD was stably expressed in $p65^{-/-}$ MEFs along with wild-type or mutant forms of p65 (Fig. 2 E). Consistent with reporter assays, myotubes were completely absent in $p65^{-/-}$ MEFs expressing wild-type p65, whereas some myotubes formed in cells reconstituted with the (1–313) mutant (Fig. 2 F). This suggested that residues within TA (521–551) are largely dispensable for repressing myogenesis, whereas residues (313–521) play a more significant role in this regulation. However, because the (1–313) mutant only partially rescued myogenesis, it further suggested that residues within the Rel domain contributed to p65-suppressive activity. Because the phosphorylation of serine 276 is required for p65 transactivation (Zhong et al., 1998), we examined the involvement of this residue in regulating myogenesis. Reconstitution of $p65^{-/-}$ cells with p65 containing a 276 serine to alanine mutation was less effective in inhibiting myogenic activity, whereas mutations outside the Rel domain in residues 529 and 536 had no effect (Fig. 2 G). This is consistent with the aforementioned data showing that the deletion of TA (521–551) is not required for this regulation. In addition, the generation of serine to alanine 276 in the p65 (1–313) mutant caused a full rescue of myogenic activity (Fig. 2 H), demonstrating that NF-κB regulation of myogenesis is dependent on p65 transcriptional activity mediated from both serine 276 and other residues lying within the 313–521 domain.

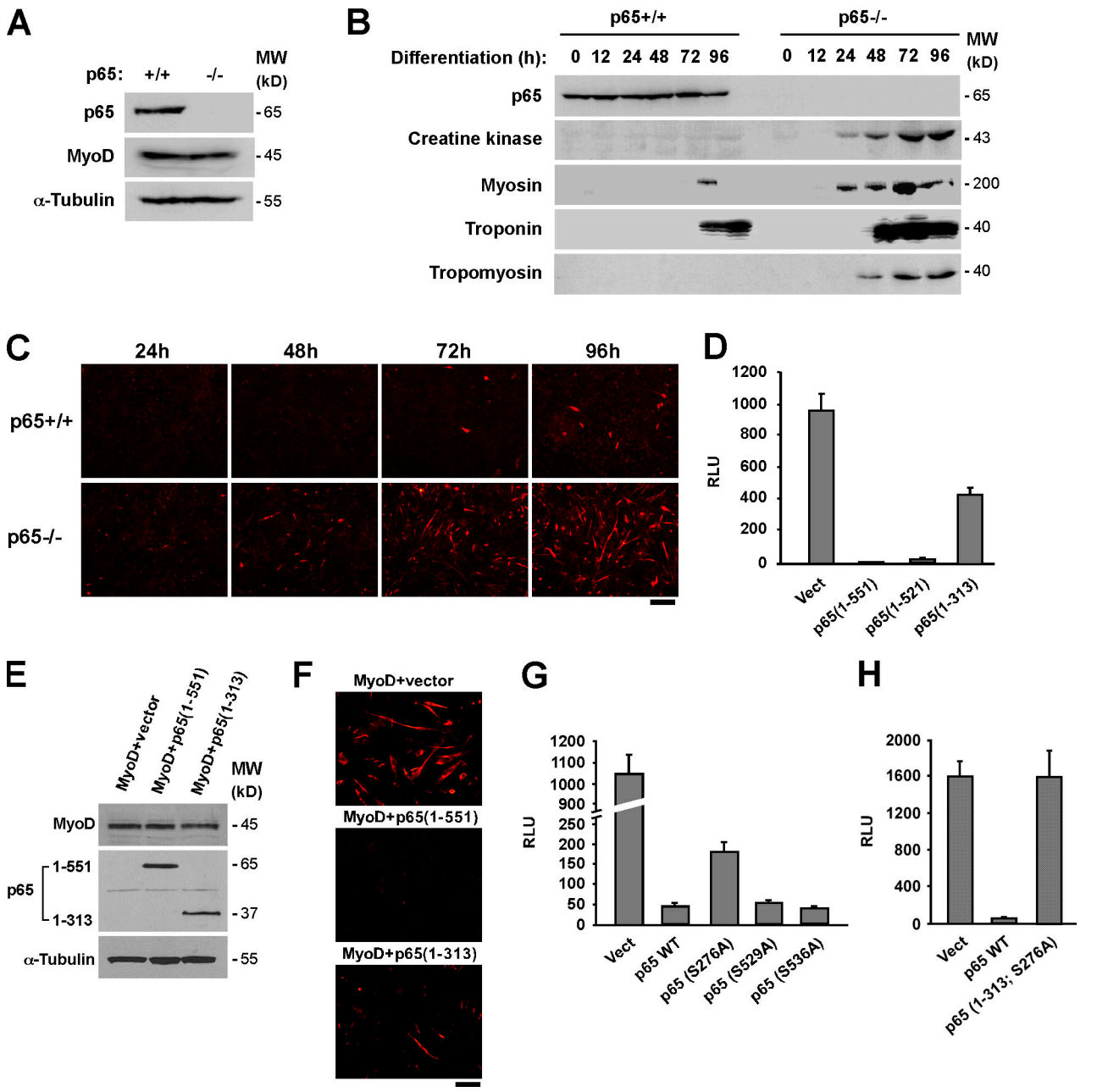


Figure 2. **Loss of p65 accelerates the myogenic program in MEFs.** (A) $p65^{+/+}$ and $p65^{-/-}$ MEFs were infected with MSCV-MyoD and, after puromycin selection, were sorted for GFP to ensure equal MyoD levels. Cells were then probed for p65 and MyoD (45 kD) protein. α-Tubulin (55 kD) was used as a loading control. (B) $p65^{+/+}$ and $p65^{-/-}$ MEFs stably expressing MyoD were differentiated, and lysates were then probed for the indicated myogenic differentiation markers. (C) Cells were differentiated as in B, and MyHC immunofluorescence was performed. (D) $p65^{-/-}$ MEFs were transfected with Tnl-luc and either vector plasmid, wild-type p65 (1–551), or p65 TA mutants (1–521 and 1–313) along with MyoD. Cells were then differentiated, and lysates were prepared for luciferase assays. RLU, relative light units. (E) $p65^{-/-}$ MEFs were reconstituted with either vector and full-length or truncated p65 along with MSCV-MyoD. After selection, whole cell lysates were prepared and probed for p65, MyoD, and α-tubulin. (F) Cells were infected as in E, differentiated for 72 h, fixed, and stained for MyHC. (G) $p65^{-/-}$ MEFs were transfected with MyoD, Tnl-luc, and either vector control, wild-type p65 (WT), or p65 constructs containing S/A mutation at positions 276, 529, and 536. MEFs were differentiated and harvested after 48 h for luciferase assays. (H) Relative luciferase activities from $p65^{-/-}$ MEFs transfected with MyoD, Tnl-luc, and either vector control, wild-type p65, or p65 (1–313) containing the S276A mutation. Error bars represent SEM. Bars: (C) 200 μm; (F) 80 μm.

Myogenesis is accelerated in p65-deficient myoblasts

To determine the physiological relevance of our findings, myogenesis was further explored in $p65^{-/-}$ myoblasts. Although mice lacking p65 are embryonically lethal (Beg et al., 1995b), this phenotype can be rescued with the additional deletion of TNFα (Doi et al., 1999). Thus, $TNF\alpha^{-/-};p65^{+/-}$ mice were crossed, and primary myoblasts were prepared from 2–4-d-old neonates (Fig. 3 A). Transfections with TnI or MyHCIIB reporters showed that myogenic activity was substantially elevated in $p65^{-/-}$ myoblasts, and, as in primary MEFs, this regulation appeared to be gene dosage dependent (Fig. 3 B). In comparison,

