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A role of DNA-dependent protein kinase for the activation of AMP-activated protein kinase in response to glucose deprivation

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ABSTRACT

The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) plays an essential role in double-strand break repair by initially recognizing and binding to DNA breaks. Here, we show that DNA-PKcs interacts with the regulatory γ1 subunit of AMP-activated protein kinase (AMPK), a heterotrimeric enzyme that has been proposed to function as a "fuel gauge" to monitor changes in the energy status of cells and is controlled by the upstream kinases LKB1 and Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK). In co-immunoprecipitation analyses, DNA-PKcs and AMPKγ1 interacted physically in DNA-PKcs-proficient M059K cells but not in DNA-PKcs-deficient M059J cells. Glucose deprivation-stimulated phosphorylation of AMPKα on Thr172 and of acetyl-CoA carboxylase (ACC), a downstream target of AMPK, is substantially reduced in M059J cells compared with M059K cells. The inhibition or down-regulation of DNA-PKcs by the DNA-PKcs inhibitors, wortmannin and Nu7441, or by DNA-PKcs siRNA caused a marked reduction in AMPK phosphorylation, AMPK activity, and ACC phosphorylation in response to glucose depletion in M059K, WI38, and IMR90 cells. In addition, DNA–DNA-PKcs$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited decreased AMPK activation in response to glucose-free conditions. Furthermore, the knockdown of DNA-PKcs led to the suppression of AMPK (Thr172) phosphorylation in LKB1-deficient HeLa cells under glucose deprivation. Taken together, these findings support the positive regulation of AMPK activation by DNA-PKcs under glucose-deprived conditions in mammalian cells.

1. Introduction

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated by DNA double-strand breaks (DSBs) [1–3]. DNA-PK plays a central role in nonhomologous DNA end-joining (NHEJ) by initially recognizing and binding to damaged DNA and then targeting other repair activities to the site of damage. DNA-PK is composed of a 470-kDa catalytic subunit (DNA-PKcs) and Ku70/Ku80 heterodimeric regulatory components [4,5]. DNA-PKcs belongs to the family of phosphatidyl-inositol 3-OH kinase-related kinases (PIKKs), which also includes ataxia telangiectasia mutated (ATM) and ATM- and RAD-3-related (ATR) kinase in mammals [4,6,7]. Members of this family are high-molecular-weight polypeptides sharing a conserved C-terminal region with homology to the catalytic domain of phosphatidylinositol-3-OH kinase. ATM, ATR, and DNA-PKcs are implicated in cellular responses to DSBs, resulting in the phosphorylation of targets such as H2AX, Chk1, Chk2, Nbs1, and MDC1 [8].

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that serves as an energy sensor in all eukaryotic cell types [9]. AMPK is a heterotrimer consisting of a catalytic α subunit and two regulatory and targeting subunits, β and γ. In mammals, there are two α subunits, three γ subunits, and two β subunits (α1, α2, γ1, γ2, γ3, β1 and β2) [10]. The α subunits each contain an N-terminal catalytic core (1–312) and a C-terminal sequence (313–548) responsible for autoregulation and binding to the β and γ subunits [11]. The γ subunits each contain four tandem cystathione β-synthase (CBS) domains that together bind two molecules of AMP and allow the kinase complex to sense adenine nucleotide levels. The β subunits have been reported to serve as a scaffold for

Abbreviations: DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; AMPK, AMP-activated protein kinase; AMPKγ1, regulatory γ1 subunit of AMP-activated protein kinase; CaMMK, Ca^{2+}/calmodulin-dependent kinase kinase; ACC, acetyl-CoA carboxylase; DSBs, DNA double-strand breaks; NHEJ, nonhomologous DNA end-joining; PIKK1s, phosphatidyl-inositol 3-OH kinase-related kinases; ATM, ataxia telangiectasia mutated; ATR, ATM- and RAD-3-related; CBS, cystathione β-synthase

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the α and γ subunits as well as to target the complex to intracellular sites such as the cell membrane, through myristoylation of its N-terminus [12,13], and to intracellular glycogen, through a glycogen binding domain [14]. The binding of AMP to the CBS domain of the AMPKγ subunit leads to activation of the AMPK complex, and this activation is antagonized by a high concentration of ATP, enabling the system to monitor changes in the cellular AMP:ATP ratio [15,16]. When activated by a rise in the cellular AMP concentration, AMPK inhibits anabolic pathways and promotes catabolic pathways to restore cellular energy levels. The activation of AMPK leads to the phosphorylation of a number of proteins, resulting in increased glucose uptake, glycolysis, and fatty acid oxidation plus the simultaneous inhibition of fatty acid synthesis, cholesterol synthesis, and glucose production [17–19].

At least two kinases have been shown to phosphorylate AMPK: LKB1, a tumor suppressor kinase, in complex with two accessory subunits, STRAD and MO25 [20–23]; and Ca^{2+}-calmodulin-dependent protein kinase β (CaMKKβ) [24–26]. LKB1 is not stimulated by AMP but is constitutively active [23,27,28]. In vitro studies have suggested that the binding of AMP to AMPK is likely to be the principle regulatory mechanism for stimulating the phosphorylation of AMPK by LKB1 [20]. AMPK can also be activated in an AMP-independent manner, by hyperosmotic stress or with the antidiabetic drug metformin [29]. The finding that CAMKKβ acts upstream of AMPK suggests that an increase of intracellular Ca^{2+} may act as a second pathway to activate AMPK, in addition to changes of the AMP:ATP ratio.

In the present study, we investigated the possible role of DNA-PKcs in the activation of AMPK, using DNA-PKcs-proficient and DNA-PKcs-deficient human glioma cells. We first showed that DNA-PKcs could interact with AMPKγ1. We observed that AMPK and ACC phosphorylation as well as AMPK activity were significantly suppressed in DNA-PKcs-deficient M059J cells in response to glucose depletion. In addition, the knockdown or inhibition of DNA-PKcs by, respectively, a DNA-PKcs-specific small interfering RNA (siRNA) or wortmannin and Nu7441, chemical inhibitors of DNA-PKcs, reduced the phosphorylation of AMPK. In addition, DNA-PKcs$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited decreased AMPK activation in response to glucose-free conditions. Finally, in LKB1-deficient HeLa cells, the knockdown of DNA-PKcs led to the suppression of AMPK activation. Our combined results suggest that DNA-PKcs may play a physiological role in the activation of AMPK in mammalian cells in response to energy depletion.

2. Materials and methods

2.1. Reagents and cell culture

Human glioma M059K and M059J cells were maintained in Dulbecco's modified Eagle medium with Ham's F-12 nutrient mixture (DMEM/F12) (1:1) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA). HeLa human cervical cancer cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen). Human primary fibroblasts IMR90 and WI38 cells were maintained in Earle's MEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen). Cells were maintained in a humidified incubator with an atmosphere of 5% CO_2 at 37 °C. All cell lines were from the American Type Culture Collection (ATCC, Rockville, MD). DNA-PKcs$^{+/+}$ and DNA-PKcs$^{-/-}$ MEFs were kindly provided by Prof. Penny A. Jeggo (Genome damage and Stability Center, University of Sussex, UK). DNA-PKcs$^{+/+}$ and DNA-PKcs$^{-/-}$ MEFs were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Wortmannin was from Cell Signaling Technology (Danvers, MA). Nu7441 and Ku55933 were from TOCRIS bioscience (Bristol, UK).

