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AMP-activated protein kinase phosphorylates CtBP1 and down-regulates its activity

Jae-Hwan Kim [a,1], Soo-Youn Choi [a,1], Byung-Hee Kang [a,1], Soon-Min Lee [a], Hyung Soon Park [b], Gum-Yong Kang [b], Joo Young Bang [b], Eun-Jung Cho [c], Hong-Duk Youn [a,d,*]

[a] National Creative Research Center for Epigenome Reprogramming Network, Departments of Biomedical Sciences & Biochemistry & Molecular Biology, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[b] Center for Biomedical Mass Spectrometry, Diatech Korea Co., Ltd., Seoul, Republic of Korea
[c] National Research Laboratory for Chromatin Dynamics, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[d] WCU Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence and Technology, Seoul National University, Seoul, Republic of Korea

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ABSTRACT

CtBP is a transcriptional repressor which plays a significant role in the regulation of cell proliferation and tumor progression. It was reported that glucose withdrawal causes induction of *Bax* due to the dissociation of CtBP from the *Bax* promoter. However, the precise mechanism involved in the regulation of CtBP still remains unclear. In this study, we found that an activated AMP-activated protein kinase (AMPK) phosphorylates CtBP1 on Ser-158 upon metabolic stresses. Moreover, AMPK-mediated phosphorylation of CtBP1 (S158) attenuates the repressive function of CtBP1. We also confirmed that triggering activation of AMPK by various factors resulted in an increase of *Bax* gene expression. These findings provide connections of AMPK with CtBP1-mediated regulation of Bax expression for cell death under metabolic stresses.

1. Introduction

Carboxyl-terminal binding protein (CtBP) is a highly conserved protein that shares an amino acid sequence homology with D-isomer-specific 2-hydroxy acid dehydrogenases (D2-HDH) [1]. CtBP acts as a transcriptional corepressor by recruiting histone modifying enzymes such as histone deacetylases, histone lysine methyl transferases and histone lysine-specific demethylase to sequence-specific DNA-binding proteins [2–7]. The binding of NAD^+/NADH causes structural changes in CtBP, which is linked to the transcriptional regulatory activity of CtBP. Due to the importance of the NAD^+/NADH ratio on CtBP activity, the metabolic status of the cell is implicated in CtBP target gene expression [8–10].

Previous studies have revealed that CtBP plays pivotal roles in gene regulation during development and oncogenesis [11–16]. Microarray assays using mouse embryo fibroblasts (MEFs) derived from mutant mice deficient in CtBP expression showed several target genes of CtBP, including *PERP* (p53-effector related to pmp-22), *p21*, *Noxa*, and *Bax*, that are closely related to apoptosis [17].

We have previously shown that CtBP1 controls gene transcription in response to metabolic stress [14]. Glucose deprivation leads to dissociation of CtBP1 from the *Bax* promoter which in turn causes ablation of mitochondrial morphology, reduction of mitochondrial activities and finally, apoptotic cell death.

Of note, there is a study that reported glucose deprivation activates AMP-activated protein kinase (AMPK) and induces cell death [18]. AMPK is a heterotrimeric serine/threonine protein kinase complex that acts as an energy sensor whose activity is dependent on the cellular energy status. Beside its role in maintaining cellular energy homeostasis, AMPK plays important roles under conditions of hypoxia, energy deprivation, and genotoxic stress [19,20].

Both AMPK and CtBP1 act as energy sensors and cause cell death upon glucose deprivation. However, the functional relevance of these two proteins is unknown and the precise mechanism of cell death upon glucose deprivation is also not quite elucidated. Scanning of the CtBP1 protein sequence revealed the presence of several sites that have a similar sequence with the consensus motif phosphorylated by AMPK. Therefore, we sought to determine if CtBP1 is a target of phosphorylation by AMPK.

In this study, we found that AMPK activation triggered CtBP1 phosphorylation on Ser-158 and subsequent ubiquitination, inducing *Bax* expression upon AMPK activating conditions.

2. Materials and methods

2.1. Cell culture

HEK293 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) containing 10% (v/v) FBS and penicillin–streptomycin. Transient transfection was done using lipofectamine reagent (Invitrogen).