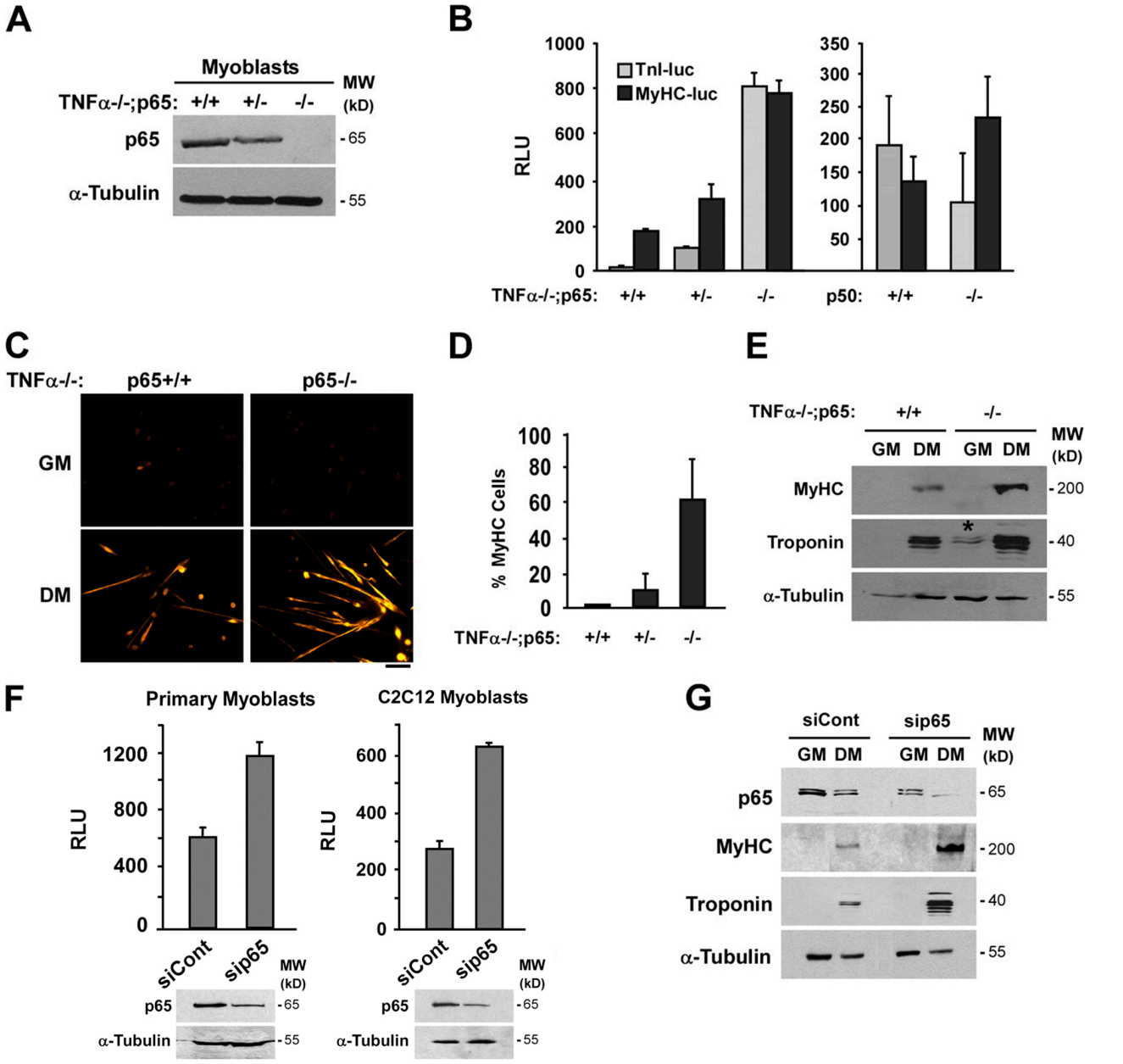


Figure 3. **Loss of p65 enhances the differentiation of primary myoblasts.** (A) Primary myoblasts were prepared from 2–4-d-old *TNFα*$^{-/-}$;*p65*$^{+/+}$, *TNFα*$^{-/-}$;*p65*$^{+/-}$, and *TNFα*$^{-/-}$;*p65*$^{-/-}$ neonates, and genotypes were verified by Western blots for p65. (B) p65 and p50 primary myoblasts were transfected with Tnl-luc or MyHC-luc plasmids, differentiated for 48 h, and subsequently harvested for luciferase assays. RLU, relative light units. (C) *TNFα*$^{-/-}$;*p65*$^{+/+}$ and *TNFα*$^{-/-}$;*p65*$^{-/-}$ myoblasts were differentiated for 0 h (GM) or 48 h (DM) and subsequently stained for MyHC. (D) Quantification of myogenesis was performed by scoring MyHC-positive cells from a minimum of 25 fields normalized to total cell number as determined by Hoechst staining. (E) Myoblasts were differentiated for 0 (GM) and 48 h (DM), and lysates were probed for MyHC and Tn. The asterisk indicates Tn expression under GM conditions. (F) Primary or C2C12 myoblasts were transfected with siControl (siCont) or siRNA against p65 (sip65) along with Tn-luc reporter. Cells were switched to DM, and luciferase assays were performed. (G) C2C12 myoblasts were transfected with siControl or siRNA against p65 and switched to DM for 48 h, after which lysates were prepared and Western blots were performed. Error bars represent SEM. Bar, 80 μm.

myogenic activity in *p50*$^{-/-}$ myoblasts was not significantly different from wild-type cells. Furthermore, under differentiation conditions, *p65*$^{-/-}$ myoblasts formed 58% more myotubes (Fig. 3, C and D) and expressed higher levels of myofibrillar proteins (Fig. 3 E) compared with *p65*$^{+/+}$ cells. It is also noteworthy that the modest but reproducible expression of Tn was detectable in *p65*$^{-/-}$ myoblasts even under growth conditions (growth medium [GM]; Fig. 3 E, asterisk). Given that myofibrillar genes are silent in myoblasts, this suggested that p65 functions as a transcriptional repressor of Tn, which is consistent with our recent report that NF-κB is capable of silencing Tn enhancers through the production of YY1 and recruitment of Ezh2 and HDAC-1 (Wang et al., 2007). Because TNFα has recently been shown to be required for muscle regeneration (Chen et al., 2007), admittedly it was possible that the increase in muscle differentiation in *p65*$^{-/-}$ myoblasts occurred secondary to

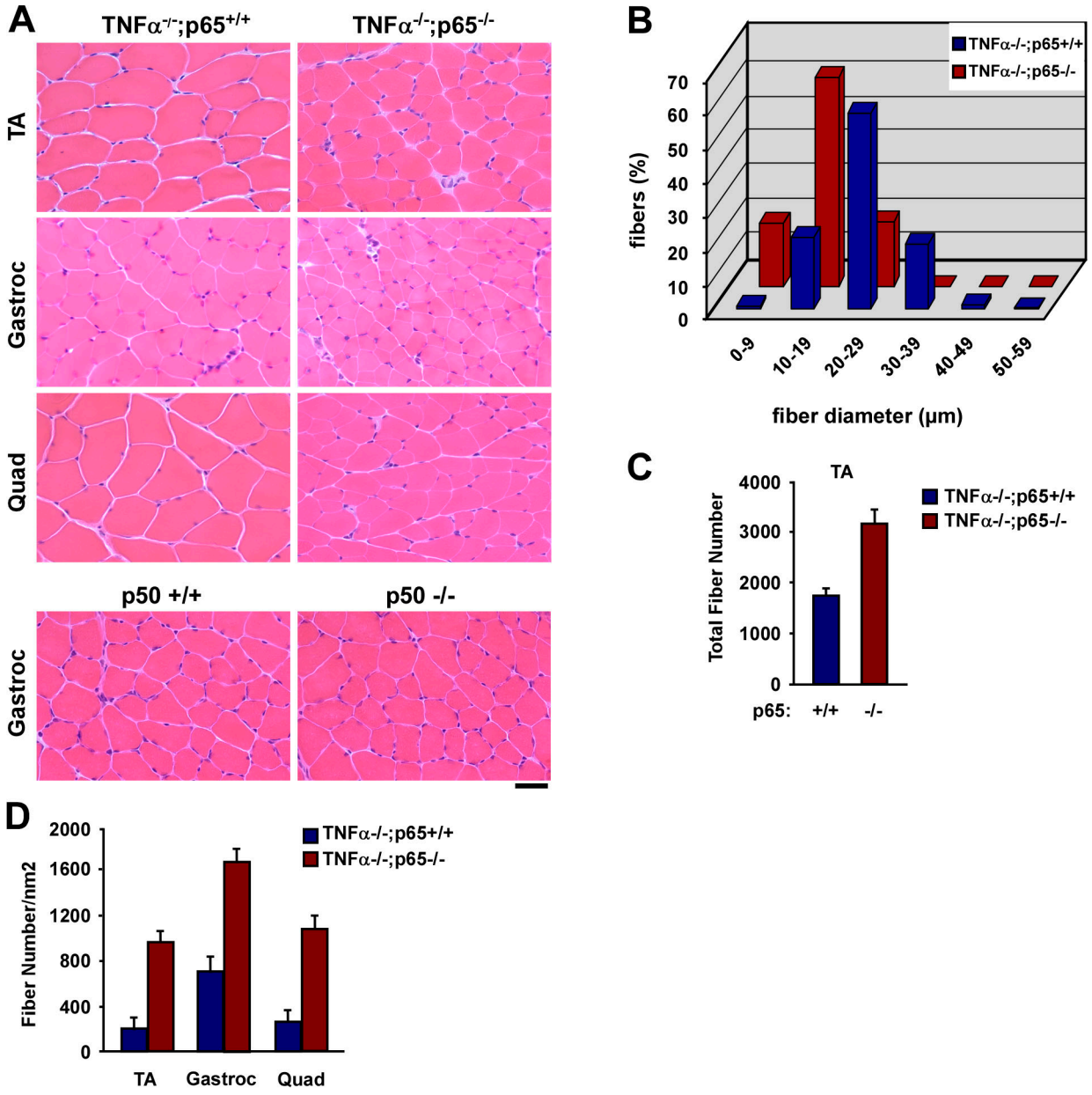


Figure 4. **Myogenesis is enhanced in p65-deficient mice.** (A) Hematoxylin- and eosin-stained cryosections of tibialis anterior (TA), gastrocnemius (Gastroc), and quadriceps (Quad) muscles from *TNFα*$^{-/-}$;*p65*$^{+/+}$ and *TNFα*$^{-/-}$;*p65*$^{-/-}$ mice or *p50*$^{+/+}$ and *p50*$^{-/-}$ gastrocnemius. (B) Fiber diameters were measured from gastrocnemius muscle sections from a total of 1,500 fibers ($n = 5$ mice per group). (C) Fiber numbers were determined in whole cross sections from tibialis anterior muscles from *TNFα*$^{-/-}$;*p65*$^{+/+}$ and *TNFα*$^{-/-}$;*p65*$^{-/-}$ mice ($n = 3$). (D) Fiber numbers were recorded from premeasured randomly selected areas (minimum of 25 per animal) throughout the tibialis anterior, gastrocnemius, and quadriceps muscles ($n = 5$ mice per genotype). Error bars represent SEM. Bar, 50 μm.

