Molecules and Cells

SKP2 Contributes to AKT Activation by Ubiquitination Degradation of PHLPP1, Impedes Autophagy, and Facilitates the Survival of Thyroid Carcinoma

Yuan Shao[1,4], Wanli Ren[1,4], Hao Dai[1], Fangli Yang[1], Xiang Li[1], Shaoqiang Zhang[1], Junsong Liu[1], Xiaobao Yao[1], Qian Zhao[1], Xin Sun[2], Zhiwei Zheng[3], and Chongwen Xu[1,*]

[1]Department of Otorhinolaryngology-Head and Neck Surgery, The First Affiliated Hospital of Xi'An Jiaotong University, Xi'an, China, [2]Department of Thoracic Surgery, The First Affiliated Hospital of Xi'An Jiaotong University, Xi'an, China, [3]The Third Ward of General Surgery Department, Rizhao People's Hospital, Rizhao, China, [4]These authors contributed equally to this work.
*Correspondence: henao22@xjtu.edu.cn
https://doi.org/10.14348/molcells.2022.2242
www.molcells.org

Papillary thyroid carcinoma (PTC) is the most common subtype of thyroid carcinoma. Despite a good prognosis, approximately a quarter of PTC patients are likely to relapse. Previous reports suggest an association between S-phase kinase-associated protein 2 (SKP2) and the prognosis of thyroid cancer. SKP1 is related to apoptosis of PTC cells; however, its role in PTC remains largely elusive. This study aimed to understand the expression and molecular mechanism of *SKP2* in PTC. *SKP2* expression was upregulated in PTC tissues and closely associated with clinical diagnosis. *In vitro* and *in vivo* knockdown of *SKP2* expression in PTC cells suppressed cell growth and proliferation and induced apoptosis. *SKP2* depletion promoted cell autophagy under glucose deprivation. SKP2 interacted with PH domain leucine-rich repeat protein phosphatase-1 (PHLPP1), triggering its degradation by ubiquitination. Furthermore, SKP2 activates the AKT-related pathways via PHLPP1, which leads to the cytoplasmic translocation of SKP2, indicating a reciprocal regulation between SKP2 and AKT. In conclusion, the upregulation of SKP2 leads to PTC proliferation and survival, and the regulatory network among SKP2, PHLPP1, and AKT provides novel insight into the molecular basis of SKP2 in tumor progression.

Keywords: AKT, molecular mechanism, papillary thyroid carcinoma, PH domain leucine-rich repeat protein phosphatase-1, S-phase kinase-associated protein 2

INTRODUCTION

Thyroid cancer is the most common cancer of the endocrine system. There are four subtypes: papillary thyroid carcinoma (PTC), follicular thyroid cancer, medullary thyroid cancer, and anaplastic thyroid cancer (Wang et al., 2015). PTC is the most common type, accounting for approximately 88% of all thyroid cancers (Aschebrook-Kilfoy et al., 2011). Reports suggest that the rise in PTC cases accounts for the increase in thyroid cancer cases in recent years (Cho et al., 2013; Elisei et al., 2010; McNally et al., 2012). PTC has a good prognosis after surgical treatment, with a 99% survival rate for a 20-year (Kakudo et al., 2004). Despite the high survival rate of PTC, the long-term follow-up shows a 25% high recurrence rate (Grogan et al., 2013). Surgical treatment and fear of recur-

Received September 22, 2021; revised February 18, 2022; accepted March 31, 2022; published online January 24, 2023

eISSN: 0219-1032

rence are traumatizing and cause psychological distress and burden to PTC patients. Hence, understanding the underlying molecular mechanism of PTC development, progression, and recurrence will aid in enhancing treatment and preventing recurrences in PTC patients.

S-phase kinase-associated protein 2 (SKP2) is an F-Box protein (FBP) that contains at least one F-box domain. The F-box domain is a 50 amino acid protein motif that mediates the interaction between proteins. FBP is one of the components of the SCF (SKP1-cullin-F-box) ubiquitin-protein ligase complex (Bai et al., 1996), which plays an important role in signal transduction and cell cycle regulation (Craig and Tyers, 1999). SKP2 is a substrate recognition factor in the SCF complex (Nakayama and Nakayama, 2005). Recent studies have shown the role of SKP2 in various cancers, which makes SKP2 a potential therapeutic and drug target (Chan et al., 2010). Overexpression of SKP2 was reported in the initiation, growth, and metastasis of many cancers. Further *in vitro* and *in vivo* studies show SKP2 has a carcinogenic effect (Chan et al., 2010). Altered expression of SKP2 has been reported in various cancers, including lymphoma (Seki et al., 2003), prostate cancer (Wang et al., 2012b), melanoma (Rose et al., 2011), nasopharyngeal carcinoma (Fang et al., 2009), pancreatic cancer (Schüler et al., 2011), breast cancer (Radke et al., 2005; Zheng et al., 2005), and non-small cell lung cancer (Yokoi et al., 2004). *SKP2* overexpression is associated with a poor prognosis of breast cancer (Sonoda et al., 2006). In xenograft tumor models, *SKP2* overexpression promotes tumorigenesis and tumor growth (Lin et al., 2009). SKP2 inactivation triggers cellular senescence and apoptosis in a p19Arf/P53-independent and p27-dependent manner, which significantly inhibits the growth and development of cancer (Lin et al., 2010). Further, the association between SKP2 expression and the prognosis of thyroid cancers has been reported. *SKP2* regulates tumor growth by triggering p27 degradation (Chiappetta et al., 2007). In addition, the inhibition of SKP2 expression in cells enhances the synergistic lethal effect of TNF-mediated apoptosis-inducing ligand (TRAIL) (Pratheeshkumar et al., 2018). These results indicate that SKP2 promotes tumor growth in thyroid cancer. Despite these reports, the role of SKP2 in thyroid cancer and the mechanism associated is not fully understood and requires further exploration. Therefore, this study aimed to understand the expression and the role of SKP2 in thyroid cancer. The results show high expression of SKP2 in thyroid cancer, which regulates cell growth and proliferation. Further analysis revealed that SKP2 plays a role in thyroid cancer by regulating the AKT-associated pathways via the ubiquitination and degradation of PH domain leucine-rich repeat protein phosphatase-1 (PHLPP1). This is the first time the regulatory role of SKP2 and PHLPP1 have been deciphered which adds to our understanding of the role of SKP2 in cancers.

MATERIALS AND METHODS

PTC tissues and their paired normal thyroid tissue (n = 98) were collected from PTC patients during surgical resection at The First Affiliated Hospital of Xi'An Jiaotong University. Written informed consent was obtained from all the participating patients. The patients who underwent chemotherapy or radiotherapy before the surgery were excluded from this study. All tissue samples were frozen in liquid nitrogen or paraffin-embedded immediately after surgical resection, and two independent pathologists evaluated the tissue biopsies. This study was approved by the institutional ethics committee of The First Affiliated Hospital of Xi'An Jiaotong University (No. 2021-1407). All the experiments were performed in accordance with the principles of the Declaration of Helsinki (2013).

Cell culture and transfection

Human embryonic kidney (HEK293T) cells were purchased from the National Infrastructure of Cell Line Resource (NICR, China), and Normal Human thyroid follicular epithelial cells (Nthy-ori 3-1) were purchased from American Type Culture Collection (ATCC, USA). Three human PTC cell lines, TPC-1, BCPAP, and IHH-4, were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Nthy-ori 3-1 cells and all three PTC cell lines were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). HEK293T cells were cultured in DMEM medium supplemented with 10% FBS.

SKP2 expression was knockdown in PTC cells and Nthy-ori 3-1 using lentivirus containing shRNAs targeting SKP2 (refer to as shSKP2#1 and shSKP2#2). PTC cells and Nthy-ori 3-1 were transfected with scrambled shRNA and were used as control. The sequences of shRNAs used in this study were listed as follows: shSKP2#1 (5′-GATAGTGTCATGCTAAAGAAT-3′), shSKP2#2 (5′-GCCTAAGCTAAATCGAGAGAA-3′), shNC (5′-GGAATCTCATTCGATGCATAC-3′). *PHLPP1* expression was knockdown in cells using two shRNAs, shPHLPP1#1 (5′-CCAGACTTACTACATTTGCTT-3′) and shPHLPP1#2 (5′-CGAGGTCTTTCCCGAAGTTAT-3′).

Myc-tagged SKP2 and Flag-tagged PHLPP1 were cloned in pcDNA vectors using the Gateway recombination system (Invitrogen, USA).

Cell proliferation

Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Japan) method per the manufacturer's protocol. Briefly, cells with the indicated treatment were seeded in 96-well plates at a density of 2 × 10^3 cells/well and cultured for 24, 48, and 72 h at 37°C. Further, 10 μl CCK-8 solution (Dojindo Molecular Technologies) was added to each well and incubated at 37°C for 2.5 h. The absorbance was measured at 450 nm wavelength using a microplate reader (Bio-Tek, USA).

For colony forming assay, cells in the logarithmic phase were digested using 0.25% trypsin. TPC-1 (1×10^3 cells) and IHH-4 (500 cells) cells were seeded in a 6-well plate and cultured for 10-14 days at 37°C. The colonies were fixed with methanol, stained with crystal violet, and manually counted.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was used to study DNA synthesis. Cells were seeded in a 24-well plate, incubated with EdU solution at 37°C for 2 h, followed by incubation with Apollo 488 dye for another 30 min. The fluorescence was observed and captured using a fluorescence microscope (Nikon, Japan).

Cell apoptosis

Cell apoptosis was measured using flow cytometry. Approximately 1 × 10^6 cells with the indicated treatment were collected and digested with trypsin and were stained using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) per the manufacturer's instructions. After staining, the cells were sorted using a FACSCalibur flow cytometer (BD Biosciences). Apoptosis was analyzed using FlowJo software (TreeStar, USA).

In vivo tumorigenicity assay

BALB/c female nude pathogen-free mice (6-8 weeks old, 20-25 g) were purchased from Shanghai experimental animal center China. Mice were housed at 22°C ± 2°C in a light-dark cycle of 12 h. PTC cells (TPC-1 and IHH-4) transfected with *SKP2* shRNA or scrambled shRNA were prepared as described above. Approximately 2 × 10^6 cells were suspended in 0.1 ml phosphate-buffered saline (PBS) and subcutaneously injected into the flank of each nude mice. Tumor growth was monitored every five days using calipers, and tumor volumes were calculated per the equation described previously (He et al., 2020). After 30 days, the xenografts were harvested and photographed. One-half of the xenograft tissue was frozen in liquid nitrogen and stored at −80°C for further use, while the others were fixed in formalin and paraffin-embedded for immunohistochemical staining.