* Corresponding author at: National Creative Research Center for Epigenome Reprogramming Network, Departments of Biomedical Sciences & Biochemistry & Molecular Biology, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea. Fax: +82 2 3668 7622.
E-mail address: hdyoun@snu.ac.kr (H.-D. Youn).
[1] These authors contributed equally to this work.

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2.2. Plasmids

The plasmids used in this study were described previously [2,14]. CtBP1 mutants (S158A, S158E) in the pcDNA-Myc-His vector were generated by site-directed mutagenesis using iPfu (iNtRON).

2.3. Reagents and antibodies

AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) and 2-Deoxy-D-glucose (2-DG) were purchased from Sigma–Aldrich Inc. (St. Louis, MO). An AMPK inhibitor, compound C, was purchased from Merck Millipore.

The following antibodies were used: HA (16B12, Covance), MYC (9E10, Covance), β-actin (Sigma–Aldrich), Bax (sc-493, Santa Cruz Biotechnology), CtBP1 (sc-17759, Santa Cruz Biotechnology), AMPK (Cell Signaling), and phosphor-AMPK (Cell Signaling).

2.4. AMPK kinase assay

HEK293 cells were transiently transfected with HA-tagged AMPK wild-type or kinase-dead mutant. The next day, the cell lysates were prepared and immunoprecipitated with anti-HA antibody and protein G beads. Immunoprecipitated HA-AMPK or purified recombinant AMPK (Millipore) was incubated with His_6-CtBP1 (1 μg) in kinase reaction buffer (20 mM HEPES (pH 7.0), 0.4 mM dithiothreitol, 0.01% Brij-35, 18.75 mM MgCl2, 6.25 mM β-glycerophosphate, 1.25 mM EGTA, and 125 μM ATP) and 2 μCi of radiolabeled ATP, with or without 150 μM AMP, at 30 °C for 15 min.

2.5. Purification of recombinant His_6-tagged CtBP1

His_6-tagged CtBP1 proteins were expressed in the BL21 (DE3) pLysS strain of *Escherichia coli*. After 4 h of induction of IPTG (1 mM), bacterial cells were harvested and lysed. Purification method using TALON metal affinity resin (Clontech) was performed as described previously [2].

2.6. Mass spectrometry

His_6-tagged CtBP1 was incubated with or without purified active AMPK in AMPK kinase reaction buffer with cold ATP. After 15 min of incubation at 30 °C, samples were prepared and separated by SDS–PAGE, digested with trypsin and subjected to MS analysis (Diatech Korea, Inc.).

2.7. Fluorescence microscopy

HEK293 cells were grown on coverslips for 16 h and transfected with Myc-tagged CtBP1. Immunohistochemistry was performed as described previously (14). Labeled cells were visualized with FV1000 (Olympus).

2.8. RNA purification and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA as described previously [21].

Primers used for human *Bax* RT-PCR were: forward, 5′-GAGGATGATTGCCGCCGT GGAC-3′ and reverse, 5′-CGGTGGTGGGGGTGAGGAGG-3′. Primers used for *β-actin* RT-PCR were: forward, 5′-GGCATCCACGAAACTACCTT-3′ and reverse, 5′-CTGTGTGGACTTGGGAGAGG-3′.

2.9. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described previously (14). Primers used for *Bax* were: forward, 5′-GCGCCACTGCTGGCACTTATC-3′ and reverse, 5′-CACGTGAGAGCCCCGCTGAAC-3′.

3. Results

3.1. CtBP1 interacts with AMPK

To test whether CtBP1 physically interacts with AMPK, we transfected HEK293 cells with Myc-tagged CtBP1 and HA-tagged AMPK for binding assay. We found that Myc-tagged CtBP1 readily co-immunoprecipitated with HA-tagged AMPK and vice versa (Fig. 1A and B). We also observed that over-expressed CtBP1 bound to the catalytic domain of AMPK (Fig. 1C). To identify the region of the CtBP1 protein involved in the interaction with AMPK, we performed Co-IP assays with HA-tagged AMPK and Myc-tagged CtBP1 deletion mutants. Myc-tagged CtBP1 fragments carrying 1–300 a.a. regions showed strong binding ability with AMPK, whereas the mutant carrying 181–300 a.a. did not. These results demonstrate that 1–180 a.a. regions of CtBP1 are responsible for binding with AMPK.

