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DAPK1 modulates a curcumin-induced G2/M arrest and apoptosis by regulating STAT3, NF-κB, and caspase-3 activation

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ABSTRACT

Curcumin, an active polyphenol extracted from the perennial herb *Curcuma longa*, controls various molecules involved in tumor cell death. In this study, we found that the tumor suppressor death-associated protein kinase 1 (DAPK1) plays a vital role in the anti-carcinogenic effects of curcumin. We found that curcumin increased DAPK1 expression at the mRNA and protein levels in U251 cells, and that the siRNA-mediated knockdown of DAPK1 attenuated the curcumin-induced inhibition of STAT3 and NF-κB. Moreover, DAPK1 suppression diminished curcumin-induced caspase-3 activation. In addition, we confirmed that DAPK1 was required for a curcumin-induced G2/M cell cycle arrest and apoptosis. Thus, DAPK1 is involved in curcumin-mediated death pathways. Our data suggest novel mechanisms for curcumin in cancer therapy.

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1. Introduction

Interest in using dietary phytochemicals to treat human cancer is on the rise. Curcumin, an active component of the perennial herb *Curcuma longa* (turmeric), is widely used as a spice in Asian cuisine. Recent studies have shown that curcumin possesses potent anti-inflammatory, antioxidant, chemopreventive, and chemotherapeutic activities, as well as a broad spectrum of tumor-suppressive activities, including in glioblastoma (GBM), lung cancer, and hepatocarcinoma [1]. Curcumin suppresses cell proliferation and metastasis, and induces tumor apoptosis [2]. These effects are mediated through various transcription factors, growth factors, cytokines, protein kinases, and other bioactive molecules [3].

Regulation of the cell cycle and apoptosis contributes to important carcinogenic mechanisms. A cell cycle arrest and apoptosis may occur in response to a wide variety of physiological and pathological stimuli and conditions. Curcumin inhibits cancer cell proliferation by inducing an arrest at different stages of the cell cycle or by inducing apoptosis. Extensive research has revealed that multiple signaling pathways, including caspase activation pathways, tumor suppressor pathways, death receptor pathways, mitochondrial pathways, and protein kinase pathways, underlie the therapeutic potential of curcumin [1,3].

Death-associated protein kinase 1 (DAPK1), a newly identified calcium/calmodulin (Ca^{2+}/CaM)-dependent serine/threonine kinase, has been recognized as a multi-domain protein. In addition to its kinase domain and CaM regulatory domain, DAPK1 contains a C-terminal death domain, which acts as a protein interaction domain in cell death, survival, and proliferation [4]. The knockdown of DAPK1 by RNA interference or knockout of *DAPK1* through gene targeting attenuates cytokine-induced cell death, suggesting a death-promoting role for DAPK1 [4,5]. Moreover, the forced expression of DAPK1 is sufficient to induce apoptosis in several cell lines, providing additional evidence for its death-inducing function [6,7].

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that integrates signals from extracellular stimuli and regulates genes involved in many key cellular processes. It plays important roles in cell growth and apoptosis, and is constitutively activated in a variety of tumor cells, including glioma cells [8]. NF-κB is another pivotal anti-apoptotic transcription factor that acts as a key mediator of carcinogenesis [9]. Its constitutive activation is a hallmark of several types of tumors, whereas its inhibition is required for the induction of apoptosis [10]. Additionally, caspase-3, an effector caspase, is responsible for apoptosis [11]. Thus, the suppression of STAT3 and NF-κB, or activation of caspase-3, offers a promising therapeutic approach for medical intervention.

Abbreviations: Cur, curcumin; DAPK1, death-associated protein kinase 1; GBM, glioblastoma; siRNA, small interfering RNA.

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Studies targeting curcumin have revealed its inhibitory role in both STAT3 and NF-κB activation, as well as its positive effect on caspase-3 activity [1,2]; however, the underlying mechanisms are not fully understood. In the present study, we demonstrate for the first time that DAPK1 mediates a curcumin-induced G2/M cell cycle arrest and apoptosis by modulating STAT3 and NF-κB signaling and caspase-3 activation in GBM U251 cells, indicating that DAPK1 is a potential target in the treatment of tumors.

