Skeletal muscle PI3K p110 β regulates expression of AMP-activated protein kinase

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Running title: *PI3K p110 β regulates AMPK expression*

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Abstract

Skeletal muscle metabolic homeostasis is maintained through numerous biochemical and physiological processes. Two principal molecular regulators of skeletal muscle metabolism include AMP-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3K); however, PI3K exists as multiple isoforms, and specific metabolic actions of each isoform have not yet been fully elucidated in skeletal muscle. Given this lack of knowledge, we performed a series of experiments to define the extent to which PI3K p110 β mediated expression and (or) activation of AMPK in skeletal muscle. To determine the effect of p110 β inhibition on AMPK expression and phosphorylation in cultured cells, C2C12 myoblasts were treated with a pharmacological inhibitor of p110 β (TGX-221), siRNA against p110 β , or overexpression of kinase-dead p110 β . Expression and phosphorylation of AMPK were unaffected in myoblasts treated with TGX-221 or expressing kinase-dead p110 β . However, expressions of total and phosphorylated AMPK at T172 were reduced in myoblasts treated with p110 β siRNA. When normalized to expression of total AMPK, phosphorylation of AMPK S485/491 was elevated in p110 β -deficient myoblasts. Similar results were observed in tibialis anterior muscle from mice with conditional deletion of p110 β (p110 β -mKO mice). Analysis of AMPK transcript expression revealed decreased expression of *Prkaa2* in p110 β -deficient myoblasts and in p110 β -mKO muscle. Loss of p110 β had no effect on oligomycin-stimulated phosphorylation of AMPK or phosphorylated Acetyl-CoA carboxylase (ACC), although oligomycin-induced AMPK and ACC phosphorylation were increased in p110 β -deficient myoblasts compared to oligomycin-stimulated control myoblasts when normalized to levels of total AMPK or ACC. Overall, these results suggest that p110 β positively regulates expression of AMPK in cultured myoblasts and in skeletal muscle in vivo; moreover, despite the reduced abundance of AMPK in p110 β -deficient

myoblasts, loss of p110 β does not appear to impair AMPK activation following stimulus. These findings thus reveal a novel role for p110 β in mediating skeletal muscle metabolic signaling.

Key Words: AMP-activated Protein Kinase (AMPK); phosphoinositide 3-kinase (PI3K); myoblast; skeletal muscle

Introduction

Metabolic homeostasis in skeletal muscle is maintained through the balanced integration of numerous biochemical, genetic, and cell-signaling processes. One well-studied molecular mediator of skeletal muscle energy metabolism is AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase that regulates cellular energy homeostasis by modulating metabolic and energetic processes [1]. AMPK is activated under conditions of low cellular energy (high AMP:ATP ratio) such as seen with strenuous physical activity [2,3,4]. The AMPK complex itself is composed of α , β , and γ subunits, each encoded by two or more genes, thus allowing for a number of heterotrimeric combinations [5]. Among these subunits, the α subunit contains the kinase domain, the β subunit contains the glycogen binding domain, and the γ subunit contains adenine nucleotide binding domains. Activation of AMPK is controlled in part by various upstream kinases that phosphorylate specific residues on the α subunit; namely, threonine 172 (T172) and serine 485 in AMPK α 1 and serine 491 in AMPK α 2 (S485/491) [6]. Phosphorylation at T172 is generally associated with activation of AMPK, whereas phosphorylation at S485/491 is generally associated with AMPK inhibition [7,8,9]. While a number of molecules that regulate AMPK expression and activation have been identified, much still remains unknown.

Other molecules that regulate skeletal muscle metabolism have been identified, including phosphoinositide 3-kinase (PI3K). Class IA PI3Ks are heterodimeric enzymes composed of a 110 kDa catalytic subunit (p110) and a 85 kDa regulatory subunit (p85) that regulate multiple cellular processes, including metabolism [10]. Three Class IA PI3K p110 catalytic isoforms have been identified in skeletal muscle (α , β , and δ) [11], and while PI3K p110 α is the principal p110 isoform regulating signaling through receptor tyrosine kinases (RTKs) such as the insulin