3.2. CtBP1 is phosphorylated by AMPK

To determine whether AMPK directly phosphorylated CtBP1, we performed *in vitro* kinase assays using purified AMPK and bacterially purified His_6-CtBP1 as a substrate. The purified CtBP1 protein was strongly phosphorylated by the constitutively-active AMPK (Fig. 2A). For IP-kinase assay, we then stimulated with 2-deoxyglucose (2-DG) with HEK293 cells transfected with HA-tagged AMPK. After immunoprecipiation of HA-AMPK, we carried out *in vitro* kinase assay with IPed-AMPK. We found that IPed-AMPK from 2-DG-treated cells strongly phosphorylates CtBP1 (Fig. 2B). To further confirm the AMPK-mediated phosphorylation of CtBP1, we first showed that CtBP1 bound to both wild type (WT) and catalytic-dead (KD) mutant of AMPK (Fig. 2C). However, only IP-ed wild type of AMPK significantly phosphorylates His_6-CtBP1 *in vitro* (Fig. 2D), indicating that activation of AMPK and its catalytic activity is important for phosphorylation of CtBP1.

3.3. Identification of AMPK phosphorylation sites in CtBP1

We next sought to determine the residue of CtBP1 that was phosphorylated by AMPK. For mass spectrometry analysis, we incubated the purified His_6-tagged CtBP1 with or without purified constitutively-active AMPK in kinase reaction buffer; this process was fairly similar with *in vitro* kinase assay except for the presence of cold ATP instead of radioactive γ-^{32}P-ATP. Mass spectrometry analysis results identified serine 158 (S-158) as the principal phosphorylation site in CtBP1 which is mediated by constitutively-active AMPK (Fig. 3A).

3.4. Phosphorylation of S158 induces ubiquitination of CtBP1

To investigate the effect of S-158 phosphorylation on CtBP1 protein stability, we performed ubiquitination assays using wild-type or mutant CtBP1 constructs. We observed that the ubiquitination of Myc-tagged CtBP1 was enhanced by AICAR treatment. Mutation of this critical serine to alanine (S158A) reduced CtBP1 ubiquitination even in response to AICAR treatment, suggesting that phosphorylation of this residue is required for CtBP1 ubiquitination (Fig. 3B). Surprisingly, a S158E substitution in CtBP1 caused extensive ubiquitination of CtBP1, compared with the wild-type

