

# A novel human TINP1 gene promotes cell proliferation through inhibition of p53 and p21 expression

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**Abstract.** Transforming growth factor (TGF)- $\beta$ -inducible nuclear protein 1 (TINP1) is a novel gene, which is localized at chromosome 5q13 where frequent abnormalities in hairy cell leukemia (HCL) occur. The present study investigated the effects of TINP1 knockdown or overexpression on the viability and gene expression of various tumor cell lines. siTINP1 was designed to knock down TINP1 expression. Reverse transcription polymerase chain reaction (RT-PCR) and western blotting were performed to assess gene expression; the cell counting kit-8 (CCK-8) assay was used to detect cell viability, and luciferase and flow cytometry assays were used to determine gene activity. *TINP1* was widely expressed in various cell lines. In addition, TINP1 siRNA was able to knock down TINP1 expression in HeLa cells. TINP1 overexpression significantly promoted tumor cell proliferation, which may be associated with the downregulation of p53 expression. Furthermore, *TINP1* promoted a number of cell lines to the S phase of the cell cycle. *TINP1* promotes cell proliferation and significantly reduces p53 and p21 expression.

## Introduction

The novel proliferation-related gene transforming growth factor (TGF)- $\beta$ -inducible nuclear protein 1 (TINP1) was recently identified and cloned by using the gene-function screening platform of the fluorescein reporter gene-phenotype system (1). TINP1 (GenBank accession number: NM\_014886) is localized at chromosome 5q13, which shows frequent abnormalities in hairy cell leukemia (HCL), indicating that this chromosomal region harbors a gene involved in the transformational events of HCL (2). Indeed, the cell viability and the cell cycle analysis have shown that *TINP1* promotes HeLa cell

proliferation, which provides a basis for the further study of this gene's function. The *TINP1* gene covers 1,105 bp with 6 exons and 5 introns for coding a protein containing 260 amino acids (1,3,4). The PSORT II program predicted that *TINP1* may be a nuclear protein (91.3%) and that the *TINP1* gene contains a Ribosomal\_S8e conserved domain at amino acids 1-237 after searching the Pfam database. Ribosomal\_S8e is a very highly conserved domain in eukaryotes, and its function is involved in RNA-protein interactions that are associated with mRNA translation and protein synthesis (3,5). In addition, a previous study indicated that the human *TINP1* gene is homologous to the *NSA2* gene in *Saccharomyces cerevisiae* (6). The *NSA2* gene has been demonstrated to play an important role in *Saccharomyces cerevisiae* growth, and it has been shown that the *TINP1* gene exhibits similar functions to those of *NSA2*, which significantly promotes the growth of gene-deficient *Saccharomyces cerevisiae* (2). However, the role of TINP1 in human carcinogenesis and cancer progression remains largely unknown. It is clear that cell proliferation is an essential characteristics of tumor initiation and progression. Cell proliferation is due to cell cycle progression, which is specifically regulated in a time- and space-dependent manner, and abnormal cell proliferation induces the development of multiple diseases (4,7,8), including human cancer. Thus, the present study investigated the signaling transduction pathway involved in human *TINP1* gene promotion of cell proliferation and the association between *TINP1* expression and multiple proliferation-related signaling pathways. Furthermore, the molecular mechanisms responsible for the *TINP1*-mediated gene pathway were explored to provide a potential drug target for cancer treatment.

## Materials and methods

**Cell lines and culture.** A cervical cancer HeLa cell line, an osteosarcoma U2OS cell line, a colorectal cancer HCT-16 cell line, a lung cancer H1299 cell line, and a hepatocellular carcinoma HepG2 cell line were maintained in our laboratory. A lung cancer A549 cell line, a human embryonic kidney HEK293T cell line, a Jurkat cell line and a THP-1 cell line were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). These cell lines were

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cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO<sub>2</sub> and 95% air.

