PTPN18 functions as a tumor suppressor in breast cancer by negatively regulating cyclin E

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Received September 13, 2025; Accepted December 16, 2025

DOI: 10.3892/ijo.2025.5843

Abstract. Protein tyrosine phosphatase non-receptor 18 (PTPN18) is widely expressed in breast cancer (BC) cell lines. Additionally, high levels of PTPN18 facilitate an improved overall survival and prognosis in patients with BC. However, the effects and mechanisms of PTPN18 in BC remain unclear. In the present study, it was found that PTPN18 serves a tumor suppressor role in BC cells by promoting apoptosis, inhibiting proliferation and metastasis and inducing cell cycle arrest. Bioinformatics analysis showed that PTPN18 was significantly negatively correlated with the cell cycle and downregulated cyclin E expression, which was consistent with the experimental results. Subsequent co-immunoprecipitation assay results showed that PTPN18 could bind to cyclin E and promote its degradation through the ubiquitin-proteasome pathway. Moreover, the addition of cyclin E2 did not reduce the binding of PTPN18 to cyclin E1. In the present study, the signaling pathways involved in cell cycle regulation were further investigated and it was found that PTPN18 may regulate the expression levels of cyclin-dependent kinase (CDK) inhibitor 1A and CDK inhibitor 1B proteins through phosphatidylinositol 3-kinase/protein kinase B signaling pathway, which leads to cell cycle arrest and tumor inhibition in BC. Thus, analysis of the tumor suppressor mechanism of PTPN18 not only helps us to understand its biological function but also provides a theoretical basis for the development of new therapeutic strategies for BC.

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Abbreviations: Co-IP, co-immunoprecipitation; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; HER2, human epidermal growth factor receptor 2; MCF7, Michigan Cancer Foundation-7; MDA-MB-231, Metastatic Derivative of Anaplastic Breast Cancer-231; WB, western blot; TBST, Tris-buffered saline with Tween 20; RIPA, radioimmunoprecipitation assay; PBS, phosphate-buffered saline; FBS, fetal bovine serum; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EMT, epithelial-mesenchymal transition; mTOR, mechanistic target of rapamycin kinase; CDK, cyclin-dependent kinase; RB, retinoblastoma protein; GEPIA, Gene Expression Profiling Interactive Analysis; CHX, cycloheximide; PDPK1, phosphoinositide-dependent protein kinase 1; PI3CG, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit γ; FOXO, forkhead box O; MAPK8, mitogen-activated protein kinase 8; GADD45A, growth arrest and DNA damage-inducible protein α; APC, adenomatous polyposis coli protein; EP300, E1A-binding protein p300; CDKN1A (p21Cip1), CDK inhibitor 1A; CDKN1B (p27Kip1), CDK inhibitor 1B

Key words: PTPN18, breast cancer, cyclin E, cell cycle arrest, PI3K/AKT

Introduction

Breast cancer (BC), a common malignancy, is the leading cause of cancer-related death in women worldwide (1). Although some progress has been made in understanding the pathogenesis and treatment of BC (2-6), its pathogenesis is complex and involves abnormal regulation of a variety of genes and signaling pathways; thus, further studies are still needed. The family of protein tyrosine phosphatases (PTPs) rich in proline, glutamic acid, serine and threonine includes PTP non-receptor type 18 (PTPN18), PTPN22 and PTPN12 (7). Members of this family have a key role in various physiological and pathological processes of cells (7). For instance, previous studies by the authors confirmed the notable role of PTPN22 in immune regulation (8,9) as well as signaling events triggered by the synergistic inactivation of Src kinase by PTPN18 and C-Src kinase (10). As a classical PTP, PTPN18 can also regulate multiple signal transduction pathways by regulating the phosphorylation of cellular Abelson tyrosine kinase, a key signaling protein tyrosine kinase (11). The interaction between PTPN18 and proline-serine-threonine phosphatase interacting

protein 1/2 or P190 Rho GTPase-activating protein confirms its function in regulating the cytoskeleton (11-14). Additionally, it has been shown that PTPN18 negatively regulates human epidermal growth factor receptor 2 (HER2) to inhibit BC progression (15,16). Furthermore, a recent study by the authors found that nuclear PTPN18 exerts antitumor effects in BC by targeting ETS proto-oncogene 1 (ETS1) to inhibit transforming growth factor-β signaling and epithelial-mesenchymal transition (EMT) (17). Notably, patients with BC harboring high PTPN18 expression have an improved prognosis and longer overall survival (OS) (17), which suggests that PTPN18 may play an important role in BC progression.

Cyclin E, a key molecule of the cell cycle regulatory network, functions in the G1 and S phases and mainly binds to and activates cyclin-dependent kinase 2 (CDK2) (18). Cyclin E/CDK2 complexes phosphorylate numerous substrates to control important cellular processes (18,19). There are two E-type cyclins, E1 and E2, which are considered to primarily exert overlapping functions (18). However, it has been reported that cyclin E1 and cyclin E2 have different emphasis on regulatory patterns and functions (20). High levels of cyclin E protein lead to poor prognosis, reduced survival and treatment resistance in patients with cancer (21,22). Additionally, cyclin E amplification/upregulation is one of the mechanisms of trastuzumab resistance in patients with $HER2^+$ BC (23). HER2 is an important target of PTPN18 in inhibiting BC (15,16), suggesting that there may be a link between PTPN18 and cyclin E.

Based on the potential importance of PTPN18 in the field of oncology as well as its broad role in the regulation of cellular function, it is necessary to further explore its role in BC and its underlying mechanisms. Therefore, through phenotypic functional experiments, the present study first confirmed that PTPN18 can exert antitumor effects by promoting apoptosis, inhibiting metastasis and proliferation and causing S phase cell cycle arrest in BC cells. Subsequently, it was found that S-phase arrest caused by PTPN18 may be via the downregulation of cyclin E expression. Further mechanistic dissection revealed that cyclin E is an important substrate for PTPN18 in exerting antitumor effects. Taken together, the mechanism of BC development and progression identified in the present study provides an additional comprehensive and extensive theoretical basis for the prevention and treatment of this disease.

Materials and methods

Cell culture and transfection. The Michigan Cancer Foundation-7 (MCF7) and Metastatic Derivative of Anaplastic BC-231 (MDA-MB-231) cell lines were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and maintained in Dulbecco's modified Eagle's medium (HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva) and 1% penicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2. Interference with PTPN18 expression levels was achieved by transfecting (37˚C, 6 h) cells with PTPN18-3HA (1 μg/μl) and PTPN18 small interfering RNA (20 μM) (siRNA; Suzhou GenePharma Co., Ltd.) using Lipo6000 (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. To achieve an improved knockdown efficiency, the knockdown siRNA was transfected again after 24 h of initial transfection. The siRNA sequences used are listed in Table SI.

Western blotting. First, total protein was extracted from cells using precooled radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology) containing 1% protease inhibitor and phosphatase inhibitor (Selleck Chemicals) and then quantified by bicinchoninic acid assay. Equal amounts of protein (10 μg) were separated by 10% SDS-PAGE at a constant voltage (80 V) until the blue band of the loading buffer approached the lower edge of the gel. Proteins were then transferred to PVDF membranes using the sandwich method at a constant voltage of 100 V for 100 min at 4˚C. Membranes were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, then incubated overnight at 4˚C with blocking buffer plus primary antibodies. Subsequent washing with TBST was followed by incubation with secondary antibodies for 1 h at room temperature. Goat anti-rabbit IgG (heavy and light chain) antibody or horse anti-mouse IgG antibody conjugated with horseradish peroxidase secondary antibody was selected for chemiluminescence detection according to the primary antibody species. Finally, the protein bands were visualized and semi-quantified using the ChemiDoc XRS + Gel Imaging Analysis System (Bio-Rad Laboratories, Inc.) and Image Lab 5.2.1 software (6). The antibody information is listed in Table SII.

Co-immunoprecipitation (Co-IP) assay. Treated cells were lysed in pre-chilled RIPA lysis buffer containing protease inhibitor and phosphatase inhibitor cocktails for 10-30 min. Experiments involving tyrosine phosphorylation or PTPN18 C229S were stimulated with EGF 100 ng/ml for 30 min before cells were collected. The samples were centrifuged at 12,000 x g for 15 min at 4˚C to remove cell debris, then the supernatant was incubated with the appropriate primary antibody for 2-4 h or overnight at 4˚C. The conjugated product was incubated with 30-50 μl Protein A/G agarose beads (Beyotime Biotechnology) for 2-4 h at 4˚C the following day. The agarose beads were washed five times with cold lysis buffer, the supernatant was discarded and the beads were boiled with 1X SDS loading buffer for 10 min prior to western blotting.

Flow cytometry apoptosis assay. The apoptosis of BC cells was observed using flow cytometry. Cells were washed twice with pre-chilled phosphate-buffered saline (PBS) and centrifuged at 500 x g for 5 min at 4˚C to collect 1-2.5x10^6 cells. The cells were resuspended in 100 μl of 1X Binding Buffer (BD Biosciences) and divided into two aliquots, one without Annexin V-FITC/PI and the other with 5 μl Annexin V-FITC and 5 μl PI Staining Solution (BD Biosciences), then mixed gently. The individual groups were stained with PI or FITC only for subsequent adjustment of compensation. The samples were protected from the light and the reaction was allowed to proceed at room temperature for 15 min. Next, 400 μl of 1X Binding Buffer was added and mixed well with the samples, which were then filtered with gauze and detected by BD Accuri™ C6 Plus flow cytometry (BD Biosciences) within 1 h. For each sample, 10,000 events were analyzed and the experiment was independently repeated three times (24). Data were

analyzed and processed using the FlowJo 10.8.1 (FlowJo LLC; BD Biosciences) software.

Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). *In vitro* apoptosis was examined by DAPI staining. MCF-7 cells that had previously been transfected for 48 h were seeded into 24-well plates for overnight incubation and attachment. The cells were then washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The fixative was removed, then the cells were washed with PBS and stained with 2 μg/ml DAPI (Beyotime Institute of Biotechnology) for 15 min at room temperature in the dark. Changes in nuclear morphology were examined by fluorescence microscopy and images were collected.

