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Antagonistic control of muscle cell size by AMPK and mTORC1

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Nutrition and physical activity have profound effects on skeletal muscle metabolism and growth. Regulation of muscle mass depends on a thin balance between growth-promoting and growth-suppressing factors. Over the past decade, the mammalian target of rapamycin (mTOR) kinase has emerged as an essential factor for muscle growth by mediating the anabolic response to nutrients, insulin, insulin-like growth factors and resistance exercise. As opposed to the mTOR signaling pathway, the AMP-activated protein kinase (AMPK) is switched on during starvation and endurance exercise to upregulate energy-conserving processes. Recent evidence indicates that mTORC1 (mTOR Complex 1) and AMPK represent two antagonistic forces governing muscle adaption to nutrition, starvation and growth stimulation. Animal knockout models with impaired mTORC1 signaling showed decreased muscle mass correlated with increased AMPK activation. Interestingly, AMPK inhibition in p70S6K-deficient muscle cells restores cell growth and sensitivity to nutrients. Conversely, muscle cells lacking AMPK have increased mTORC1 activation with increased cell size and protein synthesis rate. We also demonstrated that the hypertrophic action of *MyrAkt* is enhanced in AMPK-deficient muscle, indicating that AMPK acts as a negative feedback control to restrain muscle hypertrophy. Our recent results extend this notion by showing that AMPKα1, but not AMPKα2, regulates muscle cell size through the control of mTORC1 signaling. These results reveal the diverse functions of the two catalytic isoforms of AMPK, with AMPKα1 playing a predominant role in the control of muscle cell size and AMPKα2 mediating muscle metabolic adaptation. Thus, the crosstalk between AMPK and mTORC1 signaling is a highly regulated way to control changes in muscle growth and metabolic rate imposed by external cues.

Key words: muscle cell size, protein synthesis, mTOR, AMPK, muscle hypertrophy, energy metabolism, knockout mice

Abbreviations: ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CSA, cross-sectional area; HSA, human skeletal actin; IRS, insulin receptor substrate; LKB1, liver kinase B1; mTORC1, mammalian target of rapamycin complex 1; TA, tibialis anterior muscle; TAK1, TGFβ-activated protein kinase 1

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Skeletal muscle comprises about 40–50% of total body mass and contributes to nutrient storage and supply. The major role of mammalian target of rapamycin complex 1 (mTORC1) in regulating muscle mass in response to a wide range of upstream inputs, including nutrients, growth factors and insulin, has long been established.[1] The control of muscle cell size by mTORC1 relies on a dynamic balance between anabolic processes, such as protein synthesis and nutrient storage, and catabolic processes, like the utilization of energy stores. More recently, the energy sensor 5'-AMP-activated protein kinase (AMPK), an energy-sensing kinase, has emerged as another key player in controlling muscle cell size.[2-4] Interestingly, AMPK directly phosphorylates TSC1/2,[5] mTOR[5] and raptor,[6] leading to the suppression of mTORC1 signaling. AMPK is a monitor of cellular energy homeostasis present in a wide variety of organisms, from yeast to mammals.[7] 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. There are a number of isoforms known for each subunit (α1, α2, β1, β2, γ1, γ2, γ3), which are

encoded by different genes and give rise to a variety of heterotrimeric combinations. The phosphorylation of a conserved threonine residue (Thr172) within the kinase domain of the α-catalytic subunit is absolutely required for AMPK activation by upstream kinases identified as the protein kinase LKB1 (a tumor suppressor whose germline mutations in humans are the cause of Peutz-Jeghers syndrome), the CaMKKβ (Ca^{2+}/calmodulin-dependent protein kinase kinase β) and TAK1 (transforming growth factor β-activated kinase).[7] When the cellular AMP/ATP ratio is high, AMPK is activated, switching off ATP-consuming anabolic pathways and switching on ATP-producing catabolic pathways,[7] the net result being suppression of protein synthesis and cell growth.[8] In skeletal muscle, AMPK activation causes metabolic changes that assist muscle cells in adapting to energy deprivation (hypoxia, osmotic stress, endurance exercise and electrically stimulated contraction) by increasing oxidative capacity via increased mitochondrial biogenesis, enzyme expression and nutrient uptake.[9,10]