2. Materials and methods

2.1. Chemicals, reagents, and cell culture

Curcumin, DMSO, an Annexin V-FITC Apoptosis Detection Kit, and mouse monoclonal IgG antibodies against β-actin were purchased from Sigma (St. Louis, MO). Curcumin diluted in DMSO was prepared as a stock solution of 50 mM and stored at −20 °C until use. Human DAPK1 small interfering RNA (siRNA) and scrambled control siRNA were obtained from GenePharm (Shanghai, China). Double-stranded gel shift oligonucleotides were obtained from Viagene Biotech (Beijing, China). The human neuroblastoma cell line U251 was obtained from the Chinese Academy of Medical Sciences (Beijing, China). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Valencia, CA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. The cells were passaged twice weekly and used for experiments when in the exponential growth phase.

2.2. Cell transfection

U251 cells were cultured in six-well plates (2×10^5 cells per well) for 24 h and then transfected with DAPK1 siRNA (30 nM) or control siRNA (30 nM) using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The specific target sequences of the DAPK1 siRNA oligonucleotides were: CAAGAAACGTTAGCAAATG (si-DAPK1-1) and GGTCAAGGATCCAAAGAAG (si-DAPK1-2), respectively. The sequence of the control siRNA for human DAPK1 was CACCAGAACCATGGCCAAC.

2.3. RNA preparation and real-time RT-PCR

Total RNA was extracted using an E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA), and was reverse-transcribed using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). For validation, real-time RT-PCR was performed using a SYBR Premix Ex Taq Kit (Takara) and an ABI Vii7 detection system (Applied Biosystems, Foster City, CA). The reaction conditions were: 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s (40 cycles). The nucleotide sequences of the primers used for amplification were: DAPK1 (forward, 5′-CGAGGTGATGGTGTATGGTG-3′; reverse, 5′-CTGTGCTTTG CTGGTGGA-3′) [12] and β-actin (forward, 5′-AGCGCGGCTACAGCT TCA-3′; reverse, 5′-GGCCATCTCTTGCTCGAAGT-3′).

2.4. Western Blot analyses

Western Blotting was performed as described previously [13]. Briefly, whole-cell extracts were prepared using ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas, Burlington, Canada) supplemented with protease and phosphatase inhibitors (Fermentas) according to the manufacturer's instructions. Protein (20–40 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 8–10% gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The blots were blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma) in Tris-buffered saline/0.1% Tween-20. Next, the blots were probed with anti-DAPK1, anti-STAT3, anti-phospho-STAT3 (pY705), anti-NF-κB, anti-phospho-NF-κB (pS536) (all from Epitomics, Burlingame, CA), anti-caspase-3 (Cell Signaling Technology Inc., Danvers, MA), or β-actin antibodies overnight at 4 °C. The blots were then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000 dilution) for 1 h at room temperature. After additional washes, signals were detected using SuperSignal ECL (Pierce, Rockford, IL).

2.5. Nuclear extract preparation and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared using a ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) according to the manufacturer's instructions. EMSAs for STAT3 and NF-κB were performed using a non-radioactive gel shift assay system (Pierce). Briefly, nuclear extracts were incubated with biotin-labeled STAT3 and NF-κB oligonucleotides (STAT3, 5′-GATCCTTCTGGGAATTCCTAGATC-3′; NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′), electrophoresed on 6% native polyacrylamide gels, transferred to positive nylon membranes, UV-crosslinked, and probed with a streptavidin-HRP conjugate. The gels were exposed to X-ray film, which was developed and digitized using a ChemiDoc XRS+ with Image Lab software (Bio-Rad, Hercules, CA).

2.6. Cell cycle analyses

Cell cycle distribution was analyzed by flow cytometry. Cells were plated at $1.5–2 \times 10^5$ cells per well in six-well plates, transfected for 48 h with siRNA, and then treated with 40 μM curcumin for another 24 h before harvesting. After permeabilization with ice-cold 70% ethanol overnight, the cells were washed with phosphate-buffered saline (PBS), resuspended in 0.5 ml of propidium iodide (PI)/RNase Staining Buffer reagent (BD, Franklin Lakes, NJ), and stained for 15 min at room temperature before analysis using an Accuri C6 flow cytometer system (BD).

