Coordinated maintenance of muscle cell size control by AMP-activated protein kinase

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ABSTRACT **Skeletal muscle mass is regulated by signaling pathways that govern protein synthesis and cell proliferation, and the mammalian target of rapamycin (mTOR) plays a key role in these processes. Recent studies suggested the crucial role of AMP-activated protein kinase (AMPK) in the inhibition of protein synthesis and cell growth. Here, we address the role of AMPK in the regulation of muscle cell size *in vitro* and *in vivo*. The size of AMPK-deficient myotubes was 1.5-fold higher than for controls. A marked increase in p70S6K Thr^{389} and rpS6 Ser-235/236 phosphorylation was observed concomitantly with an up-regulation of protein synthesis rate. Treatment with rapamycin prevented p70S6K phosphorylation and rescued cell size control in AMPK-deficient cells. Importantly, myotubes lacking AMPK were resistant to further cell size increase beyond AMPK deletion alone, as MyrAkt-induced hypertrophy was absent in these cells. Moreover, in skeletal muscle-specific deficient AMPKα1/α2 KO mice, soleus muscle showed a higher mass with myofibers of larger size and was associated with increased p70S6K and rpS6 phosphorylation. Our results uncover the role of AMPK in the maintenance of muscle cell size control and highlight the crosstalk between AMPK and mTOR/p70S6K signaling pathways coordinating a metabolic checkpoint on cell growth.—Lantier, L., Mounier, R., Leclerc, J., Pende, M., Foretz, M., Viollet, B. Coordinated maintenance of muscle cell size control by AMP-activated protein kinase. *FASEB J.* 24, 3555–3561 (2010). www.fasebj.org**

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Regulation of muscle mass depends on a thin balance between growth-promoting and growth-suppressing factors. Alterations in skeletal muscle mass are controlled by the regulation of complex cell signaling pathways that govern muscle protein synthesis, breakdown, and cell proliferation. Signaling through the mammalian target of rapamycin (mTOR) plays a key role in cell growth regulation and particularly in the control of protein synthesis (1). The activation of mTOR by the GTPases rheb and rag and the sequential activation of its downstream targets enhance mRNA translation, initiation, and elongation, which results in an increase of muscle protein synthesis and growth (2). mTOR is part of two distinct multiprotein complexes: mTORC1 and mTORC2. The mTORC1 complex (containing mTOR, mLST8/GβL, PRAS40, and raptor) is responsible for cell growth. It is rapamycin sensitive and is activated by amino acids, hormones/growth factors, and energy signals (2). In contrast, the mTORC2 complex (containing mTOR, mLST8, and rictor) is important in the organization of actin cytoskeleton (3). Clear evidence suggests that signaling through mTOR is required for muscle hypertrophy (4, 5), and this process is mediated in part *via* activation of skeletal muscle p70S6K (6, 7). Phosphorylation of p70S6K is associated with increased muscle mass following hypertrophic stimuli, whereas muscle atrophy is associated with reduced p70S6K phosphorylation and an overall decrease in protein abundance (4).

A number of recent studies have revealed the key role of AMP-activated protein kinase (AMPK) in the inhibition of protein synthesis by suppressing the function of multiple translation regulators of the mTORC1 signaling complex in response to cellular energy depletion and low metabolic conditions (8, 9). AMPK is an evolutionary conserved serine/threonine kinase that regulates energy homeostasis and metabolic stress (10). AMPK is a heterotrimeric complex, consisting of a catalytic α subunit and the regulatory β and γ subunits, which functions as a fuel sensor to coordinate the balance between energy-consuming and energy-producing processes. When the cellular AMP/ATP ratio is high, AMPK is activated, switching off ATP-consuming anabolic pathways and switching on ATP-producing catabolic pathways. This action would typically occur when AMPK is activated because of energy deprivation, and the net result would be suppression of protein synthesis and cell growth. AMPK could, therefore, modulate skeletal muscle mass and limit overloading-induced muscle hypertrophy (11–13). Several lines of evidence suggest that AMPK reduces both the initiation and the elongation of ribosomal peptide synthesis

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(14–16). One mechanism is linked to the ability of AMPK to suppress mTORC1 signaling effectively (17). AMPK directly phosphorylates the TSC2 tumor suppressor (9), mTOR (18), as well as the critical mTORC1 binding subunit raptor to induce rapid suppression of mTORC1 activity.