Use of knockout models for upstream and downstream components of mTORC1 signaling has helped to demonstrate its crucial role in the regulation of muscle cell size. Defects in mTORC1 signaling have severe consequences on muscle cell size regulation, as revealed by decreased muscle mass and fiber cross-sectional area in IRS1/2, raptor, p70S6K and mTOR knockout mouse models.[11-15] Downregulation of the mTORC1 signaling in these models is confirmed by the decreased phosphorylation of downstream targets, such as p70S6K or 4E-BP1.[11-15] The double IRS1/2 muscle-specific knockout had also decreased protein content in the skeletal muscle, suggesting the atrophy in IRS1/2-deficient muscles is due, at least partly, to the inhibition of mTORC1-mediated muscle growth.[13] Interestingly, in all these models with impaired mTORC1 signaling, AMPK is highly activated, suggesting a negative crosstalk between these two pathways. In this context, activation of AMPK is induced by an increase in the AMP:ATP ratio.[11,13] Thus, mTORC1 signaling appears to be essential for normal ATP production, and its disruption clearly induces energetic stress, subsequent activation of AMPK and then inhibition of muscle growth. Indeed, p70S6K-knockout mice show a robust $AMPK^{Thr172}$ phosphorylation, as well as of its downstream target acetyl CoA carboxylase (ACC), and mimic the metabolic adaptations to a low-calorie diet.[11] Moreover, knockdown of p70S6K in myotubes induces AMPK activation and a concurrent decrease in cell size, indicating that activation of AMPK is accountable for muscle cell atrophy.[11] Similarly, the muscle-specific knockout of IRS1/2 exhibits increased AMPK phosphorylation, associated with increased phosphorylation of ACC and raptor.[13] All together, these results indicate that, while mTORC1 promotes muscle growth and protein synthesis, the role of AMPK might be to limit muscle hypertrophy.

As could be expected, muscle cells deficient in p70S6K are resistant to a hypertrophic stimulus, whether it is mediated by nutrients, by a constitutively active form of Akt (*MyrAkt*) (an effector known to enhance skeletal muscle protein synthesis through activation of mTORC1 signaling) or by IGF1.[14] Indeed, lack of a key protein of the mTORC1 pathway, such as p70S6K, renders the muscle cell unresponsive to a growth stimulus, even if it is able to sense it.[14] Interestingly, most of the studies focusing on inactivation of the mTORC1 pathway in muscle show an increase in the phosphorylation of Akt (Thr^{308} and Ser^{473}) in vivo.[11,12,15] Nevertheless, this activation does not have any effect downstream of mTORC1 and does not induce hypertrophy because of the lack of a key signaling protein downstream of Akt (either raptor, p70S6K or mTOR). The phosphorylation of Akt in these muscles compared with control muscle is likely due to the suppression of the inhibitory feedback of mTORC1 on insulin signaling. The negative feedback involves P70S6K phosphorylation of IRS-1, leading to its degradation, thereby suppressing insulin signaling.[16]

To verify the counter-regulatory crosstalk between mTORC1 and AMPK, we have generated muscle-specific AMPK-deficient mice as well as AMPK-deficient myotubes.[2] We have shown that AMPK deletion is sufficient to activate mTORC1 signaling, leading to increased muscle cell size in cultured primary myotubes and muscle fibers. Activation of mTORC1 in AMPK-deficient myotubes promotes a high protein synthesis rate, leading to a 1.5-fold increase in myotube diameter. We have also demonstrated that selective inactivation of mTORC1 by rapamycin reduces protein synthesis to control levels and limits cell growth, rescuing AMPK-deficient myotube cell size.[2] Mirroring this result, inhibition of AMPK activity in p70S6K-deficient myotubes, either by expression of a kinase-dead mutant (AMPK-KD) or by using siRNA targeting both α1 and α2 catalytic subunits, rescues p70S6K-deficient myotubes' cell size, thereby highlighting the role of AMPK in muscle cell size control.[11] In addition, expression of AMPK-KD in WT myotubes and muscle induces the phosphorylation of p70S6K and 4E-BP1, with a concomitant increase in myotube diameter and fiber cross-sectional area, consistent with the results obtained with AMPK-deficient myotubes.[11] Taken together, these studies clearly demonstrate the reciprocity in the crosstalk between AMPK and mTORC1, as downregulating one pathway is sufficient to upregulate the other. Thus, downregulation of either the AMPK or mTORC1 signaling pathway directly induces an abnormal muscle cell size, showing that the AMPK/mTORC1 crosstalk constitutes a gauge essential for the precise adjustment of muscle cell size.

