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Role of the α2 subunit of AMP-activated protein kinase and its nuclear localization in mitochondria and energy metabolism-related gene expressions in C2C12 cells

Shiki Okamoto [a,b,c], Nur Farehan Asgar [a,b], Shigefumi Yokota [a], Kumiko Saito [a], Yasuhiko Minokoshi [a,b,*]

[a] Division of Endocrinology and Metabolism, Department of Homeostatic Regulation, National Institute for Physiological Sciences, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan
[b] Department of Physiological Sciences, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan
[c] Second Department of Internal Medicine (Endocrinology, Diabetes and Metabolism, Hematology, Rheumatology), Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

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ABSTRACT

Background: AMP-activated protein kinase (AMPK), a heterotrimer with α1 or α2 catalytic subunits, acts as an energy sensor and regulates cellular homeostasis. Whereas AMPKα1 is necessary for myogenesis in skeletal muscle, the role of AMPKα2 in myogenic differentiation and energy metabolism-related gene expressions has remained unclear. We here examined the specific roles of AMPKα1 and AMPKα2 in the myogenic differentiation and mitochondria and energy metabolism-related gene expressions in C2C12 cells.
Materials and Methods: Stable C2C12 cell lines expressing a scramble short hairpin RNA (shRNA) or shRNAs specific for AMPKα1 (shAMPKα1), AMPKα2 (shAMPKα2), or both AMPKα1 and AMPKα2 (shPanAMPK) were generated by lentivirus infection. Lentiviruses encoding wild-type AMPKα2 (WT-AMPKα2) or AMPKα2 with a mutated nuclear localization signal (ΔNLS-AMPKα2) were also constructed for introduction into myoblasts. Myogenesis was induced by culture of C2C12 myoblasts for 6 days in differentiation medium.
Results: The amount of AMPKα2 increased progressively, whereas that of AMPKα1 remained constant, during the differentiation of myoblasts into myotubes. Expression of shPanAMPK or shAMPKα1, but not that of shAMPKα2, attenuated the proliferation of myoblasts as well as the phosphorylation of both acetyl-CoA carboxylase and the autophagy-initiating kinase ULK1 in myotubes. Up-regulation of myogenin mRNA, a marker for the middle stage of myogenesis, was attenuated in differentiating myotubes expressing shPanAMPK or shAMPKα1. In contrast, up-regulation of gene expression for muscle creatine kinase (MCK), a late-stage differentiation marker, as well as for genes related to mitochondrial biogenesis including the transcriptional coactivator peroxisome proliferator–activated receptor–γ coactivator–1α1 and α4 (PGC-1α1 and PGC-1α4) and mitochondria-specific genes such as cytochrome c were attenuated in myotubes expressing shAMPKα2 or shPanAMPK. The diameter of myotubes expressing shPanAMPK or shAMPKα2 was reduced, whereas that of those expressing shAMPKα1 was increased, compared with myotubes expressing scramble shRNA. A portion of AMPKα2 became localized to the nucleus during myogenic differentiation. The AMPK activator AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) and 2-deoxyglucose (2DG) each induced the nuclear translocation of WT-AMPKα2, but not that of ΔNLS-AMPKα2. Finally, expression of WT-AMPKα2 increased the mRNA abundance of PGC-1α1 and MCK mRNAs as well as cell diameter and tended to increase that of PGC-1α4, whereas that of ΔNLS-AMPKα2 increased only the abundance of MCK mRNA, in myotubes depleted of endogenous AMPKα2.
Conclusion: TAMPKα1 and AMPKα2 have distinct roles in myogenic differentiation of C2C12 cells, with AMPKα1 contributing to the middle stage of myogenesis and AMPKα2 to the late stage. AMPKα2 regulates gene expressions including MCK, PGC-1α1 and PGC-1α4 and mitochondria-specific genes such as cytochrome c during the late stage of differentiation. Furthermore, the nuclear translocation of AMPKα2 is necessary for maintenance of PGC-1α1 mRNA during myogenesis.

Abbreviations: 2DG, 2-deoxyglucose; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Dnm1l, dynamin-1-like protein; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HDAC5, histone deacetylase 5; MCK, muscle creatine kinase; MFF, mitochondrial fission factor; mTOR, mammalian target of rapamycin; MyoD, myoblast determination protein; NLS, nuclear localization signal; NRF-2, nuclear respiratory factor–2; Opa1, optic atrophy 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGC-1α, peroxisome proliferator–activated receptor–γ coactivator–1α; qPCR, quantitative PCR; RT, reverse transcription; RT-qPCR, Real time-quantitative PCR; shRNA, short hairpin RNA; Tfam, mitochondrial transcription factor A; TFB2M, mitochondrial transcription factor B2; TMRM, tetramethylrhodamine methyl ester; ULK1, Unc51-like autophagy-activating kinase 1; WT, wild-type.

* Corresponding author.
E-mail address: minokosh@nips.ac.jp (Y. Minokoshi).

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1. Introduction

AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status and regulates cell polarity, fatty acid oxidation, mitochondrial biogenesis, and autophagy [1]. AMPK is a heterotrimeric complex that comprises a catalytic α subunit and regulatory β and γ subunits [2–6]. In mammals, the catalytic α subunit exists in two isoforms, α1 and α2. The AMPKα2 subunit is highly expressed in skeletal muscle, and α2-containing complexes (but not α1-containing complexes) appear to be important for the activated AMPK during muscle contraction [6–8]. Transgenic mice that express an inactive (dominant negative) mutant form of AMPKα2 specifically in skeletal muscle manifest reduced voluntary activity and exercise tolerance [9,10]. Knockout of AMPKα2, but not that of AMPKα1, was also shown to reduce the expression of various genes related to mitochondrial biogenesis, including that for the transcriptional coactivator PGC-1α, in mouse skeletal muscle [11]. In contrast, AMPKα1 knockout mice manifest reduced activation of satellite cells and impaired muscle regeneration [12]. Knockout of AMPKα1, but not that of AMPKα2, also attenuated myogenin expression and myogenic differentiation in C2C12 cells [13,14]. These various observations thus suggest that AMPKα1 is necessary for myogenesis in skeletal muscle and cell lines. However, the role of AMPKα2 in myogenesis has remained unknown. Furthermore, the specific roles of AMPKα1 and AMPKα2 in mitochondria and energy metabolism-related gene expressions in muscle cells including PGC-1α isoforms (PGC-1α1 to PGC-1α4) have remained elusive.

We have now examined the isoform-specific roles of AMPKα during myogenesis in C2C12 cells. We have previously shown that AMPKα2, but not AMPKα1, possesses a putative nuclear localization signal (NLS) that appears to be functional in C2C12 cells [15]. Previous studies have also shown that AMPKα1 and AMPKα2 have different subcellular localizations in skeletal muscle cells, with AMPKα2 being enriched in the nucleus and AMPKα1 being localized predominantly to the cytoplasm [16,17]. We now show that AMPKα1 and AMPKα2 play distinct roles in myogenic differentiation of C2C12 cells, with AMPKα1 contributing to the middle stage of differentiation and AMPKα2 to the late stage. Our results indicate that AMPKα2 is essential for maintenance of cell diameter and for the expression of genes related to energy metabolism and mitochondrial biogenesis including those for MCK, PGC-1α1 and PGC-1α4. Furthermore, nuclear translocation of AMPKα2 is necessary to maintain the abundance of PGC-1α1 mRNA, but not that of MCK mRNA, in C2C12 myotubes.

2. Materials and Methods

2.1. Cell Culture

The mouse myoblast cell line C2C12 was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing glucose at 4500 mg/l (Sigma-Aldrich, St. Louis, MO) and supplemented with 10% heat-activated fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA). Myogenic differentiation was induced by exposure of C2C12 cells at 80% confluence for 6 days to DMEM containing glucose at 4500 mg/l and supplemented with 2% horse serum (Life Technologies). To induce the differentiation of all cell lines at 80% confluence, we extended the culture of myoblasts expressing shAMPKα1 and shPanAMPK for 24 h, compared with that of myoblasts expressing scramble shRNA and shAMPKα2.

2.2. RNA Interference by Lentivirus Transduction

Coding sequences for short hairpin RNAs (shRNAs) targeted to AMPKα1 (shAMPKα1), AMPKα2 (shAMPKα2), or both AMPKα isoforms (shPanAMPK) (Supplementary Figs. 1 and 2), or for a control scrambled shRNA, were inserted under the control of the mouse U6 gene promoter into pFUGW (kindly provided by P. Osten, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), which also encodes enhanced green fluorescent protein (EGFP). The shRNA target sequences were as follows: AMPKα1, 5′-GTACTTAAACCCTTCAGTA-3′; AMPKα2, 5′-GAAGCGAGCGACTATCAAA-3′; and PanAMPK, 5′-GATGTCAGATG GTGAATTT-3′ (Supplementary Figs. 1 and 2). Lentiviruses were produced by transfection of Lenti-X 293T HEK cells (Takara, Shiga, Japan) with the shRNA vector, the HIV-1 packaging vector Δ8.9 (pΔ8.9), and a vector for the envelope glycoprotein of vesicular stomatitis virus (pVSV-G) with the use of polyethylenimine (Polysciences, Warrington, PA). The virus particles were collected by centrifugation, and C2C12 myoblasts at 70% confluence in six-well plates were infected with the lentiviruses for 16 h in the presence of Polybrene (8 μg/ml). The cells were then cultured in 100-mm tissue culture dishes for an additional 48 h. The infected myoblasts were harvested, suspended in ice-cold phosphate-buffered saline (PBS) containing 5% FBS and propidium iodide (2 μg/ml), and subjected to fluorescence-activated cell sorting (FACS) with a Cell Sorter SH800 (Sony, Tokyo, Japan) for the isolation of EGFP-positive cells. All recombinant DNA experiments were approved by the relevant committee of the National Institute for Physiological Sciences and were performed under biosafety level 2 containment for lentiviruses.

2.3. Assay of Cell Proliferation

C2C12 myoblasts infected with lentiviruses were seeded in 12-well plates at a density of 1×10^4 cells per well and cultured for up to 72 h. The number of cells were counted at 24-h intervals.

2.4. Measurement of Myotube Diameter

The diameter of lentivirus-infected cells was measured 6 days after the induction of differentiation in 100-mm tissue culture dishes. Images were captured for seven independent fields. Selection criteria for differentiated myotubes included the presence of at least three nuclei in a single alignment and a length of more than three times that of myoblasts. Three measurements of the short axis were taken along the length of a given myotube, and the average diameter was calculated. A total of 150 myotubes was measured for each culture dish.

