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Important role for AMPK α 1 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 AMPK α 1 catalytic subunit on muscle cell size control and adaptation to muscle hypertrophy. We found that AMPK α 1 (-/-) primary cultured myotubes and myofibers exhibit larger cell size compared to control cells in response to chronic Akt activation. We next subjected the *plantaris* muscle of AMPK α 1 (-/-) and control mice to mechanical overloading in order to induce muscle hypertrophy. We observed significant elevations of AMPK α 1 activity in the control muscle at days 7 and 21 following the overload. Overloading-induced muscle hypertrophy was significantly accelerated in AMPK α 1 (-/-) mice than in control mice (+32% vs +53% at day 7 and +57% vs +76% at day 21 in control vs AMPK α 1 (-/-) mice, respectively). This enhanced growth of AMPK α 1-deficient muscle was accompanied by increased phosphorylation of mTOR signaling downstream targets and decreased phosphorylation of eukaryotic elongation factor 2. These results demonstrate that AMPK α 1 plays an important role in limiting skeletal muscle overgrowth during hypertrophy through inhibition of mTOR signaling pathway.

Key Words : muscle functional overload, protein synthesis, mTOR-S6K signaling.

Introduction

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/ATP ratio by lowering ATP production following 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 (α 1, α 2, β 1, β 2, γ 1, γ 2 and γ 3). 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, CaMKK β (Ca²⁺/calmodulin-dependent protein kinase kinase β) and TAK1 (TGF β -activated kinase-1, a member of the mitogen-activated protein kinase kinase family)(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 phosphatase(s) (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 AMPK α 2 but not via AMPK α 1 isoform (5-7). These results suggest distinct physiological

roles for α 1- and α 2-containing AMPK complexes in the regulation of skeletal muscle adaptations. Activation of the various AMPK complexes may depend on the specificity of upstream kinase for α -catalytic subunits phosphorylation. Indeed, in LKB1-deficient muscle, AMPK α 2 activation is severely blunted whereas AMPK α 1 activation is substantially induced in response to contraction, indicating discordant regulation of α 1- and α 2-containing AMPK complexes (8). Similar results have been obtained in LKB1-deficient heart in response to ischemia or anoxia (9).

One consequence of endurance exercise training is a partial fiber type transition towards 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 (eEF-2) kinase at Ser³⁹⁸ (15), leading to increase Thr⁵⁶ 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 leads also 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 mTORC1 signaling pathway and phosphorylation of these proteins have been associated with increased muscle mass following 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 Thr³⁸⁹ 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 AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside) infusion inhibits overloading-induced muscle hypertrophy (31). Interestingly, McGee *et al.* have shown that the hypertrophy produced by overload was associated with a marked activation of AMPK α 1 in both WT and LKB1-deficient mice and they proposed that AMPK α 1 might play an important role in the regulation of skeletal muscle growth (32).

Therefore, the aim of this investigation was to examine the potential role of AMPK α 1 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 AMPK α 1-deficient muscle cells in response to cell growth stimulation and determined the degree of muscle hypertrophy following muscle overload in AMPK α 1-deficient mice.

Methods

Animals

The generation of AMPK α 1 (-/-) mice has been described previously (33). AMPK α 1 (-/-) and WT mice (20-26 weeks old) were maintained on a 12h:12h 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'-ACCAGAAGCGGTGCCGAAAGCTGG-3' and reverse 5'-TGTAGTCGGTTTATGCAGCAACGAG-3' primers for AMPK α 1 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-week-old mice as described (34). Myotubes at day 2 of differentiation were infected with an adenovirus expressing GFP or a constitutively active Akt, NH(2)-terminally myristoylation signal-attached Akt (*MyrAkt*) (28) at the multiplicity of infection of 75 and incubated for 2 days in DMEM/Ham's F12 medium containing 2% horse serum. Then, bright-field images were taken and analysed using Metamorph software (version 7.04, Molecular Devices). 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**

Hyaluronidase (0.4 U) was injected along the TA muscle length and after two hours, animals anaesthetized with xylazine-ketamine were injected with 15 μ g of plasmids encoding GFP or *MyrAkt*. Electrical pulses were then applied by two stainless steel spatula placed on each side

of the muscle (130 Volts/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 *plantaris* (PLN) muscle by functional overload in AMPK α 1 (-/-) and control mice, as previously described (35). On the day of surgery (D0), animals were anaesthetized with xylazine-ketamine and maintained in the surgical plane. A longitudinal incision on the dorsal aspect of the lower hindlimb 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 prior to being returned to their cages. Animals were monitored for signs of pain or post-operative infection.

