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# Suppression of death-associated protein kinase 2 by interaction with 14-3-3 proteins

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ABSTRACT

Death-associated protein kinase 2 (DAPK2), a $Ca^{2+}$/calmodulin-regulated serine/threonine kinase, induces apoptosis. However, the signaling mechanisms involved in this process are unknown. Using a proteomic approach, we identified 14-3-3 proteins as novel DAPK2-interacting proteins. The 14-3-3 family has the ability to bind to phosphorylated proteins via recognition of three conserved amino acid motifs (mode 1-3 motifs), and DAPK2 contains the mode 3 motif ((pS/pT)$X_{1-2}$-COOH). The interaction of 14-3-3 proteins with DAPK2 was dependent on the phosphorylation of $Thr^{369}$, and effectively suppressed DAPK2 kinase activity and DAPK2-induced apoptosis. Furthermore, we revealed that the 14-3-3 binding site $Thr^{369}$ of DAPK2 was phosphorylated by the survival kinase Akt. Our findings suggest that DAPK2-induced apoptosis is negatively regulated by Akt and 14-3-3 proteins.



## 1. Introduction

The death-associated protein kinase (DAPK) family is a group of serine/threonine kinases associated with a wide spectrum of apoptotic signaling pathways, including interferon γ, tumor necrosis factor-α, and anoikis [1,2]. The family consists of five members: DAPK1, DAPK2/DAPK-related protein kinase (DRP-1), DAPK3/zipper-interacting protein kinase (ZIPK)/DAP-like kinase (DLK), DAP kinase-related apoptosis-inducing protein kinase 1 (DRAK1), and DRAK2. The members of the DAPK family show high sequence homology in their N-terminal catalytic domains, whereas the structures of their C-terminal regions are different. Although both DAPK1 and DAPK2 possess a $Ca^{2+}$/calmodulin (CaM)-binding domain and are activated by $Ca^{2+}$/CaM, DAPK1 also contains other unique domains such as eight ankyrin repeats, a cytoskeletal binding domain, and a death domain, which are involved in protein–protein interactions [3]. The cytoskeletal binding domain defines the intracellular localization of the protein through its interaction with actin. The ankyrin repeats are involved in association with DAPK-interacting protein-1, promoting tumor necrosis factor-induced apoptosis [4], and the death domain regulates the proapoptotic function of DAPK1 by interacting with netrin-1 receptor UNC5H2 [5]. In contrast, DAPK3 lacks a $Ca^{2+}$/CaM-binding domain; however, it has a leucine zipper domain, which is required for dimerization and interactions with other leucine zipper-containing proteins such as the apoptosis antagonizing transcription factor [6]. Although DAPK2 contains a unique C-terminal tail involved in homodimerization, functional proteins interacting with DAPK2 have not yet been identified.

14-3-3 proteins bind to a number of functionally diverse signaling proteins including protein kinases and protein phosphatases, and are involved in important cellular processes such as signal transduction, cell cycle control, and apoptosis [7,8]. The members of this family (seven isotypes in mammals: β, γ, ε, η, σ, τ, and ζ) recognize specifically phosphorylated serine/threonine residues of the target proteins. Most of the 14-3-3-binding proteins contain either of the three consensus motifs: RSXpSXP (mode 1), RX(Y/F)XpSXP (mode 2), or pS/pT$X_{1-2}$-COOH (mode 3), where pS and pT denote phosphoserine and phosphothreonine, respectively. To date, more than 300 proteins have been shown to interact with 14-3-3 family members; these interactions can affect the biological activity or the localization of the binding proteins. For example, 14-

*Abbreviations:* DAPK, death-associated protein kinase; CaM, calmodulin; MLC2, myosin light chain 2; GST, glutathione S-transferase; CnA-α, calcineurin A-α; PARP, poly (ADP-ribose) polymerase.

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3-3 proteins interact with BAD, a proapoptotic member of the Bcl-2 family, and suppress its binding to and inactivation of the pro-survival protein Bcl2/Bcl-xL, thus preventing BAD-induced apoptosis [9]. The binding of 14-3-3 proteins to BAD depends on its phosphorylation by serine/threonine kinase Akt, which is a central node in the downstream cell signaling of growth factors and cytokines [10]. On the other hand, $Ca^{2+}$/CaM-regulated protein phosphatase, calcineurin, dephosphorylates BAD, resulting in dissociation from 14-3-3, BAD/Bcl-xL heterodimerization, and induction of apoptosis [11]. Thus, binding of 14-3-3 proteins to target proteins is controlled by a balance between protein kinase and protein phosphatase activities.

In this study, we identified 14-3-3 proteins as novel DAPK2 binding proteins using a proteomic approach. We revealed that 14-3-3 proteins were associated with DAPK2 phosphorylated at $Thr^{369}$ by Akt. In addition, we showed that 14-3-3 proteins suppressed the kinase activity of the proapoptotic protein DAPK2, blocking DAPK2-induced apoptosis. Our findings provide a novel mechanism for the regulation of DAPK2-induced apoptosis.

