



# DAPK2 activates NF- $\kappa$ B through autophagy-dependent degradation of I- $\kappa$ B $\alpha$ during thyroid cancer development and progression

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**Background:** Death-associated protein kinase 2 (DAPK2) is a serine/threonine kinase, which has been implicated in autophagy and apoptosis. DAPK2 functions as a tumor suppressor in various cancers. However, the role of DAPK2 in thyroid cancer (TC) is unclear.

**Methods:** RNA sequencing of human TC samples was performed to identify differentially expressed genes that may play a role in TC development. The messenger RNA (mRNA) expression of DAPK2 was verified by quantitative real-time polymerase chain reaction (qRT-PCR). To investigate the role of DAPK2 in TC development, DAPK2 was knocked down and overexpressed in a TTA1 cell line. The effect of DAPK2 on cell proliferation, sensitization of TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and tumor growth was examined. The effect of DAPK2 on autophagy and NF- $\kappa$ B activation was investigated to address the underlying mechanism.

**Results:** DAPK2 was upregulated in TC. Knockdown of DAPK2 in TTA1 cells led to reduced cell proliferation, sensitization of TRAIL-induced apoptosis, and restricted tumor growth both *in vitro* and *in vivo*, while overexpression of DAPK2 exhibited the opposite effect. Mechanistically, DAPK2 promoted autophagy as demonstrated by the accumulation of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II, which correlated with the level of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation. Knockdown of inhibitory- $\kappa$ B $\alpha$  (I- $\kappa$ B $\alpha$ ) in short hairpin (sh) DAPK2 TTA1 cells restored the activity of NF- $\kappa$ B, suggesting DAPK2 activated NF- $\kappa$ B through autophagy-mediated I- $\kappa$ B $\alpha$  degradation.

**Conclusions:** Our findings revealed a pivotal role of DAPK2 in thyroid carcinogenesis, being required for tumor growth and for resistance to TRAIL-induced apoptosis through autophagy-mediated I- $\kappa$ B $\alpha$  degradation. This result provides a novel target for the therapy of TC.

**Keywords:** Death-associated protein kinase 2 (DAPK2); autophagy; thyroid cancer (TC); nuclear factor- $\kappa$ B (NF- $\kappa$ B); I- $\kappa$ B $\alpha$

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## Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, and its incidence has risen worldwide over the past few decades (1). Although most thyroid carcinomas have well-differentiated papillary histology and good prognosis, patients with more aggressive tumor subtypes, such as anaplastic thyroid cancer (ATC), already show local and distant metastasis at the time of diagnosis, and are usually resistant to standard chemotherapy. Consequently, understanding the molecular mechanisms of these carcinomas is a key research imperative in TC therapeutics.

Autophagy, a process of self-digestion, is essential for the maintenance of cellular homeostasis (2). Autophagy has been shown to be involved in several steps of TC pathogenesis, including tumor development and progression, as well as resistance to chemo- and radiotherapy (3). Intervention of this process may therefore present a novel approach in the management of TC therapy.

Death-associated protein kinase 2 (DAPK2) belongs to a family of  $Ca^{2+}$ /calmodulin-regulated serine/threonine kinases, which regulate various cellular activities, including cell death, cytoskeletal dynamics, and immune functions. Recent studies suggest that DAPK2 is involved in several steps of autophagy. DAPK2 functions as a tumor suppressor in several cancers like hepatocellular carcinoma (HCC) and acute myeloid leukemia (AML). However, the role of DAPK2 in TC development has not yet been investigated.

Much progress has been made in understanding the molecular mechanisms of TC in recent years (4). Emerging evidence has shown that deregulated nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation is associated with thyroid tumor initiation and progression, especially in ATC in which constitutive NF- $\kappa$ B activity has been found (5-8). NF- $\kappa$ B promotes TC progression by controlling proliferative and antiapoptotic signaling pathways in TC cells (9,10).

Here, we show that DAPK2 is upregulated in human TC samples and plays a critical role in TC pathogenesis, being required for tumor growth and for the resistance to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, through selective autophagy mediated inhibitory- $\kappa$ B $\alpha$  (I- $\kappa$ B $\alpha$ ) degradation, which correlates with strong of NF- $\kappa$ B activity. This makes DAPK2 a target for the development of novel therapeutic strategies for TC.

We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-2062>).

## Methods

### Cell lines

Human anaplastic TC cell line TTA1 and human embryonic kidney cell line HEK293T were purchased from the American Type Culture Collection (ATCC) and grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (GIBCO, USA).

### RNA interference of DAPK2

The following oligos were used for the construction of short hairpin (sh) RNAs against DAPK2: shDAPK2-1: 5'-TGCTGTTGACAGTGAGCGACCGGAATTTGT TGCTCCAGAATAGTGAAGCCACAGATGTATTC TGGAGCAACAAATTCCGGCTGCCTACTGCCTC GGA-3', shDAPK2-2: 5'-TGCTGTTGACAGTGAGC GCCAGAGAGAGACCATATCCAAATAGTGAAGCC ACAGATGTATTTGGATATGGTCTCTCTCTGTT GCCTACTGCCTCGGA-3'. The oligos were amplified using the primer pair 5'-CAGAAGGCTCGAGAAGGT ATATTGCTGTTGACAGTGAGCG-3' and 5'-CTAAA GTAGCCCCTTGAATTCGAGGCAGTAGGCA-3' to introduce the miR-30 sequence, and XhoI and EcoRI restriction sites, and then were inserted into pMLP vector (Transomics, Shanghai, China). Recombinant MLP vector containing shDAPK2-1, shDAPK2-2, or non-targeting control combined with pCL-10A1 retrovirus packaging vector were transfected into HEK293T cells using Lipofectamine 2000 (Life Technologies, USA) according to manufacturer's instructions. After 48-hour incubation, lentiviruses were collected from culture supernatant. TTA1 cells were transfected with retroviruses in the presence of 10  $\mu$ g/mL of polybrene and selected by 1  $\mu$ g/mL of puromycin. The efficiency of RNA interference was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot.