and IGF-I receptors [12,13], p110 β is the principal p110 isoform regulating G-protein coupled receptor (GPCR) signaling [14]. Following ligand binding to receptors, PI3K is recruited to activated RTKs or GPCRs via regulatory subunit binding to adaptor proteins. Once in proximity to inositol lipids within the cell membrane, the p110 catalytic subunit phosphorylates the D3 position of the inositol ring, thus generating phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃) from phosphatidylinositol-(4,5)-bisphosphate (PIP₂). Generation of PIP₃ then allows for recruitment and activation of signaling molecules such as Akt, whose activation regulates growth, differentiation, and metabolism [15]. In general, the role of PI3K in regulating skeletal muscle metabolism is beginning to be elucidated; however, the roles of the specific PI3K p110 catalytic subunit isoforms in regulating muscle metabolism are not well defined.

While both PI3K and AMPK are known regulators of skeletal muscle metabolism, there is currently little information linking specific PI3K p110 catalytic isoforms to AMPK expression or activation. While a recent report has shown that PI3K p110 α negatively regulates phosphorylation of AMPK at T172 and positively regulates phosphorylation of AMPK at S485/491 in C2C12 myoblasts [16], it is unknown whether the other Class IA PI3K isoforms, such as p110 β , regulate skeletal muscle AMPK in a similar fashion. We therefore performed a series of experiments designed to determine whether p110 β regulates AMPK expression and phosphorylation in skeletal muscle.

Materials and Methods

Materials and Reagents. C2C12 cells and DMSO were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal

bovine serum (FBS), penicillin/streptomycin, trypsin, Lipofectamine 2000, and Opti-mem medium were purchased from Life Technologies (Carlsbad, CA). TGX-221 was from Selleck Chemicals (Houston, TX). Antibodies for p-AMPK Thr 172 (#2535), p-AMPK Ser 485/491 (#4185), total AMPK α (#2603), p-Akt Ser 473 (#4060), total (pan) Akt (#4685), p110 β (#3011), GAPDH (#2118), α -tubulin (11H10) (#2125) and oligomycin (#9996) were purchased from Cell Signaling Technologies (Danvers, MA).

Cell culture and pharmacological inhibitors. C2C12 were grown in DMEM containing 10% FBS and 1% antibiotics at 37° C in 5% CO₂. All experiments were performed within six passages of receipt from the vendor (ATCC). TGX-221 was dissolved in DMSO and diluted in culture medium such that cells were exposed to a final DMSO concentration of 0.02%. Control cells received an equivalent concentration of DMSO.

Small-interfering RNA (siRNA). Non-targeting control siRNA (catalog number 4390483) and siRNA directed against *Pik3cb* (p110 β ; catalog number s93108) were purchased from Life Technologies. siRNA transfections were carried out as previously described [17].

Baculoviruses and transductions. Generation and transduction of baculoviral constructs were performed exactly as described previously [18].

Animals and tissue harvest. Mice with conditional deletion of *Pik3cb* in skeletal muscle ('p110 β -mKO') have been described previously [18]. Tibialis anterior muscles were removed from 21 month old p110 β -mKO and floxed littermate control mice, immediately snap-frozen in liquid nitrogen, and stored at -80°C until use. All experimental procedures utilizing mice were conducted under the guidelines on humane use and care of laboratory animals for research and in

accordance with the Animal Care and Use Committee of the US Army Research Institute of Environmental Medicine.

Protein extraction and immunoblotting. Extraction of protein from cells was performed as described [17], and protein concentrations were determined by the method of Bradford [19]. Western immunoblotting was performed exactly as described previously [18], and densitometric analysis was performed using NIH Image J 1.60 (<http://rsbweb.nih.gov/ij/>).

RNA isolation, cDNA synthesis, and real-time PCR. RNA isolation from cells and tissues and cDNA synthesis was performed as described previously [18,20] using Trizol reagent (Life Technologies). Real time PCR was performed as previously described [18] normalizing to *Hprt1* gene expression. Primers/probes for real-time PCR were purchased from Life Technologies: *Prkaa1* (Mm01296700_m1), *Prkaa2* (Mm01264789_m1), and *Hprt1* (Mm01545399_m1).

Statistics. Data are presented as mean $\pm$ S.E.M. Statistics were performed using Student's t-test or one-way ANOVA with Tukey's test performed *post hoc*. A P-value<0.05 was considered significant.

