

**AMP-Activated Protein Kinase  $\alpha$ 1 but Not  $\alpha$ 2 Catalytic Subunit Potentiates Myogenin Expression and Myogenesis**

Xing Fu, Jun-Xing Zhao, Mei-Jun Zhu, Marc Foretz, Benoit Viollet, Mike V. Dodson and Min Du  
*Mol. Cell. Biol.* 2013, 33(22):4517. DOI:  
10.1128/MCB.01078-13.  
Published Ahead of Print 16 September 2013.

---

Updated information and services can be found at:  
<http://mcb.asm.org/content/33/22/4517>

---

*These include:*

**REFERENCES**

This article cites 41 articles, 19 of which can be accessed free at: <http://mcb.asm.org/content/33/22/4517#ref-list-1>

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](#)

---

---

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>  
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

---

# AMP-Activated Protein Kinase $\alpha$ 1 but Not $\alpha$ 2 Catalytic Subunit Potentiates Myogenin Expression and Myogenesis

Xing Fu,<sup>a</sup> Jun-Xing Zhao,<sup>b</sup> Mei-Jun Zhu,<sup>c</sup> Marc Foretz,<sup>d,e</sup> Benoit Viollet,<sup>d,e,f</sup> Mike V. Dodson,<sup>a</sup> Min Du<sup>a</sup>

Department of Animal Sciences, Washington State University, Pullman, Washington, USA<sup>a</sup>; College of Animal Science and Veterinary Medicine, Shanxi Agriculture University, Taigu, Shanxi, China<sup>b</sup>; School of Food Sciences, Washington State University, Pullman, Washington, USA<sup>c</sup>; INSERM U1016, Institut Cochin, Paris, France<sup>d</sup>; Université Paris Descartes, Sorbonne Paris Cité, Paris, France<sup>e</sup>; CNRS UMR 8104, Paris, France<sup>f</sup>

**The link between AMP-activated protein kinase (AMPK) and myogenesis remains poorly defined. AMPK has two catalytic  $\alpha$  subunits,  $\alpha$ 1 and  $\alpha$ 2. We postulated that AMPK promotes myogenesis in an isoform-specific manner. Primary myoblasts were prepared from AMPK knockout (KO) mice and AMPK conditional KO mice, and knockout of the  $\alpha$ 1 but not the  $\alpha$ 2 subunit resulted in downregulation of myogenin and reduced myogenesis. Myogenin expression and myogenesis were nearly abolished in the absence of both AMPK $\alpha$ 1 and AMPK $\alpha$ 2, while enhanced AMPK activity promoted myogenesis and myotube formation. The AMPK $\alpha$ 1-specific effect on myogenesis was likely due to the dominant expression of  $\alpha$ 1 in myoblasts. These results were confirmed in C2C12 cells. To further evaluate the necessity of the AMPK $\alpha$ 1 subunit for myogenesis *in vivo*, we prepared both DsRed AMPK $\alpha$ 1 knockout myoblasts and enhanced green fluorescent protein (EGFP) wild-type myoblasts, which were cotransplanted into tibialis anterior muscle. A number of green fluorescent muscle fibers were observed, showing the fusion of engrafted wild-type myoblasts with muscle fibers; on the other hand, very few or no red muscle fibers were observed, indicating the absence of myogenic capacity of AMPK $\alpha$ 1 knockout myoblasts. In summary, these results indicate that AMPK activity promotes myogenesis through a mechanism mediated by AMPK $\alpha$ 1.**

Skeletal muscle, which comprises about 40% of the body mass of adults, is the main peripheral tissue responsive to insulin-stimulated uptake of glucose (1) and is critical in the development of type 2 diabetes (2). Proper myogenesis is critical for fetal muscle development (3–5) and postnatal muscle growth and regeneration, which relies heavily on the myogenic differentiation of satellite cells (6–8). In improper myogenesis and muscle regeneration, damaged muscle fibers are replaced with fibric tissue, leading to muscle atrophy and aging (9–11).

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme, composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, which plays an important role in energy metabolism (12–14). In addition to its capacity to acutely regulate the activity of metabolic enzymes through phosphorylation, AMPK is increasingly recognized for its regulatory role in gene expression, cell differentiation, and tissue development (15, 16). The role of AMPK in muscle fiber atrophy has been well defined; a number of studies demonstrated that AMPK promotes muscle protein degradation and autophagy (17) and inhibits protein synthesis (18). To date, however, the role of AMPK in myogenesis (formation of muscle fibers) has been sparsely studied. Our previous studies showed that low AMPK activity due to obesity is correlated with attenuated myogenic differentiation during fetal muscle development (19–21). We further observed that AMPK promotes myogenin expression and myogenesis through phosphorylation of histone deacetylase 5 (HDAC5) (22), suggesting that AMPK has a critical role in myogenesis.

The catalytic  $\alpha$  subunit of AMPK has two isoforms,  $\alpha$ 1 and  $\alpha$ 2, which display differential expression during myogenic cell differentiation (23). Knockout (KO) of either the  $\alpha$ 1 or  $\alpha$ 2 subunit results in visually normal mice, but KO of both  $\alpha$ 1 and  $\alpha$ 2 subunits is lethal at around embryonic day 9.5 (E9.5) (24), showing that  $\alpha$ 1 and  $\alpha$ 2 subunits have compensatory roles in regulating fetal growth and development. We found that AMPK $\alpha$ 1 KO but not AMPK $\alpha$ 2 KO is associated with reduced muscle mass (22),

which points to the isoform-specific role of AMPK in myogenesis. However, there is no direct evidence detailing such an effect. Here we demonstrate that AMPK $\alpha$ 1 but not AMPK $\alpha$ 2 has a major regulatory role in myogenic differentiation, and AMPK $\alpha$ 1 KO results in a dramatic reduction in myogenesis.

## MATERIALS AND METHODS

**Mice.** All animals were handled in accordance with protocols approved by the Animal Use and Care Committees of Washington State University and the University of Wyoming. Wild-type (WT) C57BL/6 mice, enhanced green fluorescent protein (EGFP) mice [B6-Tg(CAG-EGFP)131Osb/LeySop], catalog number 006567], red fluorescent (DsRed) mice [B6.Cg-Tg(CAG-DsRed\*MST)1Nagy/J, catalog number 006015], and B6;129-Gt(ROSA)26Sor<sup>tm1(Cre/ERT)Nat</sup>/J mice (catalog number 004847), in which tamoxifen-inducible Cre recombinase is driven by the endogenous mouse Gt(ROSA)26Sor promoter, were obtained from the Jackson Laboratory (Bar Harbor, ME). AMPK $\alpha$ 1<sup>-/-</sup> 129S2/SvPas (AMPK $\alpha$ 1 KO) and AMPK $\alpha$ 2<sup>-/-</sup> C57BL/6 (AMPK $\alpha$ 2 KO) mice were generated as previously described (25, 26). Mice with AMPK $\alpha$ 1 floxed, mice with AMPK $\alpha$ 2 floxed, and mice with both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 floxed were generated as previously described (26) and cross-bred with tamoxifen-inducible Cre mice to generate AMPK conditional KO mouse strains in which AMPK $\alpha$ 1, AMPK $\alpha$ 2, or both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 can be deleted in response to tamoxifen. Red fluorescent (DsRed) mice [B6.Cg-Tg(CAG-DsRed\*MST)1Nagy/J, catalog number 006015; Jackson Laboratory] were cross-bred with AMPK $\alpha$ 1 conditional KO mice to generate DsRed-labeled AMPK $\alpha$ 1 conditional KO mice.