2.2. Antibodies

Monoclonal antibodies against DNA-PKcs, AMPKγ1, and phospho-DNA-PKcs on T2609 were from Abcam (Cambridge, MA). Monoclonal antibodies against AMPKα and phospho-AMPKα on Thr172, mTOR, ATM, LKB1 and polyclonal antibodies against phospho-acetyl-CoA carboxylase on Ser79, were from Cell Signaling Technology. Antibodies to α-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.3. Yeast two-hybrid assay

A yeast two-hybrid screen was performed using a Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) following the manufacturer's directions. *Saccharomyces cerevisiae* strain AH109, containing distinct ADE2, HIS3, lacZ and MEL1 reporter constructs, was used for the assay. The AH109 strain was transformed with the bait plasmid pGBT9, encoding an in-frame fusion of the GAL4 DNA-binding domain with DNA-PKcs (amino acid residues 2908–3538). Transformants were plated onto selective SD medium lacking Trp and incubated for 3–5 days at 30 °C. Yeast strains containing the bait plasmid were transformed with a prostate cDNA library fused to the GAL4 activation domain in pACT2 vector. Transformants were plated on selective SD medium lacking Leu, Trp, and His and were further screened on SD medium with X-α-Gal to identify the expression of α-galactosidase, as indicated by the formation of blue colonies. Plasmid DNA was isolated from positive clones and sequenced to identify the genes encoding the interacting proteins. The sequences were analyzed using NCBI's BLAST program. One of the positive clones, identified as AMPKγ1, was cloned into pACT2 vector and transformed into *Escherichia coli* DH5α. After expression was confirmed, AMPKγ1 in pACT2 was transformed into yeast strain AH109 containing the bait plasmid. Assays were repeated for growth on selective medium and blue colony formation, and the clone was reconfirmed by sequencing.

2.4. Immunoprecipitation

Whole-cell lysates were obtained by incubating cells in lysis buffer (25 mM Tris, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 5% glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) for 30 min at 4 °C. Cellular debris was removed by centrifugation at 13,000 ×*g* for 30 min at 4 °C. One milligram of protein from the cell lysates was immunoprecipitated overnight at 4°C, using anti-DNA-PKcs and anti-AMPKγ1 antibodies and protein G-agarose beads (Amersham Biosciences, Piscataway, NJ). The beads were washed extensively with lysis buffer and boiled in SDS sample buffer; the proteins were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-DNA-PKcs, anti-AMPKα, and anti-AMPKγ1 antibodies.

2.5. Western blot

Cultured cells were washed with phosphate-buffered saline and lysed at 0 °C for 30 min in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, and 5 mM NaF). Cellular protein content was quantitated using a dye-binding microassay (Bio-Rad, Hercules, CA). Protein samples (20 μg) were boiled for 5 min in Laemmli sample buffer, loaded onto 10% SDS-polyacrylamide gels, and electrophoresed. The separated proteins were electroblotted onto Bio-trace PVDF membranes (PALL Life Sciences, Pensacola, FL, UAS) and the membranes were blocked with Tris-buffered saline (10 mM Tris–HCl, pH 7.6, 150 mM NaCl) containing 0.1% Tween-20 and 5% milk. The membranes were incubated for overnight with the appropriate primary antibodies and α-tubulin antibody, diluted according to the manufacturer's protocol, in blocking buffer.

The membranes were washed, incubated for 2 h with the appropriate secondary antibodies (1:4000) in blocking buffer, and washed again. The blotted proteins were detected using an enhanced chemiluminescence detection system (iNtRON Biotech, Seoul, Korea).

2.6. Small interfering RNA (siRNA)

DNA-PKcs siRNA (#1) was purchased from Santa Cruz Biotechnology (sc-35200). This DNA-PKcs siRNA #1 contains two different siRNA sequences of DNA-PKcs. The sequences of sense siRNAs are as follows: 5′-GAU CGCACCUUACUCUGUUdTdT-3′ for the DNA-PKcs gene (nt 352–370) and 5′-CUU UAUGGUGGCCAUGGAGdTdT-3′ for the DNA-PKcs gene (nt 11,836–11,854). Another DNA-PKcs siRNA (#2) was designed using siRNA target Finder program (Ambion, Austin, TX). The sequence of the DNA-PKcs #2 duplexes was 5′-UUGGAGAAUACGACGUC CUUU-3′ for the DNA-PKcs gene (nt 8,853-8871). M059K and HeLa cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Scrambled siRNA was purchased from Bioneer (Daejeon, Korea) and was used as the negative control.

2.7. AMPK assay

AMPK was immunoprecipitated from cell extracts prepared in triplicate, each representing a pooling of two wells treated under identical conditions. Whole-cell lysates containing 200 μg of protein were incubated with protein G-sepharose beads (Amersham Biosciences) and anti-AMPKα antibody (Cell Signaling). Following 2 h incubation at 4 °C, the extracts were centrifuged for 20 s at 12,000 rpm to gently pellet the beads. The supernatant was carefully removed, and the beads were washed once with buffer containing 1% Triton X-100 and then two times with the same buffer but containing no detergent. The beads were resuspended in 50 μl of this buffer, and the resulting immune complexes were immediately assayed, in duplicate, for AMPK activity. The AMPK activity was determined by measuring the incorporation of ^{32}P into 5 μg synthetic peptide substrate SAMS (HMRSAMSGLHLVKRR), using 5 μCi [γ-^{32}P]ATP [30]. The assay mixtures were incubated at 30 °C for 10 min, and the reaction was terminated by spotting the mixtures onto P-81 phosphocellulose paper and washing 3 × 5 min in 1% phosphoric acid. The samples were dried, and the incorporation of labeled phosphate was quantified using a liquid scintillation counter (Packard Instruments, Meriden, CT).

2.8. Data analysis

All values shown represent means ± S.D. Where indicated, we performed analyses of significance by the two-tailed Student's *t*-test. We considered P 0.05 (indicated * in figures) as significant and P 0.01 (**) as highly significant.

3. Results

3.1. Identification of AMPKγ1 as a DNA-PKcs-interacting protein

To explore DNA-PKcs-interacting proteins, we performed yeast two-hybrid screening analysis using a GAL4 transcription activation domain (AD)-fused human prostate cDNA library. With GAL4 DNA binding domain (BD)-fused the C-terminus of DNA-PKcs (amino acids 2908 to 3538) as the bait, we identified several independent real positive clones out of 2×10^6 transformants; real positive clones exhibit a blue color on media containing X-gal, growth on medium lacking leucine, tryptophan, and histidine. Sequence analysis of cDNAs from the positive colonies identified AMPKγ1. To further confirm an interaction between AMPKγ1 and DNA-PKcs, we cotransfected yeast cells with the DNA-PKcs (amino acids 2908 to 3538) and AMPKγ1 constructs. Blue–white color selection in medium lacking leucine, tryptophan, and histidine was used to identify the transformed colonies (data not shown), suggesting that the AMPKγ1 is sufficient for DNA-PKcs binding in the yeast two-hybrid system.