Although AMPK activation is emerging as a powerful way to suppress protein synthesis through the down-regulation of mTOR signaling in skeletal muscle (17, 19, 20), the regulation of these signaling pathways has not been studied in muscle cells lacking AMPK. Therefore, the present study aimed to define whether the deletion of AMPK catalytic subunits affects muscle cell size control in unstimulated as well as in response to hypertrophic stimuli. We tested the hypothesis that AMPK deletion would result in enhanced activation of signaling pathways that promote protein synthesis and cell hypertrophy. Herein, we found that AMPK-deficient myotubes have a greater cell size, a marked increase in p70S6K Thr^{389} and rpS6 Ser-235/236 phosphorylation, and an enhanced protein synthesis rate. Following transfection with an adenovirus expressing MyrAkt, an effector known to enhance protein synthesis through activation of the mTOR pathway, we have observed an increase in wild-type (WT) myotube size, whereas no difference was found in AMPK-deficient myotubes. Furthermore, we report that soleus muscle from skeletal muscle AMPK-deficient mice showed a shift of fiber size distribution toward higher values correlated with increased p70S6K Thr^{389} and rpS6 Ser-235/236 phosphorylation. Our findings highlight the crosstalk between AMPK and mTOR/p70S6K signaling pathway in the adaptative response of skeletal muscle for the control of muscle cell size.

MATERIALS AND METHODS

Animals

To obtain skeletal muscle AMPK-deficient mice ($AMPK\alpha1^{-/-}\alpha2^{fl/fl}$ HSA-Cre^{+} mice), $AMPK\alpha1^{-/-}\alpha2^{fl/fl}$ mice (21, 22) were interbred with transgenic mice expressing the Cre recombinase under the control of the human skeletal actin (HSA) promoter, which is expressed in differentiated multinucleated skeletal fibers (23). Animals were maintained on a 12:12-h light-dark cycle and received standard rodent chow and water *ad libitum*. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Animal studies described herein were reviewed and approved (agreement no. 75-886) by the Directeur Départemental des Services Vétérinaires of the Préfecture de Police de Paris.

Cell cultures, adenovirus infection, and immunostaining

Primary muscle cell cultures were derived from mice as described previously (13). WT and $AMPK\alpha1^{-/-}\alpha2^{fl/fl}$ myoblasts were infected with either GFP or Cre adenovirus at the multiplicity of infection (m.o.i.) of 50. After 24 h, myoblasts were submitted to a second round of infection in the same conditions. Diameter of myotubes was measured after 4 d of differentiation using Metamorph software (Molecular Devices, Sunnyvale, CA, USA) as described previously (13). Diameter of myotubes was measured in a region where myonuclei were absent and diameter was constant. For immunostaining, myotubes were fixed with 4% formaldehyde, permeabilized with Triton X-100, and exposed to P-rpS6 (Ser-235/236) antibody (Cell Signaling Technology, Danvers, MA, USA).

Tissue collection

Animals were anesthetized with xylazine-ketamine, and soleus muscles of both legs were removed, cleaned, and precisely weighed. Muscles were then frozen in liquid nitrogen for protein extraction or in liquid nitrogen-chilled isopentane for preservation of fiber morphology and stored at −80°C until processed. The fiber cross-sectional area of soleus muscle was determined by staining serial transverse sections (12–16 μm thick) with mouse antidystrophin Dys2 antibody (Novocastra, Newcastle upon Tyne, UK) as described previously (13).

Protein isolation and immunoblotting

Total protein from soleus muscles and myotubes were extracted as described previously (13) and subjected to immunoblot. Blots were probed with antibodies against total AMPKα1, AMPKα2 (a kind gift from Grahame Hardie, University of Dundee, Dundee, UK), β-actin (Sigma, St. Louis, MO, USA), phosphorylated and total forms of AMPK (Thr^{172}), ACC (Ser-79), raptor (Ser-792), rpS6 (Ser-235/236), and p70S6K (Thr^{389}) (Cell Signaling Technology).

Protein synthesis measurements

After 4 d of differentiation, myotubes were serum starved for 2 h and then exposed to rapamycin (30 mM, 1 h). Protein synthesis rate was then measured as described previously by incorporation of ^{35}S-methionine and ^{35}S-cysteine (EasyTag Express Protein Labeling mix; Perkin Elmer, Waltham, MA, USA) (16). We conducted identical incubations on parallel plates that contained no cells for blanks measurement.

Statistical analysis

Results are expressed as means ± SE. We used Student's *t* test for unpaired data. Differences were considered significant at values of $P < 0.05$.