The animal study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Xi'An Jiaotong University prior to the experiments being conducted (No. 2020AM88). All the animal experiments were performed per the Guide for the Care and Use of Laboratory Animals.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from the thyroid tissue or PTC cells was extracted using Trizol reagent (TaKaRa Bio, Japan) per the manufacturer's protocol. One microgram RNA was used to carry out cDNA synthesis using ReverTra Ace qPCR-RT Master mix kit (Toyobo, Japan) according to the manufacturer's protocol. PCR was performed using SYBR Green II kit (TaKaRa Bio). The PCR program was set as: denaturation at 94°C for 3 min, followed by 30 cycles including denaturation at 94°C for 30 s, annealing at 59.5°C for 30 s, and extension at 72°C for 60 s.The housekeeping gene *GAPDH* was used as the internal control. The primer sequences are as follows: GAPDH (forward primer [FP]: 5′-CAATGACCCCTTCATTGACC-3′; reverse primer [RP]: 5′-GATCTCGCTCCTGGAAGATG-3′), SKP2 (FP: 5′-TTGCCCTGCAGACTTTGCTA-3′; RP: 5'-CAGCTGGGTGATGGTCTCTG-3′), PHLPP1 (FP: 5′-GACAAGACGCCAAGTCATCC-3′; RP: 5′- GTTCCAGGCTACAACACGAGA-3′).

Western blot analysis

For protein extraction, ground tissue or cells were lysed using RIPA lysis buffer, and the debris was removed by centrifuge. The protein in the supernatant was collected and quantified using a bicinchoninic acid assay (BCA) kit (Beyotime, China). The extracted proteins were separated on 12% SDS-PAGE gels, the voltage was first 80 V for ~30 min and subsequent 120 V, for ~60 min, and then and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) (400 mA, 90 min). The membranes were blocked using skimmed milk in Tris-buffered saline Tween 20 (TBST) and probed using primary antibodies for 12 h at 4°C. After washing; the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for another 1 h at room temperature. Immunoreactive bands were detected using chemiluminescence detection kits (Millipore).

The primary antibodies used were as follows: SKP2 (ab183039, 1:500; Abcam, UK), β-actin (ab6276, 1:3,000; Abcam), LC3B (ab192890, 1:2,000; Abcam), p62 (#5114, 1:1,000; CST), PHLPP1 (ab135957, 1:1,000; Abcam), β-tubulin (#2146, 1:1,000; CST), AKT (#4691, 1:1,000; CST), phosphorylated-AKT (#4060, 1:2,000; CST), mTOR (#2972, 1:1,000; CST), phosphorylated-mTOR (#5536, 1:1,000; CST). Antibodies against HA tag (ab18181, 1:2,000; Abcam), Myc tag (ab9106, 1:1,000; Abcam), Flag tag (ab125243, 1:2,000; Abcam).

Immunohistochemistry (IHC) analysis

Four micrometers thick paraffin-embedded sections of human tissues or xenografts were prepared. The sections were dewaxed, incubated with citric buffer at 95°C for 10 min, and blocked using H_2O_2 at room temperature for 20 min. The sections were incubated with normal goat serum for another 30 min at room temperature, followed by overnight incubation at 37°C with primary antibodies (SKP2, 1:100, ab183039 [Abcam]; Ki-67, 1:150, ab16667 [Abcam]). The sections were washed using PBS and incubated with HRP-conjugated-secondary antibody. For peroxidase detection, the sections were stained with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) for approximately 15 s and counterstained with hematoxylin.

Immunofluorescence (IF)

PTC cells after indicated treatment were washed using PBS and fixed with 4% polyformaldehyde (PFA) for 20 min. The excess PFA was removed by washing; the cells were permeabilized using 0.1% Triton X-100 for 10 min and incubated with 5% normal goat serum for 60 min to prevent nonspecific binding. Cells were probed with primary antibodies at 4°C overnight, followed by incubation for 1 h with fluorescent secondary antibodies (1:200, Alexa-488-conjugated goat anti-rabbit IgG, ab150077; Abcam). The cells were counterstained using 4',6-diamidino-2-phenylindole (DAPI; Sigma, USA) for 10 min at room temperature and visualized. The images were captured using a confocal microscope (Olympus, Japan). The primary antibodies used were anti-SKP2 (ab183039, 1:200; Abcam) and LC3B (ab192890, 1:100; Abcam).

Immunoprecipitation (IP)

Scrambled shRNA, *SRP2,* and *PHLPP1* knockdown HEK-293T cells or PTC cells were lysed using RIPA buffer and precleared with protein A/G beads (Sigma). The supernatant was incubated with protein A/G beads and specific antibodies at 4°C for 3 h, followed by centrifugation at 1,000 × *g*. The pellets were then collected and washed with RIPA buffer. After resuspension, samples were used for western blot analysis.

Ubiquitination analysis

In vitro ubiquitination assays were performed on PTC cells and HEK-293T cells. Briefly, cells were treated with proteasome inhibitor MG132 (50μg/ml) for 6 h. Cells were then lysed with RIPA buffer and boiled for 15min, and the protein samples were purified with nickel beads overnight. Ubiquitinated proteins were immunoprecipitated with anti-PHLPP1 or anti-Flag antibodies and detected with the anti-HA antibody using immunoblot.

Statistical analysis

Data were represented as the mean ± SD. Statistical analysis was performed using IBM SPSS Statistics software (ver. 19.0; IBM, USA). Student's *t*-test and one-way ANOVA test was used to calculate the differences between groups. $P < 0.05$ were considered statistically significant.

RESULTS

Expression of SKP2 in PTC

Expression of *SKP2* has been reported in various human carcinomas, especially thyroid cancer, and acts as an oncogene (Chiappetta et al., 2007; Pratheeshkumar et al., 2018). Hence, our study examined the expression and molecular mechanism of SRP2. The expression of *SKP2* was evaluated in 98 PTC and paired normal thyroid tissue. As expected, RNA and protein expression of SKP2 was higher in PTC tissues compared to the paired normal thyroid tissue (Fig. 1, Supplementary Fig. S1). As shown in Table 1, a positive association was observed between high *SKP2* expression and the tumor size ($P = 0.002$), TNM stage ($P = 0.009$), and lymph node metastasis ($P = 0.036$).

Knockdown of *SKP2* expression suppressed PTC cell proliferation

SKP2 expression was evaluated in three PTC cell lines and Nthy-ori3-1 cells as the normal thyroid cells for comparison. As shown in Fig. 2A, *SKP2* expression was higher in PTC cell lines compared to Nthy-ori3-1 cells. Two lentiviral vectors with shRNAs against *SKP2* were used to knockdown the endogenous expression of *SKP2* in TPC-1 and IHH-4 cells (Fig. 2B). *In vitro* studies suggest the altered levels of SKP2 reduced the prolif-

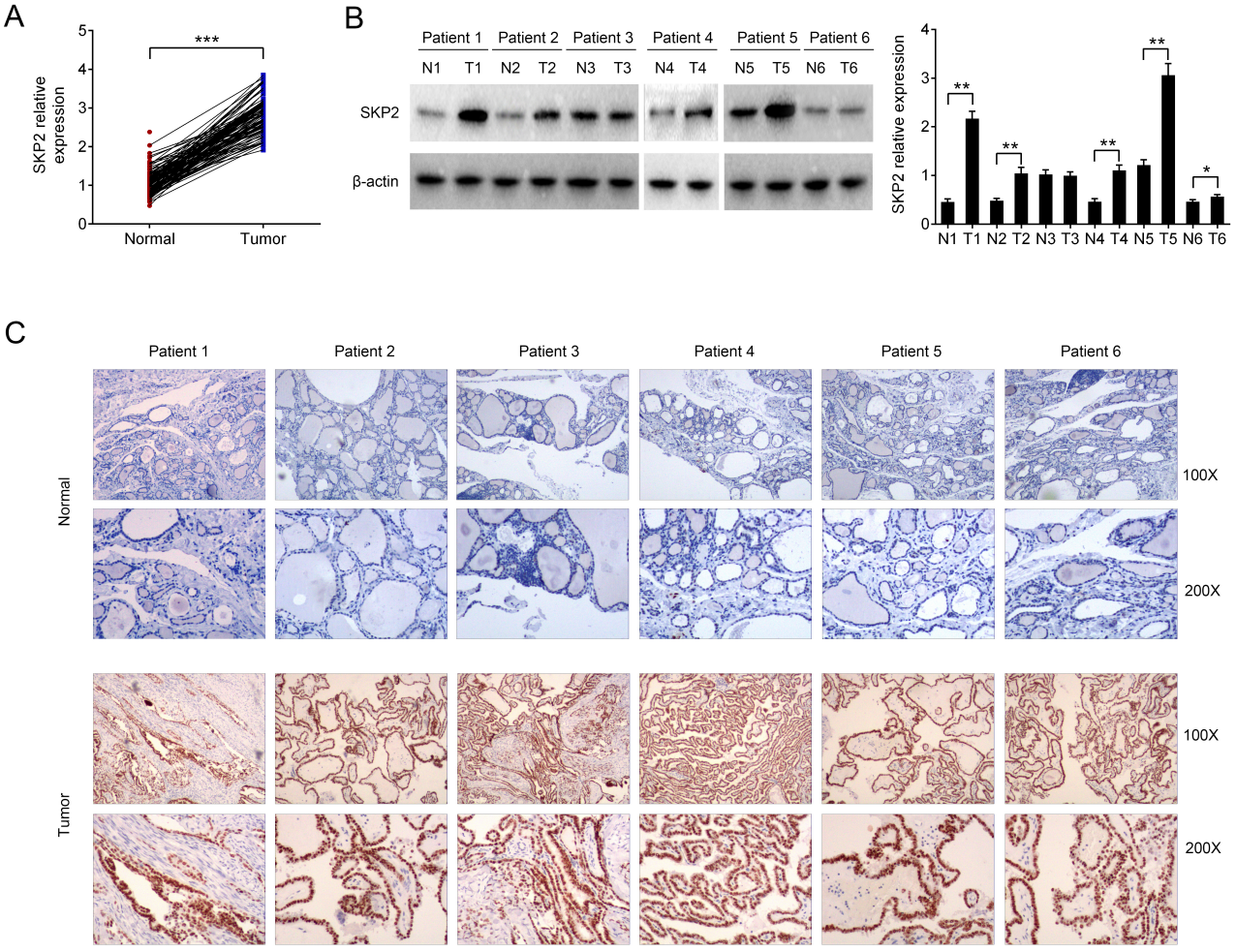

Fig. 1. Expression of *SKP2* in PTC tissues. (A) mRNA expression of *SKP2* in PTC and adjacent tumor tissues (n = 98). Protein expression of SKP2 in PTC and adjacent tumor tissues from patients 1-6 was detected by western blot (B) and immunohistochemistry (C), respectively. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 1. Relationship between *SKP2* expression in papillary thyroid carcinoma and clinicopathological features of patients