## 2. Materials and methods

### 2.1. Plasmid construction

cDNAs encoding mouse DAPK2, human 14-3-3 isoforms, mouse Akt, and mouse calcineurin A-α (CnA-α) were cloned by PCR using the respective specific primers. PCR products were cloned into the TA-cloning vector pGEM-T Easy (Promega), and the inserted DNA sequences were confirmed by DNA sequencing. A cDNA encoding mouse DAPK2 was subcloned into the N-terminal FLAG-tagged expression vector pFLAG-CMV-2 (Sigma) and N-terminal Strep-tagged expression vector pEXPR-IBA105 (IBA), resulting in pFLAG-DAPK2 and p105-DAPK2, respectively. Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the manufacturer's instructions. The mutations were confirmed by DNA sequence analysis.

### 2.2. Cell culture and transfection

COS-7 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% $CO_2$. Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions.

### 2.3. Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MCF-7 cells were transfected with the p105-DAPK2. After 24 h, cells were scraped in cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 10 μM leupeptin, and 10 μg/ml aprotinin). The cell extracts were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were incubated with Strep-Tactin Sepharose (IBA) over night at 4 °C. The beads were washed five times with wash buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% NP40, and 1 mM EDTA), and the bound proteins were eluted with 2 x SDS-loading buffer. The eluates were resolved by 12% SDS-PAGE, followed by silver staining (Silver Staining MS Kit, Wako Pure Chemical). The bands in the SDS-PAGE gel were cut off and destained twice in de-staining solution (Wako Pure Chemical) for 10 min. After dehydration in 100% acetonitrile, the gel pieces were dried using a centrifugal evaporator. To reduce disulfide bonds, the dried gel pieces were incubated with reducing reagent (10 mM dithiothreitol and 25 mM ammonium bicarbonate) at 56 °C for 45 min. After removing the reducing reagent, the gel pieces were incubated with alkylating reagent (55 mM iodoacetamide and 25 mM ammonium bicarbonate) for 30 min, washed with 25 mM ammonium bicarbonate, and dehydrated (using 25 mM ammonium bicarbonate/50% acetonitrile followed by 100% acetonitrile). The gel pieces dried using a centrifugal evaporator were rehydrated with 20 ng/μl of trypsin (Promega) in 25 mM ammonium bicarbonate, and incubated in 25 mM ammonium bicarbonate over night at 37 °C. The trypsin-digested peptides were extracted using extraction buffer (5% trifluoroacetic acid/50% acetonitrile) by sonication for 3 min and vortex-mixing for 30 min. After centrifugation, the supernatant was collected and concentrated using a centrifugal evaporator. The samples were desalted using C18 ZipTip according to the manufacturer's instructions (Millipore), mixed with the matrix, and then spotted on AnchorChip sample target (Bruker Daltonics). The mass spectra of peptides were acquired by MALDI-TOF MS (Autoflex Speed, Bruker Daltonics). The mass spectra were processed using FlexAnalysis 3.3 and Biotools 3.2 (Bruker Daltonics), and the generated data were analyzed using the Mascot server (Matrix Science).

### 2.4. Co-immunoprecipitation and Strep pull-down assays

Co-immunoprecipitation assay has been described previously [12]. For a Strep pull-down assay, COS-7 cells were co-transfected with pFLAG-DAPK2 and p105-14-3-3. After 24 h, cells were scraped in lysis buffer. Cell lysates were incubated with Strep-Tactin Sepharose over night at 4 °C. The bound proteins were analyzed by immunoblot analysis using anti-FLAG M2 antibody (Sigma).

### 2.5. In vitro kinase assay

*In vitro* phosphorylation by DAPK2 was performed as described previously [13]. GST-MLC2 was used as a substrate for DAPK2 activity assay. COS-7 cells transfected with pFLAG-DAPK2 in the presence or absence of pFLAG-14-3-3 were harvested in lysis buffer. Cell lysates were incubated with anti-FLAG M2 antibody and protein G Sepharose (GE Healthcare) overnight at 4 °C. The beads were washed three times with wash buffer and twice with 50 mM Tris–HCl, pH7.5. The kinase reaction was carried out by resuspending the complexes in 100 μl of kinase buffer (50 mM Tris–HCl pH 7.5, 20 mM magnesium acetate, 50 μM ATP, 2 μCi [$\gamma$-$^{32}$P]ATP, Phosphatase inhibitor cocktail (Nacalai Tesque), 30 μg/ml purified GST-MLC2, 100 mM $CaCl_2$ and 10 nM CaM), and incubating for 30 min at 30 °C. Phosphorylated GST-MLC2 was separated by SDS-PAGE and observed using a BAS-1500 Bioimaging Analyzer (Fuji Film).

### 2.6. Statistical analysis

All experiments were performed multiple times to confirm their reproducibility. One representative set of data was shown in the figures. The results were quantified using Image J software (NIH). Data were expressed as means ± S.E., and statistical significance was determined by Student's t-test or ANOVA.

