Important role for AMPKα1 in limiting skeletal muscle cell hypertrophy

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ABSTRACT **Activation of AMP-activated protein kinase (AMPK) inhibits protein synthesis through the suppression of the mammalian target of rapamycin complex 1 (mTORC1), a critical regulator of muscle growth. The purpose of this investigation was to determine the role of the AMPKα1 catalytic subunit on muscle cell size control and adaptation to muscle hypertrophy. We found that AMPKα1(−/−) primary cultured myotubes and myofibers exhibit larger cell size compared with control cells in response to chronic Akt activation. We next subjected the *plantaris* muscle of AMPKα1(−/−) and control mice to mechanical overloading to induce muscle hypertrophy. We observed significant elevations of AMPKα1 activity in the control muscle at days 7 and 21 after the overload. Overloading-induced muscle hypertrophy was significantly accelerated in AMPKα1(−/−) mice than in control mice [+32 *vs.* +53% at day 7 and +57 *vs.* +76% at day 21 in control *vs.* AMPKα1(−/−) mice, respectively]. This enhanced growth of AMPKα1-deficient muscle was accompanied by increased phosphorylation of mTOR signaling downstream targets and decreased phosphorylation of eukaryotic elongation factor 2. These results demonstrate that AMPKα1 plays an important role in limiting skeletal muscle overgrowth during hypertrophy through inhibition of the mTOR-signaling pathway.—Mounier, R., Louise Lantier, Leclerc, J., Sotiropoulos, A., Pende, M., Daegelen, D., Sakamoto, K., Foretz, M., Viollet, B. Important role for AMPKα1 in limiting skeletal muscle cell hypertrophy. *FASEB J.* 23, 2264–2273 (2009)**

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The 5′-AMP-activated protein kinase (AMPK) is a sensor of cellular energy homeostasis well conserved in all eukaryotic cells (1). AMPK is activated by metabolic stresses that elevate the AMP-to-ATP ratio by lowering ATP production after inhibition of mitochondrial oxidative phosphorylation (*e.g.*, metabolic poisons) or increasing ATP consumption (*e.g.*, muscle contraction). Once activated, at the level of the individual cell, AMPK switches on catabolic pathways (such as fatty acid oxidation and glycolysis) and switches off ATP-consuming pathways (such as lipogenesis) with short-term effects on phosphorylation of regulatory proteins and long-term effects on gene expression. Mammalian AMPK is a heterotrimeric enzyme complex consisting of one catalytic subunit (α) and two regulatory (β and γ) subunits, and isoforms of all three subunits have been identified (α1, α2, β1, β2, γ1, γ2, and γ3). The phosphorylation of a conserved threonine residue (Thr172) within the kinase domain of the α-catalytic subunit is absolutely required for AMPK activation. Three upstream kinases have been recently identified and correspond to the tumor suppressor LKB1 (STK11) kinase, $Ca2^{+}$/calmodulin-dependent protein kinase kinase-β (CaMKKβ), and TGFβ-activated kinase-1, a member of the mitogen-activated protein kinase kinase family (TAK1; ref. 2). Binding of AMP to the γ-subunits leads to allosteric activation of AMPK as well as to enhanced phosphorylation of Thr172 by inhibiting its dephosphorylation by protein phosphatases (3).

In skeletal muscle, AMPK is activated by multiple stimuli including hypoxia, osmotic stress, endurance exercise, and electrically stimulated contraction (4). AMPK activation causes metabolic changes that assist muscle cells in making chronic adaptations to energy deprivation, such as an increase in oxidative capacity *via* increased mitochondrial biogenesis, enzyme expression, and uptake of nutrients such as glucose and fatty acids. Importantly, some of these metabolic adaptations seem to preferentially occur through the regulation of AMPKα2 but not *via* the AMPKα1 isoform (5–7). These results suggest distinct physiological roles for α1- and α2-containing AMPK complexes in the regulation of skeletal muscle adaptations. Activation of the various AMPK complexes may depend on the specificity of

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upstream kinase for α-catalytic subunit phosphorylation. Indeed, in LKB1-deficient muscle, AMPKα2 activation is severely blunted, whereas AMPKα1 activation is substantially induced in response to contraction, indicating discordant regulation of α1- and α2-containing AMPK complexes (8). Similar results have been obtained in the LKB1-deficient heart in response to ischemia or anoxia (9).

One consequence of endurance exercise training is a partial fiber-type transition toward a slow-oxidative phenotype associated with mitochondrial biogenesis but no growth. In contrast, resistance exercise training mainly stimulates muscle protein synthesis and increases muscle fiber volume resulting in hypertrophy. Since protein synthesis can account for up to 30–50% of the cellular energy expenditure, a reduction in protein synthesis seems an efficient mechanism to save energy. Activation of skeletal muscle AMPK during conditions of energetic stress is thought to act as a negative regulator of protein synthesis and may therefore modulate skeletal muscle mass and hypertrophy (10, 11). AMPK activation has been also reported to inhibit protein synthesis associated with hypertrophy in cardiac muscle (12, 13). Several lines of evidence suggest that AMPK reduces both the initiation and the elongation of ribosomal peptide synthesis (14). One potential mechanism is through AMPK-mediated phosphorylation of the eukaryotic elongation factor 2 (eEF2) kinase at Ser-398 (15), leading to an increase in Thr^{56} phosphorylation and inactivation of eEF2, a key component in protein elongation (16). Another mechanism is linked to the ability of AMPK to block the activation of the mammalian target of rapamycin (mTOR) signaling pathway (10, 17–20). While AMPK is active in response to nutrient deprivation and inactive under nutrient-rich conditions, mTOR is activated in the inverse pattern. mTOR is part of two distinct multiprotein complexes, mTORC1 (containing mTOR, mLST8, and Raptor), which is responsible for cell growth and mTORC2 (containing mTOR, mLST8, protor, and Rictor), which is important in glucose metabolism and cytoskeletal organization (20, 21). mTORC1 is activated in skeletal muscle by a variety of anabolic signals, such as resistance exercise, insulin, growth factors, and amino acids, to stimulate protein synthesis and hence muscle cell growth, and hypertrophy (17, 22, 23). It has been well established that insulin primarily regulates mTOR through PI3K-Akt signaling to stimulate cell growth (20). It was recently shown that Rag GTPases play a primary role in amino acids signaling to mTOR (24). The mTORC1-signaling pathway contains multiple potential sites for regulatory integration with AMPK (10, 25). AMPK phosphorylates and activates the tuberous sclerosis complex 2 (TSC2) kinase, which subsequently inactivates the G-protein Rheb thereby blocking activation of mTOR (25, 26). Recently, it has been shown that AMPK-dependent phosphorylation of Raptor also leads to the inhibition of mTORC1 (27). Skeletal muscle ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor (eIF)-4E binding protein 1 (4E-BP1) are well-characterized downstream components of the mTORC1-signaling pathway, and phosphorylation of these proteins has been associated with increased muscle mass after hypertrophic stimuli. Conversely, deletion of p70S6K reduces muscle cell size to the same extent as that observed with the mTORC1 inhibitor rapamycin. Interestingly, the suppression of the p70S6K kinases is sufficient to trigger an energy stress response associated with AMPK activation, leading to coordinated changes in cell growth and metabolic rates (28).