Previous studies have implicated mTORC1 and its downstream target p70S6K in the control of protein synthesis rate during muscle adaptation to increased contractile loading. During mechanically induced skeletal muscle growth, AMPK activation and phosphorylation has been negatively correlated with phosphorylation of p70S6K at the mTOR-specific Thr^{389} residue ($p70S6K^{Thr389}$), implicating AMPK as a major negative regulator of overload-induced skeletal muscle hypertrophy.[11,17-19] Recently, Paturi et al. have reported that insulin resistance and related comorbidities may be associated with decreases in the ability of muscle to undergo hypertrophy, and that this alteration was associated with decreased $p70S6K^{Thr389}$ phosphorylation and increased $AMPK^{Thr172}$ phosphorylation.[20] It has also been reported that chronic AMPK activation by continuous infusion of AICAR

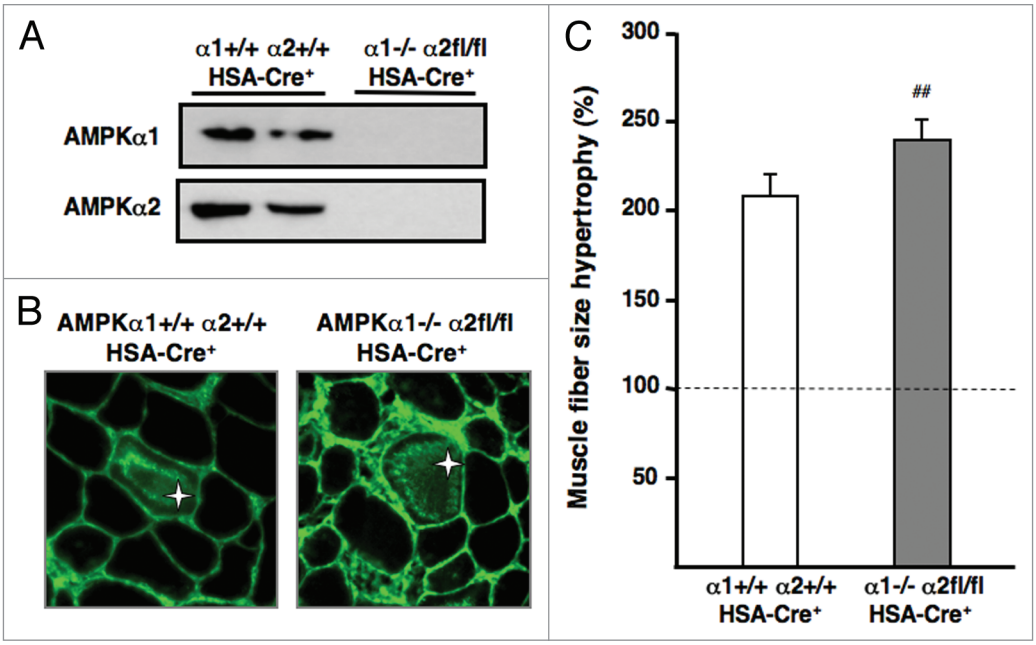

Figure 1. Increased hypertrophic response in AMPK-deficient muscle fibers. (A) AMPKα1 and AMPKα2 protein expression in tibialis anterior (TA) muscle from AMPKα1$^{+/+}$α2$^{+/+}$ HSA-Cre^{+} and AMPKα1$^{-/-}$α 2$^{fl/fl}$ HSA-Cre^{+} mice. (B) Cross-sectional area (CSA) of AMPKα1$^{+/+}$α2$^{+/+}$ HSA-Cre^{+} and AMPKα1$^{-/-}$α2$^{fl/fl}$ HSA-Cre^{+} TA myofibers transfected with *MyrAkt*. *MyrAkt* (HA-tagged *MyrAkt*)-transfected myofibers were visualized by immunostaining with anti-HA antibody. Positive cells are marked with a star in the representative image. (C) Myofiber hypertrophy is represented as percent change to GFP-transfected myofibers CSA. TA muscles from α1$^{+/+}$α2$^{+/+}$ HSA-Cre^{+} or α1$^{-/-}$α2$^{fl/fl}$ HSA-Cre^{+} mice were transfected by electroporation with plasmids encoding *MyrAkt* or GFP as control. The muscle was removed ten days later and muscle fiber CSA was analyzed. The CSA was obtained from at least three different muscle areas of four animals in each group. Results are presented as means ± SD, $^{\#\#}p < 0.01$ vs. WT.