## 3. Results

### 3.1. Identification of the proteins interacting with DAPK2

To identify novel DAPK2 binding partners, we used the Strep pull-down approach to capture the endogenous binding proteins. Our previous study has shown that DAPK2 overexpression induces apoptosis in human breast cancer MCF-7 cells [13]. Therefore, full-length mouse DAPK2 was expressed in MCF7 cells as an N-terminal Strep-tagged protein. The Strep pull-down proteins were resolved

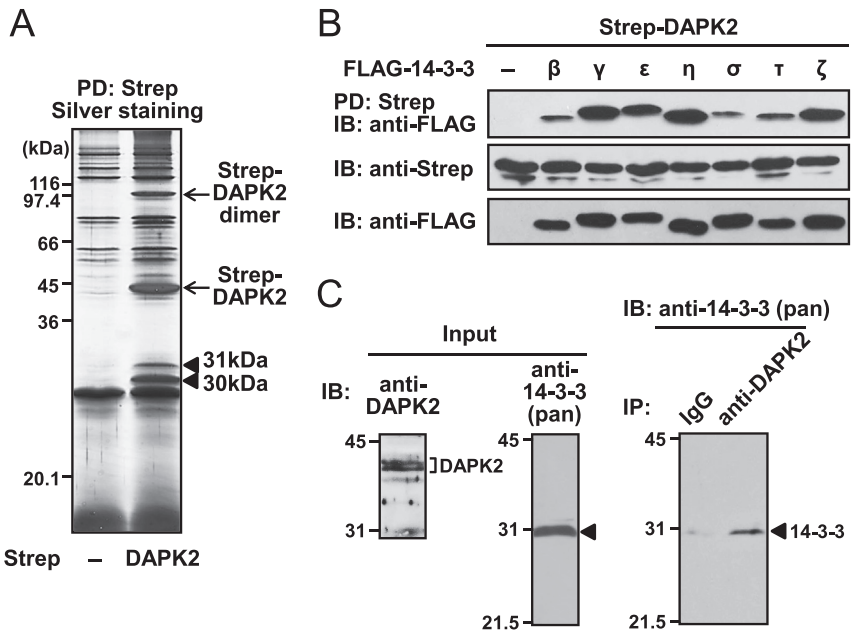


**Fig. 1.** Identification of 14-3-3 proteins as DAPK2-interacting proteins. (A) MCF-7 cells were transiently transfected with either Strep empty vector or Strep-DAPK2. After 24 h, the cells were harvested and Strep pull-down assay was performed. The precipitated proteins were subjected to SDS-PAGE and were visualized by silver staining. (B) Strep-DAPK2 was expressed in COS-7 cells with FLAG-tagged 14-3-3 family members. Cell lysates were pulled down with Strep-Tactin, followed immunoblotting with anti-FLAG antibody. Protein expression was confirmed by immunoblotting of total cell lysates. (C) The cell lysates from MCF-7 cells were immunoprecipitated using either anti-DAPK2 antibody or normal rabbit IgG (*IgG*). The immunoprecipitates were analyzed by immunoblotting with anti-14-3-3 (pan) antibody. The endogenous expression of DAPK2 and 14-3-3 proteins was confirmed by immunoblotting of MCF-7 cell lysates with the appropriate antibodies. All experiments were performed multiple times with similar results.

by SDS-PAGE followed by silver staining. To exclude non-specific binding, lysates of the cells transfected with empty Strep vector were used as negative controls. Fig. 1A shows a typical gel. The specific protein bands at 30, 31, 44 and 88 kDa were found in the lysates of the cells transfected with Strep-DAPK2. The bands at 44 and 88 kDa were close to the molecular masses of the Strep-tagged DAPK2 and its SDS-resistant dimer, respectively. Therefore, the bands at 30 and 31 kDa were excised and analyzed by MALDI-TOF