Cell cycle assay. Cell cycle distribution was determined using an Accuri C6 flow cytometer (BD Biosciences). The collected cells were fixed with pre-chilled 70-80% ethanol overnight at 4˚C, centrifuged to remove the fixative and washed once with 1 ml PBS. The samples were then divided into two aliquots after the supernatant had been removed: One aliquot was incubated at room temperature without PI/RNase for 15 min; the other was incubated at room temperature with 500 μl PI/RNase (BD Biosciences) in the dark for 15 min. The cells were then filtered with a 200-mesh filter and detected by flow cytometry. FlowJo 10.8.1 (FlowJo LLC; BD Biosciences) or ModFit LT 4.0 software (Verity Software House) was used to process the data (25).

Cell viability assay. Cell viability was monitored using the MTS assay, a modified version of the classical MTT assay that has the same core principle (26). The treated cells (2,000 cells/100 μl medium) were digested and transferred to 96-well plates, with four duplicate wells set up for each treatment group, and the absorbance was detected after 0, 12, 24, 36 and 48 h. In each well, an average of 20 μl of CellTiter 96® AQueous One Solution Reagent (Promega Corporation) was added to 100 μl of medium. After gentle mixing and 2 h of reaction in a 37˚C and 5% CO_2 incubator, the absorbance at 490 nm was measured.

Colony formation assay. Treated cells were seeded into 6-well plates at a density of 2,000 cells/well. After the cells adhered overnight, the medium was replaced every 3 days for 14 days. The cells were then fixed with 4% paraformaldehyde (room temperature, 30 min) and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) for 20 min at room temperature. Finally, the plates were washed with water and air dried at room temperature. The colonies were recorded using ImageJ 1.53e software (National Institutes of Health).

Transwell invasion assay. Conditioned medium (500 μl) containing 20% FBS was added to the lower chamber of a 24-well plate. Next, 100 μl of diluted BD Matrigel was added to each upper chamber. After the Matrigel had solidified at 37˚C, a digested and diluted serum-free cell suspension was added to the upper chamber, and the plates were incubated at 37˚C for 24-48 h. The inserts (8 μm) were then washed with PBS and fixed with a 5% paraformaldehyde solution for 30 min. The cells were stained with crystal violet (0.1%) at room temperature for 30 min, then observed and counted under an inverted fluorescence microscope (Leica Microsystems GmbH) operating in white light mode.

Wound healing assay. After creating a scratch with a 200-μl sterilized tip, the cells were washed three times with sterilized PBS, the impurities and cell debris were washed off, and fresh low serum (1%) culture medium was added to continue the cell culture. Images were collected at 0, 24 and 48 h and the change in scratch size was recorded. The experiment was repeated three times. ImageJ software was used to assess the scratch area and for subsequent experimental data processing.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted using TRIzol and cDNA synthesis was performed using the All-in-One cDNA Synthesis SuperMix kit (Selleck Chemicals) following the manufacturer's instructions. In a total reaction volume of 20 μl per well, the 2X SYBR Green qPCR Master Mix kit (Selleck Chemicals) was used for qPCR. The PCR amplification protocol consisted of pre-denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 30 sec. A total of 3-5 replicate wells were set up per group. The mean quantification cycle (Cq) values were collected for each reaction and relative expression was quantified using the $2^{-\Delta\Delta Cq}$ method (27). The sequences of the qPCR primers used in the present study are listed in Table SIII.

Associated database analysis. Changes in the expression levels of total PTPN18 protein were analyzed through the 'Proteomics' catalog of the UALCAN database (https://ualcan.path.uab.edu/cgi-bin/CPTAC-Result.pl?genenam=PTPN18&ctype=Breast) (28). 'Expression DIY' in the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia2.cancer-pku.cn/#index) was used to obtain gene expression related information in the box plot and stage plot formats. Correlation analysis (GEPIA) was used to test the association of PTPN18 or CDKN1A/B with CCNE1/2 gene. Survival analysis (GEPIA) was used to obtain gene and OS or disease-free survival data (29). Additionally, gene expression levels and OS or relapse-free survival data were obtained from the BC category of the Kaplan-Meier Plotter database (https://kmplot.com/analysis/index.php?p=service&cancer=breast) (30). PTPN18 correlation analysis was performed using breast invasive carcinoma and HiSeq RNA data from the LinkedOmics platform (https://www.linkedomics.org/admin.php) (31). Protein interaction networks for cyclin E1 and cyclin E2 were obtained from the STRING database (https://cn.string-db.org/) (32).

Statistical analysis. Data are presented as the mean ± SD of at least three independent experiments. Data analysis was performed using GraphPad Prism software 8.0.2 (Dotmatics). Data were analyzed using unpaired Student's t-test or one-way analysis of variance with Tukey's multiple comparison test, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

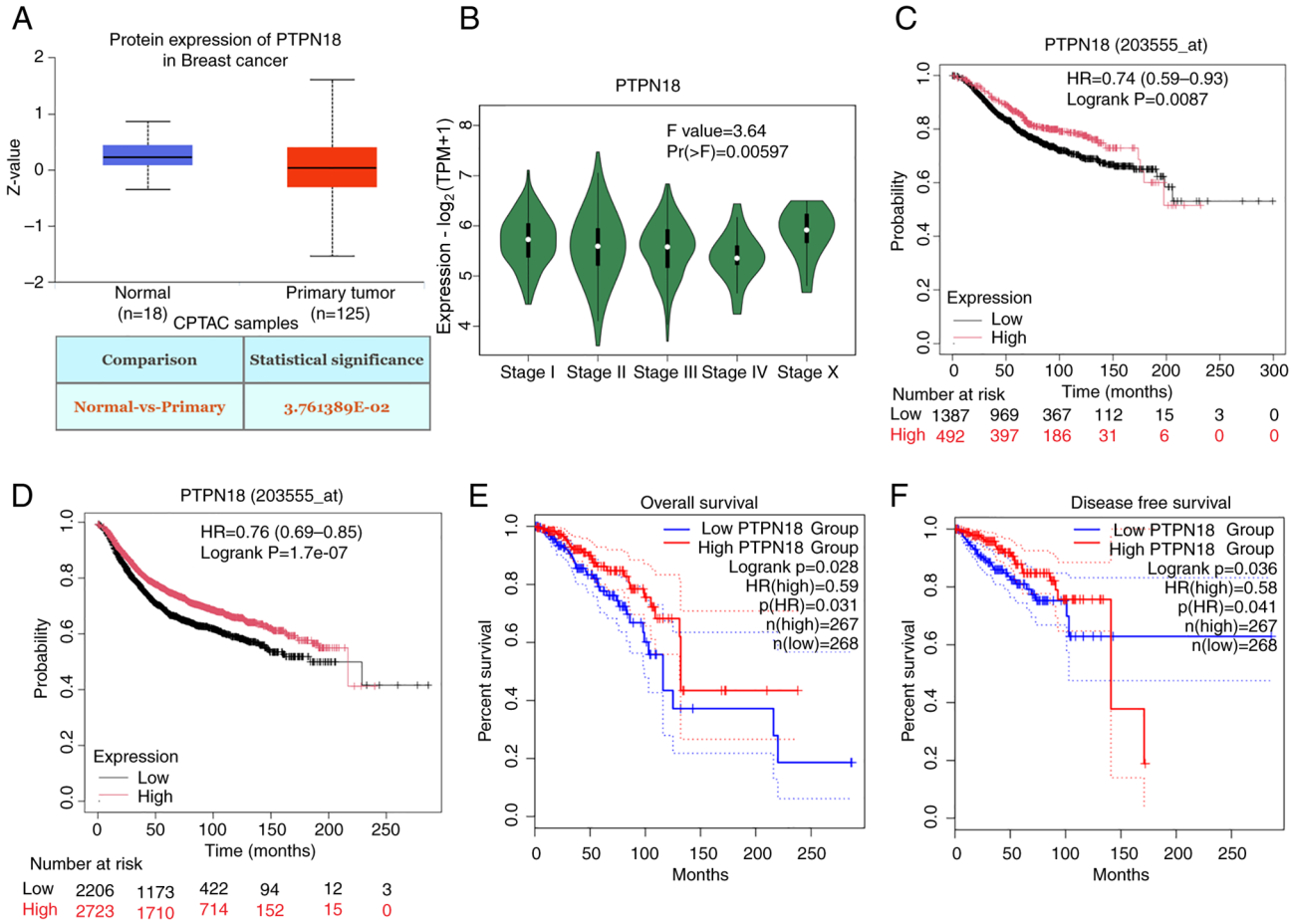

Figure 1. High expression of PTPN18 improves the survival and prognosis of patients with BC. (A) The UALCAN database was used to analyze the protein expression changes of PTPN18 in BC. (B) Box plots of PTPN18 expression in different tumor stages using data from the GEPIA database. (C) Kaplan-Meier plotter was used to analyze the effect of high and low PTPN18 expression on the OS of patients with BC. (D) Effect of PTPN18 expression on the relapse-free survival of patients with BC. (E) GEPIA database was used to analyze the effect of high and low PTPN18 expression on the OS of patients with BC. (F) GEPIA database was used to analyze the influence of PTPN18 expression on disease-free survival in patients with BC. BC, breast cancer; PTPN18, protein tyrosine phosphatase non-receptor 18; GEPIA, Gene Expression Profiling Interactive Analysis. OS, overall survival.

Results

PTPN18 expression is associated with BC. Proteomic expression profiling using the UALCAN database showed that PTPN18 protein expression levels are decreased in BC (Fig. 1A). In addition, a close link between PTPN18 gene expression and the stage of BC was identified (Fig. 1B). Statistical analyses using the Kaplan-Meier Plotter and GEPIA databases showed that high levels of PTPN18 improved the survival and prognosis of patients with BC (Fig. 1C-F), indicating that PTPN18 may play a tumor suppressor role in this disease. Therefore, the function and mechanism of PTPN18 in BC was further investigated.