RESULTS

Generation of myotubes lacking both AMPKα1 and α2 catalytic subunits

In undifferentiated WT myoblasts, only a low AMPKα1 subunit expression was noticeable, whereas the AMPKα2 subunit was not detected (**Fig. 1*A***). However, as differentiation progressed, levels of both AMPKα1 and α2 catalytic subunits gradually increased and reached a maximum by d 4 (Fig. 1*A*). In $AMPK\alpha1^{-/-}AMPK\alpha2^{fl/fl}$ myotubes, AMPKα1 subunit was absent in both undifferentiated myoblasts and differentiated myotubes, whereas AMPKα2 subunit expression levels showed an increase during differentiation without any significant difference with WT myotubes (Fig. 1*A*). To obtain myotubes lacking both AMPKα1 and AMPKα2 catalytic subunits, we infected primary $AMPK\alpha1^{-/-}AMPK\alpha2^{fl/fl}$ myoblasts with Cre-expressing adenovirus (Ad-Cre). Myoblasts were infected with GFP-expressing adenovirus as a control. Expression of Cre resulted in the loss of AMPKα2 protein

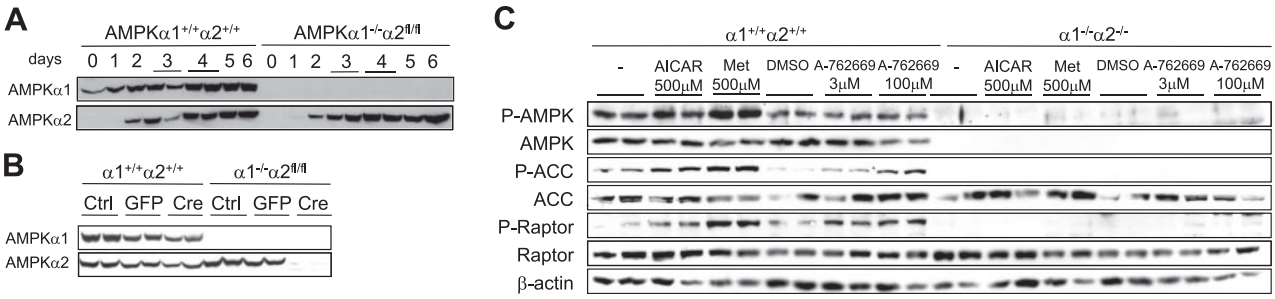

Figure 1. Generation of AMPKα1$^{-/-}$α2$^{-/-}$ myotubes, and induction of AMPK and ACC phosphorylation by AICAR, metformin, and A-769662 in muscle cells. *A*) AMPKα1 and α2 expression in WT and AMPKα1$^{-/-}$α2$^{fl/fl}$ myotubes during differentiation. Myoblasts were differentiated and proteins were harvested every 24 h during 6 d of differentiation. *B*) Myoblasts were twice subjected to AdGFP or AdCre infection (50 m.o.i.). At 24 h after the second infection, myoblasts were split and differentiated in myotubes. Cells were harvested on d 4 of differentiation, and proteins were subjected to α1 or α2 immunoblotting. *C*) Western blot of P-AMPK, AMPK, P-ACC, ACC, P-raptor, raptor, and β-actin on myotubes incubated with AICAR (500 μM, 5 h), metformin (500 μM, 5 h), or A-769662 (10 or 50 μM, 2 h).

during the differentiation process, and AMPKα2 protein was absent after 4 d of differentiation (Fig. 1*B*). In addition, AMPKα1 protein expression was undetectable in Ad-Cre-infected myoblasts and 4 d after initiation of differentiation (Fig. 1*B*). AMPK-deficient myoblasts efficiently initiate the differentiation program, as evidenced by the typical up-regulation of myogenin expression after 1 d of differentiation to about equal levels in both WT and AMPK-deleted cells (data not shown). AMPK-deficient myotubes were fully differentiated at d 4 as assessed by morphology (**Fig. 2*A***).

Lack of ACC and raptor phosphorylation following AICAR, metformin, and A-762669 treatment in AMPK-deficient myotubes

Myotubes lacking both expression of AMPKα1 and α2 catalytic subunits (referred to as AMPKα1$^{-/-}$α2$^{-/-}$) were treated with the AMPK activators AICAR (5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside) (24), metformin (25), and A-762669 (26). Phosphorylation of AMPK at Thr^{172} was increased in response to AMPK activators in WT myotubes but was undetectable in AMPK-deficient myotubes (Fig. 1*C*). The effect of AMPK activators on ACC phosphorylation was abolished in AMPKα1$^{-/-}$α2$^{-/-}$ myotubes, which confirmed that induction of ACC phosphorylation by AICAR, metformin, and A-762669 depends completely on AMPK activation in muscle cells. We also examined phosphorylation of raptor, a recently identified AMPK target in the regulation of protein synthesis pathway (8). Raptor is phosphorylated at Ser-792 following AICAR, metformin, and A-762669 treatment in WT but not in AMPKα1$^{-/-}$α2$^{-/-}$ myotubes, precisely paralleling the ACC phosphorylation pattern (Fig. 1*C*).