Received 19 August 2013 Returned for modification 3 September 2013

Accepted 9 September 2013

Published ahead of print 16 September 2013

Address correspondence to Min Du, min.du@wsu.edu.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/MCB.01078-13

**Antibodies and chemicals.** Antibodies against AMPK $\alpha$  (catalog number 2532), phospho-AMPK $\alpha$  at Thr172 (number 2535), acetyl coenzyme A carboxylase (ACC) (catalog number 3662), phospho-ACC at Ser79 (catalog number 3661), tag (catalog number 2368), mouse IgG (catalog number 7076) and  $\beta$ -tubulin (catalog number 2146 and goat anti-mouse antibody–Alexa Fluor 555 (catalog number 4409), goat anti-rat antibody–Alexa Fluor 488 (catalog number 4416), and goat anti-rabbit antibody–Alexa Fluor 555 (catalog number 4413) were purchased from Cell Signaling (Danvers, MA). Antimyogenin (F5D), anti-embryonic myosin heavy chain (anti-EMH) (F1.652), and anti-myosin heavy chain (anti-MHC) (MF20) mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit antidesmin antibody (ab15200) and chick anti-GFP antibody (ab13970) were purchased from Abcam (Cambridge, MA). Rat antilaminin antibody (4H8-2) was purchased from Enzo (Farmingdale, NY). Rabbit anti-DsRed antibody (catalog number 632496) was purchased from Clontech (Mountain View, CA). Goat anti-chick antibody–Alexa Fluor 488 (catalog number A11039) was purchased from Life Technologies (Grand Island, NY). IRDye 800CW–goat anti-rabbit secondary antibody and IRDye 680–goat anti-mouse secondary antibody were purchased from Li-Cor Biosciences (Lincoln, NE). Puromycin, compound C, cardiotoxin (CTX), and 4-hydroxytamoxifen (TM) were purchased from Sigma (St. Louis, MO). MC1568 was purchased from Selleck (Houston, TX). AICAR (5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribose nucleoside) (5 mM) was purchased from Toronto Research Chemicals (Toronto, Canada). Lipofectamine was purchased from Invitrogen (Carlsbad, CA). Basic fibroblast growth factor (FGF2) (catalog number 233-FB-025) was purchased from R&D Systems (Minneapolis, MN).

**Cell culture.** Myogenic C2C12 cells were grown at 37°C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a 1% antibiotic mixture. Primary myoblasts were extracted from neonatal mice according to a procedure described previously, with modifications (27). Briefly, muscle from hind limbs was minced and digested in DMEM with collagenase D and dispase II (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for about 30 min. The slurry was then passed through a 100- $\mu$ m cell strainer. Cells were collected by centrifugation at 350  $\times$  g for 5 min. The cell pellet was then resuspended in F-10 medium with 20% FBS, a 1% antibiotic mixture, and 5 ng/ml FGF2. Primary myoblasts were seeded onto collagen-coated plates and enriched by preplating. The purity of enriched primary myoblasts was checked by fluorescence-activated cell sorter (FACS) analysis using an antidesmin antibody. When cells reached 80% confluence, culture medium was switched to DMEM supplemented with 2% horse serum and a 1% antibiotic mixture to induce myogenic differentiation. AMPK conditional KO cells were supplemented with 1  $\mu$ M 4-hydroxytamoxifen for 48 h to KO the AMPK $\alpha$  subunit.

**Immunoblotting analyses.** Immunoblotting analysis was performed as previously described, using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) (28). Band density was normalized to  $\beta$ -tubulin content.

**Real-time quantitative PCR.** Total RNA was extracted by using TRIzol (Sigma, St. Louis, MO) followed by DNase (NEB, Ipswich, MA) treatment, and cDNA was synthesized by using a reverse transcription kit (Bio-Rad, Hercules, CA). Real-time PCR (RT-PCR) was carried out by using a CFX RT-PCR detection system (Bio-Rad) with a SYBR green RT-PCR kit from Bio-Rad (Hercules, CA). The following cycle parameters were used: 34 three-step cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s. Primer sequences (with their respective PCR fragment lengths in parentheses) were as follows: forward primer 5'-GAGATCCTGCGCAGCGCCAT-3' and reverse primer 5'-CCCCGCTCTGTAGCGGAGA-3' for myogenin (97 bp), forward primer 5'-TGCTGTCCCTGTATGCCTCT-3' and reverse primer 5'-TGTAGCCACGCTCCGGTCA-3' for 18S rRNA (110 bp), forward primer 5'-CTCAGTTCCTGGAGAAAGATG-3' and reverse primer 5'-CTGCCGTTGAGTATCTTACAC-3' for AMPK $\alpha$ 1 (174 bp), forward primer 5'-CATGGCTGAGAAAGCAGAAGC

AC-3' and reverse primer 5'-CTTAAGTCCACTTTATGGCCTG-3' for AMPK $\alpha$ 2 (136 bp), forward primer 5'-GTGGACCCCTACAGCTACAAACC-3' and reverse primer 5'-TGGAAGAAAGGCGCTGAAGAC-3' for MRF4 (181 bp), forward primer 5'-TCTGGAGCCCTCCTGGCACC-3' and reverse primer 5'-CGGAAGGGGGAGAGTGGGG-3' for MyoD (100 bp), and forward primer 5'-AAACTCCGGGAGCTCCGCT-3' and reverse primer 5'-GGCAGCCGTCCGTTCATGTCC-3' for Myf5 (125 bp). After amplification, a melting curve (0.01°C/s) was used to confirm product purity, and agarose gel electrophoresis was performed to confirm that only a single product of the right size was amplified. Relative mRNA content was normalized to 18S rRNA content.

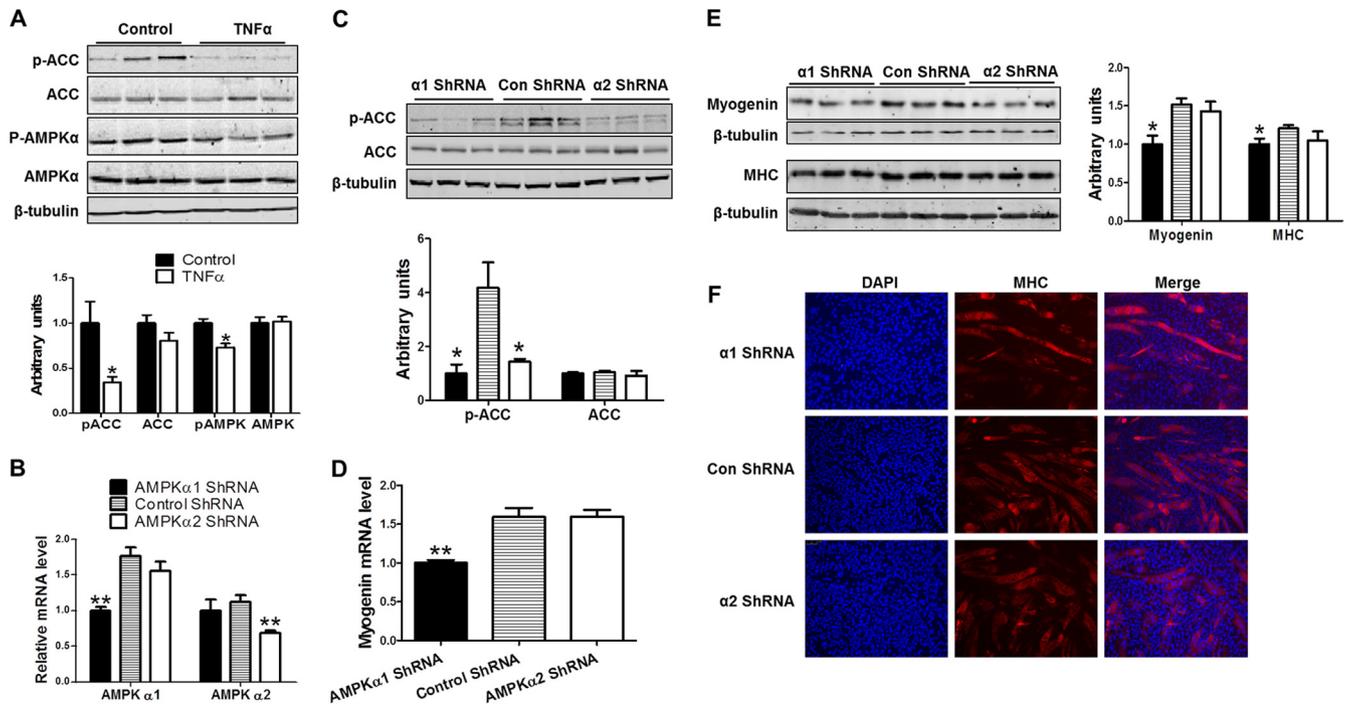
**Transfection.** Plasmid transfection of C2C12 cells was performed by using Lipofectamine according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Briefly, 12 h before transfection, cells were switched to medium without antibiotics. Transfections were carried out when cells reached 80% confluence, using a 1:3 ratio of DNA ( $\mu$ g) to Lipofectamine ( $\mu$ l); medium was switched to DMEM containing 10% FBS and 1% antibiotics 12 h following transfection.