A strong negative correlation has been reported between the degree of AMPK phosphorylation and the degree of hypertrophy in overloaded *plantaris* (PLN) muscles, implicating AMPK as a potentially important negative regulator of overload-induced skeletal muscle hypertrophy (29). AMPK phosphorylation was negatively correlated with the amount of phosphorylated p70S6K at the mTOR-specific Thr^{389} residue, and it was postulated that AMPK inhibited p70S6K phosphorylation *via* its reported suppression of mTOR pathway (10, 28, 30). It has recently been reported that continuous 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) infusion inhibits overloading-induced muscle hypertrophy (31). Interestingly, McGee *et al.* (32) have shown that the hypertrophy produced by overload was associated with a marked activation of AMPKα1 in both wild-type (WT) and LKB1-deficient mice, and they proposed that AMPKα1 might play an important role in the regulation of skeletal muscle growth.

Therefore, the aim of this investigation was to examine the potential role of the AMPKα1 catalytic subunit in the size control of muscle cells in unstimulated and in response to hypertrophic stimuli. For this purpose, we analyzed the size of AMPKα1-deficient muscle cells in response to cell growth stimulation and determined the degree of muscle hypertrophy after muscle overload in AMPKα1-deficient mice.

MATERIALS AND METHODS

Animals

The generation of AMPKα1(−/−) mice has been described previously (33). AMPKα1(−/−) and WT mice (20–26 wk old) were maintained on a 12:12-h light-dark cycle and received standard rodent chow and water *ad libitum*. Genotyping was performed by PCR using DNA from a tail-piece (forward: 5′-AGCACAGTACCTGGTATCTTATAGG-3′ and reverse: 5′-GGACTTATTACTAAACAGACCTCTG-3′ primers for WT allele; forward: 5′-ACCAGAAGCGGTGCCGGAAAGCTGG-3′ and reverse: 5′-TGTAGTCGGTTTATGCAGCAACGAG-3′ primers for AMPKα1 deleted allele). All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Cell culture and adenovirus infection

Primary muscle cell cultures were derived from gastrocnemius and tibialis anterior (TA) muscles of 4-wk-old mice as described previously (34). Myotubes at day 2 of differentia-

tion were infected with an adenovirus expressing GFP or a constitutively active Akt, NH(2)-terminally myristoylation signal-attached Akt (*MyrAkt*; ref.28) at the multiplicity of infection of 75, and then they were incubated for 2 days in DMEM/Ham's F-12 medium containing 2% horse serum. Then, bright-field images were taken and analyzed using Metamorph software (version 7.04, Molecular Devices, Sunnyvale, CA, USA). The diameter of at least 400 myotubes was measured at day 4 of differentiation in a region where myonuclei were absent and diameter was constant. Cells were harvested the same day for Western blot analysis.

In vivo transfection of mouse skeletal muscle by electroporation

Hyalurodinase (0.4 U) was injected along the TA muscle length, and after 2 h, animals anesthetized with xylazine-ketamine were injected with 15 μg of plasmids encoding GFP or *MyrAkt*. Electrical pulses were then applied by two stainless steel spatulas placed on each side of the muscle (130 V/cm, 6 pulses, 100 ms interval). Muscles were removed and analyzed 10 days later.

Bilateral synergist ablation model

The bilateral synergist ablation model was used to induce hypertrophy of the PLN muscle by functional overload in AMPKα1(−/−) and control mice, as described previously (35). On the day of surgery (D0), animals were anesthetized with xylazine-ketamine and maintained in the surgical plane. A longitudinal incision on the dorsal aspect of the lower hind limb was made, exposing the *gastrocnemius* muscle. The tendons of the *gastrocnemius* and *soleus* muscles were isolated and used to guide in the excision of these muscles without disturbing the PLN muscle. The overlying skin was closed using sterile suture, and the mice were allowed to recover in a temperature-controlled area before being returned to their cages. Animals were monitored for signs of pain or postoperative infection.

Tissue collection

Animals were anesthetized with xylazine-ketamine, and overloaded TA muscles or transfected PLN 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.

Protein extraction, Western blotting, and AMPK activity assays

Total protein extracts from PLN muscles and muscle cell samples were obtained as described previously (8). Fifty micrograms of protein was subjected to SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with antibodies against total AMPKα1 and AMPKα2 (a kind gift from Grahame Hardie, University of Dundee, Dundee, UK), and phosphorylated forms of eEF2 (Thr56), S6 (Ser-235/236), p70S6K (Thr389), and 4E-BP1 (Thr37/46; Cell Signaling, Danvers, MA, USA). Measurement of AMPKα1 and -α2 activities was performed as described previously (36).

