ORIGINAL RESEARCH

Polo-like kinase 2 acting as a promoter in human tumor cells with an abundance of TAp73

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Background: TAp73, a member of the p53 tumor suppressor family, is frequently overexpressed in malignant tumors in humans. TAp73 abundance and phosphorylation modification result in variations in transcriptional activity. In a previous study, we found that the antitumor function of TAp73 was reactivated by dephosphorylation in head and neck squamous cell carcinomas. Polo-like kinase 2 (PLK2) displayed a close relationship with the p53 family in affecting the fate of cells. Herein, we investigate the hypothesis that PLK2 phosphorylates TAp73 and inhibits TAp73 function.

Materials and methods: Head and neck squamous cell carcinoma cell lines and osteosarcoma cell lines were used as natural models of the different expression levels of TAp73. Phosphorylation predictor software Scansite 3.0 and the predictor GPS-polo 1.0 were used to analyze the phosphorylation sites. Coimmunoprecipitation, phosphor-tag Western blot, metabolic labeling, and indirect immunofluorescence assays were used to determine the interactions between PLK2 and TAp73. TAp73 activity was assessed by Western blot and reverse transcription polymerase chain reaction, which we used to detect P21 and PUMA, both downstream genes of TAp73. The physiological effects of PLK2 cross talk with TAp73 on cell cycle progress and apoptosis were observed by flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling assays.

Results: PLK2 binds to and phosphorylates TAp73. PLK2 phosphorylates TAp73 at residue Ser48 and prohibits TAp73 translocation to the nucleus. Additionally, PLK2 inhibition combined with a DNA-damaging drug upregulated p21 and PUMA mRNA expression to a greater extent than DNA-damaging drug treatment alone. Inhibiting PLK2 in TAp73-enriched cells strengthened the effects of the DNA-damaging drug on both G1 phase arrest and apoptosis. Pretreatment with TAp73-siRNA weakened these effects.

Conclusion: These findings reveal a novel PLK2 function (catalyzed phosphorylation of TAp73) which suppresses TAp73 functions. PLK2 promotes the survival of human tumor cells, a novel insight into the workings of malignant tumors characterized by TAp73 overexpression, and one that could speed the development of therapies.

Keywords: antitumor therapy, DNA damaging reagent, phosphorylation, PLK2, TAp73

Introduction

TAp73 is a member of the p53 family, the most important tumor-suppressing family, and has a structure similar to p53. It is able to activate some of the p53 target genes, such as p21 (a cell cycle inhibitor) and PUMA (a proapoptosis gene), which regulate cell survival.[1] TAp73 is frequently overexpressed in carcinomas and sarcomas[1] and is, under certain conditions, an accepted marker of malignant tumors. These mechanisms are not yet completely clear. Among the inconsistencies, we noted differences in expression levels of TAp73 between human tumor cell lines such as head and neck squamous cell carcinoma (HNSCC) cell lines originating from epithelial carcinoma

and osteosarcoma cell lines originating from mesenchymal sarcoma.

Previous studies have demonstrated a close interaction between the p53 family and the Polo-like kinase (PLK) family in a number of human tumors, including osteosarcoma. However, the mechanisms, as well as the effects, remain to be elucidated. Since Polo-like kinase 2 (PLK2) was first reported in 1991,[2] five members of the PLK family (PLKs 1–5) have been reported.[3] PLKs play a key role in the regulation of cell cycle progression. Although many studies have determined that PLK1 plays a prosurvival role in human tumors,[4,5] controversy still surrounds the functions of PLK2.[6,7] We identified several phosphorylation sites on TAp73 using the PhosphoSite database. Interestingly, these TAp73 phosphorylation sites appear to have differing functions in certain cells. As is well known, the reversible protein phosphorylation mediated by phosphokinase helps harmonize a large number of principal cell processes.[8–10] We have previously demonstrated that this kinase 2 (CK2) phosphorylates and inhibits the tumor-suppressing function of TAp73 in HNSCCs.[11] Prior to our study, Songyang et al[12] found that CK2 displays a consensus sequence identical to PLK2. More recently, studies have demonstrated that PLK2 can recognize substrates similar to CK2 even more efficiently.[13,14] We speculated that PLK2, similar to CK2, may have a kinase-like function in its effect on TAp73.

We conducted this research so as to determine whether PLK2 activates or deactivates TAp73.

Materials and methods
Bioinformatics analysis
We used the online bioinformatics phosphorylation predictor Scansite 3.0 (http://scansite3.mit.edu) to predict the phosphorylation sites of TAp73.[15] The predictor GPS-polo 1.0 was used to identify PLK binding and phosphorylation sites in TAp73.[16] The known phosphorylation sites in TAp73 were collected from www.PhosphoSitePlus.org.[17]

Cell lines
We used four human tumor cell lines such as osteosarcoma cell lines Saos2 and MG63 (ATCC), UM-SCC1, and UM-SCC22A (squamous cell carcinoma cell lines were previously described in our research)[18] from the University of Michigan. The Ethical Commission of South Medical University stated that these cell lines are commercialized and can be purchased from many cell banks; thus, ethical permission was not required for the use of these cell lines in this study. Cells were cultured in McCoy's 5A medium (Thermo Fisher

Scientific, Waltham, MA, USA) or Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. The medium was replaced with fresh medium as necessary, and the cultures were maintained at 37°C in the presence of 5% CO_2.

Transfection
Forty-eight hours before the experiments, the small interfering RNA (200 pmol of siTAp73 or siPLK2; Thermo Fisher Scientific Dharmacon Products, Lafayette, CO, USA) and appropriate expression vectors (20 µg of Flag-pcDNA3-PLK2, Flag-pcDNA3-TAp73, or Flag-pcDNA3-TAp73S48A, obtained from Dr Zhi-Min Yuan, Harvard University, USA) were transfected into cells as described previously[19] using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) following the manufacturer's protocols.[11] The TAp73 S48A mutant, in which Ser-48 was substituted with Ala (GENEWIZ, Inc., USA), was confirmed by DNA sequencing. Empty pcDNA3 (Thermo Fisher Scientific) and control siRNA (contr-si) were used as control.

Drug treatment and antibodies
Cells were seeded at a density of 60%–70% and cultured for 24 hours before 12-hour drug treatments. DNA-damaging reagents, cisplatin (CDDP, Qiru Corp., People's Republic of China), adriamycin (ADM, Sigma-Aldrich, St Louis, MO, USA), and ifosfamide (Sigma-Aldrich), were diluted to the indicated concentrations in cell culture medium. The PLK2 inhibitor ELN582646[20,21] (Elan Pharmaceuticals, San Francisco, CA, USA) was diluted to 5 µg/mL in cell culture medium. Dimethyl sulfoxide was used as a control. The following antibodies were used for Western blot (WB) and coimmunoprecipitation (co-IP): anti-PLK2 (EPR10070, ab154794, Abcam, USA), anti-TAp73 (38C674.2, ab79078, Abcam, USA), anti-PUMA (4976S, CST, MA, USA), anti-p21 (SC-397, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Flag (F3165, Sigma-Aldrich), and anti-β-actin (A5441, Sigma-Aldrich). The following antibodies were used for immunofluorescence assay: primary antibodies against PLK2 (EPR10070, ab154794, Abcam, MA, USA) and TAp73 (1E8, ab118985, Abcam, USA) and secondary antibodies against PLK2 (anti-rabbit 594, ZF-0416, ZSGB-BIO, People's Republic of China) and TAp73 (anti-mouse-488, ZF-0412, ZSGB-BIO, People's Republic of China).