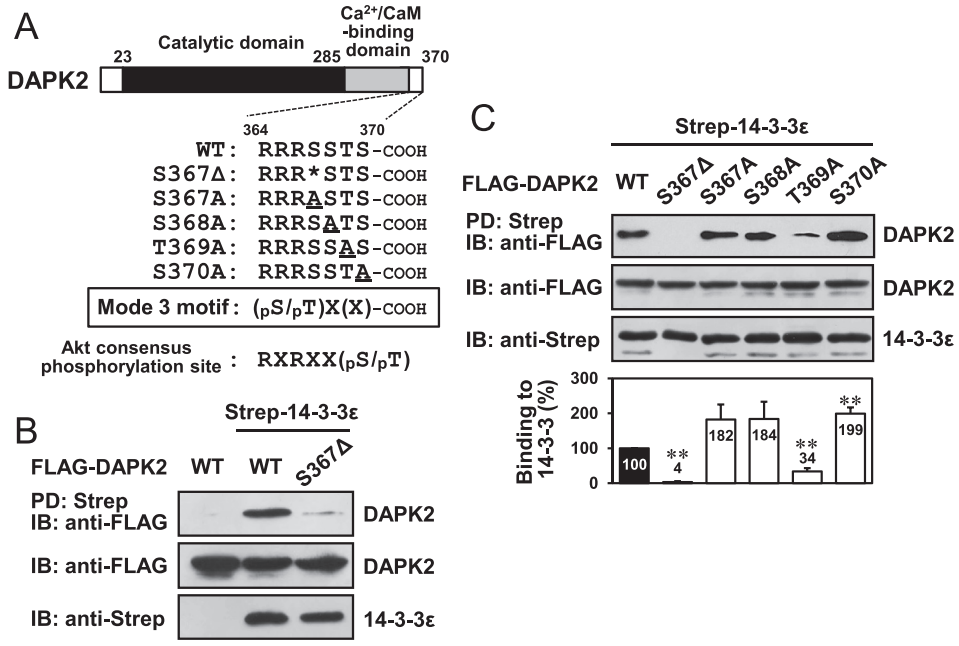


**Fig. 2.** DAPK2 interacts with 14-3-3ε via Thr[369] located at the C-terminus. (A) The structure of DAPK2 is illustrated. The C-terminal truncated (S367Δ) or alanine substituted mutants (S367A, S368A, T369A, and S370A) are shown. (B) Strep-14-3-3ε was expressed in COS-7 cells with either FLAG-tagged wild-type DAPK2 (*WT*) or its mutant, DAPK2 S367Δ, in which Ser[367] was replaced by a stop codon. Total cell lysates were pulled down with Strep-Tactin, followed immunoblotting with anti-FLAG antibody. Protein expression was confirmed by immunoblotting of total cell lysates. (C) COS-7 cells were transiently transfected with Strep-14-3-3ε together with either FLAG-DAPK2 wild type (*WT*) or mutants. Total cell lysates were pulled down with Strep-Tactin, followed immunoblotting with anti-FLAG antibody. The binding to 14-3-3ε was quantified by Image J software. The binding of wild-type DAPK2 to 14-3-3ε was taken as 100%. Results are expressed as means ± S.E. of three different experiments. Statistical significance compared with the wild-type was determined by Student's t-test. **, $p < 0.01$.

MS after in-gel tryptic digestion. The analysis showed that 30- and 31-kDa proteins were 14-4-3ζ and 14-3-3ε, respectively.

The mammalian 14-3-3 protein family consists of seven members (β, γ, ε, η, σ, τ, and ζ) [8,9]. We analyzed which members of the 14-3-3 family bind to DAPK2. COS-7 cells were transfected with Strep-tagged DAPK2 and FLAG-tagged human 14-3-3 family members. Cell lysates were pulled down with Strep-Tactin, and immunoblotted with anti-FLAG antibody. As shown in Fig. 1B, all 14-3-3 family members interacted with DAPK2. Furthermore, the endogenous interaction between DAPK2 and 14-3-3 proteins was investigated. The cell lysates from MCF-7 cells were immunoprecipitated using anti-DAPK2 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-14-3-3 (pan) antibody, which recognizes all 14-3-3 isoforms. As shown in Fig. 1C, 4-3-3 proteins were detected in the DAPK2 immunoprecipitates, indicating endogenous interactions between DAPK2 and 14-3-3 proteins.

### 3.2. 14-3-3 proteins interact with DAPK2 via phosphorylated Thr$^{369}$

14-3-3 proteins associate with phosphopeptides containing consensus motifs, RSXpSXP (mode 1), RX(Y/F)XpSXP (mode 2), or pS/pTX$_{1\text{-}2}$-COOH (mode 3), where X stands for any amino acid. Amino acid sequence analysis revealed that DAPK2 has only a mode 3 motif in its C terminus ($^{364}$RRRSSTS-COOH) (Fig. 2A). To identify 14-3-3 binding sites for DAPK2, we created DAPK2 S367Δ mutant, in which Ser$^{367}$ was replaced by a stop codon. As shown in Fig. 2B, Strep pull-down assay demonstrated that binding of 14-3-3ε to DAPK2 S367Δ was much weaker than to wild-type DAPK2. To analyze the binding site in more detail, we constructed four non-phosphorylatable mutants of DAPK2, in which the serine or threonine at the C terminus was replaced by alanine (Fig. 2A), and performed the pull-down experiment. As shown in Fig. 2C, the interaction of 14-3-3ε with the T369A mutant was significantly reduced as compared with that with wild-type DAPK2, whereas binding to the S367A, S368A, and S370A mutants was rather increased. These results suggest that 14-3-3 proteins recognize phosphorylated Thr$^{369}$ of DAPK2 and interact with it.

### 3.3. The activity of DAPK2 is inhibited by 14-3-3 proteins

14-3-3 proteins can regulate enzyme activity, alter subcellular localization, and mediate protein–protein interaction through conformational changes or by masking/revealing functional motifs of the interacting proteins [8,9]. We examined the effect of 14-3-3 binding on the kinase activity of DAPK2. Myosin light chain 2 (MLC2) was used as a substrate for DAPK2 activity as described previously [13]. An *in vitro* kinase assay revealed that $Ca^{2+}$/CaM-activated DAPK2 phosphorylated GST-MLC2, whereas the DAPK2 activity was significantly reduced by all members of the 14-3-3 family, particularly by 14-3-3σ (Fig. 3A).