Variable	*SKP2* expression		*P* value
	Low expression	High expression	
Age (y)			
≤45	29	27	0.683
>45	20	22	
Sex			
Male	24	23	0.840
Female	25	26	
Tumor size (cm)			
≤1	30	15	0.002*
>1	19	34	
TNM stage			
I/II	45	35	0.009*
III/IV	4	14	
Extra thyroidal			
Negative	33	29	0.402
Positive	16	20	
Lymph node metastasis			
Negative	36	26	0.036*
Positive	13	23	
Nodular goiter			
Negative	42	37	0.201
Positive	7	12	

Values are presented as median.
*$P < 0.05$.

eration of anaplastic thyroid carcinoma cells (Chiappetta et al., 2007). Therefore the effect of *SKP2* knockdown on PTC cell proliferation was studied. Knockdown of *SKP2* expression in PTC cells reduced the cell viability, colony formation capacity, and DNA synthesis compared to cells transfected with scrambled shRNA, as assessed by CCK-8, colony formation, and EdU assays, respectively (Figs. 2C-2E). Further, an increase in apoptosis was observed in *SRP2* knockdown PTC cells compared to cells transfected with scrambled shRNA (Fig. 2F).

To assess the effects of loss of *SKP2* in normal cells, *SKP2* expressed was knockdown in Nthy-ori3-1 cells. As shown in Supplementary Fig. S2, a decrease in cell viability and DNA synthesis was observed in cells knockdown with shRNA#1, while no difference in apoptosis was observed between the three groups.

Additionally, a tumorigenicity assay performed in nude mice proved that knockdown of *SKP2* expression slowed down the growth of PTC cells *in vivo*, as indicated by the reduction in tumor size and Ki67 immunohistochemical staining (Figs. 2G and 2H).

These results suggested the oncogenic role of SKP2 in PTC cells as *SKP2* knockdown inhibited carcinogenesis.

Knockdown of *SKP2* expression facilitated PTC cell autophagy

Several recent studies show the role of SKP2 in autophagy. For instance, Gassen et al. (2019) demonstrated loss of *SKP2* promoted autophagy and restricted coronavirus infection. Autophagy is an evolutionarily conserved mechanism for maintaining cell homeostasis. The role of autophagy in cancer progression is complicated; on the one hand, autophagy-dependent energy and nutrition recycling is indispensable for cancer progression; on the other hand, excessive autophagy causes cell death, especially under stressful conditions (Yu et al., 2019a). Hence, the effect of *SKP2* knockdown on autophagy of PTC cells in glucose deprived medium was studied. PTC cells were cultured in the normal or glucose-free medium for 12 h, and autophagy-related proteins were then assessed. In *SKP2* knockdown PTC cells cultured in a normal medium, the loss of SKP2 facilitated the transformation from LC3I to LC3II, which correlates to autophagosome formation. Further, a slight decline in p62 expression (p62 is an indicator of autophagic flux, whose decrease implies the enhancement of autophagic flux) was also observed in *SKP2* knockdown cells compared to cells transfected with scrambled shRNA (Fig. 3A). When cells were cultured in a glucose-free medium, a loss of *SKP2* dramatically exacerbated autophagy by altering the formation of both autophagosome and autophagic flux (Figs. 3A and 3B). We next evaluated autophagosome formation using immunofluorescence. An enhanced autophagosome formation was observed in *SKP2* knockdown PTC cell in a normal medium, and the absence of glucose further strengthened the autophagosome formation (Fig. 3C). These results show the role of *SKP2* in autophagy, especially under stressful conditions.

SKP2 interacts with *PHLPP1*

SKP2, an FBP, forms the SCF (Skp1-Cullin 1-F-box) complex to participate in the ubiquitination-mediated degradation of proteins (Wang et al., 2012b). Various proteins have been identified as the SKP2 substrate, which was degraded by ubiquitination, including p27 (Bochis et al., 2015), FOXO1 (Huang et al., 2005), PDCD4 (Li et al., 2019), etc. Previous studies have shown that β-TRCP (F-box family protein) degrades PHLPP1 via ubiquitination (Li et al., 2009). Therefore, the correlation between SKP2 and PHLPP1 was examined using HEK-293T cell overexpressing Myc-tagged SKP2 and Flag-tagged PHLPP1. Co-immunoprecipitation assay was performed to evaluate the interaction between SKP2 and PHLPP1. As shown in Fig. 4A, SKP2 interacted with PHLPP1. Immunoprecipitation on TPC-1 cell lysates and using SKP2 antibody revealed an endogenous interaction between SKP2 and PHLPP1 (Fig. 4B). Next, the effect of *SKP2* knockdown on PHLPP1 expression was evaluated. Knockdown of *SKP2* expression in both PTC cell lines did not alter *PHLPP1* mRNA expression (Fig. 4C). However, an increase in PHLPP1 protein was observed in a concentration-dependent manner (Figs. 4D and 4E). Besides, we analyzed the correlation between SKP2 and PHLPP1 protein abundance in 30 tumor samples, and the result showed an inverse correlation between the expression of SKP2 and PHLPP1 (Supplementary Fig. S3). The results show that SKP2 regulates PHLPP1 post-transcriptionally, and an inverse correlation between the two proteins was observed.

SKP2 prompted ubiquitination-dependent PHLPP1 degradation

The stability of PHLPP1 protein was investigated in the absence or presence of SKP2. PTC cells were incubated with a

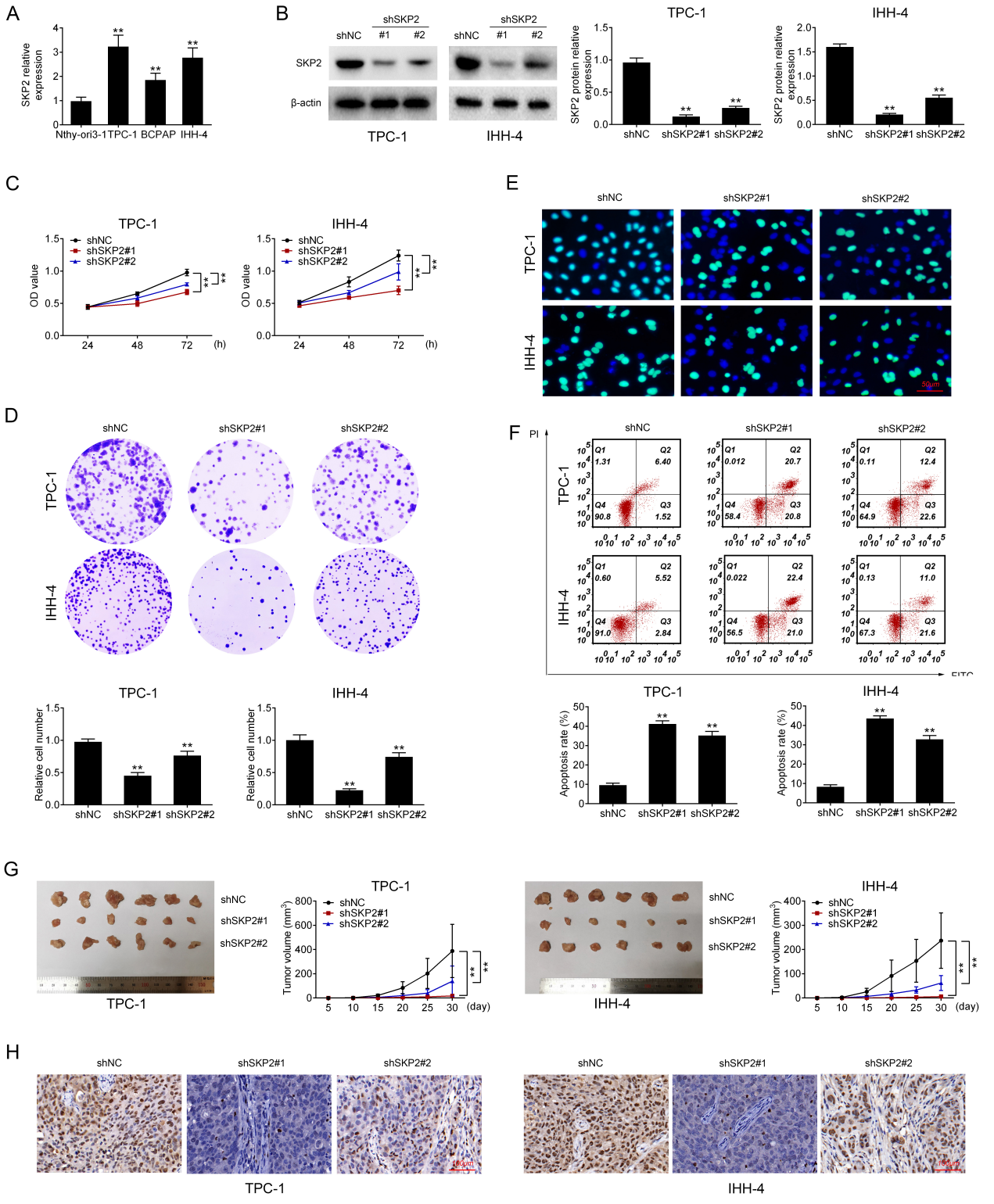

Fig. 2. *SKP2* knockdown suppressed PTC cell proliferation and promoted apoptosis. (A) Expression of SKP2 in PTC cells (TPC-1, BCPAP, IHH-4) and normal thyroid cells (Nthy-ori3-1). (B) SKP2 expression in PTC cells transfected with scrambled shRNA (shNC) or *SKP2* shRNA (shSKP2). (C) Cell viability of PTC cells transfected with shNC or shSKP2 assessed by CCK-8 assay. (D) Colony formation assay of PTC cells transfected with shNC or shSKP2. (E) DNA synthesis in *SKP2* knockdown PTC cells was detected using Ethynyl deoxyuridine (EdU) incorporation assay. (F) Apoptosis in PTC cells was detected using flow cytometric analysis. (G) *In vivo* tumor growth was evaluated by injecting SKP2 knockdown PTC cells in nude mice. (H) IHC assay was carried out to examine the levels of Ki-67 in xenograft tumors. $^{**}P < 0.01$.