**Design of siTINP1 and gene transfection.** TINP1 small interfering RNA (si-TINP1) and non-silencing control siRNA were designed and purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The non-silencing siRNA was confirmed to have no matches with the complete human genome by a BLAST search in NCBI (www.ncbi.nlm.nih.gov). These siRNAs were used to transfect tumor cells, i.e., tumor cells were seeded into 6-well plates at a concentration of  $2.5 \times 10^5$  cells/ml and grown in DMEM containing 10% FBS for 24 h. siTINP1 and TINP1 cDNA containing plasmid or non-silencing siRNA (negative control) were transfected with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total cellular RNA from cultured tumor cells was isolated using the TRIzol™ reagent (Invitrogen), according to the manufacturer's instructions, and reversely transcribed into single-strand cDNA using the Reverse transcript™ kit (Invitrogen). Next, PCR amplification was performed using specific primers for the *TINP1* gene: 5'-CTG CTG GAC AGA GAG GGA CAA-3' and 5'-TTT CTC CCT GGG CAC GTA CTT-3'. The housekeeping gene GAPDH was used as a loading control of PCR amplification using the following primers: 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'. The RT-PCR amplification was performed with the ThermoScript RT-PCR system (Invitrogen), according to the manufacturer's protocol, with an initial cycle of 94°C for 5 min and 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were separated in 2% agarose gel and semi-quantified according to the levels of GAPDH mRNA. Then, the *TINP1* expression was compared with that of the negative control siRNA or vector only-transfected cells.

**Protein extraction and western blot analysis.** Total cellular protein was extracted from the cultured cells using a RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% deoxycholate Na; 1% NP-40; 0.1% sodium dodecyl sulfate, with freshly added protease inhibitor cocktail). Protein concentrations were determined using the BCA protein assay reagent (Pierce, Biotechnology, Inc., Rockford, IL, USA). Following quantification, 20 µg of total protein samples were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Hybond™, ECLTM; Amersham Pharmacia, Little Chalfont, UK). After blocking for 2 h in 5% non-fat milk in Tris-buffer supplemented with 0.05% Tween-20 (TBS-T), the membrane was incubated with the corresponding primary antibody at 4°C overnight. Polyclonal antibodies against p53, Erk 1/2, JNK/SPAK, p38, and β-actin were obtained from Cell Signaling Technology (Boston, MA, USA). The membrane was then rinsed in TBS-T three times for 10 min each and incubated with the secondary antibody at room temperature for 2 h. The membrane was rinsed again in TBS-T three times for 10 min

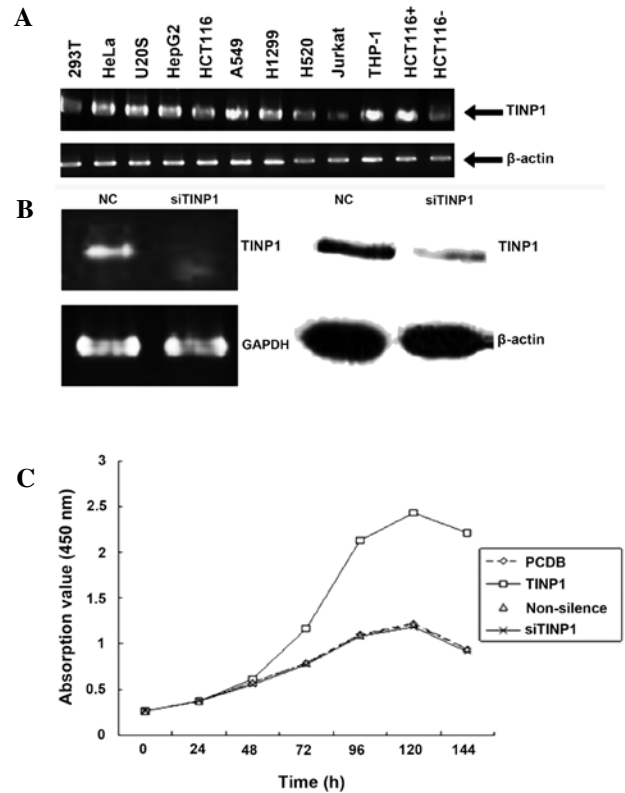


Figure 1. Knockdown of TINP1 expression in HeLa cells. (A) Expression of TINP1 mRNA in multiple cell lines; (B) knockdown of TINP1 mRNA and protein expressions in HeLa cells using siTINP1 nucleotides; (C) knockdown of TINP1 expression using siTINP1 reduced HeLa cell viability.

each and visualized using an ECL chemiluminescence system (Bio-Rad Laboratories, Hercules, CA, USA).