Furthermore, we examined the effect of14-3-3 proteins on apoptosis induced by ectopically expressed DAPK2. During apoptosis, poly (ADP-ribose) polymerase (PARP), 116-kDa nuclear protein involved in DNA repair, is cleaved by active caspases including caspase 3, into 85- and 25-kDa fragments [14]. Therefore, the extent of PARP cleavage is used as a marker of apoptosis. We transfected COS-7 cells with DAPK2 in the presence and absence of 14-3-3σ, and immunoblotted the total cell lysates using anti-PARP and anti-cleaved PARP antibodies. As shown in Fig. 3B, overexpression of DAPK2 induced the cleavage of PARP, and this cleavage was significantly reduced by coexpression of 14-3-3σ. These results suggest that DAPK2 is suppressed by association with 14-3-3 proteins.

### 3.4. DAPK2 is phosphorylated by Akt at Thr$^{369}$

Further, we identified the protein kinase that phosphorylates DAPK2 at Thr$^{369}$. The site ($^{364}$RRRSSpTS) including Thr$^{369}$ matched the consensus motif for Akt phosphorylation (RXRXX(pS/pT)) (Fig. 2A) [15]. Thus, we examined whether DAPK2 is phosphorylated by Akt. Because DAPK2 can be autophosphorylated [13], a kinase-dead mutant of DAPK2, DAPK2 K52A, was expressed with constitutively active Akt, Akt T308D/S473D [16], in COS-7 cells. Cell lysates were immunoblotted with anti-phospho-Akt substrate antibody, which recognizes the consensus Akt phosphorylation sequence RXRXX(pS/pT). As shown in Fig. 4A, DAPK2 K52A was efficiently phosphorylated by constitutively active Akt. However, Akt failed to phosphorylate DAPK2 K52A/T369A, in which Thr$^{369}$ was replaced by alanine. Furthermore, we investigated the effect of Akt on the interaction between DAPK2 and 14-3-3 proteins. COS-7 cells transiently transfected with Strep-DAPK2 and FLAG-14-3-3γ were treated with an Akt-specific inhibitor, MK2206, in increasing concentrations, and Strep pull-down assay was performed. When the cells were treated with 10 μM MK2206, binding of DAPK2 to 14-3-3γ was significantly reduced to approximately 30% of that of cells treated with dimethyl sulphoxide alone (Fig. 4B). These results

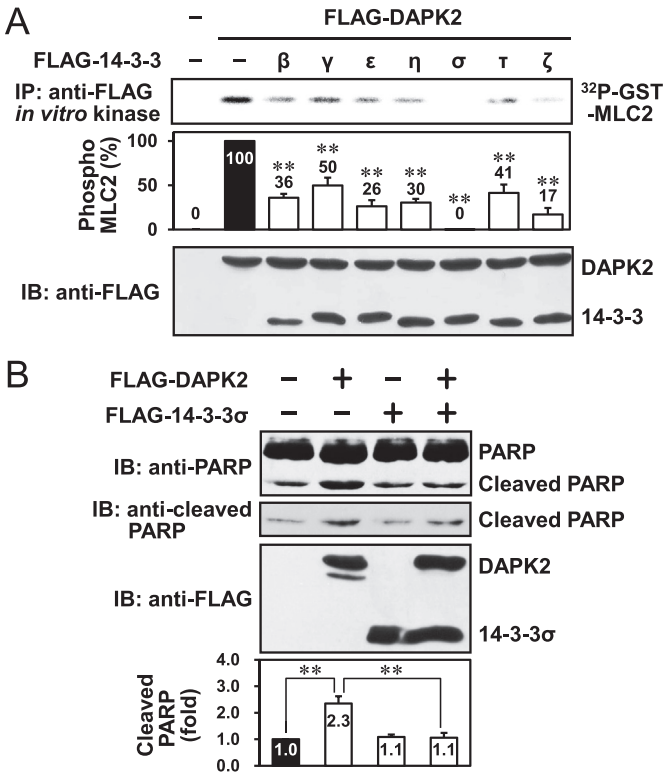


**Fig. 3.** 14-3-3 blocks DAPK2-induced apoptosis through suppression of DAPK2 activity. (A) FLAG-DAPK2 was transfected with or without FLAG-tagged 14-3-3 family members into COS-7 cells. FLAG-tagged proteins were immunoprecipitated and used in an *in vitro* kinase assay with recombinant GST-MLC2 as a substrate. GST-MLC2 was separated on SDS-PAGE, after which the gel was analyzed by a bioimaging analyzer. The kinase activity of DAPK2 in the absence of 14-3-3 was taken as 100%. The data shown are the means ± S.E. derived three independent experiments. **, $p < 0.01$ indicates significant difference from the DAPK2 activity in the absence of 14-3-3, as analyzed by Student's t-test. (B) COS-7 cells were transiently transfected with FLAG-DAPK2 and FLAG-14-3-3σ. After 24 h, the cells were harvested and cell lysates were analyzed by immunoblot analysis with anti-PARP, anti-cleaved PARP, and anti-FLAG antibodies. The amount of cleaved PARP in mock-transfected cells was taken as 1. Results are expressed as means ± S.E. of three different experiments. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. **, $p < 0.01$.