(5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), a potent AMPK activator, inhibits overload-induced muscle hypertrophy.[21] In order to determine the role of AMPK on mTOR-induced muscle hypertrophy, we analyzed the hypertrophic action of *MyrAkt* in AMPK-deficient and control tibialis anterior (TA) muscle (**Fig. 1A**). The cross-sectional area (CSA) of TA fibers was significantly increased but was more pronounced in AMPK-deficient muscle, in which the CSA was 32% larger than in control muscle (**Fig. 1B and C**). These data indicate that AMPK acts as a negative feedback control to restrain muscle hypertrophy.

Interestingly, McGee et al. have recently shown that skeletal muscle hypertrophy is normal in response to chronic mechanical overload in the absence of LKB1, the primary upstream kinase for AMPK in muscle. In this context, skeletal muscle hypertrophy is associated with a marked activation of AMPKα1, which still occurs in LKB1-deficient muscle, whereas the activity of AMPKα2 is completely abolished.[3] These data suggest that the α1 and α2 catalytic isoforms of AMPK have distinct functions in the regulation of overload-induced skeletal muscle hypertrophy. Consistent with this hypothesis, we recently reported that the AMPKα1 catalytic isoform plays an important role in the regulation of skeletal muscle growth. First, we showed that AMPKα1-deficient myotubes are larger than AMPKα1-expressing control cells, indicative of a unique role for AMPKα1 in the control of cell size.[4] Similarly, two recent studies have also reported a role for AMPKα1 in the regulation of pancreatic β cell size through the inhibition of mTOR signaling.[22,23] Second, the hypertrophic action of *MyrAkt* caused a higher increase both in the diameter of myotubes deleted for AMPKα1 and in the fiber cross-sectional area of AMPKα1-deficient muscle.[4] Importantly, we also demonstrated that overload-induced muscle hypertrophy is greater in AMPKα1-knockout mice compared with control mice.[4] We observed a greater increase in p70S6K and 4E-BP1 phosphorylation in AMPKα1-deficient muscle compared with control during overload-induced hypertrophy of plantaris muscle,[4] consistent with the correlation between 4E-BP1 phosphorylation levels and muscle hypertrophy.[19] Our data confirms that, in response to mechanical overload, mTORC1 signaling is unaffected by the presence of AMPKα2 in AMPKα1-deficient muscle and provides evidence that AMPKα1 activation limits its muscle mass and cell size.[4] To further evaluate the role of AMPKα2 in the regulation of muscle cell hypertrophy, we investigated the hypertrophic action of *MyrAkt* in AMPKα2-deficient and control TA muscles. Interestingly, *MyrAkt*-induced hypertrophy was slightly lower in muscle fibers lacking AMPKα2 than in control fibers but does not reach statistical significance (**Fig. 2A and B**). To address whether AMPKα2 contributes to the control of muscle cell size, we measured the size of myotubes isolated from AMPKα2-knockout and control mice. The diameter of AMPKα2-deficient myotubes was 25% smaller than control myotubes (**Fig. 2C**). Furthermore, the degree of cell hypertrophy in response to *MyrAkt* action is significantly lower in AMPKα2-deficient myotubes compared with control myotubes (+ 7% vs. 17%