For short hairpin RNA (shRNA) interference, AMPK $\alpha$ 1 shRNA, AMPK $\alpha$ 2 shRNA, and control shRNA (Santa Cruz Biotech, Santa Cruz, CA) were delivered into cells, and transfected cells were selected with puromycin (2  $\mu$ g/ml).

**Induction of muscle regeneration, fluorescent myogenic cell transplantation, and *in vivo* tracing.** Myogenic cells were separated from neonatal mice. DsRed AMPK $\alpha$ 1 conditional KO myoblasts were treated with tamoxifen for 48 h to induce AMPK $\alpha$ 1 KO. WT EGFP myoblasts were treated simultaneously. Because in these mice, both DsRed and EGFP are under the control of a chicken  $\beta$ -actin promoter and the overall transgenic constructs between these two fluorescent mice are very similar and are in the same C57BL/6 background, DsRed and EGFP transgenesis does not affect the myogenic capacity of myogenic cells. Three-month-old C57BL/6 mice were used as the recipient mice. Briefly, 1 day before cell transplantation, recipient mice were anesthetized by administration of a mixture of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg) (intraperitoneally [i.p.]). Next, 100  $\mu$ l of 10  $\mu$ M cardiotoxin was injected into the tibialis anterior (TA) muscle to induce muscle regeneration (9). After 1 day, mice were ready for cell transplantation; 6  $\times$  10<sup>4</sup> cells (3  $\times$  10<sup>4</sup> WT green cells and 3  $\times$  10<sup>4</sup> AMPK $\alpha$ 1 KO red fluorescent cells, which were mixed before injection) in 50  $\mu$ l phosphate-buffered saline (PBS) were slowly injected into the TA muscle by using a 29-gauge needle (9, 29). Fourteen days after transplantation, mice were sacrificed, and TA muscle was collected, fixed in 4% paraformaldehyde, and frozen in isopentane cooled in liquid nitrogen. Frozen tissue was sectioned (5 to 10  $\mu$ m thick). Sections were blocked in 5% goat serum in Tris-buffered saline (TBS) containing 0.3% Triton X-100 and stained with chick anti-GFP antibody (1:1,000), rabbit anti-DsRed antibody (1:500), and the corresponding fluorescent secondary antibodies. Green fluorescent muscle fibers and red fluorescent muscle fibers on sections at 100- $\mu$ m intervals were counted.

**Immunocytochemical staining.** Cells grown on coverslips or multiple-well plates were fixed in 4% paraformaldehyde for 10 min, permeabilized with cold methanol for 5 min, quenched with 0.1% sodium borohydride for 5 min, and incubated with anti-MHC or mouse IgG (1:100) at 4°C overnight. Fluorescent secondary antibody (1:1,000) was then added, and stained cells were incubated at room temperature for 1 h. Fluorescence was examined by using a Leica inverted microscope (28). Fusion indexes of differentiating myoblasts were calculated by dividing the number of nuclei in MHC-positive myotubes by the total number of nuclei.

**Statistics.** For all studies, at least 3 independent experiments were conducted. All data are expressed as means  $\pm$  standard errors of the mean (SEM). Data were analyzed by using the general linear model (GLM) of SAS (SAS Institute Inc., Cary, NC), and Tukey's Studentized range test was used to determine the significance of differences among means. A *P* value of <0.05 was considered significant.



**FIG 1** AMP-activated protein kinase knockdown reduces myogenesis in C2C12 cells. (A) C2C12 cells were treated with TNF- $\alpha$  (10 ng/ml) or vehicle (PBS) for 6 h, and AMPK, ACC, phospho-AMPK (p-AMPK), and phospho-ACC (p-ACC) protein contents were analyzed by immunoblotting. (B to F) Endogenous AMPK $\alpha$ 1 and AMPK $\alpha$ 2 were knocked down by shRNA in C2C12 cells. (B) AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA contents were analyzed by RT-PCR. (C) ACC and p-ACC protein contents were analyzed by immunoblotting. (D) Myogenin mRNA content (2 days after induction of myogenesis). (E) Myogenin and MHC protein contents (3 days and 6 days after induction of myogenesis, respectively). (F) Immunocytochemical staining of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 knockdown C2C12 cells using an anti-MHC antibody 6 days after induction of myogenesis. Data shown are means  $\pm$  SEM ( $n \geq 3$ ).  $\alpha$ 1, AMPK $\alpha$ 1;  $\alpha$ 2, AMPK $\alpha$ 2; DAPI, 4',6-diamidino-2-phenylindole. \*,  $P < 0.05$  versus the control; \*\*,  $P < 0.01$  versus the control.

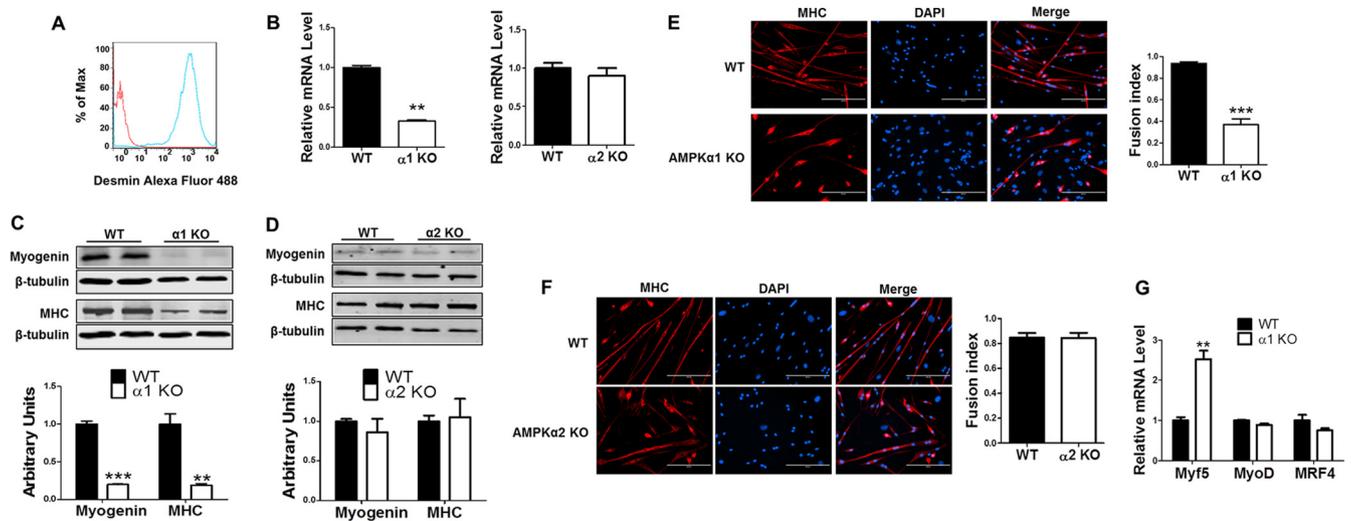
**RESULTS**

**AMPK $\alpha$ 1 inhibition attenuates myogenesis in C2C12 cells.** The reduction of AMPK activity has significant physiological relevance, because obesity and its associated chronic low-grade inflammation reduce AMPK activity via elevating tumor necrosis factor alpha (TNF- $\alpha$ ) levels (30, 31). To test if TNF- $\alpha$  inhibits AMPK in myoblasts, we treated C2C12 cells with TNF- $\alpha$  (10 ng/ml). TNF- $\alpha$  treatment reduced AMPK phosphorylation at Thr172, a marker of AMPK activity. We further analyzed phosphorylation of Ser79 in acetyl coenzyme A carboxylase (ACC), a site exclusively phosphorylated by AMPK, and found that, indeed, ACC Ser79 phosphorylation was reduced in cells treated with TNF- $\alpha$  (Fig. 1A).

To assess the relationship between decreased AMPK activity and myogenesis, we stably transfected C2C12 cells with plasmids expressing either AMPK $\alpha$ 1 or AMPK $\alpha$ 2 short hairpin RNA (shRNA). We found that about 40% of endogenous AMPK $\alpha$ 1 and AMPK $\alpha$ 2 were knocked down by their respective shRNAs (Fig. 1B); the activity of both subunits was also decreased, as indicated by decreased phosphorylation of ACC (Fig. 1C).

We further observed that AMPK $\alpha$ 1 but not AMPK $\alpha$ 2 knockdown was associated with decreased myogenin mRNA content (Fig. 1D). Consistently, myogenin protein content was decreased when AMPK $\alpha$ 1 was knocked down (Fig. 1E). As expected, both myosin heavy chain (MHC) protein content and the number of mature myotubes were decreased only when AMPK $\alpha$ 1 was knocked down (Fig. 1E and F).