the loss of this cytokine. However, siRNA knockdown of p65 in primary myoblasts and C2C12 cells again led to increases in myogenic activity and muscle markers (Fig. 3, F and G), supporting results that negative regulation of myogenesis is specific to p65 and is unlikely to be related to the absence of TNFα.

The absence of p65 enhances myogenesis in vivo

Next, we explored muscles from *TNFα*$^{-/-}$;*p65*$^{-/-}$ mice in an attempt to correlate our in vitro findings with an in vivo phenotype. To our surprise, p65-null muscles displayed a large number of fibers that were noticeably smaller in size than their wild-type counterpart (Fig. 4 A). In p65-deficient tibialis anterior muscles from 4-wk-old male or female mice, mean fiber diameter was reduced by 39% as compared with wild-type littermates (25.6 μm for wild type vs. 15.5 μm for null; $n = 5$; Fig. 4 B). This phenotype was common to multiple hind limb muscles and was selective to p65 because no such differences were observed in *p50*$^{-/-}$ mice (Fig. 4 A). Slow MyHC staining from gastrocnemius muscles confirmed that the absence of p65 did not affect fiber type (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200707179/DC1). Although muscle atrophy is

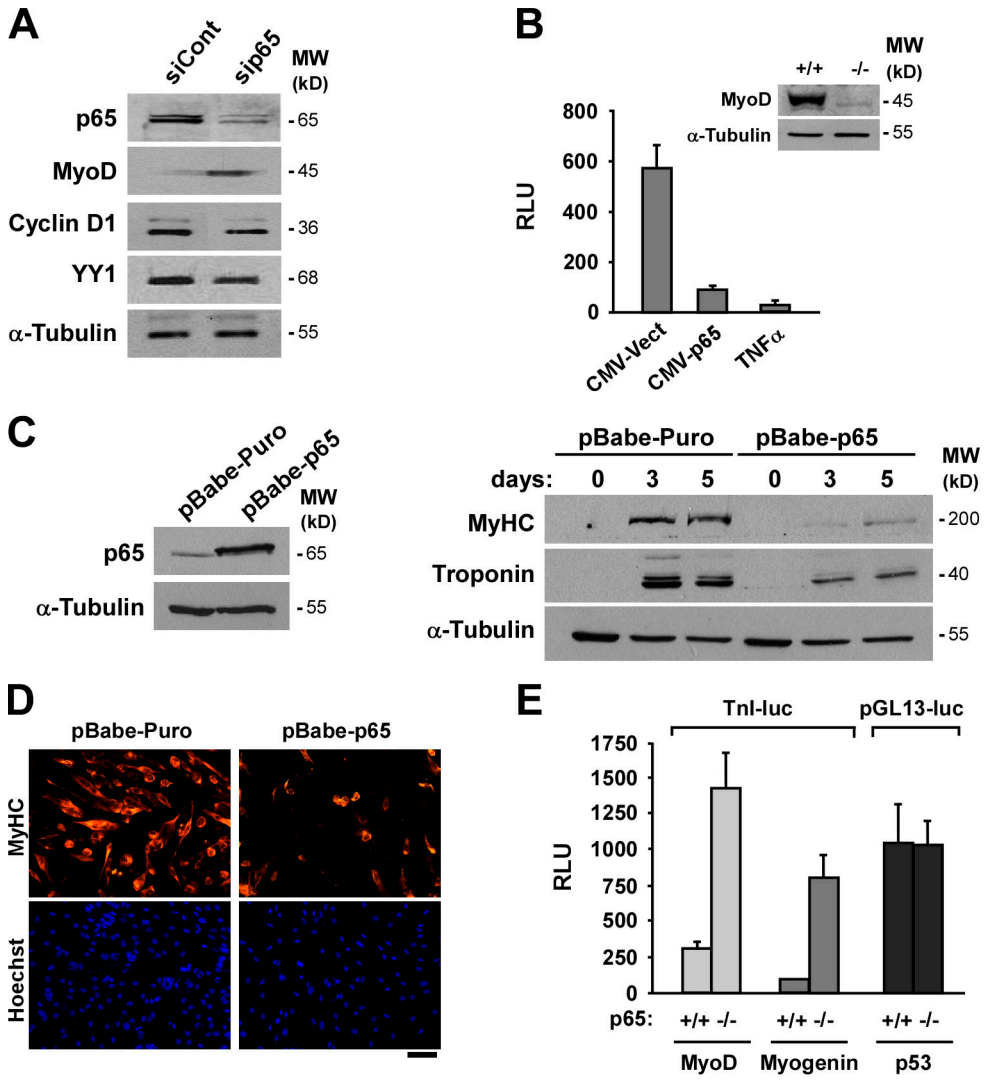


Figure 5. **p65 regulation of myogenesis occurs through multiple mechanisms.** (A) C2C12 myoblasts were transfected with control and p65 siRNA, and lysates were harvested for Western analysis. (B) *MyoD*$^{-/-}$ myoblasts were transfected with Tnl-luc along with either an empty vector (CMV-Vect) or a p65 expression plasmid (CMV-p65) or were transfected with vector and subsequently treated with 5 ng/ml TNFα. Cells were differentiated for 2 d, and luciferase assays were performed. RLU, relative light units. (inset) Western blot for MyoD wild-type and null myoblasts. (C) *MyoD*$^{-/-}$ myoblasts were infected with pBabe-Puro or pBabe-p65 retroviruses. After selection and differentiation for 3 and 5 d, protein lysates were prepared for Western analysis. (D) *MyoD*$^{-/-}$ myoblasts stably expressing pBabe-Puro and pBabe-p65 were differentiated, fixed, and stained for MyHC (red) and nuclei (Hoechst; blue). (E) *p65*$^{+/+}$ and *p65*$^{-/-}$ MEFs were transfected with Tnl-luc and either MyoD or myogenin plasmids. As a control, transfections were also performed with a p53 expression plasmid and responsive reporter (pGL13-luc). Cells were subsequently differentiated, and luciferase assays were performed. Error bars represent SEM. Bar, 80 μm.

an underlying cause of fiber reduction associated with induction of the E3 ubiquitin ligases MuRF1 and atrogene-1/MAFbx, muscles depleted for p65 displayed no evidence of this regulation (Fig. S1 B). However, given our findings with primary myoblasts lacking p65, we considered the possibility that changes in *p65*$^{-/-}$ muscles might result from an increase in overall myotube number. Indeed, fiber counts from entire cross sections of tibialis muscles revealed a 76% increase in *p65*$^{-/-}$ mice compared with control littermates (Fig. 4 C). Similar results were found when counts were extended to gastrocnemius and quadriceps, demonstrating that this regulation is not muscle type specific ($n = 5$; Fig. 4 D). In addition, the *p65*$^{-/-}$ phenotype was not related to a compromised immune function in adult mice because increases in fiber number were also observed in postnatal day (P) 7 and 9 neonates (Fig. S1, C and D). These findings are consistent with cell culture data suggesting that p65 absence in vivo leads to enhanced myogenesis, a phenotype highly reminiscent of dystrophin-deficient or acutely injured muscles depleted of p65 (Acharyya et al., 2007).

p65 regulates myogenesis through multiple mechanisms

Next, we sought to address the mechanism by which p65 negatively regulates myogenesis. Previous use of the IκBα-SR inhibitor revealed that p65 can inhibit C2C12 differentiation through the suppression of MyoD synthesis (Guttridge et al., 2000). Such analysis also revealed that NF-κB is capable of inhibiting myogenesis through cyclin D1 (Guttridge et al., 1999),

limiting myoblasts from exiting the cell cycle or through YY1 to silence myofibrillar promoters in myoblasts (Wang et al., 2007). Consistent with these findings, MyoD was elevated in $p65^{-/-}$ myoblasts, whereas both YY1 and cyclin D1 levels declined (Fig. 5 A). Thus, it is likely that p65 negatively regulates myogenesis through multiple mechanisms.