2.5. Construction and Expression of a FLAG-Tagged NLS Mutant of AMPKα2

Site-specific mutagenesis for generation of an NLS mutant of AMPKα2 (ΔNLS-AMPKα2) was performed by the polymerase chain reaction (PCR) with KOD+ DNA polymerase (Toyobo, Osaka, Japan) and with a cDNA for NH_2-terminally FLAG epitope–tagged mouse wild-type (WT) AMPKα2 as the template. Two fragments (A and B) were generated, with fragment A containing the ATG initiation codon and FLAG sequence, and fragment B containing the site-directed mutation in the NLS sequence (-K**K**IR- → -K**A**IR-). The mutagenesis primers were 5′-CATGGATTACAAGGACGATGACGACAAGGCT GAGAAGCAGAAGACGACG-3′ (forward) and 5′-GGATGTAAAACACACC CCCTCGGATCGCCTTGAAGAGCGTAGGCACGTGCTC-3′ (reverse, ΔNLS position) for fragment A, and 5′-GAGCACGTGCCTACGCTCTTCAAGGC GATCCGAGGGGGTGTGTTTTACATCC-3′ (forward, ΔNLS position) and 5′-GATAATCAACGAGCTAAAGCAGTGATAAGACTGGCGCAC-3′ (reverse) for fragment B (Supplementary Fig. 3). The two fragments were combined by denaturation at 94 °C and annealing at the ΔNLS primer positions at 30 °C, with the gaps being filled in by KOD+ DNA polymerase. The mutation of the NLS sequence was confirmed by DNA sequencing. Myoblasts expressing shAMPKα2 or scramble shRNA were infected with lentiviruses encoding WT or ΔNLS mutant forms of AMPKα2 for 16 h and were then transferred to 100-mm tissue culture dishes, cultured to 80% confluence, and induced to differentiate for 6 days.

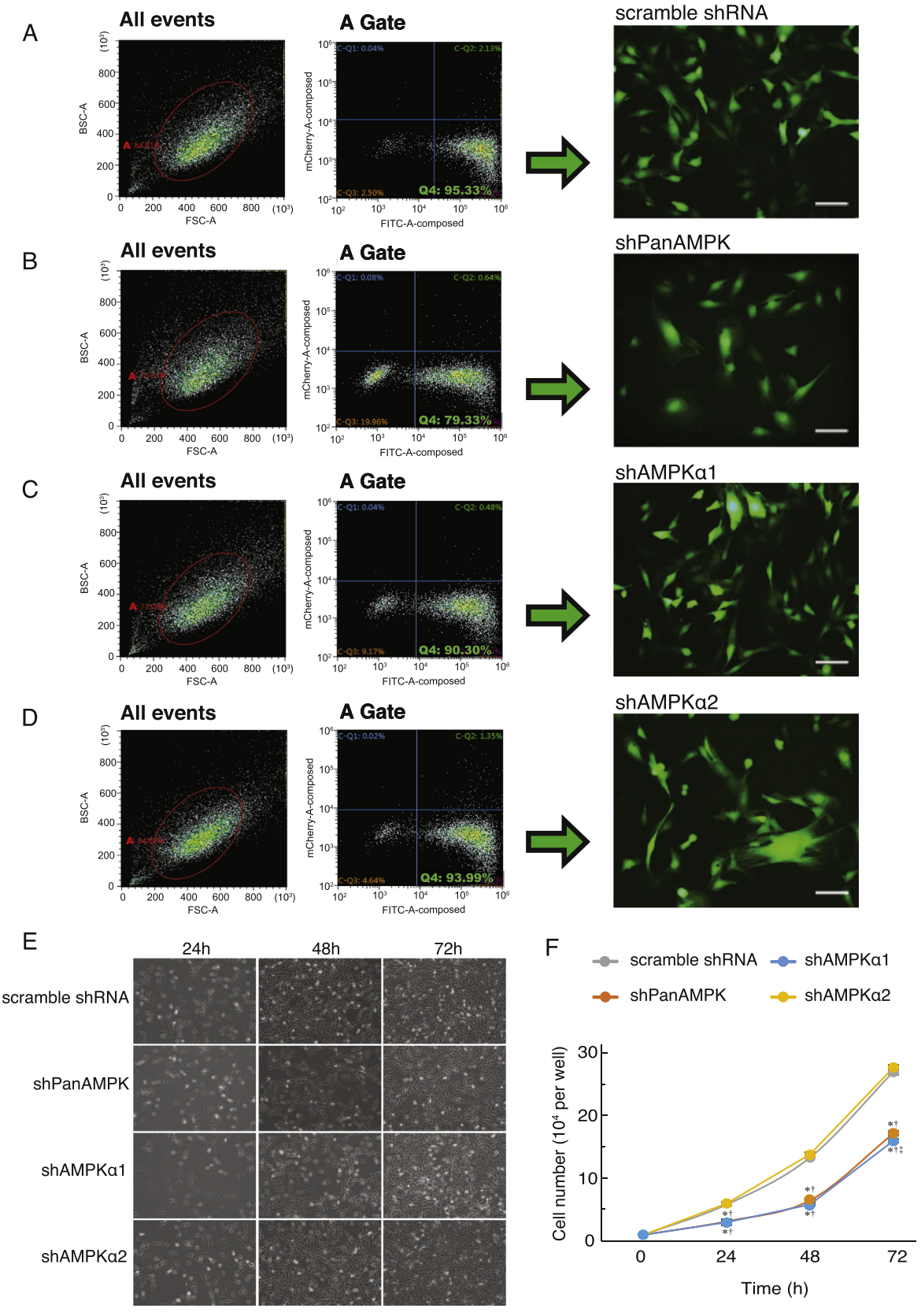


Fig. 1. Effects of depletion of AMPKα isoforms on C2C12 myoblast proliferation. A–D) FACS analysis of C2C12 myoblasts infected with lentiviruses encoding EGFP and either scramble shRNA (A), shPanAMPK (B), shAMPKα1 (C), or shAMPKα2 (D). Cells selected from a backscatter (BSC) versus forward scatter (FSC) density plot (left panels) were gated on the basis of a bivariate histogram (middle panels; mCherry versus FITC), the lower right quadrant (Q4) of which represented EGFP-positive cells that were isolated for subsequent culture. Representative fluorescence images of the sorted EGFP-positive myoblasts are shown in the right panels (scale bars, 200 μm). Scale bar, 100 μm. E) Representative phase-contrast images of C2C12 myoblasts expressing scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 at 24, 48, and 72 h of culture. F) Growth curves for C2C12 myoblasts expressing scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 that were cultured for up to 72 h. Data are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus corresponding value for scramble shRNA, †$p < 0.05$ versus corresponding value for shAMPKα2, ‡$p < 0.05$ versus corresponding value for shPanAMPK (ANOVA followed by the Tukey-Kramer post hoc test).

2.6. Transient Expression of WT-AMPKα2 and ΔNLS-AMPKα2, Stimulation with AICAR, and Energy Deprivation

C2C12 myoblasts cultured in six-well plates were transfected with a pCAGGS vector (Addgene) encoding FLAG-tagged WT-AMPKα2 or ΔNLS-AMPKα2 (Supplementary Figs. 2 and 3) with the use of the Lipofectamine 2000 reagent (Life Technologies). The transfected myoblasts were then cultured for 48 h before incubation for 1 h with 0.5 mM 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Toronto Research Chemicals, Toronto, Ontario, Canada) or with DMEM containing glucose at 1000 mg/l (Sigma-Aldrich) and supplemented with 21.3 mM 2DG (Sigma-Aldrich). The cells were finally subjected to immunofluorescence staining.

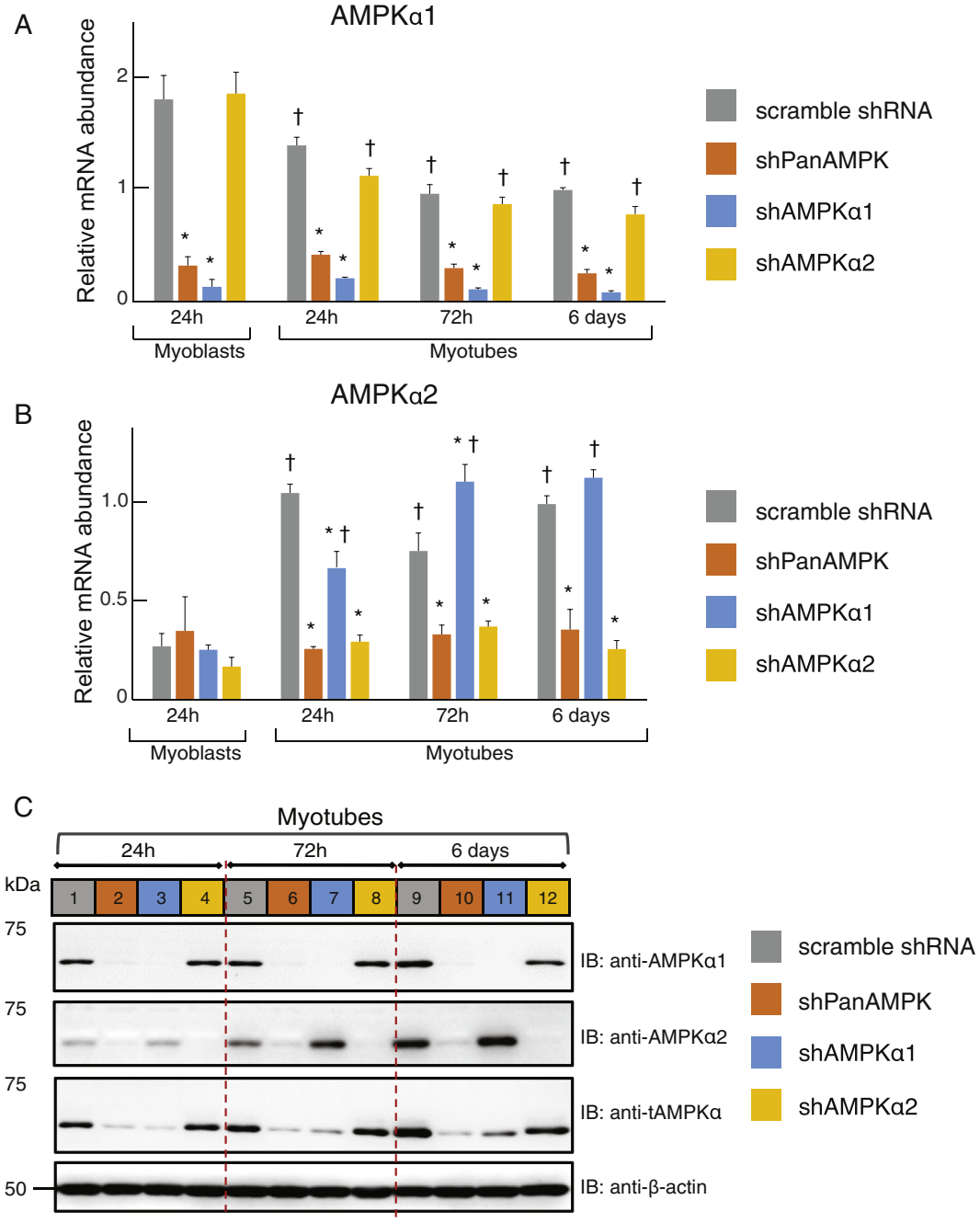


Fig. 2. Expression of AMPKα isoforms during the differentiation of C2C12 myoblasts into myotubes and their contributions to AMPK activity and to ACC and ULK1 phosphorylation. A and B) RT-qPCR analysis of AMPKα1 (A) and AMPKα2 (B) mRNA abundance in C2C12 myoblasts cultured for 24 h and in myotubes at 24 h, 72 h, or 6 days of differentiation. The cells expressed scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 as indicated. Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus corresponding value for scramble shRNA, †$p < 0.05$ versus corresponding value for myoblasts (ANOVA followed by the Tukey-Kramer post hoc test). C) Immunoblot (IB) analysis of myotubes as in (A) at 24 h, 72 h, and 6 days of differentiation with antibodies to AMPKα1, to AMPKα2, to total AMPKα (tAMPKα), and to β-actin. D) Immunoblot analysis of AMPKα phosphorylated on Thr^{172} (pAMPK), total AMPKα, AMPKα1, AMPKα2, and β-actin in C2C12 myotubes expressing scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 at 6 days of differentiation. E and F) Lysates of myotubes expressing scramble shRNA or shPanAMPK at 6 days of differentiation were subjected to immunoprecipitation (IP) with antibodies specific for AMPKα1 (E) or for AMPKα2 (F), and the resulting precipitates were subjected to immunoblot analysis with antibodies to AMPKα phosphorylated on Thr^{172} or to the corresponding AMPKα isoform. G and H) Immunoblot analysis of ACC phosphorylated on Ser^{79} (pACC) and total ACC (G) as well as of ULK1 phosphorylated on Ser^{555} (pULK1) and total ULK1 (H) in C2C12 myotubes expressing scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 at 6 days of differentiation.