Tissue collection

Animals were anaesthetized 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 cells samples were obtained as described previously (8). Fifty μ g of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with antibodies against total AMPK α 1, AMPK α 2 (a kind gift from G. Hardie, University of Dundee, Scotland), phosphorylated forms of eEF2 (Thr56), S6 (Ser235/236), p70S6K (Thr389) and 4E-BP1 (Thr37/46) (Cell Signaling). Measure of AMPK α 1 and α 2 activities was performed as described previously (36).

Immunohistochemistry

Serial transverse sections (12-16 μm thick) were cut from TA and PLN muscles in a cryostat microtome (Leica, 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) and anti-HA (Roche) antibodies and staining with Hoechst dye. The fiber cross-sectional area (CSA) was determined using Metamorph software (version 7.04, Molecular Devices).

Statistical analysis

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

Results

Cell size control in AMPK α 1-deficient muscle cells

In order to examine the role of AMPK α 1 in the control of muscle cell size, we isolated and analyzed primary muscle cells from AMPK α 1 (-/-) and control mice. Western blotting analysis revealed that AMPK α 1 protein was undetectable in AMPK α 1 (-/-) myotubes and the levels of AMPK α 2 protein were similar between AMPK α 1 (-/-) and WT myotubes (Figure 1A). The deletion of AMPK α 1 caused a 15% increase in myotube diameter (Figure 1B). Furthermore, myotubes size distribution for AMPK α 1 (-/-) cells showed fewer small-diameter myotubes and increased number of large-diameter myotubes compared to WT cells (Figure 1C). To evaluate the functional consequence of AMPK deletion on mTOR-pathway-mediated hypertrophy, we introduced WT and AMPK α 1 (-/-) 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 two-fold higher in AMPK α 1 (-/-) myotubes compared to WT cells (Figure 1B). The diameter of myotubes increased significantly by 29% in AMPK α 1 (-/-) 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 AMPK α 1 (-/-) cells in response to *MyrAkt* overexpression (Figures 1C). Finally, we observed an increase in p70S6K phosphorylation (+39%) in AMPK α 1 (-/-) myotubes introduced with *MyrAkt*, consistent with a negative action of AMPK α 1 on mTOR signaling network (Figure 1D).

MyrAkt-induced cell growth in AMPK α 1-deficient muscle

In order to gain further insights into the role of AMPK α 1 in the regulation of muscle cell growth *in vivo*, we transfected AMPK α 1 (-/-) and control TA muscles with a plasmid expressing *MyrAkt* or GFP by electroporation. The hypertrophic action of *MyrAkt* was

significantly higher in AMPK α 1 (-/-) skeletal muscle fibers compared to WT fibers (Figure 2). Indeed, the cross sectional area of AMPK α 1 (-/-) myofibers increased significantly by 125% compared to 68% for WT myofibers (Figure 2).

Changes in AMPK activity and expression following overload-induced hypertrophy

Since robust activation of AMPK α 1 has recently been reported during muscle hypertrophy (32), we examined the overloading-induced muscle growth in mice lacking AMPK α 1. Following 7 days of overload, AMPK α 1 activity was markedly increased in the PLN of WT mice (+446%; Figure 3A) and remained significantly higher at D21 compared to D0 (+106%; Figure 3A), although it was significantly lower compared to D7 (Figure 3A). Coincidentally, AMPK α 1 expression increased dramatically at D7 (+441%; Figure 4) and at D21 (+336%; Figure 4) in WT mice. In the PLN muscles of the AMPK α 1 (-/-) mice, AMPK α 1 activity was ablated and only background counts were detected (Figure 3A). The absence of AMPK α 1 expression in PLN muscle of AMPK α 1 (-/-) mice was confirmed by western blot analysis (Figure 4). Loss of AMPK α 1 resulted in an increase in the basal level of AMPK α 2 activity (Figure 3B). In contrast to AMPK α 1 activity, AMPK α 2 activity was reduced in both WT (-67%; Figure 3B) and AMPK α 1 (-/-) mice at D7 (-65%; Figure 3B). The activity of AMPK α 2 at D21 for both WT and AMPK α 1 (-/-) mice returned to a value closed to the basal level of WT mice (Figure 3B). AMPK α 2 expression was unaffected in PLN from AMPK α 1 (-/-) and WT mice at D7, but significantly decreased in AMPK α 1 (-/-) mice at D21 (Figure 4).