### Ectopic expression of DAPK2

Human DAPK2 cDNA was cloned into the pCDH vector. PCDH-DAPK2 or vector control with pSPAX2 and pMD2.G lentivirus packaging vectors were transfected into HEK293T cells using Lipofectamine 2000 (Life

Technologies). After 48-hour incubation, lentiviruses were collected from culture supernatant. TTA1 cells were infected with the lentiviruses in the presence of 10 µg/mL of polybrene. The infected cells were screened by 1 µg/mL of puromycin. DAPK2 expression levels were assessed by both qRT-PCR and western blot.

#### *qRT-PCR analysis*

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). Complement DNA (cDNA) was prepared using Reverse Transcriptase kit (Takara, China). RNA levels were determined using SYBR Green Real-time PCR master mix (Takara) on a 7900 HT qRT-PCR machine (Applied Biosystems, USA). The following pair of primers of DAPK2 were used for amplification: 5'-AGGCGTCATCACCTACATCC-3' and 5'-GAGCCTCTTGGATTGTGAGC-3'; beta Actin (ACTB) gene was used as an internal control. The primers of ACTB were 5'-CACCAATTGGCAATGAGCGGTTTC-3' and 5'-AGGTCCTTGGCGGATGTCCACGT-3'. Relative messenger RNA (mRNA) expression levels were calculated as the ratio of DAPK2 to ACTB using the  $\Delta\Delta C_t$  method.

#### *Western blot analysis*

Cells were lysed with radio immunoprecipitation assay buffer (RIPA, Beyotime Biotech, China) supplemented with 1% protease inhibitor and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA). For detecting phosphorylated proteins, phosphatase inhibitors (1 mM NaF, 1 mM sodium vanadate, 25 mM  $\beta$ GPO4) were also added. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Tiangen, China). Equal amounts of protein were resolved onto SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) nonfat milk, incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies, and then detected with an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, USA). The following antibodies were used to probe the corresponding proteins: DAPK2 (Abcam, USA), microtubule-associated protein 1A/1B-light chain 3 (LC3) B (Cell Signaling Technology, USA),  $\beta$ -actin (CST, USA), and HRP-conjugated goat anti-rabbit immunoglobulin G (IgG; Cell Signaling Technology, USA).

#### *Cell proliferation assay*

TTA1 cells were seeded into 12-well plates at a concentration of  $1 \times 10^4$  cells/well. Cell numbers were recorded using a cell counter (Bio-Rad, USA) for 7 consecutive days. Growth curves of the cells were plotted as the number of cells versus days.

#### *Apoptosis assay*

TTA1 cells were seeded into 24-well plates ( $1 \times 10^5$  cells/well). Cells were treated with TRAIL (100 ng/mL) for 24 hours, harvested, washed with phosphate-buffered saline (PBS) and then stained with Annexin V and 1 µL of propidium iodide (PI; eBioscience, USA) according to the manufacturer's instructions. Cell apoptosis was detected and analyzed by Accuri C6 (BD Biosciences, USA).

#### *Soft agar colony-formation assay*

Subsequently,  $1 \times 10^3$  of TTA1 cells was resuspended in DMEM medium containing 10% FBS and 0.3% low-melting agarose (Sangon Biotech, China). Cells were plated onto 60-mm culture dishes with the solidified bottom layer in DMEM medium containing 10% FBS and 0.5% low-melting agarose. On day 14, the colonies were fixed with methanol, stained with 0.05% crystal violet, and imaged.

#### *Tumor growth in nude mice*

Six-week-old male nude mice were purchased from Shanghai Laboratory Animal Center (SLAC) Laboratory Animal Company (Shanghai, China). Mice were housed in a specific pathogen-free (SPF) room at  $24 \pm 1$  °C and with a relative humidity of  $55\% \pm 5\%$ . Control, shDAPK2, or DAPK2 OE TTA1 cells were subcutaneously injected ( $1 \times 10^6$ /mouse) into the back of nude mice (6 mice/group). Tumor growth was monitored for 6 weeks. Tumors were weighted and imaged after surgical removal. Animal experiments were performed under a project license (No. SH9H-2020-T346-1) granted by institutional ethics committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, in compliance with Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine institutional guidelines for the care and use of animals.

### ***Immunofluorescence staining and confocal laser microscopy***

The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 1% bovine serum albumin (BSA). The cells were then incubated with primary antibodies against LC3B (Abcam, USA) overnight at 4 °C. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibodies (Abcam, USA) for 0.5 hours, stained with DAPI for 2 minutes, and then observed by confocal fluorescence microscopy.

### ***NF- $\kappa$ B luciferase reporter assay***

TTA1 cells were transfected with firefly luciferase gene reporter plasmid encoding pNF- $\kappa$ B Luc using Lipofectamine 2000 (Life Technologies). Transfection efficiency was normalized by cotransfection with the Renilla luciferase plasmid. The cells were lysed 48 hours after transfection, and luciferase activity was measured following the manufacturer's instructions (Promega, USA). NF- $\kappa$ B transcriptional activity was analyzed as the ratio of firefly luciferase activity to Renilla luciferase activity.