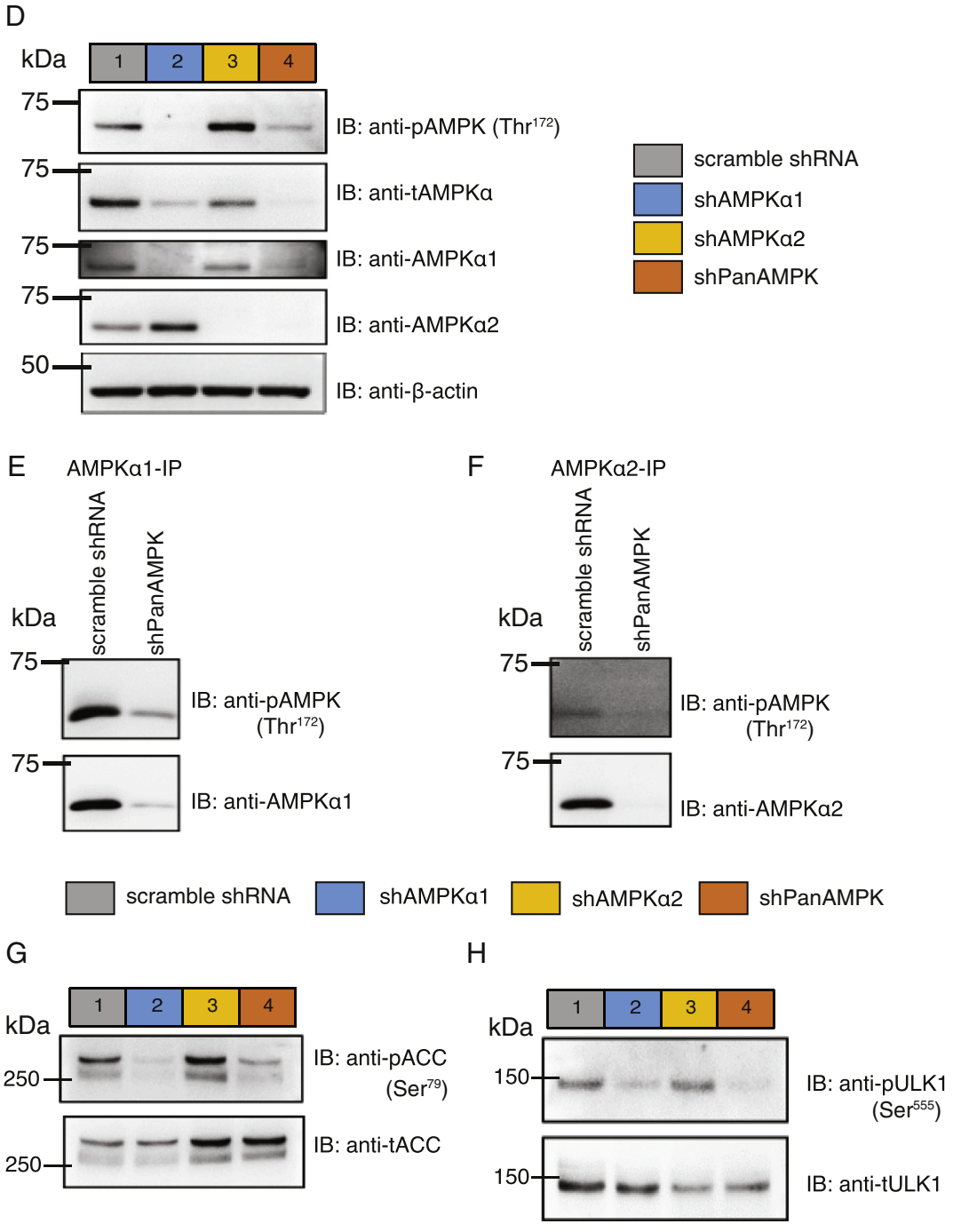


Fig. 2 (*continued*).

2.7. Immunofluorescence Staining and Analysis of the Nuclear Localization of AMPKα2

C2C12 myoblasts or myotubes cultured on cover slips were fixed with 4% paraformaldehyde for 20 min, permeabilized by exposure to ice-cold acetone, and treated with 5% FBS in PBS at room temperature. The cells were then incubated overnight at 4 °C with rabbit polyclonal antibodies to AMPKα2 or to FLAG (Supplementary Table 1). Immune complexes were detected with Alexa Fluor 488–labeled goat antibodies to rabbit immunoglobulin G (Life Technologies), and nuclei were visualized by mounting slides with Fluoromount-G containing 4′,6-diamidino-2-phenylindole (DAPI) (Southern Biotech, Birmingham, AL). Fluorescence of Alexa Fluor 488 and DAPI was detected with a fluorescence microscope (DMI4000B; Leica Microsystems, Wetzlar, Germany). A total of 500 transfected cells from six independent fields was counted for each group.

2.8. Tetramethylrhodamine Methyl Ester (TMRM) Staining

C2C12 myotubes at 6 days after culture in differentiation medium were incubated for 30 min at 37 °C with 20 nM TMRM, washed once with PBS, and then overlaid with PBS for live-cell imaging with a fluorescence microscope (DMI4000B, Leica Microsystems). TMRM and EGFP images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD) to determine the level of fluorescence in

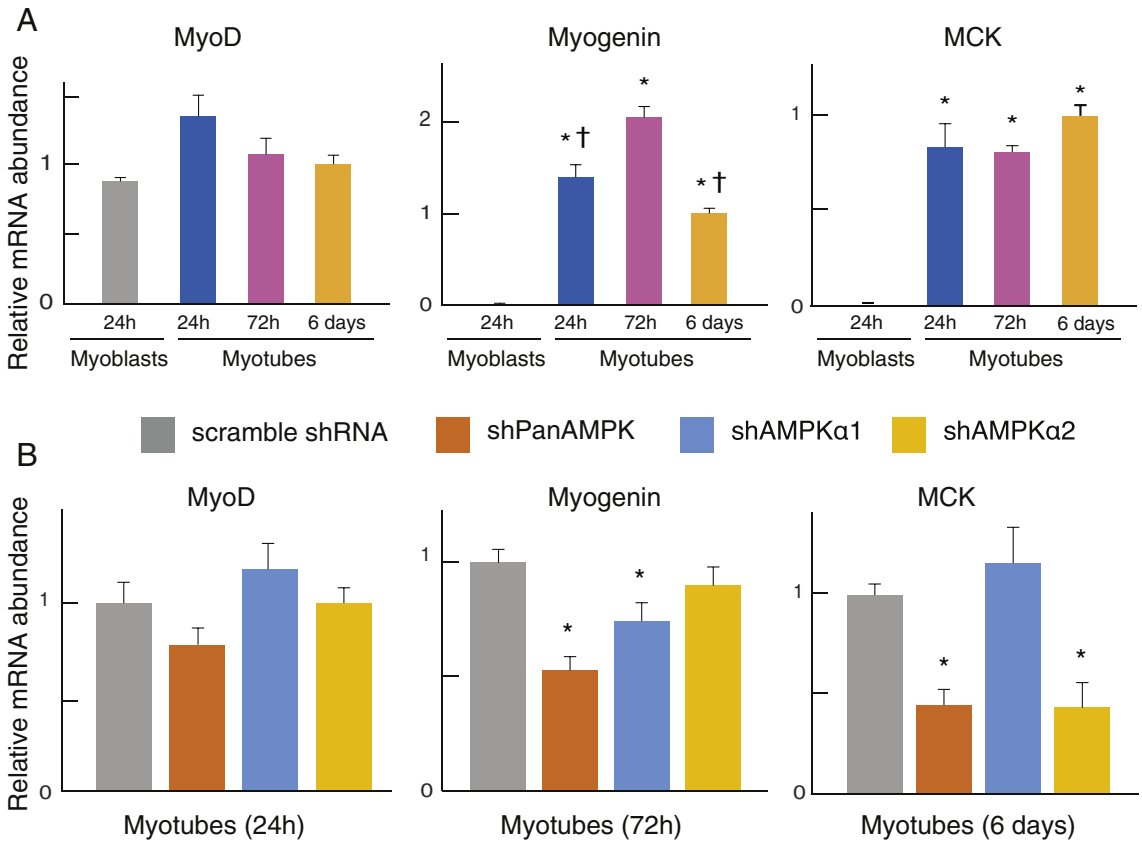


Fig. 3. Effects of depletion of AMPKα isoforms on the expression of myogenic markers during differentiation of C2C12 cells. A) RT-qPCR analysis of MyoD1, myogenin, and MCK mRNA abundance in myoblasts expressing scramble shRNA cultured for 24 h as well as in corresponding myotubes at 24 h, 72 h, and 6 days of culture in differentiation medium. Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus corresponding value for myoblasts, †$p < 0.05$ versus corresponding value for myotubes at 72 h (ANOVA followed by the Tukey-Kramer post hoc test). B) RT-qPCR analysis of MyoD1, myogenin, and MCK mRNA abundance in C2C12 myotubes at 24 h, 72 h, and 6 days of differentiation, respectively. The cells expressed scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 as indicated. Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus corresponding value for scramble shRNA (ANOVA followed by the Tukey-Kramer post hoc test).

regions of interest [18,19]. Twenty independent regions were selected from each TMRM and EGFP image for each cell line.

2.9. RNA Extraction, Reverse Transcription (RT), and Real-Time PCR Analysis

Total RNA was extracted from C2C12 myoblasts or myotubes with the use of the Trizol reagent (Life Technologies). The purified RNA (500 ng) was subjected to RT (reverse transcription) with avian myeloblastosis virus (AMV) reverse transcriptase and an oligo(dT) primer (Takara). Transcripts encoding FLAG-tagged AMPKα2 (WT or ΔNLS) (Supplementary Figs. 2 and 3) were detected by RT-PCR analysis with ExTaq polymerase (Takara) and a Veriti 96-Well Thermal Cycler (Life Technologies). Real-time (quantitative, q) PCR (RT-qPCR) analysis was performed with SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara) and a StepOne Real-Time PCR System (Life Technologies). The abundance of target mRNAs was normalized by that of 36B4 mRNA. Primer sequences for PCR analysis are listed in Supplementary Table 2.