**Cell viability cell counting kit-8 (CCK-8) assay.** The cells were seeded into 96-well plates at a density of  $2 \times 10^3$  cells/ml in a volume of 100 µl/well and placed in an incubator for 18-24 h. After reaching 40-60% confluency, the cells were transfected with 20 ng of blank vector as a negative control, TINP1 plasmid, and H-Ras (as a positive control), respectively, using the VigoFect transfection reagent in triplicate and then cultured for 5 days. At the end of the experiments, 10 µl of CCK-8 reagent was added to each well, and the cells were further incubated for 2 h. Subsequently, the absorbance value was determined using a multifunctional plate reader (GENios Pro™). Cell viability was summarized as the percentage of control.

**Flow cytometry.** The cells were seeded into 6-well plates at a density of  $3 \times 10^5$  cells/ml and transfected with different plasmids for up to 3 days. At the end of the experiments, the cells were digested with 2.5% pancreatin for 24 h, harvested and prepared into single-cell suspensions. After washing with pre-cooled phosphate-buffered saline (PBS) twice, the cell suspension was rotated on a Vortex rotator; cooled 75% ethanol was added dropwise to the suspension and the cells were fixed at -20°C overnight. The next day, the cell suspension was washed with PBS twice, incubated with RNase (10 µg/ml) at 37°C for 30 min and stained with propidium iodide. The cells were then analyzed by a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