Real-time reverse transcription polymerase chain reaction
Reverse transcription polymerase chain reaction (RT-PCR) was performed as described in our previous research.[11,18]

Briefly, the isolated RNA was subjected to reverse transcription and PCR, and β-actin mRNA was used as an internal control. The relative gene expression levels ($2^{-\Delta\Delta Ct}$) were normalized. Table 1 shows the primer and siRNA sequences.

Coimmunoprecipitation, Western blot, and Phos-tag Western blot

Co-IP and WB were performed as described in our previous research.[11,18] Phosphorylated TAp73 was tested by Phos-tag WB, as previously described.[22] Briefly, cell lysates were harvested using 1× RIPA (radioimmunoprecipitation assay) lysis buffer (0.5% Nonidet P-40, 50 mM NaCl containing protease inhibitors). The protein concentration was determined by protein assay reagents (Bio-Rad Laboratories Inc., Hercules, CA, USA) and spectrophotometer quantification by Beckman DU-640 (Beckman Coulter, Pasadena, CA, USA). Total protein (25 µg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a pure nitrocellulose membrane. Primary antibodies (1:500) were incubated in Tris-buffered saline (pH 7.4) with 0.1% Tween-20 at 4°C for 12 hours. Signals were generated by horseradish peroxidase-conjugated secondary antibodies (1:2,000; Bio-Rad Laboratories Inc.) in Tris-buffered saline (pH 7.4) with 5% nonfat milk and 0.1% Tween-20 at room temperature for 1 hour. Immunoblots were detected. For immunoprecipitation, cell lysate (500 µg) was incubated at 4°C for 12 hours with TAp73 (1:100), PLK2 (1:200), flag (1:200) antibody, 20 µL protein G+/protein A agarose (Oncogene Research Products), or anti-flag affinity agarose gel (Sigma-Aldrich). The immunocomplex subsets were subjected to SDS-PAGE and immunoblotting. For TAp73 Phos-tag WB, the immunocomplex subsets were subjected to SDS-PAGE in which gels were made using a Phos-tag™ Acrylamide (50 µmol/L; Wako Pure Chemical Industries, Ltd., Osaka, Japan), containing $MnCl_2$ (30 µmol/L), and then transferred to membranes and immunoblotted with TAp73 antibody. For testing exogenous TAp73 phosphorylate, both TAp73 and PLK2 immunocomplex subsets (ratio 1:5) from an anti-flag affinity agarose gel were incubated together for indicated times at 37°C in kinase buffer. The reactions were stopped using 2× loading buffer and the mixtures were used for phos-tag WB, as described earlier.

Indirect immunofluorescence microscopy

Indirect immunofluorescence experiments were performed according to the experimental protocols in the previous research.[23] Briefly, UM-SCC1 cells with or without transfection cultivated on coverslips in six-well plates were treated with ADM for 2 hours and then rinsed with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde at room temperature for 20 minutes. They were washed again with PBS and blocked with 3% bovine serum albumin (Sigma-Aldrich) for 10 minutes. Cells were then incubated with primary antibody (1:100) at room temperature for 60 minutes, washed three times using PBS, and then incubated with secondary antibody (1:200) at room temperature for 50 minutes. After a final PBS wash, cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, UK) for 10 minutes, and the coverslips were mounted using Dako Fluorescence Mounting Medium (Dako Denmark A/S, Glostrup, Denmark). At least 200 cells were observed overall within discrete areas of each sample, and the samples were prepared from three independent wells of examined cell lines. Samples were observed using an Olympus BH2-RFCA microscope and an Olympus LSM-GB200 confocal microscope.

Metabolic labeling of PLK2 assay

To determine the half-life of endogenous PLK2, pulse-chase experiments were performed in Saos2 cells following previously reported protocols.[24] Briefly, 2.5 mL of Saos2, transfected Saos2, or TAp73-Knockdown Saos2 cells (5×10^5 mL) per well was planted in six-well plates, which were then cultivated for 24 hours. Total protein was collected after cultivation in methionine-free medium containing 100 µCi/mL trans ^{35}S-labeled methionine for 30 minutes. The newborn PLK2 from Saos2 and TAp73-knockdown Saos2 cells, stimulated by ADM (25 µg/mL) at indicated time points, was harvested to SDS-PAGE and radioautography, respectively.

Table 1 The siRNA and PCR primer sequence

Item	Gene	Sequence
Primer	*PLK2*	Forward, 5′-ATCACCACCATTCGCACTCG-3′; reverse, 5′-AAATGGGGGCCTCCCTAGTA-3′
	TAp73	Forward, 5′-CCATCAAGGAGGAGTTCACGGA-3′; reverse, 5′-TCGGTGTTGGAGGGGATGACA-3′
	p21	Forward, 5′-AGCGACCTTCCTCATCCACC-3′; reverse, 5′-AAGACAACTACTCCCAGCCCCATA-3′
	puma	Forward, 5′-TCTCCTCTCGGTGCTCCTTCACT-3′; reverse, 5′-ACGTTTGGCTCATTTGCTCTTCA-3′
	β-actin	Forward, 5′-ACGTGGACATCCGCAAAG-3′; reverse, 5′-GACTCGTCATACTCCTGCTTG-3′
siRNA	*PLK2*	Sequence (549–571): 5′-UCUUUGUCAAUCUUUU CCCUUGGGAAAAGAUUGACAAAGAAA-3′
	TAp73	Sequence (4,927–4,949): 5′-AUUAAAGUGCUUU AACUGGUACCAGUUAAAGCACUUUAAUGC-3′

Abbreviations: siRNA, small interfering RNA; PCR, polymerase chain reaction; PLK2, Polo-like kinase 2.

Cell cycle analysis

Flow cytometric analysis of the cell cycle phase was conducted as described previously.[25] Briefly, cell samples were washed in PBS, trypsinized, harvested by centrifugation, washed in PBS, and fixed in ethanol (50%) overnight at 4°C. Cells were harvested and resuspended in PBS. Fifty microliters of RNAase (100 µg/mL) was added to each sample and they were then incubated for 30 minutes at 37°C. Thereafter, propidium iodide (2 mg/mL) was added to each sample, and they were analyzed by flow cytometry (Becton Dickinson Coulter Epic XL flow cytometer), where over 10,000 events were recorded.

Apoptosis assay

Cell apoptosis was assessed according to the manufacturer's instructions provided in the TUNEL kit (a terminal deoxynucleotidyl transferase dUTP nick end labeling kit; Hoffman-La Roche Ltd., Basel, Switzerland).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between two groups were compared using either a chi-squared test or a two-tailed Student's t-test. Differences between more than two groups were analyzed using one-way analysis of variance in conjunction with Dunnett's post hoc test or a chi-squared test, and for repeated measurements, analysis of variance was used. Each sample was assayed in triplicate. Differences were considered significant when $P<0.05$.

Results

Theoretically, Ser48 within TA1 domain of TAp73 is the most feasible target after integrating predictor software and online data

The Scansite 3.0 program provides predictions for all of the known kinases that can phosphorylate a given site. TAp73 was analyzed as a substrate. A total of 21 sites were identified under matching with high thresholds (Figure S1A).

TAp73 was also considered as a substrate using the software GPS-polo 1.0 based on the high threshold match. The results predicted PLKs that could phosphorylate a given site. A total of two binding and four phosphorylation sites were identified (Figure S1B). Table 2 summarizes the predicted sites distributed in TAp73 domains. Integrating the aforementioned predictions with the PLK2 functions and characteristics, the residue Ser48 within the TA1 domain of TAp73 was deemed to have the most potential.