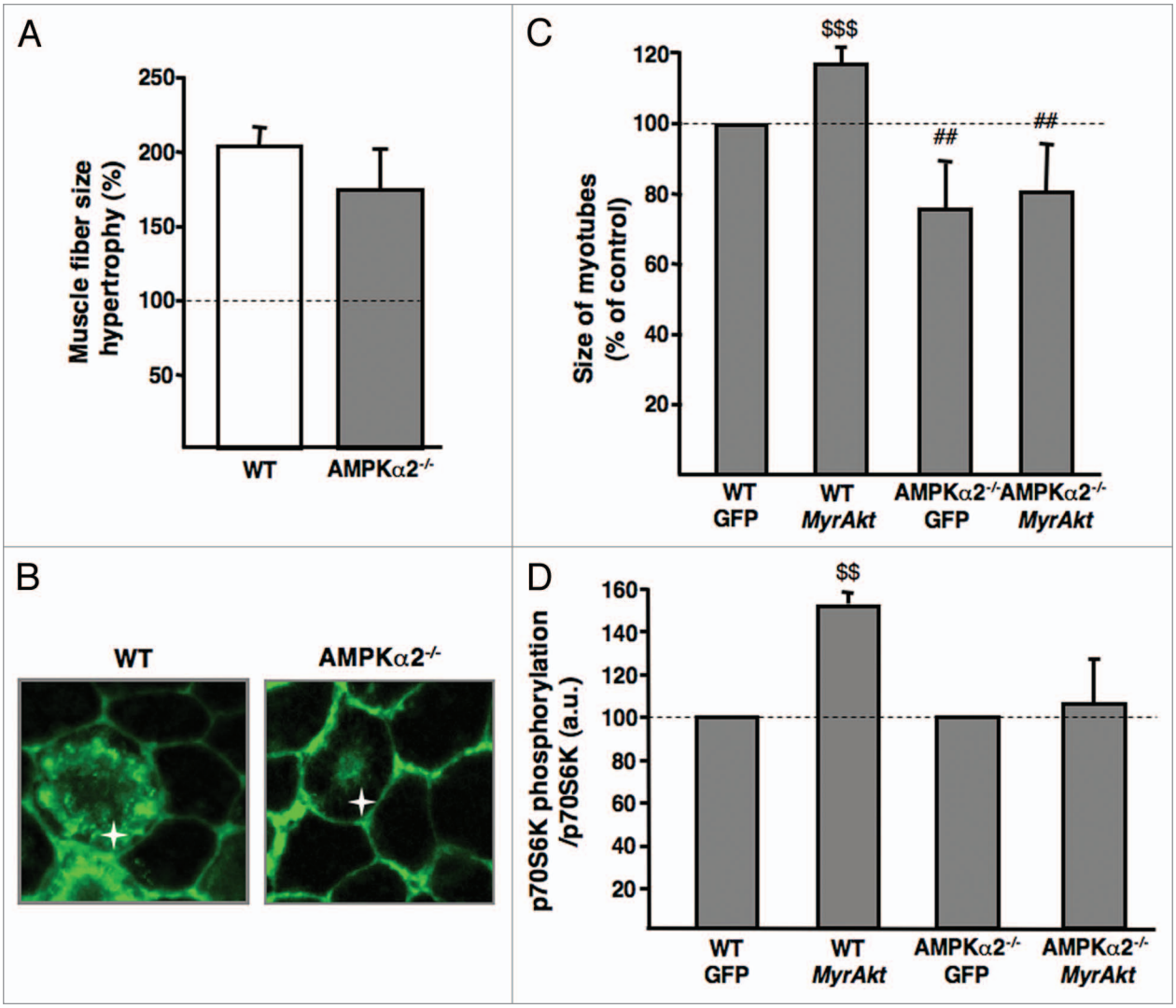

Figure 2. Cell size control in muscle cells lacking AMPKα2. (A) Skeletal muscle fiber hypertrophy is evaluated with variations in size of CSA of WT and AMPKα2$^{-/-}$ myofibers overexpressing *MyrAkt* in TA muscle, represented as percent change to non-transfected myofibers CSA. The CSA of myofibers was determined from at least four different muscle areas of four animals in each group. (B) *MyrAkt* (HA-tagged *MyrAkt*)-transfected myofibers were visualized by immunostaining with anti-HA antibody. Positive cells are marked with a star in the representative image. (C) Primary muscle cells isolated from AMPKα2$^{-/-}$ and control mice were differentiated into myotubes. WT and AMPKα2$^{-/-}$ myotubes were transduced with 75 moi GFP and *MyrAkt* adenoviruses as indicated. Size of myotubes is presented as a percentage of WT muscle cells transduced with GFP adenovirus. Size of myotubes was measured in a region where myonuclei were absent and diameter was constant. (D) ^{Thr389}p70S6K phosphorylation was determined 2 days after infection. Results are presented as a percentage of WT and AMPKα2$^{-/-}$ muscle cells transduced with GFP adenovirus, respectively. Data from four experiments on three different cultures are shown in (C and D). Results are represented as means ± SD in (A, C and D). Different from GFP cells of the same genotype, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$; Different for the same conditions of infection, $^{\#\#}p < 0.01$.