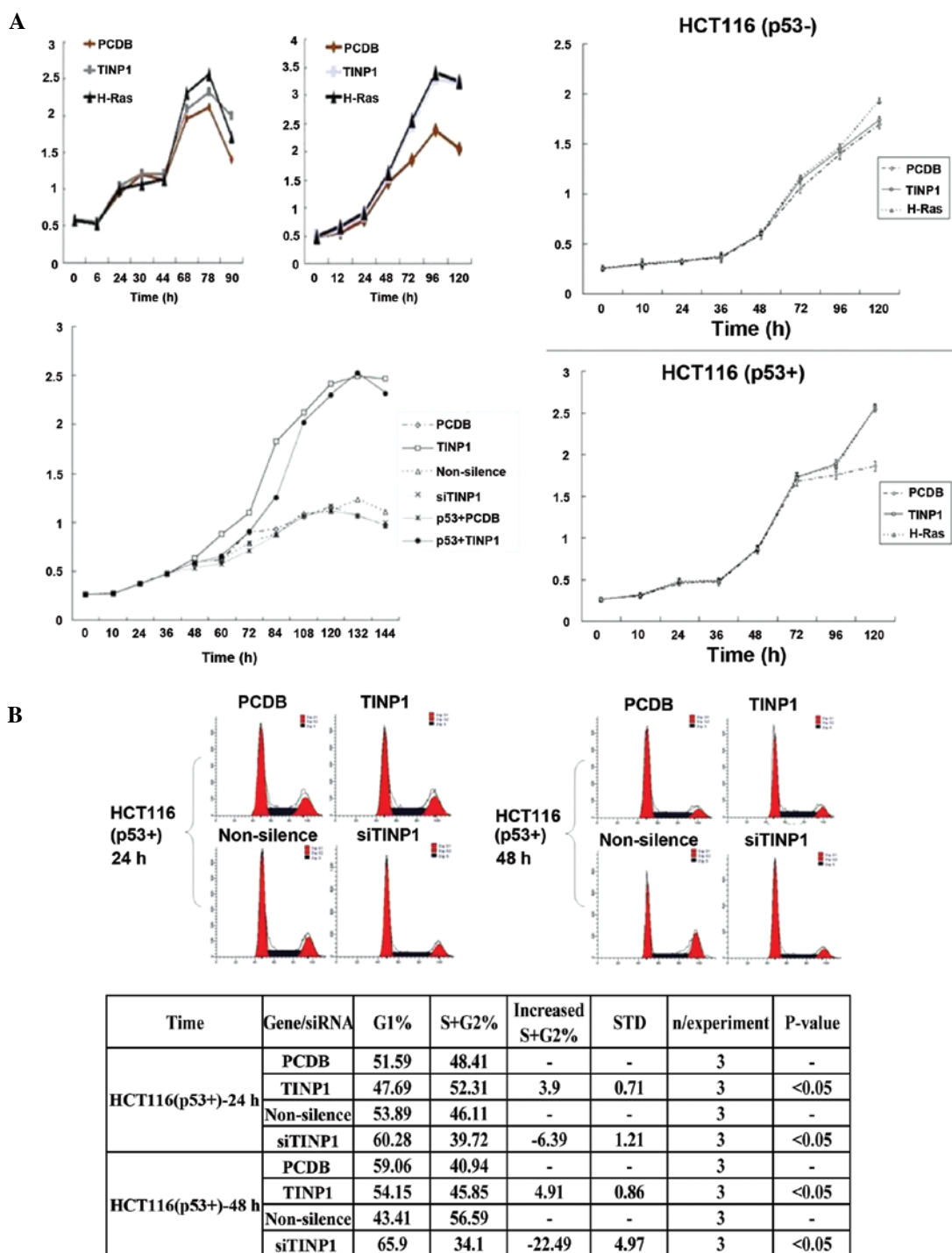


Figure 2. Effect of TINP1 cDNA transfection in promoting tumor cell viability and cell cycle progression. (A) Cell viability assay; (B) flow cytometry assay.

**Luciferase reporter assay.** The cells were seeded into 96-well plates at a density of  $1.1 \times 10^6$  cells/ml in a volume of 100  $\mu$ l/well and placed in an incubator for 18-24 h. After reaching 40-60% confluency, the cells were co-transfected with the cell proliferation-related pathway reporters: 50 ng of NF- $\kappa$ B-LUC, pWNT-LUC, pP53-LUC, pC/EBP-LUC, or pT-bet-LUC plus 50 ng of pAP-1-LUC, 5 ng of pRL-TK-LUC, and 50 ng of TINP1 or the control plasmid using the VigoFect transfection reagent (Beijing Vigorous, Beijing, China). Cells transfected with blank vector served as blank controls, while cells transfected with TNF- $\alpha$ ,  $\beta$ -catenin, p53, PKA, aj41 and MEKK served as positive controls. These transfections were performed in triplicate

and repeated at least once. Following incubation for 24 h, the luciferase activity was determined using a multifunctional plate reader (GENios Pro<sup>TM</sup>). The relative fluorescence value of the cells transfected with blank vector was defined as 100%, and the results were expressed as the mean value of three replicate wells.

**Statistical analyses.** The data are summarized as means  $\pm$  standard deviation (SD) as appropriate. The Student's t-test was used to generate a P-value using SPSS software (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant difference.