### ***Human TC specimens***

Three pairs of TC and adjacent noncancerous tissue samples of TC patients were obtained from the Shanghai Ninth People's Hospital for RNA sequencing (RNA-seq) and qRT-PCR analysis. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine institutional ethics committee (No. SH9H-2020-T346-1) and informed consent was taken from all the patients.

### ***Statistical analysis***

Data are presented as mean  $\pm$  standard deviation (SD). Statistical differences were determined by two-tailed Student's *t*-test using GraphPad Prism 5.0 software. A *P* value  $<0.05$  was considered statistically significant.

## **Results**

### ***DAPK2 was upregulated in TC***

To identify the novel functional genes involved in TC, we

performed RNA-seq using an Illumina HiSeq Sequencer to select differentially expressed genes (*Figure 1A*). Among the upregulated genes, DAPK2 was picked due to its role in autophagy. We validated the DAPK2 mRNA expression level using qRT-PCR (*Figure 1B*). The relationship between DAPK2 and TC has not been previously reported. Therefore, we sought to investigate the role and the underlying mechanism of DAPK2 in TC pathogenesis.

### ***DAPK2 silencing inhibited the proliferation and tumorigenicity of TTA1 cells***

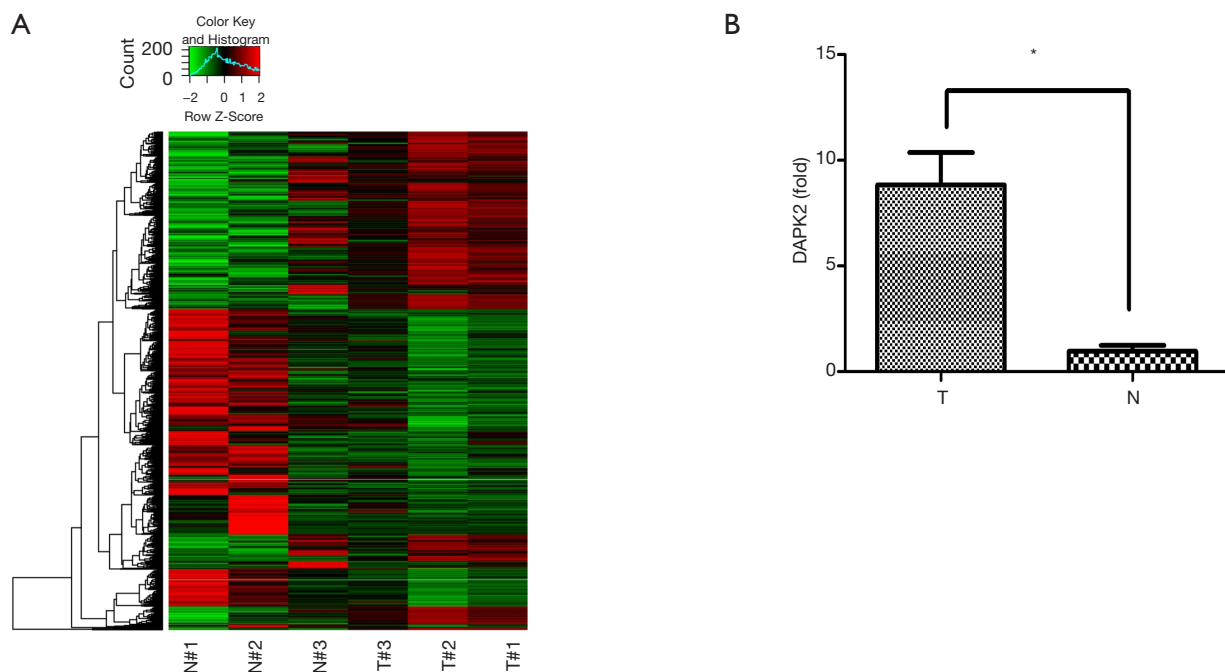
To explore the role of DAPK2 in TC, we used shRNA to silence DAPK2 expression in TTA1 cells. The shRNAs effectively reduced the expression of DAPK2 in TTA1 cells compared to the non-targeting control, as indicated by western blot and qRT-PCR results (*Figure 2A,B*).

We first examined whether DAPK2 is involved in thyroid tumor growth control. An equal number of non-targeting control TTA1 cells and shDAPK2 cells were cultured for 7 days. As shown in *Figure 2C*, silencing of DAPK2 decreased the proliferation of TTA1 cells. It has been long held that carcinogenesis occurs as a result of the imbalance between proapoptotic or antiapoptotic signaling. TRAIL, a member of the TNF family has been shown to trigger apoptosis and selectively kill TC cells without damaging normal cells (11). However, some cancers, including TC, have developed resistance to TRAIL, limiting its anticancer potential. We thus investigated the effect of DAPK2 knockdown on sensitivity to TRAIL-induced cell death. Cells were challenged with TRAIL, and cell apoptosis were measured with flow cytometer, silencing DAPK rendered TTA1 cells more susceptible to the killing effect of TRAIL (*Figure 2D*). Next, we examined whether DAPK2 plays a role in anchorage-independent growth of TTA1 cells. As shown in *Figure 2E*, silencing of DAPK2 abolished TTA1 cell colony formation ability in a semisolid medium. To confirm these observations in vivo, an equal number of non-targeting control TTA1 and shDAPK2 TTA1 cells were implanted s.c. into nude mice. As shown in *Figure 2F*, the weight and size of tumors developed from shDAPK2 TTA1 cells were significantly decreased. These results suggest that DAPK2 plays a critical role in the tumorigenicity of TTA1 cells.