There are 14 additional known phosphorylation sites in TAp73, according to the PhosphoSitePlus database (Figure 1). Our analysis of these known sites indicates that a phosphorylation site within the TA domain would negatively regulate TAp73 function (Table 3). Accordingly, we hypothesized that PLK2 phosphorylates the Ser48 residue of TAp73 and inhibits TAp73 transcriptional functionality.

PLK2 binds to enriched TAp73

To test our hypothesis, co-IP experiments between endogenous PLK2 and TAp73 were conducted in human tumor cells to determine whether PLK2 and TAp73 are capable of physically binding to each other. UM-SCC cell lines (UM-SCC1 and UM-SCC22A) originate from epithelial tumor cells, whereas osteosarcoma cell lines (Saos2 and MG63) originate from mesenchymal tumor cells. Counter to our expectation, co-IP between PLK2 and TAp73 was negative in Saos2 and UM-SCC1 under normal culture conditions (Figure 2A). However, PLK2 does bind directly to TAp73 in MG63 and UM-SCC22A. In addition, the process was also apparent in Saos2 and UM-SCC1 when pretreated for 12 hours with the DNA-damaging drugs CDDP or ADM (Figure 2B). This was surprising because we previously found high levels of TAp73 in UM-SCC 22A cell lines and low levels of TAp73 in UM-SCC1 cell lines.[18] We measured the expression level of TAp73 in these cells under normal culture conditions and found that MG63 was similar to UM-SCC22A, expressing a high level of TAp73 compared

Table 2 Distribution of predicted sites from Scansite 3.0 and GPS 1.0

Domain	Predicted sites by Scansite 3.0 (sum: 21 sites)	Predicted sites by GPS 1.0 (sum: six sites)
TA1	T13*, Y28, S48*	S48
Gap1	S81, T86, Y99, P113	
DBD	T141, S145, I275#, S289, I294#	T136#, T167#
OD	Y356	
TA2		
Gap2	Y407, S412, S471	
SAM	T524, T540	
Other	P335, T568, T593	S319, S467, T631

Notes: *Represents the sites matched to acidic kinases. #Represents sites matched to protein binding.
Abbreviations: TA, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile-alpha motif domain.

TAp73 (human)–636 amino acids
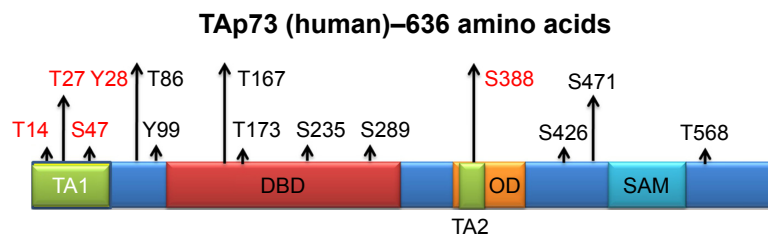
Figure 1 Known phosphorylation sites in TAp73.
Notes: The 14 known sites collected from www.PhosphoSitePlus.org.[56] Phosphorylation at the sites of residues within the TA domains (in red) had a negative effect on TAp73 function. Excepting site S235, phosphorylation at the sites in domains other than TA (in black) had a positive effect on TAp73 function. TA, DBD, OD, and SAM are different function domains in TAp73. Adapted with permission from PhosphoSite Plus®, www.PhosphoSitePlus.org.[56]
Abbreviations: TA, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile-alpha motif domain.

with Saos2, a result that was similar to the levels found in UM-SCC1 (Figure 3A and B).

Previous research has demonstrated that DNA damage upregulates endogenous TAp73[26] and PLK2[27] expression. We determined that DNA-damaging drugs significantly upregulated both TAp73 and PLK2 in a dose-dependent manner (Figure 3C). We then applied, at random, the DNA-damaging drugs CDDP, ADM, and ifosfamide to stimulate upregulation of endogenous TAp73 and PLK2. Our co-IP results indicated that PLK2 binds to TAp73 in cells with high levels of TAp73.

PLK2 does catalyze TAp73 phosphorylation at Ser48 residue

We investigated whether PLK2 was able to phosphorylate TAp73 protein via Phos-tag™ WB assay. We tested this in UM-SCC1 cells with low expression levels of TAp73. Endogenous phosphorylation of TAp73 in UM-SCC1 clearly occurred when cells whose Tap73 was lowered by PLK2 inhibition were stimulated using CDDP or ADM (50 µg/mL). We confirmed these results in MG63 cells with high expression levels of TAp73. In fact, in MG63 cells treated with or without DNA-damaging drugs, PLK2 inhibition using siRNA or the selective inhibitor ELN582646 reduced endogenous phosphorylation of TAp73 (Figure 4A). Because many kinases promote TAp73 phosphorylation, an in vitro phosphorylation assay was performed. Flag-PLK2 and Flag-TAp73 were detected (Figure 4B), purified, and then incubated together in kinase buffer for the indicated amount of time. Identical amounts of these samples were

subjected to Phos-tag WB assay, which demonstrated that ectopic Flag-TAp73 phosphorylation occurred and also increased in a time-dependent way (Figure 4C). To the contrary, we also found that phosphorylation levels were close to zero in purified mutant TAp73 (S48A) incubated with Flag-PLK2 when it was subjected to Phos-tag™ WB assay (Figure 4D).

PLK2 suppresses the transcriptional activity of enriched TAp73

Although previous experiments have shown that PLK2 phosphorylates higher levels of TAp73, it remains to be determined whether or not the transcriptional activity of TAp73 is suppressed. As mentioned earlier, two of the main target genes of TAp73 are p21 and PUMA, which are related to the cell cycle and cell apoptosis. We used RT-PCR to assess the transcriptional activity of TAp73 in MG63 and Saos2. The mRNA expression levels of p21 and PUMA increased in response to PLK2 inhibition in all cells treated with CDDP or ADM (Figure 5A–D). In MG63 cells, PLK2 inhibition alone was able to increase the mRNA expression levels of p21 and PUMA (Figure 5C and D). Moreover, the increases in p21 and PUMA expression were greater in the copresence of PLK2 inhibition and DNA-damaging drugs when compared with the increase in the presence of DNA-damaging drugs alone, and this increase was markedly attenuated by pretreatment with siTAp73. However, the PLK2-dependent increase in p21 and PUMA expression did not occur in Saos2 cells that are left untreated with DNA-damaging drugs (Figure 5A and B).

Table 3 Distribution of total 14 known phosphorylation sites

Domain	TA1	Gap1	DBD	OD	TA2	Gap2	SAM	Other	Sum
Sum	4	2	4	0	1	2	0	1	14
Positive or negative	N	P	P or N		N	P		P	