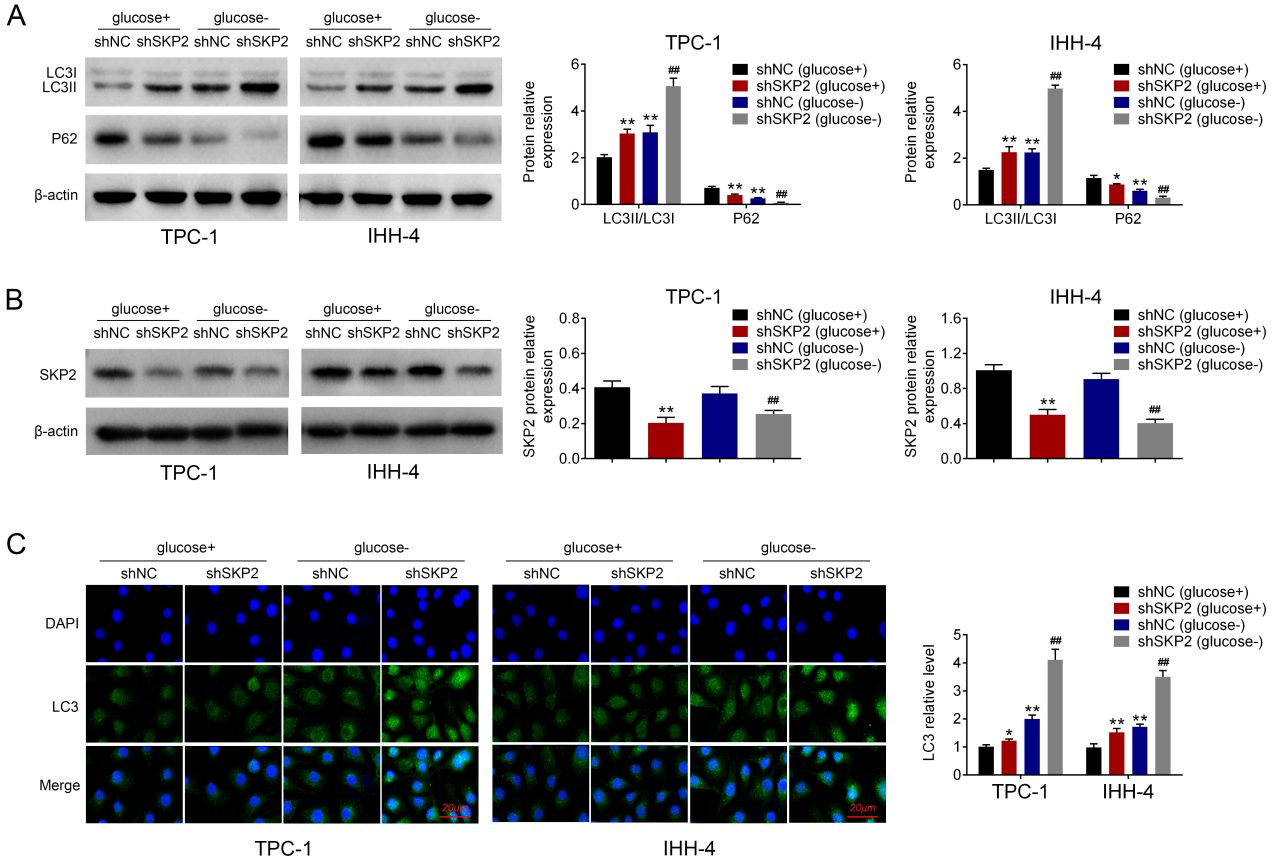

Fig. 3. *SKP2* knockdown facilitated PTC cell autophagy under glucose deprivation. PTC cells transfected with shNC or shSKP2 were cultured in normal glucose or glucose deprivation medium for 12 h; autophagosome markers (LC3I and LC3II), autophagic flux marker (p62) (A), and SKP2 (B) were detected by western blot. (C) Immunofluorescence assay was performed to examine the formation of LC3 puncta in PTC cells. $*P < 0.05$, $**P < 0.01$, vs shNC (glucose+), $^{\#\#}P < 0.01$, vs shNC (glucose-).

proteasome inhibitor (MG132), and the PHLPP1 expression was evaluated by western blot. As indicated in Fig. 5A, the PHLPP1 expression increased moderately over time and was independent of SKP2 expression, thus suggesting a proteasome-dependent degradation of PHLPP1. Next, the PTC cells were treated with a protein synthesis inhibitor (cycloheximide, CHX). A decrease in PHLPP1 expression was observed in cells treated with CHX. Similarly, in *SKP2* knockdown cells, a reduction of PHLPP1 expression was also observed, but to a lesser extent (Fig. 5B), which indicates enhanced stability of PHLPP1 protein on *SKP2* knockdown. In a review of these results, a ubiquitination assay was performed on two PTC cells to evaluate the ubiquitination of PHLPP1. An increase in ubiquitination of PHLPP1 was observed in TPC-1 cells transfected with scrambled shRNA compared to SKP2 knockdown cells (Fig. 5C, left, line 2). In contrast, a reduction in PHLPP1 ubiquitination of PHLPP1 was observed in *SKP2* knockdown cells (Fig. 5C, left, line 3). Similar results were observed in IHH-4 cells (Fig. 5C, right). Ubiquitination assay was performed in Flag-PHLPP1 and Myc-SKP2 was transfected in HEK-293T cells, and PHLPP1 ubiquitination was assessed using HA-lagged ubiquitin. An increase in PHLPP1 ubiquitination was observed in the presence of Myc-SKP2 (Fig. 5D). These results indicate that SKP2 mediated ubiquitination-dependent degradation of PHLPP1.

SKP2 mediated AKT activation via PHLPP1

PHLPP1 belongs to PH domain leucine-rich repeat protein phosphatase family. Increasing evidence has suggested the role of PHLPP1 in various cancers (Wang et al., 2013). Multiple studies suggest that PHLPP1 regulates the AKT pathway by dephosphorylating AKT (Chen et al., 2011; Nitsche et al., 2012). Therefore, it is likely that SKP2 may regulate the AKT pathway via PHLPP1. Hence, AKT and mTOR phosphorylation was assessed in *SKP2* knockdown PTC cells. As expected, a significant decrease in phospho-AKT and the phospho-mTOR level were observed in SKP2 knockdown compared to cells transfected with scrambled shRNA (Fig. 6A). Knockdown of *SKP2* or *PHLPP1* alone or in combination suggested the dephosphorylation of AKT and mTOR could be largely abrogated by PHLPP1 knockdown in PTC cells (Fig. 6B). In contrast with *SKP2* knockdown, *PHLPP1* knockdown increased cell proliferation (Fig. 6C). and decreased apoptosis in PTC cells (Fig. 6D). Further, the effect of SKP2 and PHLPP1 on autophagy was investigated under glucose deprivation. As shown in Figs. 6E and 6F, knockdown of *SKP2* promoted autophago-

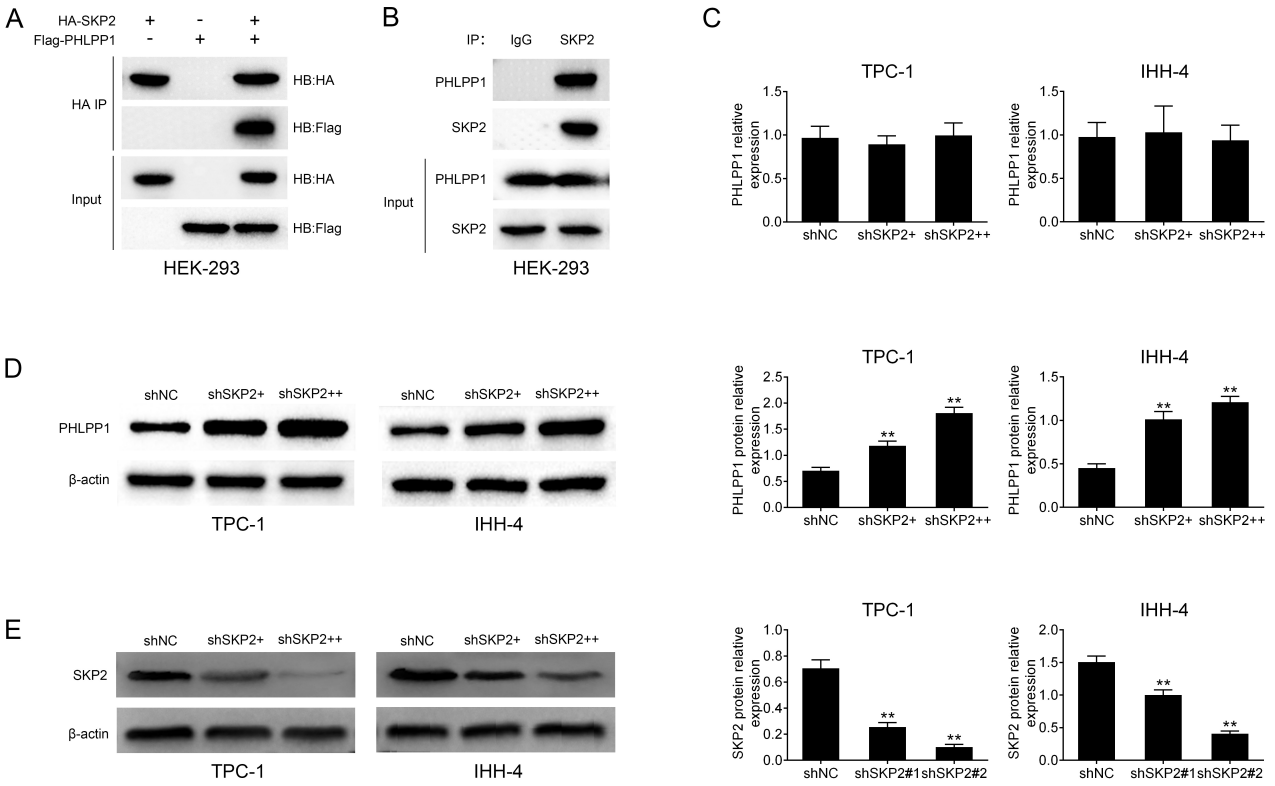

Fig. 4. SKP2 interacted with PHLPP1 and negatively regulated PHLPP1 protein expression. (A) HEK-293 cells were co-transfected with Myc-SKP2 and Flag-PHLPP1, followed by anti-HA immunoprecipitation. Further immunoblot was performed using specific antibodies. PTC cells were subjected to immunoprecipitation with IgG and SKP2 antibodies (B) and subsequent immunoblot with specific antibodies. mRNA expression (C), protein abundance (D) of PHLPP1 and SKP2 protein (E) in two PTC cells transfected with shNC or shSKP2. $**P < 0.01$.

some formation, whereas knockdown of *PHLPP1* depletion restricted autophagosome formation, which was assessed by the variation in LC3I/II and LC3 puncta. These results show that SKP2 negatively regulates PHLPP1, activating AKT and mTOR pathways, which influence cell fates, like cell proliferation, apoptosis, and autophagy.