PTPN18 suppresses the metastasis of BC cells. PTPN18 is widely expressed in BC cell lines (15,16). In the present study, the western blot results showed that PTPN18 was expressed at higher levels in MCF7 and MDA-MB-231 cells (Fig. S1A), which represent estrogen receptor and progesterone receptor double-positive and triple-negative BC cell lines, respectively. Overexpression and knockdown of PTPN18 in MCF7 cells at different time points after transfection is shown in Fig. S1B and C. Next, the effect of PTPN18 on cancer cell invasion was investigated using a Transwell chamber assay. The results showed that overexpression of PTPN18 effectively inhibited the invasion of cancer cells, while knockdown of endogenous PTPN18 expression enhanced invasion (Fig. 2A and B). Consistent with these results, *in vitro* cell wound healing assays demonstrated that PTPN18 expression inhibited tumor cell migration (Fig. 2C and D). Analysis of the mRNA levels of genes involved in cell metastasis revealed that E-cadherin gene expression increased and Snail family transcriptional repressor 1 expression decreased in the PTPN18 overexpression group compared with the vector group, while the TWIST basic helix-loop-helix transcription factor 1 and Notch receptor 3 levels remained essentially unchanged (Fig. 2E). The trend was roughly the opposite between the knockdown and overexpression groups (Fig. 2E). Subsequent verification of the translation levels of the E-cadherin EMT marker protein revealed changes consistent with the transcription levels (Fig. 2F).

PTPN18 inhibits BC cell proliferation and induces apoptosis. The key to cancer treatment is inhibiting the metastasis and proliferation of cancer cells and inducing apoptosis. The MTS assay results demonstrated that PTPN18 overexpression

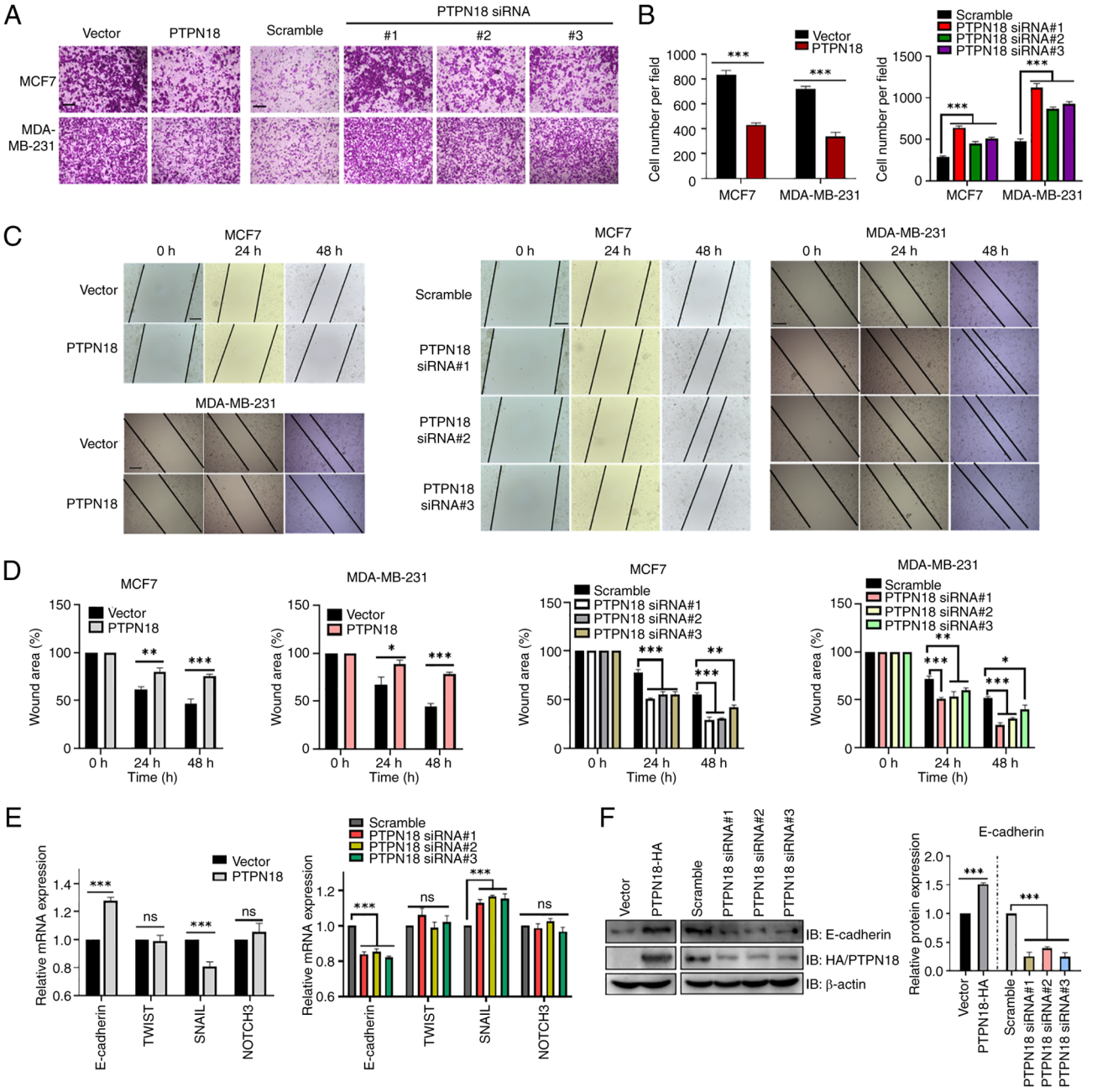

Figure 2. PTPN18 suppresses the invasion and migration of BC cells. (A) Representative images of the Transwell invasion assays of BC cells with PTPN18 overexpression or knockdown. Cells were collected after 48 h of culture. Scale bars, 200 μm. (B) Statistical analysis of the number of invasive cells. (C) Cell migration capability was determined with a wound healing assay. Scale bars, 200 μm. (D) Statistical analysis of the wound area. (E) Expression of genes associated with cell migration and invasion measured by reverse transcription-quantitative polymerase chain reaction. (F) Immunoblot analysis of the epithelial-mesenchymal transition marker, E-cadherin, following interference with PTPN18 expression. RNA or protein was extracted 48 h after overexpression and 72 h after knockdown transfections. Data are shown as the mean ± SD from three technical replicates. *P<0.05, **P<0.01 and ***P<0.001. PTPN18, protein tyrosine phosphatase non-receptor 18; BC, breast cancer; ns, not significant; siRNA, small interfering RNA.

inhibited cell viability, an inhibitory effect that exhibited an increasing cumulative trend over time (Fig. 3A). Although the MCF7 and MDA-MB-231 cells in the PTPN18 knockdown groups showed a high cell viability at different time points, the overall trend was the opposite to that of the overexpression group (Fig. 3B). This conclusion was further verified by a colony formation assay (Fig. 3C and D).

The flow cytometric results identified that PTPN18 overexpression significantly increased the proportion of BC cells undergoing apoptosis, while the three knockdowns of PTPN18 revealed different degrees of apoptosis' inhibition (Fig. 3E and F). In addition, the MCF7 BC cell line was stained with DAPI and subsequent fluorescence microscopy showed significantly increased signs of apoptotic cell death, such as chromatin condensation and fragmentation, in the PTPN18 overexpression group compared with the control cells (Fig. 3G). Furthermore, RT-qPCR (Fig. 3H) and western blot (Fig. 3I) results showed that PTPN18 inhibited the expression of mechanistic target of rapamycin kinase (mTOR) mainly at the protein level. Additionally, PTPN18 promoted Bax