Immunohistochemistry

Serial transverse sections (12- to 16-μm thick) were cut from TA and PLN muscles in a cryostat microtome (Leica, Wetzlar, Germany) maintained at −25°C and stained with hematoxylin and eosin to visualize tissue morphology. We analyzed fiber size by incubating muscle sections with mouse anti-dystrophin Dys2 (Novocastra, Newcastle, UK) and anti-HA (Roche, Indianapolis, IN, USA) antibodies and staining with Hoechst dye. The fiber cross-sectional area was determined using Metamorph software (version 7.04, Molecular Devices).

Statistical analysis

Results are expressed as means ± SD. We used a Student's *t* test for unpaired data. Differences were considered significant if $P < 0.05$.

RESULTS

Cell size control in AMPKα1-deficient muscle cells

To examine the role of AMPKα1 in the control of muscle cell size, we isolated and analyzed primary muscle cells from AMPKα1(−/−) and control mice. Western blotting analysis revealed that AMPKα1 protein was undetectable in AMPKα1(−/−) myotubes and that the levels of AMPKα2 protein were similar between AMPKα1(−/−) and WT myotubes (**Fig. 1*A***). The deletion of AMPKα1 caused a 15% increase in myotube diameter (Fig. 1*B*). Furthermore, myotube size distribution for AMPKα1(−/−) cells showed fewer small-diameter myotubes and an increased number of large-diameter myotubes compared with WT cells (Fig. 1*C*). To evaluate the functional consequence of AMPK deletion on mTOR-pathway-mediated hypertrophy, we introduced WT and AMPKα1(−/−) muscle cells with an adenovirus expressing *MyrAkt*, an established way of activating the mTOR-signaling pathway (22, 23). We found that the hypertrophic action of *MyrAkt* was 2-fold higher in AMPKα1(−/−) myotubes compared with WT cells (Fig. 1*B*). The diameter of myotubes increased significantly by 29% in AMPKα1(−/−) cells *vs.* 16% in WT cells. Furthermore, the degree of hypertrophy (determined by the shift on the right of the cumulated percentage of myotube size distribution) was greater for AMPKα1(−/−) cells in response to *MyrAkt* overexpression (Fig. 1*C*). Finally, we observed an increase in p70S6K phosphorylation (+39%) in AMPKα1(−/−) myotubes introduced with *MyrAkt,* consistent with a negative action of AMPKα1 on the mTOR-signaling network (Fig. 1*D*).

MyrAkt-induced cell growth in AMPKα1-deficient muscle

To gain further insights into the role of AMPKα1 in the regulation of muscle cell growth *in vivo*, we transfected AMPKα1(−/−) and control TA muscles with a plasmid expressing *MyrAkt* or GFP by electroporation. The hypertrophic action of *MyrAkt* was significantly higher in AMPKα1(−/−) skeletal muscle fibers compared with WT fibers (**Fig. 2**). Indeed, the cross-sectional area