2.10. Immunoblot Analysis

Lysates of C2C12 myoblasts or myotubes were subjected to immunoblot analysis with specific primary antibodies (Supplementary Table 1). Immune complexes were detected with horseradish peroxidase–conjugated goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, UK). Images of protein bands were captured with an ImageQuant LAS 1000 Mini instrument (GE Healthcare) and were processed with ImageJ software (National Institutes of Health). β-Actin was examined as a loading control.

2.11. Immunoprecipitation

Cell lysates (200 μg of protein) were incubated overnight at 4 °C in a solution containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, 250 mM sucrose, protease and phosphatase inhibitor cocktails (Sigma-Aldrich and Calbiochem, respectively), antibodies to AMPKα1 or to AMPKα2 (Abcam, Cambridge, UK), and Protein A/G Sepharose mix (GE Healthcare). The Protein A/G Sepharose beads were then separated by centrifugation and washed twice with ice-cold PBS, after which the bound proteins were subjected to immunoblot analysis.

2.12. Statistical Analysis

Data are presented as means ± SEM. Comparisons among multiple groups were performed by analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test, whereas those between two groups were performed with the unpaired or paired Student's *t*-test (two-tailed). A *p* value of <0.05 was considered statistically significant. The cell diameter of C2C12 myobutes were statistically analyzed after logarithmic conversion to the normal distribution.

3. Results

3.1. Depletion of AMPKα1 Slows C2C12 Cell Proliferation

C2C12 myoblasts expressing scramble shRNA (Fig. 1A), shPanAMPK (Fig. 1B), shAMPKα1 (Fig. 1C), or shAMPKα2 (Fig. 1D) were isolated by

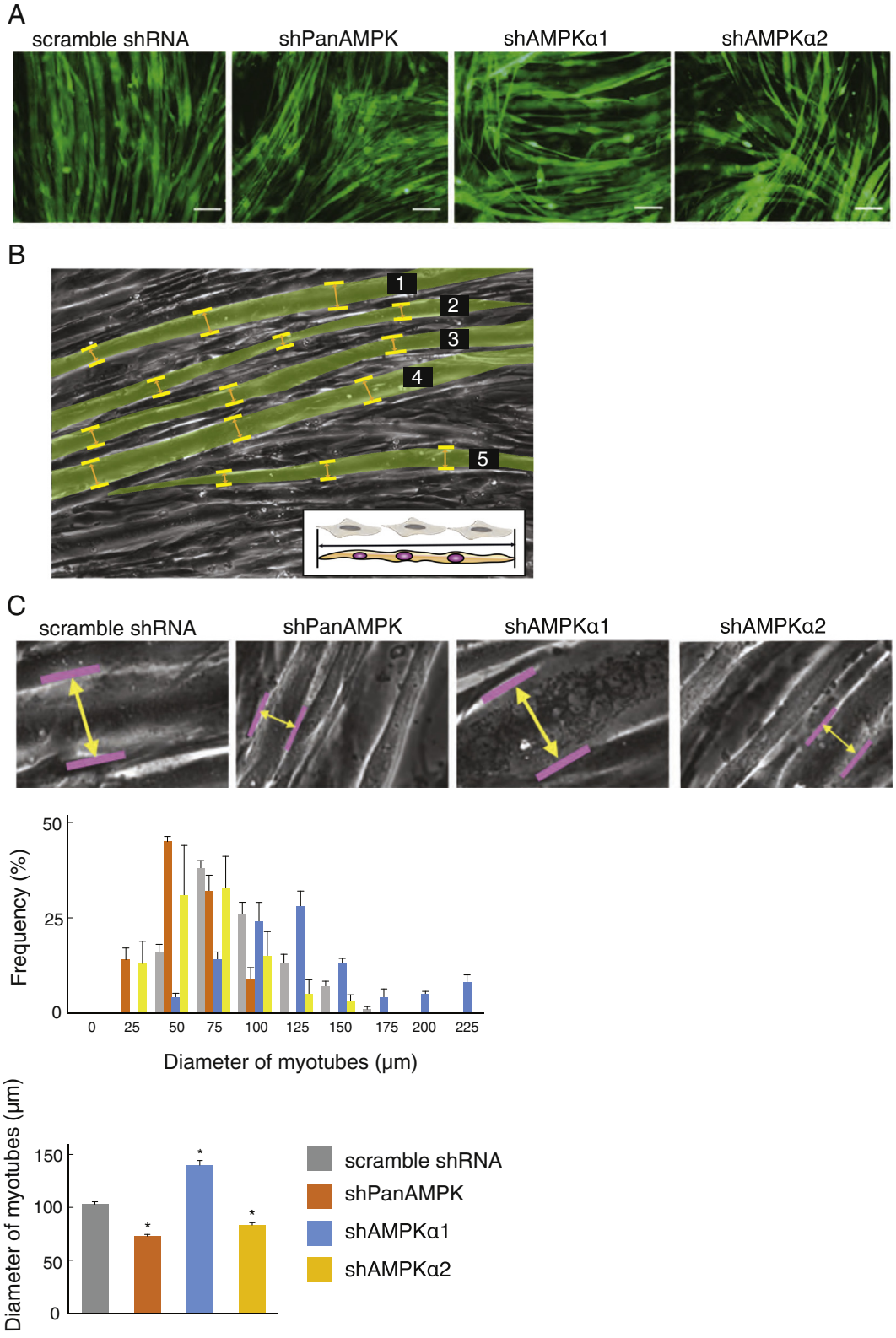
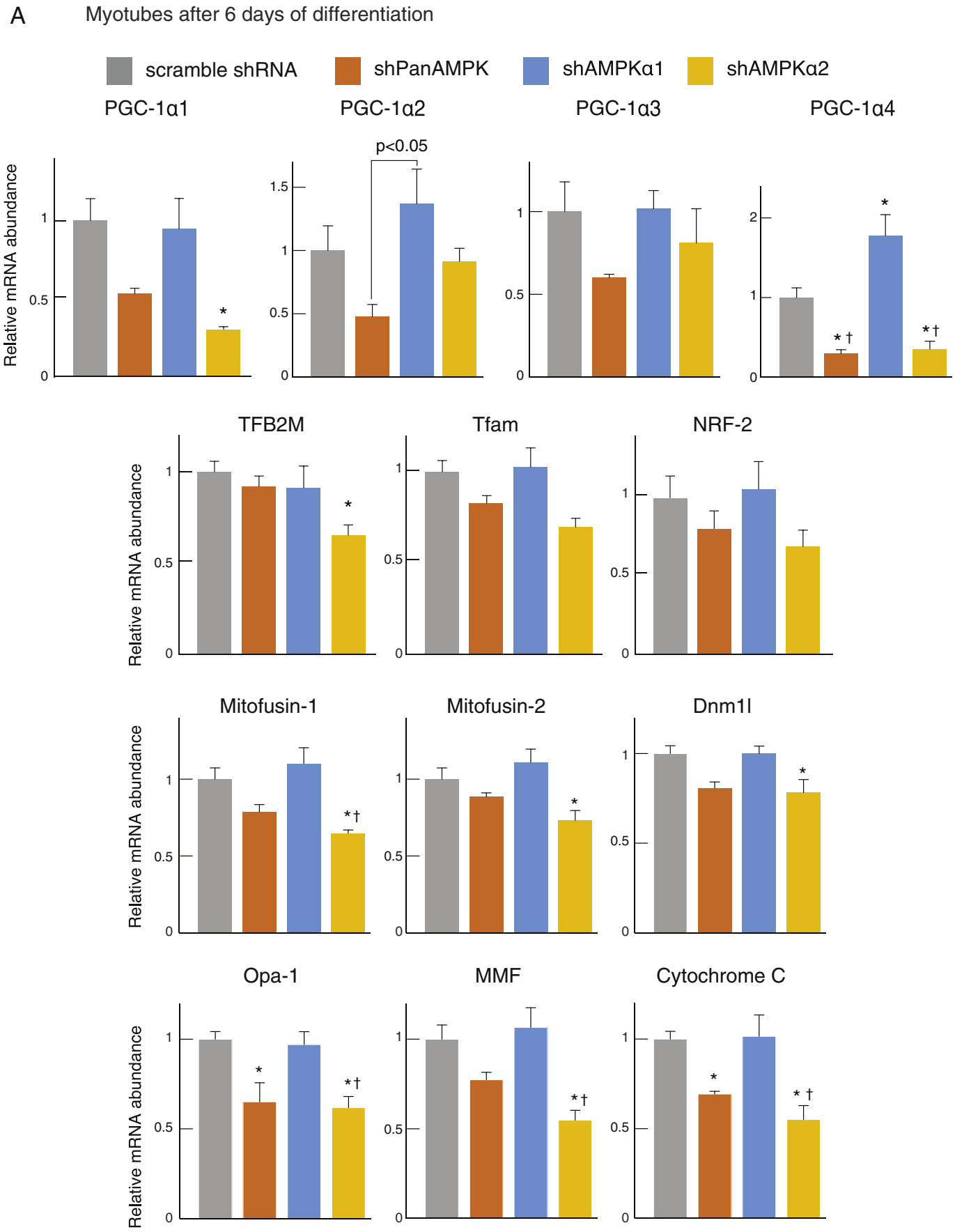


Fig. 4. AMPKα1 and AMPKα2 reciprocally regulate the volume of C2C12 myotubes. A) Representative images of EGFP fluorescence in C2C12 myotubes incubated in differentiation medium for 6 days. The cells expressed scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 as indicated. Scale bars, 200 μm. B) Representative fluorescence image showing measurement of the diameter of individual myotubes in one field. Three short-axis measurements were taken along the length of a selected myotube, and the average value was calculated. The inset depicts the selection criteria for measurement of myotube diameter: a length that is more than three times that of myoblasts, and the presence of at least three nuclei in a single alignment. C) Representative fluorescence images of myotubes as in (A) depicting the differences in cell diameter among the cell lines (upper panels). Histogram of cell diameter for 150 myotubes of each line (middle panel). Mean diameter calculated from the data in the middle panel (lower panel). Data are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus scramble shRNA (ANOVA followed by the Tukey-Kramer post hoc test).