We performed immunoprecipitation experiments to determine whether DNA-PKcs would interact with AMPKγ1 in DNA-PKcs-proficient M059K and in DNA-PKcs-deficient M059J cells. After the M059K and M059J cells were cultured in normal or glucose-free medium for 12 h, the cells were lysed. The proteins were immunoprecipitated from the lysate with a DNA-PKcs-specific antibody and were subjected to immunoblotting with an anti-AMPKγ1 antibody, revealing that endogenous DNA-PKcs was bound to AMPKγ1 in M059K cells but not in M059J cells. Glucose-free conditions did not significantly change the amount of DNA-PKcs bound to AMPKγ1 (Fig. 1A). In a reciprocal co-immunoprecipitation assay with anti-AMPKγ1 antibody, endogenous DNA-PKcs was readily immunoprecipitated from the cell lysates (Fig. 1B). We also confirmed that endogenous AMPKγ1 was not bound to DNA-PKcs in M059J cells. The binding was specific, as no DNA-PKcs was detected in samples immunoprecipitated with rabbit IgG. These results identify AMPKγ1 as an endogenous interacting partner for DNA-PKcs.

To further investigate whether DNA-PKcs regulates AMPK complex association under normal and glucose-deprived conditions, we performed immunoprecipitation experiments. After M059K and M059J cells were cultured in the presence or absence of glucose, the cells were lysed. The proteins were immunoprecipitated from the lysate with an anti-AMPKγ1 antibody and were subjected to immunoblotting with an anti-AMPKα antibody. Under our experimental conditions, endogenous AMPKγ1 was bound to AMPKα1 in both M059K and M059J cells (Supplementary Fig. S1), indicating that DNA-PKcs did not affect AMPK complex association under normal and glucose-deprived conditions.

3.2. DNA-PK contribute to the glucose deprivation-induced AMPK phosphorylation in human glioma cells

Energy depletion, defined as an increase in the AMP/ATP ratio, can be caused by a variety of factors such as glucose deprivation, hypoxia, and oxidative stress [13]. The cellular AMP/ATP ratio is one of the major determinants of AMPK activity, and thus we tested the ability of DNA-PKcs-deficient and DNA-PKcs-proficient cells to activate AMPK under glucose-deprived conditions. DNA-PKcs-proficient M059K and DNA-PKcs-deficient M059J human glioma cells were cultured in glucose-free conditions for 0, 6, 12, and 24 h. Whole-cell lysates were prepared by a rapid lysis procedure and were subjected to immunoblotting with anti-phospho-Thr172-AMPKα antibody. The blots were re-probed with anti-AMPKα and anti-DNA-PKcs antibodies. AMPK phosphorylation at Thr172 was significantly greater in M059K cells than in M059J cells under glucose deprivation (Fig. 2A, B). Similarly, when both cell types were cultured with a series of decreasing glucose concentrations for 12 h, the absence of glucose had a greater effect on AMPKα phosphorylation in M059K cells than in M059J cells (Fig. 2C, D), while the expression level of total AMPKα remained constant. AMPK activity is indicated by the phosphorylation level of Ser79 in its critical substrate ACC, which is the rate-limiting enzyme of long-chain fatty acid synthesis [31]. Thus, we also analyzed the phosphorylation of ACC as a marker of AMPK activity. We observed that both basal and stimulated ACC phosphorylation at Ser79 were higher in M059K cells than in M059J cells (Fig. 2A, 3rd panel), suggesting that DNA-PK might be involved in AMPK activation. Because LKB1 is an important upstream kinase active on AMPK [14], we compared the LKB1 expression levels between M059K and M059J cells. We saw no differences in the expression level of LKB1 between M059K and M059J cells (Fig. 2A, 4th panel); thus, the different patterns of AMPK activity between these cells cannot be attributed mainly to the expression of LKB1. We also checked whether glucose depletion affected DNA-PKcs phosphorylation; however, DNA-PKcs was not phosphorylated in M059K cells in response to glucose deprivation (Fig. 2A, 6th panel).

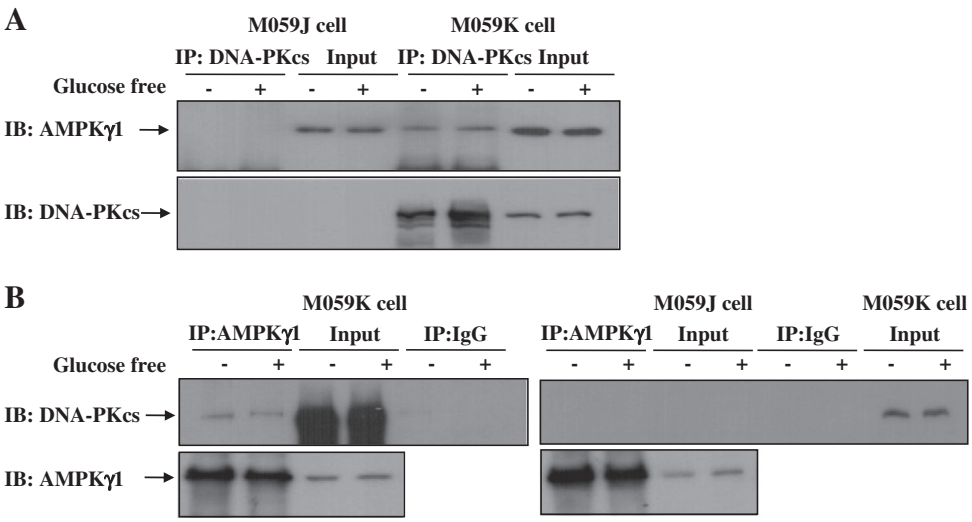

Fig. 1. Interaction of DNA-PKcs with AMPKγ1 in intact human glioma M059K cells. (A) DNA-PKcs-proficient M059K and DNA-PKcs-deficient M059J human glioma cells were cultured in the presence or absence of glucose for 24 h, and then whole-cell lysates were prepared. Proteins were immunoprecipitated from the lysates with anti-DNA-PKcs antibody, and the immunoprecipitates were subjected to immunoblot analysis with an antibody specific for AMPKγ1 or DNA-PKcs. (B) Whole-cell lysates prepared from M059K and M059J cells, maintained in the presence or absence of glucose for 12 h, were immunoprecipitated with monoclonal anti-AMPKγ1 antibody or control immunoglobulin (normal rabbit IgG), and the resulting precipitates were immunoblotted with an antibody specific for DNA-PKcs or AMPKγ1.