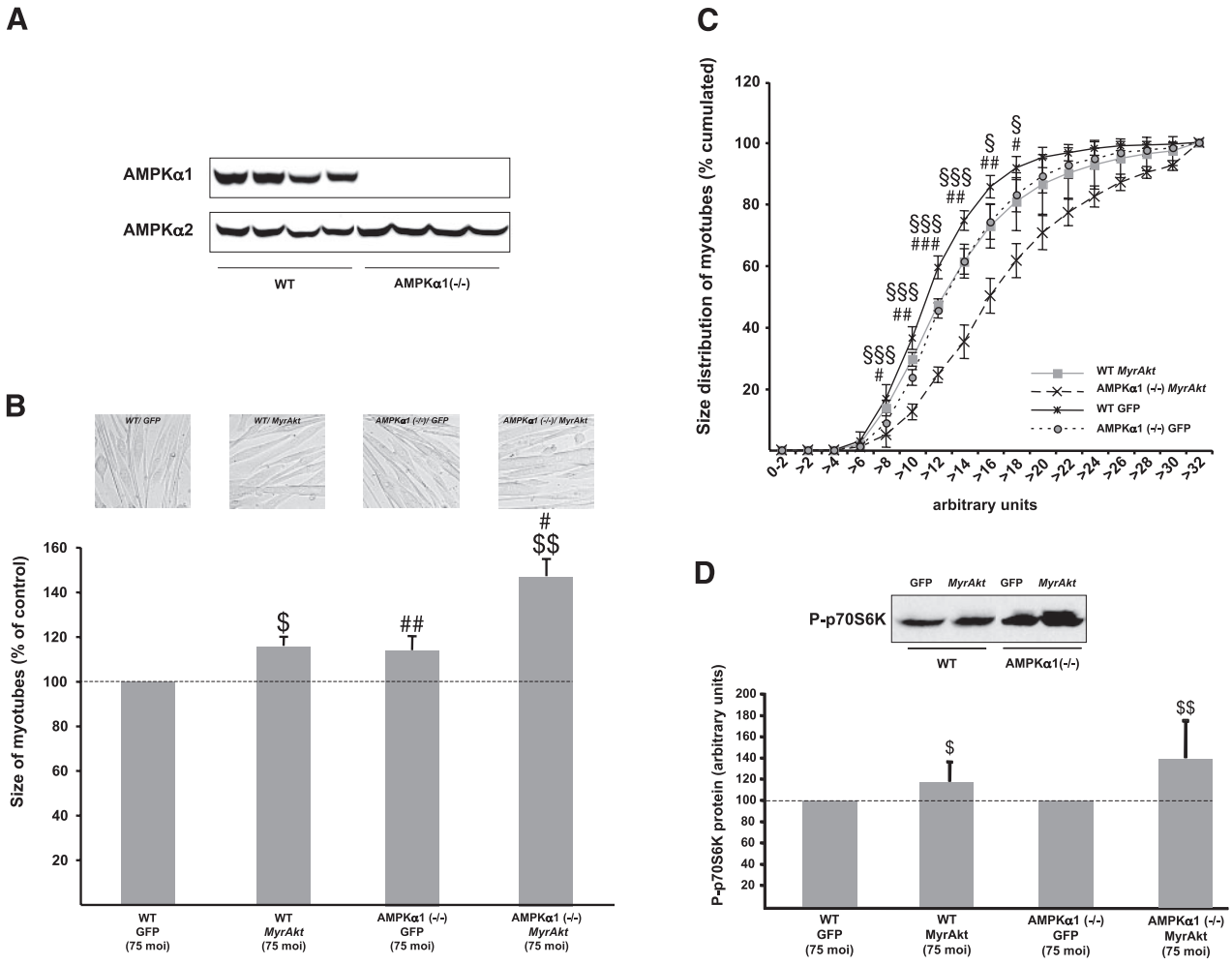

Figure 1. Cell size control in primary muscle cells lacking AMPKα1. *A*) Primary muscle cells isolated from AMPKα1(−/−) and control mice were differentiated into myotubes, and AMPKα1 and -2 protein content was assessed by Western blot analysis. Top panel: representative AMPKα1 immunoblot. Bottom panel: representative AMPKα2 immunoblot. *B*) Top panels: representative bright-field images of myotubes transduced with GFP and *MyrAkt* adenoviruses. Bottom panel: size of WT and AMPKα1(−/−) myotubes transduced with 75 multiplicity of infection GFP and *MyrAkt* adenoviruses as indicated. Results are presented as percentage of WT muscle cells transduced with GFP adenovirus. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *vs.* corresponding GFP cells. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *vs.* same conditions of infection. *C*) Frequency distribution of size of WT and AMPKα1(−/−) myotubes transduced with GFP and *MyrAkt* adenoviruses. Size of myotubes was determined with the diameter of at least 400 myotubes measured in a region where myonuclei were absent and diameter was constant. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; WT GFP *vs.* AMPKα1(−/−) GFP. $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$; WT *MyrAkt vs.* AMPKα1(−/−) *MyrAkt*. Data from 5 experiments on 2 different cultures (*B*, *C*). *D*) Thr389 p70S6K phosphorylation was determined 2 days after infection. Top panel: representative immunoblot. Protein content is expressed in arbitrary units relative to GFP-infected cells for each genotype. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *vs.* corresponding GFP cell. Results are represented as means ± SD.

of AMPKα1(−/−) myofibers increased significantly by 125% compared with 68% for WT myofibers (Fig. 2).

Changes in AMPK activity and expression after overload-induced hypertrophy

Since robust activation of AMPKα1 has recently been reported during muscle hypertrophy (32), we examined the overloading-induced muscle growth in mice lacking AMPKα1. At day 7 (D7) of overload, AMPKα1 activity was markedly increased in the PLN of WT mice (+446%; **Fig. 3*A***) and remained significantly higher at day 21 (D21) compared with D0 (+106%; Fig. 3*A*), although it was significantly lower compared with D7 (Fig. 3*A*). Coincidentally, AMPKα1 expression increased dramatically at D7 (+441%; **Fig. 4**) and at D21 (+336%; Fig. 4) in WT mice. In the PLN muscles of the AMPKα1(−/−) mice, AMPKα1 activity was ablated and only background counts were detected (Fig. 3*A*). The absence of AMPKα1 expression in PLN muscle of AMPKα1(−/−) mice was confirmed by Western blot analysis (Fig. 4). Loss of AMPKα1 resulted in an increase in the basal level of AMPKα2 activity (Fig. 3*B*). In contrast to AMPKα1 activity, AMPKα2 activity was reduced in both WT (−67%; Fig. 3*B*) and AMPKα1(−/−) mice at D7 (−65%; Fig. 3*B*). The activity of AMPKα2 at D21 for both WT and AMPKα1(−/−) mice returned to a value close to the basal level of WT mice (Fig. 3*B*). AMPKα2 expression

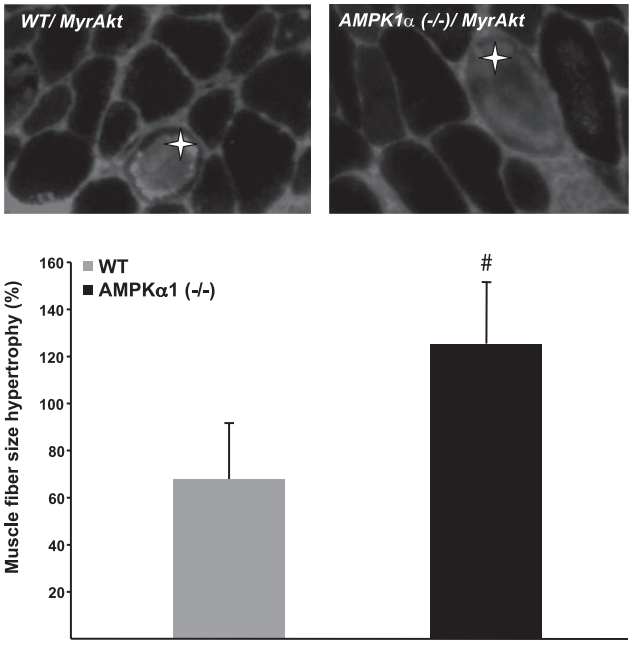

Figure 2. Change in cross-sectional area of WT and AMPKα1(−/−) myofibers in TA muscle transfected with *MyrAkt*. Changes in size of WT and AMPKα1(−/−) myofibers overexpressing *MyrAkt* represented as percentage changes to nontransfected myofibers. Detection of *MyrAkt* (HA-tagged *MyrAkt*) transfected myofibers was realized by immunostaining with anti-HA antibody. Top panels: representative images; star indicates positive cells. Fiber cross-sectional area of 180 fibers was determined from at least 3 different muscle areas of 4 animals/group. Results are represented as means ± SD. $^{\#}P < 0.05$ *vs.* WT.

was unaffected in PLN from AMPKα1(−/−) and WT mice at D7 but significantly decreased in AMPKα1(−/−) mice at D21 (Fig. 4).