Notes: Gap1 refers to the area between the TA1 and DBD domains. Gap2 refers to the area between the TA2 and SAM domains. "Negative" refers to negative regulation of TAp73 functions, and "positive" refers to positive regulation of TAp73 functions. Adapted from PhosphoSite Plus®, www.PhosphoSitePlus.org.
Abbreviations: TA, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile-alpha motif domain; N, negative; P, positive.
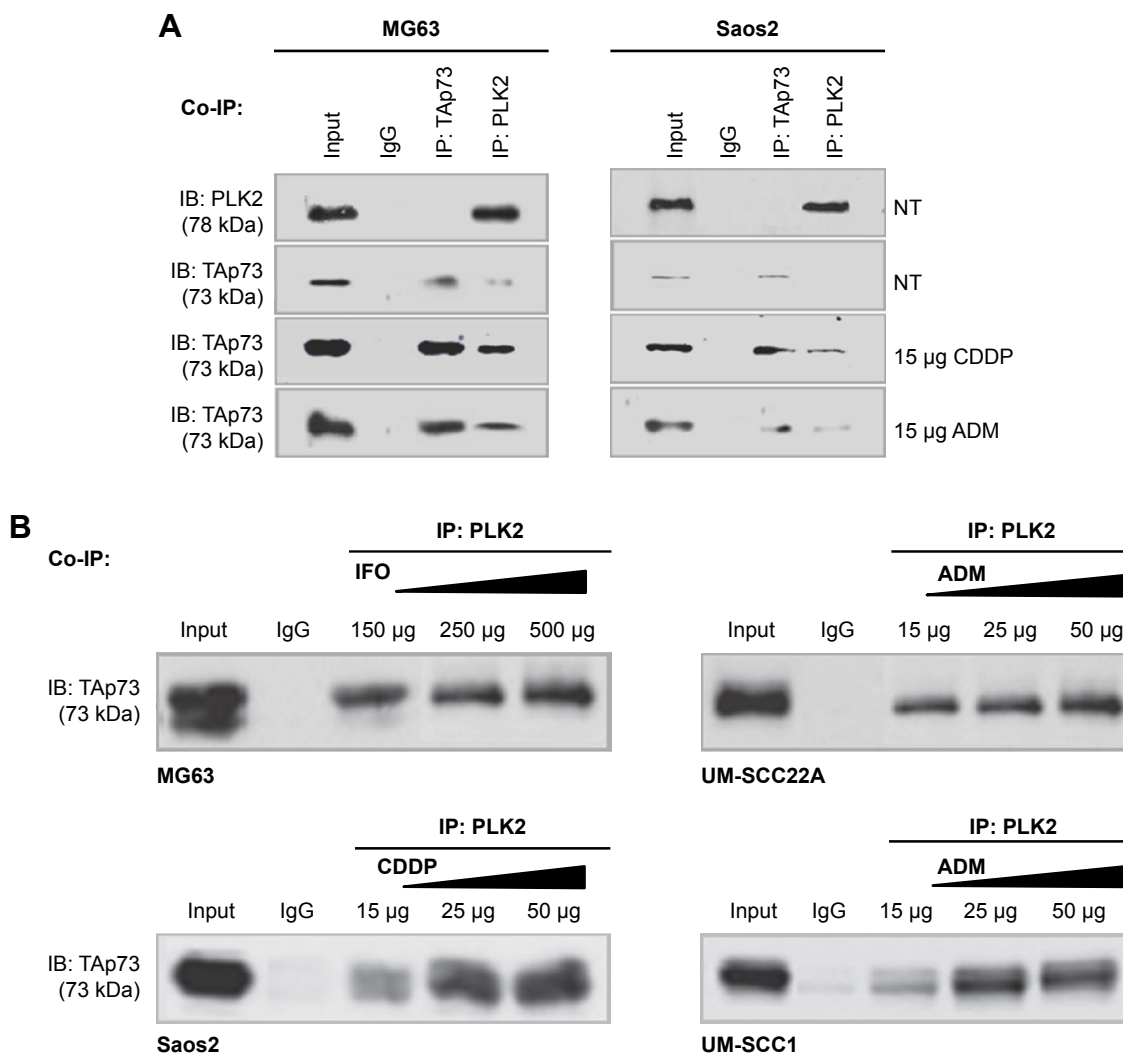
Figure 2 PLK2 binds to TAp73 depending on the expression level of TAp73.
Notes: (**A**) Normally, PLK2 binds to TAp73 in MG63 cells expressing high levels of TAp73 and does not bind to Saos2 cells expressing low levels of TAp73. (**B**) After stimulation with moderate doses of DNA-damaging drugs, PLK2 binds to TAp73 in MG63, Saos2, UM-SCC-22A, and UM-SCC1 cells.
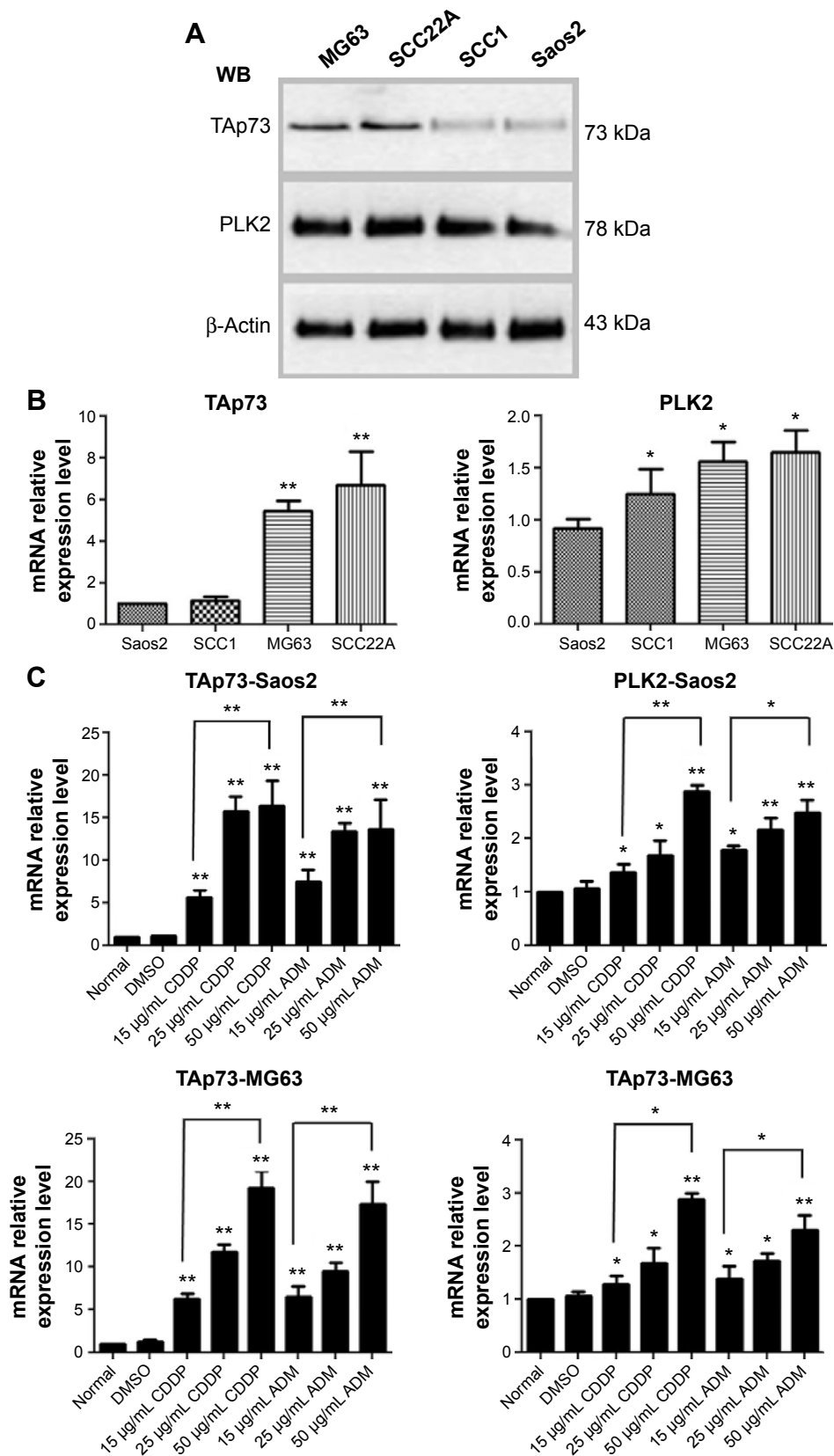**Abbreviations:** Co-IP, coimmunoprecipitation; IP, immunoprecipitation; IB, immunoblot; PLK2, Polo-like kinase 2; MG63, MG63 cell; SCC22A, UM-SCC-22A cell; SCC1, UM-SCC-1 cell; Saos2, Saos2 cell; NT, no treatment; CDDP, cisplatin; ADM, adriamycin; IFO, ifosfamide.