Muscle mass and myofiber size in AMPK α 1-deficient mice

The ratio of PLN mass to tibia length was significantly lower in AMPK α 1 (-/-) mice compared to WT mice (Figure 5A). Myofiber size distribution of AMPK α 1 (-/-) PLN exhibits increased number of small myofibers and fewer large myofibers compared to WT PLN

(Figures 5B). Moreover, the number of nuclei per fiber in AMPK α 1 (-/-) mice was smaller (-22%) compared to WT mice ($P < 0.01$).

Muscle mass and myofiber size following 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 is more pronounced in AMPK α 1 (-/-) mice than in WT mice (Figure 5A). PLN mass of AMPK α 1 (-/-) mice was increased by 53% ($P < 0.01$) and 76% ($P < 0.01$) at D7 and D21 respectively, whereas PLN mass of WT mice was significantly increased by 32% ($P < 0.05$) and 57% ($P < 0.01$) during the same period (Figure 5A). The degree of hypertrophy, as determined by the shift of myofibers size distribution between D0 and D7 and between D0 and D21, was more pronounced in AMPK α 1 (-/-) mice than in WT mice (Figures 5B and 5C). Furthermore, analysis of myofiber size distribution shows a clear shift to fibers with larger size at D21 compared to D0 and D7 for both AMPK α 1 (-/-) and WT mice (Figures 5C and 5D).

mTOR signaling in AMPK α 1 (-/-) mice following overload-induced hypertrophy

After 7 days of overload, we observed a significant increase of p70S6K phosphorylation in both WT and AMPK α 1 (-/-) mice (Figure 6A). This increase in p70S6K phosphorylation between D0 and D7 was greater for AMPK α 1 (-/-) mice compared to WT mice (Figure 6A). In addition, the enhanced phosphorylation p70S6K (Figure 6A) was still present at D21 in AMPK α 1 (-/-) mice, whereas it returned to basal level in WT mice (Figure 6A). Consequently, phosphorylation of p70S6K was significantly higher in AMPK α 1 (-/-) mice compared to WT mice at D21. Following 7 and 21 days of overload, phosphorylation of S6 was also significantly increased in AMPK α 1 (-/-) and WT mice (Figure 6A). The deletion of AMPK α 1 caused an enhanced increase in 4E-BP1 phosphorylation at D7 ($P < 0.001$) and D21 ($P = 0.051$) (Figure 6B). Finally, phosphorylation of eEF2 was significantly higher in AMPK α 1 (-/-) mice compared to WT mice at D0 (Figure 6B). Furthermore, we observed a

significant increase in phosphorylation of eEF2 in response to 21 days of overload in WT mice (Figure 6B), whereas phosphorylation of eEF2 significantly decreased in AMPK α 1 (-/-) mice (-59%; $P < 0.01$; Figure 6B).

Discussion

The major aim of this study was to investigate the role of AMPK α 1 in the control of muscle cell size and growth. Previous studies have demonstrated that AMPK integrates fuel sensing with cell growth control and contributes to the atrophic adaptations of p70S6K-deficient muscle (28). Here, we show that the size of AMPK α 1-deficient myotubes is larger than AMPK α 1-expressing control cells, demonstrating a unique role for AMPK α 1 in the control of cell size. However unexpectedly, the mass of PLN muscle in basal state was significantly lower in AMPK α 1 (-/-) mice compared to WT mice. One potential reason for this lower muscle mass in AMPK α 1 (-/-) mice is a reduced ATP production, thus providing less energy for protein synthesis and growth. However, this should not be the case in cultured AMPK α 1 (-/-) myotubes as their size is larger than AMPK α 1-expressing control myotubes. Interestingly, the role for AMPK α 1 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 AMPK α 2 than on AMPK α 1 activation (32). Furthermore, we previously reported that the lack of AMPK α 2 isoform was associated with a lower ATP content in skeletal muscle (33), indicating that remaining AMPK α 1 isoform was not able to compensate for the loss of AMPK α 2 isoform to maintain muscle energy balance. Lastly, it should be also noted that in AMPK α 1 (-/-) PLN muscle, AMPK α 2 activity is increased compared to WT muscle in the basal state, resulting probably in a compensatory response for ATP production. Another hypothesis for the lower basal PLN mass in AMPK α 1 (-/-) 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 AMPK α 1, as

illustrated by the lower number of nuclei per fiber in AMPK α 1 (-/-) PLN muscle, might explain this unexpected result (39).