To determine whether these mechanisms could function independently of each other, we examined the regulation of myogenesis by p65 in $MyoD^{-/-}$ myoblasts. Although myotube formation is impaired in these cells (Sabourin et al., 1999), myogenic activity was nevertheless retained under differentiation conditions (Fig. 5 B). However, the addition of p65 or TNFα strongly repressed this activity. Likewise, retroviral expression of p65 in $MyoD^{-/-}$ myoblasts caused a noticeable reduction of myogenic markers (Fig. 5, C and D), demonstrating that p65 can inhibit myogenesis independently of MyoD. To substantiate this finding, reporter assays were repeated in $p65^{+/+}$ and $p65^{-/-}$ MEFs where MyoD was substituted with myogenin. Like MyoD, myogenin is capable of converting fibroblasts to a muscle lineage, albeit with lower efficiency (Gerber et al., 1997). Indeed, myogenin-induced myogenic activity was less than that for MyoD, but these levels were nonetheless greater in $p65^{-/-}$ MEFs compared with wild-type cells (Fig. 5 E). This regulation also appeared specific to these muscle regulatory factor proteins because a p53-responsive reporter was not affected by the absence of p65.

Myogenesis is regulated by a temporal switch in IKK signaling pathways

Having gained insight into the role of p65 in muscle differentiation, we now turned our attention to its upstream regulator, the IKK complex. Recently, our group elucidated that chronic activation of IKKβ in *mdx* muscles inhibits muscle differentiation (Acharyya et al., 2007). Interestingly, Mourkioti et al. (2006) have also reported that skeletal muscle deletion of IKKβ increased intermediate fiber numbers in 4-mo-old mice, a phenotype that closely matched that of younger $p65^{-/-}$ mice (Fig. 4). Such results suggested that p65 and IKKβ share overlapping functions in skeletal muscle differentiation. Indeed, analogously to p65, myogenic activity was increased in primary fibroblasts and myoblasts deleted for IKKβ floxed (f/f) alleles using Cre recombinase (Fig. 6 A and Fig. S2, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200707179/DC1). Likewise, 4-wk-old mice lacking skeletal muscle IKKβ exhibited an increase in total fiber number (Fig. 6, B and C), reaffirming that IKKβ, like p65, functions as a negative regulator of myogenesis. Because IKKβ and p65 are components of classical NF-κB signaling, myogenesis was also tested in $IKK\gamma^{-/-}$ MEFs to address whether this pathway acts as a general inhibitor of differentiation. Consistent with this thinking, myogenic activity was increased in $IKK\gamma^{-/-}$ MEFs but decreased in MEFs lacking IKKα, the latter of which is not considered part of the classical pathway (Fig. 6 D; Senftleben et al., 2001).

To further explore the myogenic functions of IKK, we measured its activity in differentiating myoblasts. Results showed that IKK activation was relatively low in undifferentiated cells but became induced at 48 h into the myogenic program (Fig. 6 E), a time when cell fusion and contractile myofibrillar expression is well underway. This activity was specific to IKK because phosphorylation was undetectable when assays were repeated with a mutated substrate, and the increase in activity was not a consequence of altered protein expression because IKK subunits remained unchanged during myogenesis (Fig. 6 E).

Next, we analyzed endogenous IKK substrates to ascertain which IKK complex became activated during late stage myogenesis. As part of the classical pathway, IKKβ activation results in the phosphorylation of IκBα and p65 (Hayden and Ghosh, 2004). On the other hand, IKKα predominantly phosphorylates p100, leading to its proteolysis and conversion to p52. Results revealed that levels of phosphorylated IκBα and p65 decreased during C2C12 differentiation, whereas total protein levels remained unchanged (Fig. 6 F). Consistent with this decrease, nuclear p65 levels declined, and, by chromatin immunoprecipitation (ChIP), p65 binding activity on the IκBα promoter was also lost (Fig. 6 F). In comparison, the processing of p100 to p52 was induced during myogenesis, with similar kinetics to C2C12 as well as primary myoblast differentiation (Fig. 6 G and not depicted). Because IKKα activation results in the formation of RelB–p52, we also investigated the contribution of these NF-κB subunits by repeating myogenic assays in $RelB^{-/-}$ and $p52^{-/-}$ MEFs. Consistent with findings in $IKK\alpha^{-/-}$ MEFs, myogenic activities were reduced in both $p52^{-/-}$ and $RelB^{-/-}$ fibroblasts (Fig. 6 H). Together, these data suggest that skeletal myogenesis is characterized by a temporal switch in NF-κB signaling pathways whereby the reduction of classical NF-κB is followed by activation of the alternative pathway relatively late in the myogenic program.

IKKα functions as a regulator of myotube maintenance under metabolic stress

The aforementioned data suggested that components of the alternative pathway might function to promote myogenesis. However, stable expression of an HA-tagged version of IKKα in C2C12 myoblasts did not affect induction of the early and late myogenic markers myogenin and Tn, respectively (Fig. 7 A), nor was myogenic activity affected when IKKα or a kinase-dead version (K/M) of this kinase was overexpressed in MyoD-expressing 10T1/2 fibroblasts (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200707179/DC1). In comparison, the expression of classical signaling components IKKβ, IKKγ, or p65 led to clear reductions in myogenic activity in these same cells. Furthermore, no differences in skeletal muscle gene expression were detected by Affymetrix microarray between control and HA-IKKα–expressing C2C12 myotubes (not depicted), and siRNA-mediated depletion of IKKα from differentiating myoblasts also revealed little change in myogenic markers (Fig. 7 B). Therefore, although results from $IKK\alpha^{-/-}$, $RelB^{-/-}$, and $p52^{-/-}$ MEFs suggested that the alternative pathway is promyogenic, overall, the data do not support that activation of this NF-κB signaling pathway is necessary for myotube formation (see Discussion).

However, under long-term differentiation conditions (6 d) without medium replenishment, we observed that myotubes expressing HA-IKKα were better maintained compared with control cells (Fig. 7 C). Specifically, IKKα-expressing myotubes