2.7. Annexin V-FITC staining

Cells were plated at $1.5–2 \times 10^5$ cells per well in six-well plates, transfected for 48 h with siRNA, treated with 40 μM curcumin for another 24 h, and collected and stained with Annexin V-FITC as per the manufacturer's instructions (Sigma). Briefly, the cells were washed twice in cold PBS, resuspended in binding buffer at 5×10^5 cells/500 μl, stained with 5 μl of Annexin V and 10 μl of PI, and incubated for 15 min at room temperature in the dark before flow cytometric analysis.

2.8. Statistical analysis

All data are given as the mean ± standard deviation (SD). Statistical analyses were conducted using two-tailed paired student's *t*-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin upregulates DAPK1 expression in U251 cells

A dose-dependent rise in the *DAPK1* mRNA level was detected by real-time RT-PCR in response to different concentrations of curcumin (Fig. 1A, $P < 0.05$). Next, we confirmed the rise at the protein level by Western Blot analyses, which demonstrated a time-dependent (Fig. 1B) and dose-dependent effect (Fig. 1C). These findings show that curcumin increased DAPK1 expression at both the mRNA and protein levels in U251cells.

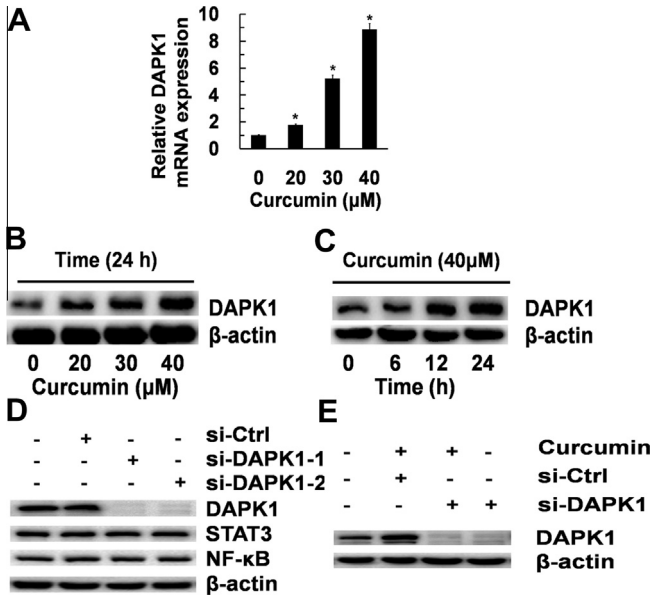

Fig. 1. Effects of curcumin on DAPK1 expression and the knockdown of DAPK1 in U251 cells. (A) Real-time RT-PCR analysis of DAPK1 mRNA expression in U251 cells. Cells were treated with various concentrations of curcumin for 24 h. Total RNA was prepared and quantified using specific primers. (B,C) Western Blot (WB) analyses of the DAPK1 protein levels in curcumin-treated U251 cells. Cells were treated with the indicated concentration of curcumin for 24 h (B) or treated with 40 μM curcumin for the indicated times (C). At the indicated time points, cells were harvested and whole-cell extracts were prepared and probed for DAPK1. (D,E) Cells were transfected with control siRNA or DAPK1-specific siRNA. Seventy-two hours after transfection, whole-cell extracts were prepared and subjected to WB analyses to determine the DAPK1 knockdown efficiency. Probing for STAT3 and NF-κB revealed that the knockdown of DAPK1 did not affect the expression of STAT3 or NF-κB (D). (E) Cells were transfected as described in panel D. After 48 h, cells were treated with curcumin (40 μM) for 24 h and WB analyses were performed. β-Actin was used as a loading control. All experiments were repeated at least three times with similar results. The values are the mean ± SD. $^{*}P < 0.05$ based on paired *t*-tests.

3.2. The knockdown of DAPK1 attenuated STAT3 and NF-κB dephosphorylation

Curcumin dephosphorylates STAT3 and NF-κB and upregulates DAPK1 expression. To determine whether DAPK1 upregulation is involved in the curcumin-induced inhibition of STAT3 and NF-κB phosphorylation, we suppressed DAPK1 expression using siRNA transfection. We successfully suppressed DAPK1 expression using both si-DAPK1-1 and si-DAPK1-2 in U251 cells, in contrast to the nonspecific control siRNA (Fig. 1D). We verified that the knockdown of DAPK1 did not alter STAT3 or NF-κB expression (Fig. 1D). In addition, we found that curcumin failed to elevate DAPK1 expression in DAPK1 siRNA-transfected cells (Fig. 1E).