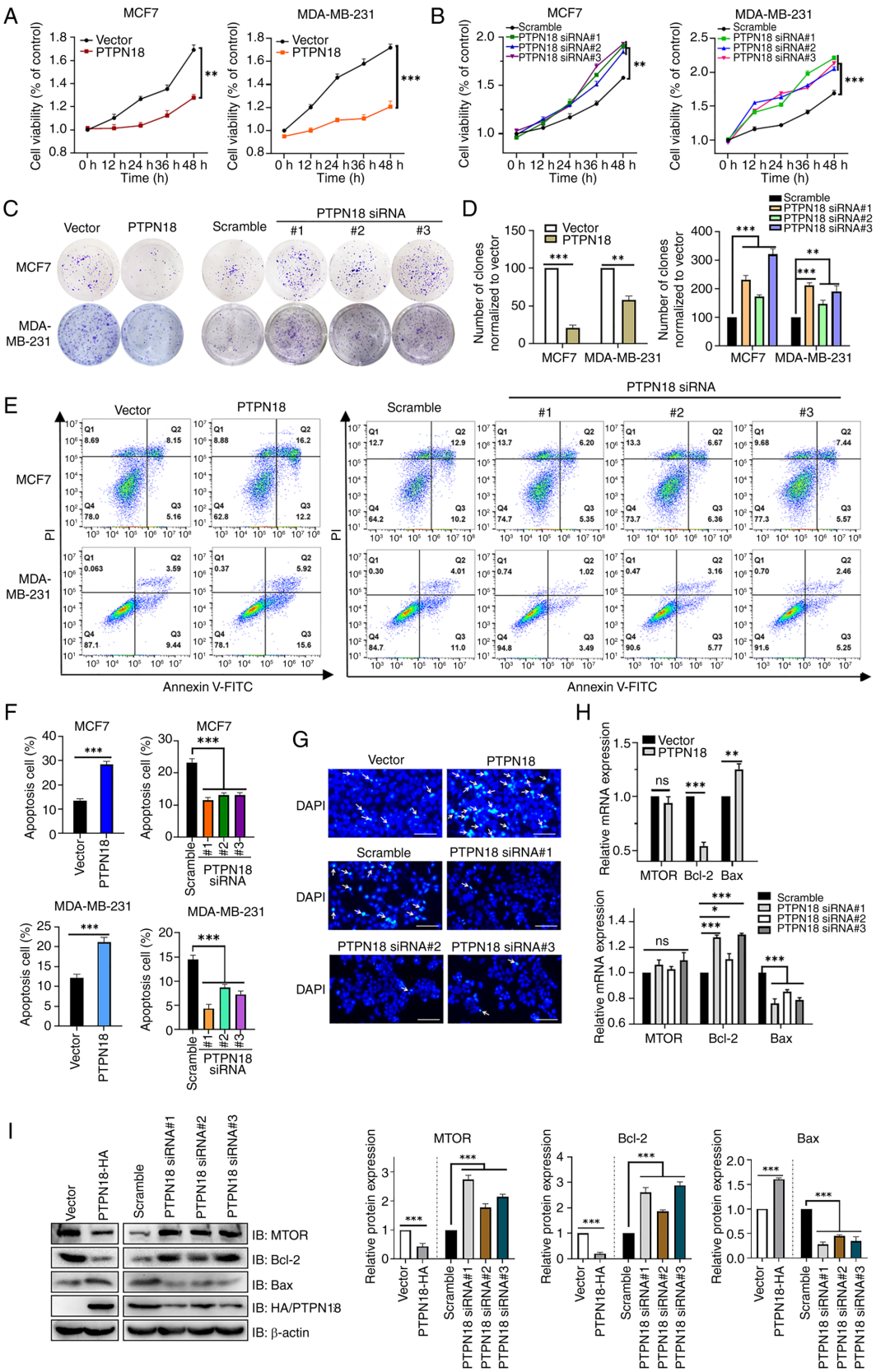

Figure 3. PTPN18 inhibits cell proliferation and induces apoptosis in breast cancer cells. (A) Cell viability analysis at different time points following PTPN18 overexpression. (B) Assessment of cell viability over time upon PTPN18 knockdown. (C) Representative images from the colony formation assays after 2 weeks of cultivation. (D) Statistical analysis of the number of colonies counted using ImageJ. (E) Apoptosis scatter plots based on PI and Annexin V-FITC staining were obtained by flow cytometry. (F) Quantitative analysis of apoptosis rate measured by flow cytometry. (G) DAPI was used to stain the nuclei. The nuclei of apoptotic cells showed dense hyperchromatic bright spots under a fluorescence microscope. Scale bars, 100 μm. (H) Reverse transcription-quantitative polymerase chain reaction analysis of the mTOR, Bcl-2 and Bax mRNA expression levels. (I) Protein expression levels of mTOR, Bcl-2 and Bax were analyzed by western blotting following interference with PTPN18 expression. Data are shown as the mean ± SD from three technical replicates following overexpression (48 h) or knockdown (72 h) treatment. *P<0.05, **P<0.01 and ***P<0.001. PTPN18, protein tyrosine phosphatase non-receptor 18; mTOR, mechanistic target of rapamycin kinase; ns, not significant; siRNA, small interfering RNA.

expression and suppressed Bcl-2 expression (Fig. 3H and I). Collectively, these results confirmed that PTPN18 inhibited proliferation and promoted the apoptosis of BC cells.

PTPN18 can induce S phase cell cycle arrest in BC by downregulating the expression of cyclin E. Next, the effect of PTPN18 on the cell cycle of BC cells was further investigated. The results of the cell cycle flow cytometry experiments revealed that PTPN18 overexpression significantly increased the number of cells in the S phase and decreased the number of cells in the G2/M phase but did not significantly change the number of cells in the G0/G1 phase (Fig. 4A and C). However, the PTPN18 knockdown groups showed the opposite trend (Fig. 4B and D), indicating that PTPN18 could induce cell cycle arrest in the S phase and affect the cell cycle progression of BC cells. In addition, PTPN18 overexpression significantly decreased cyclin E1 expression and increased cyclin D1, cyclin B1 and CDK4 expression at both the transcriptional and translational levels (Fig. 4E-H). These results suggested that significant downregulation of cyclin E may be an important factor in PTPN18 overexpression-induced S phase cell cycle arrest in BC. The aforementioned phenotypic functional experiments demonstrated that PTPN18 could play a tumor suppressive role in BC cells by promoting apoptosis and inhibiting proliferation, cell cycle, migration and invasion.

PTPN18 and the cell cycle are significantly and negatively correlated in BC. To illustrate the biological function of PTPN18 in BC development, RNA sequencing gene enrichment analysis of PTPN18 in BC was performed using the LinkedOmics database. The results revealed that PTPN18 showed a close and significantly negative correlation with the cell cycle in BC (Fig. 5A). Additionally, the results of the cell cycle gene enrichment analysis showed that PTPN18 may affect cell cycle progression by inhibiting gene expression of cell cycle pathways (Fig. 5B). Volcano plots revealed the genes that were positively or negatively correlated with PTPN18 (Fig. 5C). Pearson correlation coefficient analysis suggested that PTPN18 was significantly negatively correlated with cyclins E1 and E2 (Fig. 5C). Furthermore, the GEPIA database gene correlation analysis results also demonstrated that PTPN18 was negatively correlated with cyclins E1 and E2 (Fig. 5D). The cyclin E1 and E2 expression levels were found to be significantly increased in BC tissues compared with normal tissues (Fig. 5E). Cyclin E1 expression was significantly different in different clinical stages of BC and may play a role in disease stage progression; however, cyclin E2 expression was weakly associated with stage, suggesting that the functions of these two proteins in disease progression may be different (Fig. 5F). The results of the survival analyses using the Kaplan-Meier Plotter and GEPIA databases identified that high expression of cyclin E was associated with a reduced OS and the poor prognosis of patients with BC (Fig. 5G and H; Fig. S2A and B). These analyses support the hypothesis that PTPN18 may affect the BC cell cycle through regulation of the cyclin E pathway. These results echo the aforementioned experimental results and reinforce the rationality and necessity of studying the mechanism of cyclin E downregulation due to PTPN18 overexpression.

PTPN18 can interact with cyclin E1 and promote its proteasomal degradation. In view of the notable role of PTPN18 in the BC cell cycle, the present study further explored the molecular mechanism by which PTPN18 causes cell cycle arrest in the S phase. First, treatment with cycloheximide (CHX), an inhibitor of protein synthesis, revealed that overexpression of PTPN18 promoted cyclin E1 degradation and knockdown of PTPN18 inhibited cyclin E1 degradation (Fig. 6A and B). Furthermore, MG132 proteasome inhibitor inhibited cyclin E1 degradation induced by PTPN18 overexpression and enhanced the inhibitory effect on cyclin E1 degradation in the knockdown group (Fig. 6C and D). When CHX and MG132 were combined, the effect of PTPN18 on the cyclin E1 protein levels was not significantly different from that of the control (Fig. 6E and F).

Next, the effect of PTPN18 on cyclin E1 ubiquitination was examined. The results demonstrated that PTPN18 overexpression markedly increased the ubiquitination level of cyclin E1 and that PTPN18 knockdown reduced the cyclin E1 ubiquitination level after MG132 treatment (Fig. 6G and H). Since PTPN18 is a PTP, it was found that the ubiquitination of cyclin E1 by PTPN18 requires enzymatic activity (Fig. 6I). Using western blotting to detect the pan-tyrosine phosphorylation level of cyclin E1, it was found that PTPN18 was able to dephosphorylate the tyrosine of cyclin E1, indicating that cyclin E1 is a substrate of PTPN18 (Fig. 6J and K). Next, the interaction between PTPN18 and cyclin E1 was verified by forward and reverse Co-IP experiments as well as by examination of the endogenous interaction in MCF7 cells (Fig. 6L-N). In summary, the results revealed that PTPN18 can bind to cyclin E1 and promotes its degradation through the ubiquitin-proteasome pathway.

PTPN18 downregulates the expression of cyclin E2 protein via the ubiquitin-proteasome pathway. Next, the present study explored the effect of PTPN18 on cyclin E2, another isoform of cyclin E. It was found that overexpression of PTPN18 decreased the cyclin E2 protein levels after treatment with CHX (Fig. 7A). However, the cyclin E2 protein levels were restored when MG132, a proteasome inhibitor, was added (Fig. 6C). When PTPN18 expression was knocked down with siRNA, the opposite effects on the cyclin E2 protein levels were observed (Fig. 7B and D). The combined use of CHX and MG132 nearly abolished the effect of PTPN18 on the cyclin E2 protein levels compared with the control (Fig. 7E and F). Examination of the cyclin E2 ubiquitination levels revealed that MG132 enhanced the PTPN18-mediated protein ubiquitination of cyclin E2, whereas knockdown of PTPN18 reduced the cyclin E2 ubiquitination levels (Fig. 7G and H). Similar to the cyclin E1 results, inactivation treatment with PTPN18 C229S reduced the ubiquitination and increased the phosphorylation of cyclin E2, indicating that cyclin E2 is also a substrate for PTPN18 (Fig. 7I-K). Additionally, forward and reverse Co-IP experiments verified the interaction of PTPN18 with cyclin E2 (Fig. 7L and M). These results indicated that PTPN18 can also promote the degradation of cyclin E2 through the ubiquitin-proteasome pathway. Given that both cyclins E1 and E2 can bind PTPN18, it was further explored whether there is a competitive relationship between their interactions. The Co-IP results showed that the addition of cyclin E2 did not reduce the binding of PTPN18 to cyclin E1, suggesting cyclin E2 did not interfere with the binding of cyclin E1 to PTPN18 (Fig. 7N).