### ***Ectopic expression of DAPK2 promoted proliferation and tumorigenicity of TTA1 cells***

As knockdown of DAPK2 suppressed the proliferation





**Figure 1** DAPK2 was upregulated in TC. (A) Heat map of clustering of genes with  $\log_2$  fold change  $>0.5$  in thyroid tumor (T) and paired normal tissues (N); (B) qRT-PCR was used to compare expression level of DAPK2 between paired tumorous tissues (T) and normal tissues (N). \*,  $P < 0.05$ . DAPK2, death-associated protein kinase 2; TC, thyroid cancer; qRT-PCR, quantitative reverse transcription- polymerase chain reaction.

and tumorigenicity of TTA1 cells, overexpression of DAPK2 might have an opposite effect. Therefore, DAPK2-expressing plasmid or vector control was transfected into TTA1 cells using the lentivirus system. As indicated by western blot and qRT-PCR analysis (Figure 3A,B), DAPK2 was highly expressed. In contrast with DAPK2 knockdown, overexpression of DAPK2 promoted proliferation of TTA1 cells (Figure 3C). Also, overexpression of DAPK2 led to resistance of TTA1 cells to TRAIL-induced apoptosis (Figure 3D). DAPK2 overexpression enhanced tumorigenicity of TTA1 cells both *in vitro* and *in vivo*, as indicated by soft agar colony-formation assay (Figure 3E) and xenograft tumor model (Figure 3F).

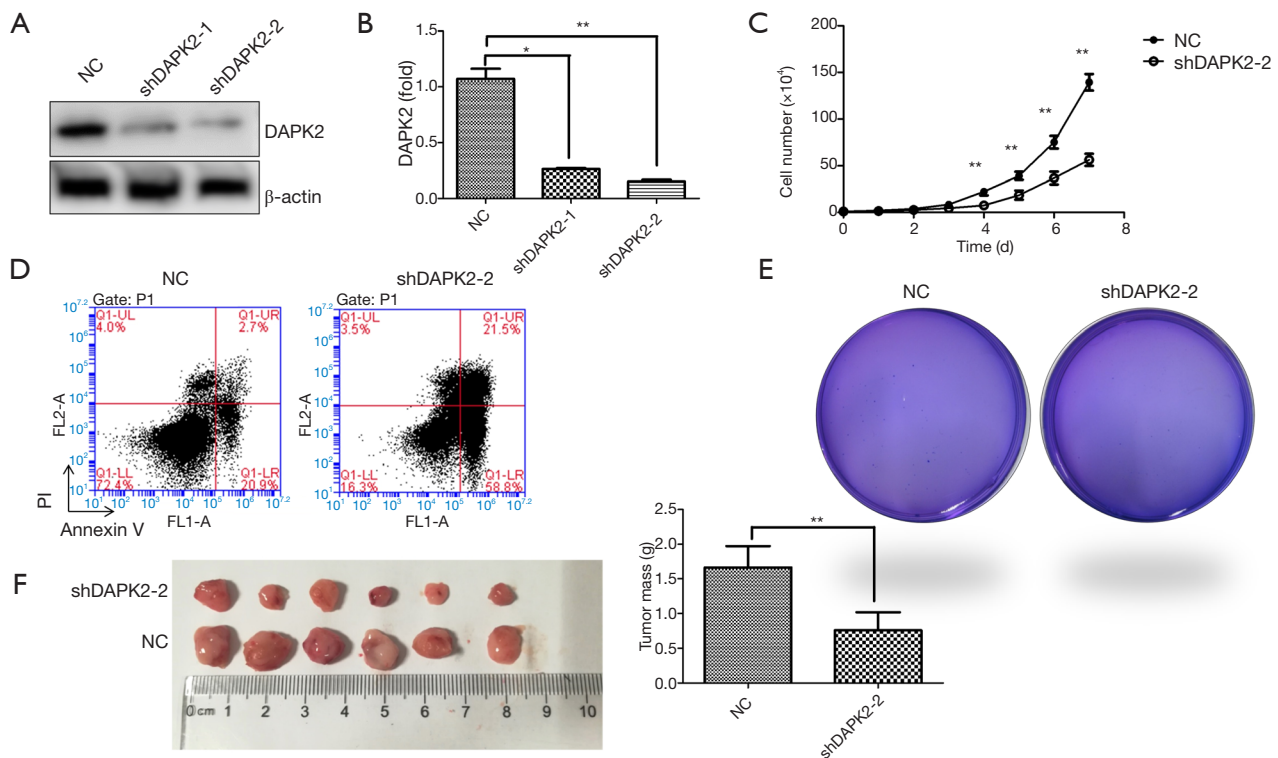
#### **DAPK2 activated NF- $\kappa$ B through triggering degradation of I- $\kappa$ B $\alpha$**

As increased NF- $\kappa$ B activity has been shown to be associated with TC development and tumor progression, we aimed to determine whether enhanced NF- $\kappa$ B activation is involved in the tumorigenic effect of DAPK2. Western blot results showed that knockdown of DAPK2 decreased

p65 phosphorylation, which correlated with increased I- $\kappa$ B protein level, while overexpression of TTA1 exhibited the opposite effect (Figure 4A,B). Dual luciferase reporter assay demonstrated that overexpression of DAPK2 significantly enhanced the transcriptional activity of NF- $\kappa$ B (Figure 4C), suggesting that DAPK2 positively regulates NF- $\kappa$ B signaling. We used small interfering (siRNA) to knockdown I- $\kappa$ B $\alpha$  in shDAPK2 TTA1 cells. Luciferase reporter assay showed that knockdown of I- $\kappa$ B $\alpha$  in shDAPK2 TTA1 cells restored the activity of NF- $\kappa$ B (Figure 4C), suggesting that DAPK2 activates NF- $\kappa$ B through promoting I- $\kappa$ B $\alpha$  degradation.