A
Myotubes after 6 days of differentiation
scramble shRNA
shPanAMPK
shAMPKα1
shAMPKα2
PGC-1α1
PGC-1α2
PGC-1α3
PGC-1α4
p<0.05
Relative mRNA abundance
TFB2M
Tfam
NRF-2
Mitofusin-1
Mitofusin-2
Dnm1l
Opa-1
MMF
Cytochrome C

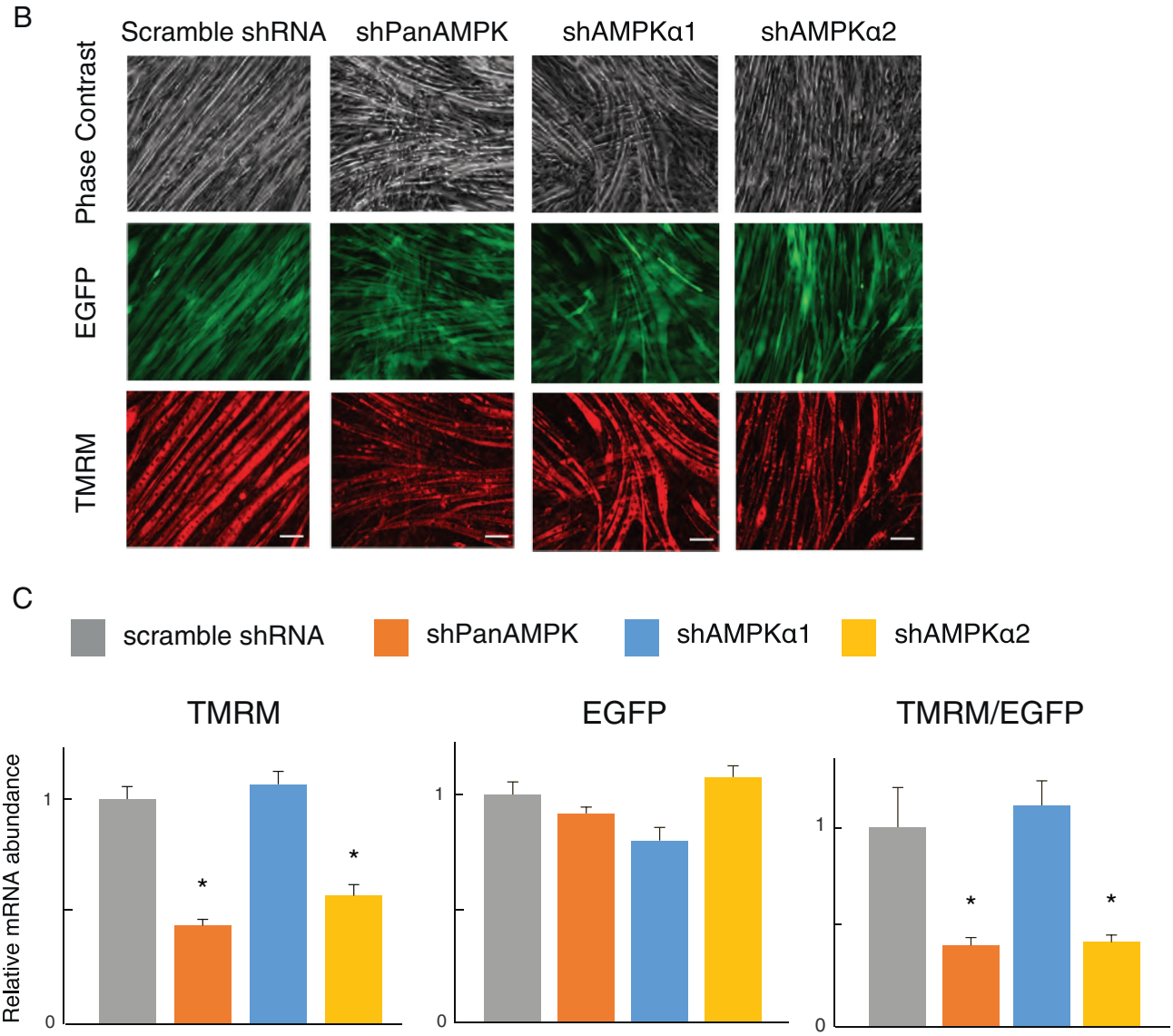


Fig. 5. Effects of depletion of AMPKα1 or AMPKα2 on mitochondrial biogenesis in C2C12 myotubes. A) RT-qPCR analysis of PGC-1α1, PGC-1α2, PGC-1α3, PGC-1α4, TFB2M, Tfam, NRF-2, mitofusin-1 and 2, Dnm1l, Opa-1, MMF, cytochrome c mRNA abundance in C2C12 myotubes cultured for 6 days in differentiation medium. The cells expressed scramble shRNA, shPanAMPK, shAMPKα1, or shAMPKα2 as indicated. Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 3–5 independent experiments). $^{*}p < 0.05$ versus corresponding value for scramble shRNA, †$p < 0.05$ versus corresponding value for shAMPKα1 (ANOVA followed by the Tukey-Kramer post hoc test). B) Phase-contrast, EGFP fluorescence, and TMRM fluorescence images of myotubes cultured as in (A). Scale bars, 200 μm. C) Quantification of TMRM and EGFP fluorescence intensities for 20 independent regions of interest in images similar to those in (B). Data are means ± SEM (n = 20). $^{*}p < 0.05$ versus corresponding value for scramble shRNA (ANOVA followed by the Tukey-Kramer post hoc test).

FACS on the basis of their expression of EGFP. >95% of the sorted myoblasts were positive for EGFP. Myoblasts expressing shPanAMPK or shAMPKα1 were found to proliferate more slowly compared with those expressing shAMPKα2 or scramble shRNA (Fig. 1E and F), suggesting that AMPKα1 is necessary for the normal proliferation of C2C12 myoblasts.

3.2. Up-Regulation of AMPKα2 Abundance During Differentiation of C2C12 Myoblasts

RT-qPCR analysis revealed that AMPKα1 mRNA was abundant in myoblasts but that its amount declined in cells expressing scramble shRNA or shAMPKα2 during their differentiation (Fig. 2A). As expected, the amount of AMPKα1 mRNA was greatly reduced in cells expressing shPanAMPK or shAMPKα1. In contrast, the abundance of AMPKα2 mRNA increased significantly during the differentiation of C2C12 myoblasts expressing scramble shRNA or shAMPKα1 but not in those expressing shPanAMPK or shAMPKα2 (Fig. 2B).

Immunoblot analysis revealed that the amount of AMPKα1 protein remained relatively constant in C2C12 cells expressing scramble shRNA or shAMPKα2 during culture for 6 days in differentiation medium, whereas that in cells expressing shPanAMPK or shAMPKα1 was almost undetectable (Fig. 2C). In contrast, the amount of AMPKα2 protein increased gradually during the differentiation of myotubes expressing scramble shRNA or shAMPKα1, whereas that in cells expressing shPanAMPK or shAMPKα2 was again undetectable. Immunoblot analysis with antibodies to total AMPKα yielded results largely similar to those obtained with antibodies specific for AMPKα1. The amount of β-actin remained unchanged during myogenesis. These results suggested that, whereas the amount of AMPKα1 protein remains constant, that of AMPKα2 increases during the differentiation of C2C12 myoblasts into myotubes.

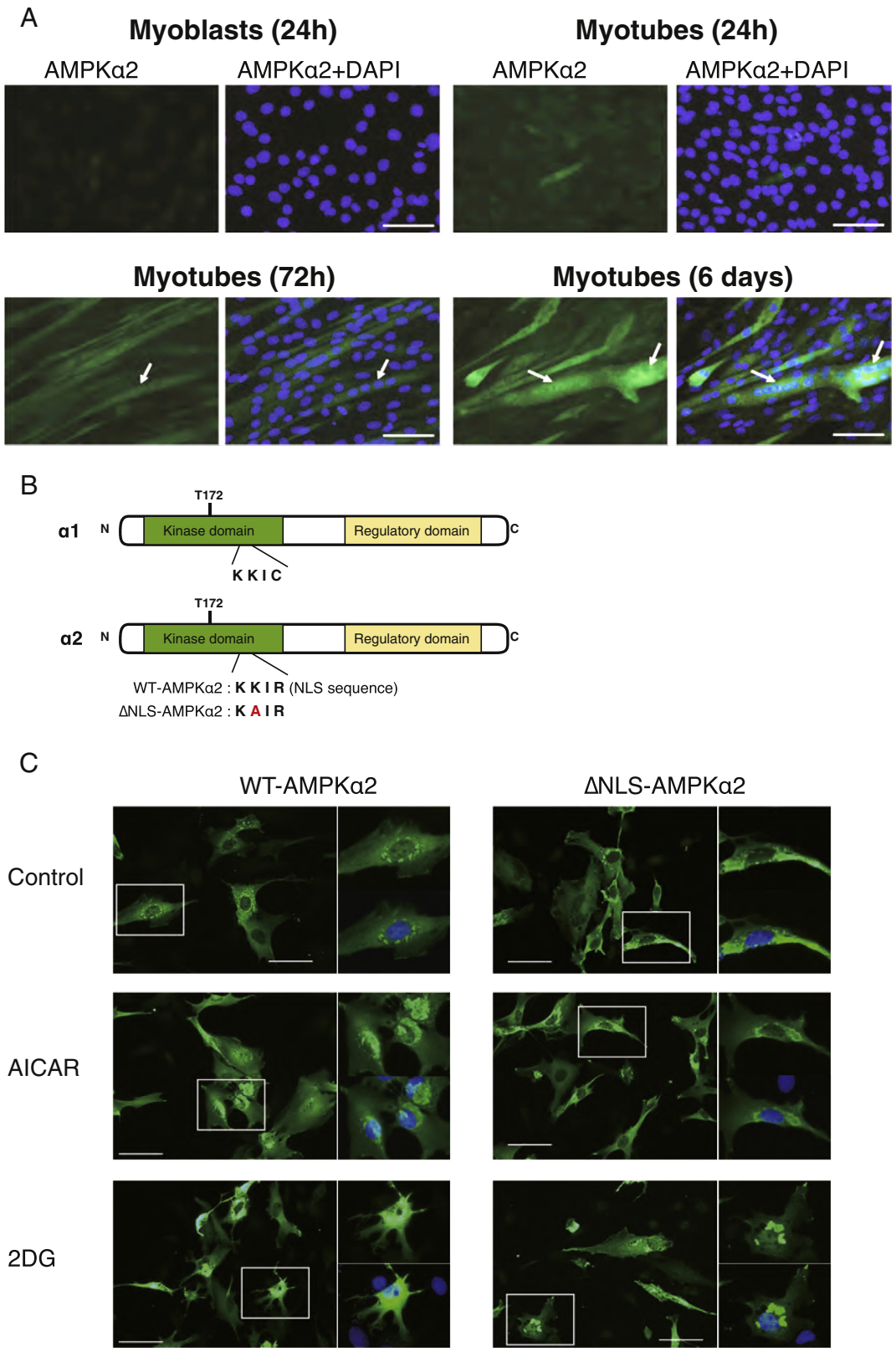


Fig. 6. Subcellular distribution of AMPKα2 during myogenesis and effects of AMPK activators on the nuclear translocation of WT-AMPKα2 and ΔNLS-AMPKα2 in C2C12 myoblasts. A) Immunofluorescence staining of endogenous AMPKα2 (green) in C2C12 myoblasts cultured for 24 h as well as in myotubes at 24 h, 72 h, or 6 days after the induction of differentiation. Nuclei were stained with DAPI (blue). Arrows indicate colocalization of AMPKα2 and DAPI fluorescence. Scale bars, 200 μm. B) Domain organization of AMPKα1 and AMPKα2 as well as the amino acid sequence of the putative NLS present only in AMPKα2. A point mutation (K224A) was introduced into the NLS sequence by site-directed mutagenesis to generate the ΔNLS-AMPKα2 mutant. C) Immunofluorescence staining with antibodies to FLAG of C2C12 myoblasts transiently expressing FLAG-tagged WT-AMPKα2 or ΔNLS-AMPKα2 and incubated in the absence or presence of AICAR (0.5 mM) or 2DG (21.3 mM) for 1 h. Cells in the insets are shown at higher magnification on the right without (upper) or with (lower) DAPI staining. Scale bars, 200 μm. D) Numbers of cells in which FLAG-tagged WT-AMPKα2 or ΔNLS-AMPKα2 was detected in the cytoplasm only or in both the cytoplasm and nucleus of myoblasts stimulated with AICAR or 2DG as in (C). A total of 500 transfected cells from six independent fields was counted for each group. Data are means ± SEM (n = 6). $^{*}p < 0.05$ versus corresponding value for cytoplasm, †$p < 0.05$ versus corresponding value for no stimulation (ANOVA followed by the Tukey-Kramer post hoc test).