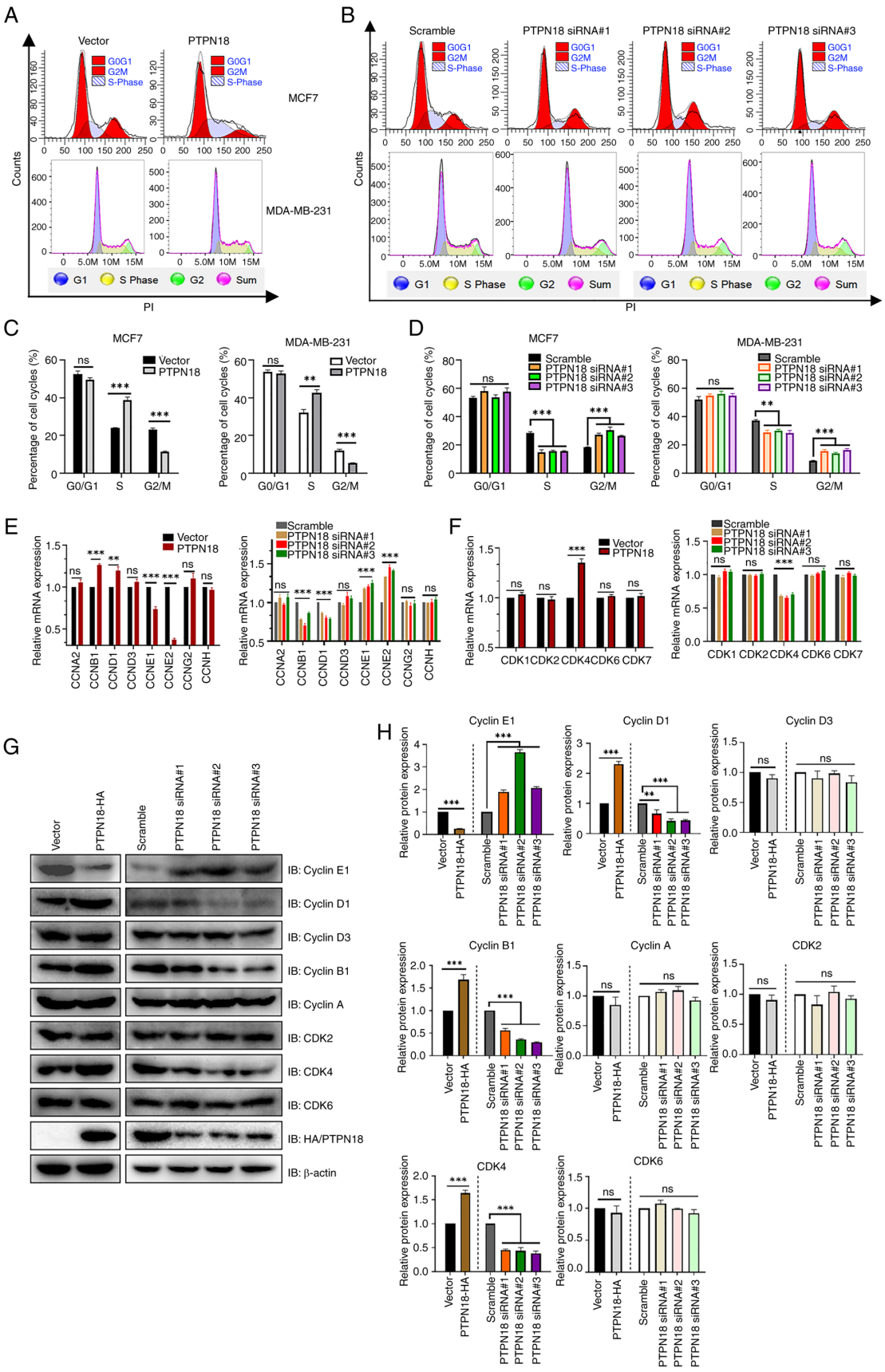

Figure 4. PTPN18 induces S phase cell cycle arrest in breast cancer cells by downregulating cyclin E expression. (A and B) DNA content was measured by flow cytometry to analyze cell cycle distribution under PTPN18 (A) overexpression and (B) knockdown conditions. (C and D) Statistical analysis percentage of cells at different cell cycle stages in (C) Panel A and (D) Panel B. (E) Reverse transcription-quantitative polymerase chain reaction was used to analyze the effect of PTPN18 on cyclin mRNA expression levels. (F) Impact of PTPN18 on the transcriptional levels of CDKs. (G) Effect of PTPN18 on cyclin and CDK protein expression levels. (H) Statistics of changes in cell cycle-related protein expression. The bar graph was obtained by normalizing the expression levels to β-actin. All data were acquired 48 h after overexpression or 72 h after knockdown and are shown as the mean ± SD from three technical replicates. **P<0.01 and ***P<0.001. PTPN18, protein tyrosine phosphatase non-receptor 18; CDKs, cyclin-dependent kinases; ns, not significant.

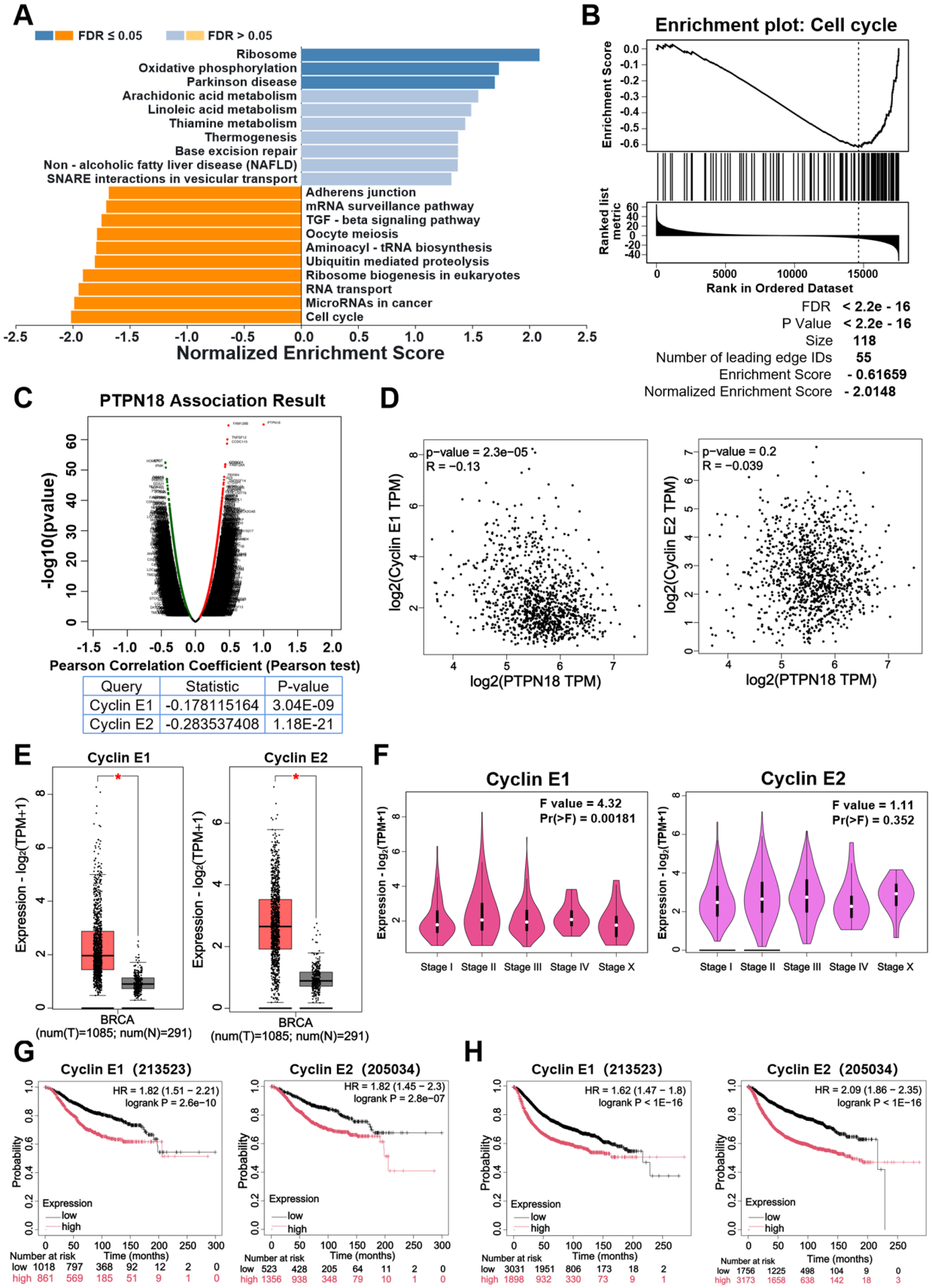

Figure 5. Bioinformatic statistical analysis showed a significant negative correlation between PTPN18 and the cell cycle in BC. (A) Bar chart of the standardized enrichment score of PTPN18 in BC. (B) Cell cycle gene set enrichment analysis of PTPN18 in BC. (C) Volcano plot showing the genes associated with PTPN18 in BC samples analyzed by LinkedOmics. (D) Correlation analysis of PTPN18 with cyclins E1 and E2 using data from the Gene Expression Profiling Interactive Analysis database. (E) Expression profiles of the cyclin E1 and cyclin E2 genes in normal and tumor tissues of the breast (T, red; N, gray). (F) Box plots of gene expression changes in different tumor stages. (G and H) Kaplan-Meier Plotter database was used to analyze the effect of the cyclin E1 or cyclin E2 expression levels on the (G) overall survival and (H) relapse-free survival of patients with BC. PTPN18, protein tyrosine phosphatase non-receptor 18; BC, breast cancer; T, tumor; N, normal.