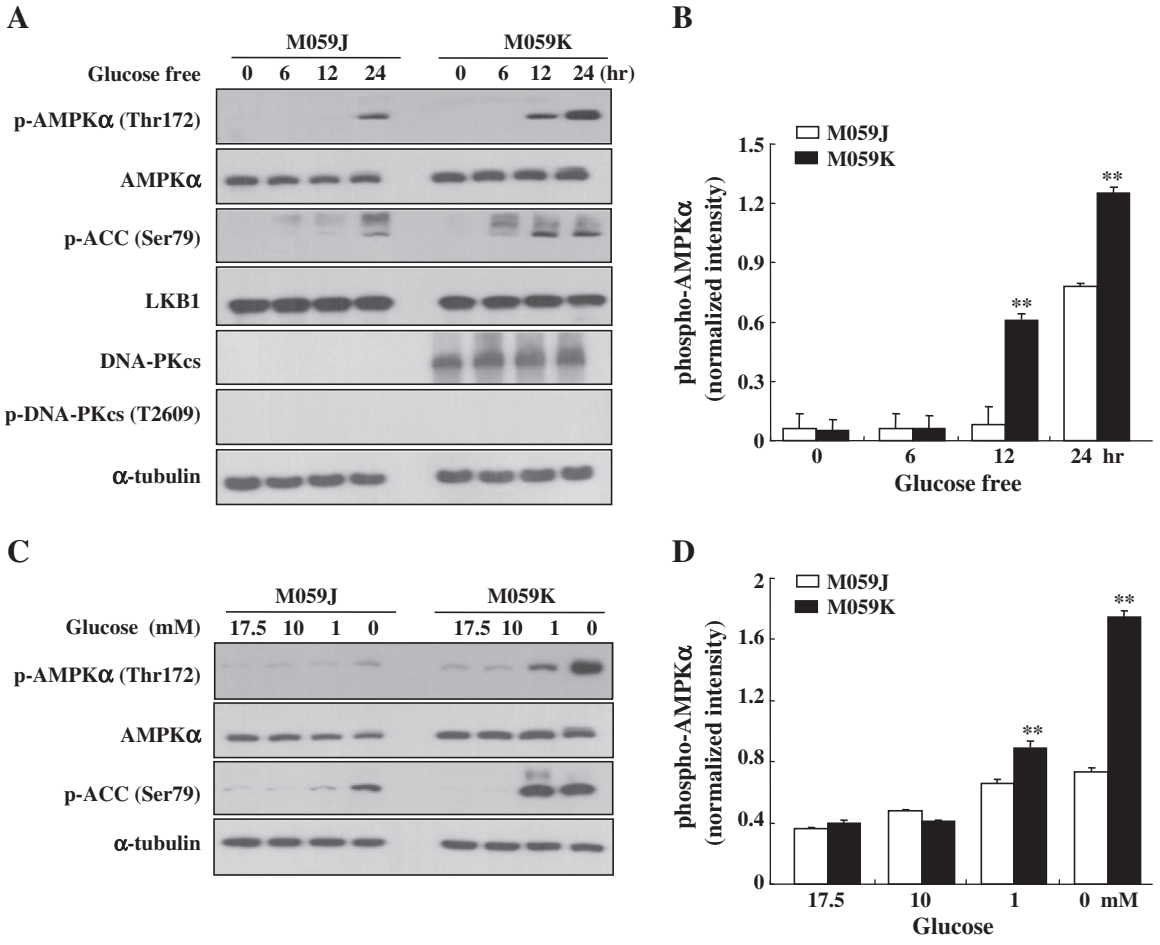

Fig. 2. DNA-PK contributes to the phosphorylation AMPK in response to glucose deprivation. (A) M059K and M059J human glioma cells were cultured under glucose-free conditions for the indicated time periods. Whole-cell lysates were prepared and subjected to immunoblotting with anti-phospho-AMPKα, anti-AMPKα, anti-phospho-ACC, anti-DNA-PKcs, anti-phospho-DNA-PKcs, and anti-α-tubulin antibodies. (B) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01. (C) M059J and M059K cells were incubated in 17.5, 10, 1, and 0 mM glucose for 24 h. Whole-cell lysates were prepared and subjected to immunoblotting with anti-phospho-AMPKα, anti-AMPKα, anti-phospho-ACC, and anti-α-tubulin antibodies. (D) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean ± s.d. from three separate experiments. $^{**}p < 0.01$.

Phosphorylation of AMPK has been recently been observed following DNA damage [32,33]. This phosphorylation occurs independent of LKB1, but is dependent upon DNA damage sensor, ATM. Since DNA-PKcs is activated by DNA damage induced by both ionizing and UV radiation and is regarded as a DNA damage sensor, we examined the effect of DNA-PKcs on ionizing radiation-induced AMPK phosphorylation. At the indicated time points following ionizing radiation, whole-cell lysates were prepared and analyzed for the expression levels of phosphorylated AMPK by immunoblotting. The results showed that the AMPK phosphorylation at Thr172 was significantly greater in M059K cells than in M059J cells in response to ionizing radiation (Supplementary Fig. S2), indicating that DNA-PKcs also contributes to the DNA damage-induced AMPK activation.

3.3. DNA-PK positively regulates AMPK activity in response to Glucose deprivation

To further explore the role of DNA-PKcs in AMPK activation, we cultured both M059J and M059K cells with and without glucose, immunoprecipitated total AMPK from the cell lysates, and assayed the phosphorylation activity of the immune complex, using a SAMS peptide assay. As shown in Fig. 3A, the basal AMPK activities of M059J and M059K cells were 995 22 and 1020 25 cpm, respectively. However, AMPK activity significantly increased in M059K cells after the depletion of glucose for 12 h compared with that in the M059J cells. The AMPK activities were 1160 26 and 1480 36 cpm in M059J and M059K cells exposed to glucose depletion, respectively.

We next investigated the effect of a reduced level of DNA-PKcs on glucose deprivation-stimulated AMPK activity by transfecting M059K cells with control siRNA or DNA-PKcs siRNA. M059K cells were transfected with control siRNA or DNA-PKcs siRNAs. After 48 h, a significant decrease in the DNA-PKcs mRNA level was confirmed by RT-PCR (data not shown), and a decrease in the protein level of more than 80% was observed by immunoblotting (example, Fig. 4C). The AMPK activity after DNA-PKcs-siRNA transfection was next examined. The results showed that the M059K cells transfected with the DNA-PKcs-siRNA had significantly lower AMPK activity than control siRNA-transfected cells. The AMPK activity in the control siRNA transfected cells was increased by approximately 46% (1010 22 versus 1470 31) with glucose deprivation, whereas the AMPK activity was increased by only 24% (820 19 versus 1020 27) in the DNA-PKcs-knockdown cells under the same conditions (Fig. 3B). These results suggest that DNA-PKcs increases glucose deprivation-stimulated AMPK activity.

3.4. Glucose deprivation-stimulated phosphorylation of AMPK is decreased by inhibition or knockdown of DNA-PKcs

To explore its role in the phosphorylation of AMPK under glucose-deprived conditions, the activity of DNA-PKcs was inhibited using wortmannin, a potent and irreversible inhibitor of the enzyme, or the level of DNA-PKcs was knocked down using siRNA. We initially investigated the effect of wortmannin on glucose deprivation-induced AMPK phosphorylation in M059K cells treated with 20 μM wortmannin for 1 h and then maintained under glucose-free conditions for different amounts of time. Whole-cell lysates were prepared and analyzed by immunoblotting. The increase in AMPK phosphorylation stimulated by glucose deprivation was less in wortmannin-treated cells than in untreated control cells (Fig. 4A, B). These results imply that DNA-PKcs contributes to the phosphorylation of AMPK under glucose-free conditions. However, wortmannin also inhibits other PI3 kinases such as ATM. ATM phosphorylate the AMPK in response to insulin-like growth fator-1 (IGF-1) [34] and 5-aminoimidazoe-4-carboxamide-1-β-4-ribofuranoside (AICAR) [35], independently of LKB1. Thus, we used highly specific small-molecule inhibitors of DNA-PKcs (Nu7441) and ATM (Ku55933). We found that treatment of Nu7441 and Ku55933 caused decreased phosphorylation AMPK following glucose deprivation, and both inhibitors were equally effective in the suppression of the AMPK activation under glucose-depleted condition (Supplementary Fig. S3). Thus, DNA-PKcs and ATM may redundantly the participation of the phosphorylation of AMPK in response in response to glucose depletion.

To further determine whether DNA-PKcs is involved in the phosphorylation of AMPK, we tested the effect of DNA-PKcs knockdown on glucose deprivation-induced AMPK phosphorylation in M059K cells.