respectively; **Fig. 2C**). This was associated with a lack in increased p70S6K phosphorylation in AMPKα2-deficient mytotubes following *MyrAkt* expression (**Fig. 2D**). Taken together, these results suggest that hypertrophic action of *MyrAkt* is impaired in the absence of AMPKα2. Since growth requires ATP, one possibility is that AMPKα2 deficiency limits ATP generation for cell growth in response to *MyrAkt*-induced hypertrophy. Particularly, the ability of Akt activity to increase cell size depends on the activity of mTOR[24] and the increase in intracellular ATP level to activate the mTOR signaling.[25] It has been proposed that mTOR activity requires high intracellular ATP concentrations due to its relatively high K_m for ATP.[26] Defect in ATP production has been observed in mice expressing a kinase-dead AMPKα2 subunit in skeletal muscle following chronic energy deprivation[27] and exercise.[28] In addition, a marked energy disturbance was also reported during exercise in skeletal muscle of AMPKα2-knockout mice.[29] Clearly, more experiments are required to delineate the additional mechanism(s) by which AMPKα2 deficiency resulted in an attenuated hypertrophic response.

The catalytic AMPKα1 isoform has been shown to be preferentially activated following overload-induced hypertrophy without causing metabolic adaptations (without any change in mitochondrial mass or GLUT4 expression), suggesting

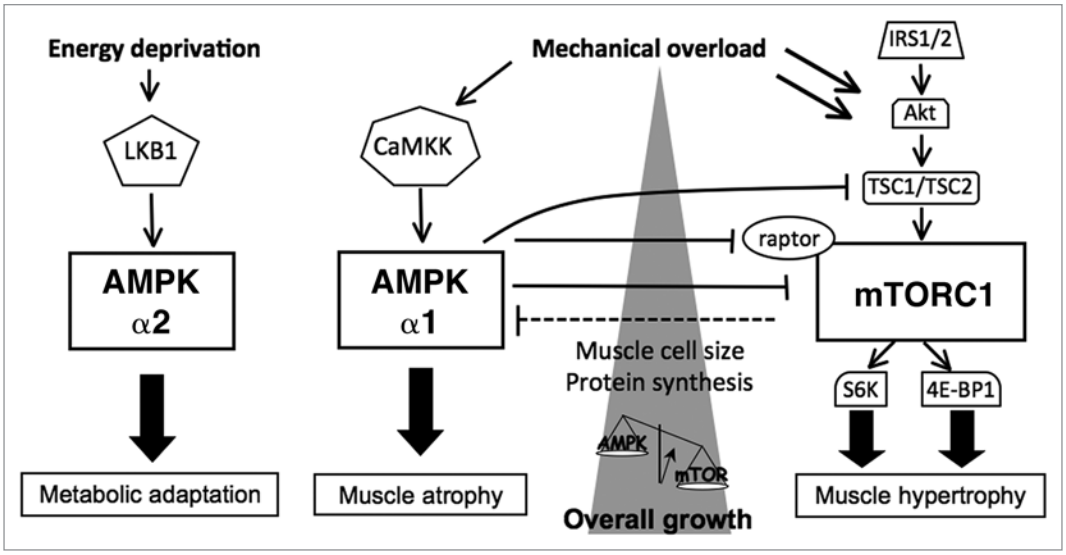

Figure 3. The AMPK/mTORC1 gauge tightly controls muscle cell size. AMPKα1 and α2 catalytic isoforms perform diverse functions in skeletal muscle. In response to energy deprivation, AMPKα2 is activated by LKB1 to regulate metabolic adaptation of skeletal muscle, whereas, in response to mechanical overload, AMPKα1 is activated by CaMKK to control muscle cell size. AMPKα1 inhibits mTORC1 signaling by the direct phosphorylation of raptor, mTOR and TSC1/TSC2, consequently shutting down protein synthesis and muscle cell growth. On the other hand, activation of mTORC1 in response to resistance exercise or under high nutritional status inhibits AMPK activity by affecting intracellular energy levels and stimulates protein synthesis by the phosphorylation of p70S6K and 4E-BP1, effectively promoting muscle cell growth.