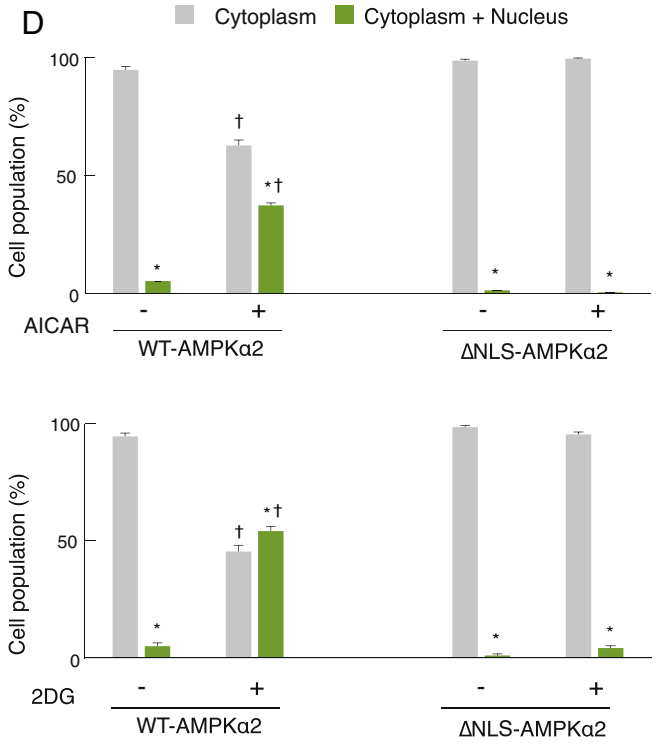


Fig. 6 *(continued).*

3.3. Phosphorylation of ACC and ULK1 is Mediated Mostly by AMPKα1

We next examined the phosphorylation of AMPKα subunits, which reflects AMPK activation, in C2C12 myotubes at 6 days after culture in differentiation medium. Myotubes expressing scramble shRNA or shAMPKα2 showed a high level of phosphorylated AMPKα (pAMPK), whereas the amount of pAMPK was greatly reduced in those expressing shAMPKα1 or shPanAMPK (Fig. 2D). The pattern of band intensity obtained with the antibodies to pAMPK was similar to that obtained with those to total AMPKα or to AMPKα1. These results thus suggested that AMPKα1 accounts for most pAMPK in C2C12 myotubes at 6 days of differentiation. However, immunoprecipitation of AMPKα1 and AMPKα2 separately revealed that both isoforms are phosphorylated in myotubes at this time (Fig. 2E and F), indicating that both AMPKα1 and AMPKα2 are activated.

Activated AMPK mediates the phosphorylation of acetyl-CoA carboxylase (on Ser^{79} for ACC1 and Ser^{212} for ACC2) and thereby promotes fatty acid oxidation [5,8,20,21]. AMPK also regulates autophagy, which is initiated as a result of phosphorylation of ULK1 (Unc51-like autophagy-activating kinase 1) on Ser^{555} [22–26]. C2C12 myotubes expressing scramble shRNA or shAMPKα2 showed a high level of phosphorylation of ACC and ULK1 at 6 days of differentiation (Fig. 2G and H). In contrast, the extent of ACC and ULK1 phosphorylation was greatly reduced in myotubes expressing shAMPKα1 or shPanAMPK. These results suggested that AMPKα1 rather than AMPKα2 is the major regulator of ACC and ULK1 activity in C2C12 myotubes.

3.4. Roles of AMPKα1 and AMPKα2 in the Middle and Late Stages of Myogenesis, Respectively

We determined the mRNA abundance for myogenic markers in C2C12 myoblasts or myotubes expressing scramble shRNA. MyoD, myogenin, and MCK are considered as markers of early, middle, and late stages of myogenic differentiation, respectively [27–32]. The abundance of MyoD mRNA remained relatively constant throughout myogenesis (Fig. 3A). Myogenin mRNA was detected in myotubes but not in myoblasts, with its abundance peaking at 72 h and then declining by 6 days of differentiation. MCK mRNA was also detected only in myotubes, with its abundance tending to be highest at 6 days.

The abundance of MyoD mRNA in myotubes at 24 h of differentiation was not significantly affected by depletion of AMPKα1, AMPKα2, or both AMPKα isoforms (Fig. 3B). In contrast, the amount of myogenin mRNA at 72 h of differentiation was significantly reduced in myotubes expressing shPanAMPK or shAMPKα1, but not in those expressing shAMPKα2. In addition, the amount of MCK mRNA at 6 days of differentiation was significantly reduced in myotubes expressing shPanAMPK or shAMPKα2, but not in those expressing shAMPKα1. These results thus suggested that AMPKα1 preferentially regulates myogenin mRNA abundance and the middle stage of myogenic differentiation, whereas AMPKα2 preferentially regulates the amount of MCK mRNA and the late stage of myogenesis.

3.5. AMPKα1 and AMPKα2 Reciprocally Regulate the Volume of C2C12 Myotubes

After incubation in differentiation medium for 6 days, almost all C2C12 cells had differentiated into multinucleated myotubes regardless of depletion status for AMPKα isoforms (Fig. 4A). However, some myotubes expressing shPanAMPK or shAMPKα2 appeared thinner, whereas some myotubes expressing shAMPKα1 appeared thicker, compared with those expressing scramble shRNA. To examine further whether AMPKα1 and AMPKα2 differentially regulate the volume of C2C12 myotubes, we measured the diameter of individual myotubes at 6 days of differentiation. Three short-axis measurements were taken along the length of a given myotube, and the average value was calculated (Fig. 4B). The distribution and mean value of cell diameter revealed that myotubes expressing shPanAMPK or shAMPKα2 were indeed thinner, whereas those expressing shAMPKα1 were thicker, compared with those expressing scramble shRNA (Fig. 4C). These results thus indicated that AMPKα1 and AMPKα2 regulate the diameter

of C2C12 myotubes in a reciprocal manner. The thin phenotype of myotubes expressing shPanAMPK also suggested that AMPKα2 is necessary for the increase in cell volume induced by the depletion of AMPKα1.

3.6. Depletion of AMPKα2 Attenuates the Expression of Genes Related to Mitochondrial Biogenesis in C2C12 Myotubes

Given that the mRNA abundance for MCK, a key enzyme in the control of cellular energy metabolism, was down-regulated in response to depletion of AMPKα2 in C2C12 myotubes, we examined whether the thin phenotype of myotubes expressing shAMPKα2 at the late stage of differentiation might result from reduced expression of genes related to energy metabolism and mitochondrial biogenesis. AMPK plays an important role in mitochondrial biogenesis through regulation of PGC-1α–dependent gene transcription [33–40]. We found that C2C12 myotubes expressing shAMPKα2 showed a significantly reduced abundance of PGC-1α1 and PGC-1α4, but not PGC-1α2 or PGC-1α3, at 6 days of differentiation (Fig. 5A). The amounts of mitochondrial transcription factor