Recent evidence supports the existence of distinct regulatory pathways and functions for AMPK α 1 and AMPK α 2 isoforms. The overloading-induced hypertrophy is associated with a significant increase in AMPK α 1 expression and activity in WT muscle, whereas the activity of AMPK α 2 is reduced at D7 and returns close to basal value at D21 for both WT and AMPK α 1 (-/-) muscles. This marked activation of AMPK α 1 following 7 and 21 days of chronic overload has been suggested to limit muscle mass growth (32). AMPK α 2 appears to be preferentially activated in response to acute exercise (40-46) or by endurance training program (47) whereas low intensity contraction stimulation activates AMPK α 1 in skeletal muscle (48). Interestingly, it has been shown that only AMPK α 1 activation is required for stimulation of glucose uptake by twitch contraction (49). In this condition, AMPK α 1 activation was not accompanied by an increase in AMP level and AMP/ATP ratio, suggesting that AMPK α 1 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 AMPK α 2 activity in different muscle types whereas AMPK α 1 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 significant increase in the activation of the AMPK α 1 isoform (32). One mechanism for AMPK α 1 activation could be through the Ca²⁺/calmodulin-dependent protein kinase kinases (CaMKKs) and the TGF β -activated protein kinase (TAK-1) (32).

In the present study, we provide genetic evidence for a negative role for AMPK α 1 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 AMPK α 1. However, the effect of rapamycin or Torin1 (potent allosteric mTORC1 inhibitors) in AMPK α 1-deficient muscle would bring additional evidence for a negative role on mTOR signaling. We also show that muscle hypertrophy produced by functional overload is greater in mice deleted for AMPK α 1 catalytic subunit compared to 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 at the mTOR-specific Thr³⁸⁹ 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 AMPK α 1 (-/-) muscle is associated with higher activation of mTORC1 signaling pathway, as determined by increased phosphorylation of p70S6K and 4E-BP1 at D7 and D21. These results are in accordance with studies showing the importance of mTOR-p70S6K signaling and 4E-BP1 phosphorylation in the hypertrophic response of chronically-overloaded muscle (17, 19, 30). In addition, we observed a decrease in eEF2 phosphorylation in AMPK α 1 (-/-) muscle following 7 and 21 days of overload, indicating an improvement of protein elongation. Thus, in the absence of AMPK α 1, unphosphorylated (active) eEF2 levels are higher in response to chronic overload with a parallel increase in mTORC1 signaling leading to a greater muscle hypertrophy. Hence, AMPK α 1 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 AMPK α 1 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

AMPK α 1 catalytic subunit compared to control mice; (2) phosphorylation of mTORC1 signaling downstream targets, controlling protein synthesis, is enhanced in the absence of AMPK α 1 following chronic overload and is associated with a greater muscle hypertrophy. Our study provides important knowledge on 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.

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Figure legends

Figure 1 : Cell size control in primary muscle cells lacking AMPK α 1. (A) Primary muscle cells isolated from AMPK α 1 (-/-) and control mice were differentiated into myotubes and AMPK α 1 and AMPK α 2 protein content was assessed by western blot analysis. A representative immunoblot of AMPK α 1 (upper panel) and AMPK α 2 (lower panel) is shown. **(B)** Size of WT and AMPK α 1 (-/-) myotubes transduced with 75 moi GFP and *MyrAkt* adenoviruses as indicated. Results are presented as a percentage of WT muscle cells transduced with GFP adenovirus. Representative bright-field images of myotubes transduced with GFP and *MyrAkt* adenoviruses are shown in the upper part of the figure. **(C)** Frequency distribution of size of WT and AMPK α 1 (-/-) 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. Data from five experiments on two different cultures are shown in B and C. Results are represented as means \pm SD in B and C. Different from GFP cells of the same genotype in B : $^{\$}P<0.05$, $^{\$\$}P<0.01$; Different for the same conditions of infection in B : $^{\#}P<0.05$, $^{\#\#}P<0.01$. WT myotubes different from AMPK α 1 (-/-) myotubes both transduced with GFP adenovirus in C : $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$. WT myotubes different from AMPK α 1 (-/-) myotubes both transduced with *MyrAkt* adenovirus in C : $^{\$}P<0.05$, $^{\$\$\$}P<0.001$. **(D)** Thr389 p70S6K phosphorylation was determined 2 days after infection. Representative immunoblot is shown in the upper part of the figure. Protein content is expressed in arbitrary units relative to GFP infected cells for each genotype. Results are represented as means \pm SD. Different from GFP cells of the same genotype : $^{\$}P<0.05$, $^{\$\$}P<0.01$.