that AMPKα1 might be more important in growth than in metabolism regulation.[3] These data indicate that AMPKα1 and α2 catalytic isoforms perform diverse functions, with AMPKα1 playing a predominant role in the control of cell size and AMPKα2 primarily involved in energy metabolism regulation. Indeed, skeletal muscle metabolic adaptation preferentially occurs through the activation of AMPKα2 rather than AMPKα1.[30-32] While AMPKα1 activity is similar in muscle from lean and obese animals and is unaffected by rosiglitazone, AMPKα2 activity was 25% lower in obese vs. lean animals and was normalized to control values after rosiglitazone treatment.[33] Abbott et al. have shown that AICAR, caffeine and muscle contraction increase AMPKα2 activity but do not affect AMPKα1 activity in skeletal muscle.[34] In contrast, Egawa et al. have shown that of the two AMPK α isoforms, AMPKα1 is most activated by caffeine in rat skeletal muscle both ex vivo and in vivo through an energy-independent mechanism associated with increased ACC phosphorylation and glucose transport. Furthermore, recent evidence indicates that AMPKα1 and AMPKα2 isoforms are differentially activated. AMPKα2 appears to be preferentially activated in response to acute exercise[35-37] or endurance training,[38] whereas low intensity contraction and oxidative stress activate AMPKα1 in skeletal muscle.[39-41] Interestingly, it has been shown that twitch contraction stimulates glucose uptake through AMPKα1 activation but not AMPKα2.[42] AMPKα1 activation was not accompanied by an increase in AMP level or AMP/ATP ratio, suggesting that AMPKα1 activation induced by low-intensity contraction is regulated by an AMP-independent[40,41] and/or LKB1-independent mechanism.[43] 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.[3,44,45] CaMKK and TAK1 could therefore be the upstream kinases responsible for AMPKα1 activation during overload-induced muscle hypertrophy.[3]

AMPK has emerged over the last decade as a central integrator of signals that control energy balance. Our results extend this notion by showing that AMPKα1 but not AMPKα2 controls muscle cell size and is involved in the cell size maintenance through the regulation of mTORC1 signaling (**Fig. 3**). The recent findings that mTORC1 regulates skeletal muscle metabolism[12] in addition to protein synthesis suggest that AMPK's metabolic effects might be mediated, at least in part, through modulation of mTORC1 activity and vice versa. It has been proposed that the mTORC1-AMPK gauge adjusts muscle plasticity to environmental signals by altering the glycolytic-oxidative properties of the muscle.[11] All muscle types seem to be equally affected by p70S6K deficiency, which induces a shift toward an oxidative metabolism without affecting myosin expression.[11,14] In contrast, oxidative muscles are the most affected by raptor deficiency and behave metabolically like fast-twitch glycolytic muscles while exhibiting structural features and contraction properties indicative of slow-twitch oxidative muscle fibers.[12] Loss of mTOR in muscle results in a myopathy similar to that observed in raptor-deficient muscles, but the observed pathology is more severe and affects all muscle types.[15] However, fast-twitch muscles are the most affected by mTOR deficiency regarding mass and cross-sectional area reduction. Similar to raptor-deficient muscles, muscles lacking mTOR have an impaired oxidative function as well as increased slow myosin expression.[15] Interestingly, AMPK also appears to be an important mediator

of training-induced muscle fiber type changes.[46] In addition, sedentary mice carrying an AMPK-activating mutation show increases in the oxidative profile of individual muscle fibers, highlighting the function of AMPK in skeletal muscle metabolism and fiber type adaptations.[46,47] Thus, these data support the view that the crosstalk between of AMPK and mTOR signaling is a highly regulated way to gauge cellular energy and nutrition content, leading to changes in growth and metabolic rates. Accordingly, it has been shown that in skeletal muscle, AMPK is activated during low-calorie diet and endurance exercise, while the mTORC1 pathway is activated during high-calorie diet and resistance exercise.[48,49] This tight control likely serves the purpose of enforcing a particular metabolic response following a particular energy stress and avoiding futile cycles in which synthesis and degradation are simultaneously activated (**Fig. 3**). Lastly, AMPK and mTOR signaling pathways act as two antagonistic forces governing adaptive programs to nutrition and exercise and may represent attractive targets for the treatment of not only the metabolic syndrome, but also sarcopenia (atrophy and cell loss).[50] The use of AMPK agonist (AICAR) and mTORC1 antagonist (rapamycin) has proven beneficial in preventing nutrient-induced insulin resistance and improving obesity-related muscle mass loss.[7,51,52] This is consistent with the notion that chronic overactivation of mTOR can lead to cell senescence driving both growth and aging.[53,54] Noteworthy, calorie restriction, which leads to AMPK activation and deactivates mTOR in skeletal muscle, also mitigates sarcopenia.[55,56] Future experiments will tell whether the crosstalk between AMPK and mTORC1 signaling can offer new therapeutic perspectives.

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