Results

Loss of PI3K p110 β is associated with altered AMPK protein expression and phosphorylation

Previous research has shown that in cultured myoblasts, PI3K p110 α negatively regulates AMPK phosphorylation at T172 and positively regulates AMPK phosphorylation at S485/491 [16]. To determine the degree to which p110 β also regulates phosphorylation of AMPK in

myoblasts, we first treated C2C12 myoblasts with TGX-221, a pharmacological inhibitor of p110 β catalytic activity [21,22]. We found that phosphorylation of AMPK at T172, phosphorylation of AMPK at S485/491, and expression of total AMPK were all unaffected by 4-hours treatment with 1 μ M TGX-221 (Figure 1A). Treatment with TGX-221 for 24-hours yielded similar results to those observed following 4-hours treatment (data not shown). Next, C2C12 myoblasts were transduced with cDNA constructs encoding either wild-type p110 β ('p110 β -WT'), kinase-inactive p110 β ('p110 β -K805R'), or vector control. Myoblasts expressing p110 β -WT or p110 β -K805R showed no differences in phosphorylation or expression of AMPK compared to myoblasts transduced with vector control (Figure 1B). Finally, myoblasts were treated with siRNA directed against p110 β . Compared to myoblasts treated with control siRNA, p110 β -deficient myoblasts possessed 52% less phosphorylated AMPK T172 ($P<0.05$) and 43% less total AMPK ($P<0.001$) when normalized to GAPDH expression (Figure 1C and 1D). Phosphorylation at S485/491 was increased 40% p110 β -deficient myoblasts as compared to control myoblasts when expressed relative to GAPDH ($P<0.05$). When phosphorylated AMPK T172 and S485/491 were normalized to total AMPK, there was no difference in T172 phosphorylation between control and p110 β -deficient myoblasts; however, phosphorylation at S485/491 was increased ~2.5-fold in cells deficient in p110 β compared to control cells (Figure 1E).

Phosphorylation of AMPK at S485/491 has been shown to be mediated by Akt [8,16], and p110 β -deficient myoblasts have previously been shown to possess increased levels of phosphorylated Akt compared to control myoblasts [23]. As figures 1C and 1F show, there was a 76% increase in Akt phosphorylation at S473 in p110 β -deficient myoblasts compared to control myoblasts ($P<0.01$), suggesting that the increased AMPK S485/491 phosphorylation is

secondary to increased Akt activation. All together, these data reveal that inhibition of p110 β catalytic activity alone (through pharmacological inhibition or overexpression of kinase inactive p110 β) is not sufficient to alter phosphorylation or expression of AMPK. Instead, it is the loss of p110 β protein (through siRNA directed against p110 β) that is associated with these phenomena.

To assess whether these cell-based observations also occurred in vivo, we analyzed levels of phosphorylated and total AMPK in tibialis anterior (TA) muscles of mice with conditional deletion of p110 β in skeletal muscle (p110 β -mKO) [18]. Similar to our observations in p110 β -deficient C2C12 cells, total and phosphorylated AMPK at T172 were each reduced by 37% in muscles of p110 β -mKO mice compared to floxed controls when normalized to α -tubulin ($P < 0.05$ for both; Figure 1G and 1H). There was no significant difference in phosphorylation of AMPK at S485/491 between p110 β -mKO and p110 β -flox TA when normalized to α -tubulin; however, when normalized to total AMPK, TA muscles from p110 β -mKO mice showed a 2.4-fold increased AMPK S485/491 expression compared to TA muscles from p110 β -flox mice ($P < 0.01$; Figure 1I). These data reveal that, similar to cultured p110 β -deficient myoblasts, AMPK phosphorylation and expression are modified in skeletal muscle lacking p110 β in vivo.

mRNA expression of Prkaa2 is decreased in p110 β -deficient myoblasts and p110 β -mKO muscle

We next asked whether the reduced AMPK protein expression observed in p110 β -deficient myoblasts and in p110 β -null mouse skeletal muscle was associated with reduced expression of AMPK RNA transcripts. The α -subunit of AMPK is encoded by two genes: AMPK α 1 (*Prkaa1*) and AMPK α 2 (*Prkaa2*). We found that expression of *Prkaa2*, but not

Prkaa1, was reduced by 19% in p110 β -deficient myoblasts as compared to control myoblasts (P<0.001; Figure 2A). Moreover, expression of *Prkaa2* was reduced by 9% in TA muscle of p110 β -mKO mice as compared to p110 β -flox control mice (P<0.05; Figure 2B).