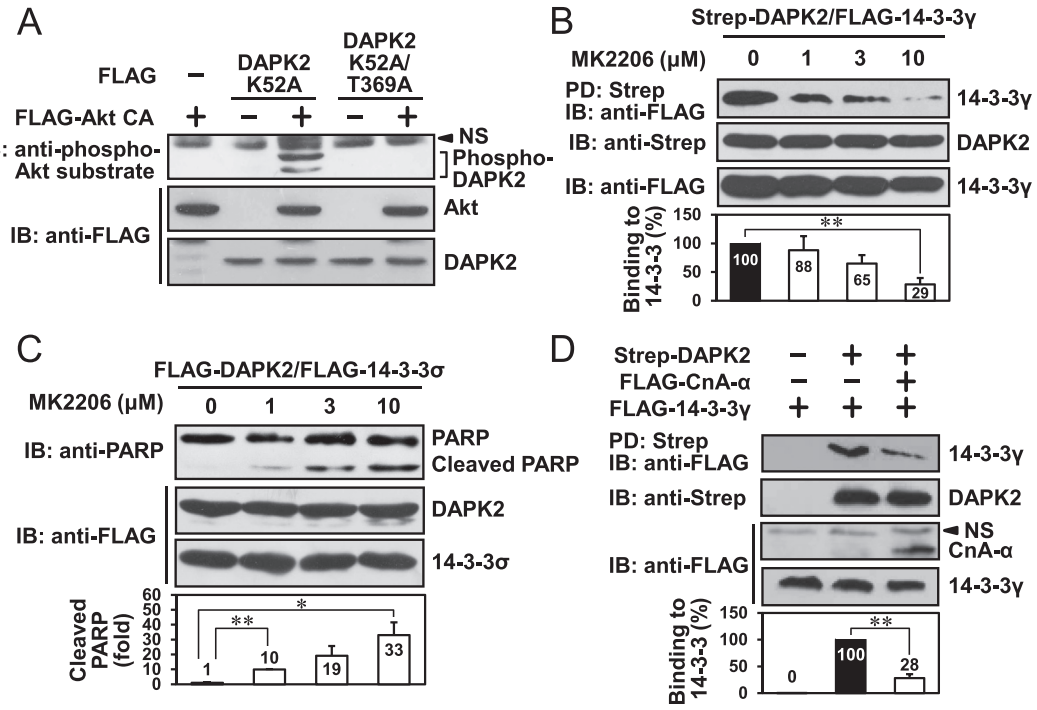


**Fig. 4.** Interaction between DAPK2 and 14-3-3 is promoted by Akt. (A) The kinase-dead mutant of DAPK2 (DAPK2 K52A) or DAPK2 K52A/T369A was transfected with or without constitutively active Akt (*Akt CA*). Total cell lysates were subsequently analyzed by immunoblotting with anti-phospho-Akt substrate (phosphor-RXRXXpS/pT) or anti-FLAG antibody. "NS" indicates a nonspecific band. (B) COS-7 cells transfected with Strep-DAPK2 and FLAG-14-3-3γ were treated with MK2206 at the indicated concentrations for 18 h. Cell extracts were pulled down with Strep-Tactin, and precipitates were subsequently analyzed by immunoblotting with anti-FLAG antibody. Protein expression was confirmed by immunoblotting total cell lysates. The binding of DAPK2 to 14-3-3γ without MK2206 was taken as 100%. (C) FLAG-DAPK2 and FLAG-14-3-3σ were transfected into COS-7 cells. After treatment with MK2206 at the indicated concentrations, cells were harvested and extracts were subjected to immunoblot analysis for PARP. The amount of cleaved PARP without MK2206 was taken as 1. (D) FLAG-14-3-3γ was transfected in combination with Strep-DAPK2 and FLAG-CnA-α into COS-7 cells. Total cell lysates were pulled down with Strep-Tactin, followed immunoblotting with anti-FLAG antibody. To monitor the expression level of the Strep- and FLAG-tagged proteins, cell lysates were blotted with anti-Strep and anti-FLAG antibodies, respectively. "NS" indicates a nonspecific band. The binding of DAPK2 to 14-3-3γ without CnA-α was taken as 100%. All experiments were performed three times independently. Results are expressed as means ± S.E. Statistical significance was determined by Student's t-test. **, $p < 0.01$, *, $p < 0.05$.

indicate that Akt regulates the interaction between DAPK2 and 14-3-3 proteins via phosphorylation of $Thr^{369}$ in DAPK2. In addition, we examined the effect of Akt on the DAPK2-induced apoptosis. DAPK2-induced PARP cleavage was suppressed by 14-3-3σ (Fig. 3B). Treatment with MK2206 prevented 14-3-3σ from blocking DAPK2-induced PARP cleavage in a dose-dependent manner (Fig. 4C).