**AMPK $\alpha$ 1 activity is necessary for myogenesis in primary myoblasts *in vitro*.** Because only about a 40% knockdown of AMPK $\alpha$  subunits was achieved by shRNA, we assessed myogenin mRNA expression in differentiating primary myoblasts from neonatal WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO mice. Desmin staining showed that the primary myogenic cells which we prepared had very high purity, eliminating the possibility that the detected difference was due to an impurity of primary myogenic cells (Fig. 2A). We observed that myogenin mRNA expression was profoundly reduced in AMPK $\alpha$ 1 KO myoblasts but not in AMPK $\alpha$ 2 KO myoblasts (Fig. 2B). Consistently, AMPK $\alpha$ 1 KO was associated with a dramatic decrease in myogenin protein content (Fig. 2C), whereas AMPK $\alpha$ 2 KO showed no significant difference (Fig. 2D). We also found that AMPK $\alpha$ 1 KO but not AMPK $\alpha$ 2 KO was associated with a decreased MHC level (Fig. 2C and D). In addition, myotube formation in AMPK $\alpha$ 1 KO myoblasts but not in AMPK $\alpha$ 2 KO myoblasts was reduced (Fig. 2E and F). Two days after induction of myogenesis, the mRNA levels of additional myogenic regulatory factors (Myf5, MyoD, and MRF4) in WT myoblasts and AMPK $\alpha$ 1 KO myoblasts were also tested. While no difference was seen in MyoD and MRF4 mRNA levels, the Myf5 mRNA level was higher in AMPK $\alpha$ 1 KO myoblasts than in WT myoblasts, which suggests a specific regulatory effect of AMPK on myogenin expression (Fig. 2G). Myf5 is known to express prior to myogenin and is able to induce myogenin expression (32, 33), and thus, it is possible that the elevated level of Myf5 in AMPK $\alpha$ 1 KO myoblasts was due to compensatory effects. Another possibility is that the halted fusion of AMPK $\alpha$ 1 KO myoblasts into myotubes



**FIG 2** AMP-activated protein kinase activity is necessary for myogenesis in primary myoblasts. WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO primary myoblasts were prepared, purified, and induced for myogenesis. (A) The purity of enriched myoblasts was evaluated by FACS analysis. The blue line shows myoblasts stained with rabbit antidesmin primary and Alexa Fluor 488-conjugated secondary antibodies. The red line shows control staining without primary antibody. (B) Myogenin mRNA content (2 days after induction of myogenesis). (C and D) Myogenin and MHC protein contents (3 days and 4 days after induction of myogenesis, respectively). (E and F) Immunocytochemical staining using an anti-MHC antibody and fusion index 4 days after induction of myogenesis. (G) Myf5, MyoD, and MRF4 mRNA contents (2 days after induction of myogenesis). Data shown are means  $\pm$  SEM ( $n \geq 3$ ).  $\alpha$ 1, AMPK $\alpha$ 1;  $\alpha$ 2, AMPK $\alpha$ 2. \*\*,  $P < 0.01$  versus the WT; \*\*\*,  $P < 0.0001$  versus the WT.

rendered more abundant myoblasts which express Myf5, resulting in higher Myf5 expression levels in AMPK $\alpha$ 1 KO than in WT differentiating myoblasts.

We reasoned that if AMPK was necessary for myogenesis, then knocking out both AMPK $\alpha$ 1 and  $\alpha$ 2 should prevent the formation of myotubes. Because the double knockout (DK) of AMPK $\alpha$ 1 and  $\alpha$ 2 subunits is embryonically lethal, we prepared primary myoblasts from tamoxifen-inducible AMPK $\alpha$ 1 and AMPK $\alpha$ 2 conditional KO mice (AMPK DK mice). AMPK DK primary myoblasts were treated with 1  $\mu$ M 4-hydroxytamoxifen (TM) for 2 days to achieve complete depletion of AMPK $\alpha$  protein (Fig. 3A). As expected, the absence of AMPK activity was associated with dramatic reductions in myogenin mRNA expression levels (Fig. 3B), myogenin protein content (Fig. 3C), MHC levels (Fig. 3C), and myotube formation (Fig. 3D), clearly illustrating the critical role of AMPK in myogenesis.

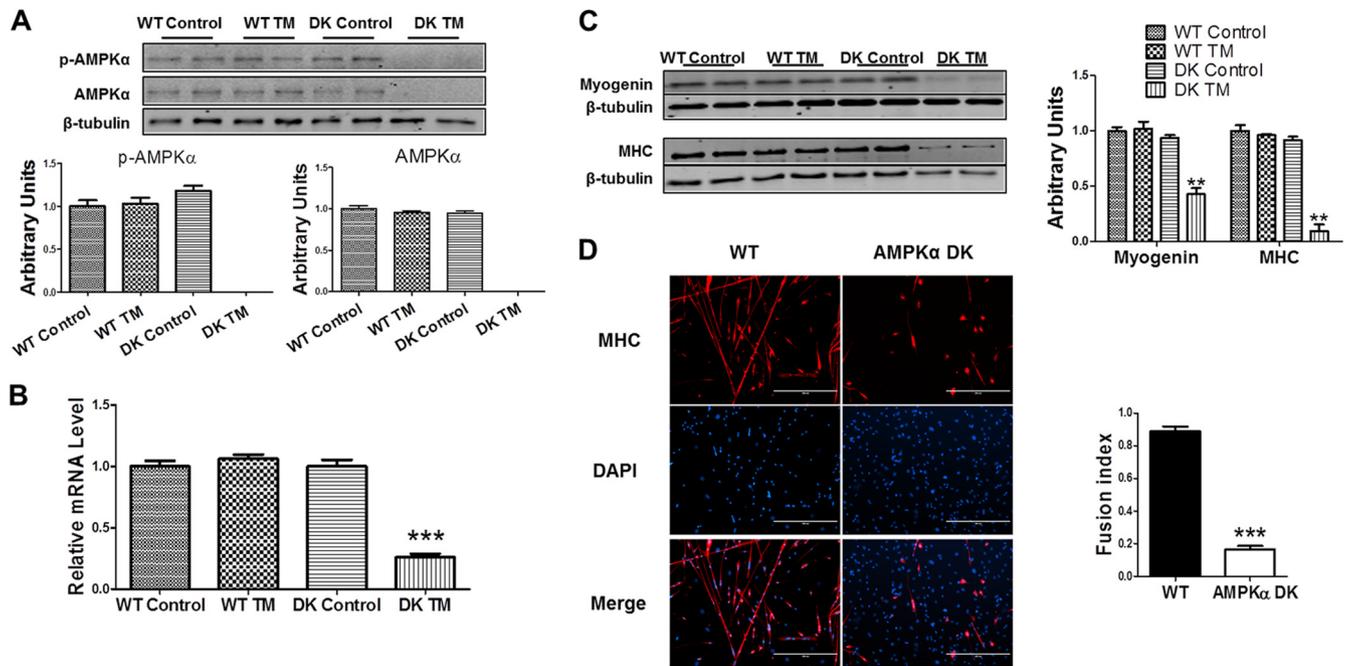
**Expression of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 subunits during myogenesis.** To dissect the effects of isoform-specific depletion of AMPK $\alpha$ 1 and  $\alpha$ 2 subunits on myogenesis, we tested whether the difference was caused by the differential expression of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 in myoblasts. While the AMPK $\alpha$ 1 mRNA level remained constant during 6 days of myogenic differentiation in C2C12 cells, the AMPK $\alpha$ 2 mRNA level increased about 4-fold (Fig. 4A). In primary myoblasts, 2 days after myogenic induction, the AMPK $\alpha$ 1 mRNA level decreased approximately 30%, while the AMPK $\alpha$ 2 mRNA level increased 4-fold (Fig. 4B). The mRNA levels of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 in the hind-limb muscles from neonatal (2 days old, with highly active myogenic differentiation) and adult (2 months old, with mature muscle fibers and only limited myogenesis) WT mice were further compared. The AMPK $\alpha$ 1 mRNA level was about 9-fold higher in neonatal mice than in adult mice, whereas the AMPK $\alpha$ 2 mRNA level was about 50% lower in neonatal mice than in adult mice (Fig. 4C). These data show that the AMPK $\alpha$ 1 level decreases gradually during

myogenic differentiation, while the AMPK $\alpha$ 2 level increases during this process, indicating the importance of AMPK $\alpha$ 1 in early myogenesis.