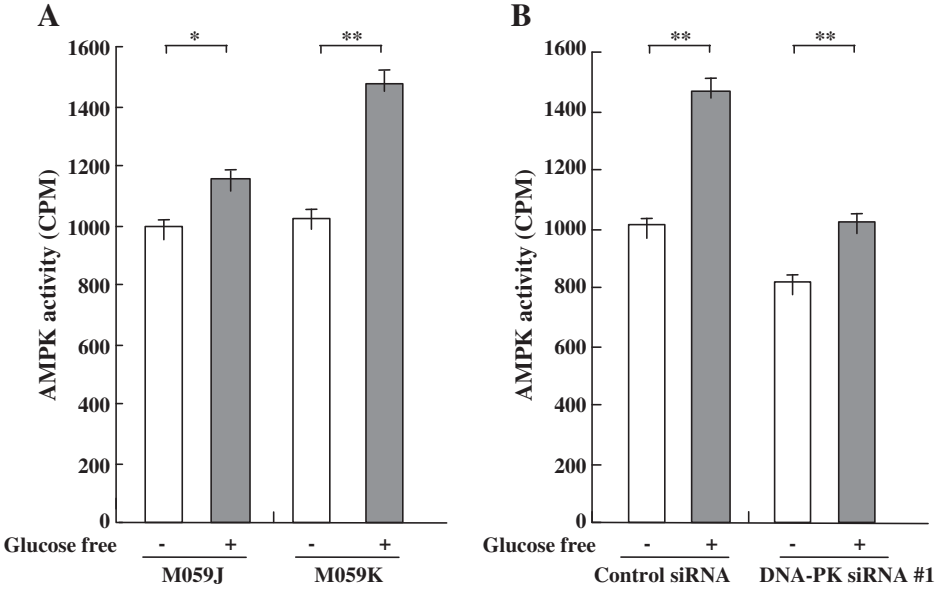

Fig. 3. AMPK activity stimulated by glucose deprivation is decreased in DNA-PKcs-deficient cells. (A) M059J and M059K cells were maintained in the absence or presence of glucose for 12 h. Whole-cell lysates were prepared and AMPK was immunoprecipitated. The activity of the immune complexes was measured using a SAMS peptide assay. Each value is the mean ± s.d. from three separate experiments. $^{**}p<0.01$, and *, $p<0.05$. (B) M059K cells were transfected with control siRNA or DNA-PKcs siRNA #1. After 48 h, the cells were maintained in the absence or presence of glucose for 12 h. Whole-cell lysates were prepared and AMPK was immunoprecipitated. The activity of the immune complexes was measured using a SAMS peptide assay. Each value is the mean s.d. from three separate experiments. $^{**}p<0.01$.

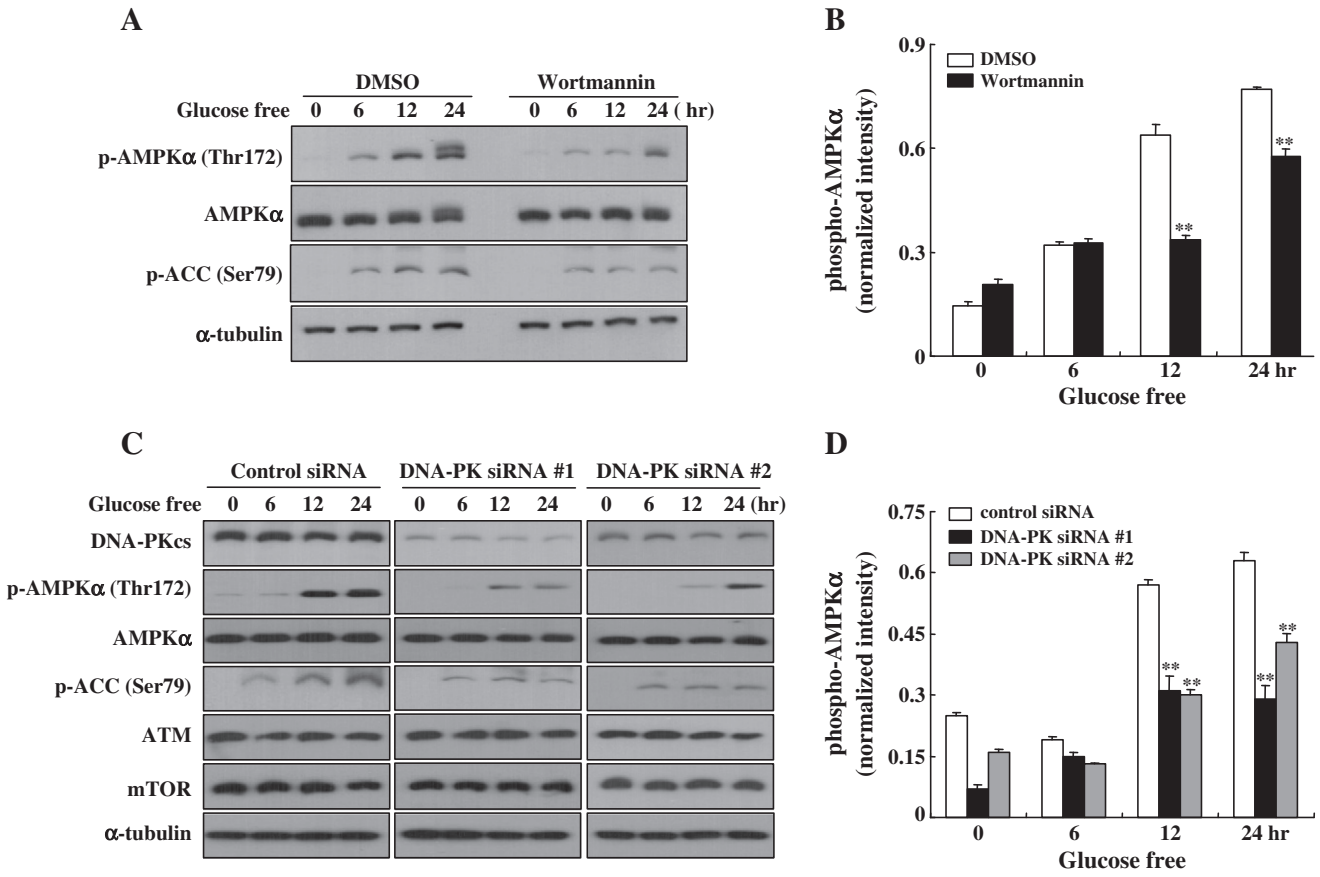

Fig. 4. Inhibition or down-regulation of DNA-PKcs in M059K cells decreases AMPK activity in response to glucose depletion. (A) M059K cells were treated with or without 20 μM of wortmannin for 1 h and then maintained in the presence or absence of glucose for the indicated times. Whole-cell lysates were prepared and subjected to immunoblotting with anti-phospho-AMPKα, anti-AMPKα, anti-pACC, and anti-α-tubulin antibodies. (B) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01. (C) M059K cells were transfected with control siRNA or DNA-PKcs siRNA #1 and #2. After 48 h, the cells were cultured under glucose-free conditions for the indicated time periods. Whole-cell lysates were prepared and subjected to immunoblotting with anti-DNA-PKcs, anti-phospho-AMPKα, anti-AMPKα, anti-mTOR, anti-ATM, anti-ACC, and anti-α-tubulin antibodies. (D) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01.