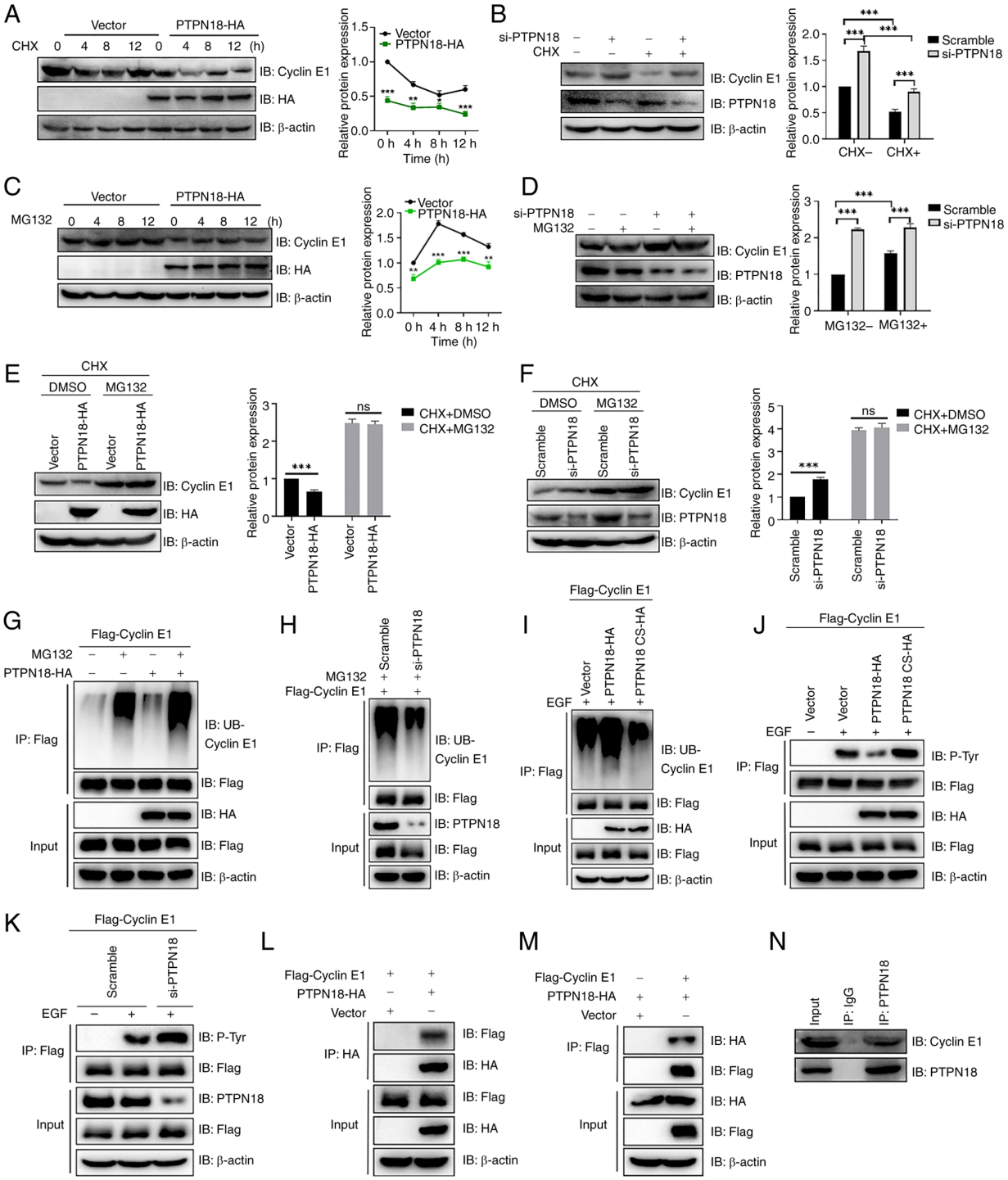

Figure 6. PTPN18 can interact with cyclin E1 and promote its proteasomal degradation. (A) Effect of PTPN18 on the cyclin E1 protein levels at different time points of CHX (10 μg/ml) treatment. (B) Knockdown of PTPN18 suppressed cyclin E1 degradation under CHX-treated conditions. (C) Proteasome inhibitor MG132 (10 μM) significantly decreased the effects of PTPN18 on cyclin E1 degradation. (D) Effect of PTPN18 knockdown on cyclin E1 expression in the absence or presence of MG132 treatment. The bar graph was obtained by normalizing the levels to β-actin. (E and F) Combined treatment with CHX and MG132 almost abolished the effects of PTPN18 (E) overexpression and (F) knockdown on cyclin E1 protein levels compared with controls. (G) PTPN18 overexpression elevated the ubiquitination level of cyclin E1. (H) PTPN18 knockdown decreased cyclin E1 ubiquitination. (I) Ubiquitination of cyclin E1 by PTPN18 requires enzymatic activity. (J) Cyclin E1 is dephosphorylated by PTPN18 upon EGF (100 ng/ml, 30 min) stimulation. (K) PTPN18 knockdown upregulated the tyrosine phosphorylation levels of cyclin E1. (L and M) Analysis of the results from (L) forward and (M) reverse co-immunoprecipitation showed the interaction between ectopic PTPN18 and cyclin E1. (N) The physical association between endogenous PTPN18 and cyclin E1 was validated using co-immunoprecipitation. Data are shown as the mean ± SD values from three technical replicates following 48 h of overexpression or 72 h of knockdown treatment. *P<0.05, **P<0.01 and ***P<0.001. PTPN18, protein tyrosine phosphatase non-receptor 18; CHX, cycloheximide; ns, not significant.

PTPN18 can regulate the protein expression levels of CDK inhibitor 1A (CDKN1A, also known as p21Cip1) and CDK inhibitor 1B (CDKN1B, also known as p27Kip1) through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway leading to cell cycle arrest. To further explore the signaling pathways through which PTPN18

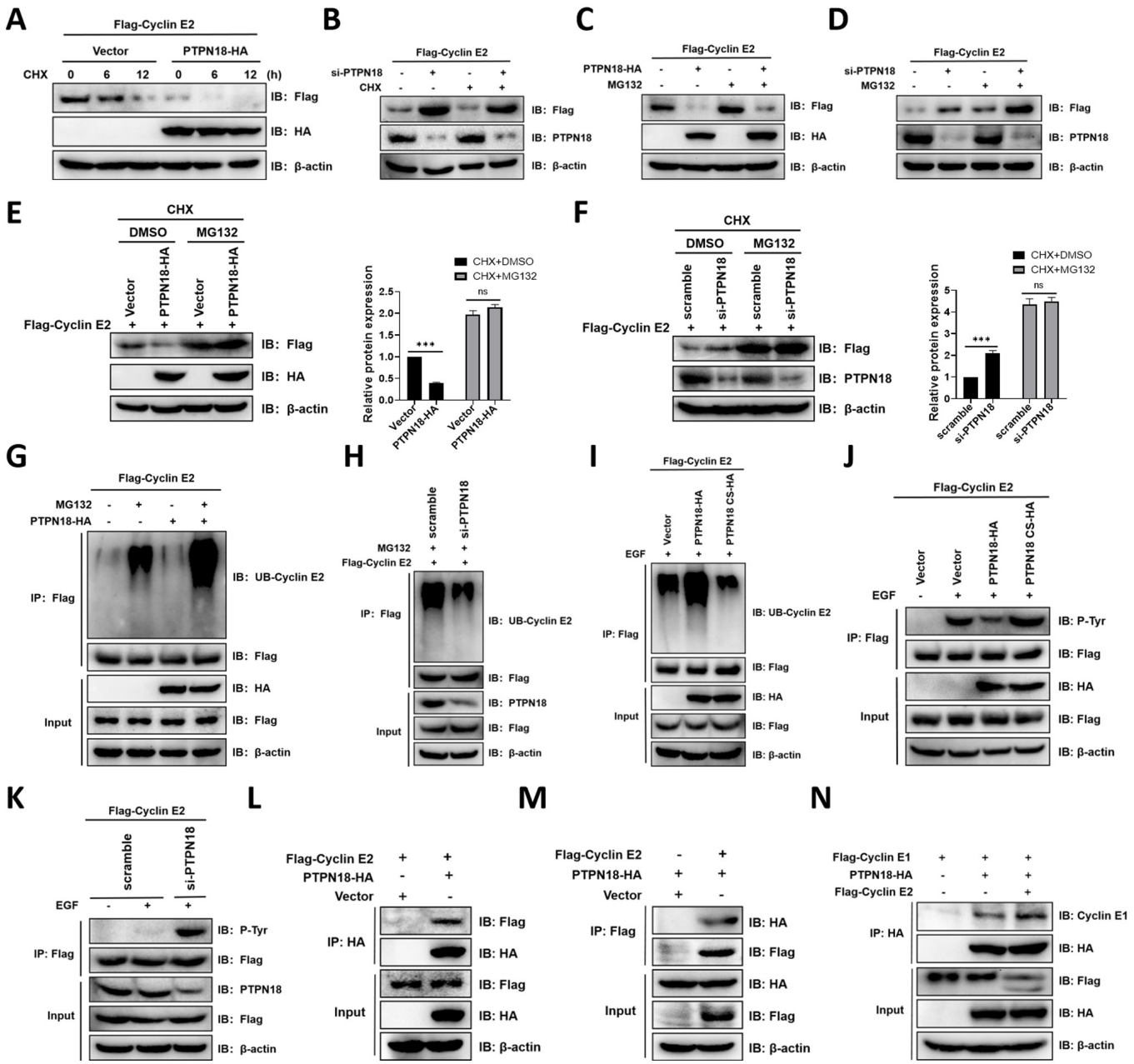

Figure 7. PTPN18 promotes the ubiquitinated degradation of cyclin E2. (A) Effect of PTPN18 on cyclin E2 protein expression at different time points of CHX (10 μg/ml) treatment. (B) Knockdown of PTPN18 inhibits the degradation of cyclin E2. (C) Effect of PTPN18 overexpression on cyclin E2 expression in the absence or presence of MG132 (10 μM, 6 h) treatment. (D) Effect of PTPN18 knockdown on cyclin E2 expression in the absence or presence of MG132 (10 μM, 6 h) treatment. (E) Overexpression or (F) knockdown of PTPN18 had no significant effect on the cyclin E2 protein levels compared with the controls when CHX and MG132 were combined. (G) Overexpression of PTPN18 enhanced cyclin E2 ubiquitination. (H) PTPN18 knockdown decreased the ubiquitination level of cyclin E2. (I) PTPN18 CS reduced the ubiquitination of cyclin E2. (J) PTPN18 regulated the pan-tyrosine phosphorylation levels of cyclin E2. (K) PTPN18 knockdown increased the tyrosine phosphorylation levels of cyclin E2. The binding between exogenous PTPN18 and cyclin E2 was verified by co-immunoprecipitation: (L) immunoprecipitation with PTPN18-HA and (M) immunoprecipitation with flag-cyclin E2. (N) Cyclin E2 had no significant effect on the binding of PTPN18 to cyclin E1. Data are shown as the mean ± SD from three technical replicates following 48 h of overexpression or 72 h of knockdown transfection. ***P<0.001. ns, not significant; PTPN18, protein tyrosine phosphatase non-receptor 18; CHX, cycloheximide; CS, C229S; ns, not significant; si-, small interfering.