Activation of AMPK is not impaired in p110 β -deficient myoblasts.

Given the altered phosphorylation and reduced abundance of AMPK in unstimulated p110 β -deficient myoblasts and in p110 β -mKO mouse skeletal muscle, we next asked whether stimulation of AMPK was affected by p110 β loss. To test this, we treated control or p110 β -deficient myoblasts with oligomycin, a known activator of AMPK. Oligomycin stimulated AMPK T172 phosphorylation to similar degrees in both control myoblasts and in p110 β -deficient myoblasts when normalized to GAPDH expression (~1.6-fold and ~1.8-fold for control and p110 β -deficient myoblasts, respectively) (Figure 3A and 3B). Additionally, oligomycin-induced phosphorylation of AMPK S485/491 was increased ~1.6-fold in control myoblasts (P<0.05) and ~2.2-fold in p110 β -deficient myoblasts (P<0.001) when normalized to GAPDH (Figure 3A and 3B). Levels of total AMPK did not differ between control cells treated with oligomycin and control cells treated with solvent; however, in p110 β -deficient myoblasts, oligomycin treatment further reduced expression of total AMPK beyond that seen with p110 β deficiency alone (si-p110 β : 0.54; si-p110 β +Oligo: 0.29; P<0.05) (Figure 1A and 1C). When normalized to expression of total AMPK, levels of phosphorylated AMPK at T172 and S485/491 were increased ~6-fold and ~7.9-fold, respectively, in oligomycin-stimulated p110 β -deficient myoblasts as compared to control myoblasts (P<0.001 for each; Figure 3C).

To determine whether activation of AMPK was altered in response to oligomycin, we examined the expression and phosphorylation of acetyl-CoA carboxylase (ACC), a direct substrate of AMPK. We found that oligomycin treatment increased phosphorylation of ACC at S79 by ~2.7-fold in both control myoblasts and in p110 β -deficient myoblasts when normalized to expression of GAPDH ($P<0.05$ for each) (Figure 3A and 3D). Interestingly, we also observed a 35% and a 45% decrease in expression of total ACC in unstimulated and in oligomycin-treated p110 β -deficient myoblasts, respectively, when normalized to GAPDH expression ($P<0.05$ for each). When normalized to expression of total ACC, levels of phosphorylated ACC at S79 were increased 5.4-fold in oligomycin-stimulated p110 β -deficient myoblasts as compared to control myoblasts ($P<0.01$; Figure 3D). These data demonstrate that oligomycin-stimulated phosphorylation and activation of AMPK is not impaired in p110 β -deficient myoblasts, even though these cells possess significantly less total AMPK than control myoblasts.

Discussion

The role of PI3K in regulating skeletal muscle metabolism is only beginning to be defined. In this study, we found that PI3K p110 β regulated expression of phosphorylated and total AMPK in cultured myoblasts and in mouse skeletal muscle. Of significant interest were our findings that AMPK phosphorylation was not modified in C2C12 myoblasts treated with TGX-221 or expressing kinase-dead (K805R) p110 β , but that AMPK phosphorylation was modified in myoblasts treated with siRNA against p110 β (Figure 1). These distinct findings may be explained by the methods used to inhibit p110 β ; in particular, the manner in which these methods affect the abundance of the p110 β protein itself. Indeed, while pharmacological

inhibition of p110 β inhibits p110 β catalytic activity, it does not affect total p110 β protein levels. Likewise, over-expression of kinase-dead p110 β results in a greater number of p110 β molecules overall due to the expression of endogenous p110 β in addition to the ectopically-expressed p110 β . However, targeting p110 β by means of siRNA reduces not only p110 β molecules, but also the kinase activity associated with the molecules subjected to the knockdown. Thus, of these three experimental manipulations (chemical inhibition, ectopic expression, and siRNA), only siRNA reduces target protein abundance below endogenous levels. Given the properties of these three experimental manipulations, and given that only siRNA treatment was associated with altered AMPK phosphorylation and expression, our observations suggest that p110 β may regulate AMPK through a mechanism independent of its kinase activity in myoblasts.