M059K cells transfected with control siRNA, DNA-PKcs siRNA #1, or DNA-PKcs siRNA #2 were cultured under in glucose-free conditions for 0, 6, 12, and 24 h. Whole-cell lysates were prepared and analyzed by immunoblotting for phosphorylated AMPK and phosphorylated ACC. As shown in Fig. 4C and D, the stimulation of AMPK (Thr172) phosphorylation by glucose deprivation was significantly less in the DNA-PKcs-knockdown cells than in the control cells. ACC phosphorylation at Ser79 was also lower in DNA-PKcs siRNA transfected cells than in control cells. To confirm the specificity of DNA-PKcs siRNA constructs, we checked the protein levels of homologues of DNA-PKcs in the P13K superfamily, such as ATM or mTOR. We observed that two different DNA-PKcs siRNAs (#1 and #2) did not affect the levels of mTOR and ATM (Fig. 4C, 5th and 6th panel).

To further confirm the role of DNA-PKcs for the activation of AMPK in response to glucose free conditions, we compared the AMPK activity between DNA-PKcs$^{+/+}$ and DNA-PKcs$^{-/-}$ MEFs. As shown in Fig. 5A and B, the basal and glucose deprivation-stimulated AMPK phosphorylation was significantly less in DNA-PKcs$^{-/-}$ MEFs than in DNA-PKcs$^{+/+}$ MEFs. The AMPK activity analysis also revealed that DNA-PKcs$^{-/-}$ MEFs had significantly lower AMPK activity than DNA-PKcs$^{+/+}$ MEFs (Fig. 5C). The basal AMPK activities of DNA-PKcs$^{-/-}$ and DNA-PKcs$^{+/+}$ MEFs were 995 22 and 1020 25 cpm, respectively. The glucose deprivation-stimulated AMPK activities were 1160 ± 26 and 1480 ± 36 cpm in DNA-PKcs$^{-/-}$ and DNA-PKcs$^{+/+}$ MEFs, respectively. These results suggest that DNA-PKcs increases glucose deprivation-stimulated AMPK activity.

3.5. Glucose deprivation-stimulated phosphorylation of AMPK is decreased by knockdown of DNA-PKcs in human fibroblast WI38 and IMR90 cells

In order to determine if DNA-PKcs indeed contributes to the enhancement of the AMPK activation, we examined the effect of DNA-PKcs knockdown by siRNA on glucose depletion-induced AMPK activation in primary human diploid fibroblast WI38 and IMR90 cells. Western blot analysis was performed 48 h after treating the WI38 and IMR90 cells with either the DNA-PKcs siRNA or control siRNA. The DNA-PKcs siRNA treatment resulted in a significant decrease in the DNA-PKcs protein level, compared with control siRNA transfected cells (Fig. 6A). The levels of AMPKα phosphorylation after DNA-PKcs-siRNA transfection were next examined. The results showed that the glucose depletion-induced AMPKα phosphorylation was markedly suppressed in the WI38 and IMR90 cells treated with the DNA-PKcs siRNA (Fig. 6A, B). The ACC phosphorylation analysis experiments confirmed that the transfection of DNA-PKcs siRNA reduces the level of ACC phosphorylation in response to glucose depletion compared with the control siRNA transfection (Fig. 6A, 4th panel). These results suggest that DNA-PKcs is involved in the glucose deprivation-stimulated AMPK activity in primary human diploid fibroblasts.

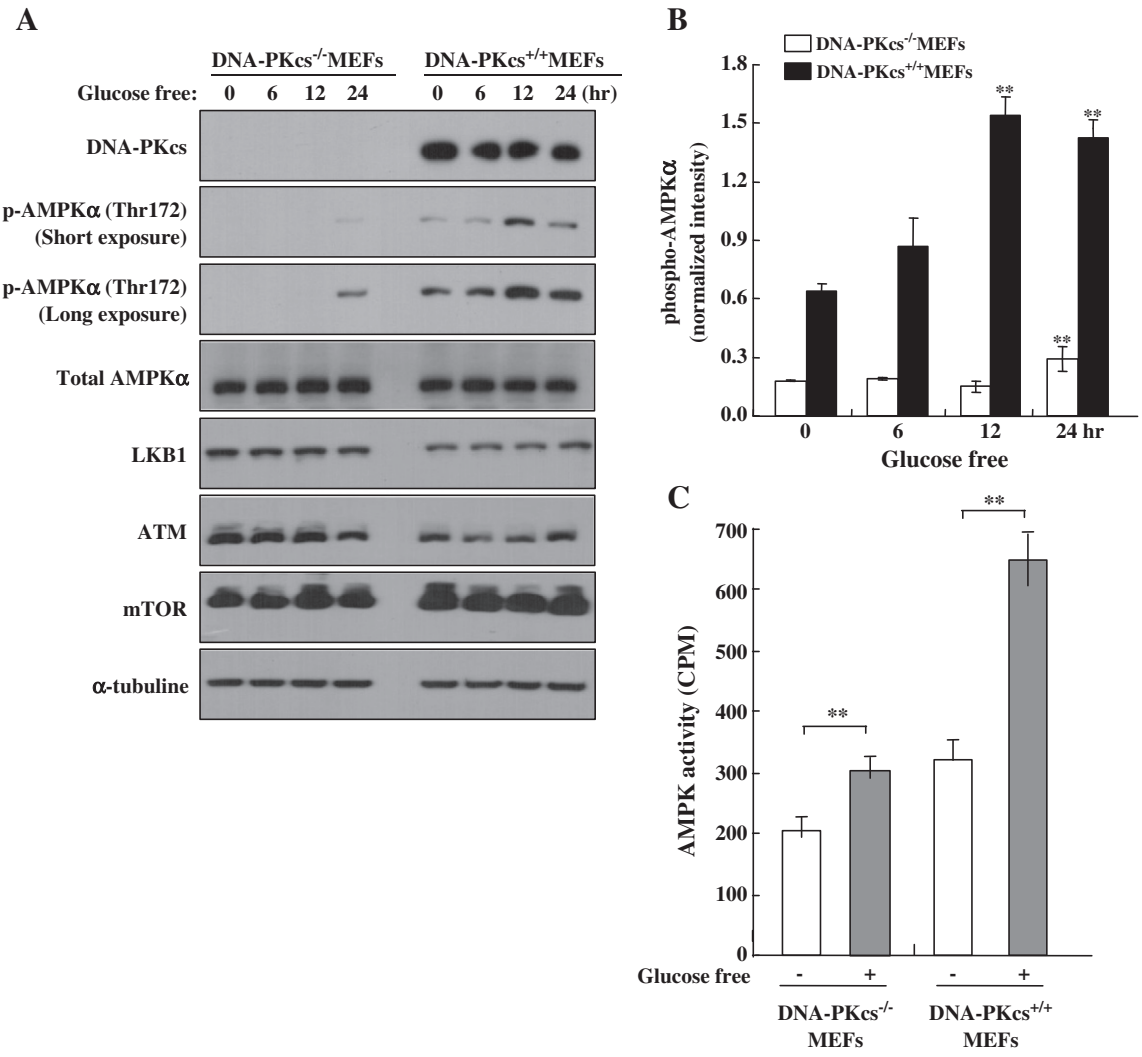

Fig. 5. AMPK activation is suppressed in DNA-PKcs$^{-/-}$ MEFs. (A) DNA-PKcs$^{-/-}$ and DNA-PKcs$^{+/+}$ MEFs were cultured under glucose-free conditions for the indicated time periods. Whole-cell lysates were prepared and subjected to immunoblotting with anti-DNA-PKcs, anti-phospho-AMPKα, anti-AMPKα, anti-LKB1, anti-ATM, anti-mTOR, and anti-α-tubulin antibodies. (B) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01. (C) DNA-PKcs$^{-/-}$ and DNA-PKcs$^{+/+}$ MEFs were incubated were maintained in the absence or presence of glucose for 12 h. Whole-cell lysates were prepared and AMPK was immunoprecipitated. The activity of the immune complexes was measured using a SAMS peptide assay. Each value is the mean s.d. from three separate experiments. $^{**}p<0.01$, and $^{*}p<0.05$.