regulates the cell cycle, key genes associated with the cell cycle according to the KEGG database were selected to examine the effects of PTPN18 on their transcript levels. The results demonstrated that PTPN18 overexpression produced significant changes in the phosphoinositide-dependent protein kinase 1 (PDPK1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit γ (PI3CG), forkhead box O3 (FOXO3), mitogen-activated protein kinase 8 (MAPK8), CDKN1A, growth arrest and DNA damage-inducible protein α (GADD45A), adenomatous polyposis coli protein (APC) and E1A-binding protein p300 (EP300) transcript levels, indicating that PTPN18 may have a wide range of multi-target and multi-pathway roles in gene transcription regulation (Fig. S3A-H). Analysis using STRING, a functional protein association network database, showed that CDKN1A and CDKN1B closely interacted with cyclins E1 (CCNE1) and

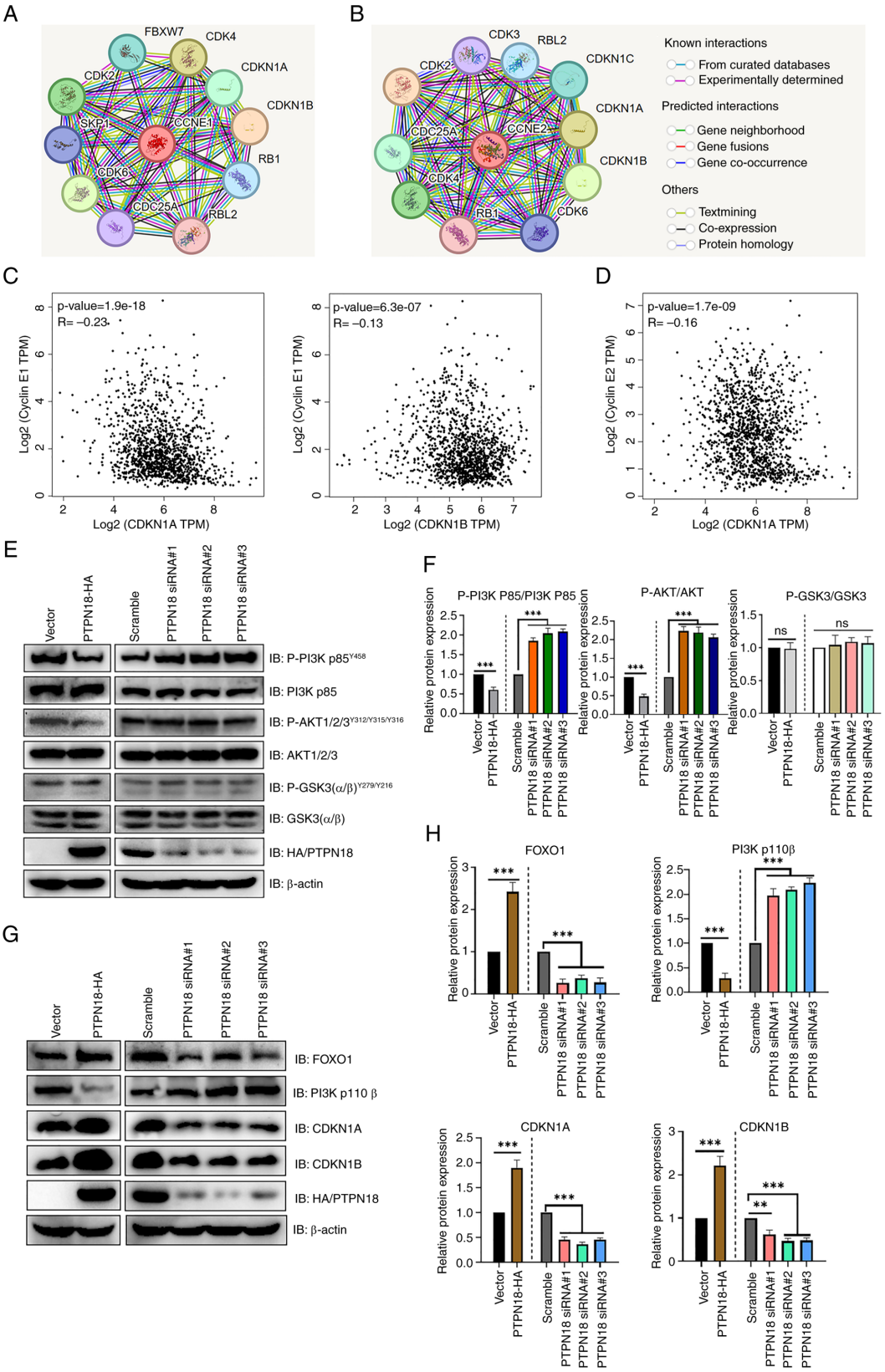

Figure 8. PTPN18 can regulate the expression levels of CDKN1A and CDKN1B proteins through the PI3K/AKT signaling pathway leading to cell cycle arrest. (A and B) STRING database analysis showed proteins that interact with (A) cyclin E1 (CCNE1) and (B) E2 (CCNE2). (C) GEPIA database analysis of the CDKN1A and CDKN1B association with cyclin E1. (D) Correlation analysis between CDKN1A and cyclin E2 using the GEPIA database. (E) Western blot analysis of the PI3K/AKT signaling pathway protein expression levels in MCF-7 cells. Levels of P-PI3K p85, P-AKT and P-GSK3 were monitored after a 30-min stimulation with EGF (100 ng/ml). (F) Relative quantitative analysis of the PI3K/AKT signaling pathway protein expression levels. (G) Western blotting demonstrated the expression levels of FOXO1, PI3K p110β, CDKN1A and CDKN1B. (H) The bar graph was obtained by normalizing the levels to β-actin. Data are shown as the mean ± SD from three technical replicates following 48 h of overexpression or 72 h of knockdown. **P<0.01 and ***P<0.001. PTPN18, protein tyrosine phosphatase non-receptor 18; GEPIA, Gene Expression Profiling Interactive Analysis; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; CDKN1A (p21Cip1), cyclin-dependent kinase inhibitor 1A; CDKN1B (p27Kip1), cyclin-dependent kinase inhibitor 1B; P-, phosphorylation; FOXO1, forkhead box O1; ns, not significant; P-, phosphorylated; siRNA, small interfering RNA.

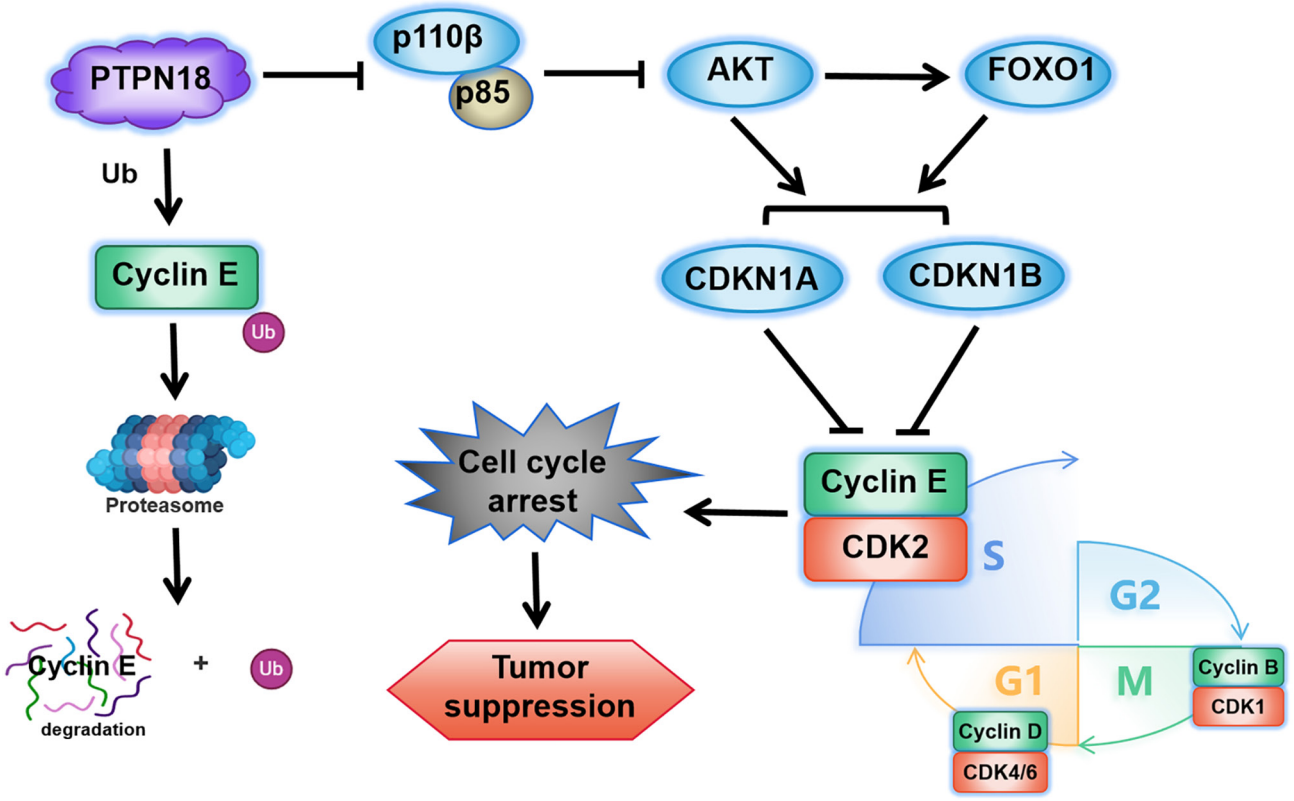

Figure 9. Proposed working model in which PTPN18 negatively regulates cyclin E to interfere with cell cycle progression to exert tumor suppressive effects in BC. The present study found that PTPN18 could inhibit the AKT signaling pathway by downregulating the expression of PI3K p110β as well as the tyrosine phosphorylation of PI3K p85 and AKT. The inhibited AKT pathway weakens the negative regulation of downstream FOXO1, CDKN1A and CDKN1B. Increased expression of CDKN1A and CDKN1B enhances the inhibition of the cell cycle and cyclin E expression, ultimately leading to cell cycle arrest and BC suppression. PTPN18 can also enhance cell cycle arrest by downregulating cyclin E expression through the ubiquitin-proteasome pathway. PTPN18, protein tyrosine phosphatase non-receptor 18; BC, breast cancer; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; CDKN1A (p21Cip1), cyclin-dependent kinase inhibitor 1A; CDKN1B (p27Kip1), cyclin-dependent kinase inhibitor 1B; FOXO1, forkhead box O1.