The levels of protein expression of p21 and PUMA in MG63 were also tested by WB (Figure 6A). The resultant levels were similar to that of mRNA. A further immunofluorescence confocal microscopy experiment on UM-SCC1 treated with 50 µg/mL ADM showed that PLK2 interferes with TAp73 translocation to the nucleus (Figure 7). These results suggest that PLK2 suppresses TAp73 transcriptional activity even when there is a high level of TAp73.

Cross talk between PLK2 and TAp73 proteins occurs at a posttranslational level

A previous study reported that the TAp73 family protein p53 can directly transcribe PLK2 expression under certain conditions. However, whether or not TAp73 directly transcribes PLK2 expression remains unclear. When compared with cells under normal conditions, PLK2 mRNA expression did not vary when TAp73 was either overexpressed or silenced by siRNA in MG63 and Saos2 (Figure 5E) and vice versa (Figure 5F), indicating that TAp73 does not directly transcribe PLK2 mRNA expression. Likewise, PLK2 expression did not regulate TAp73 mRNA expression. We recorded similar results in HNSCC cell lines (data not shown). Thus, PLK2 and TAp73 do not directly cross talk at gene transcriptional and translational levels (Figures 5 and 6). Since PLK2 is able to phosphorylate TAp73 at the posttranslational level, we considered whether TAp73 had the capability directly to affect PLK2 at a posttranslational level. We measured nascent PLK2

Figure 3 Expression levels of TAp73 and PLK2 under different conditions.
Notes: (**A** and **B**) Under normal culture conditions, high levels of TAp73 at the protein and mRNA levels appeared in MG63 and SCC22A cells compared with Saos2 and SCC1 cells. (**C**) TAp73 and PLK2 mRNA expression levels were proportional, in a DNA-damaging drug dose-dependent manner, in cells treated with drugs for 2 hours. *$P<0.05$; **$P<0.01$ versus the experimental value. Samples were tested at least in triplicate.
Abbreviations: WB, Western blot; PLK2, Polo-like kinase 2; MG63, MG63 cell; SCC22A, UM-SCC-22A cell; SCC1, UM-SCC-1 cell; Saos2, Saos2 cell; normal, under normal culture; DMSO, dimethyl sulfoxide; CDDP, cisplatin; ADM, adriamycin.
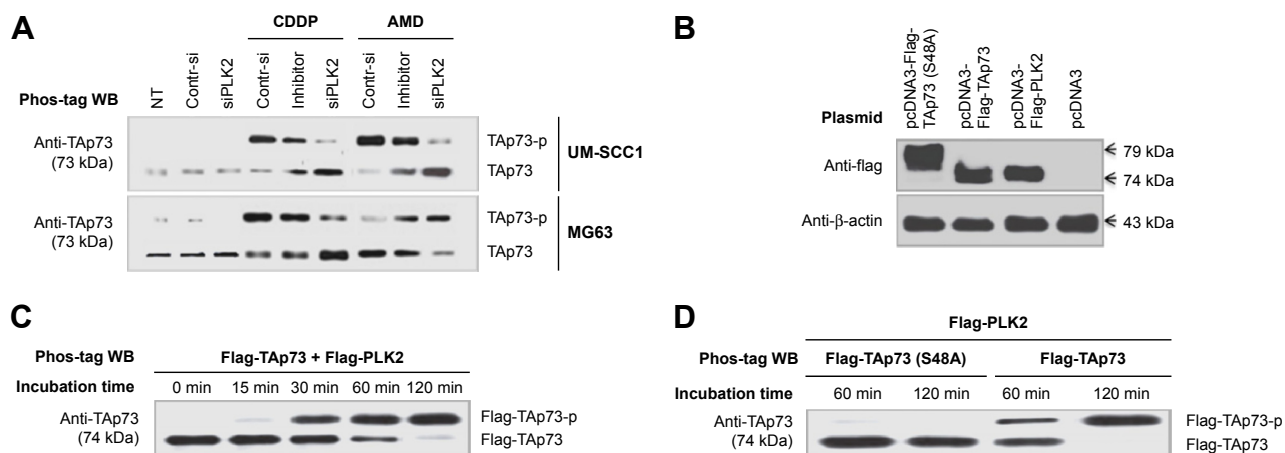
Figure 4 PLK2 phosphorylates TAp73 at Ser48.

Notes: (**A**) Phosphorylation of endogenous TAp73 was observed in cell samples with an abundance of TAp73. TAp73 phosphorylation was prohibited by PLK2 inhibition. DNA-damaging drugs used for upregulating TAp73 were administered at a concentration of 50 μg/mL and treatment lasted 8 hours. (**B**) After plasmid transfection for 48 hours, ectopic PLK2, TAp73, and TAp73 (S48A) were tested. (**C**) In Phos-tag™ WB assays, phosphorylation of exogenous Flag-TAp73 occurred at the indicated time points in 2 hours after incubation with Flag-PLK2. (**D**) Only nonphosphorylated protein was observed when analyzing TAp73 point mutant Flag-TAp73 (S48A) at the indicated time points after incubation with Flag-PLK2.

Abbreviations: WB, Western blot; NT, no treatment; Contr-si, control siRNA; siPLK2, PLK2-siRNA; PLK2, Polo-like kinase 2; inhibitor, PLK2 inhibitor EPR582646; CDDP, cisplatin; ADM, adriamycin; UM-SCC1, UM-SCC-1 cell; MG63, MG63 cell; siTAp73, TAp73-siRNA; TAp73-p, phosphorylation TAp73; min, minutes.

protein levels by autoradiography assay of Saos2 in the presence of CDDP stimuli and found that the half-life of nascent PLK2 from Saos2 was prolonged when compared with that of nascent PLK2 from Saos2 cells where PAP73 had been silenced (Figure 6B). This suggested that the cross talk between PLK2 and TAp73 may occur at a post-translational level.

PLK2 inhibition arrests cell cycle in the G1 phase and increases apoptosis through rescuing enriched TAp73 activity

Because PLK2 regulates the cell cycle G1/S transition and PLK2 prohibition in the presence of rich abundance of TAp73 increased p21 and PUMA expression, both of which play a key role in cell cycle arrest and cell apoptosis, we determined the physiological effects of PLK2 on TAp73 in MG63 with high levels of TAp73. The results of this analysis showed a significant increase in the proportion of G1 phase cells in the copresence of CDDP and PLK2 inhibition when compared with that resulting from CDDP treatment or PLK2 inhibition alone (Figure 8A and B). Consistent with FCM results, PLK2 inhibition increased MG63 cell apoptosis (Figure 8C). Additionally, we found that PLK2 inhibition enhances the effect of DNA-damaging drug-induced cell cycle arrest and apoptosis (Figure 8B and C). Cosilencing of PLK2 and TAp73 in cells resulted in a weakening of PLK2 inhibition of cell cycle arrest and cell apoptosis. These results suggest that PLK2 inhibition sensitized tumor cells to DNA-damaging drugs by restoring TAp73 activity.

Discussion

Variations in TAp73 abundance and phosphorylation of TAp73 can induce changes in tumor activity. In this study, we found that a range of DNA-damaging drugs stimulates the upregulation of TAp73 and PLK2 in several human tumor cell lines, indicating that their effect is broadly similar across these cell lines. Regarding TAp73 phosphorylation, we demonstrated that, like CK2, PLK2 and TAp73 directly bind to each other, and PLK2 phosphorylates TAp73 both in vivo and in vitro.