3.6. Glucose deprivation-stimulated activation of AMPK is reduced by the inhibition of DNA-PKcs in LKB1-deficient HeLa cells

LKB1 is a major protein kinase involved in the phosphorylation of AMPK under hypoxia, oxidative stress, and nutrient deprivation [14]. As our results suggested that DNA-PKcs may contribute to the phosphorylation of AMPK, we examined the effect of DNA-PKcs on AMPK phosphorylation in HeLa cells, which do not express LKB1 [25]. To test the hypothesis that DNA-PKcs functions as an activator of AMPK in HeLa cells, HeLa cells were transfected with either control siRNA or DNA-PKcs siRNA. After 48 h, the cells were maintained under glucose-free conditions for various times. Whole-cell lysates were prepared, and the phosphorylation of AMPKα was analyzed by immunoblotting. As shown in Fig. 7A and B, under glucose-free conditions, the phosphorylation of AMPK was significantly reduced in DNA-PKcs-knockdown HeLa cells compared with that in the control cells. Our results provide strong support for the involvement of DNA-PKcs in the phosphorylation of AMPK under glucose-free conditions.

4. Discussion

Our present work provides evidence that DNA-PKcs is an important regulator of AMPK activation in response to energy depletion. We established that AMPK phosphorylation and AMPK activity under glucose-depleted conditions is more pronounced in DNA-PKcs-proficient and DNA-PKcs$^{+/+}$ cells than in DNA-PKcs-deficient and DNA-PKcs$^{-/-}$ cells. Using DNA-PKcs siRNA and the DNA-PKcs inhibitors, wortmannin and Nu7441, we showed that there was a marked reduction in both AMPKα phosphorylation at Thr172 and AMPK activity in response to glucose depletion in DNA-PKcs-inhibited M059K cells and human primary fibroblast WI38 and IMR90 cells, as compared with control cells. These results suggest that DNA-PKcs is involved in AMPK activation under glucose-depleted conditions.

The depletion of glucose, the major source of energy in a cell, is marked by an increase in the concentration of AMP and a decrease in the concentration of ATP, and can result from various stresses such as nutrient deprivation, hypoxia, and oxidative stress [13]. AMPK, a protein kinase activated by glucose depletion, is a cellular energy sensor and

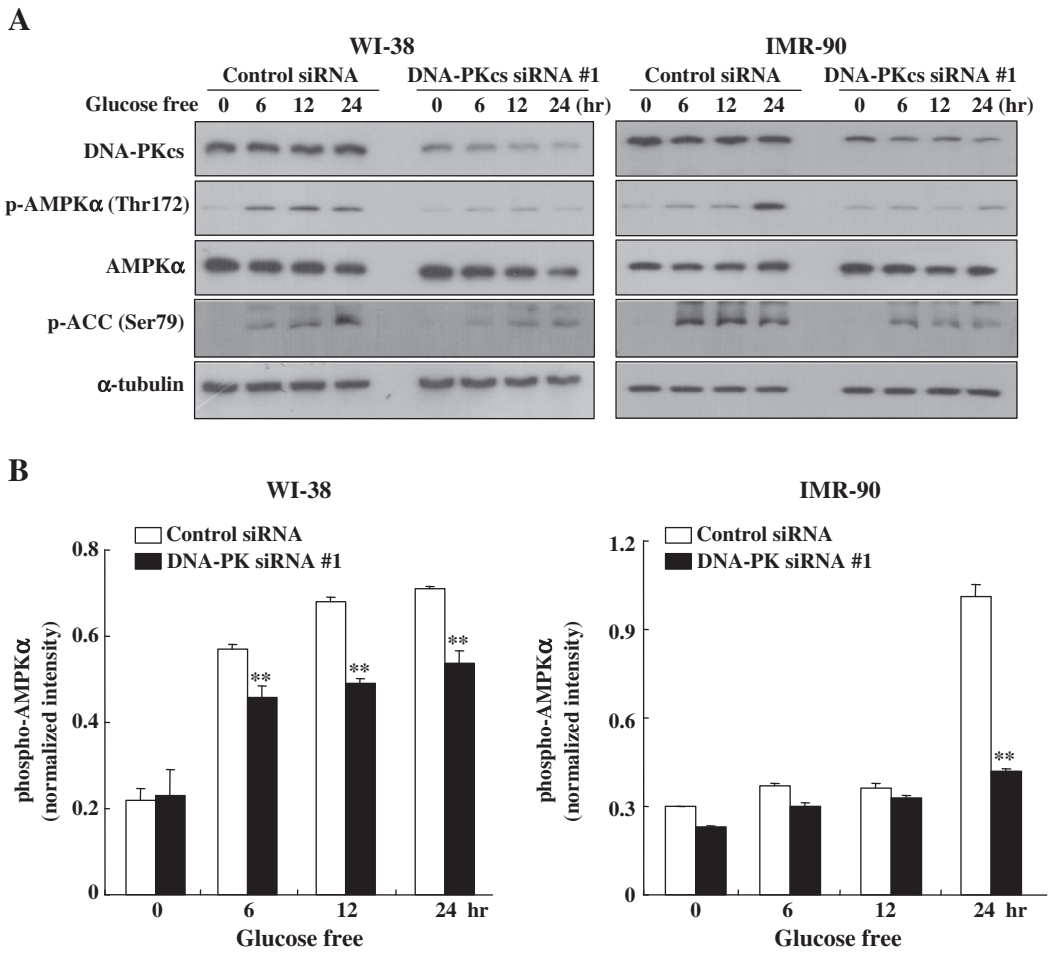

Fig. 6. AMPK activation is suppressed in DNA-PKcs-deficient primary human diploid fibroblasts. (A) WI38 and IMR90 cells were transfected with control siRNA or DNA-PKcs siRNA #1. After 48 h, the cells were cultured under glucose-free conditions for the indicated time periods. Whole-cell lysates were prepared and subjected to immunoblotting with anti-DNA-PKcs, anti-phospho-AMPKα, anti-AMPKα, anti-pACC, and anti-α-tubulin antibodies. (B) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01.

plays an important role in cellular energy homeostasis [19]. AMPK is a heterotrimer consisting of catalytic α subunits and regulatory β and γ subunits [36]. The AMPKγ1 subunit binds directly to the α2 subunit at two sites [37]. The γ subunit is essential to enzyme activity by virtue of its binding to the C-terminus of the α subunit and its role in determining AMP sensitivity. AMPK is activated by phosphorylation at Thr172 within the activation loop of the kinase domain, by AMPK kinases [38]. Two AMPK kinases are known to be involved in the phosphorylation of AMPK, LKB1 and CaMKKβ [14,31,39]. LKB1, which is a tumor suppressor gene, is the major AMPK kinase and mutation of LKB1 is responsible for