Regulation of muscle cell size in the absence of AMPK

To determine whether AMPK contributes to the control of muscle cell size, we measured the effect of AMPK deletion on myotube size at differentiation d 4 (Fig. 2*A*). The size of AMPKα1$^{-/-}$α2$^{-/-}$ myotubes was 1.5-fold greater than for WT myotubes (Fig. 2*B*). Accordingly, the distribution of AMPKα1$^{-/-}$α2$^{-/-}$ myotubes size showed a higher number of large cells and a smaller proportion of small cells (Fig. 2*C*). Myoblast fusion was similar in WT and AMPKα1$^{-/-}$α2$^{-/-}$ muscle cultures, indicating that cell hypertrophy promoted in the absence of AMPK is due to enhanced cytoplasmic growth rather than increased myoblasts fusion (data not shown).

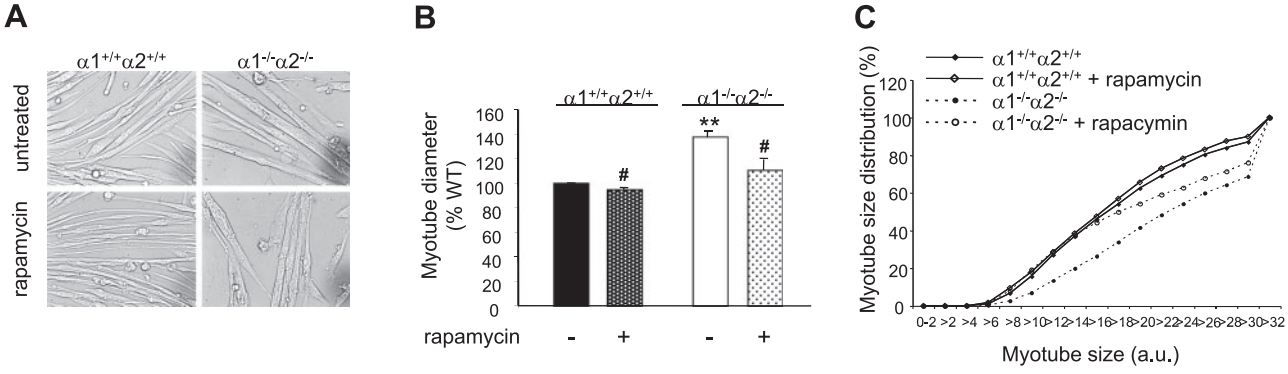

Figure 2. Cell size control in muscle cells lacking AMPK. *A*, *B*) Cells on d 2 of differentiation were treated with 20 nM rapamycin or vehicle. After another 2 d, bright-field images were taken (*A*), and myotube size was measured and expressed as percentage of WT muscle cells (*B*). *C*) Frequency distribution of size of WT and AMPKα1$^{-/-}$α2$^{-/-}$ myotubes. Results are represented as means ± SE. $^{**}P < 0.01$ *vs.* same treatment conditions; $^{\#}P < 0.05$ *vs.* untreated cells of same genotype.

To address whether inactivation of AMPK signaling was sufficient to up-regulate the mTOR pathway, we measured the phosphorylation of p70S6K as a surrogate marker of mTOR kinase activity. Interestingly, deletion of AMPK results in a rise in p70S6K phosphorylation at Thr^{389} and of its downstream target ribosomal protein S6 at Ser-235/236 as assessed by Western blot and immunofluorescence analysis (**Fig. 3*A*, *B***). Interestingly, the protein quantity recovered per well was twice as high in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes compared to WT myotubes (Fig. 3*C*). The rate of protein synthesis measured by ^{35}S-methionine incorporation was increased 2-fold in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes (Fig. 3*D*) compared to WT myotubes. The protein synthesis rate was sensitive to rapamycin in both WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes, as it was decreased by half after rapamycin treatment (30 μM, 1 h), decreasing the protein synthesis rate in AMPK-deficient myotubes to the level of WT myotubes in basal conditions (Fig. 3*D*). Accordingly, treatment with rapamycin abolished phosphorylation of p70S6K at Thr^{389} in both WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes (Fig. 3*E*). We therefore wished to investigate the effect of rapamycin on cell size and mTOR downstream components leading to the stimulation of skeletal muscle protein synthesis in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes. This mTOR pathway blunting significantly affected the size of both WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes and decreased the myotube diameter of AMPK-deficient cells to the level of WT cells (Fig. 2).