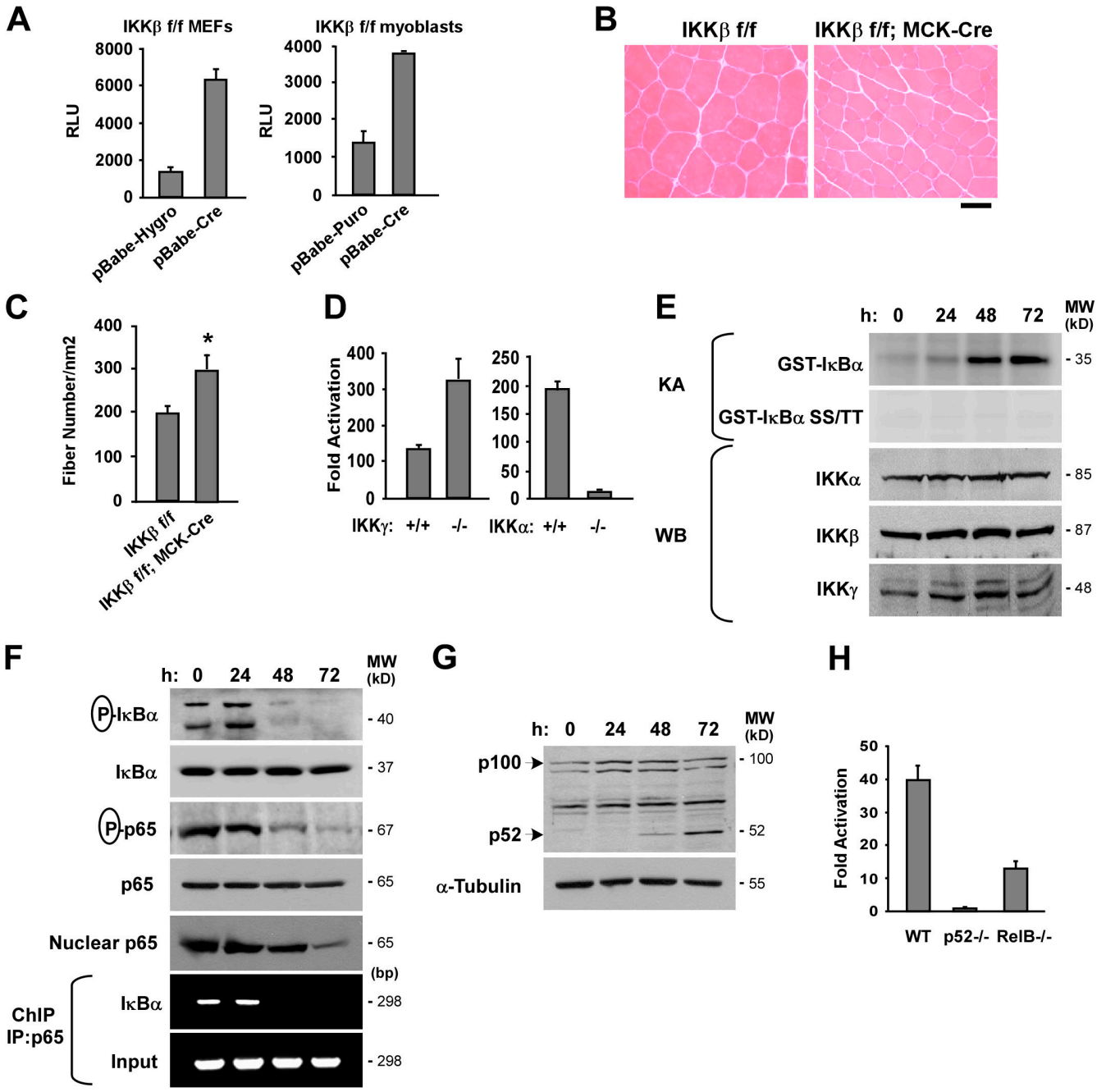


Figure 6. **IKK signaling is temporally regulated and functionally distinct in myogenesis.** (A) IKKβ (f/f) MEFs or myoblasts prepared from E13.5 embryos or 3-d-old pups, respectively, were infected with pBabe-Puro or pBabe-Cre retrovirus. After selection, cells were transfected with MyoD and Tnl-luc, and luciferase assays were performed after 2 d in DM. (B) Hematoxylin- and eosin-stained cryosections from tibialis anterior muscles of 4–6-wk-old IKKβ f/f and IKKβ f/f;muscle creatine kinase–Cre mice. (C) Fiber numbers were determined from premeasured randomly selected areas (minimum of 25 per animal) throughout the tibialis anterior muscle (n = 3 mice per genotype). *, P = 0.005. (D) IKK wild-type and null MEFs were transiently transfected with MyoD and Tnl-luc, and, after 2 d in DM, lysates were prepared for luciferase assays. (E) C2C12 myoblasts were differentiated, and, at the indicated times, cells were harvested, and lysates were prepared for IKK kinase assays using wild-type or serine to alanine mutant IκBα proteins as substrates (KA, kinase assay; WB, Western blot). (F) C2C12 cells were differentiated, and, at the indicated time points, extracts were prepared to probe for phosphorylated IκBα, total IκBα, phosphorylated p65, and total p65. Parallel samples were prepared for nuclear extraction, and Western blots were performed for nuclear p65. Parallel differentiated C2C12 cells were immunoprecipitated with a p65 antibody and processed for chromatin immunoprecipitation (ChIP). Fragments from the IκBα promoter were amplified by PCR before (input) or after immunoprecipitation. (G) Lysates from differentiating C2C12 cells were prepared and used to probe for p100–p52 and α-tubulin. (H) MEFs wild type or null for p52 and RelB were transfected with MyoD and Tnl-luc. Cells were differentiated for 48 h and prepared for luciferase assays. Error bars represent SEM. Bar, 0.5 μm.

were 48% less atrophic (26.0 ± 5.7 μm in fiber diameter compared with 13.5 ± 3.5 μm in control cells) and overall exhibited a healthier morphological appearance. In addition, IKKα-expressing myotubes were also more resistant to low glucose but not to heat shock, oxidative stress, or DNA damage (Fig. 7 C and not depicted), suggesting a selective resistance to metabolic

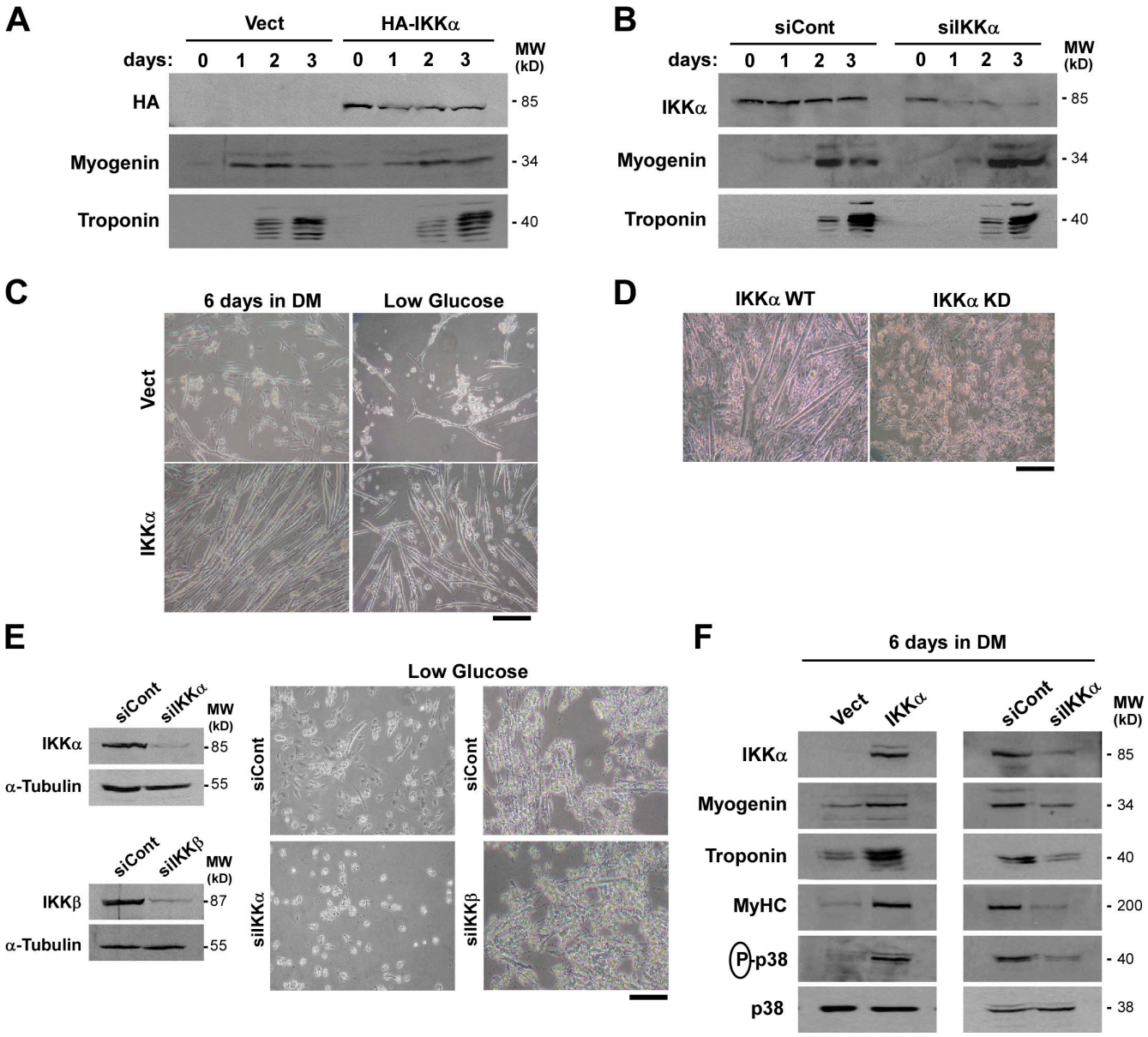


Figure 7. **IKKα regulates myotube maintenance.** (A) C2C12 cells were transfected with vector or HA-IKKα expression plasmids. After selection, cells were differentiated and harvested for Western analysis probing for HA and myogenic markers. (B) Myoblasts were transfected with siControl or siIKKα oligonucleotides and differentiated, and Western blotting was performed as in A. (C) 3-d differentiated myotubes stably expressing vector or IKKα were subjected to varying stress conditions, including no media replenishment for 6 d (6 d in DM) or low glucose (1 g/L glucose in DM for 48 h). Cells were then fixed and photographed by phase contrast at 20× magnification. (D) Differentiated myotubes stably expressing wild-type (WT) or a kinase-dead (KD) version of IKKα were switched to low glucose for 24 h and photographed by phase contrast. (E) Myotubes expressing siControl, siIKKα, or siIKKβ were differentiated for 3 d and switched to low glucose for 20 h before fixation. Parallel samples were harvested for Western blots to confirm knockdown efficiency. (F) C2C12 cells expressing vector or IKKα were differentiated for 6 d. Lysates were subsequently prepared for Western blots probing for IKKα and myogenic markers. Bars, 200 μm.

stress. This effect was dependent on the kinase activity of IKKα because myotube maintenance was lost upon expression of a kinase-dead mutant (Fig. 7 D). Moreover, siRNA deletion of the α but not the β subunit of IKK negated this protective effect upon glucose deprivation, confirming the specificity of IKKα in this regulation (Fig. 7 E). Furthermore, 6-d starved IKKα-expressing myotubes displayed higher levels of the myogenic markers myogenin, Tn, MyHC, and activated p38, whereas these markers were reduced upon IKKα knockdown (Fig. 7 F). Together, these data suggest that activation of IKKα and the alternative pathway during myogenesis functions to maintain myotubes in response to metabolic stress.