It has already been established that proteins such as TAp73 contain many functional domains.[28,29] An analysis of previous studies indicated that changes to the phosphorylation activity of the TA1 domain in TAp73 often impair the transcriptional activity of TAp73.[30–39] On the other hand, phosphorylation of other domains usually results in an increment in TAp73 antitumor activity. Thus, the identification of the PLK2 phosphorylation site within TAp73 domains is a vital clue to TAp73 function. In this research, in agreement with our computational analysis, we found that PLK2 phosphorylates Ser48, which is located in the TA1 domain. Furthermore, RT-PCR and WB results also demonstrated that PLK2 inhibition significantly increases the activity of TAp73 even where it is at high levels. A previous study[40] has demonstrated that the TAp73 isoform ΔNp73, without the TA domain, has the opposite function to TAp73. This manifestation may, in part, explain variations in functionality after TAp73 phosphorylation in the TA1 domain.

Accumulating evidence, such as gene knockout research,[41–43] DNA damaging response in human tumor cells,[26,44]
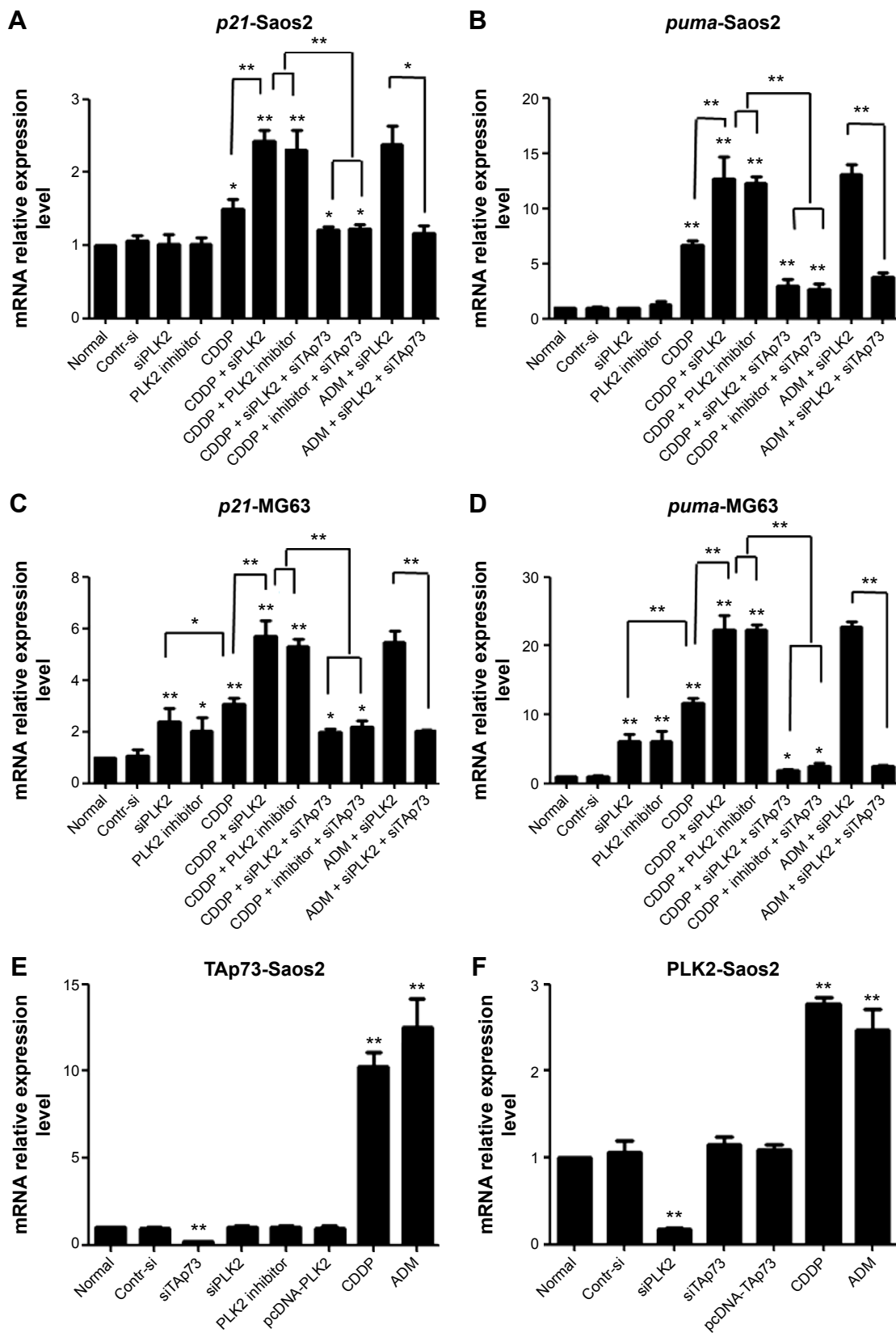
Figure 5 PLK2 suppresses TAp73 transcriptional activity.

Notes: (**A**–**D**) Without CDDP (50 μg/mL) or ADM (50 μg/mL) stimulation for 2 hours, PLK2 inhibition did not increase *p21* or *puma* mRNA expression in Saos2, but did in MG63. PLK2 inhibition enhanced the DNA-damaging drug-dependent increase, which weakened when TAp73 was downregulated. (**E** and **F**) TAp73 did not affect PLK2 mRNA expression and vice versa. *$P<0.05$; **$P<0.01$ versus the experimental value. At least three separate samples were tested.

Abbreviations: Saos2, Saos2 cell; MG63, MG63 cell; normal, under normal culture; Contr-si, control siRNA; siPLK2, PLK2-siRNA; PLK2, Polo-like kinase 2; inhibitor, PLK2 inhibitor EPR582646; CDDP, cisplatin; ADM, adriamycin; siTAp73, TAp73-siRNA.

A

B
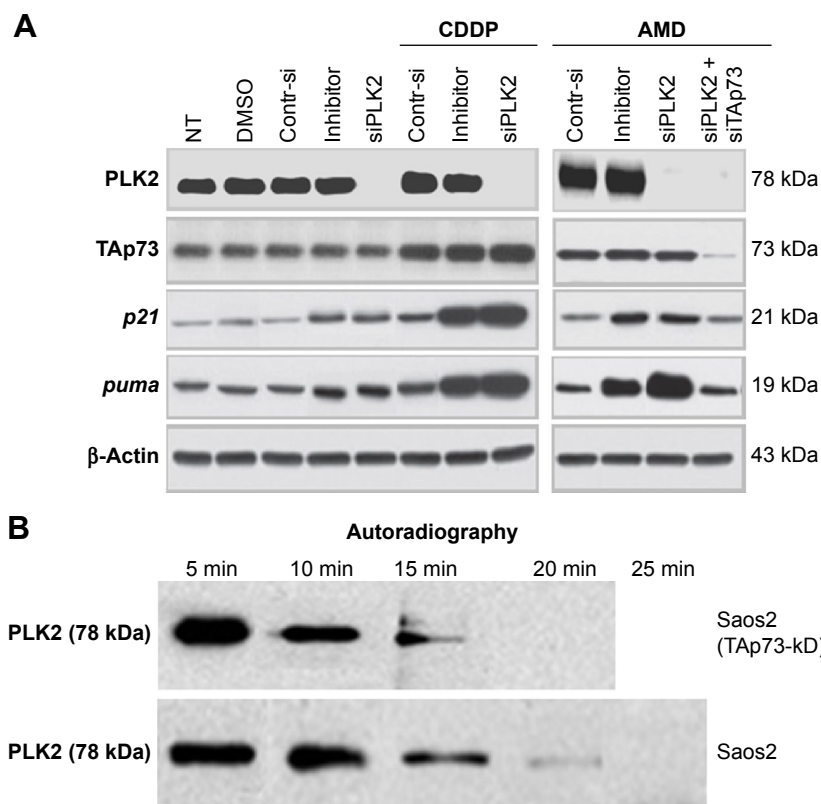
Figure 6 PLK2 and TAp73 affect each other at the posttranslational level.