Next, we examined the phosphorylation levels of the transcription factors. As shown in Fig. 2, curcumin suppressed both STAT3 and NF-κB phosphorylation, whereas the knockdown of DAPK1 resulted in an attenuated response. We then explored whether si-DAPK1 transfection could regulate different time courses of curcumin-induced dephosphorylation. Western Blot analyses demonstrated that the curcumin-mediated inhibition of STAT3 phosphorylation at different time points was rescued by si-DAPK1 transfection. Interestingly, although NF-κB phosphorylation was intensively inhibited by curcumin, si-DAPK1 rescued that phosphorylation to a comparative level (Fig. 2).

3.3. The knockdown of DAPK1 rescued the DNA-binding abilities of STAT3 and NF-κB

Because activated transcription factors such as STAT3 and NF-κB harbor DNA-binding ability, we evaluated the effect of DAPK1

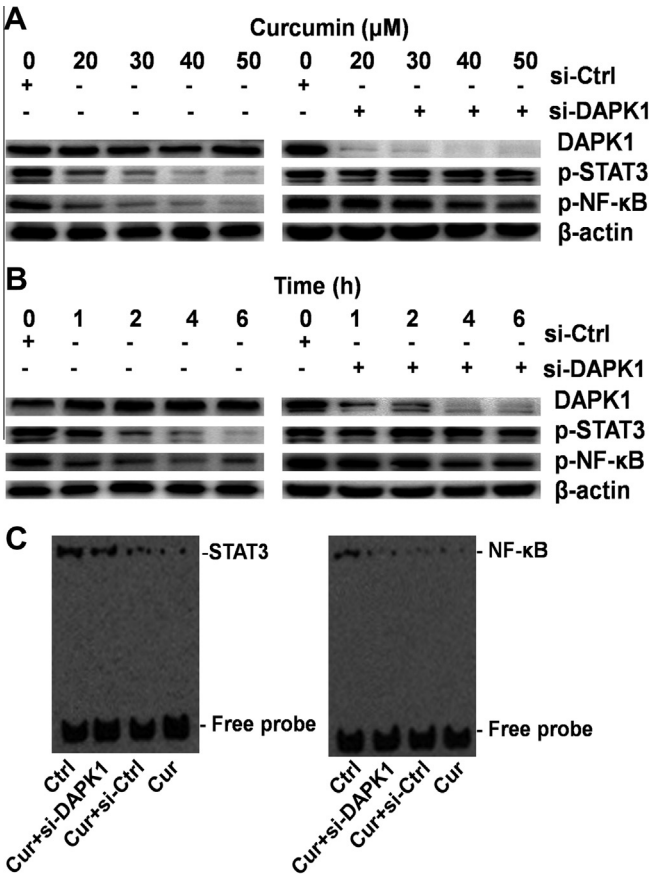

Fig. 2. Attenuated STAT3 and NF-κB inhibition in DAPK1-suppressed U251 cells. Cells were transfected with control siRNA or DAPK1-specific siRNA as described in Section 2. (A) Effect of DAPK1 suppression upon treatment with different doses of curcumin on STAT3 and NF-κB dephosphorylation. Three days after transfection, cells were treated with the indicated concentrations of curcumin for 4 h, after which whole-cell extracts were prepared and probed for the designated proteins. (B) Effects of DAPK1 suppression at different time points on curcumin-induced STAT3 and NF-κB dephosphorylation. Three days after transfection, cells were treated with curcumin (40 μM) for the indicated times, after which whole-cell extracts were prepared and probed for the designated proteins. β-Actin was used as a loading control. (C) At 72 h after transfection, transfected and untransfected cells were treated with curcumin (40 μM) for 4 h, after which nuclear extracts were prepared. The STAT3/DNA- and NF-κB/DNA-binding abilities were evaluated using EMSAs. The results shown are representative of three independent experiments.

on the curcumin-induced inhibition of STAT3 and NF-κB using EMSAs. STAT3 and NF-κB DNA-binding activity was blocked by exposure to 40 μM curcumin for 4 h (Fig. 2C). However, although the DNA-binding ability was not fully rescued, si-DAPK1-treated cells showed alleviation of that repression (Fig. 2C). These results indicate that DAPK1 enhanced the curcumin-induced inhibition of STAT3 and NF-κB DNA binding.