To further clarify AMPK $\alpha$  subunit expression, total AMPK $\alpha$  protein levels ( $\alpha$ 1 and  $\alpha$ 2 subunits were included because the antibody does not differentiate these two subunits) and activity, based on the phosphorylation of AMPK $\alpha$  Thr172, were measured in differentiating WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO myoblasts. Both AMPK $\alpha$  protein levels and activity were lower in AMPK $\alpha$ 1 KO myoblasts than in WT myoblasts 3 days after induction of myogenesis, despite the finding that the AMPK $\alpha$ 2 mRNA level increased dramatically 2 days after induction of myogenesis, which suggests an extremely low expression level of AMPK $\alpha$ 2 in undifferentiated and differentiating myoblasts (Fig. 4B and D). There were no differences in AMPK $\alpha$  protein levels and activity between WT myoblasts and AMPK $\alpha$ 2 KO myoblasts (Fig. 4D), further showing that AMPK $\alpha$ 2 expression is minor in myoblasts.

AMPK $\alpha$  protein levels and activities in the hind-limb muscle from neonatal and adult WT and AMPK $\alpha$  KO mice were further measured. Both AMPK $\alpha$  protein levels and activities were lower in neonatal AMPK $\alpha$ 1 KO mice than in neonatal WT mice, showing that AMPK $\alpha$ 1 is the dominant form in neonatal muscle (Fig. 4E); however, the difference was reduced in adult mice (Fig. 4F). There was no significant difference in AMPK $\alpha$  protein levels and activities between neonatal AMPK $\alpha$ 2 KO mice and neonatal WT mice (Fig. 4E), whereas the difference was significant in adult mice, which indicates that the  $\alpha$ 2 subunit was the dominant form in adult mice (Fig. 4F). These data suggest that AMPK $\alpha$ 1 is abundantly expressed in myoblasts, while AMPK $\alpha$ 2 is preferentially expressed in mature muscle fibers.

During muscle regeneration, satellite cells proliferate and then fuse to damaged muscle fibers or form new muscle fibers to replace damaged ones. To test the levels of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 in regenerating muscle, we induced muscle regeneration in TA



**FIG 3** Double-knockout AMPK $\alpha$  subunits dramatically reduce myogenesis. WT and AMPK $\alpha$ 1 and AMPK $\alpha$ 2 double-KO (DK) primary myoblasts were prepared, purified, and induced for myogenesis. (A) AMPK $\alpha$  subunit contents in AMPK $\alpha$  DK myoblasts following 4-hydroxytamoxifen (TM) treatment. (B) Myogenin mRNA expression in AMPK $\alpha$  DK myoblasts following TM treatment (2 days after induction of myogenesis). (C) Myogenin and MHC protein contents in AMPK $\alpha$  DK myoblasts following TM treatment (3 days and 4 days after induction of myogenesis, respectively). (D) Immunocytochemical staining using an anti-MHC antibody and fusion index 4 days after induction of myogenesis following TM treatment. Data shown are means  $\pm$  SEM ( $n \geq 3$ ).  $\alpha$ 1, AMPK $\alpha$ 1;  $\alpha$ 2, AMPK $\alpha$ 2. \*\*,  $P < 0.01$  versus the WT; \*\*\*,  $P < 0.0001$  versus the WT.

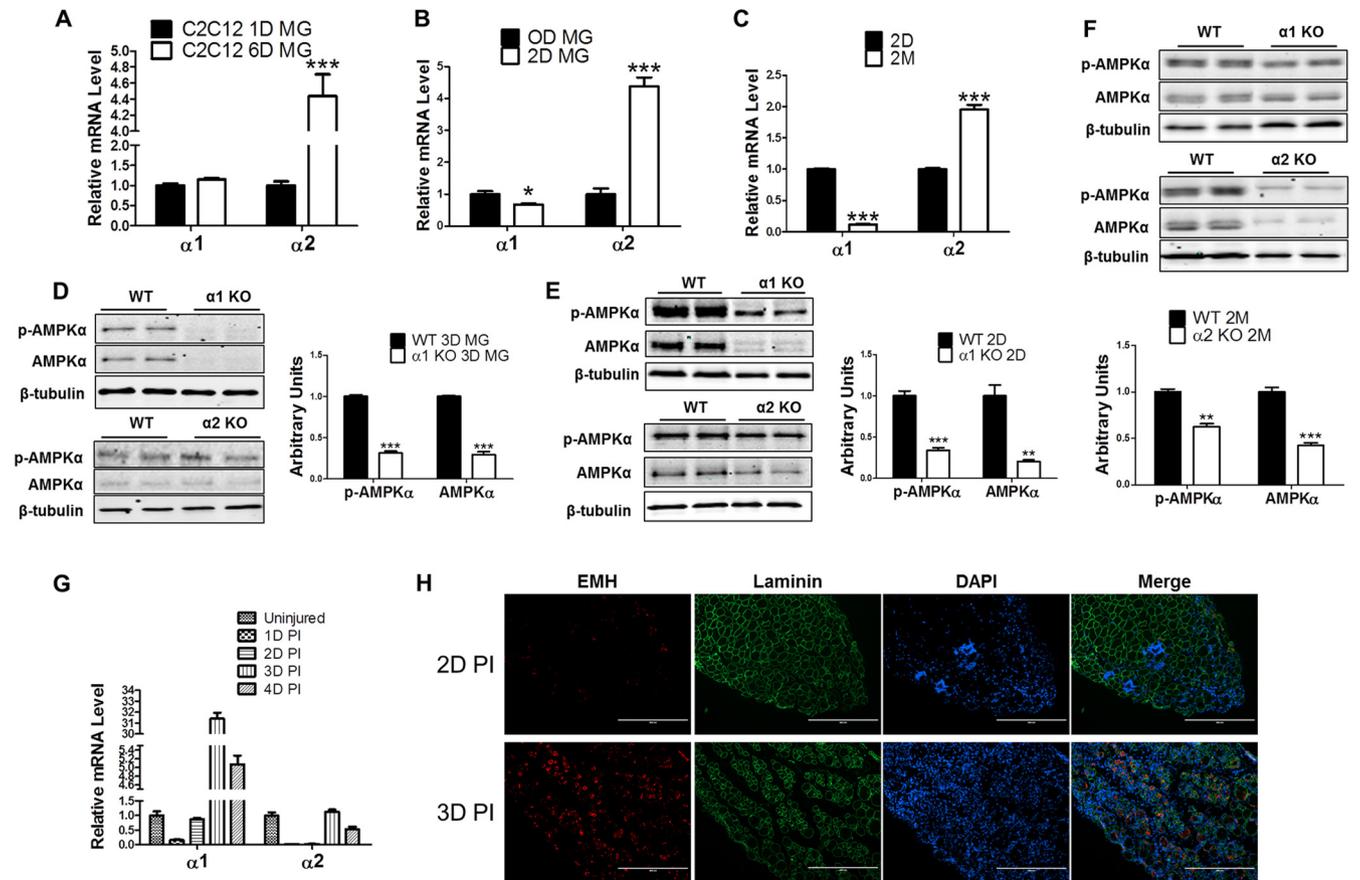
muscle by cardiotoxin (CTX) injection. AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA levels at different days post-CTX injection were tested. Both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA levels decreased at 1 day post-CTX injection, possibly due to the severe harmful effect of CTX on cell physiology. Surprisingly, a  $>30$ -fold increase in the AMPK $\alpha$ 1 mRNA level was observed in muscle at 3 days post-CTX injection, which correlates with the expression of embryonic myosin heavy chain (EMH), a marker of muscle regeneration (9). However, AMPK $\alpha$ 2 levels only returned to the level prior to damage at 3 days post-CTX injection (Fig. 4G and H). The increased AMPK $\alpha$ 1 mRNA level in regenerating muscle strongly indicates its importance in myogenesis.