#### **DAPK2 enhanced NF- $\kappa$ B activation through autophagy**

It was previously reported that DAPK2 is involved in selective autophagy (12). We therefore aimed to determine whether the effect of DAPK2 on I- $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation occurs through autophagy. TTA1 cells were treated with 100 nM of rapamycin to induce autophagy. As expected, depletion of DAPK2 caused a decrease in the number of autophagosomes compared with

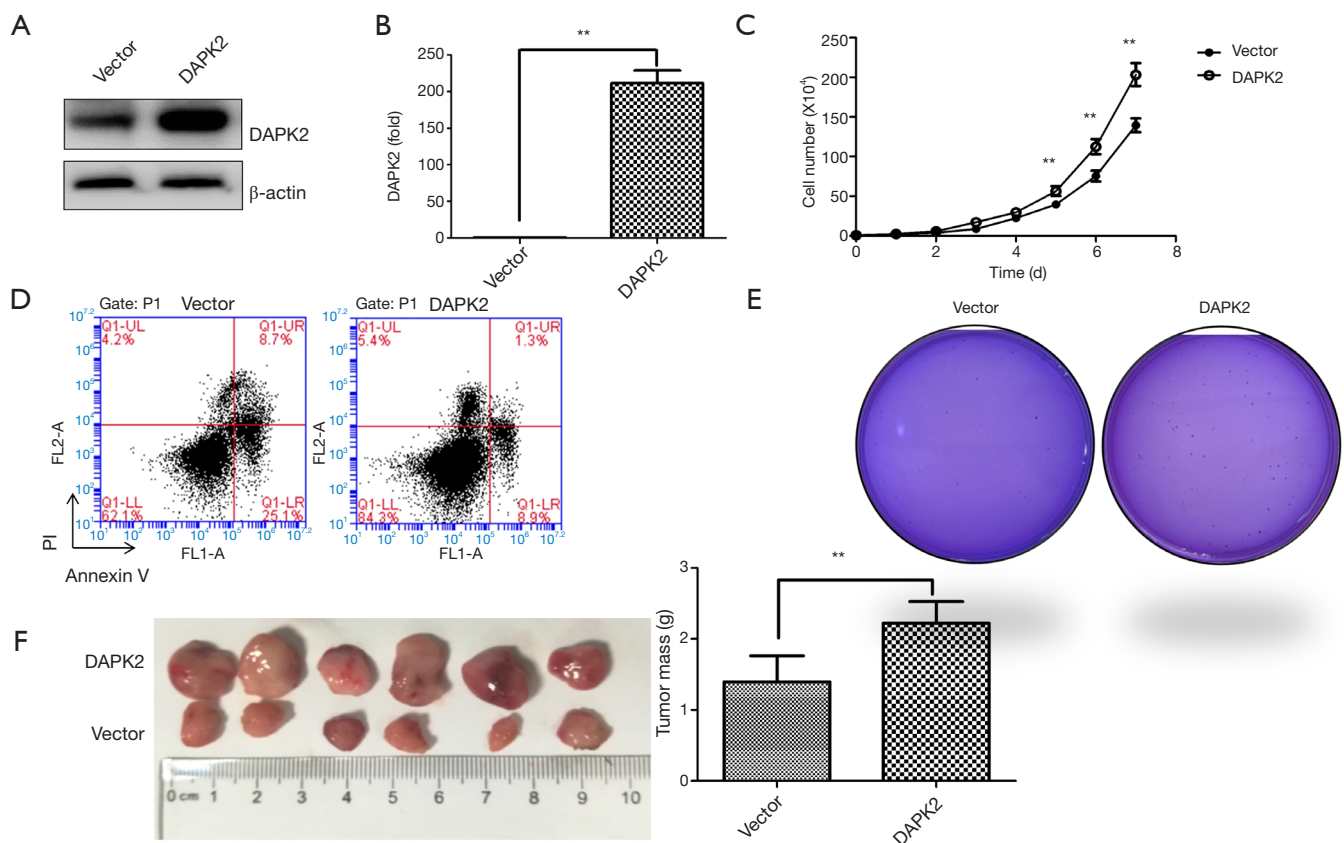


**Figure 2** DAPK2 silencing inhibited the proliferation and tumorigenicity of TTA1 cells. (A) Western blot analysis of DAPK2 protein levels in the non-targeting control (NC), shDAPK2-1, and shDAPK2-2 TTA1 cells; (B) qRT-PCR analysis to confirm DAPK2 knockdown efficiency; (C) effect of DAPK2 silencing in TTA1 cells on cell proliferation. TTA1 cells were plated on 12-well plates and cell numbers were counted every day until day 7. The assay was performed in triplicate (\*\*,  $P < 0.01$ ); (D) effect of DAPK2 knockdown on sensitivity to TRAIL-induced cell death. Cells were treated with TRAIL (100 ng/mL) for 24 hours. Cells were stained with Annexin V and PI and analyzed by flow cytometry; (E) soft agar colony formation assay of controls (NC) or shDAPK2 TTA1 cells. On day 14, the colonies were fixed with methanol, stained with 0.05% crystal violet, and imaged (1 $\times$ ). Representative photographs of the colonies are shown; (F) NC and shDAPK2-2 TTA1 cells were implanted subcutaneously into the back of nude mice. Xenografts were collected, and tumor mass were recorded. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . DAPK2, death-associated protein kinase 2; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TRAIL, tumor necrosis factor- $\alpha$  related apoptosis-inducing ligand; PI, propidium iodide.