IKKα regulates mitochondrial biogenesis

Finally, we attempted to address the process by which IKKα controls myotube maintenance. Because IKKα regulation appeared selectively linked to starvation stress, we speculated that this kinase was involved in regulating the energy capacity of differentiating muscle. Energy production during myogenesis occurs through a switch from glycosidic to oxidative phosphorylation

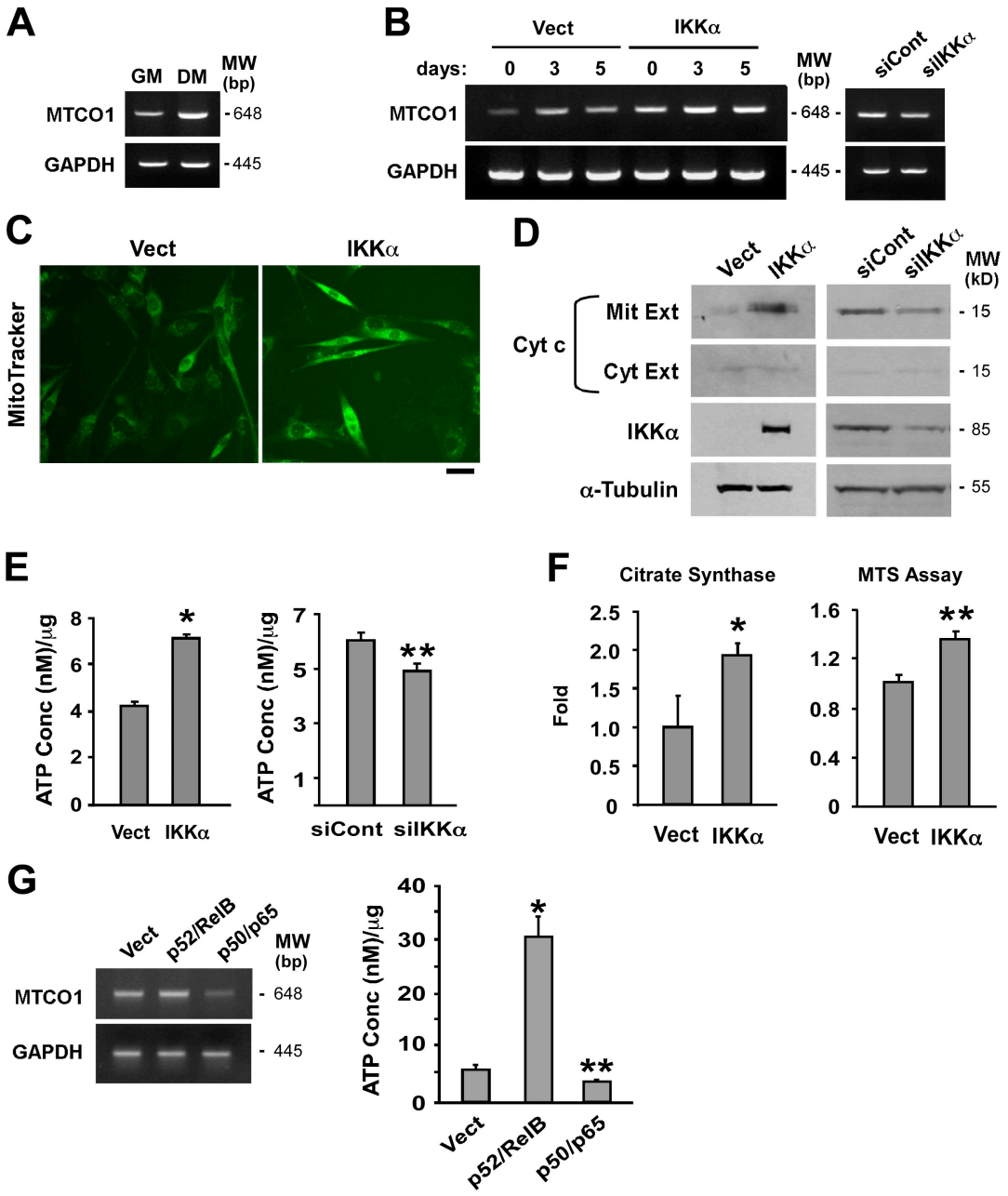
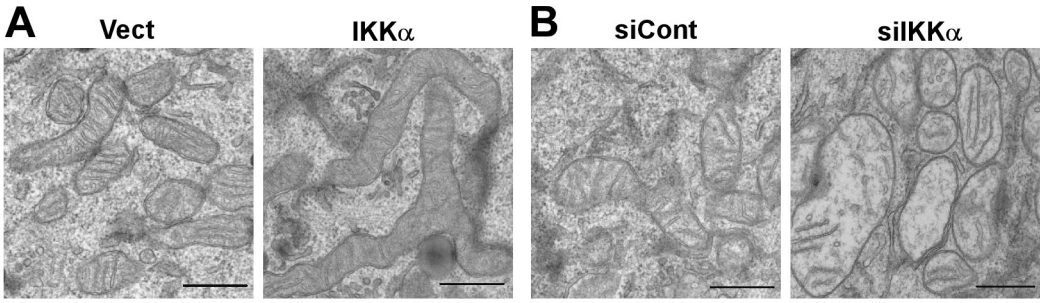


Figure 8. **IKKα regulates mitochondrial biogenesis.** (A) DNA was prepared from GM or 3-d DM C2C12 cells, diluted, and used to amplify a 648-bp fragment from MTCO1. Separate PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize for loading. (B) C2C12 cells stably expressing vector (Vect) or IKKα were differentiated, and DNA samples were prepared for the determination of mitochondrial number as in A. (C) Vector and IKKα-overexpressing cells were differentiated for 3 d and stained for mitochondria with MitoTracker green. Staining was viewed by fluorescence at 20× magnification. (D) Mitochondrial and cytoplasmic extracts were prepared from HA-IKKα or IKKα-depleted myotubes, and lysates were probed for cytochrome *c*. (E) IKKα-expressing or -depleted cells were differentiated for 3 d and lysed, and ATP production was measured by luminescence. An ATP standard curve was generated in parallel to convert luminescence readings into [ATP] (*, P = 0.02; **, P = 0.001). (F) Vector and IKKα cells were differentiated, lysed, and prepared for a citrate synthase assay. All experiments were initiated with equal protein and were performed during the linear phase of the reaction to ensure adequate substrate amounts (*, P = 0.01). A parallel set of myotubes expressing vector or IKKα was cultured and switched to DM, and dehydrogenase activity was measured by the conversion of MTS tetrazolium into aqueous formazan. Readings were taken at 15 min before saturation (**, P = 0.001). (G) C2C12 cells were transfected with vector, p52–RelB, or p50–p65 and differentiated for 3 d. DNA was prepared for the determination of mitochondrial numbers as in A or was processed for the determination of ATP production as in E (*, P = 0.001; **, P = 0.03). Error bars represent SEM. Bar, 40 μm.

resulting from an increase in mitochondrial content (Moyes et al., 1997; Lyons et al., 2004). Using semiquantitative PCR and the mitochondrial marker gene cytochrome oxidase 1 (MTCO1), we readily detected an increase in mitochondrial DNA during C2C12 myogenesis (Fig. 8 A). Examination of HA-IKKα differentiating myoblasts also revealed significantly higher levels of MTCO1 DNA compared with vector cells, whereas the depletion of IKKα led to a reduction of MTCO1 (Fig. 8 B). This suggested the possible novel finding that IKKα is a regulator of mitochondrial biogenesis. Consistent with this notion, myotubes



C

Enriched biological processes, p<0.05

	Occurrences	Percentage
Mitochondria/Metabolism	62	48
RNA related	17	13
Transcription/translation	15	12
Cell cycle	15	11
Other	15	10
Myogenesis	7	6

Figure 9. **IKKα controls mitochondrial structure.** (A) Ultrathin sections from vector or IKKα-expressing myotubes were analyzed by EM at 18,500× direct magnification. (B) Myotubes expressing control or IKKα siRNA were sectioned and visualized by EM as in A. (C) Microarray analysis was performed on vector and IKKα-expressing myotubes using the murine MG430.20 Affymetrix chip. Genes up-regulated in IKKα myotubes as compared with vector were analyzed by L2L analysis (http://depts.washington.edu/l2l/) for statistically significant enriched biological processes. Bars, 500 nm.

overexpressing IKKα contained higher levels of the mitochondrial dye MitoTracker (Fig. 8 C). In addition, mitochondrial fractions from these cells were also enriched in cytochrome *c*, which were, in turn, reduced upon the expression of IKKα siRNA (Fig. 8 D). Furthermore, total ATP was elevated by 72% in IKKα-expressing myotubes ($P = 0.001$), whereas IKKα knockdown caused an 18% reduction in ATP levels ($P = 0.02$; Fig. 8 E). To address whether the increase in mitochondrial content by IKKα reflected mitochondrial function, biochemical assays were performed for citrate synthase and dehydrogenase enzymes. Results showed that enzyme activities were significantly increased in IKKα-expressing myotubes (Fig. 8 F). This function appeared selective to the NF-κB alternative pathway because p52–RelB but not p50–p65 increased MTCO1 and ATP (Fig. 8 G). Furthermore, ATP levels were also increased in MEF and HeLa cells overexpressing IKKα (unpublished data), suggesting that IKKα regulation of mitochondria is not specific to skeletal muscle.