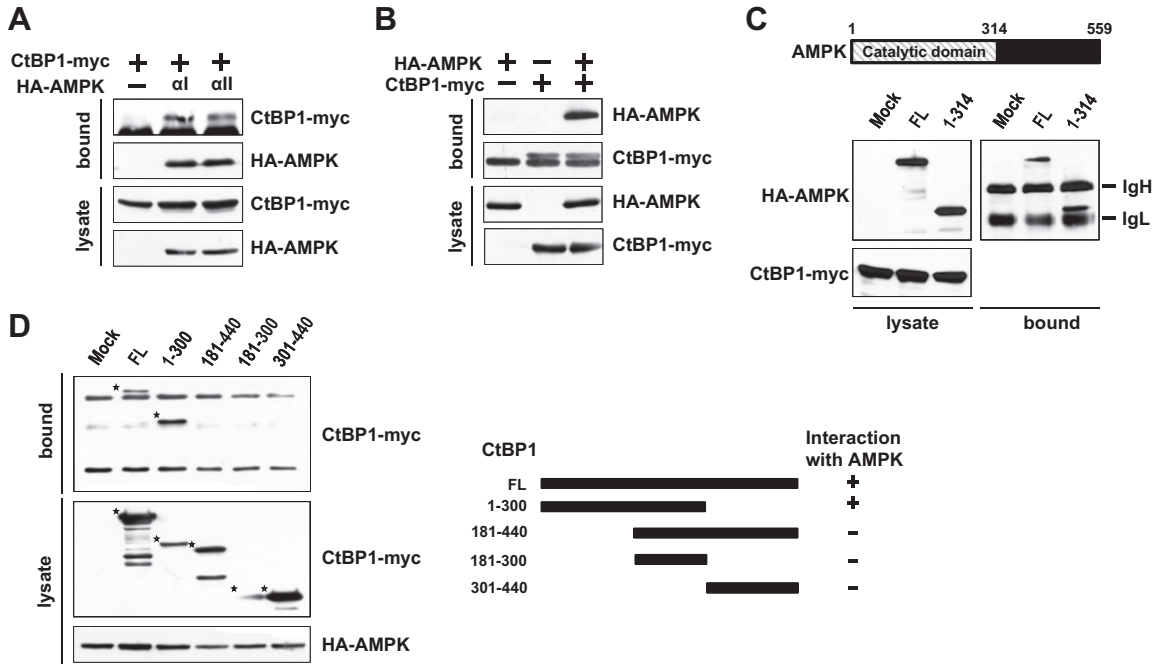

Fig. 1. CtBP1 interacts with AMPK. (A) HEK293 cells were co-transfected with Myc-tagged CtBP1 and HA-tagged AMPK αI, αII or empty vector. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc or anti-HA antibody. (B) HEK293 cells were co-transfected with HA-tagged AMPK and Myc-tagged CtBP1 or with the corresponding empty vectors. Immunoprecipitation was performed using anti-Myc antibody. Immunoblotting was performed with the indicated antibodies. (C) To test whether CtBP1 can bind to the kinase domain of AMPK, cells were co-transfected with Myc-tagged CtBP1 and HA-tagged AMPK wild type or deletion mutant carrying 1–314 amino acids. Cell lysates were immunoprecipitated with anti-Myc antibody. (D) To verify the binding region, the indicated various deletion mutants of Myc-tagged CtBP1 were co-transfected with HA-tagged AMPK and subjected to immunoprecipitation and immunoblotting. The following illustration shows CtBP1 deletion mutants and the results of their interaction with AMPK. *Asterisks indicate Myc-tagged CtBP1.