Muscle mass and myofiber size in AMPKα1-deficient mice

The ratio of PLN mass to tibia length was significantly lower in AMPKα1(−/−) mice compared with WT mice (**Fig. 5*A***). Myofiber size distribution of AMPKα1(−/−) PLN exhibits an increased number of small myofibers and fewer large myofibers compared with WT PLN (Fig. 5*B*). Morever, the number of nuclei per fiber in AMPKα1(−/−) mice was smaller (−22%) compared with WT mice ($P<0.01$).

Muscle mass and myofiber size after overload-induced hypertrophy

After 7 and 21 days of overload, the degree of muscle hypertrophy (determined by PLN mass to tibia length) produced by bilateral functional overload was more pronounced in AMPKα1(−/−) mice than in WT mice (Fig. 5*A*). The PLN mass of AMPKα1(−/−) mice was increased by 53% ($P<0.01$) and 76% ($P<0.01$) at D7 and D21, respectively, whereas the PLN mass of WT mice was significantly increased by 32% ($P<0.05$) and 57% ($P<0.01$) during the same period (Fig. 5*A*). The degree of hypertrophy, as determined by the shift of myofiber size distribution between D0 and D7 and between D0 and D21, was more pronounced in AMPKα1(−/−) mice than in WT mice (Fig. 5*B, C*). Furthermore, analysis of myofiber size distribution shows a clear shift to fibers with larger size at D21 compared with D0 and D7 for both AMPKα1(−/−) and WT mice (Fig. 5*C, D*).

mTOR signaling in AMPKα1(−/−) mice after overload-induced hypertrophy

After 7 days of overload, we observed a significant increase of p70S6K phosphorylation in both WT and AMPKα1(−/−) mice (**Fig. 6*A***). This increase in p70S6K phosphorylation between D0 and D7 was greater for AMPKα1(−/−) mice compared with WT mice (Fig. 6*A*). In addition, the enhanced phosphorylation p70S6K (Fig. 6*A*) was still present at D21 in AMPKα1(−/−) mice, whereas it returned to basal level in WT mice (Fig. 6*A*). Consequently, phosphorylation of p70S6K was significantly higher in AMPKα1(−/−) mice compared with WT mice at D21.

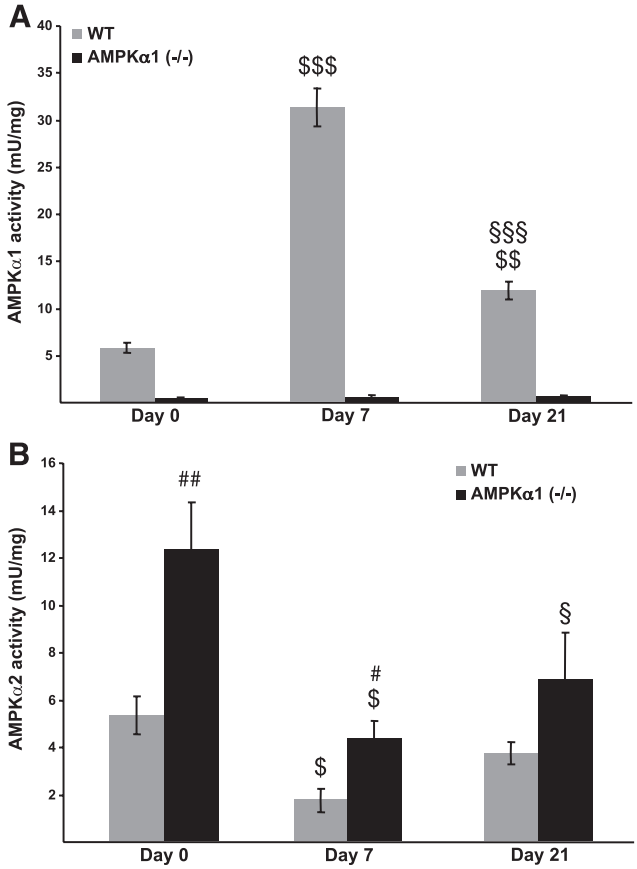

Figure 3. AMPK activity in PLN muscle after overload-induced hypertrophy in WT and AMPKα1(−/−) mice. AMPKα1 (*A*) and AMPKα2 activity (mU/mg) (*B*) measured using immunoprecipitate kinase assays in *plantaris* muscle after 0 days (D0), 7 days (D7), and 21 days (D21) of overload. Results are represented as means ± SD; $n = 6$/genotype. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* D0. $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$ *vs.* D7. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* WT.