the non-targeting control, as reflected by the appearance of LC3-FITC punctate staining (Figure 5A). Consistent with this, after rapamycin treatment, there was a reduction in the LC3II/LC3I ratio in shDAPK2 cells compared with the non-targeting control (Figure 5B). Conversely, overexpression of DAPK2 caused an increase in LC3 puncta and LC3II/LC3I ratio compared with vector control (Figure 5C,D). Furthermore, inhibition of autophagy by chloroquine diminished the effect of activating NF- $\kappa$ B by DAPK2, as indicated by luciferase reporter assay (Figure 5E). Collectively, our findings suggested that the activating effect of DAPK2 on NF- $\kappa$ B is mediated through induction of autophagy-dependent I- $\kappa$ B $\alpha$  degradation.

## Discussion

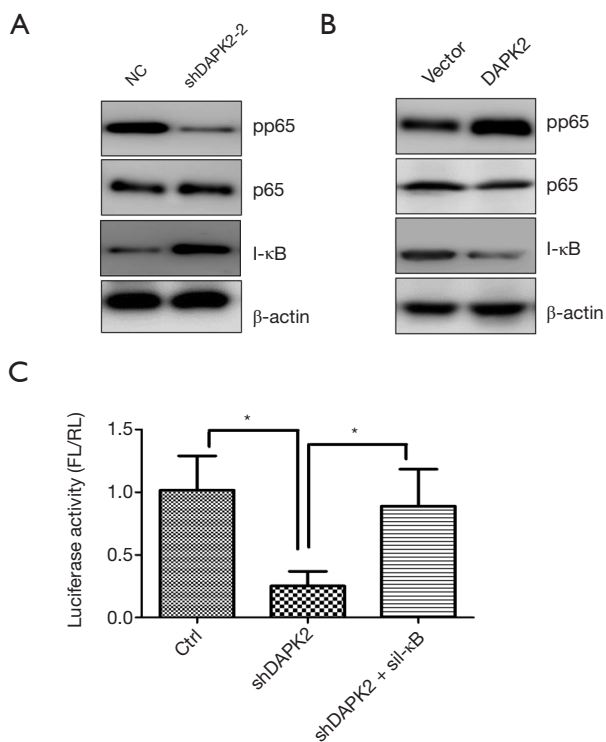
Recently, the loss of DAPK expression in multiple human cancers has sparked intense interest in this kinase family. DAPK2 was originally identified as a tumor suppressor, which is lost in several cancers, such as AML, epithelial ovarian cancer, and breast cancer, due to hypermethylation of the DAPK promoter region or micro RNA (miRNA)-mediated mRNA degradation (13-15). Increased thyroid hormone signaling leads to induction of DAPK2 which suppresses the development of HCC (12). In contrast with other malignancies, we reveal that DAPK2 is upregulated in TC. Knockdown of DAPK2 in TTA1 cells led to decreased



**Figure 3** Ectopic expression of DAPK2 promoted the proliferation and tumorigenicity of TTA1 cells. (A) Western blot analysis of DAPK2 protein expression in vector control and DAPK2 overexpression TTA1 cells; (B) qRT-PCR analysis to confirm DAPK2 expression; (C) effect of DAPK2 overexpression in TTA1 cells on cell proliferation. TTA1 cells were plated on 12-well plates, and cell numbers were counted for 7 consecutive days. The assay was performed in triplicate; (D) effect of DAPK2 overexpression on sensitivity to TRAIL-induced cell death. Cells were treated with TRAIL (100 ng/mL) for 24 hours. Cells were stained with Annexin V and PI and analyzed by flow cytometry; (E) soft agar colony formation assay of vector control or DAPK2-overexpressed TTA1 cells. On day 14, the colonies were fixed with methanol, stained with 0.05% crystal violet, and imaged (1 $\times$ ); (F) tumor formation of vector control and DAPK2-overexpressed TTA1 cells in nude mice. Vector control or DAPK2-overexpressed TTA1 cells were implanted subcutaneously into the back of nude mice. Xenografts were collected, and tumor mass was recorded. \*\*,  $P < 0.01$ . DAPK2, death-associated protein kinase 2; qRT-PCR, quantitative reverse transcription- polymerase chain reaction; TRAIL, tumor necrosis factor- $\alpha$  related apoptosis-inducing ligand; PI, propidium iodide.

cell proliferation, clonogenic formation *in vitro*, and tumor formation ability in nude mice, and enhanced sensitivity to TRAIL-induced apoptosis; meanwhile overexpression of DAPK2 exhibited the opposite effect, indicating an oncogenic role of DAPK2 in TC. Unlike DAPK2, another DAPK family member, DAPK1, is downregulated and functions as a tumor suppressor in TC (16-18), suggesting that DAPK2 and DAPK1 may have divergent functions in TC development and progression. Both DAPK1 and DAPK2 can be activated by binding of calcium-activated Calmodulin to their calmodulin auto-regulatory domains