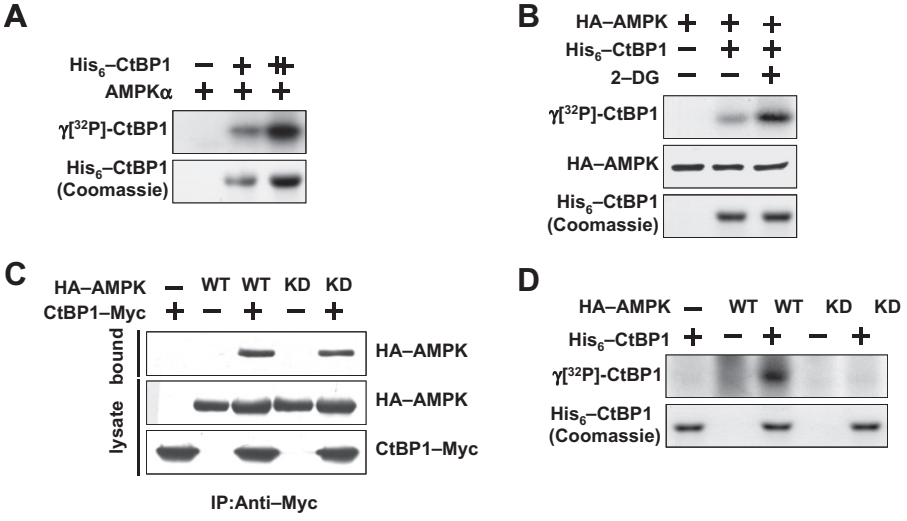

Fig. 2. CtBP1 is phosphorylated by AMPK. (A) Using bacterially expressed and purified His_6-tagged CtBP1 as a substrate and purified active AMPKα, *in vitro* kinase assay was performed and assessed by autoradiography. The bottom panel shows Coomassie staining of purified CtBP1. (B) Purified CtBP1 proteins were incubated with HA-tagged AMPK obtained from the 2-DG treated or control HEK293 cells in the AMPK assay mixture. Phosphorylation of CtBP1 was visualized by autoradiography (top panel) and the protein levels of HA-tagged AMPK and His_6-tagged CtBP1 were assessed by immunoblotting and Coomassie staining, respectively. (C) HEK293 cells were co-transfected with Myc-tagged CtBP1 and HA-tagged AMPK wild-type (WT) or kinase-dead (KD) mutant. Total lysates were immunoprecipitated using anti-Myc antibody and subjected to immunoblot analysis. (D) HEK293 cells were transfected either with HA-tagged AMPK wild-type or kinase-dead mutant. Using IP-ed HA-AMPK from transiently transfected cells and purified His_6-tagged CtBP1, *in vitro* kinase assay was performed and assessed by autoradiography.

construct (Fig. 3C). We also observed that the phosphomimic mutant S158E localized in the cytoplasm, rather than in the nucleus Fig. 3D). These results indicate that S-158 phosphorylation on CtBP1 causes ubiquitination and nuclear export of CtBP1.

3.5. Activation of AMPK induces Bax transcription

We have previously shown that Bax is the transcriptional repression target of CtBP1. In this study, we observed that the