MyrAkt induced cell hypertrophy in WT but not in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes

To investigate the functional consequence of AMPK deletion on mTOR-mediated hypertrophy, WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ muscle cells were infected with an adenovirus expressing constitutive active Akt (MyrAkt). The diameter of myotubes increased by 20% in WT cells, whereas it was not affected in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes (**Fig. 4*A*, *B***). In addition, we measured the phosphorylation of p70S6K as surrogate marker of mTOR kinase activity. We observed a significant increase in p70S6K phosphorylation only in WT myotubes introduced with MyrAkt, consistent with an absence of negative action of AMPK on mTOR/p70S6K signaling pathway in response to hypertrophy in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes (Fig. 4*C*).

Increased muscle fiber size in skeletal muscle AMPK-deficient mice

To extend our findings *in vivo*, AMPK$\alpha1^{-/-}\alpha2^{fl/fl}$ mice were interbred with transgenic mice expressing the Cre recombinase under the control of the human skeletal actin (HSA) promoter, which is exclusively expressed in differentiated multinucleated skeletal fibers (23). The resulting skeletal muscle AMPK-deficient mice were viable and appeared normal in all respects as compared to HSA-Cre transgenic controls. The body weight of AMPK$\alpha1^{-/-}\alpha2^{fl/fl}$ HSA-Cre^{+} mice was similar to that of control mice (data not shown). Skeletal muscle-specific deletion of AMPKα2 was examined by Western blot analysis. AMPKα2 expression was undetectable in soleus muscle from muscle AMPK-deficient mice (**Fig. 5*A***), with no sign of deletion in liver and heart (data not shown). In addition, AMPKα1 was also absent in soleus muscle from muscle AMPK-deficient mice (Fig. 5*A*). To determine whether AMPK deletion affects skeletal muscle mass, we compared the soleus muscle weight from AMPK$\alpha1^{-/-}\alpha2^{fl/fl}$ HSA-Cre^{+} and HSA-Cre^{-} mice. Soleus muscle mass was significantly higher by 42% in muscle AMPK-deficient mice compared to control mice (Fig. 5*B*). Histological analysis of AMPK-deficient soleus muscle revealed increased cross-sectional area of the fibers (Fig. 5*C*). Furthermore, myofiber size distribution of AMPK-deficient soleus muscle exhibits increased number of large myofibers and fewer small myofibers compared to control soleus muscle (Figs. 5*D*). Finally, fiber hypertrophy in AMPK-