**Both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 are able to promote myogenesis.** The question that remains is whether both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 subunits are able to promote myogenin expression and, thus, myogenesis. To address this question, we treated C2C12 cells and primary myoblasts with 5-aminoimidazole-4-carboxamide 1-beta-D-ribose nucleoside (AICAR) and an AMPK inhibitor, compound C. Because high levels of AMPK activity are known to inhibit universal protein synthesis (by attenuating mTOR signaling and inhibiting myogenin and MHC expression), we sought to minimize such possible side effects by using a low concentration of AICAR (0.125 mM) to induce a low level of activation; similarly, a low concentration of compound C (1  $\mu$ M) was used. AMPK activity was assessed by measuring phosphorylation levels of AMPK $\alpha$  Thr172 and ACC Ser79 following AICAR and compound C treatments in C2C12 cells (Fig. 5A). AMPK activation by AICAR resulted in an increase in the myogenin mRNA level and protein content and an increase in the MHC protein content in C2C12 cells (Fig. 5B and C). In agreement with this finding, myo-

tube formation was enhanced in AICAR-treated C2C12 cells, which indicates that a low level of AMPK activation promotes myogenesis (Fig. 5D). A reduction in myogenin mRNA (Fig. 5B) and protein (Fig. 5C) levels was observed in C2C12 cells treated with compound C. The MHC level was also decreased following compound C treatment (Fig. 5C).

The same treatment regimens were applied to primary myoblasts. AICAR treatment resulted in increased myogenin mRNA and protein levels, while compound C treatment resulted in reduced myogenin mRNA and protein levels in WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO differentiating myoblasts (Fig. 5E and G to I). Immunofluorescence analysis showed corresponding changes in myotube formation (Fig. 5F [only images of AMPK $\alpha$ 2 KO cells are shown]). AICAR treatment increased the MHC protein levels in WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO differentiating myoblasts. Compound C treatment resulted in decreased MHC levels in WT differentiating myoblasts (Fig. 5G) and AMPK $\alpha$ 2 KO differentiating myoblasts (Fig. 5I) but resulted in an increase in MHC levels in AMPK $\alpha$ 1 KO differentiating myoblasts (Fig. 5H). Although further analysis will be necessary, the increased MHC level in AMPK $\alpha$ 1 KO differentiating myoblasts (AMPK $\alpha$ 2 remaining) following compound C treatment may be related to the importance of the AMPK $\alpha$ 2 subunit in governing protein synthesis, including MHC synthesis.

**AMPK $\alpha$ 1 potentiates myogenic differentiation of engrafted myoblasts.** To test the influence of AMPK KO on myogenic differentiation *in vivo*, myoblasts were isolated from EGFP WT mice and DsRed mice with an AMPK $\alpha$ 1 conditional KO. DsRed-expressing AMPK $\alpha$ 1 conditional KO myoblasts were treated with tamoxifen for 48 h to delete AMPK $\alpha$ 1. CTX was injected into TA



**FIG 4** AMPK $\alpha$  content in C2C12 cells, primary myoblasts, and skeletal muscle. (A) AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA contents were analyzed 1 day (1D) and 6 days (6D) after myogenic induction (MG) in C2C12 cells. (B) AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA contents in primary myoblasts before (0D) and 2 days after (2D) myogenic induction. (C) AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA contents in hind-limb muscle from 2-day-old neonatal (2D) and 2-month-old adult (2M) WT mice. (D) AMPK $\alpha$  and p-AMPK $\alpha$  levels in WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO myoblasts 3 days after myogenic induction. (E) AMPK $\alpha$  and p-AMPK $\alpha$  levels in hind-limb muscle from 2-day-old WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO mice. (F) AMPK $\alpha$  and p-AMPK $\alpha$  levels in hind-limb muscle from 2-month-old WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO mice. (G) AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNA levels in undamaged TA muscle and in TA muscle 1 day, 2 days, 3 days, and 4 days post-cardiotoxin injection (PI). (H) Immunohistochemistry staining of TA muscle sections at 2 days and 3 days post-cardiotoxin injection using anti-EMH antibody and antilaminin antibody. Data shown are means  $\pm$  SEM ( $n \geq 3$ ).  $\alpha$ 1, AMPK $\alpha$ 1;  $\alpha$ 2, AMPK $\alpha$ 2. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus the WT; \*\*\*,  $P < 0.0001$ .

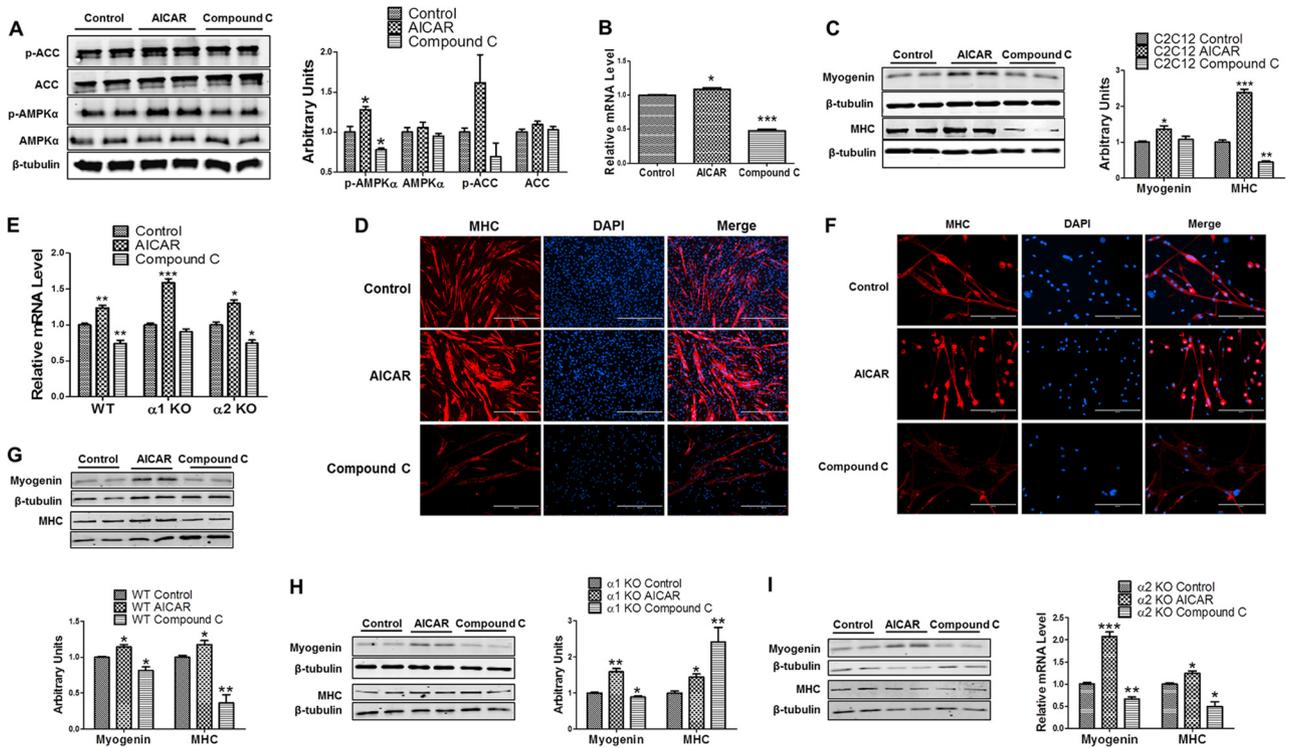
muscle of WT mice to induce muscle regeneration. Twenty-four hours after CTX injection,  $3 \times 10^4$  EGFP myoblasts and  $3 \times 10^4$  DsRed AMPK $\alpha$ 1 KO myoblasts were mixed and injected together into each damaged TA muscle. Cotransplantation of WT EGFP myoblasts and AMPK $\alpha$ 1 KO DsRed myoblasts eliminated the variations due to the transplantation procedure and physiological conditions. Fourteen days after myoblast transplantation, a number of EGFP-positive (EGFP $^+$ ) muscle fibers was observed in transplanted TA muscle, whereas a much smaller number of DsRed $^+$  muscle fibers was observed (Fig. 6). These data clearly indicate that WT myoblasts had a greater potential to differentiate into muscle fibers *in vivo* than AMPK $\alpha$ 1 KO myoblasts, showing the promoting effect of AMPK $\alpha$ 1 on myogenic differentiation.