Notes: (**A**) With or without DNA-damaging drug stimulation (50 µg/mL) for 2 hours, PLK2 variations did not affect TAp73 protein levels, but changed *P21* and *PUMA* protein levels in MG63 cells. (**B**) After ADM stimulation (25 µg/mL), the half-life of nascent PLK2 protein shortened from ~20 minutes in Saos2 to 15 minutes in TAp73-knockdown Saos2. This assay was repeated in triplicate.

Abbreviations: NT, no treatment; DMSO, dimethyl sulfoxide; Contr-si, control siRNA; inhibitor, PLK2 inhibitor EPR582646; PLK2, Polo-like kinase 2; siPLK2, PLK2-siRNA; siTAp73, TAp73-siRNA; CDDP, cisplatin; ADM, adriamycin; Saos2, Saos2 cell; TAp73-KD, TAp73 knockdown; min, minutes.
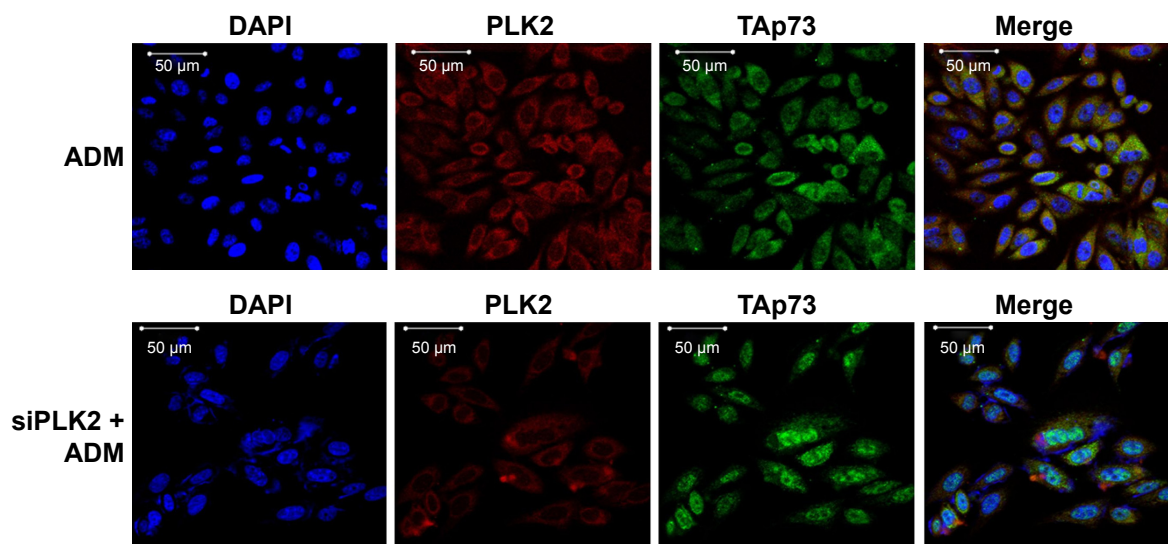
Figure 7 PLK2 affects TAp73 translocation.

Notes: The upper row shows that in UM-SCC1 cells treated with ADM (50 µg/mL), PLK2 (red) and TAp73 (green) signals are almost uniform in the cytoplasm and punctuated in the nucleus. The bottom row shows that TAp73 signal appears intense within the nucleus when PLK2 is knocked down by siRNA, suggesting that PLK2 inhibits TAp73 translocation to the nucleus. At least 200 cells were observed within discrete areas of each sample, and three independent wells of the examined cell lines were detected.

Abbreviations: ADM, adriamycin; DAPI, 4′,6-diamidino-2-phenylindole; PLK2, Polo-like kinase 2; siPLK2, PLK2-siRNA.
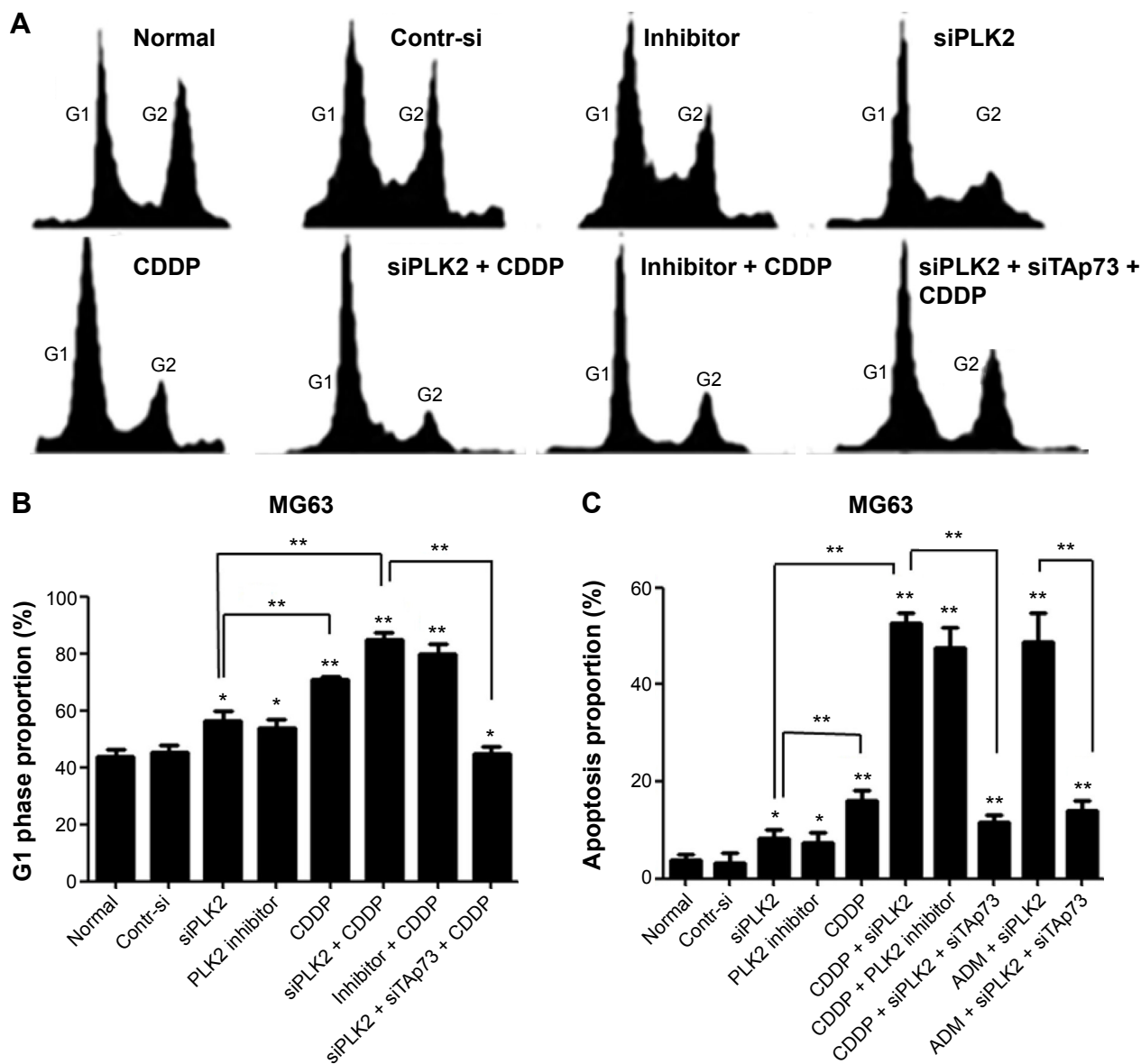
**Figure 8** PLK2 affects enriched TAp73 by regulating cell cycle progression and survival.
Notes: (**A** and **B**) PLK2 inhibition increased the proportion of cells in the G1 phase in MG63 cells. PLK2 inhibition enlarged the CDDP-dependent increase in the proportion of G1 phase cells. siTAp73 pretreatment offset this increase. At least 10,000 cells within each sample were observed, and three independent wells of the examined cell lines were detected. (**C**) A similar tendency occurred in cell apoptosis assays. This assay indicates that PLK2 can promote cell cycle progression and cell survival through phosphorylation of TAp73 which prohibits transcription activity. At least 300 cells were observed within discrete areas of each sample, and three independent wells of the examined cell lines were detected. $*P<0.05$; $**P<0.01$ versus the experimental value.
Abbreviations: MG63, MG63 cell; normal, under normal culture condition; Contr-si, control siRNA; siPLK2, PLK2-siRNA; PLK2, Polo-like kinase 2; CDDP, cisplatin; ADM, adriamycin; inhibitor, PLK2 inhibitor EPR582646; siTAp73, TAp73-siRNA.