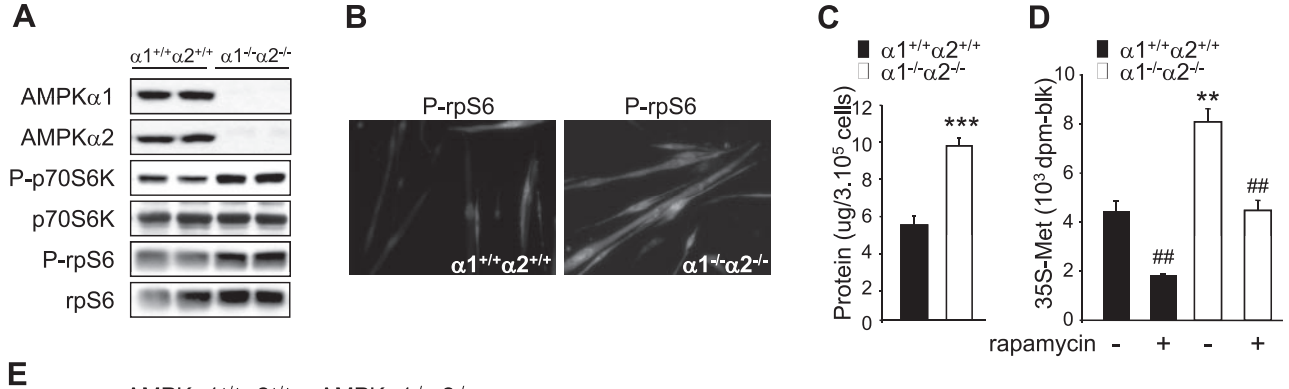

Figure 3. Up-regulation of mTOR signaling pathway in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes. *A)* Western blot of AMPKα1, AMPKα2, P-p70S6K, p70S6K, P-rpS6, and rpS6 in myotubes at d 4 of differentiation. *B)* Immunostaining for P-rpS6 in WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes. *C)* Protein quantity was measured in WT and $\alpha1^{-/-}\alpha2^{-/-}$ myotubes after 4 d of differentiation from wells seeded at the same density. *D)* Protein synthesis rate as measured before and after treatment with rapamycin (30 μM, 1 h). *E)* Western blot of P-p70S6K and p70S6K in myotubes treated with rapamycin (30 μM, 1 h) or vehicle. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* same treatment conditions; $^{\#\#}P < 0.01$ *vs.* untreated cells of same genotype.

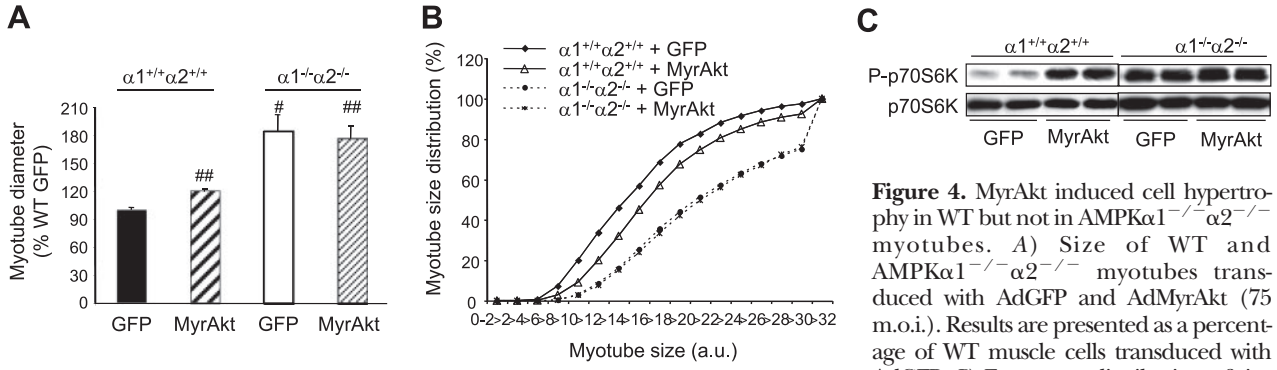

Figure 4. MyrAkt induced cell hypertrophy in WT but not in AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes. *A*) Size of WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes transduced with AdGFP and AdMyrAkt (75 m.o.i.). Results are presented as a percentage of WT muscle cells transduced with AdGFP. *B*) Frequency distribution of size of WT and AMPK$\alpha1^{-/-}\alpha2^{-/-}$ myotubes transduced with AdGFP and AdMyrAkt. *C*) Western blot of P-p70S6K and total p70S6K. Results are represented as means ± SE. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* WT cells infected with AdGFP.

deficient soleus muscle was associated with increased phosphorylation of p70S6K at Thr^{389} (Fig. 5*A*) and of its downstream target ribosomal protein S6 at Ser-235/236 (Fig. 5*A*).

DISCUSSION

The mTORC1 signaling pathway has emerged recently as a crucial point of convergence for signaling by nutrients (glucose, amino acids), growth factors, and cellular energy to promote skeletal muscle growth and fiber size (2, 4, 5, 7). As opposed to the mTORC1 signaling pathway, AMPK is activated under conditions of energy stress leading to high intracellular AMP/ATP ratio and restores cellular energy balance by promoting ATP-generating pathways, while simultaneously inhibiting ATP-utilizing pathways (10). Cell growth is energetically demanding, and its inhibition is therefore an important mechanism to save energy under metabolic stress. To suppress mTOR signaling, AMPK utilizes both direct and indirect targets *via* phosphorylation and activation of TSC2 on Thr^{1227} and Ser^{1345} (9), and/or phosphorylation and inactivation of mTOR on Thr^{2446} (18). AMPK has also been shown recently to inhibit mTORC1 directly, *via* phosphorylation of raptor on Ser-722/Ser-792 (8). Here, we confirmed that raptor is phosphorylated rapidly in muscle cells treated with AMPK activators, but induction of its phosphorylation was lost in muscle cells deleted for both AMPK catalytic subunits (Fig. 1*C*), which indicates an AMPK-dependent effect.