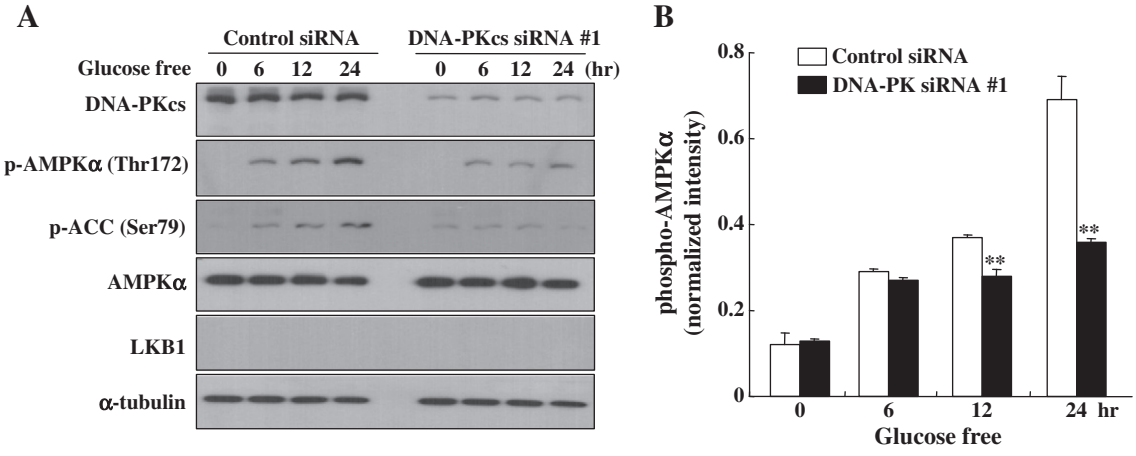

Fig. 7. Inhibition of DNA-PKcs in HeLa cells suppresses glucose-free-stimulated AMPK activity. (A) HeLa cells were transfected with control siRNA or DNA-PKcs siRNA #1. After 48 h, the cells were maintained in the presence or absence of glucose for the indicated time periods. Whole-cell lysates were prepared and subjected to immunoblotting with anti-DNA-PKcs, anti-phospho-AMPKα, anti-ACC, anti-AMPKα, and anti-α-tubulin antibodies. (B) The amount of phospho-AMPKα was quantified by densitometry and corrected for the amount of AMPKα in the corresponding lysate. The data shown are the mean s.d. from three separate experiments. $^{**}p$ 0.01.

Peutz-Jeghers syndrome [20,22,23]. In some tissues, CaMMKβ is an alternate upstream kinase that activates AMPK in a Ca^{2+}-dependent manner [24–26]. Both LKB1 and CaMKKβ directly phosphorylate Thr172 of the catalytic α subunit of AMPK.

LKB1 is not detectable in HeLa human cervical cancer cells [25]. Thus, CaMKK serves as an AMPK kinase to regulate AMPK in HeLa cells. However, the CaMKK inhibitor STO-609 did not totally block the phosphorylation and activation of AMPK in response to mannitol and 2-deoxyglucose [25], which suggests that other AMPK regulatory mechanisms may exist in these cells. We showed that glucose depletion-stimulated AMPK phosphorylation was significantly reduced, compared with control levels, when DNA-PKcs was inhibited by siRNA or chemical inhibitors in HeLa cells under glucose deprivation. These findings point to DNA-PKcs as a candidate for a regulator of AMPK activity in mammalian cells. We note that the absence of DNA-PKcs alone results in a significantly suppression of AMPK signal pathway, as phosphorylation of AMPKα and ACC was decreased in DNA-PKcs-deficient cells after glucose depletion. After energy is depleted in a cell, AMP binds to the regulatory γ subunit of AMPK, leading to the phosphorylation of AMPKα [40], which occurs in three distinct ways [15]: allosteric activation, enhanced phosphorylation by the upstream kinase, and inhibition of Thr172 dephosphorylation by protein phosphatases. These results suggest that DNA-PKcs may be a critical scaffold protein in organizing a signaling complex for AMPK activation in response to glucose deprivation. Therefore, we speculate that the binding of DNA-PKcs to AMPKγ may affect AMP-mediated allosteric activation or cause conformational changes that inhibit the inactivation by protein phosphatases. Alternatively, DNA-PKcs may affect the affinity of the AMP binding site of the γ subunit. Further studies are required to validate DNA-PKcs as a regulator of AMPK activation in mammalian cells and to determine the regulatory mechanisms of AMPK by DNA-PKcs under energy depletion.

In mammalian cells, the phosphatidylinositol 3-kinase-like kinase (PIKK) family, ATM, ATR, and DNA-PK, is a protein kinase that serves as a critical mediator of signaling pathways that facilitate the response of mammalian cells to ionizing radiation and other agents that induce DNA double-strand breaks [4,6,7]. Recent studies revealed that DNA damage activates AMPK through an LKB1-indepedent and ATM-dependent pathway [32,33], suggesting that ATM may be a candidate for a positive regulator of AMPK in response to DNA damage. We observed that ionizing radiation increased AMPK phosphorylation at Thr172 in DNA-PK-proficient cells, but not DNA-PK-deficient cells. These observations are novel and are consistent which the idea that DNA-PKcs is capable of phosphorylating and activating AMPK. We also found that specific small-molecule inhibitors of ATM (Ku55933) and DNA-PKcs (Nu7441) decreased phosphorylation of AMPK following glucose deprivation, and both inhibitors were equally effective in the suppression of the AMPK activation under glucose-depleted condition. Thus, DNA-PKcs and ATM may redundantly the participation of the phosphorylation of AMPK in response to metabolic stress and DNA damage.

A recent study reported that DNA-PK appears to be critical for the transcriptional regulation of lipogenic and gluconeogenetic genes. DNA-PK is contributed to upstream stimulating factor (USF) complex assemble and recruitment of its interacting proteins, which is required for the activation of fatty acid synthase (FAS) transcription [41]. Moreover, it has been described that activated AMPK signaling negatively regulated hepatic gluconeogenesis via DNA-PK-dependent pathway [42]. Indeed, feeding-induced de novo lipogenesis was 60 % lower in DNA-PK-deficient SCID mice after 24 hr of feeding compared to WT mice. Furthermore, SCID mice exhibit a low adipose tissue mass, indicating of a long-term defect in feeding induced lipogenesis [41]. Thus, it has been suggested that DNA-PK could possibly serve as a pharmacological target for obesity and diabetes treatment [43]. Our results suggest that DNA-PK is a positive regulator of AMPK activity. Therefore, DNA-PK may play an important role in metabolic process through transcriptional activation of metabolic genes and positive regulation of AMPK activity. Our study enlarges our understanding of the mechanisms implicated in DNA-PK's role as a regulator of the metabolic process.

In summary, we used a yeast two-hybrid system to identify AMPKγ1 as a DNA-PKcs-interacting protein. DNA-PKcs-deficient and DNA-PKcs$^{-/-}$ cells exhibited decreased AMPK activation in response to glucose-free conditions, and the inhibition of DNA-PKcs in DNA-PKcs-proficient M059K, WI38, and IMR90 cells reduced AMPK activity under glucose deprivation. The inhibitory effects of DNA-PKcs knockdown on the phosphorylation of AMPK in LKB1-deficient HeLa cells are also in accord with the possibility that DNA-PKcs contributes to glucose depletion-stimulated AMPK phosphorylation. Our data suggested a novel functional association between DNA-PKcs and AMPK, in which DNA-PKcs can act as an activator of AMPK under energy depletion. The detailed mechanism of the activation of AMPK by DNA-PKcs and its possible consequences are under investigation in our laboratory.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.08.022.

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