To further investigate this regulation, ultrastructural analysis was performed in IKKα overexpression and knockdown conditions. Remarkably, HA-IKKα–expressing myotubes displayed elongated networks of mitochondria, a hallmark of extensive proliferation (Fig. 9 A). Conversely, IKKα knockdown resulted in degenerating organelles, which was indicated by swelling and the absence of cisternae in the mitochondrial matrix (Fig. 9 B). In addition, genome-wide L2L analysis (Newman and Weiner, 2005) of microarray data from IKKα mytotubes identified 126 selectively enriched biological processes in genes that were up-regulated (>1.5-fold). From these, 48% were involved in mitochondrial and metabolic regulation (Fig. 9 C), whereas significantly fewer IKKα regulated genes associated with transcription/translation (12%) or even skeletal myogenic processes (6%). To examine whether the regulation of mitochondria is linked to myotube maintenance, we treated C2C12 myotubes with mitochondrial inhibitors chloramphenicol and oligomycin under glucose deprivation. This led to visibly less numbers of preserved myotubes (Fig. S3 B), a phenotype strikingly similar to IKKα-depleted cells. Collectively, our results strongly support that activation of NF-κB alternative signaling during myogenesis does not function to promote myotube formation but rather is important for regulating mitochondrial biogenesis and myotube homeostasis.

Discussion

Recent studies have shown that chronic activation of NF-κB is detrimental to muscle function. In skeletal muscles, NF-κB has been linked with disease states such as cachexia and various forms of muscular dystrophies and inflammatory myopathies (Baghdiguian et al., 1999; Kumar and Boriek, 2003; Monici et al., 2003; Hunter and Kandarian, 2004; Acharyya et al., 2007). Although such studies implicate NF-κB as a therapeutic target, mechanistically, relatively little is known about how this transcription factor mediates its pathological effects. Elucidation of these mechanisms might be better achieved by studying NF-κB function in basic models of skeletal myogenesis. However, even in tissue culture systems, reports have conflicted as to whether NF-κB acts as a repressor or promoter of myogenesis (Lehtinen et al., 1996; Guttridge et al., 1999; Kaliman et al., 1999; Canicio et al., 2001; Langen et al., 2001; Conejo et al., 2002; Munz et al., 2002; Baeza-Raja and Munoz-Canoves, 2004). In the present study, we describe what we believe to be a new understanding for the role of NF-κB in skeletal muscle differentiation. Our findings reveal that NF-κB is capable of functioning as both a repressor of differentiation and a promoter of

myotube maintenance depending on specific activities of IKK and NF-κB subunits.

p65 and the classical NF-κB signaling pathway function as negative regulators of myogenesis

Utilization of knockout MEFs demonstrated that myogenic activity was enhanced in cells lacking p65, and comparisons with all five NF-κB subunits showed that this activity was highest in $p65^{-/-}$ cells. Therefore, although myoblast nuclei have been shown to contain constitutive activity for p50 and p65 (Guttridge et al., 1999), our current data argue that suppression of myogenesis by NF-κB is mediated specifically through p65. This notion is consistent with results in primary myoblasts in which myogenic activity was also elevated in p65- but not p50-null cells. Together, these genetic data reaffirm that p65 activity in proliferating myoblasts functions as a negative regulator of myogenesis. This function of p65 is evident in muscle injury, in which the lack of p65 enhances myogenesis in *mdx* and toxin-treated mice (Acharyya et al., 2007). Given that p65 deficiency correlated with increases in overall fiber numbers in young and adult mice, it suggests that p65 is also relevant during postnatal muscle growth, as indicated by the high levels of NF-κB activity in muscles from neonates (Acharyya et al., 2007). Why p65 would function in this capacity at this stage of development is not yet known, and whether it functions in a similar manner during embryonic or fetal myogenesis remains to be investigated.

Our current results demonstrate that regulation of myogenesis is dependent on p65 transcriptional activity. This notion is in line with our previous findings that NF-κB inhibits myogenesis through the transcriptional activation of cyclin D1 (Guttridge et al., 1999). Repression of myogenesis by p65 has also been seen in response to TNFα, leading to the loss of MyoD (Guttridge et al., 2000) and, more recently, to the gain of YY1, resulting in silencing of myofibrillar genes (Wang et al., 2007). Thus, p65 requires its transactivation function to suppress muscle differentiation, and results from $MyoD^{-/-}$ myoblasts support that this can occur via multiple mechanisms.

Similar to p65, we discovered that myogenic activity was enhanced in MEFs lacking classical components IKKβ and IKKγ. Like p65, IKKβ deletion in muscle led to increases in fiber number and to enhanced myogenesis, as recently reported in *mdx* mice (Acharyya et al., 2007). Collectively, these data argue strongly that classical NF-κB signaling functions as a negative regulator of muscle differentiation in both physiological and disease processes.

IKKα signaling promotes myotube maintenance through mitochondrial biogenesis

With respect to alternative NF-κB signaling, our results showed that activation of IKK during myogenesis is selective to IKKα, as this activity tightly correlated with p100 processing. Such activation was preceded by a decline in classical pathway activity, which is depicted by decreases in IκBα and p65 phosphorylation, as well as p65 nuclear and DNA-bound levels. In contrast to recent findings that nuclear localization of IKKα is required in skin differentiation (Hu et al., 2001; Sil et al., 2004) or NF-κB–dependent gene expression (Anest et al., 2003; Yamamoto et al., 2003; Hoberg et al., 2004), we were unable to detect nuclear IKKα in myoblasts or myotubes (unpublished data). Although our current results do not rule out the possibility that IKKα might still phosphorylate an unknown target to modulate myogenic gene expression, we favor instead that IKKα function in skeletal muscle differentiation is represented by the alternative pathway requiring the cytoplasmic form of IKKα to activate p52–RelB complexes.

Evidence from $IKK\alpha^{-/-}$, $p52^{-/-}$, and $RelB^{-/-}$ MEFs indicated that alternative activation of NF-κB is required for myogenic activity. These results appear consistent with previous findings implicating IKKα as a positive regulator of myogenesis (Canicio et al., 2001). However, in contrast to these findings, we were unable to demonstrate by either forced expression or RNAi depletion that IKKα is essential for the induction of myogenic genes or myotube formation. Although genetic evidence from p65 and IKKβ knockout MEFs was consistent with how these classical signaling components were found to function in muscle cells, we do not yet understand why this same consistency was not present between $IKK\alpha^{-/-}$ MEFs and C2C12 cells depleted of IKKα with siRNA. Possibly, the fraction of IKKα that remains in cells after siRNA depletion is sufficient to mask a phenotype that otherwise requires its complete absence, or perhaps the increase in myogenic activity derived from established $IKK\alpha^{-/-}$ MEFs might be an indirect consequence of immortalization and continued subculturing. We suspect that additional myogenic reporter assays in primary $IKK\alpha^{-/-}$ MEFs and myoblasts will be needed to clarify this issue.

Nevertheless, our observations led to the novel discovery that IKKα acts as a regulator of mitochondrial biogenesis. Although the mechanism remains unknown, we predict that IKKα activation functions through p52–RelB to promote mitochondrial biogenesis and meet the metabolic needs of newly formed contractile myotubes. The inhibitor compounds of mitochondria were also seen to decrease myotube maintenance, suggesting that IKKα regulation of mitochondria is necessary for myotube homeostasis in response to changing metabolic conditions.