Recent evidence indicates that AMPK acts as a cellular energy checkpoint to prevent cell growth when cellular energy reserves are insufficient (8, 27). Hence, AMPK inhibits protein synthesis when nutrient conditions are limited by interfering with anabolic signaling mediated by the mTOR pathway. Our results provide strong evidence for the crucial role of AMPK in the maintenance of cell size through the control of mTOR/p70S6K signaling pathway. Deletion of AMPK in muscle cells enhanced p70S6K phosphorylation and, in turn, protein synthesis rate, contributing to the shift in muscle cell and fiber size distribution toward high values (Figs. 2*C* and 3*A*, *D*). Interestingly, treatment of AMPK-deficient cells with rapamycin com-

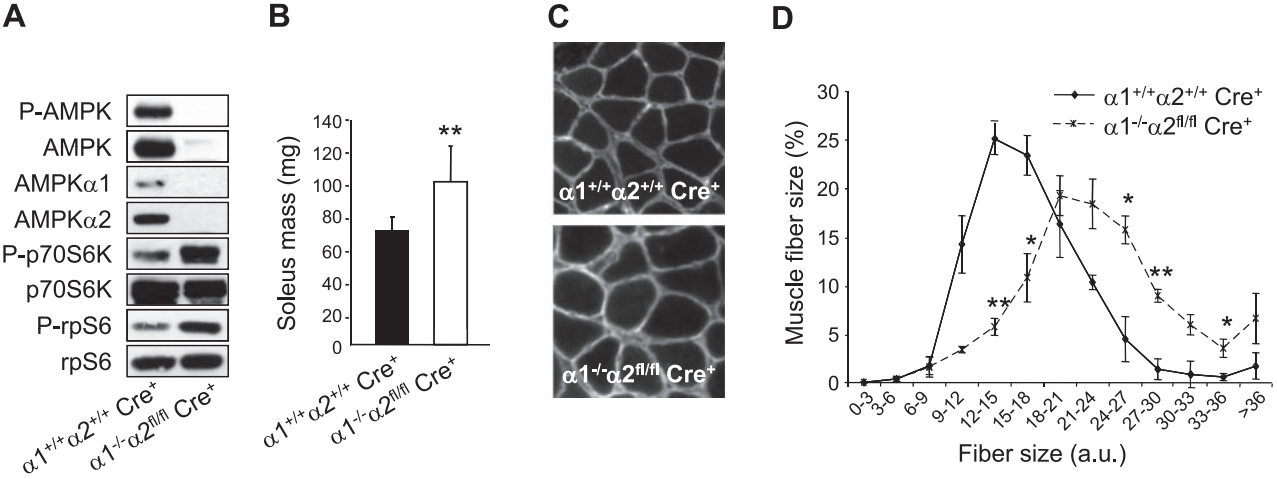

Figure 5. Increased fiber size in soleus muscle lacking AMPKα1 and α2. *A*) AMPKα1, AMPKα2, p70S6K Thr^{389}, p70S6K, rpS6 Ser-235/236, and rpS6 expression in soleus muscle from AMPK$\alpha1^{+/+}\alpha2^{+/+}Cre^{+}$ and AMPK$\alpha1^{-/-}\alpha2^{fl/fl}Cre^{+}$ mice. *B*) Mass of soleus muscle from AMPK$\alpha1^{+/+}\alpha2^{+/+}Cre^{+}$ and AMPK$\alpha1^{-/-}\alpha2^{fl/fl}Cre^{+}$ mice. Results are represented as means ± SE (n=6). *C*) Representative bright-field images of soleus muscle fibers from AMPK$\alpha1^{+/+}\alpha2^{+/+}Cre^{+}$ and AMPK$\alpha1^{-/-}\alpha2^{fl/fl}Cre^{+}$ mice. *D*) Frequency distribution of cross-sectional area fibers in soleus muscle from AMPK$\alpha1^{+/+}\alpha2^{+/+}Cre^{+}$ and AMPK$\alpha1^{-/-}\alpha2^{fl/fl}Cre^{+}$ mice (n=6). Fiber cross-sectional area of fibers (n=417 in mean) was determined from different muscle areas of 6 muscles in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* WT.