Binding of 14-3-3 proteins is dependent on the phosphorylation state of the binding protein, which is controlled by the balance of protein kinases and protein phosphatases. DAPK2, a $Ca^{2+}$/CaM-dependent serine/threonine kinase, induces apoptosis. In addition, calcineurin, which is a $Ca^{2+}$/CaM-regulated protein phosphatase, induces apoptosis through dephosphorylation of phosphoproteins such as BAD [11]. Finally, we examined the involvement of calcineurin in the regulation of the interaction between DAPK2 and 14-3-3 proteins. As shown in Fig. 4D, overexpression of the phosphatase subunit of calcineurin (calcineurin A-α, CnA-α) significantly suppressed the binding of DAPK2 to 14-3-3γ.

## 4. Discussion

Although DAPK2 is involved in promoting apoptosis, the detailed signaling mechanisms of this process remain unclear. Using a proteomic approach, we identified 14-3-3 proteins as novel DAPK2-binding proteins. During the course of this study, Gilad et al. also demonstrated a novel protein–protein interaction between DAPK2 and 14-3-3 proteins using another method [17]. 14-3-3 proteins, by interacting with phosphoserine or phosphothreonine-containing motifs, bind to and regulate the key proteins involved in various physiological processes including apoptosis [8]. Interaction with 14-3-3 proteins may alter the subcellular localization, complex formation, or enzymatic activity of target proteins. For example, 14-3-3 proteins bind to forkhead box O (FoxO) transcription factor phosphorylated by Akt, and prevent its localization in the nucleus [18]. In another case, 14-3-3 proteins inhibit apoptosis signal regulating kinase-1 (ASK1) activity by binding to phosphorylated $Ser^{967}$, blocking ASK1-mediated apoptosis [19]. We found that DAPK2-induced apoptosis was antagonized by 14-3-3 proteins. In addition, our *in vitro* kinase assay revealed that 14-3-3 proteins suppressed DAPK2 kinase activity. These results suggest that 14-3-3 proteins induce a conformation change of DAPK2 or mask its catalytic domain, resulting in the inhibition of DAPK2 activity. On the other hand, we also demonstrated the interaction of 14-3-3 proteins with DAPK2 by binding to phosphorylated $Thr^{369}$ at the C terminus of DAPK2. The last 40-amino acid C-terminal region of DAPK2 shows no homology to other DAPK family members and mediates its homodimerization [20]. Although the proapoptotic function of DAPK2 is regulated at multiple steps including transcription, phosphorylation, and CaM-binding, it can also be affected by dimerization [21,22]. This suggests that another mechanism is involved in the inactivation of DAPK2 by 14-3-3 binding; 14-3-3 proteins may affect DAPK2 dimerization by binding to its C-terminal region. Furthermore, it is intriguing that 14-3-3σ strikingly inhibited DAPK2 activity compared with the other isoforms. The 14-3-3σ is structurally divergent in dimerization and substrate-binding [23]. It would be interesting to identify a unique peptide sequence in 14-3-3σ responsible for this profound inhibition of DAPK2 in the feature study.

Akt, a major survival kinase, phosphorylates and inactivates proapoptotic proteins, preventing cell death. It has been established that Akt preferentially phosphorylates Ser or Thr in the RXRXX(S/T) motif, which is contained within consensus 14-3-3 binding motifs: RSXpSXP (mode 1) and RX(Y/F)XpSXP (mode 2). A number of 14-3-3-binding sites, in proteins such as BAD, FoxO, and Beclin 1, are created by Akt phosphorylation [10,18,24]. We demonstrated that the 14-3-3-binding site (phosphorylated $Thr^{369}$) of DAPK2 was also produced by Akt. The requirement for Akt phosphorylation was confirmed using an Akt-specific inhibitor in an interaction experiment between DAPK2 and 14-3-3; however, the binding of DAPK2 to 14-3-3 was not entirely blocked. Because the 14-3-3-binding site of DAPK2 ($^{364}$RRRSSpTS) also contains a putative phosphorylation sequence for basophilic kinases, such as p90 ribosomal S6 kinase (consensus motif: RXRXXpS/pT) and protein kinase A and G (consensus motif: RRXpS/pT) [25,26], protein kinases other than Akt may be also involved in DAPK2 phosphorylation.

In summary, we identified novel DAPK2-binding proteins implicated in the regulation of apoptosis. We revealed that DAPK2-induced apoptosis was negatively regulated by the combination of phosphorylation by Akt and interaction with 14-3-3 proteins. DAPK1, a member of the DAPK family, also interacts with 14-3-3 proteins; however, the detailed analysis of this interaction has not yet been performed, and amino acid sequence analysis indicates that DAPK1 has neither the mode 3 motif at the C terminus nor any other consensus motif [27]. Further research is required to unravel detailed DAPK2 signaling mechanisms.