3.4. The knockdown of DAPK1 inhibited caspase-3 activation

Curcumin activates caspase-3, which plays a pivotal role in STAT3- and NF-κB-related apoptotic pathways. Therefore, we next examined the impact of knocking down DAPK1 on curcumin-induced caspase-3 activation. As shown in Fig. 3, curcumin increased caspase-3 cleavage in U251 cells. Caspase-3 cleavage, characterized by the appearance of 17- and 19-kDa protein bands, was decreased in DAPK1-knockdown cells compared to control siRNA-transfected cells (Fig. 3, $P < 0.05$). These results suggest that DAPK1 plays a positive role in caspase-3 activation.

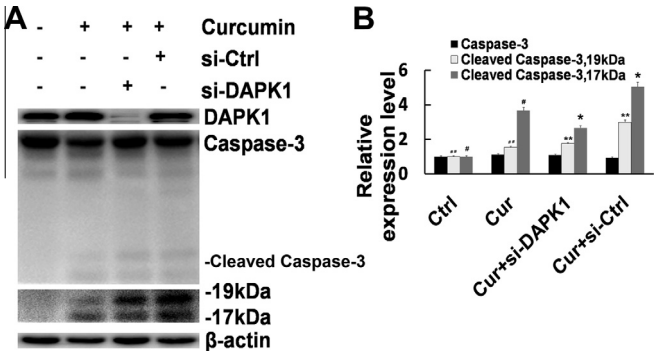

Fig. 3. The suppression of DAPK1 inhibits curcumin-induced caspase-3 activation. U251 cells were transfected with control siRNA or DAPK1-specific siRNA as described in Section 2. At 48 h after transfection, cells were treated with curcumin (40 μM) for 24 h. Whole-cell extracts were prepared and probed for caspase-3 (A). The cleaved bands (17 and 19 kDa) are highlighted with enhanced contrast (A). The relative protein levels were normalized to β-actin (B), which was used as a loading control. The results represent three independent experiments with similar results. The values are the mean ± SD. $^{*,\#}P < 0.05$ based on paired *t*-tests.

3.5. The knockdown of DAPK1 rescued the curcumin-induced cell cycle arrest and apoptosis

To determine whether DAPK1 depletion could alleviate the curcumin-induced cell cycle arrest, we first confirmed that curcumin induces a G2/M, but not G1, cell cycle arrest in U251 cells (Fig. 4A). Next, we treated DAPK1 siRNA-transfected cells with curcumin and found that the percentage of cells in G2/M phase decreased from 55.2% to 32.9%, and that the percentage of cells in G1 phase increased from 31.3% to 52.3% (Fig. 4A, $P < 0.05$). The control siRNA-transfected cells did not show any alteration in cell cycle distribution.

We also found that the knockdown of DAPK1 rescued cells from curcumin-induced apoptosis. The apoptotic cell number decreased from 58.3% to 33.0% (si-Ctrl vs. si-DAPK1) (Fig. 4B, $P < 0.05$). These findings provide additional evidence that DAPK1 is involved in the regulation of a curcumin-induced G2/M arrest and apoptosis in U251 cells.

4. Discussion

In this study, we identified a novel mechanism for DAPK1 in curcumin-induced cell cycle arrest and apoptosis in human GBM U251 cells. In agreement with previous studies, our results show that curcumin arrested cells in G2/M phase of the cell cycle and induced apoptosis extensively after 24 h of treatment. Curcumin treatment upregulated DAPK1 expression in a dose- and time-dependent manner. DAPK1 depletion using DAPK1-specific siRNA transfection attenuated the curcumin-induced cell cycle arrest and apoptosis. Furthermore, we investigated the associated molecular mechanisms and concluded that the knockdown of DAPK1 rescued the curcumin-induced inhibition of two oncogenic