(19,20). Alternatively, both can be activated by Ser289 phosphorylation, which leads to a conformational change mimicking the effect of calmodulin binding (21). Yet, Ser289 phosphorylation of DAPK1 and DAPK2 are regulated by mutually-exclusive and different signaling pathways. While DAPK1 Ser289 phosphorylation is mediated specifically by ribosomal s6 kinase (RSK) following MAPK activation (22), DAPK2 is phosphorylated on Ser289 by the metabolic sensor AMP-activated protein kinase (AMPK) (21), which is also up-regulated in TC. The different activation mode of these two similar kinases suggest the disparate roles of



**Figure 4** DAPK2 activated NF- $\kappa$ B through triggering degradation of I- $\kappa$ B. (A) Western blot analysis of P65 phosphorylation and I- $\kappa$ B degradation in DAPK2 knockdown (shDAPK2) and non-targeting control (NC) TTA1 cells; (B) Western blot analysis of p65 phosphorylation and I- $\kappa$ B analysis of p65 DAPK2-overexpressed and vector control TTA1 cells; (C) dual luciferase reporter assay to determine NF- $\kappa$ B transcriptional activity in NC, DAPK2 knockdown, and DAPK2, I- $\kappa$ B double-knockdown TTA1 cells. \*,  $P < 0.05$ . DAPK2, death-associated protein kinase 2; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I- $\kappa$ B $\alpha$ , inhibitory- $\kappa$ B $\alpha$ .

DPAK1 and DAPK2 in response to different stimuli (23), providing a perspective to selectively target DAPK2 in TC.

Autophagy contributes to tumor progression by maintaining the survival of tumor cells in starvation and hypoxic conditions and also confers therapeutic resistance to cancer cells (24,25). DAPK2 has been shown to be involved in several steps of autophagy. DAPK2 promotes autophagy induction by phosphorylating the mammalian target of rapamycin (mTOR) binding partner raptor, thus inhibiting mTOR complex 1 (mTORC1) activity (26). DAPK2 also activate the Beclin-1/Vacuolar protein sorting 34 (Vps34) complex through phosphorylating Beclin-1 on Thr119 and promoting its dissociation from B-Cell Lymphoma-extra-large (Bcl-XL) (21). Recently, DAPK2

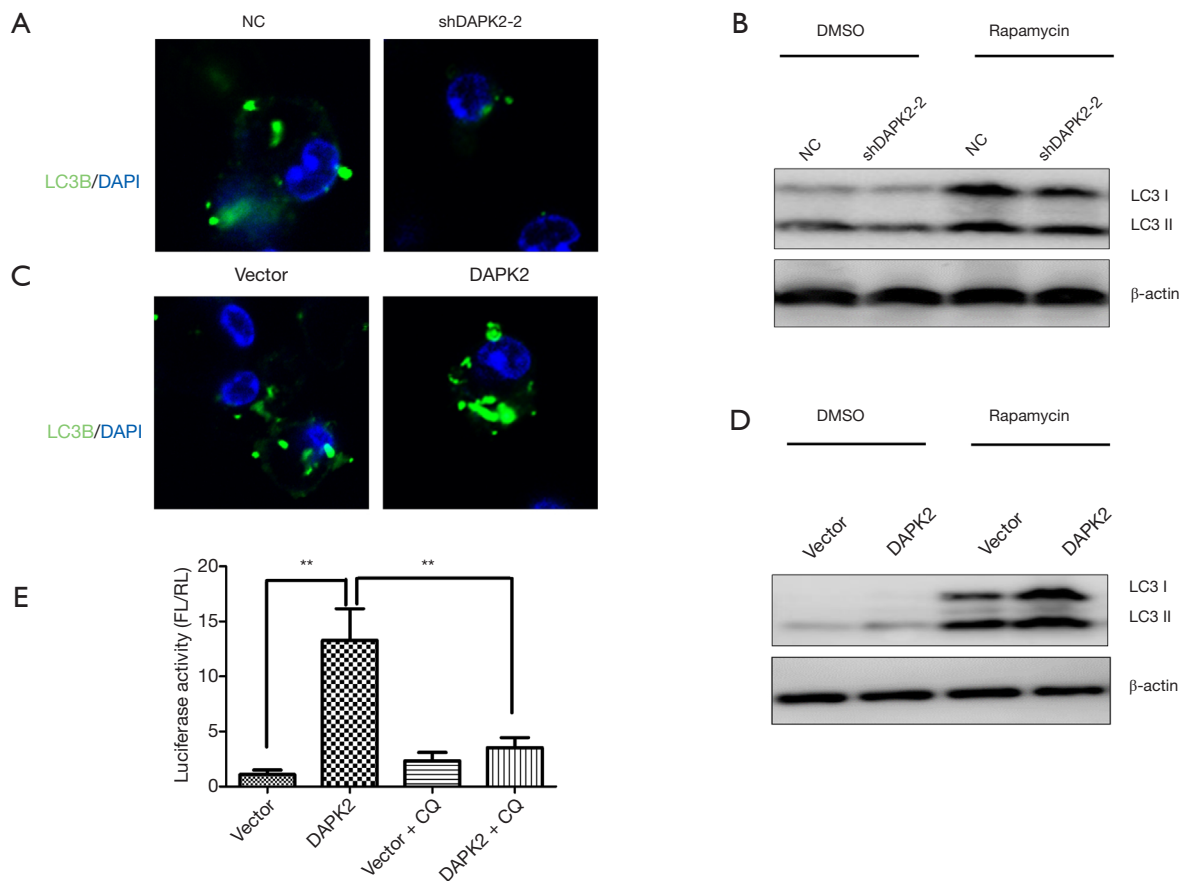
was shown to interact with the Beclin-1 complex member Autophagy-related protein 14 (Atg14) (27). In addition, DAPK2 phosphorylates the autophagy receptor protein p62 to promote selective autophagy (12).