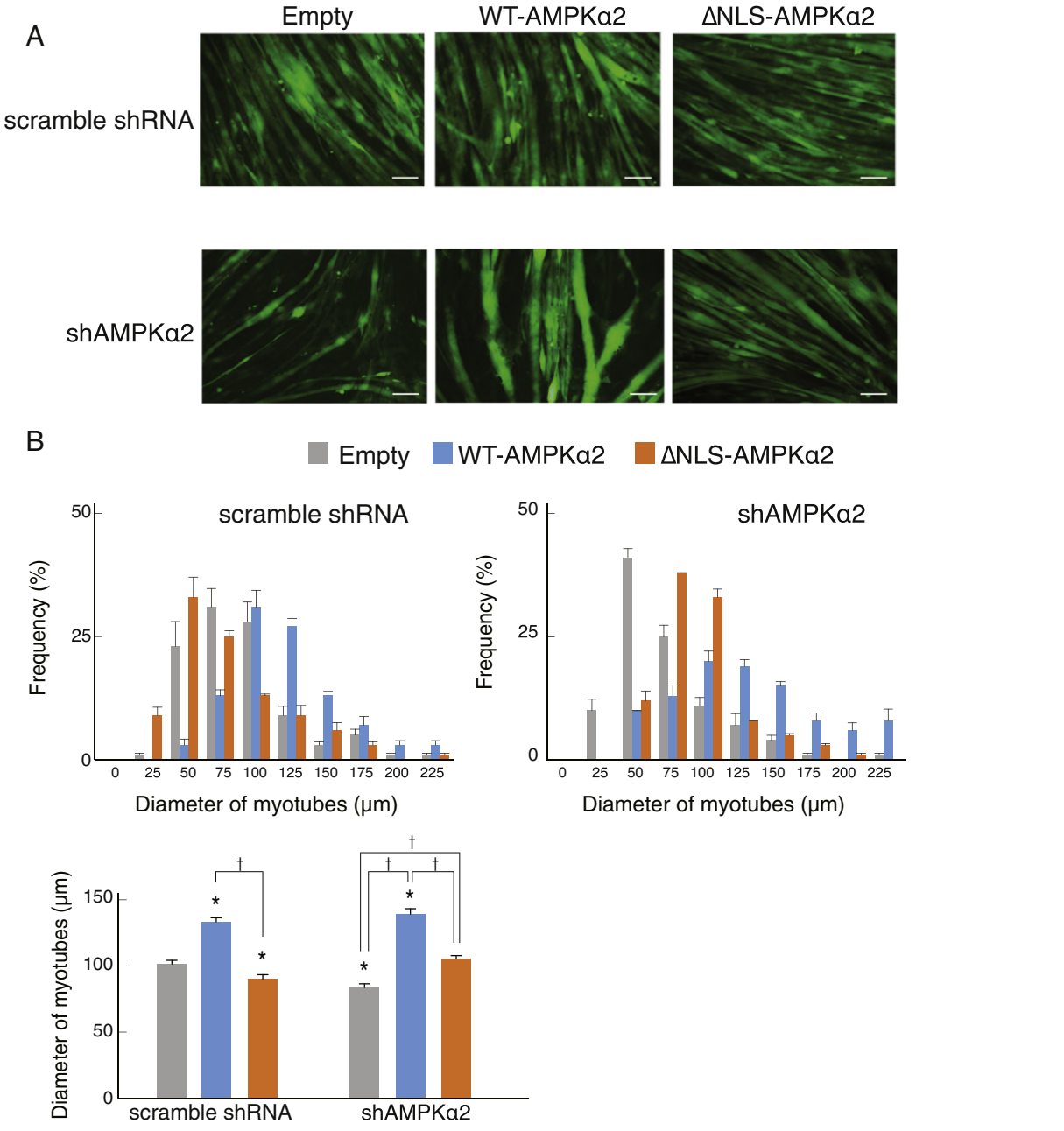


Fig. 7. Effects of WT-AMPKα2 and ΔNLS-AMPKα2 expression on cell diameter and MCK and PGC-1α mRNA abundance in C2C12 myotubes depleted of endogenous AMPKα2. A) Representative EGFP fluorescence images of myotubes expressing scramble shRNA or shAMPKα2 that had also been infected with lentiviruses encoding WT-AMPKα2 or ΔNLS-AMPKα2 (or with the empty virus) before culture in differentiation medium for 6 days. Scale bars, 200 μm. B) Histograms for the distribution of cell diameter (upper panels) and mean values of cell diameter (lower panel) for 150 myotubes determined from images similar to those in (A). Data are means ± SEM (n = 3 independent experiments). *$p < 0.05$ versus value for the empty virus in myotubes expressing scramble shRNA (ANOVA followed by the Tukey-Kramer post hoc test). †$p < 0.05$ (ANOVA followed by the Tukey-Kramer post hoc test). C) RT-qPCR analysis of AMPKα1 (left) and AMPKα2 (right) mRNA abundance in myotubes expressing shAMPKα2 that had also been infected with lentiviruses encoding WT-AMPKα2 or ΔNLS-AMPKα2 (or with the empty virus) and cultured in differentiation medium for 6 days. Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 5 independent experiments). *$p < 0.05$ versus corresponding value for the empty virus (ANOVA followed by the Tukey-Kramer post hoc test). D) Immunoblot analysis of AMPKα1, AMPKα2, and β-actin in myotubes as in (C). E) RT-PCR analysis for the FLAG coding sequence and GAPDH mRNA (internal control) in myotubes as in (C). F) RT-qPCR analysis of MCK, PGC-1α1, PGC-1α2, PGC-1α3 and PGC-1α4 mRNA abundance in myotubes as in (C). Data were normalized by the abundance of 36B4 mRNA and are means ± SEM (n = 5–8 independent experiments). *$p < 0.05$ versus corresponding value for the empty virus (ANOVA followed by the Tukey-Kramer post hoc test).

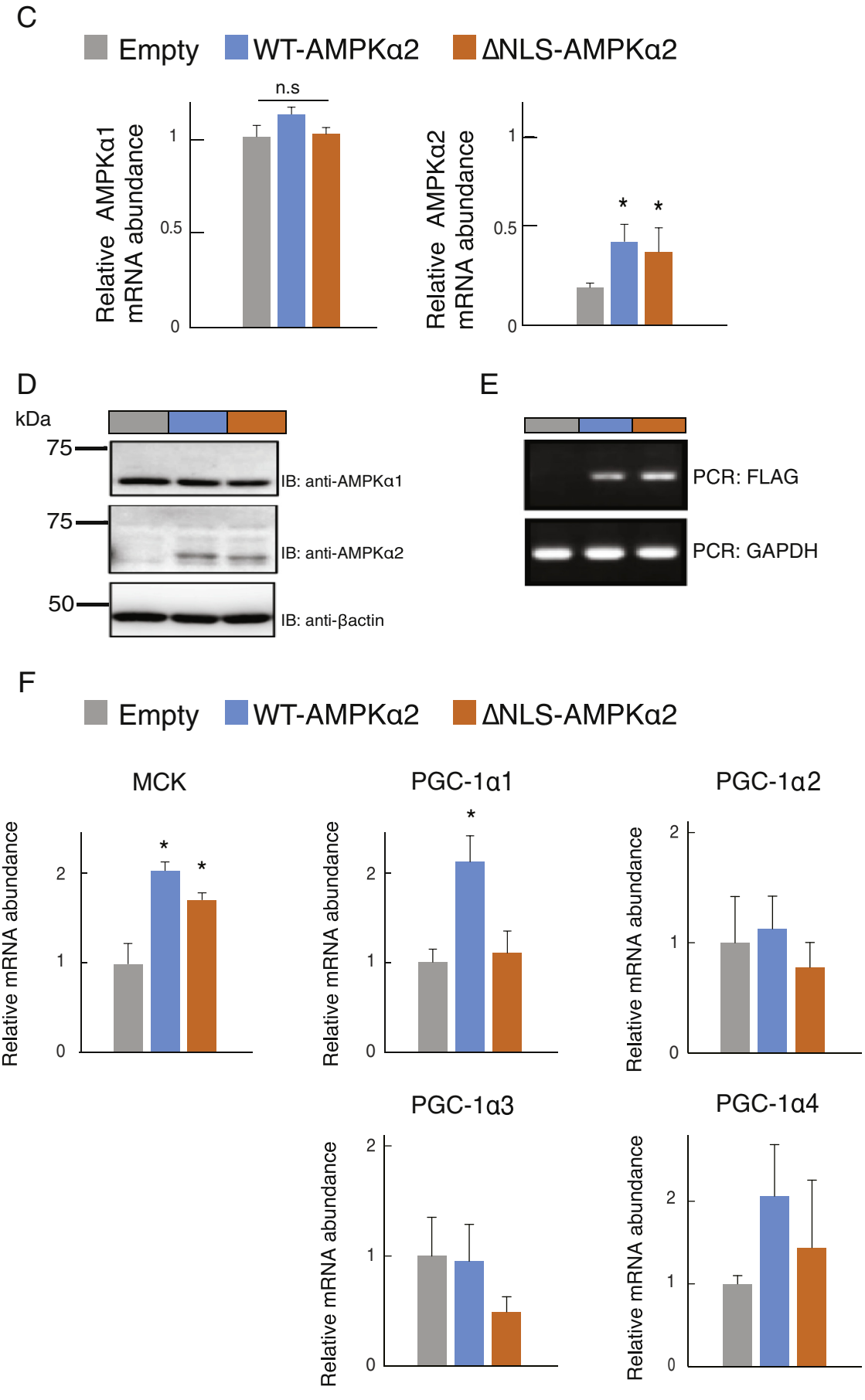


Fig. 7 *(continued).*

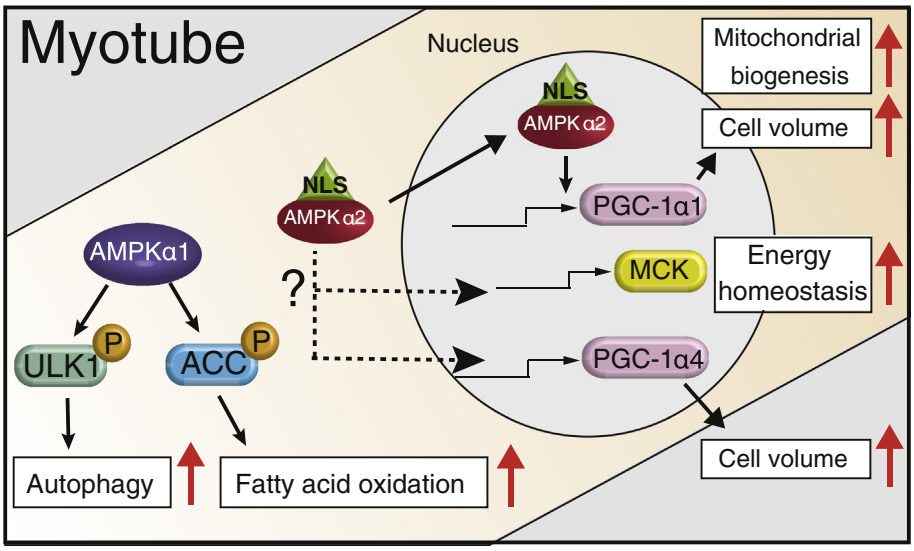


Fig. 8. Proposed roles for AMPKα1 and AMPKα2 in C2C12 myotubes. AMPKα1 is necessary for the phosphorylation of ACC and ULK1 and the consequent up-regulation of fatty acid oxidation and autophagy, respectively. On the other hand, AMPKα2 mediates the up-regulation of PGC-1α1, PGC-1α4 and MCK expression. NLS in AMPKα2 is necessary for the up-regulations of PGC-1α1 expression but not that of MCK. The action of PGC-1α1 possibly induces mitochondrial biogenesis. These effects of AMPKα2 may contribute to an increase in cell volume.

B2 (TFB2M), mitofusion-1 and -2, dynamin-1-like protein (Dnm1l), optic atrophy 1 (Opa1), mitochondrial fission factor (MFF) and cytochrome c are also reduced in myotubes expressing shAMPKα2, compared with those expressing scramble shRNA. The amounts of mitochondrial transcription factor A (Tfam) and nuclear respiratory factor–2 (NRF-2) mRNAs tended to be reduced in myotubes expressing shAMPKα2. The mRNA amounts of PGC-1α4, Opa-1 and cytochrome c were also reduced in myotubes expressing shPanAMPK, and those of PGC-1α1 and some other mitochondrial genes tended to be reduced in myotubes expressing shPanAMPK. These results suggest that AMPKα2 is necessary for maintenance of the mRNA abundance for some genes related to mitochondrial biogenesis in C2C12 myotubes. In contrast, C2C12 myotubes expressing shAMPKα1 increased the mRNA amount of PGC-1α4.

TMRM is a cell-permeant fluorescent dye that is readily sequestered by active mitochondria. Myotubes expressing shPanAMPK or shAMPKα2 showed a reduced level of TMRM staining compared with those expressing scramble shRNA or shAMPKα1 at 6 days of differentiation (Fig. 5B and C), suggesting that AMPKα2 is necessary for mitochondrial biogenesis at the late stage of myogenic differentiation in C2C12 cells.

3.7. Activated AMPKα2 Translocates to the Nucleus

Immunofluorescence analysis confirmed that the abundance of AMPKα2 gradually increased during differentiation of C2C12 myoblasts expressing scramble shRNA into myotubes as well as revealed that a portion of AMPKα2 was localized to the nucleus at the late stage of differentiation (Fig. 6A). We investigated whether AMPKα2 might translocate to the nucleus in an NLS-dependent manner in response to its activation. We thus transiently transfected C2C12 myoblasts with expression vectors for FLAG-tagged WT or ΔNLS mutant forms of AMPKα2, the latter of which contains a point mutation in the NLS (Fig. 6B). Immunofluorescence analysis with antibodies to the FLAG epitope revealed that both WT-AMPKα2 and ΔNLS-AMPKα2 remained in the cytoplasm of the myoblasts under basal conditions (Fig. 6C). In contrast, exposure of the cells to the AMPK activator AICAR or to 2DG induced the nuclear translocation of WT-AMPKα2 but not that of ΔNLS-AMPKα2 (Fig. 6C). Whereas WT-AMPKα2 was restricted to the cytoplasm in almost all (95%) cells without stimulation, it was also present in the nucleus of ~40% and ~50% of cells stimulated with AICAR or 2DG, respectively (Fig. 6D). In contrast, ΔNLS-AMPKα2 remained in the cytoplasm of cells after exposure to either agent. These results suggested that AMPKα2 indeed translocates to the nucleus in an NLS-dependent manner in response to its activation.