AKT influences the subcellular localization of SKP2

The results indicate that the loss of SKP2 promotes autophagy without glucose. Hence, we next studied the mechanism associated with this phenomenon. *SKP2* expression under glucose deprivation was studied in PTC cells. As shown in Fig. 7A, no significant difference in *SKP2* expression was observed in PTC cells deprived of glucose compared to PTC cells cultured in a normal medium. Therefore, we next checked the localization of SKP2 in PTC cells. As shown in Fig. 7B, a more distinctive cytoplasmic localization of SKP2 was observed in PTC cells cultured in glucose deprivation compared to PTC cells cultured in a normal medium. Additionally, higher ubiquitination of PHLPP1 was observed in cells cultured in glucose deprived medium accompanied by more cytoplasmic localized SKP2 (Supplementary Fig. S4). Next, the impact of glucose starvation on SKP2 and PHLPP1 binding was evaluated. The results show a stronger binding of SKP2 and PHLPP1 was observed in PTC cells cultured in glucose deprived medium compared to PTC cells cultured in a normal medium (Supplementary Fig. S5). Previous studies show the reciprocal relationship between SKP2 and AKT in some cases. AKT phosphorylates SKP2, which leads to the cytoplasmic localization of SKP2 (Gao et al., 2009; Lin et al., 2009). Therefore, next, we assessed if reciprocal regulation exists between the two proteins during glucose deprivation. PTC cells treated with AKT inhibitor LY294002 cultured in glucose-deprived media showed cytoplasmic localization of SKP2 induced by glucose deprivation was reversed by AKT inhibition (Fig. 7C). Interestingly, glucose deprivation also led to a decrease in PHLPP1 expression, irrespective of SKP2 expression.

Similarly, an increase in expression of PHLPP1 was observed in *SKP2* knockdown cells cultured in glucose-deprived media compared to cells cultured in a normal medium (Fig. 7D). Further, the activation of AKT under normal or glucose deprivation n in the absence or presence of LY294002 (AKT inhibitor) was determined. The results indicated glucose deprivation mildly suppresses the AKT activity, and the administration of LY294002 under glucose deprivation entirely blocked the AKT activation (Supplementary Fig. S6). These results show a reciprocal correlation between SKP2 and AKT, which may further enrich our understanding of the molecular basis of SKP2 in PTC.

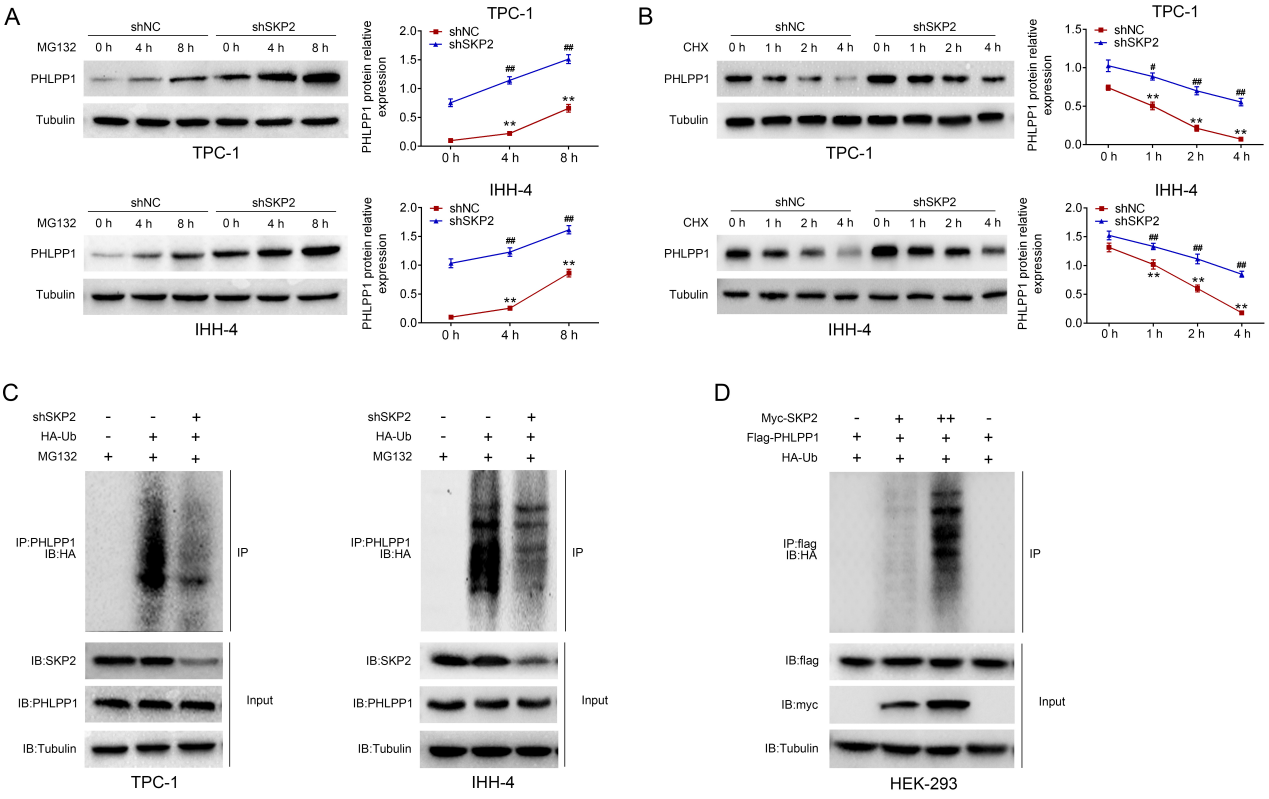

Fig. 5. *SKP2* promotes ubiquitination-dependent PHLPP1 degradation. (A) TPC-1 and IHH-4 cells transfected with shNC or shSKP2 were incubated with MG132 for the indicated time, and the PHLPP1 expression was detected by western blotting. (B) The PHLPP1 expression in TPC-1 and IHH-4 cells transfected with shNC or shSKP2 was detected after incubation with cycloheximide (CHX). TPC-1 and IHH-4 cells with indicated transfections were treated with MG132 for 6 h, and the ubiquitination of PHLPP1 was evaluated by western blotting (C). HEK-293 cells with indicated transfections were treated with MG132, and the ubiquitination of PHLPP1 was assessed by first immunoprecipitated against FLAG, then immunoblotting against HA was performed (D). $^{**}P < 0.01$, vs shNC (0 h), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs shSKP2 (0 h).

DISCUSSION

In this study, we show the overexpression of *SKP2* in PTC and its correlation with PTC prognosis. These results are consistent with the previous findings (Chiappetta et al., 2007; Pratheeshkumar et al., 2018). *In vitro* and *in vivo* studies reveal loss of *SKP2* has an antitumor effect in PTC. PHLPP1 was identified downstream mediator of SKP2, which negatively regulates the AKT pathway. Our results demonstrate that SKP2 regulates the AKT pathway via PHLPP1, and there is a reciprocal regulation between SKP2 and AKT. Further, the subcellular localization of SKP2 is dependent on AKT activation. These results show a regulatory loop between SKP2, PHLPP1, and AKT, extending the regulatory network of SKP2 in tumor progression.

SKP2 is widely known as the ubiquitin ligase subunit, involved in the substrate recognition by SCF complex and ubiquitin-dependent degradation of the proteins (Deshaies, 1999). Various proteins have been identified as the targets of SKP2, one of which is CDK inhibitor p27, which inhibits G1/S transition and promotes apoptosis (Yalcin et al., 2014). SCF-SKP2 complex recognizes and phosphorylated p27 at Thr-187 site (Bretones et al., 2011). Thus SKP2 triggered ubiquitin-dependent p27 degradation accelerates the cell cycle, leading to dysregulation in cell proliferation. SKP2 is also involved in post-transcriptional regulation of forkhead transcription factors; for instance, Akt-dependent phosphorylation of FOXO1at Ser-256 is mediated by SKP2 and degrades FOXO1 via ubiquitination (Huang et al., 2005). Further, FOXO3 undergoes deacetylation-dependent recognition by SKP2 and succedent ubiquitination (Wang et al., 2012a). Reports suggest SKP2 mediates ubiquitination and degradation of c-Myc. Meanwhile, SKP2 also acts as a transcriptional coactivator for c-Myc, which activates downstream genes (Kim et al., 2003; von der Lehr et al., 2003). This shows the dual role of SKP2 in c-Myc regulation, which suggests a peculiar feature of SKP2 in carcinogenesis. Interestingly, the role of SKP2-mediated ubiquitination extends beyond protein degradation. It also mediates YAP nuclear localization and Hippo-YAP pathway activation (Yao et al., 2018). Similarly, reports suggest SKP2 mediated polyubiquitination of LKB1 at K63 induced its binding to the STRAD-MO25 complex, which helps cell survival under stress (Lee et al., 2015). Interestingly, reports suggest membrane translocation of SKP2 activates AKT directly and leads to subsequent protumor signaling (Chan et al., 2012). Recent reports suggest PI3K independent activation of AKT,