and up- and downregulation in tumors adapting to cellular contexts strongly suggest a close correlation between PLK2 and TAp73.[45–48] In this study, we found that PLK2 phosphorylates TAp73 even at high levels of the latter, suppresses TAp73 transcriptional activity, and consequently causes cell cycle arrest and cell apoptosis. TAp73 may also stabilize PLK2 by prolonging the half-life of PLK2, which implies that TAp73 may potentiate PLK2 to regulate cell cycle progression.

As mentioned earlier, whether PLK2 plays a promotional or suppressant role in human tumors remains unclear.

Previous research has demonstrated that deregulation of PLK2 results in a malignant phenotype.[49,50] Recently, Li et al[7] demonstrated that PLK2 is essential for the survival of tumor cells. In addition, Matthew et al[51] observed that PLK2 inhibition sensitizes tumor cells to DNA-damaging drugs, a finding mirrored in our research. Furthermore, PLK2 is able to activate PLK1, suppress p53,[52] and promote the oncogenic effects of mutant p53 in tumor cells.[53] The aforementioned studies demonstrate that PLK2 has a tumor-promoting role. Thus, our results are consistent with the finding that PLK2
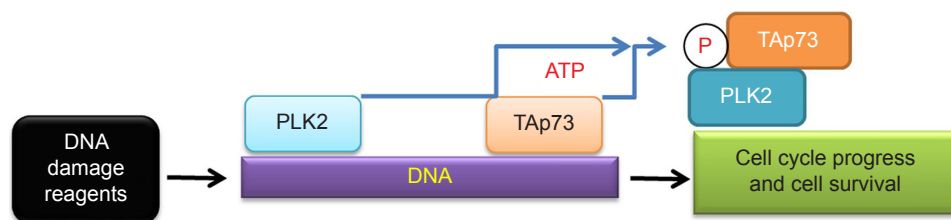
Figure 9 PLK2 regulates the fate of human tumor cells in a TAp73 concentration-dependent manner.
Abbreviations: PLK2, Polo-like kinase 2; ATP, adenosine triphosphate.

promotes tumors by prohibiting TAp73 activity, even where there is a rich abundance of TAp73.

Because numerous malignant tumors exhibited TAp73 overexpression in clinical patients,[54,55] high levels of TAp73 are usually regarded as a marker of malignant tumors. One possible explanation for this is that TAp73 expression in tumors is upregulated under certain conditions, but its activity is inhibited. To confirm this observation, researchers have used several methods to restore TAp73 activity in tumors.[55] In our study, we found that PLK2 inhibition is capable of restoring TAp73 activity, a finding that provides extra insight into this phenomenon.

Conclusion

In summary, we reveal here a novel mechanism of PLK2 as a prosurvival agent in tumor cells with a rich abundance of TAp73, where PLK2 phosphorylates the Ser48 residue of TAp73 and blocks TAp73 transcriptional activity (Figure 9). PLK2 inhibition may thus sensitize tumor cells to the effects of DNA-damaging drugs by rescuing TAp73 activity. Our research may aid the quest for antitumor therapies in tumors characterized by TAp73 overexpression.

Acknowledgments

Disclosure

ZH, ZX, and XL are equal contributors in this work and should be considered as first co-authors. AJ and HL contributed equally to this work as senior authors. The authors report no conflicts of interest in this work.

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Supplementary material
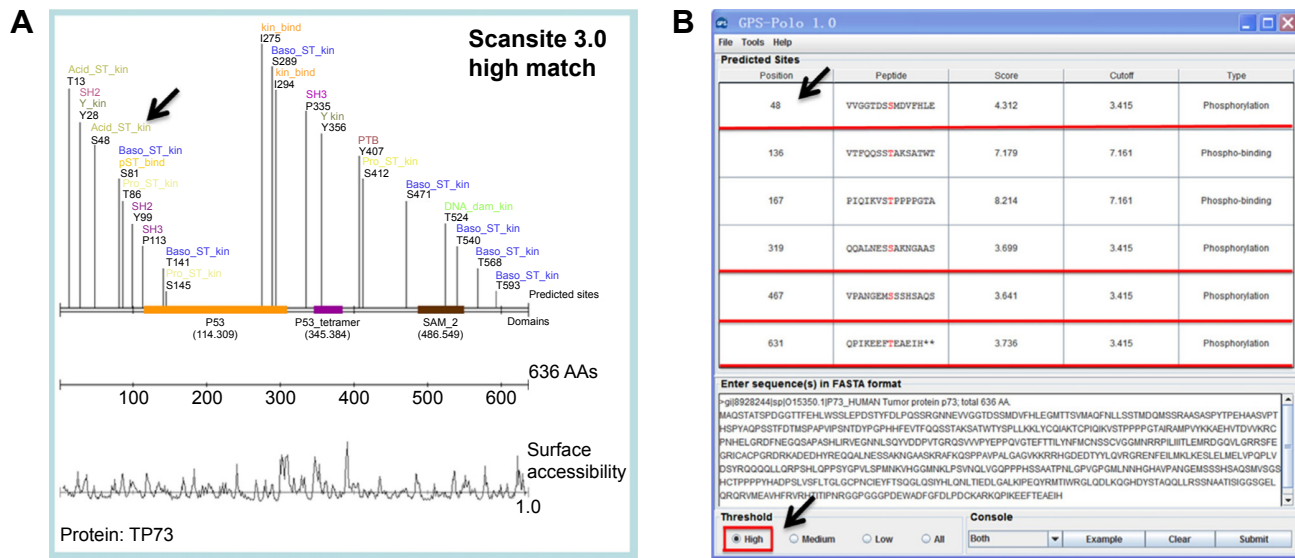
Figure S1 The most likely target, according to Scansite 3.0 and GPS 1.0 prediction results.
Notes: (**A**) The prediction results using Scansite 3.0 with a high match threshold: residue Ser48 (black arrow) is one of the candidate sites within the TA1 domain. (**B**) The prediction results using GPS 1.0 with a high match threshold: residue Ser48 is one of the candidate sites (black arrows).