It has been previously shown that loss of p110 β protein results in compensatory upregulation of p110 α and Akt activities in C2C12 myoblasts [23]. It is this p110 α hyper-activation that likely underlies the decreased phosphorylation of AMPK T172 and increased phosphorylation of AMPK S485/491 we observed in p110 β -deficient myoblasts (Figure 1). Indeed, it has been reported that phosphorylation of AMPK T172 was reduced and phosphorylation of AMPK S485/491 was increased in C2C12 myoblasts expressing a constitutively-active mutant of p110 α [16]. Together, these data suggest that the altered phosphorylation of AMPK in p110 β -deficient myoblasts is modulated principally through p110 α hyper-activation resulting from the reduced expression of p110 β . Despite this similarity between p110 β -deficient myoblasts and myoblasts expressing constitutively-active p110 α in modulating AMPK phosphorylation, we found that total AMPK protein was reduced in p110 β -deficient myoblasts - an effect not observed in myoblasts expressing constitutively-active p110 α [16]. This observation supports the existence of a p110 α -independent and p110 β -specific action on

AMPK. The reduction in AMPK protein appears to be dependent on the abundance of p110 β protein and not p110 β catalytic activity, as AMPK protein levels were unaffected by pharmacological inhibition or over-expression of p110 β cDNA constructs.

To further study the mechanism underlying the reduced total AMPK protein expression in p110 β -deficient myoblasts and mouse skeletal muscle, we asked whether the reduced total AMPK protein expression might result from reduced transcript expression of AMPK α RNA. To this end, we examined expression of *Prkaa1* and *Prkaa2* in p110 β -deficient myoblasts and in muscles of mice with conditional deletion of p110 β in skeletal muscle. Our findings revealed that the mRNA abundances of *Prkaa2* were significantly reduced in p110 β -deficient C2C12 cells and in muscles of p110 β -mKO mice (Figure 2). However, this reduced expression was quite modest (19% in p110 β -deficient cells and 9% in p110 β mKO muscle), and may not fully explain the ~50% reduced expression of AMPK protein. This discrepancy between the small reductions in mRNA and larger reductions in AMPK protein may result from post-transcriptional events (such as a reduction in the rate of nuclear export of AMPK transcripts), or from translational or post-translational events affecting the rate of protein synthesis, stability, or turnover. In any case, previous evidence suggests that mRNA levels may explain only ~40% of the variability in protein levels globally [24]; thus, while our data clearly show that p110 β regulates expression of AMPK, further research must be undertaken to establish the mechanism(s) underlying this phenomenon.

In response to oligomycin treatment, phosphorylation of AMPK at T172 and at S485/491 were increased to similar degrees in p110 β -deficient myoblasts as they were in control myoblasts, even though p110 β -deficient myoblasts possessed less total AMPK protein than did control myoblasts (Figure 3). This suggests that loss of p110 β has no effect on oligomycin-

stimulated AMPK phosphorylation. However, a second and equally valid interpretation of these findings is that oligomycin-induced phosphorylation of AMPK is actually *increased* compared to control myoblasts treated with oligomycin because these phosphorylated AMPK molecules represent a greater proportion of total AMPK molecules in p110 β -deficient myoblasts than they do in control myoblasts (Figure 3C). It could therefore be argued that phosphorylation of AMPK is more sensitive to oligomycin treatment in p110 β -deficient myoblasts than in control myoblasts. Such an increased response to oligomycin in p110 β -deficient myoblasts could be caused by stimulation of an AMPK kinase or inhibition of an AMPK phosphatase, although further study is needed to determine the exact mechanism regulating the increased oligomycin-induced AMPK phosphorylation (as a function of total AMPK) in myoblasts lacking p110 β .

In conclusion, our data suggest that p110 β positively regulates expression of AMPK in cultured myoblasts and in mouse skeletal muscle, possibly through a mechanism independent of its catalytic activity. Despite the reduced abundance of AMPK in p110 β -deficient myoblasts, phosphorylation of AMPK was sensitive to oligomycin to the same degree as control myoblasts, indicating that p110 β deficiency did not impair AMPK activation in myoblasts. In all, we have identified a novel role for PI3K p110 β in regulating AMPK expression and phosphorylation in skeletal muscle, thus identifying p110 β as a contributor to metabolic signaling. This information may ultimately be used to inform treatment strategies to optimize skeletal muscle health during disease states.