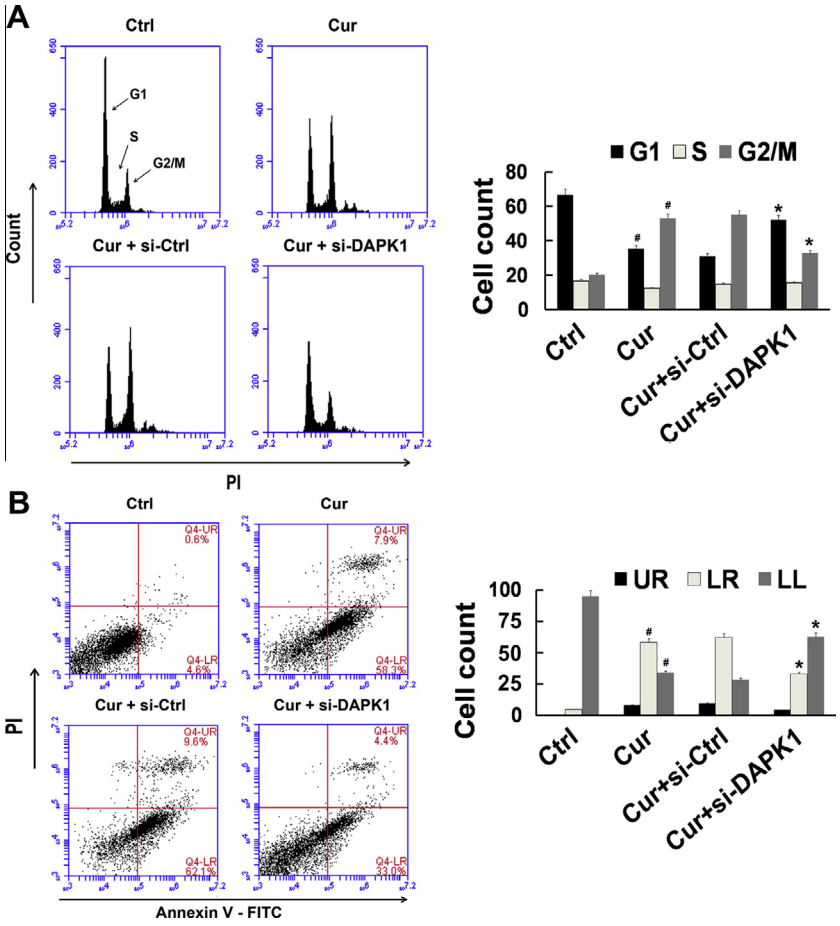

Fig. 4. DAPK1 is required for a curcumin-induced G2/M arrest and apoptosis. Cells were treated as described in Section 2. After treatment, cells were harvested and subjected to flow cytometric analyses to assess the cell cycle distribution (A) and apoptosis (B). At least 10,000 cells were analyzed per sample. UR, upper right; UL, lower left; LL, lower left quadrant. All experiments were repeated at least three times with similar results. The values are the mean ± SD. $^{*,\#}P < 0.05$ based on paired *t*-tests.

transcription factors, STAT3 and NF-κB, and diminished caspase-3 activation.

DAPK1 is a well-known tumor suppressor gene. Recent studies have shown that DAPK1 functions as a positive mediator of apoptosis induced by several stimuli [3,5]. Reports have also shown that DAPK1 is downregulated in many human cancers, and in our study curcumin raised DAPK1 expression at both the mRNA and protein levels. Promoter hypermethylation results in the weakened expression and diminished function of DAPK1 in a wide range of cancer cells [14]. A cohort of publications has shown that curcumin modulates gene expression through its activity as an epigenetic agent [15]. However, DAPK1 promoter hypermethylation was not observed in U251 cells [16]. Consequently, curcumin may not induce DAPK1 expression through epigenetic modification. Investigations on another pivotal tumor suppressor gene, *p53*, which is also activated by curcumin, have shown that p53 regulates DAPK1 expression [3,17]. Nevertheless, the molecular mechanism whereby curcumin regulates DAPK1 requires further investigation.