Figure 2 : Change in cross-sectional area of WT and AMPK α 1 (-/-) myofibers in *tibialis anterior* muscle transfected with *MyrAkt*. Changes in size of WT and AMPK α 1 (-/-)

myofibers overexpressing *MyrAkt* represented as percent changes to non-transfected myofibers. Detection of *MyrAkt* (HA-tagged *MyrAkt*) transfected myofibers was realized by immunostaining with anti-HA antibody. A representative image showing positive cells marked with a star is presented in the upper part of the figure. The fiber cross-sectional area of 180 fibers was determined from at least 3 different muscle areas of 4 animals in each group. Results are represented as means \pm SD. Different from WT : #P<0.05.

Figure 3 : AMPK activity in *plantaris* muscle following overload-induced hypertrophy in WT and AMPK α 1 (-/-) mice. (A) AMPK α 1 and (B) AMPK α 2 activity (mU/mg) measured using immunoprecipitate kinase assays in *plantaris* muscle following 0 (D0), 7 (D7) and 21 (D21) days of overload. Results are represented as means \pm SD, n = 6 for each genotype. Different from Day 0 : ^{\$}P<0.05; ^{\$\$}P<0.01, ^{\$\$\$}P<0.001; Different from Day 7 : ^{\$}P<0.05, ^{\$\$\$}P<0.001; Different from WT : #P<0.05; ##P<0.01.

Figure 4 : AMPK expression in *plantaris* muscle following overload-induced hypertrophy in WT and AMPK α 1 (-/-) mice. AMPK α 1 and AMPK α 2 expression measured by western blot analysis in *plantaris* muscle following 0 (D0), 7 (D7) and 21 (D21) days of overload. Quantification by densitometry of immunoblots is reported alongside the figure. Results are represented as means \pm SD, n = 4 for each genotype. Different from Day 0 : ^{\$\$\$}P<0.001; Different from WT : ##P<0.01.

Figure 5 : Muscle mass and fiber size in response to overload-induced hypertrophy in WT and AMPK α 1 (-/-) mice. (A) Mass (g) of *plantaris* muscles normalized to the tibia length (cm) following 0 (D0), 7 (D7) and 21 (D21) days of overload is shown for WT and AMPK α 1 (-/-) mice. Percentage of *plantaris* muscles mass increase following 7 and 21 days

of overload in WT and AMPK α 1 (-/-) mice relative to day 0 counterparts is presented on the right part of the figure (Inset). Results are represented as means \pm SD, n = 6 for each group. Different from Day 0 : ^sP<0.05, ^{ss}P<0.01; Different from WT : ^{###}P<0.001. Frequency distribution of cross sectional area fibers in *plantaris* muscle from WT and AMPK α 1 (-/-) mice before (B) and after 7 days (C) and 21 days (D) of overload. The fiber cross-sectional area of 318 fibers was determined from at least 9 different muscle areas of 3-6 animals in each group. Results are represented as means \pm SD. Different from WT : [#]P<0.05, ^{##}P<0.01, ^{###}P<0.001.

Figure 6 : Changes in phosphorylation levels of p70S6K, S6K, 4E-BP1 and eEF2 in *plantaris* muscle following overload-induced hypertrophy in WT and AMPK α 1 (-/-) mice. (A) p70S6K phosphorylation at Thr389 (upper part) and S6 ribosomal protein phosphorylation at Ser235/236 (lower part) measured by western blot analysis in *plantaris* muscle before (D0) and following 7 (D7) and 21 (D21) days of overload. (B) 4E-BP1 phosphorylation at Thr37/46 (upper part) and eEF2 phosphorylation at Thr56 (lower part) measured by western blot analysis in *plantaris* muscle before (D0) and following 7 (D7) and 21 (D21) days of overload. Quantification by densitometry of all immunoblots is reported alongside the figure. Results are represented as means \pm SD, n = 4 for each genotype. Different from Day 0 : ^sP<0.05, ^{ss}P<0.01, ^{sss}P<0.001; Different from WT : [#]P<0.05, ^{##}P<0.01, ^{###}P<0.001.