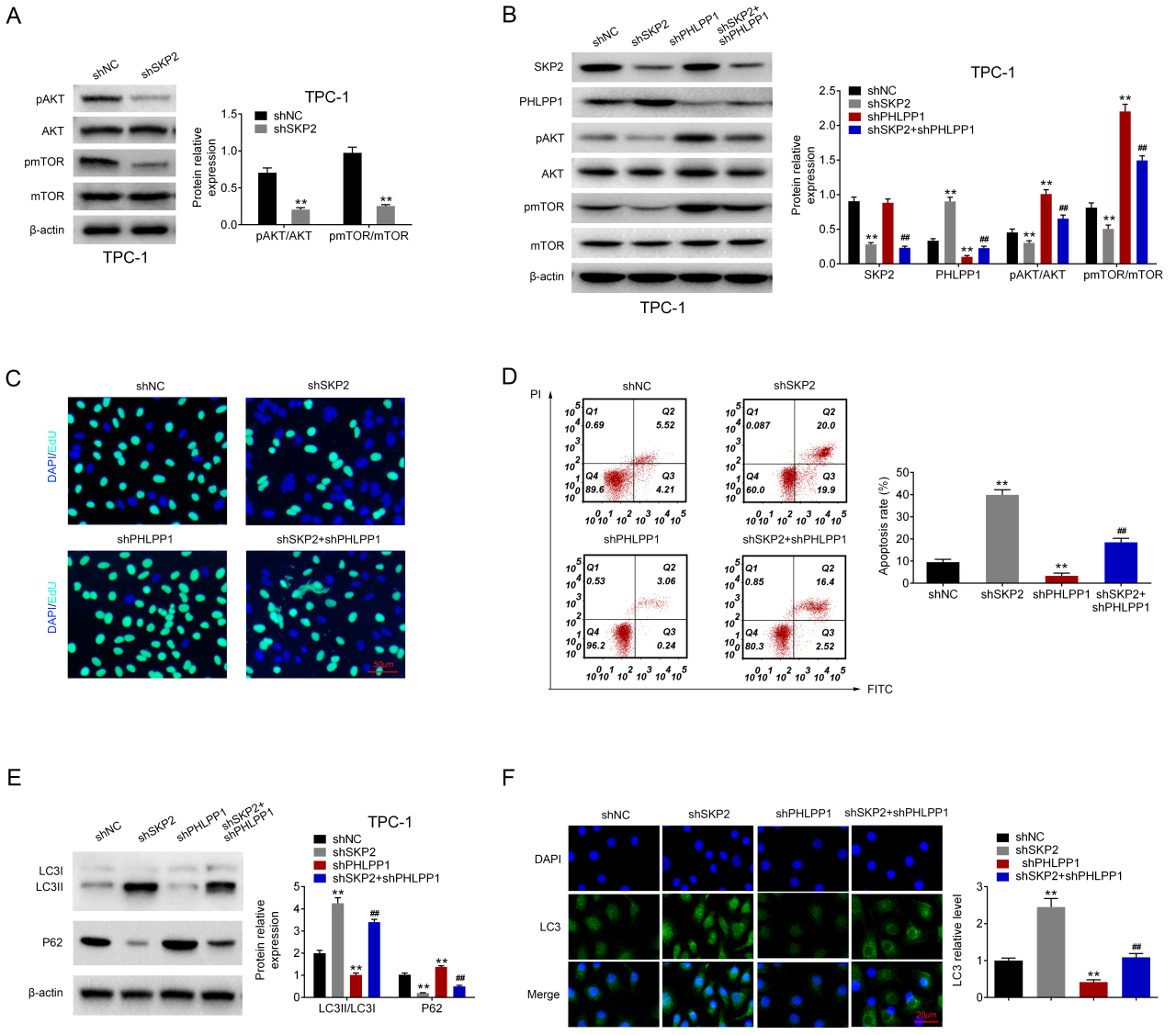

Fig. 6. PHLPP1 is involved in SKP2-mediated activation of AKT. (A) TPC-1 cells were transfected with shNC or shSKP2, and the expression of pAKT, AKT, pmTOR, and mTOR was determined using western blotting, $^{**}P < 0.01$. (B) TPC-1 cells were transfected with shSKP2 and shPHLPP1 alone or in combination, and the expression of pAKT, AKT, pmTOR, and mTOR was determined. The DNA synthesis (C), apoptosis (D), autophagosome and autophagic flux markers (E), and LC3 puncta (F) in TPC-1 transfected with shSKP2 and shPHLPP1 alone or in combination were examined using EdU incorporation assay, flow cytometry, western blotting, and immunofluorescence, respectively. $^{**}P < 0.01$, vs shNC, $^{\#\#}P < 0.01$, vs shPHLPP1.

triggered by SKP2-mediated ubiquitination (Clement et al., 2018). Additionally, SKP2-mediated ubiquitination activates K63 which further interacts with HK2. Notably, the ubiquitination of AKT leads to mitochondrial translocation induced by EGF (Yu et al., 2019b). In this study, we demonstrated that SKP2 acts as a proto-oncogene in PTC and subsequently identified PHLPP1as a substrate for SKP2-mediated ubiquitination.

PHLPP1 is a serine/threonine phosphatase and plays a crucial role in tumor suppression by inactivating AKT. There are three AKT isoforms, AKT1, AKT2, and AKT3, encoded by different genes (Moc et al., 2015). Among these three isoforms, AKT2 and AKT3 are believed to be targets of PHLPP1, as PHLPP1 regulates dephosphorylation of AKT2 and AKT3, but not Akt1 (Dong et al., 2014). For instance, in pancreatic cancers, PHLPP1 selectively dephosphorylated and negatively correlated with AKT1 (Nitsche et al., 2012). Similarly, in metastatic melanoma, the selective regulation of AKT2 by PHLPP1 was shown (Yu et al., 2018). Despite multiple reports suggesting an indirect relationship between PHLPP1 and AKT2, few reports show a direct regulation between PHLPP1 and AKT1. A decrease in PHLPP1 expression increased the phosphorylation of AKT1 at P473 in lung cancer cells (Zhiqiang et al., 2012). Another study demonstrated that AKT1 binds

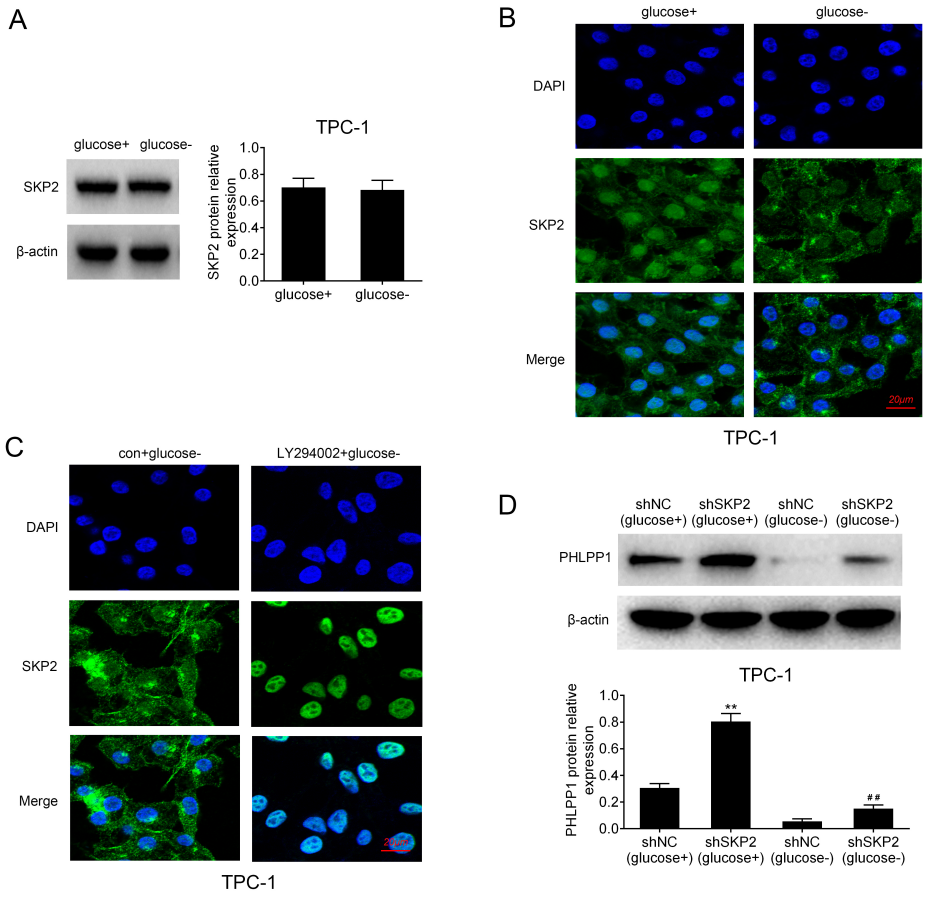

Fig. 7. Involvement of AKT in the subcellular localization of SKP2. (A) SKP2 expression in TPC-1 cells cultured in normal glucose or glucose deprivation. (B) The subcellular localization of SKP2 in TPC-1 cells cultured in normal glucose or glucose deprivation. (C) The subcellular localization of SKP2 in TPC-1 cells cultured in glucose deprivation in the absence or presence of AKT inhibitor, LY294002. (D) The protein expression of PHLPP1 in TPC-1 cells cultured in normal glucose or glucose deprivation medium with shNC or shSKP2 transfection. $^{**}P < 0.01$, vs shNC (glucose+), $^{\#\#}P < 0.01$, vs shNC (glucose-).

to PHLPP1, which was verified by an increase in PHLPP1 and Scrib complex expression. This binding was not observed with other AKT isoforms (Li et al., 2011). Additionally, the blocking of the PHLPP1 catalytic site by a specific inhibitor promoted phosphorylation of AKT1 at Ser473 under serum starvation, suggesting the role of PHLPP1 in chaperone-mediated autophagy (Arias et al., 2015). These contradictory results indicate an alternative mechanism regulating the AKT pathway via PHLPP1. This study revealed that SKP2 indirectly regulated the AKT pathway triggered by ubiquitin-induced degradation of PHLPP1. However, our results indicate that *PHLPP1* knockdown could partly rescue AKT phosphorylation and enhance autophagy induced by *SKP2* knockdown. Therefore, it is likely that other factors are involved in SKP2-mediated AKT activation and autophagy. For instance, SKP2 directly regulated AKT's ubiquitination and mitochondrial localization (Yu et al., 2019b). Besides, *PHLPP1* knockdown by shRNA could only partially reduce PHLPP1 expression in PTC cells. Therefore, it is likely that SKP2 could still mediate the activation of AKT via PHLPP1 in knockdown cells. We also demonstrated that SKP2 inhibited autophagy under glucose deprivation. Considering the well-recognized inhibitory role of AKT in autophagy, it is tempting to postulate that SKP2 exerts a protective effect on cell survival under stress by promoting AKT activation. As starvation triggers autophagy, that could mildly suppress the AKT activation. Further, the cytoplasmic localization of SKP2 is induced by glucose starvation, which contradicts our previous hypothesis. Therefore, it is likely that other mechanisms are involved in the starvation-triggered deactivation of the AKT pathway, which leads to SKP2 subcellular localization. However, additional studies are required to understand the mechanism.