3.8. Forced Expression of AMPKα2 Increases the Abundance of MCK and PGC-1α mRNAs in Myotubes Depleted of Endogenous AMPKα2

We next examined the effects of stable expression of WT-AMPKα2 or ΔNLS-AMPKα2 in C2C12 myotubes depleted of endogenous AMPKα2 by RNA interference. Expression of WT-AMPKα2 significantly increased the diameter of myotubes expressing scramble shRNA and shAMPKα2 (Fig. 7A and B). In contrast, expression of ΔNLS-AMPKα2 decreased that of myotubes expressing scramble shRNA and shAMPKα2, compared with that of myotubes expressing WT-AMPKα2. However, expression of ΔNLS-AMPKα2 slightly but significantly increased the diameter of myotubes expressing shAMPKα2, compared with that of myotubes infected empty vector, while expression of ΔNLS-AMPKα2 decreased the diameter of myotubes expressing scramble shRNA. The latter effect may be due in part to a dominant-negative effect of ΔNLS-AMPKα2 to endogenous AMPKα2.

Infection with lentiviruses encoding WT-AMPKα2 or ΔNLS-AMPKα2 increased the abundance of AMPKα2 mRNA (Fig. 7C) and protein (Fig. 7D) in myotubes expressing shAMPKα2, whereas it had no effect on that of AMPKα1 mRNA or protein. RT-PCR analysis of the coding sequence for FLAG also indicated that the constructs for WT-AMPKα2 and ΔNLS-AMPKα2 were expressed at similar levels in myotubes expressing shAMPKα2 (Fig. 7E).

Finally, we examined the effects of WT-AMPKα2 and ΔNLS-AMPKα2 on the mRNA abundances of MCK and PGC-1α isoforms, and PGC-1α isoforms (PGC-1α1 to PGC-1α4), in myotubes expressing shAMPKα2. RT-qPCR analysis showed that expression of WT-AMPKα2 increased the amounts of both MCK and PGC-1α1 mRNAs, whereas that of ΔNLS-AMPKα2 increased the amount of MCK mRNA but not that of PGC-1α1 mRNA (Fig. 7F). The amounts of PGC-1α2 and PGC-1α3 mRNAs did not change in the myotubes expressing WT-AMPKα2 or ΔNLS-AMPKα2. These results suggest that the nuclear translocation of AMPKα2 is necessary for maintenance of PGC-1α1 mRNA abundance during the late stage of myogenic differentiation in C2C12 cells, whereas the maintenance of MCK mRNA abundance by AMPKα2 is independent of its nuclear translocation. The amount of PGC-1α4 mRNA tended to

increase in myotubes expressing WT-AMPKα2, whereas the amount in myotubes expressing ΔNLS-AMPKα2 showed the intermediate level between those of myotubes infected empty vector and expressing WT-AMPKα2. There was a large variation of PGC-1α4 mRNA amounts in myotubes expressing ΔNLS-AMPKα2.

4. Discussion

We have here shown that AMPKα1 and AMPKα2 have distinct roles in myogenesis, with AMPKα1 contributing to the middle stage and AMPKα2 to the late stage of myogenic differentiation. Our results indicate that selective depletion of AMPKα1 attenuated the proliferation of C2C12 myoblasts as well as the up-regulation of myogenin mRNA abundance and the phosphorylation of ACC and ULK1 in myotubes. In contrast, selective depletion of AMPKα2, but not AMPKα1, attenuated the up-regulation of MCK mRNA abundance during the late stage of muscle differentiation as well as the expression of genes related to mitochondrial biogenesis including those for PGC-1α1, PGC-1α4, TFB2, mitofusion-1 and -2, Dnm1l, Opa-1, MFF, and cytochrome c. Selective depletion of AMPKα2 also decreased diameter of C2C12 myotubes. Furthermore, the nuclear translocation of AMPKα2 was necessary for the maintenance of PGC-1α1 mRNA abundance and cell diameter in C2C12 myotubes but not for that of MCK mRNA abundance. Together, our findings thus suggest that AMPKα2 plays a key role in the cell diameter and expressions of mitochondria and energy homeostasis-related genes during the late stage of myogenesis in C2C12 cells. Furthermore, the effects of AMPKα2 are mediated by the nuclear translocation-dependent and -independent mechanisms. PGC-1α1 and PGC-1α4 distinctly regulate expressions of mitochondria-specific gene and muscle hypertrophy-related genes [41]. Thus, PGC-1α1 and PGC-1α4 may regulate the diameter of C2C12 myotubes through distinct mechanisms.

We found that expression of ΔNLS-AMPKα2 slightly but significantly increased the diameter of C2C12 myotubes depleted endogenous AMPKα2, suggesting that the cell diameter is partly regulated by AMPKα2 in the cytoplasm. The increase in the expressions of MCK and PGC-1α4 induced by ΔNLS-AMPKα2 probably contributes the increase in the diameter of C2C12 myotubes. In addition, improvement of energy homeostasis by MCK expression may also contribute to the partial increase in PGC-1α4 mRNA expression in myotubes expressing ΔNLS-AMPKα2. Further investigations are necessary to clarify the mechanism for PGC-1α4 gene expression in C2C12 myotubes.

Activated AMPK was previously shown to stimulate gene transcription through phosphorylation of histone H2B on Ser^{36} [42]. AMPK was also found to mediate the phosphorylation of class IIa histone deacetylase in human primary myotubes and thereby to trigger its nuclear export and consequent abrogation of its transcriptionally repressive function [43]. AMPK also phosphorylates cAMP response element–binding protein (CREB) at the same site targeted by cAMP-dependent protein kinase (PKA) in cultured cells [44]. AMPK was also found to control the expression of PRDM16, a key determinant of brown adipogenesis, via regulation of the Krebs cycle and demethylation of its gene promoter [45]. PGC-1α is regulated by AMPK at the levels of gene expression [37] and posttranslational modification, with the latter including direct phosphorylation of PGC-1α by AMPK and its deacetylation by SIRT1 [36,38]. Whereas the mechanism underlying AMPK-dependent mitochondrial biogenesis remains to be fully elucidated, it likely relies at least in part on the ability of nuclear AMPKα2 to stimulate expression of the PGC-1α1 gene. In addition, a recent study revealed that activated AMPK phosphorylates glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at Ser122 in the cytoplasm, promoting translocation of GAPDH into the nucleus where it directly interacts with and activates Sirt1, thereby initiating autophagy via Atg8 (LC-3 in mammals) [46]. Thus, AMPKα2 in the cytoplasm may induce MCK mRNA expression via a mediator such as GAPDH.

Our results revealed that depletion of AMPKα1 resulted in a significant increase in the diameter of C2C12 myotubes cultured for 6 days in differentiation medium. Primary cultured myotubes derived from AMPKα1 knockout mice were also found to be larger than control cells [47]. We also found that the diameter of myotubes expressing shPanAMPK or shAMPKα2 was smaller than that of those expressing scramble shRNA. These results suggest that the hypertrophic action of shAMPKα1 requires AMPKα2 at the late stage of myogenic differentiation. Our results showing that the diameter of C2C12 myotubes during the late stage of differentiation were paralleled with the expressions of PGC-1α4, suggest that PGC-1α4 as well as PGC-1α1 is necessary for the hypertrophy of C2C12 myotubes. However, knockout of both AMPKα1 and AMPKα2 genes in skeletal muscle was previously shown to result in an increase in muscle mass in vivo and in vitro [48]. This apparent discrepancy in in vitro findings may be due to the facts that the previous study examined myotubes 4 days after the induction of differentiation and that AMPKα1 may be the dominant isoform during this period, with the influence of AMPKα2 being smaller at this time than at 6 days (Figs. 2 and 3). In addition, muscle hypertrophy in the muscle-specific AMPKα1 and AMPKα2 double-knockout mice might be due to AMPK-independent mechanisms such as those mediated by hormones, growth factors, or muscle contraction [5,8,11].

Depletion of AMPKα1 markedly attenuated the proliferation of C2C12 myoblasts, consistent with the previous finding that the lack of AMPKα1 reduced the proliferation and myogenic capacity of satellite cells during muscle regeneration [12]. Ablation of AMPKα1, but not that of AMPKα2, was also previously shown to attenuate myogenin expression and myogenic differentiation in C2C12 cells [13,14]. Moreover, AMPKα1 directly controls hepatocyte proliferation by regulating cyclin A2 expression, which is tightly synchronized with progression of the cell cycle [49]. AMPKα1 thus appears to function as an effector in the control of cell proliferation.

We found that the phosphorylation of ACC on Ser^{79} and that of ULK1 on Ser^{555} were greatly attenuated in C2C12 myotubes expressing shAMPKα1, suggesting that AMPKα1 is the major contributor to AMPK-dependent fatty acid oxidation and autophagy in these cells. However, it remains unclear whether our results indicate that regulation of cell proliferation and phosphorylation of ACC and ULK1 in C2C12 cells are mediated by AMPKα1 alone or whether they simply reflect the higher expression level of AMPKα1 than of AMPKα2. Indeed, voluntary exercise and leptin treatment preferentially activate AMPKα2 in skeletal muscle in vivo, resulting in increased phosphorylation of ACC and fatty acid oxidation in the tissue [5,7,8,50].

Three (α1β2γ1, α2β2γ1, and α2β2γ3) of the 12 theoretically possible heterotrimeric combinations of AMPK subunits were previously shown to account for AMPK activity in human skeletal muscle [6,8,51–53]. We found that β1 and γ2 are the most abundant β and γ subunit mRNAs in C2C12 myoblasts, whereas β2, γ1, and γ3 mRNAs are the most abundant in mature C2C12 myotubes (data not shown). It will be of interest to determine the subcellular localization and function of different combinations of AMPK subunits in skeletal muscle and muscle cell lines.

Collectively, our data show that AMPKα1 and AMPKα2 have distinct roles in myogenic differentiation in C2C12 cells (Fig. 8). AMPKα1 is necessary for the normal proliferation of C2C12 myoblasts and limits the volume of myotubes. AMPKα1 also mediates the phosphorylation of ACC and ULK1 in C2C12 myotubes. On the other hand, AMPKα2 up-regulates MCK, PGC-1α1 and PGC-1α4 mRNA abundance and cell volume in C2C12 myotubes. The nuclear localization of AMPKα2 is necessary for maintenance of the amount of PGC-1α1 mRNA but not for that of MCK mRNA abundance. Our findings have thus uncovered a specific role for AMPKα2 in mitochondriogenesis during myogenic differentiation of skeletal muscle cells.

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Author Contributions

SO and NFA designed the study, performed experiments, and analyzed data. SY and NFA contributed to immunoblot analysis, and KS contributed to cell culture. YM and SO wrote the paper.

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Disclosure

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2018.10.003.

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