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Figure Legends

Figure 1. Reduced expression of AMPK in p110 β -deficient myoblasts and in mouse skeletal muscle lacking p110 β . (A) C2C12 myoblasts were treated with DMSO or 1 μ M TGX-221 for 4-hours. Western blotting was performed on lysates using the indicated antibodies. (B) Myoblasts were transduced with vector control ('Vector'), wild-type p110 β ('p110 β -WT'), or kinase-inactive p110 β ('p110 β -K805R') as described in the Methods section. Lysates were immunoblotted with the indicated antibodies. Blots are representative of four independent experiments. (C) Myoblasts were treated with 5 nM non-targeting control siRNA ('si-Con') or 5 nM siRNA directed against p110 β ('si-p110 β ') as described in Methods. Lysates were immunoblotted with the indicated antibodies. Blots are representative of three independent experiments. (D) Quantification of phosphorylated and total AMPK normalized to GAPDH (shown in panel C) (means $\pm$ SEMs; n = 3; asterisks indicate significant difference from si-Con myoblasts; *, P<0.05; ***, P<0.001). (E) Quantification of phosphorylated AMPK normalized to expression of total AMPK (all shown in panel C) (means $\pm$ SEMs; n = 3; asterisks indicate significant difference from si-Con myoblasts; *, P<0.05). (F) Quantification of phosphorylated Akt normalized to expression of total Akt shown in panel C (means $\pm$ SEM; n = 3; asterisks indicate significant difference from si-Con myoblasts; **, P<0.01). (G) Western immunoblotting was performed on lysates from TA muscle from p110 β -flox and p110 β -mKO mice using the indicated antibodies. (H) Quantification of phosphorylated and total AMPK normalized to α tubulin (all shown in panel G) (means $\pm$ SEMs; n = 9 p110 β -flox and 11 p110 β -mKO; asterisks indicate significant difference from p110 β -flox; *, P<0.05). (I) Quantification of phosphorylated AMPK normalized to total AMPK (all shown in panel G) (means $\pm$ SEMs; n = 9

p110 β -flox and 11 p110 β -mKO; asterisks indicate significant difference from p110 β -flox; **, P<0.01).

Figure 2. Expression of *Prkaa1* and *Prkaa2* in p110 β -deficient myoblasts and in TA muscle of p110 β -mKO mice.

(A) Real-time PCR was performed on cDNA derived from control or p110 β -deficient myoblasts using the indicated primers/probes (means $\pm$ SEMs; n = 3; asterisks indicate significant difference from si-Con-treated myoblasts; ***, P<0.001). (B) Real-time PCR was performed on cDNA derived from TA muscle of p110 β -flox or p110 β -mKO mice using the indicated primers/probes (means $\pm$ SEMs; n = 6 p110 β -flox and 7 p110 β -mKO; asterisks indicate significant difference from p110 β -flox; *, P<0.05).

Figure 3. Effects of oligomycin on AMPK activation in p110 β -deficient myoblasts.

(A) Control or p110 β -deficient myoblasts were treated with 0.5 μ M oligomycin for 30-min, harvested for protein lysates, and Western blotting performed using the indicated antibodies. Blots are representative of three independent experiments. (B) Quantification of phosphorylated and total AMPK normalized to GAPDH from panel A (means $\pm$ SEMs; n = 3; one-way ANOVA with Tukey test; asterisks indicate significant difference from non-targeting siRNA-treated control ('si-Con') myoblasts *, P<0.05; **, P<0.01; ***, P<0.001). (C) Quantification of phosphorylated AMPK normalized to expression of total AMPK from panel A (means $\pm$ SEMs; n = 3; one-way ANOVA with Tukey test; asterisks indicate significant difference from control (si-Con) myoblasts ***, P<0.001). (D) Quantification of phosphorylated ACC normalized to expression of GAPDH and to total ACC from panel A (means $\pm$ SEMs; n = 3; one-way ANOVA with Tukey test; asterisks indicate significant difference from control (si-Con) myoblasts; *, P<0.05; **, P<0.01).

A **B** **C** **D** **E** **F** **G** **H** **I**

A **B**

A

B

C

D