pletely blocked the up-regulation of p70S6K phosphorylation and rescued AMPK$\alpha 1^{-/-}\alpha 2^{-/-}$ muscle cell size (Figs. 2*C* and 3*E*). Consistent with our results, analysis of signaling downstream of mTORC1 has revealed the contribution of p70S6K phosphorylation in the control of muscle cell size and muscle hypertrophy (7). Muscle cells lacking p70S6K exhibited reduced cell size and failed to adapt their size to the inhibitory effect of rapamycin or amino acid starvation (7). Suppression of p70S6K signaling triggers an energy stress response defined by an increase in the AMP/ATP ratio, which leads to the activation of AMPK (6). This increase in AMPK activity blunts the growth responses to nutrient availability and inhibits signals that affect the regulation of muscle size. In this context, AMPK inhibition restores muscle cell growth and sensitivity to nutrient signals (6). Of note, inactivation of mTORC1 has been demonstrated previously to be critical for the ability of AMPK to integrate energy sensing with cell growth and proliferation requirement (9, 28). In contrast, loss of AMPK induces an absence of p70S6K inhibition and leads to higher protein synthesis rate and ultimately to increased muscle cell size. Interestingly, AMPK$\alpha 1^{-/-}\alpha 2^{-/-}$ myotubes are resistant to MyrAkt hypertrophic action (Fig. 4*A*, *B*), and p70S6K phosphorylation was not increased further in the absence of AMPK, which suggests a ceiling effect (Fig. 4*C*). One possible explanation is that mTOR signaling pathway is activated already at its maximal level in the absence of AMPK and that no further increase is possible in response to Akt stimulation. Furthermore, it is possible that AMPK deletion also limits energy production for cell growth in response to MyrAkt-induced hypertrophy. Impaired mitochondrial function has been demonstrated in skeletal muscle of mice expressing a kinase-dead AMPK$\alpha 2$ subunit in skeletal muscle (29). Accordingly, impaired ATP generation was observed in skeletal muscle of these mice following chronic energy deprivation (30) and exercise (29). In addition, a marked energy disturbances were also reported during exercise in skeletal muscle of AMPK$\alpha 2^{-/-}$ mice (31), which supports the role of AMPK$\alpha 2$ in metabolic adaptations of skeletal muscle (32). These observations are completely consistent with the model that AMPK plays an essential role in the coordination between cell growth and cellular energy levels.

The inability of AMPK-deficient muscle cells to sense the cellular energy status triggers a signal equivalent to high energy content and a nutrient-rich environment, leading to the hypertrophy of AMPK$\alpha 1^{-/-}\alpha 2^{-/-}$ myotubes. These results show the major impact of AMPK deletion on muscle cell size in our model. The physiological relevance of these data has been investigated in our skeletal muscle AMPK-deficient mice model. Interestingly, the increase in muscle mass and fiber size found in the soleus muscle is associated with an activation of the p70S6K/mTOR pathway (Fig. 5). These findings demonstrate that *in vivo* as well as *in vitro* AMPK is highly implicated in the maintenance of muscle cell size. Thus, a crosstalk between p70S6K and AMPK establishes a thin balance that integrates cellular energy and nutrition content and leads to changes in growth and metabolic rates. Accumulating evidence indicates that the molecular interplay between AMPK and mTOR signaling also limits hypertrophy in the heart to various stimuli. Treatment of neonatal rat cardiomyocytes with metformin, AICAR, or resveratrol activated AMPK and inhibited the development of hypertrophy during phenylephrine treatment (33, 34). Notably, increased heart hypertrophy was observed in AMPK$\alpha 2$-deficient mice following isoproterenol treatment (35) or transverse aortic constriction (36), which correlated with dramatic increases in mTORC1 signaling.

Recent studies have also linked AMPK and protein degradation in the muscle. Indeed, AICAR and metformin treatments decreased protein synthesis and increased protein degradation in an AMPK-dependant manner in C2C12 myotubes (37). Another study, also in C2C12 myotubes, has shown that AMPK activation stimulates myofibrillar protein degradation by increasing FOXO transcription factors (38). In our AMPK-deficient models, protein degradation may well be down-regulated, thus emphasizing the observed phenotype. The increased protein quantity and associated hypertrophy observed may result from both an increase in protein synthesis and a decrease in protein degradation.

AMPK has emerged over the past decade as a central integrator of signals that control energy balance. Our results extend this notion by showing that AMPK controls muscle cell size and is involved in the cell size maintenance through the regulation of mTOR/p70S6K pathway. The recent findings that mTORC1 regulates muscle insulin signaling (39) and metabolism (40) in addition to protein synthesis suggest that AMPK metabolic effects might be mediated, at least in part, through modulation of mTORC1 activity. Thus, our data support the view that crosstalk between of AMPK and mTOR/p70S6K defines a metabolic program coordinating muscle plasticity. FJ

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