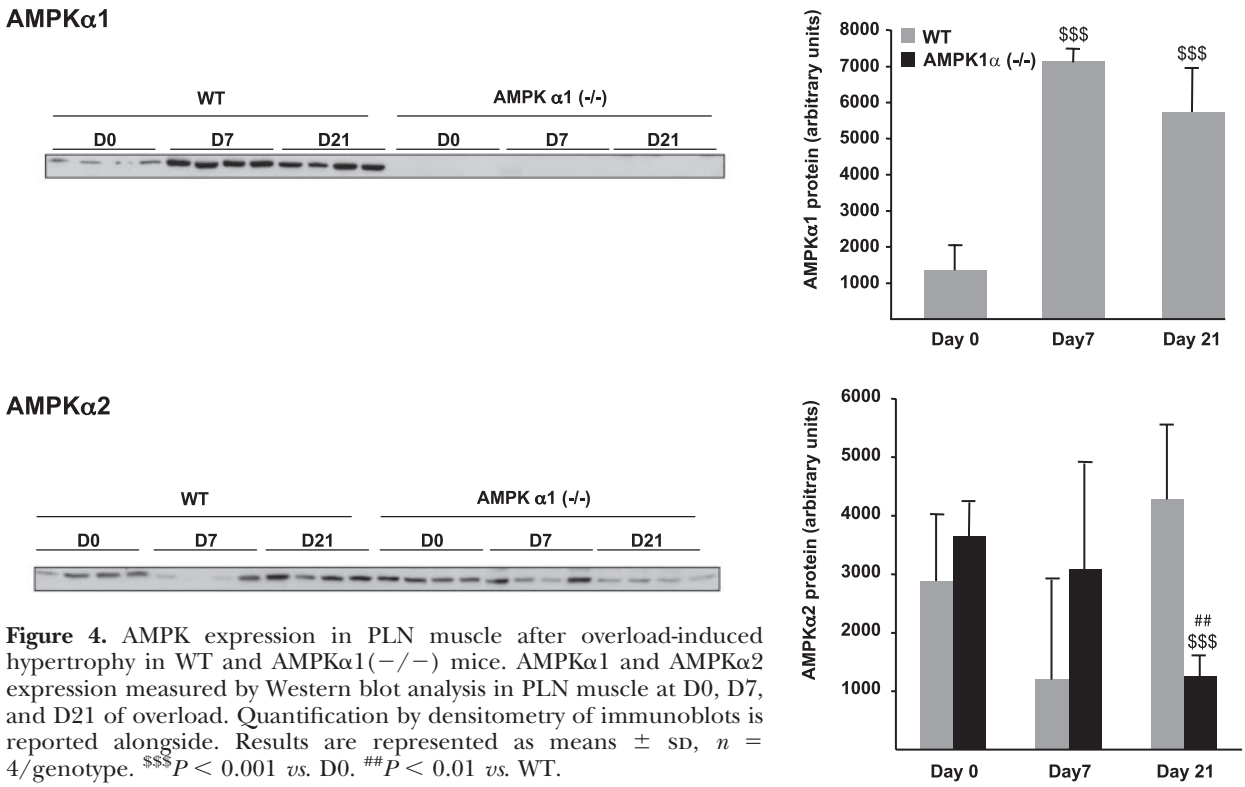

Figure 4. AMPK expression in PLN muscle after overload-induced hypertrophy in WT and AMPKα1(−/−) mice. AMPKα1 and AMPKα2 expression measured by Western blot analysis in PLN muscle at D0, D7, and D21 of overload. Quantification by densitometry of immunoblots is reported alongside. Results are represented as means ± SD, n = 4/genotype. $^{\$\$\$}P < 0.001$ *vs.* D0. $^{\#\#}P < 0.01$ *vs.* WT.

After 7 and 21 days of overload, phosphorylation of S6 was also significantly increased in AMPKα1(−/−) and WT mice (Fig. 6*A*). The deletion of AMPKα1 caused an enhanced increase in 4E-BP1 phosphorylation at D7 ($P<0.001$) and D21 ($P=0.051$; Fig. 6*B*). Finally, phosphorylation of eEF2 was significantly higher in AMPKα1(−/−) mice compared with WT mice at D0 (Fig. 6*B*). Furthermore, we observed a significant increase in phosphorylation of eEF2 in response to 21 days of overload in WT mice (Fig. 6*B*), whereas phosphorylation of eEF2 significantly decreased in AMPKα1(−/−) mice (−59%; $P<0.01$; Fig. 6*B*).

DISCUSSION

The major aim of this study was to investigate the role of AMPKα1 in the control of muscle cell size and growth. Previous studies (28) have demonstrated that AMPK integrates fuel sensing with cell growth control and contributes to the atrophic adaptations of p70S6K-deficient muscle. Here, we show that the size of AMPKα1-deficient myotubes is larger than AMPKα1-expressing control cells, demonstrating a unique role for AMPKα1 in the control of cell size. However unexpectedly, the mass of PLN muscle in basal state was significantly lower in AMPKα1(−/−) mice compared with WT mice. One potential reason for this lower muscle mass in AMPKα1(−/−) mice is a reduced ATP production, thus providing less energy for protein synthesis and growth. However, this should not be the case in cultured AMPKα1(−/−) myotubes, as their size is larger than AMPKα1-expressing control myotubes. Interestingly, the role for AMPKα1 isoform in the control of muscle energy balance has been recently challenged and data obtained from LKB1-deficient muscles suggest that skeletal muscle metabolic adaptation is rather dependent on AMPKα2 than on AMPKα1 activation (32). Furthermore, we previously reported that the lack of AMPKα2 isoform was associated with a lower ATP content in skeletal muscle (33), indicating that the remaining AMPKα1 isoform was not able to compensate for the loss of AMPKα2 isoform to maintain muscle energy balance. Lastly, it should also be noted that in AMPKα1(−/−) PLN muscle, AMPKα2 activity is increased compared with WT muscle in the basal state, resulting probably in a compensatory response for ATP production. Another hypothesis for the lower basal PLN mass in AMPKα1(−/−) mice is a role for satellite cells that are partly responsible for postnatal muscle growth (37, 38). Although AMPK function in satellite cells has not been investigated yet, an impaired or reduced satellite cell proliferation in the absence of AMPKα1, as illustrated by the lower number of nuclei per fiber in AMPKα1(−/−) PLN muscle, might explain this unexpected result (39).

Recent evidence supports the existence of distinct regulatory pathways and functions for AMPKα1 and AMPKα2 isoforms. The overloading-induced hypertrophy is associated with a significant increase in AMPKα1 expression and activity in WT muscle, whereas the activity of AMPKα2 is reduced at D7 and returns close to the basal value at D21 for both WT and AMPKα1(−/−) muscles. This marked activation of AMPKα1 after 7 and 21 days of chronic overload has been suggested to limit muscle mass growth (32). AMPKα2 appears to be preferentially