## DISCUSSION

To date, the relationship between AMPK and myogenesis has been controversial. A previous study suggested that AMPK activation blocked myogenesis through a PGC-1 $\alpha$ -dependent mechanism involving SIRT1 in C2C12 cells (34). However, a high dose of AICAR (1 mM) was used in this study. Because AMPK is known to inhibit protein synthesis, highly activated AMPK would be ex-

pected to inhibit protein synthesis globally and would likely inhibit cell proliferation. Moreover, pathophysiological changes such as obesity and chronic inflammation are known to inhibit, rather than activate, AMPK (31), suggesting that inhibition, rather than activation, is more relevant to the pathophysiological roles of AMPK (35–37). AMPK is phosphorylated by a constitutively active kinase, LKB1, which suggests that dephosphorylation rather than phosphorylation may be the main mechanism in the regulation of AMPK activity (37–39). In our previous studies, we observed that low AMPK activity correlated with impaired myogenesis (19). Moreover, we recently reported that AMPK positively regulates myogenesis through phosphorylation of HDAC5 and that AMPK $\alpha$ 1 KO but not AMPK $\alpha$ 2 KO was associated with reduced muscle mass (22).

To dissect the relationship between decreased AMPK activity and myogenesis, we used shRNA to selectively knock down either AMPK $\alpha$ 1 or AMPK $\alpha$ 2. Our results indicated that AMPK $\alpha$ 1 but not AMPK $\alpha$ 2 knockdown was associated with decreased myogenesis, as shown by decreased expression levels of myogenin and MHC and less formation of myotubes. Our data were further strengthened by using primary myoblasts isolated from neonatal WT, AMPK $\alpha$ 1 KO, and

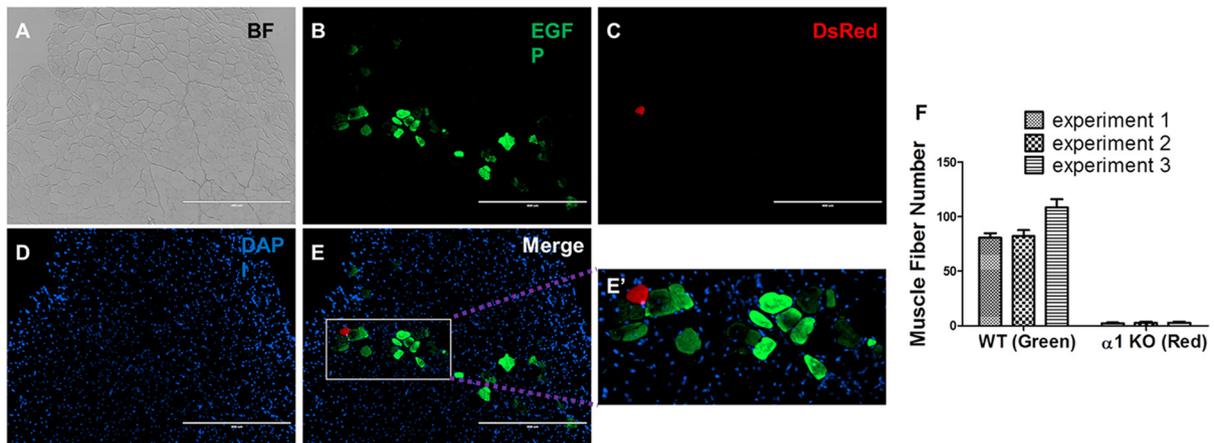


**FIG 5** Myogenin levels and myogenesis are associated with AMPK activity in C2C12 cells and primary myoblasts. (A to D) C2C12 cells were induced for myogenesis in myogenic differentiation medium. The medium was supplemented with either 0.125 mM AICAR or 1  $\mu$ M compound C. Control cultures received vehicle (dimethyl sulfoxide). (A) Immunoblot of the indicated proteins or phosphoproteins performed 2 days after myogenic induction. (B) Real-time PCR testing of myogenin mRNA content (2 days after induction of myogenesis). (C) Immunoblot of myogenin and myosin heavy chain (MHC) (3 days and 6 days after induction of myogenesis, respectively). (D) Immunocytochemical staining for MHC 6 days after induction of myogenesis. (E to I) Primary myoblasts were induced for myogenesis in myogenic differentiation medium, which was supplemented with either 0.125 mM AICAR, 1  $\mu$ M compound C, or vehicle (dimethyl sulfoxide). (E) Myogenin mRNA content assessed in WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO primary myoblasts 2 days after induction of myogenesis. (F) Immunocytochemical staining for MHC 4 days after induction of myogenesis. (G to I) Myogenin protein content and MHC protein content of myoblasts assessed by immunoblotting (3 days and 4 days after induction of myogenesis, respectively). (G) WT; (H) AMPK $\alpha$ 1 KO; (I) AMPK $\alpha$ 2 KO. Data shown are means  $\pm$  SEM ( $n \geq 3$ ).  $\alpha$ 1, AMPK $\alpha$ 1;  $\alpha$ 2, AMPK $\alpha$ 2. \*,  $P < 0.05$  versus the control; \*\*,  $P < 0.01$  versus the control; \*\*\*,  $P < 0.0001$  versus the control.

AMPK $\alpha$ 2 KO mice. During myogenic differentiation, AMPK $\alpha$ 1 KO myoblasts showed reduced expression levels of myogenin and MHC and less formation of myotubes than WT myoblasts. Also, the absence of both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 was associated with further

reductions in myogenesis, which indicates that AMPK activity is necessary for optimal myogenic differentiation.

We questioned why AMPK $\alpha$ 2 depletion failed to reduce myogenin and MHC levels. We postulated that it may be caused, in



**FIG 6** AMPK $\alpha$ 1 is critical for myogenic differentiation of engrafted myoblasts. (A to E) Immunostaining of muscle fibers formed by transplanted WT EGFP myoblasts and DsRed AMPK $\alpha$ 1 KO myoblasts. (E') Magnified image of a selected area in panel E. (F) Numbers of muscle fibers formed by transplanted myoblasts in three independent experiments. BF, bright field.

part, by the differential expression of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 in myoblasts. Indeed, AMPK $\alpha$ 1 is expressed during the early stages of myogenesis; however, the AMPK $\alpha$ 2 level increases during myogenic differentiation and becomes the dominant isoform in mature muscle, as indicated by previous reports showing that AMPK $\alpha$ 2 is the major form in skeletal muscle (40, 41). When treated with low levels of AICAR, WT, AMPK $\alpha$ 1 KO, and AMPK $\alpha$ 2 KO myoblasts all showed increased myogenesis, which indicates that both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 promote myogenesis. As the dominant AMPK isoform in early differentiating myoblasts, it was not unexpected that AMPK $\alpha$ 1 KO inhibited myogenin expression and myogenesis, while the depletion of AMPK $\alpha$ 2 did not affect myogenesis. This notion is further supported by the profound upregulation of AMPK $\alpha$ 1 but not AMPK $\alpha$ 2 mRNA expression during muscle regeneration, indicating an important role of AMPK $\alpha$ 1 in muscle regeneration.

Finally, consistent with our *in vitro* data, AMPK $\alpha$ 1 KO dramatically reduced myogenic differentiation of myoblasts transplanted into mouse TA muscle *in vivo*. Surprisingly, very few or no AMPK $\alpha$ 1 KO myoblasts fused with muscle fibers, while WT myoblasts did. These data show that AMPK $\alpha$ 1 KO significantly impaired the ability of myoblasts to participate in muscular regeneration, indicating that AMPK $\alpha$ 1 has a critical role in muscle fiber regeneration. Considering that proper muscle fiber regeneration is essential for maintaining muscle function (8), improper muscular regeneration due to attenuated AMPK activity is expected to accelerate muscle atrophy and aging (11).

Our observation that AMPK activity is necessary to maintain normal myogenesis has important physiological implications, because improper muscle regeneration is largely responsible for muscle atrophy and loss of contractile function during aging and muscular atrophy (42). AMPK likely provides a convenient molecular target to facilitate muscle development and muscle regeneration.

## ACKNOWLEDGMENTS

The work was supported by NIH grants 1R01HD067449 and 1R03HD057506 and USDA-NIFA grant 2008-35206-18826.

We declare that we have no conflicts of interest.