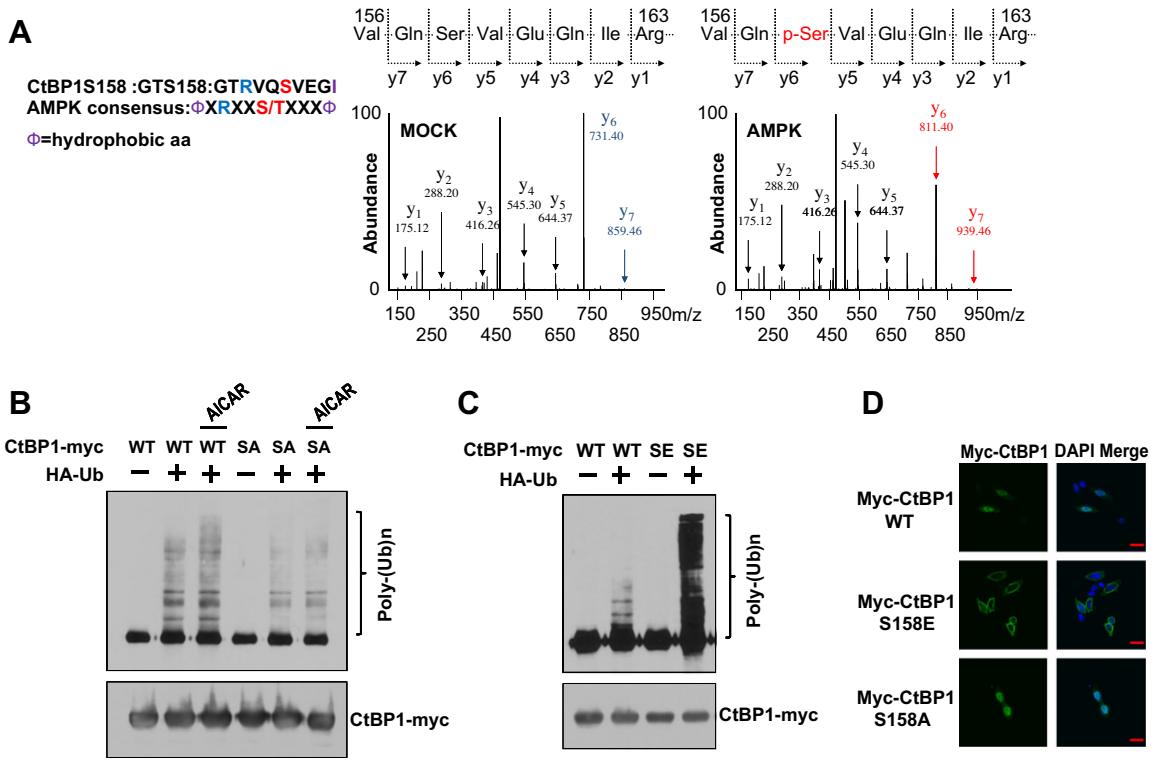

Fig. 3. Phosphorylation of S158 induces ubiquitination of CtBP1. (A) MS analysis identified serine 158 as the principal phosphorylation site in CtBP1 mediated by AMPK. Purified His_6-tagged CtBP1 was incubated with or without purified active AMPK in AMPK kinase reaction buffer at 30 °C for 15 min. The samples were separated by SDS–PAGE, digested with trypsin and subjected to MS analysis (Diatech Korea Inc.). (B) HEK293 cells were co-transfected with Myc-tagged CtBP1 wild-type (WT) or S158A (SA) mutant along with HA-tagged ubiquitin or the corresponding empty vector. Twenty four hours after transfection, cells were treated with MG132 in the presence of AICAR or mock vehicle. Immunoprecipitaion assay was performed using anti-Myc antibody and then immunoblotted with the indicated antibodies. (C) HEK293 cells were co-transfected with Myc-tagged CtBP1 wild-type or S158E (SE) mutant along with HA-tagged ubiquitin or the corresponding empty vector. Cells were treated with MG132 for 12 h and harvested, lysed and immunoprecipitated with anti-Myc antibody. Poly-ubiquitinated bands of CtBP1 were detected by anti-HA antibody. (D) Cells were grown on coverslips for 16 h and transfected with Myc-tagged CtBP1 WT, S158E and S158A, respectively. Immunohistochemistry was performed using anti-Myc antibody and FITC-conjugated goat anti-mouse antibody. The scale bar represents 20 μm.

CtBP1 protein is phosphorylated by AMPK and this leads to an increase of ubiquitination of CtBP1. To test whether AMPK activity could influence CtBP1 target gene expression, we assessed the mRNA and protein levels of Bax. AMPK activating reagents, such as AICAR and 2-DG, or glucose free medium treatments increased the expression of the *Bax* gene (Fig. 4A and B). Pre-treatment of cultures with an AMPK inhibitor, Compound C, prevented these effects (Fig. 4C and D). These results show that phosphorylation of CtBP1 by AMPK attenuates the repressive function of CtBP1 on *Bax* promoter. To confirm the promoter occupancy upon AMPK activation condition, we performed chromatin immunoprecipitation assays. As results, AICAR treatment attenuated the binding of CtBP1 on Bax promoter.

4. Discussion

CtBP serves as an energy-sensing transcriptional regulator which provides a direct link to cellular redox states and target gene expression. The activity of CtBP is regulated by the NAD^+/NADH ratio as well as post-translational modification upon various cellular signaling pathways. The CtBP protein is known to undergo sumoylation, phosphorylation and ubiquitination which in turn cause a conformational change and cellular translocation upon various cellular signaling processes.

It is reported that CtBP1 recruits the small ubiquitin-related modifier (SUMO) conjugating E2 enzyme UBC9 and a SUMO E3 ligase (HPC2) [22,23]. HPC2 enhances sumoylation of CtBP at a single site (K428) of CtBP1 and also mediates SUMO modification of CtBP-interacting PLDLS-containing proteins. Sumoylation of CtBP1 has an effect on its subcellular localization and thus attenuates the corepressive function.

Previous studies have also identified several phosphorylation sites of CtBP1. The homeodomain-interacting protein kinase-2 (HIPK2) and c-Jun NH2-terminal kinase 1 (JNK1) phosphorylate CtBP1 at Ser-422 upon UV-irradiation [24]. Phosphorylation of CtBP1 at Ser-422 directs CtBP1 toward degradation and induces cell death [25,26]. It is noteworthy that both kinases target the same residue even though they have distinct signaling pathways. However, the results of their phosphorylation result in the same outcome.

The p21-activated kinase 1 (Pak1) is also responsible for the phosphorylation of CtBP1. It acts as a CtBP1 specific kinase targeting Ser-158 phosphorylation, which in turn, causes attenuation of CtBP corepressor functions by mediating nuclear-to-cytosolic translocation [26]. Here, we present evidence that AMPK participates in a similar mode of CtBP1 regulation.