E2 (CCNE2) (Fig. 8A and B). The Kendall statistical analysis results from the GEPIA database also showed that CDKN1A and CDKN1B had significant negative correlations with cyclin E1 and CDKN1A had a negative correlation with cyclin E2 (Fig. 8C and D). Further protein level experiments showed that PTPN18 overexpression significantly decreased the tyrosine phosphorylation levels of PI3K p85 and AKT and the protein expression levels of PI3K P110β compared with the controls, whereas the protein levels of FOXO1, CDKN1A and CDKN1B were significantly increased (Fig. 8E-H). Decreased levels of AKT tyrosine phosphorylation resulted in reduced inhibition of FOXO and increased FOXO expression, followed by increased CDKN1A and CDKN1B protein levels, resulting in cyclin inhibition and cell cycle arrest (Figs. 8E-H and 9). The results of the endogenous PTPN18 knockdown experiments were generally consistent with those of the overexpression experiments (Fig. 8E-H). Taken together, the protein level results confirmed that the antitumor effect of PTPN18 may be closely related to the PI3K/AKT signaling pathway.

Discussion

PTPN18 is expressed in a variety of normal tissues and is ubiquitous in the nucleus and cytoplasm of cells, with low tissue specificity (29,33,34). The core function of PTPN18 is to act as a negative regulator of receptor tyrosine kinase signaling and regulate a variety of cellular processes including cell proliferation, differentiation, the mitotic cycle and oncogenic transformation by dephosphorylating key receptors such as HER2 (7,10-17). It has also been shown that PTPN18 is a protective factor against obesity, hyperlipidemia and type 2 diabetes (35). More notably, PTPN18 is closely related to the development of BC and its high expression improves the survival and prognosis of patients with this disease (15-17,29,30); therefore, it is necessary to dissect the specific mechanism of action of PTPN18 in BC. In the present study, through tumor phenotypic functional assays, it was found that overexpression of PTPN18 inhibited metastasis and proliferation, promoted apoptosis and led to cell cycle arrest in BC cells. Although MCF-7 cells lack caspase-3 activity, PTPN18 may still achieve pro-apoptotic effects by negatively regulating the PI3K/AKT pathway, regulating Bcl-2 family protein balance, increasing ROS levels, or activating lysosomal enzymes, triggering compensatory activation of other effector caspases or mitochondria-mediated caspase-independent apoptosis (36-38). Annexin V/PI double staining results directly verified the existence of this apoptotic phenotype.

Since the cell cycle is the key regulatory hub of cell proliferation, apoptosis and metastasis (39), as well as owing to the notable role of PTPN18 in the BC cell cycle, subsequent experiments in the present study focused on exploring the molecular mechanisms by which PTPN18 affects cell cycle changes to

dissect its tumor suppressor mechanism. It was found that PTPN18 not only interacted with cyclin E1 and downregulated its protein expression and tyrosine phosphorylation levels, but it also acted on cyclin E2 with the same effect, indicating that PTPN18 may be a specific and broad-spectrum regulator of the cyclin E family. Moreover, PTPN18 binding to cyclin E1 was not perturbed by cyclin E2. This property may allow PTPN18 to comprehensively inhibit cyclin E family-mediated abnormal proliferation, avoid isoform compensation and improve the scope and stability of its antitumor effects. Cyclin E can bind to the CDK1 and CDK2 protein kinases and participate in the regulation of the G1 phase, G1/S phase transition and S phase progression (18-23,40,41). As an important regulatory protein in the S phase, the decrease of cyclin E expression and activity may directly lead to the inability of retinoblastoma protein (RB) to maintain a highly phosphorylated state (39-41), resulting in S phase-related proteins not being successfully synthesized, the accumulation of cells in the S phase and the failure of cells to transition to the G2/M phase. The effect of PTPN18 on RB phosphorylation status and the key downstream targets of cyclin E/CDK2 activity decrease will be the next research focus to dissect the mechanism of S phase arrest caused by PTPN18 affecting DNA replication.

In the present study, it was found that PTPN18 overexpression enhanced the expression of cyclin B1, cyclin D1 and CDK4. Elevated cyclin B1 expression promotes the formation and activation of maturation-promoting factor, thereby accelerating G2/M phase transition and cell division, resulting in a decrease in the number of G2/M phase cells (42-44). Cyclin B can competitively bind to CDK2 and CDK1 with cyclin E, affecting the stability of the cyclin E/CDK complex and arresting the cell cycle in the S phase (19,42,43). The cyclin D-CDK4/6 complex is the main regulatory complex of the G1 phase of the cell cycle and phosphorylates RB (45). The increase of cyclin D1 and CDK4 may compensate for the effect of cyclin E in G1 phase and alleviate the stress trend caused by the accelerated cycle progression in G2/M phase, allowing G1 phase to transform normally and maintain dynamic balance.

In the present study, PTPN18 significantly affected the transcript levels of PDPK1, PI3CG, FOXO3, MAPK8, CDKN1A, GADD45A, APC and EP300. FOXO and CDKN1A are downstream targets of the PI3K/AKT pathway (46) and PI3K/AKT can regulate GADD45A through the FOXO pathway (47). AKT may play a role in the regulation of a variety of cellular activities through the transcriptional coactivator EP300 (48) and MAPK8 can regulate the AKT pathway (49). Additionally, dual PI3K/mTOR inhibition is a potential therapeutic strategy for APC and PIK3CA mutant colorectal cancer (50). Given that these significantly altered genes are all associated with the PI3K/AKT pathway, the present study examined the regulation of the PI3K/AKT pathway by PTPN18 at the protein level. The results of the present study indicated that PTPN18 significantly decreased the tyrosine phosphorylation levels of PI3K p85 and AKT and the protein levels of P110β as well as markedly increased the protein levels of FOXO1, CDKN1A and CDKN1B. Decreased tyrosine phosphorylation levels of PI3K p85 and AKT leads to inhibition of their function. P110β stimulates cell proliferation and upregulation in the wild-type state is oncogenic (51). A reduction in P110β expression directly leads to the weakening of its cancer-promoting effect and a relative increase in intracellular PI3K p85 content; excess free PI3K p85 inhibits the PI3K/AKT pathway (52). Combined with the characteristics of PTPN18 protein tyrosine phosphatase and experimental data, it is speculated that PTPN18 may regulate PI3K/AKT pathway by directly dephosphorylating the tyrosine sites of PI3K P85 and/or AKT, and then inhibit BC progression through FOXO1-CDKN1A/CDKN1B-Cyclin E axis (Fig. 9); this hypothesis is consistent with the consensus of field studies (15,46,51-55), but experiments are still needed to verify the specific substrate and whether there are other potential targets.

Unlike previous studies in which PTPN18 has been shown to exert antitumor effects in BC through targets such as HER2 or ETS1 (15-17), a novel anticancer target of PTPN18, cyclin E, was discovered in the present study. The current literature suggests that cyclin E depletion improves the chemosensitivity of BC cells to DNA-damaging agents (56). Patients with tumors harboring high cyclin E expression have a reduced benefit from tamoxifen treatment compared with patients with low tumor expression (57). However, the present study found that PTPN18 could downregulate cyclin E expression and activity, which is likely to be one of the key mechanisms by which PTPN18 synergistic drug treatment results in an improved prognosis and longer survival of patients with BC. Needless to say, the role and molecular mechanism of PTPN18 in the efficacy of conventional anticancer drugs is an important guide for future research. In the future, it may be possible to focus on exploring how to develop new therapeutic strategies by enhancing the activity of PTPN18 or mimicking its mechanism of action.

In summary, the antitumor effect of PTPN18 should be the result of a combination of numerous mechanistic pathways and substrates (15-17). Based on the current findings alone, it is not sufficient to clarify whether PTPN18 can be used as a reliable marker for BC diagnosis. Subsequently, a large number of experiments are required to analyze the relationship between PTPN18 expression level or activity and different clinicopathological features of BC and explore its potential as a diagnostic marker. Because the original intention of this paper was to dissect the protective mechanism of high PTPN18 expression on OS in patients with BC, and its function in normal breast cells may be at rest or physiological homeostasis, it was not investigated. However, the function and mechanism of PTPN18 in normal breast cell lines should not be ignored, which is an important supplement to comprehensively elucidate its role in breast physiological homeostasis and the development of BC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 32100992) and Key Laboratory of Bioresource Research and Development of Liaoning (grant no. 2022JH13/10200026).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NZ performed the majority of the experiments, analyzed the data and wrote and edited the manuscript. TW, BB and XZ constructed the plasmids. WX and WC performed the reverse transcription-quantitative PCR and data analysis. YY and BW directed the study, analyzed and approved all of the data, and wrote and edited the manuscript. NZ and BW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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