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## Transparency document

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## References

[1] D. Gozuacik, A. Kimchi, DAPk protein family and cancer, Autophagy 2 (2) (2006) 74–79.
[2] R. Shiloh, S. Bialik, A. Kimchi, The DAPK family: a structure-function analysis, Apoptosis 19 (2) (2014) 286–297.
[3] Y. Lin, T.R. Hupp, C. Stevens, Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death, FEBS J. 277 (1) (2010) 48–57.
[4] Y. Jin, E.K. Blue, S. Dixon, et al., A death-associated protein kinase (DAPK)-interacting protein, DIP-1, is an E3 ubiquitin ligase that promotes tumor necrosis factor-induced apoptosis and regulates the cellular levels of DAPK, J. Biol. Chem. 277 (49) (2002) 46980–46986.
[5] F. Llambi, F.C. Lourenço, D. Gozuacik, et al., The dependence receptor UNC5H2 mediates apoptosis through DAP-kinase, EMBO J. 24 (6) (2005) 1192–1201.
[6] G. Page, I. Lödige, D. Kögel, et al., AATF, a novel transcription factor that interacts with Dlk/ZIP kinase and interferes with apoptosis, FEBS Lett. 462 (1–2) (1999) 187–191.
[7] T. Obsil, V. Obsilova, Structural basis of 14-3-3 protein functions, Semin. Cell. Dev. Biol. 22 (7) (2011) 663–672.
[8] A.K. Gardino, M.B. Yaffe, 14-3-3 proteins as signaling integration points for cell cycle control and apoptosis, Semin. Cell. Dev. Biol. 22 (7) (2011) 688–695.
[9] J. Zha, H. Harada, E. Yang, et al., Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-$X_L$, Cell 87 (4) (1996) 619–628.
[10] L. del Peso, M. González-García, C. Page, et al., Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt, Science 278 (5338) (1997) 687–689.
[11] H.G. Wang, N. Pathan, I.M. Ethell, et al., $Ca^{2+}$-induced apoptosis through calcineurin dephosphorylation of BAD, Science 284 (5412) (1999) 339–343.
[12] S. Matsuda, K. Kominato, S. Koide-Yoshida, et al., PCTAIRE kinase 3/cyclin-dependent kinase 18 is activated through association with cyclin A and/or phosphorylation by protein kinase A, J. Biol. Chem. 289 (26) (2014) 18387–18400.
[13] K. Isshiki, S. Matsuda, A. Tsuji, et al., cGMP-dependent protein kinase I promotes cell apoptosis through hyperactivation of death-associated protein kinase 2, Biochem. Biophys. Res. Commun. 422 (2) (2012) 280–284.
[14] M. Tewari, L.T. Quan, K. O'Rourke, et al., Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, Cell 81 (5) (1995) 801–809.
[15] T.F. Franke, PI3K/Akt: getting it right matters, Oncogene 27 (50) (2008) 6473–6488.
[16] D.R. Alessi, M. Andjelkovic, B. Caudwell, et al., Mechanism of activation of protein kinase B by insulin and IGF-1, EMBO J. 15 (23) (1996) 6541–6551.
[17] Y. Gilad, R. Shiloh, Y. Ber, et al., Discovering protein-protein interactions within the programmed cell death network using a protein-fragment complementation screen, Cell. Rep. 8 (3) (2014) 909–921.
[18] H. Huang, D.J. Tindall, Dynamic FoxO transcription factors, J. Cell. Sci. 120 (15) (2007) 2479–2487.
[19] E.H. Goldman, L. Chen, H. Fu, Activation of apoptosis signal-regulating kinase 1 by reactive oxygen species through dephosphorylation at serine 967 and 14-3-3 dissociation, J. Biol. Chem. 279 (11) (2004) 10442–10449.
[20] B. Inbal, G. Shani, O. Cohen, et al., Death-associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis, Mol. Cell. Biol. 20 (3) (2000) 1044–1054.
[21] G. Shani, S. Henis-Korenblit, G. Jona, et al., Autophosphorylation restrains the apoptotic activity of DRP-1 kinase by controlling dimerization and calmodulin binding, EMBO J. 20 (5) (2001) 1099–1113.
[22] A.K. Patel, R.P. Yadav, V. Majava, et al., Structure of dimeric autoinhibited conformation of DAPK2, a pro apoptotic protein kinase, J. Mol. Biol. 409 (3) (2011) 369–383.
[23] E.W. Wilker, R.A. Grant, S.C. Artim, et al., A structural basis for 14-3-3sigma functional specificity, J. Biol. Chem. 280 (19) (2005) 18891–18898.
[24] R.C. Wang, Y. Wei, Z. An, et al., Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation, Science 338 (6109) (2012) 956–959.
[25] A. Carriere, H. Ray, J. Blenis, et al., The RSK factors of activating the Ras/MAPK signaling cascade, Front. Biosci. 13 (2008) 4258–4275.
[26] L.R. Pearce, D. Komander, D.R. Alessi, The nuts and bolts of AGC protein kinases, Nat. Rev. Mol. Cell. Biol. 11 (1) (2010) 9–22.
[27] D.C. Henshall, T. Araki, C.K. Schindler, et al., Expression of death-associated protein kinase and recruitment to the tumor necrosis factor signaling pathway following brief seizures, J. Neurochem. 86 (5) (2003) 1260–1270.