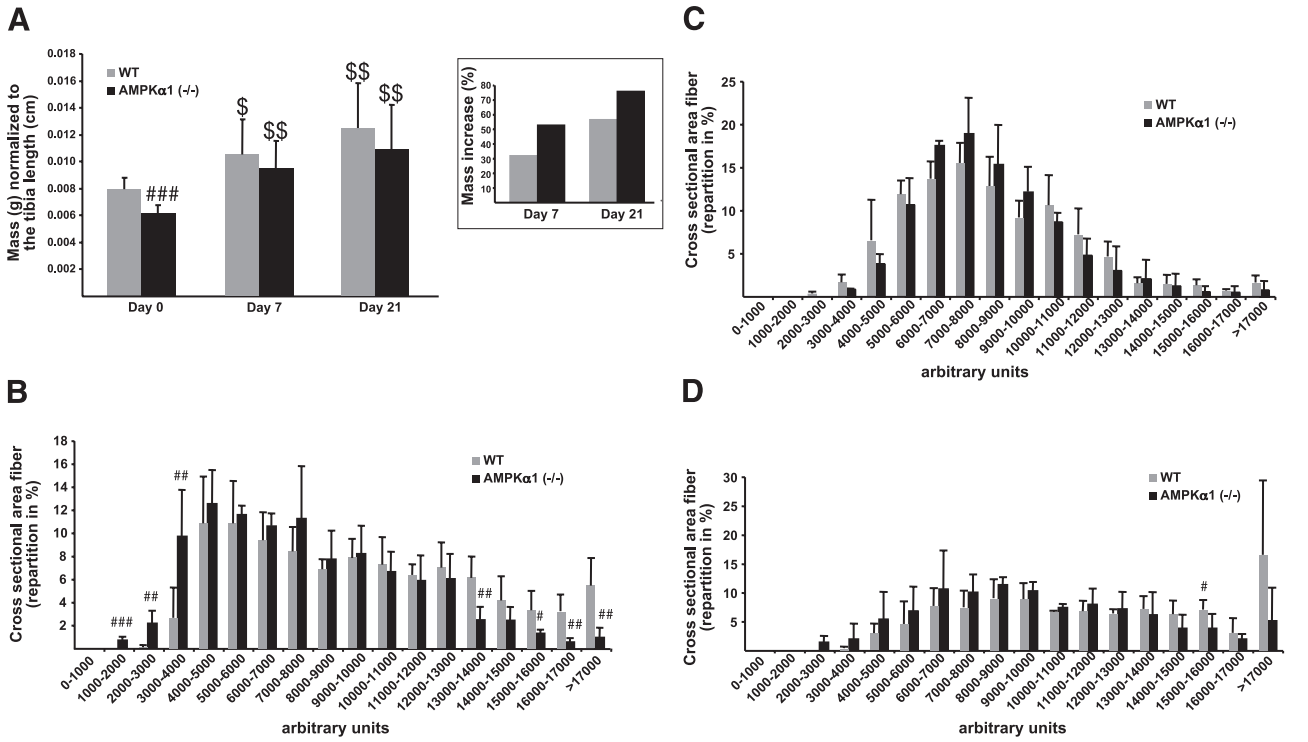

Figure 5. Muscle mass and fiber size in response to overload-induced hypertrophy in WT and AMPKα1(−/−) mice. *A*) Mass (g) of PLN muscles normalized to tibia length (cm) at D0, D7, and D21 of overload for WT and AMPKα1(−/−) mice; $n = 6$/group. Inset: percentage of PLN muscles mass increase at D7 and D21 of overload in WT and AMPKα1(−/−) mice relative to D0 counterparts. *B–D*) Frequency distribution of cross sectional area fibers in PLN muscle from WT and AMPKα1(−/−) mice at D0 (*B*), D7 (*C*), and D21 of overload (*D*). Fiber cross-sectional area of 318 fibers was determined from at least 9 different muscle areas of 3–6 animals/group. Results are represented as means ± SD. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *vs.* D0. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* WT.

activated in response to acute exercise (40–46) or by endurance training program (47), whereas low-intensity contraction stimulation activates AMPKα1 in skeletal muscle (48). Interestingly, it has been shown that only AMPKα1 activation is required for stimulation of glucose uptake by twitch contraction (49). In this condition, AMPKα1 activation was not accompanied by an increase in AMP level and AMP-to-ATP ratio, suggesting that AMPKα1 activation induced by low-intensity contraction is regulated by an AMP-independent (48) and/or LKB1-independent mechanism. Accordingly, LKB1 appears to be essential for AMPKα2 activity in different muscle types, whereas AMPKα1 activity is only partially affected in LKB1-deficient skeletal muscle (8, 32). Recently, it has been demonstrated that skeletal muscle hypertrophy is normal in response to chronic mechanical overload in the absence of LKB1 due to a significant increase in the activation of the AMPKα1 isoform (32). One mechanism for AMPKα1 activation could be through CaMKKs and TAK1 (32).

In the present study, we provide genetic evidence for a negative role for AMPKα1 in the control of mTOR signaling and growth in muscle cells. The hypertrophic action of *MyrAkt*, an effector known to stimulate skeletal muscle protein synthesis through activation of mTOR signaling, was enhanced in primary cultured myotubes and myofibers deleted for AMPKα1. However, the effect of rapamycin or Torin1 (potent allosteric mTORC1 inhibitors) in AMPKα1-deficient muscle would bring additional evidence for a negative role in mTOR signaling. We also show that muscle hypertrophy produced by functional overload is greater in mice deleted for the AMPKα1 catalytic subunit compared with WT mice. A strong correlation between increased AMPK activity in the PLN muscle from old rats and impaired overload-induced skeletal muscle hypertrophy has been recently reported (29). AMPK phosphorylation was negatively correlated with the amount of p70S6K phosphorylated in the mTOR-specific Thr^{389} residue in response to 7 days of chronic overload, supporting the notion that AMPK inhibited p70S6K phosphorylation in skeletal muscle *via* suppression of mTOR (10, 30). Conversely, enhanced hypertrophy in AMPKα1(−/−) muscle is associated with higher activation of the mTORC1 signaling pathway, as determined by increased phosphorylation of p70S6K and 4E-BP1 at D7 and D21. These results are in accordance with studies (17, 19, 30) showing the importance of mTOR-p70S6K signaling and 4E-BP1 phosphorylation in the hypertrophic response of chronically overloaded muscle. In addition, we observed a decrease in eEF2 phosphorylation in AMPKα1(−/−) muscle after 7 and 21 days of overload, indicating an improvement of protein elongation. Thus, in the absence of AMPKα1, unphosphorylated (active) eEF2 levels are higher in response to chronic overload with a parallel increase in mTORC1 signaling