## REFERENCES

- Lee RC, Wang ZM, Heymsfield SB. 2001. Skeletal muscle mass and aging: regional and whole-body measurement methods. *Can. J. Appl. Physiol.* 26:102–122.
- Lowell BB, Shulman GI. 2005. Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384–387.
- Aberle ED. 1984. Myofiber differentiation in skeletal muscles of newborn runt and normal weight pigs. *J. Anim. Sci.* 59:1651–1656.
- Handel SE, Stickland NC. 1987. The effects of low birthweight on the ultrastructural development of two myofibre types in the pig. *J. Anat.* 150:129–143.
- Bayol SA, Macharia R, Farrington SJ, Simbi BH, Stickland NC. 2009. Evidence that a maternal “junk food” diet during pregnancy and lactation can reduce muscle force in offspring. *Eur. J. Nutr.* 48:62–65.
- Brameld JM, Mostyn A, Dandrea J, Stephenson TJ, Dawson JM, Buttery PJ, Symonds ME. 2000. Maternal nutrition alters the expression of insulin-like growth factors in fetal sheep liver and skeletal muscle. *J. Endocrinol.* 167:429–437.
- Allen RE, Merkel RA, Young RB. 1979. Cellular aspects of muscle growth: myogenic cell proliferation. *J. Anim. Sci.* 49:115–127.
- Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, Antonini S, Sambasivan R, Brunelli S, Tajbakhsh S, Cossu G. 2011. Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. *Nat. Commun.* 2:499. doi:10.1038/ncomms1508.
- Uezumi A, Fukada S, Yamamoto N, Takeda S, Tsuchida K. 2010. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat. Cell Biol.* 12:143–152.
- Bernasconi P, Torchiana E, Confalonieri P, Brugnoli R, Barresi R, Mora M, Cornelio F, Morandi L, Mantegazza R. 1995. Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J. Clin. Invest.* 96:1137–1144.
- Li H, Malhotra S, Kumar A. 2008. Nuclear factor-kappa B signaling in skeletal muscle atrophy. *J. Mol. Med.* 86:1113–1126.
- Kim J, Solis RS, Arias EB, Cartee GD. 2004. Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation. *J. Appl. Physiol.* 96:575–583.
- Hardie DG. 2004. AMP-activated protein kinase: a key system mediating metabolic responses to exercise. *Med. Sci. Sports Exerc.* 36:28–34.
- Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y. 2010. AMPK and SIRT1: a long-standing partnership? *Am. J. Physiol. Endocrinol. Metab.* 298:E751–E760. doi:10.1152/ajpendo.00745.2009.
- Giri S, Rattan R, Haq E, Khan M, Yasmin R, Won JS, Key L, Singh AK, Singh I. 2006. AICAR inhibits adipocyte differentiation in 3T3L1 and restores metabolic alterations in diet-induced obesity mice model. *Nutr. Metab. (Lond.)* 3:31. doi:10.1186/1743-7075-3-31.
- Jeyabalan J, Shah M, Viollet B, Chenu C. 2012. AMP-activated protein kinase pathway and bone metabolism. *J. Endocrinol.* 212:277–290.
- Pauly M, Daussin F, Burelle Y, Li T, Godin R, Fauconnier J, Koehlin-Ramonatxo C, Hugon G, Lacampagne A, Coisy-Quivy M, Liang F, Hussain S, Matecki S, Petrof BJ. 2012. AMPK activation stimulates autophagy and ameliorates muscular dystrophy in the mdx mouse diaphragm. *Am. J. Pathol.* 181:583–592.
- Sanchez AM, Candau RB, Csibi A, Pagano AF, Raibon A, Bernardi H. 2012. The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis. *Am. J. Physiol. Cell Physiol.* 303:C475–C485. doi:10.1152/ajpcell.00125.2012.
- Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, Xiao Y, Hess BW, Ford SP, Nathanielsz PW, Du M. 2008. AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. *J. Physiol.* 586:2651–2664.
- Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, Du M. 2010. Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. *Endocrinology* 151:380–387.
- Tong JF, Yan X, Zhao JX, Nathanielsz PW, Du M. 2011. Metformin mitigates the impaired development of skeletal muscle in the offspring of obese mice. *Nutr. Diabetes* 1:e7. doi:10.1038/nutd.2011.3.
- Fu X, Zhao JX, Liang J, Zhu MJ, Viollet B, Du M. AMP-activated protein kinase mediates myogenin expression and myogenesis via histone deacetylase 5. *Am. J. Physiol. Cell Physiol.*, in press.
- Niesler CU, Myburgh KH, Moore F. 2007. The changing AMPK expression profile in differentiating mouse skeletal muscle myoblast cells helps confer increasing resistance to apoptosis. *Exp. Physiol.* 92:207–217.
- Viollet B, Athes Y, Mounier R, Guigas B, Zarrinpashneh E, Horman S, Lantier L, Hebrard S, Devin-Leclerc J, Beauloye C, Foretz M, Andreelli F, Ventura-Clapier R, Bertrand L. 2009. AMPK: lessons from transgenic and knockout animals. *Front. Biosci.* 14:19–44.
- Jorgensen SB, Viollet B, Andreelli F, Froisig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF. 2004. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside but not contraction-induced glucose uptake in skeletal muscle. *J. Biol. Chem.* 279:1070–1079.
- Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloan A, Flamez D, Mu J, Lenzner C, Baud O, Bennoun M, Gomas E, Nicolas G, Wojtaszewski JF, Kahn A, Carling D, Schuit FC, Birnbaum MJ, Richter EA, Burcelin R, Vaulont S. 2003. The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. *J. Clin. Invest.* 111:91–98.
- Rando TA, Blau HM. 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–1287.
- Zhao JX, Yue WF, Zhu MJ, Du M. 2011. AMP-activated protein kinase regulates beta-catenin transcription via histone deacetylase 5. *J. Biol. Chem.* 286:16426–16434.

29. Yang Z, Zeng Q, Ma Z, Wang Y, Xu X. 2009. Tracking dynamics of muscle engraftment in small animals by in vivo fluorescent imaging. *J. Vis. Exp.* 2009:1388. doi:10.3791/1388.
30. Subbaramaiah K, Howe LR, Bhardwaj P, Du B, Gravaghi C, Yantiss RK, Zhou XK, Blaho VA, Hla T, Yang P, Kopelovich L, Hudis CA, Dannenberg AJ. 2011. Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer Prev. Res. (Phila.)* 4:329–346.
31. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Görgün CZ, Carling D. 2006. Tumor necrosis factor  $\alpha$ -induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. *Cell Metab.* 4:465–474.
32. Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M, Lassar AB. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* 89:139–148.
33. Potthoff MJ, Olson EN. 2007. MEF2: a central regulator of diverse developmental programs. *Development* 134:4131–4140.
34. Williamson DL, Butler DC, Alway SE. 2009. AMPK inhibits myoblast differentiation through a PGC-1 $\alpha$ -dependent mechanism. *Am. J. Physiol. Endocrinol. Metab.* 297:E304–E314. doi:10.1152/ajpendo.91007.2008.
35. Brown KA, McInnes KJ, Hunger NI, Oakhill JS, Steinberg GR, Simpson ER. 2009. Subcellular localization of cyclic AMP-responsive element binding protein-regulated transcription coactivator 2 provides a link between obesity and breast cancer in postmenopausal women. *Cancer Res.* 69:5392–5399.
36. Yang Z, Kahn BB, Shi H, Xue BZ. 2010. Macrophage  $\alpha$ 1 AMP-activated protein kinase ( $\alpha$ 1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. *J. Biol. Chem.* 285:19051–19059.
37. Park S, Scheffler TL, Rossie SS, Gerrard DE. 2013. AMPK activity is regulated by calcium-mediated protein phosphatase 2A activity. *Cell Calcium* 53:217–223.
38. Sakamoto K, Goransson O, Hardie DG, Alessi DR. 2004. Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am. J. Physiol. Endocrinol. Metab.* 287:E310–E317. doi:10.1152/ajpendo.00074.2004.
39. Park S, Scheffler TL, Gerrard DE. 2011. Chronic high cytosolic calcium decreases AICAR-induced AMPK activity via calcium/calmodulin activated protein kinase II signaling cascade. *Cell Calcium* 50:73–83.
40. Chen Z, Heierhorst J, Mann RJ, Mitchelhill KI, Michell BJ, Witters LA, Lynch GS, Kemp BE, Stapleton D. 1999. Expression of the AMP-activated protein kinase  $\beta$ 1 and  $\beta$ 2 subunits in skeletal muscle. *FEBS Lett.* 460:343–348.
41. Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. 2000. Characterization of AMP-activated protein kinase  $\gamma$ -subunit isoforms and their role in AMP binding. *Biochem. J.* 346(Part 3):659–669.
42. Kudryashova E, Kramerova I, Spencer MJ. 2012. Satellite cell senescence underlies myopathy in a mouse model of limb-girdle muscular dystrophy 2H. *J. Clin. Invest.* 122:1764–1776.