Consistent with previous reports, our results show that STAT3 was constitutively activated in U251 cells, and that curcumin inhibited this activation. By evaluating STAT3 phosphorylation at tyrosine 705 and its DNA-binding ability, we found that curcumin suppressed STAT3 activation. This sup ression, however, could be attenuated by the knockdown of DAPK1, indicating a critical role for DAPK1 in curcumin-induced STAT3 suppression. However, in ovarian and endometrial cancer cells, curcumin suppresses the constitutive activation of STAT3 by upregulating protein inhibitor of activated STAT3, thereby attenuating STAT3 phosphorylation and tumor cell growth [13]. Moreover, both inducible and constitutive STAT3 activation can be blocked through the activation of tyrosine-protein phosphatase non-receptor type 6 and suppressors of cytokine signaling proteins (e.g., SOCS1 and SOCS3) [18,19]. Our study highlights an attractive aspect of curcumin in STAT3 inhibition, and suggests that DAPK1 is intimately connected with the regulation of STAT3 activity.

Similarly, we found constitutive activation of NF-κB in U251 cells, and that curcumin suppressed this activation. We found that phosphorylation of the p65 subunit of NF-κB and the DNA-binding activity of NF-κB were blocked upon curcumin exposure. Yoo et al. [5] reported that TNF-α- or INF-γ-dependent NF-κB activity was enhanced by DAPK1 inhibition. NF-κB activity was dependent on DAPK1 levels, indicating that DAPK1 is required for NF-κB activation. However, Chuang et al. [20] also examined the effects of DAPK1 on TNF-α-triggered NF-κB activation. The expression of DAPK1[K42A] failed to affect the nuclear entry of p65 induced by TNF-α. Therefore, they concluded that DAPK1 did not modulate TNF-α-mediated NF-κB activation, and that DAPK1 specifically regulated TCR-induced NF-κB activation. In our study, the knockdown of DAPK1 rescued the inhibitory effect of curcumin on NF-κB phosphorylation. Furthermore, we found that DAPK1 suppression hampered the inhibitory effect of curcumin on NF-κB DNA-binding activity. These data provide convincing evidence that DAPK1 plays an essential role in regulating NF-κB activity.

Additional data demonstrated that curcumin also activates caspase-3 in U251 cells, and that the knockdown of DAPK1 reduced this activation. Caspase activation is a hallmark of apoptosis. The inhibition of caspase-3 activation induced by the knockdown of DAPK1 provides further evidence of DAPK1 tumor-suppressive activity. Because caspase-3 can be regulated through both STAT3 [11] and NF-κB [21], one possible mechanism for the regulation of caspase-3 activity by DAPK1 involves the regulation of these transcription factors.

Our results indicate that DAPK1 suppression retards the curcumin-induced deactivation of STAT3 and NF-κB, as well as the activation of caspase-3, a cell cycle arrest, and apoptosis. Several reports have illustrated that DAPK1 regulates caspase-dependent and -independent cell death signals [22]. Of note, cotransfection with DAPK1 and programmed cell death 6 accelerates apoptosis via caspase-3-dependent pathways [23]. Moreover, DAPK1 activates DAPK3, another member of the DAPK family that physically interacts with STAT3, augmenting death-inducing signals [24,25]. Additionally, DAPK1 suppression enhances the entry of NF-κB-activating molecules into membrane rafts [20], and DAPK1 overexpression promotes a cell cycle arrest and apoptosis accompanied by reduced NF-κB activity [5]. Thus, we can infer that DAPK1 modulates the curcumin-induced G2/M arrest and apoptosis via targeting of STAT3, NF-κB, and caspase-3. However, additional studies on how DAPK1 suppression regulates STAT3, NF-κB, and caspase-3 activity should help to determine the exact participation of DAPK in cell-death pathways.

In summary, our study reveals a novel mechanism for the tumor suppressor DAPK1 in cancer treatment. Our findings demonstrate that DAPK1 mediates the anti-proliferative and pro-apoptotic effects of curcumin through the regulation of STAT3 and NF-κB signaling pathways and the inhibition of caspase-3 in U251 cells. The knockdown of DAPK1 via siRNA transfection attenuated the inhibitory effects of curcumin on these pathways, elucidating a novel mechanism for curcumin in cancer therapy. Further investigation is warranted to delineate the exact mechanisms underlying the regulation of DAPK1 by curcumin.

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