Accumulating evidence has begun to clarify the complex interplay between autophagy and NF- $\kappa$ B pathway, and developing new strategies that target both pathways may help improve the effectiveness of anticancer therapy. NF- $\kappa$ B signaling can either activate autophagy by regulating the transcription of Beclin 1 (28) or inhibit autophagy through activating mTOR (29). NF- $\kappa$ B signaling also controls the level of autophagy activators, such as reactive oxygen species (ROS) and oxidative stress (30,31). In turn, autophagy can either inactivate NF- $\kappa$ B by degradation of I $\kappa$ B kinase (IKK) or maintain the persistent activation of NF- $\kappa$ B through clearance of I- $\kappa$ B $\alpha$  (32).

NF- $\kappa$ B contributes to TC development and progression by maintaining the transformed phenotype and conferring resistance to apoptosis. Inhibition of constitutively activated NF- $\kappa$ B signaling in cancer cells may improve the efficacy of chemo- and radiotherapy. Although constitutive activation of NF- $\kappa$ B has been observed in various types of malignancies, genetic alterations in NF- $\kappa$ B members and regulatory signaling components are rare in solid tumors, suggesting cancer cells have evolved other mechanisms to activate NF- $\kappa$ B (33,34). I- $\kappa$ B $\alpha$  is a cytoplasmic inhibitor of NF- $\kappa$ B and is essential to retaining NF- $\kappa$ B in the cytoplasm. Extracellular stimuli could activate IKK to phosphorylate and degrade I- $\kappa$ B $\alpha$ , releasing NF- $\kappa$ B to induce the transcription of target genes (35,36). Constitutive degradation of I- $\kappa$ B $\alpha$  or loss-of-function mutations of I- $\kappa$ B $\alpha$  may lead to persistent NF- $\kappa$ B activation, while inhibition of I- $\kappa$ B $\alpha$  degradation promotes apoptotic cell death and inhibits tumorigenesis (37-39).

The autophagy and ubiquitin proteasome system (UPS) are the two major intracellular protein degradation systems, and have been thought to be largely distinct. While proteasomal degradation of I- $\kappa$ B $\alpha$  is essential for the initial induction of NF- $\kappa$ B activation, persistent activation of NF- $\kappa$ B is maintained through autophagy-dependent degradation of I- $\kappa$ B $\alpha$  (32). Our results showed that DAPK2 positively regulates degradation of I- $\kappa$ B $\alpha$  through autophagy, thus enhancing NF- $\kappa$ B activation. In this way, DAPK2 contributes to TC development and progression. P62 (also known as SQSTM1) is a multiple-domain protein, which binds to ubiquitinated proteins and LC3 simultaneously, mediating selective degradation of the ubiquitinated proteins by autophagy, thus coupling





**Figure 5** DAPK2 enhanced NF- $\kappa$ B activation through selective autophagy. (A) Visualization of autophagosome formation in NC and shDAPK2-2 TTA1 cells using confocal laser scanning microscopy (630 $\times$ ). Cells were treated with 100 nM rapamycin for 48 hours, fixed with 4% PFA, and stained with LC3B antibody and FITC-conjugated secondary antibody; DAPI was used to indicate nuclear localization; (B) Western blot analysis of LC3 expression in the non-targeting control (NC) and shDAPK2-2 TTA1 cells treated with 100 nM rapamycin or DMSO for 48 hours; (C) visualization of autophagosome formation in vector control and DAPK2-overexpressed TTA1 cells using confocal laser scanning microscopy (630 $\times$ ); (D) Western blot analysis of LC3 expression in vector control and DAPK2-overexpressed TTA1 cells treated with 100 nM rapamycin or DMSO for 48 hours; (E) dual luciferase reporter assay was used to determine NF- $\kappa$ B transcriptional activity in vector control and DAPK2-overexpressed TTA1 cells treated with or without CQ (50  $\mu$ M). \*\*,  $P < 0.01$ . DAPK2, death-associated protein kinase 2; NF- $\kappa$ B, nuclear factor- $\kappa$ B; LC3, microtubule-associated protein 1A/1B-light chain 3; DMSO, dimethyl sulfoxide; CQ, chloroquine.

UPS and the autophagy-dependent degradation system (40,41). DAPK2 has also been shown to regulate autophagic clearance of ubiquitinated aggregates through phosphorylating p62 (12), suggesting p62 is probably involved in DAPK2-mediated I- $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation during TC development and progression. Clearly, this study unravels an important role of DAPK2 in TC development and progression. Yet, our understanding is still in its infancy, the exact mechanism remains to be fully elucidated.

## Conclusions

The present study indicates that DAPK2-driven selective autophagy promotes I- $\kappa$ B $\alpha$  degradation, activating NF- $\kappa$ B, and promoting TC development in turn. Thus, this study reveals a mechanistic link between autophagy and NF- $\kappa$ B activation, providing a novel target for TC therapy.

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### Footnote

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine institutional ethics committee (No. SH9H-2020-T346-1) and informed consent was taken from all the patients. Animal experiments were performed under a project license (No. SH9H-2020-T346-1) granted by institutional ethics committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, in compliance with Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine institutional guidelines for the care and use of animals.

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