AMPK regulates cellular metabolism and gene expression by sensing the AMP/ATP ratio. Glucose deprivation or treatment with AMPK activators resulted in activation of AMPK and induced cell death. However, the precise mechanism of cell death mediated by AMPK activation is not fully elucidated [18,27].

In this study, we found that CtBP1 physically interacts with AMPK and serves as a phosphorylation target of AMPK under the condition of glucose deprivation. Mass spectrometry assays revealed the precise residue of phosphorylation, ser-158 of CtBP1.

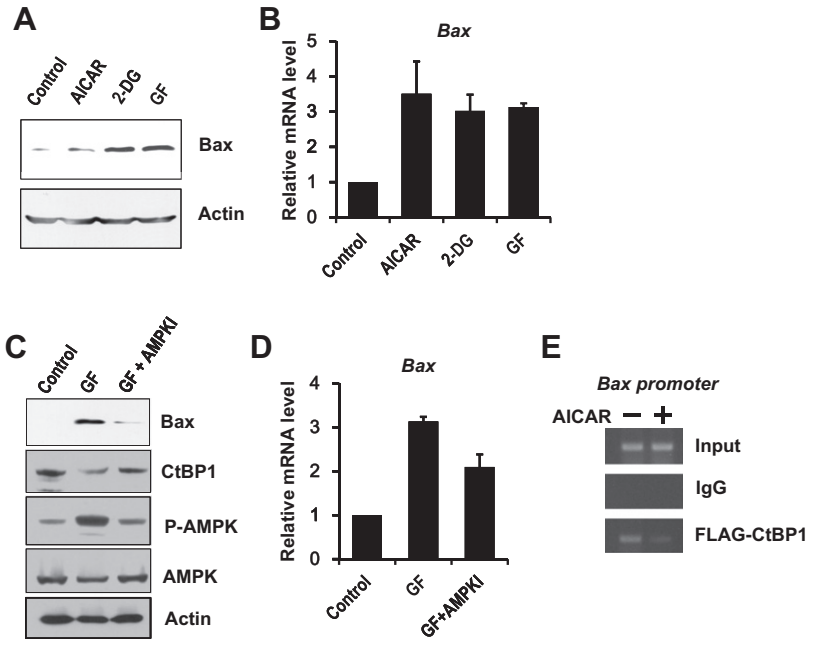

Fig. 4. Activation of AMPK induces *Bax* transcription. (A and B) HEK293 cells were treated with mock vehicle, AICAR (2 mM) for 2 h, 2-DG (10 mM) for 30 min, and glucose free (GF) media for 6 h, respectively. Cells were harvested for immunoblotting and RT-PCR. The mRNA and protein levels of Bax and β-actin were determined by immunoblotting and real-time PCR. (C and D) The indicated lane of cells were pre-treated with AMPK inhibitor (AMPKI; compound C) and then incubated in glucose-free medium. Cells were harvested and subjected to immunoblot analysis with the indicated antibodies and the mRNA level of *Bax* was determined by real-time PCR. The values represent mean relative change ± s.d. ($n \geqslant 3$). (E) HEK293 cells were transfected with FLAG-tagged CtBP1 and incubated for 24 h. After AICAR or mock vehicle treatment for 2 h, ChIP assays were performed using anti-FLAG antibodies. ChIPed DNA was analyzed by qRT-PCR using *Bax* ChIP primers.

We next tested if the phosphorylation was the mark for degradation. The phosphomimic mutant S158E construct showed extensive ubiquitination; on the other hand, S158A substitution reduced the ubiquitinated bands of CtBP1, compared with the wild-type construct.

On the basis of these findings, we hypothesized that glucose deprivation would cause clearance of CtBP1 by AMPK-mediated phosphorylation and therefore result in *Bax* gene transcription leading to cell death. To prove this hypothesis, we assessed the mRNA and protein levels of Bax upon AMPK-activating conditions. As expected, activity of AMPK influenced *Bax* transcription.

Our studies have elucidated the mechanism of cell death upon glucose deprivation. As a cellular energy sensor responding to low ATP levels, activated AMPK directly phosphorylates CtBP1 and down-regulates its activity. Dissociation of CtBP1 from the *Bax* promoter increases *Bax* transcription and finally results in cell death.

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