It is intriguing to note that the relationship between SKP2 and AKT is multi-directional. Previous studies propose a reciprocal regulation between SKP2 and AKT (Chan et al., 2012; Lin et al., 2009). Earlier studies show that AKT's direct phosphorylation of SKP2 forms SCF complex and E3 ligase activity (Lin et al., 2009). Gao et al. (2009) have also described that AKT1, but not AKT2, phosphorylate SKP2 at Ser72. The Ser72 phosphorylation of SKP2 impedes its nuclear translocation and leads to its accumulation in the cytoplasm. Moreover, AKT1-mediated phosphorylation also endowed SKP2 resistance to degradation triggered by the APC-Cdh1 complex, enhanced SKP2 stability and cytoplasmic distribution. This mechanism may be responsible for the tumor progression (Gao et al., 2009). However, AKT1-mediated translocation of SKP2 to the cytoplasm raises questions about its role in cell cycle progression. The mechanism underlying the degradation of p27 via cytoplasmic SKP2 and subsequent dysregulates of the cell cycle remains elusive mainly (Zhang, 2010). Taking account of the proto-oncogenic role of SKP2, there is still much to be addressed.

Further, the association of AKT and SKP2 is established, where AKT regulates SKP2 activity indirectly. For instance,

deactivating AKT suppressed SKP2 expression via FOXO3A, the latter consequently contributed to the expression of p27 by transcriptional regulation as well as repressing SKP2 (Li et al., 2018). Moreover, AKT regulated SKP2 expression at the translational level, along with mTORC1 and eIF4E (Nogueira et al., 2012). Based on these results, we conclude that survival stress induced a subcellular translocation of SKP2, but not its expression, which could be reversed by AKT inhibition, implying a possible role of AKT in SKP2 regulation.

However, the study has some limitations. The role of SKP2 as an oncogene in PTC was verified by downregulating the expression of *SKP2* by shRNA targeting rather than overexpressing *SKP2*. Functional assays based on *SKP2* overexpression would further validate our findings. Additionally, the interaction between SKP2 and PHLPP1 was not thoroughly verified as SKP2 executes ubiquitination-dependent degradation mainly via forming a SCF complex; whether there is direct binding between SKP2 and PHLPP1 needs more investigation. Besides, loss of function mutation or deletion in SKP2 or PHLPP1 protein may help to build a more comprehensive understanding of the correlation between the two proteins. As discussed, PHLPP1 preferably dephosphorylates AKT2, not AKT1, and the latter is mainly responsible for the fate of SKP2 subcellular localization. There is some uncertainty as to which AKT isoforms are responsible for SKP2 nuclear-cytoplasmic transformation. Finally, previous studies show AKT could regulate SKP2 in several ways since our study merely focused on one possibility. It would be intriguing to establish other molecular mechanisms of AKT-mediated SKP2 pro-oncogenic properties and further the regulatory network.

In summary, we identified a novel role of the SKP2/PHLPP1/AKT axis in PTC progression, including cell proliferation and survival under starvation. Further, activation of AKT was investigated to support the cytoplasmic accumulation of SKP2, as inhibition of AKT led to SKP2 nuclear location under glucose deprivation. The results added to the established understanding of SKP2 and would help to develop targeting therapy for PTC.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by grants from the institutional Foundation of the First Affiliated Hospital of Xi'An Jiaotong University (No. 2019ZYTS-04 and No. 2019ZYTS-13), Xi'An Science and Technology Project (No. 2019114613YX-001SF04(5)), Clinical Research Center for Thyroid Diseases of Shaanxi Province (No. 2017LCZX-03), the Clinical Research Award of the First Affiliated Hospital of Xi'An Jiaotong University (XJTU1AF-CRF-2020-020), The Basic Natural Science Research Program of Shaanxi Province (2021JQ-386 and 2021JQ-405), the Thyroid Research Project of Young and Middle-aged Physicians of Beijing Bethune Charitable Foundation (BQE-JZX-202103), the Key Research and Development Program of Shaanxi Province (2022SF-159) and the National Natural Science Foundation of China (No. 82103568).

AUTHOR CONTRIBUTIONS

Y.S., W.R., H.D., and C.X. designed the study, supervised the data collection, analyzed the data. F.Y., X.L., and S.Z. interpreted the data, and prepared the manuscript for publication. J.L., X.Y., Q.Z., X.S., and Z.Z. supervised the data collection, analyzed the data and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Yuan Shao https://orcid.org/0000-0003-4812-4435
Wanli Ren https://orcid.org/0000-0001-8190-4244
Hao Dai https://orcid.org/0000-0002-7679-6709
Fangli Yang https://orcid.org/0000-0003-4805-8458
Xiang Li https://orcid.org/0000-0002-5147-8368
Shaoqiang Zhang https://orcid.org/0000-0002-4794-8259
Junsong Liu https://orcid.org/0000-0003-4954-1783
Xiaobao Yao https://orcid.org/0000-0003-1070-3044
Qian Zhao https://orcid.org/0000-0002-8169-7676
Xin Sun https://orcid.org/0000-0001-5909-5786
Zhiwei Zheng https://orcid.org/0000-0001-8377-7509
Chongwen Xu https://orcid.org/0000-0002-4147-0397

REFERENCES

Arias, E., Koga, H., Diaz, A., Mocholi, E., Patel, B., and Cuervo, A.M. (2015). Lysosomal mTORC2/PHLPP1/Akt regulate chaperone-mediated autophagy. Mol. Cell *59*, 270-284.

Aschebrook-Kilfoy, B., Ward, M.H., Sabra, M.M., and Devesa, S.S. (2011). Thyroid cancer incidence patterns in the United States by histologic type, 1992–2006. Thyroid *21*, 125-134.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263-274.

Bochis, O.V., Irimie, A., Pichler, M., and Berindan-Neagoe, I. (2015). The role of Skp2 and its substrate CDKN1B (p27) in colorectal cancer. J. Gastrointestin. Liver Dis. *24*, 225-234.

Bretones, G., Acosta, J.C., Caraballo, J.M., Ferrándiz, N., Gómez-Casares, M.T., Albajar, M., Blanco, R., Ruiz, P., Hung, W.C., Albero, M.P., et al. (2011). SKP2 oncogene is a direct MYC target gene and MYC down-regulates p27KIP1 through SKP2 in human leukemia cells. J. Biol. Chem. *286*, 9815-9825.

Chan, C.H., Lee, S.W., Wang, J., and Lin, H.K. (2010). Regulation of Skp2 expression and activity and its role in cancer progression. ScientificWorldJournal *10*, 1001-1015.

Chan, C.H., Li, C.F., Yang, W.L., Gao, Y., Lee, S.W., Feng, Z., Huang, H.Y., Tsai, K.K.C., Flores, L.G., Shao, Y., et al. (2012). The Skp2-SCF E3 ligase regulates Akt ubiquitination, glycolysis, herceptin sensitivity, and tumorigenesis. Cell *149*, 1098-1111.

Chen, M., Pratt, C.P., Zeeman, M.E., Schultz, N., Taylor, B.S., O'Neill, A., Castillo-Martin, M., Nowak, D.G., Naguib, A., Grace, D.M., et al. (2011). Identification of PHLPP1 as a tumor suppressor reveals the role of feedback activation in *PTEN*-mutant prostate cancer progression. Cancer Cell *20*, 173-186.

Chiappetta, G., De Marco, C., Quintiero, A., Califano, D., Gherardi, S., Malanga, D., Scrima, M., Montero-Conde, C., Cito, L., Monaco, M., et al. (2007). Overexpression of the S-phase kinase-associated protein 2 in thyroid cancer. Endocr. Relat. Cancer *14*, 405-420.

Cho, B.Y., Choi, H.S., Park, Y.J., Lim, J.A., Ahn, H.Y., Lee, E.K., Kim, K.W., Yi,

K.H., Chung, J.K., Youn, Y.K., et al. (2013). Changes in the clinicopathological characteristics and outcomes of thyroid cancer in Korea over the past four decades. Thyroid *23*, 797-804.

Clement, E., Inuzuka, H., Nihira, N.T., Wei, W., and Toker, A. (2018). Skp2-dependent reactivation of AKT drives resistance to PI3K inhibitors. Sci. Signal. *11*, eaao3810.

Craig, K.L. and Tyers, M. (1999). The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. Prog. Biophys. Mol. Biol. *72*, 299-328.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. *15*, 435-467.

Dong, L., Jin, L., Tseng, H.Y., Wang, C.Y., Wilmott, J.S., Yosufi, B., Yan, X.G., Jiang, C.C., Scolyer, R.A., Zhang, X.D., et al (2014). Oncogenic suppression of PHLPP1 in human melanoma. Oncogene *33*, 4756-4766.

Elisei, R., Molinaro, E., Agate, L., Bottici, V., Masserini, L., Ceccarelli, C., Lippi, F., Grasso, L., Basolo, F., Bevilacqua, G., et al. (2010). Are the clinical and pathological features of differentiated thyroid carcinoma really changed over the last 35 years? Study on 4187 patients from a single Italian institution to answer this question. J. Clin. Endocrinol. Metab. *95*, 1516-1527.

Fang, F.M., Chien, C.Y., Li, C.F., Shiu, W.Y., Chen, C.H., and Huang, H.Y. (2009). Effect of S-phase kinase-associated protein 2 expression on distant metastasis and survival in nasopharyngeal carcinoma patients. Int. J. Radiat. Oncol. Biol. Phys. *73*, 202-207.

Gao, D., Inuzuka, H., Tseng, A., Chin, R.Y., Toker, A., and Wei, W. (2009a). Phosphorylation by Akt1 promotes cytoplasmic localization of Skp2 and impairs APCCdh1-mediated Skp2 destruction. Nat. Cell Biol. *11*, 397-408.

Gassen, N.C., Niemeyer, D., Muth, D., Corman, V.M., Martinelli, S., Gassen, A., Hafner, K., Papies, J., Mösbauer, K., Zellner, A., et al. (2019). SKP2 attenuates autophagy through Beclin1-ubiquitination and its inhibition reduces MERS-Coronavirus infection. Nat. Commun. *10*, 5770.