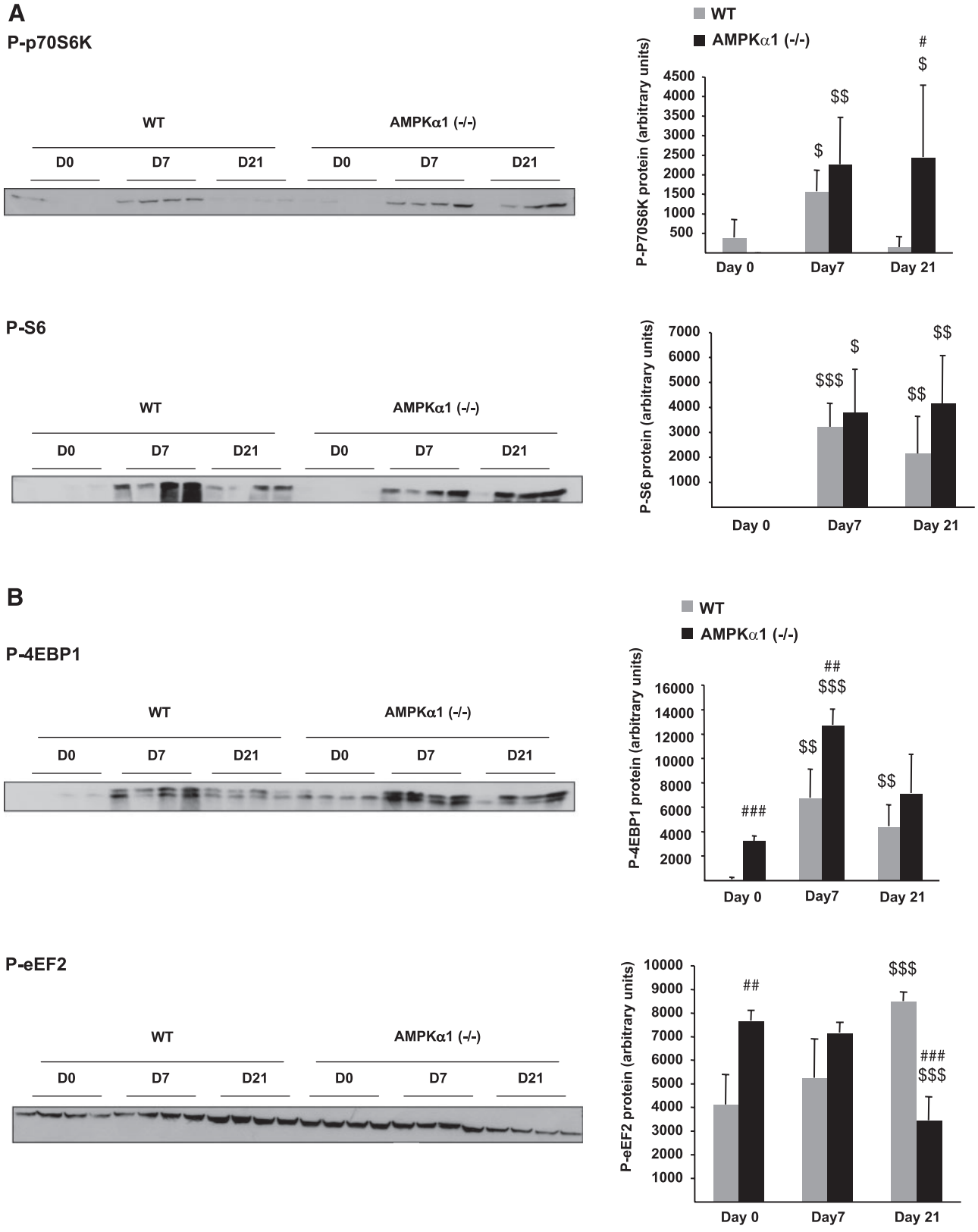

Figure 6. Changes in phosphorylation levels of p70S6K, S6K, 4E-BP1, and eEF2 in PLN muscle after overload-induced hypertrophy in WT and AMPKα1(−/−) mice. *A*) p70S6K phosphorylation at Thr389 (top panel) and S6 ribosomal protein phosphorylation at Ser-235/236 (bottom panel) measured by Western blot analysis in PLN muscle at D0, D7, and D21 of overload. *B*) 4E-BP1 phosphorylation at Thr37/46 (top panel) and eEF2 phophorylation at Thr56 (bottom panel) measured by Western blot analysis in PLN muscle at D0, D7, and D21 of overload. Quantification by densitometry of all immunoblots is reported alongside. Results are represented as means ± SD; $n = 4$/genotype. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* D0. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* WT.

leading to greater muscle hypertrophy. Hence, AMPKα1 behaves as a negative effector required to limit mTORC1 activity and to inhibit overgrowth of skeletal muscle in response to hypertrophic stimuli.

In conclusion, we demonstrate an essential role for AMPKα1 in the adaptation of skeletal muscle growth to hypertrophy. The major new findings of this study are as follows: *1*) muscle cells hypertrophy *in vitro* and *in vivo* is more pronounced in mice deleted for AMPKα1 catalytic subunit compared with control mice; and *2*) phosphorylation of mTORC1-signaling downstream targets, controlling protein synthesis, is enhanced in the absence of AMPKα1 after chronic overload and is associated with greater muscle hypertrophy. Our study provides important knowledge of the role of AMPK in the molecular mechanisms underlying the cell size control that may open new avenues of intervention against age-related skeletal muscle atrophy. Although resistance training can increase muscle size and strength, the myogenic response to exercise and the capacity for muscle hypertrophy in older humans is limited. The prevention of sarcopenia, which is the consequence of a reduction of protein synthesis and an increase in muscle protein degradation, would provide an obvious clinical benefit. FJ

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