A model for IKK/NF-κB signaling in skeletal muscle differentiation

Collectively, our data support a model whereby IKK/NF-κB signaling both inhibits and promotes the differentiation state of muscle cells (Fig. 10). This model helps unify the literature on the contradictory functions of NF-κB in myogenesis and predicts that during differentiation, a temporal switch occurs between NF-κB classical and alternative signaling pathways. In myoblasts, classical signaling is constitutively active and functions to maintain cells in an undifferentiated state. This function is regulated through the control of MyoD as well as other MyoD-independent mechanisms involving cyclin D1 and YY1. Once differentiation cues are initiated, classical signaling is turned down, whereas the alternative pathway is induced late in the myogenic program. In turn, the activation of IKKα leading to p52–RelB association regulates myogenesis by mediating the production of mitochondria necessary to satisfy the metabolic

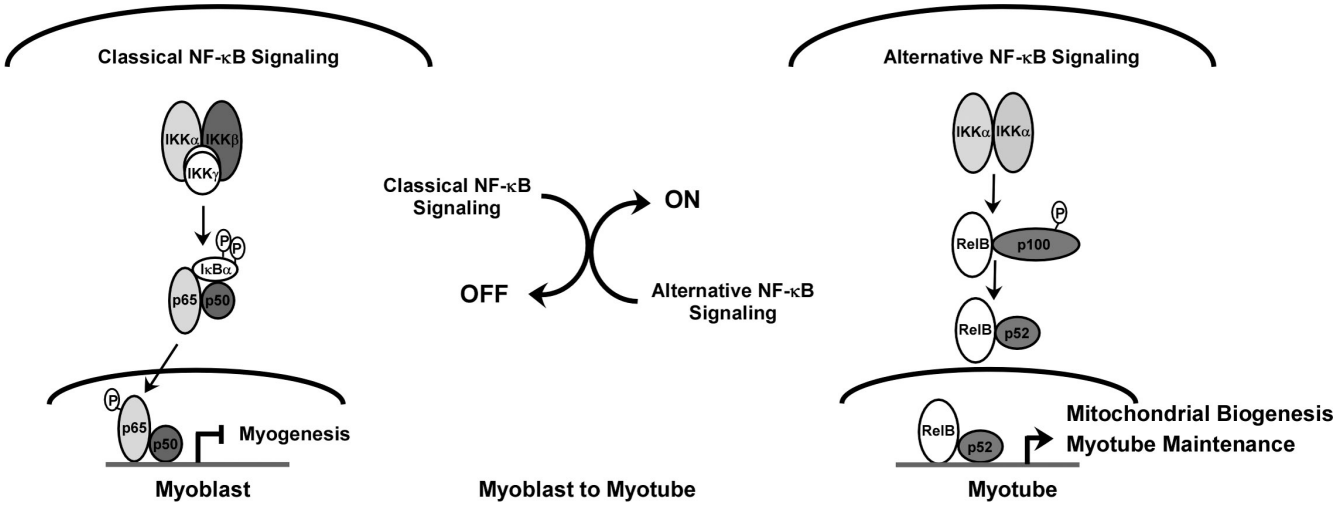


Figure 10. **A model for IKK/NF-κB signaling and function in skeletal myogenesis.** The model depicts different phases of myogenesis from proliferating myoblasts to differentiated myotubes. In proliferating myoblasts, classical NF-κB signaling mediated by IKKβ and IKKγ leads to the activation of p65 that binds DNA and regulates gene expression to inhibit myogenesis. During differentiation, classical NF-κB is down-regulated, whereas the alternative signaling becomes activated. Activation of alternative signaling occurs late in the myogenic program to regulate mitochondrial biogenesis and myotube maintenance.

needs of contractile muscle cells. Although cooperative functions of NF-κB signaling pathways are important for mammary and osteoclast tissue development (Demicco et al., 2005; Ruocco et al., 2005), skeletal muscle is, to the best of our knowledge, the first example of a differentiation system regulated through a functional switch of classical and alternative NF-κB signaling pathways.

Materials and methods

Materials

Antibodies to p100–p52, IκBα (C21), IKKβ, IKKγ (FL419), myogenin (M-225), p38, MyoD (M-318), and p65 (N terminal) were obtained from Santa Cruz Biotechnology, Inc. MyHCIIB (MY-32), MyHC slow (NOQ7.5.4D), Tn T (JLT-12), sarcomeric tropomyosin (CH1), and α-sarcomeric actin (5C5) were purchased from Sigma-Aldrich. p65 antibody was obtained from Rockland Immunochemicals, Inc., HA was purchased from Covance, and IKKα was purchased from Imgenex. Phospho-IκBα, p38, and p65 were obtained from Cell Signaling Technology, and cytochrome *c* was purchased from BD Biosciences. Bovine insulin, collagen type I, and gelatin were obtained from Sigma-Aldrich, whereas TNFα was purchased from Roche. Both collagenase P and dispase (grade II) were obtained from Boehringer Mannheim. Basic human FGF was purchased from Promega, and oligomycin was obtained from Alexis Biochemicals. MitoTracker green and secondary antibodies for immunofluorescence were obtained from Invitrogen, whereas other materials for immunohistochemical analysis were obtained from Vector Laboratories.

Plasmids

Reporter and p65 expression plasmids were previously described (Guttridge et al., 1999; Acharyya et al., 2004; Hertlein et al., 2005) with the exception of the p65(1–313; S276A) mutant, which was generated by mutating serine 276 to alanine in the p65(1–313) plasmid. MSCV-MyoD was generated by subcloning the MyoD cDNA from a pBabepuroMyoD retroviral construct (Guttridge et al., 2000). IKK plasmids were designed by subcloning IKKα, IKKβ, and IKKγ into the pBSx-HSAvpA plasmid, whereby transgene expression is driven from the human skeletal actin promoter.

Transfections, luciferase assays, and retrovirus infections

Subconfluent C2C12 cells were transfected in low serum Opti-MEM using Lipofectamine (Invitrogen) according to the manufacturer. For luciferase assays, cells were transiently transfected using Superfect (QIAGEN) for MEFs or Lipofectamine for primary myoblasts. All transfections were normalized to cytomegalovirus-βGal expression. Cells were lysed in mammalian protein extraction reagent solution (Thermo Fisher Scientific), and assays were performed as previously reported (Guttridge et al., 1999). IKKα, IKKβ, and p65 siRNAs were obtained from Dharmacon, Inc., and transfections were performed using Lipofectamine 2000. Retrovirus production and infection were performed as previously described (Guttridge et al., 1999).

Mice and genotyping

Animals were housed in the animal facility at The Ohio State University Heart and Lung Research Institute under sterile conditions maintaining constant temperature and humidity and were fed a standard diet. Treatment of mice was in accordance with the institutional guidelines of the Animal Care and Use Committee. Mice null for p65 were generated as previously described (Doi et al., 1999). p50 mice were obtained from Jackson ImmunoResearch Laboratories, and IKKβ flox mice (Li et al., 2003) were crossed to muscle creatine kinase–Cre mice to delete IKKβ in skeletal muscle. Mice genotypes were confirmed by PCR analysis from prepared tail DNA.

Cell culture

C2C12 murine myoblasts and fibroblasts were cultured as previously described (Guttridge et al., 2000). Primary myoblasts were prepared from 2-d-old neonates adopted from the described procedures (Rando and Blau, 1994). In brief, limbs from pups were skinned and incubated with collagenase/dispase mixture at 37°C for 1 h. Then, the cell suspension was further homogenized by pipetting and preplated on uncoated cell culture plates in F10 media (Invitrogen) to selectively enrich for myoblasts. After two rounds of preplating, the cell suspension was plated on gelatin-precoated plates in the presence of 20% FBS and 6 ng/ml basic FGF. Primary myoblasts were used at passage 3–5 after isolation.

Immunoblotting, Northern blots, ChIP, and kinase assays

Western and Northern blots and kinase analyses were performed as described previously (Hertlein et al., 2005). For ChIP, assays were performed as recommended by the manufacturer (Millipore).

Histology, electron microscopy, and immunofluorescence

For muscle analysis, tissues were sectioned at 10 μm on a cryostat (Leica) and stained with hematoxylin and eosin or processed for immunohistochemistry. The internal diameters (shortest diameter) from 1,200 fibers in random fields throughout the muscle were recorded using a microscope (BX50; Olympus) and MetaVue 6.2r6 software (MDS Analytical Technologies). Fiber number was recorded in 25 randomly selected fields throughout the muscle and averaged for comparisons. Muscles from three to five different animals per group were used. Immunostaining procedures on cell lines and muscle sections were performed as described previously (Acharyya et al., 2004, 2005), and all images were captured with a fluorescent microscope (Axioskop 40; Carl Zeiss, Inc.) using a

camera (AxioCam HRc; Carl Zeiss, Inc.) and AxioVision 3.1 software (Carl Zeiss, Inc.). Ultrastructural analysis was performed on fixed cells and sectioned using a microtome (EM UC6; Leica) at 70 nm. Sections were then stained and visualized using a transmission electron microscope (Spirit Tecnai; FEI) at 80 kV, and images were captured with a camera (XR60; Advanced Microscopy Techniques).

Mitochondrial assays

Both CellTiter-Glo Luminescent Assay for ATP determination and MTS cell viability assays were obtained from Promega and performed according to the manufacturer's recommendations. Citrate synthase activity was determined by using Ellman's reagent with acetyl-CoA and oxaloacetate (Leek et al., 2001). Procedures for primer design and PCR of MTCO1 as well as mitochondrial extraction for identification of cytochrome *c* were followed as described previously (Huo and Scarpulla, 2001; Liu et al., 2004).

Statistical analysis

All quantitative data are represented as means ± SEM. Analysis was performed between different groups using a two-tailed *t* test. Statistical significance was set at $P < 0.05$.

Online supplemental material

Fig. S1 shows that the absence of p65 in young mice leads to increases in fiber numbers that is independent of fiber type and muscle atrophy. Fig. S2 shows evidence for the conditional deletion of IKKβ in primary fibroblasts and adult muscles. Fig. S3 shows that the NF-κB classical pathway inhibits myogenesis in 10T1/2 fibroblasts, whereas C2C12 myotubes are not maintained with compounds that inhibit mitochondria. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707179/DC1.

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