Grogan, R.H., Kaplan, S.P., Cao, H., Weiss, R.E., Degroot, L.J., Simon, C.A., Embia, O.M.A., Angelos, P., Kaplan, E.L., and Schechter, R.B. (2013). A study of recurrence and death from papillary thyroid cancer with 27 years of median follow-up. Surgery *154*, 1436-1447.

He, J., Zhou, M., Li, X., Gu, S., Cao, Y., Xing, T., Chen, W., Chu, C., Gu, F., Zhou, J., et al. (2020). SLC34A2 simultaneously promotes papillary thyroid carcinoma growth and invasion through distinct mechanisms. Oncogene *39*, 2658-2675.

Huang, H., Regan, K.M., Wang, F., Wang, D., Smith, D.I., van Deursen, J.M.A., and Tindall, D.J. (2005a). Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl. Acad. Sci. U. S. A. *102*, 1649-1654.

Kakudo, K., Tang, W., Ito, Y., Mori, I., Nakamura, Y., and Miyauchi, A. (2004). Papillary carcinoma of the thyroid in Japan: subclassification of common type and identification of low risk group. J. Clin. Pathol. *57*, 1041-1046.

Kim, S.Y., Herbst, A., Tworkowski, K.A., Salghetti, S.E., and Tansey, W.P. (2003). Skp2 regulates Myc protein stability and activity. Mol. Cell *11*, 1177-1188.

Lee, S.W., Li, C.F., Jin, G., Cai, Z., Han, F., Chan, C.H., Yang, W.L., Li, B.K., Rezaeian, A.H., Li, H.Y., et al. (2015). Skp2-dependent ubiquitination and activation of LKB1 is essential for cancer cell survival under energy stress. Mol. Cell *57*, 1022-1033.

Li, C., Du, L., Ren, Y., Liu, X., Jiao, Q., Cui, D., Wen, M., Wang, C., Wei, G., Wang, Y., et al. (2019). SKP2 promotes breast cancer tumorigenesis and radiation tolerance through PDCD4 ubiquitination. J. Exp. Clin. Cancer Res. *38*, 76.

Li, F., Dong, X., Lin, P., and Jiang, J. (2018). Regulation of Akt/FoxO3a/Skp2 axis is critically involved in berberine-induced cell cycle arrest in hepatocellular carcinoma cells. Int. J. Mol. Sci. *19*, 327.

Li, X., Liu, J., and Gao, T. (2009). β-TrCP-mediated ubiquitination and degradation of PHLPP1 are negatively regulated by Akt. Mol. Cell. Biol. *29*, 6192-6205.

Li, X., Yang, H., Liu, J., Schmidt, M.D., and Gao, T. (2011). Scribble-mediated membrane targeting of PHLPP1 is required for its negative regulation of Akt. EMBO Rep. *12*, 818-824.

Lin, H.K., Chen, Z., Wang, G., Nardella, C., Lee, S.W., Chan, C.H., Yang, W.L., Wang, J., Egia, A., Nakayama, K.I., et al. (2010). Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. Nature *464*, 374-379.

Lin, H.K., Wang, G., Chen, Z., Teruya-Feldstein, J., Liu, Y., Chan, C.H., Yang, W.L., Erdjument-Bromage, H., Nakayama, K.I., Nimer, S., et al. (2009). Phosphorylation-dependent regulation of cytosolic localization and oncogenic function of Skp2 by Akt/PKB. Nat. Cell Biol. *11*, 420-432.

McNally, R.J., Blakey, K., James, P.W., Gomez Pozo, B., Basta, N.O., and Hale, J. (2012). Increasing incidence of thyroid cancer in Great Britain, 1976–2005: age-period-cohort analysis. Eur. J. Epidemiol. *27*, 615-622.

Moc, C., Taylor, A.E., Chesini, G.P., Zambrano, C.M., Barlow, M.S., Zhang, X., Gustafsson, Å.B., and Purcell, N.H. (2015). Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy. Cardiovasc. Res. *105*, 160-170.

Nakayama, K.I. and Nakayama, K. (2005). Regulation of the cell cycle by SCF-type ubiquitin ligases. Semin. Cell Dev. Biol. *16*, 323-333.

Nitsche, C., Edderkaoui, M., Moore, R.M., Eibl, G., Kasahara, N., Treger, J., Grippo, P.J., Mayerle, J., Lerch, M.M., and Gukovskaya, A.S. (2012a). The phosphatase PHLPP1 regulates Akt2, promotes pancreatic cancer cell death, and inhibits tumor formation. Gastroenterology *142*, 377-387.e375.

Nogueira, V., Sundararajan, D., Kwan, J.M., Peng, X.D., Sarvepalli, N., Sonenberg, N., and Hay, N. (2012). Akt-dependent Skp2 mRNA translation is required for exiting contact inhibition, oncogenesis, and adipogenesis. EMBO J. *31*, 1134-1146.

Pratheeshkumar, P., Siraj, A.K., Divya, S.P., Parvathareddy, S.K., Begum, R., Melosantos, R., Al-Sobhi, S.S., Al-Dawish, M., Al-Dayel, F., and Al-Kuraya, K.S. (2018). Downregulation of SKP2 in papillary thyroid cancer acts synergistically with TRAIL on inducing apoptosis via ROS. J. Clin. Endocrinol. Metab. *103*, 1530-1544.

Radke, S., Pirkmaier, A., and Germain, D. (2005). Differential expression of the F-box proteins Skp2 and Skp2B in breast cancer. Oncogene *24*, 3448-3458.

Rose, A.E., Wang, G., Hanniford, D., Monni, S., Tu, T., Shapiro, R.L., Berman, R.S., Pavlick, A.C., Pagano, M., Darvishian, F., et al. (2011). Clinical relevance of SKP2 alterations in metastatic melanoma. Pigment Cell Melanoma Res. *24*, 197-206.

Schüler, S., Diersch, S., Hamacher, R., Schmid, R.M., Saur, D., and Schneider, G. (2011). SKP2 confers resistance of pancreatic cancer cells towards TRAIL-induced apoptosis. Int. J. Oncol. *38*, 219-225.

Seki, R., Okamura, T., Koga, H., Yakushiji, K., Hashiguchi, M., Yoshimoto, K., Ogata, H., Imamura, R., Nakashima, Y., Kage, M., et al. (2003). Prognostic significance of the F-box protein Skp2 expression in diffuse large B-cell lymphoma. Am. J. Hematol. *73*, 230-235.

Sonoda, H., Inoue, H., Ogawa, K., Utsunomiya, T., Masuda, T.A., and Mori, M. (2006). Significance of skp2 expression in primary breast cancer. Clin. Cancer Res. *12*, 1215-1220.

von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K.I., et al. (2003). The F-Box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol. Cell *11*, 1189-1200.

Wang, F., Chan, C.H., Chen, K., Guan, X., Lin, H.K., and Tong, Q. (2012a). Deacetylation of FOXO3 by SIRT1 or SIRT2 leads to Skp2-mediated FOXO3 ubiquitination and degradation. Oncogene *31*, 1546-1557.

Wang, F., Jiang, C., Sun, Q., Yan, F., Wang, L., Fu, Z., Liu, T., and Hu, F. (2015).

miR-195 is a key regulator of Raf1 in thyroid cancer. Onco Targets Ther. *8*, 3021-3028.

Wang, Z., Gao, D., Fukushima, H., Inuzuka, H., Liu, P., Wan, L., Sarkar, F.H., and Wei, W. (2012b). Skp2: a novel potential therapeutic target for prostate cancer. Biochim. Biophys. Acta *1825*, 11-17.

Wang, Z., Shu, H., Wang, Z., Li, G., Cui, J., Wu, H., Cai, S., He, W., He, Y., and Zhan, W. (2013). Loss expression of PHLPP1 correlates with lymph node metastasis and exhibits a poor prognosis in patients with gastric cancer. J. Surg. Oncol. *108*, 427-432.

Yalcin, A., Clem, B.F., Imbert-Fernandez, Y., Ozcan, S.C., Peker, S., O'Neal, J., Klarer, A.C., Clem, A.L., Telang, S., and Chesney, J. (2014). 6-Phosphofructo-2-kinase (PFKFB3) promotes cell cycle progression and suppresses apoptosis via Cdk1-mediated phosphorylation of p27. Cell Death Dis. *5*, e1337.

Yao, F., Zhou, Z., Kim, J., Hang, Q., Xiao, Z., Ton, B.N., Chang, L., Liu, N., Zeng, L., Wang, W., et al. (2018). SKP2- and OTUD1-regulated non-proteolytic ubiquitination of YAP promotes YAP nuclear localization and activity. Nat. Commun. *9*, 2269.

Yokoi, S., Yasui, K., Mori, M., Iizasa, T., Fujisawa, T., and Inazawa, J. (2004). Amplification and overexpression of SKP2 are associated with metastasis of non-small-cell lung cancers to lymph nodes. Am. J. Pathol. *165*, 175-180.

Yu, H.I., Shen, H.C., Chen, S.H., Lim, Y.P., Chuang, H.H., Tai, T.S., Kung, F.P., Lu, C.H., Hou, C.Y., and Lee, Y.R. (2019a). Autophagy modulation in human thyroid cancer cells following aloperine treatment. Int. J. Mol. Sci. *20*, 5315.

Yu, X., Wang, R., Zhang, Y., Zhou, L., Wang, W., Liu, H., and Li, W. (2019b). Skp2-mediated ubiquitination and mitochondrial localization of Akt drive tumor growth and chemoresistance to cisplatin. Oncogene *38*, 7457-7472.

Yu, Y., Dai, M., Lu, A., Yu, E., and Merlino, G. (2018). PHLPP1 mediates melanoma metastasis suppression through repressing AKT2 activation. Oncogene *37*, 2225-2236.

Zhang, H. (2010). Skip the nucleus, AKT drives Skp2 and FOXO1 to the same place? Cell Cycle *9*, 861-869.

Zheng, W.Q., Zheng, J.M., Ma, R., Meng, F.F., and Ni, C.R. (2005). Relationship between levels of Skp2 and P27 in breast carcinomas and possible role of Skp2 as targeted therapy. Steroids *70*, 770-774.

Zhiqiang, Z., Qinghui, Y., Yongqiang, Z., Jian, Z., Xin, Z., Haiying, M., and Yuepeng, G. (2012). USP1 regulates AKT phosphorylation by modulating the stability of PHLPP1 in lung cancer cells. J. Cancer Res. Clin. Oncol. *138*, 1231-1238.