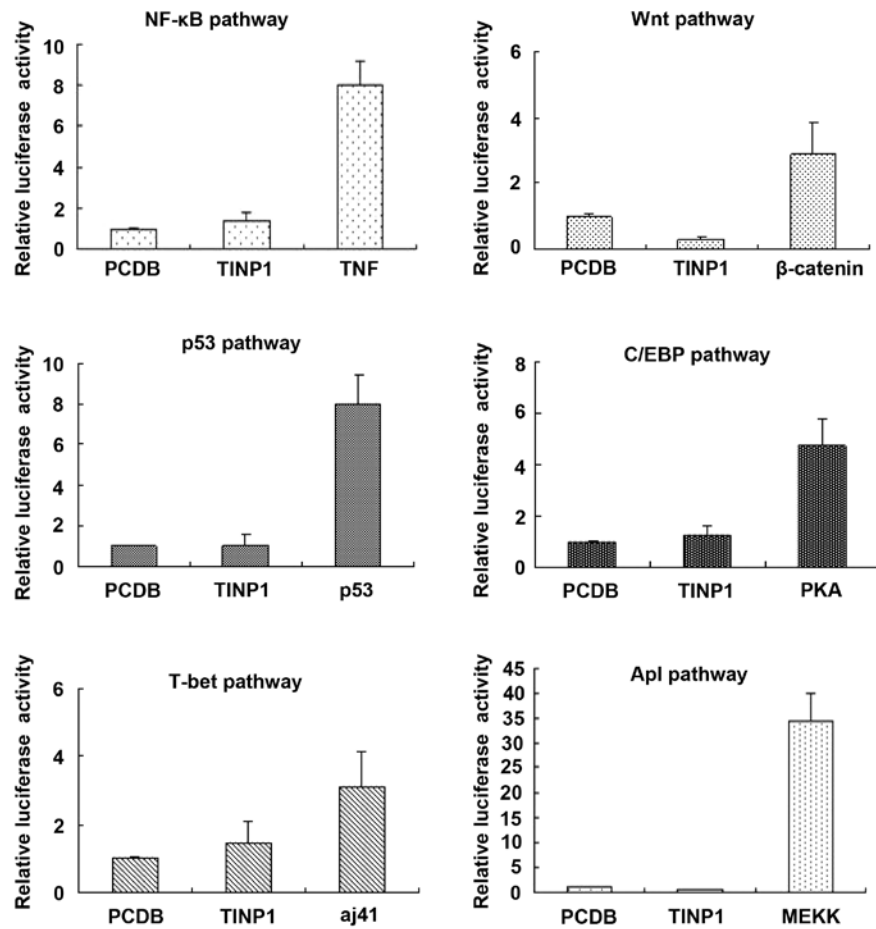


Figure 3. Effects of *TINP1* overexpression on the regulation of different gene pathways.

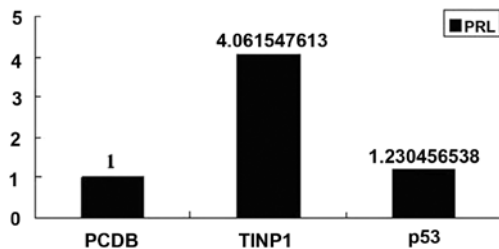


Figure 4. Effect of *TINP1* overexpression on the regulation of *p53* pathway activity.

## Results

**Expression of *TINP1* and knockdown of *TINP1* expression using *siTINP1*.** In the present study, we first detected *TINP1* expression in multiple cell lines and found that *TINP1* was expressed in most of these cells using RT-PCR (Fig. 1A). Next, we chose HeLa cells for knockdown of *TINP1* expression using *TINP1* siRNA nucleotides. After 24 and 48 h of gene transfection, the endogenous *TINP1* mRNA expression in *siTINP1*-transfected cells was significantly reduced compared to that in negative control cells (Fig. 1B). Next, we assessed the effects of *TINP1* knockdown on the regulation of HeLa cell viability and found that *siTINP1* significantly reduced tumor cell viability (Fig. 1C).

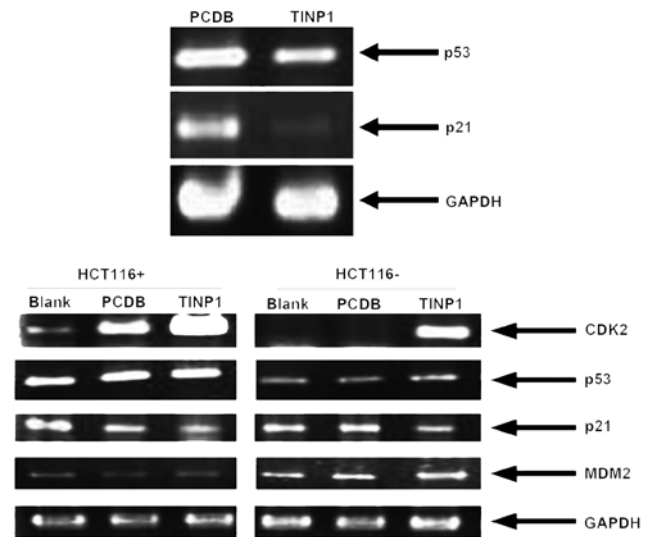


Figure 5. Effect of *TINP1* on the regulation of *p53* and *p21* mRNA expression.

**Effect of *TINP1* overexpression on tumor cell viability and cell cycle progression.** We also assessed *TINP1* overexpression on the regulation of cell proliferation by transfecting *TINP1* cDNA into HeLa cells and found that *TINP1* promoted cell growth (Fig. 2A). In addition, flow cytometry data showed that *TINP1* induced more cells at the S phase of the cell cycle, further indicating cell proliferation (Fig. 2B).

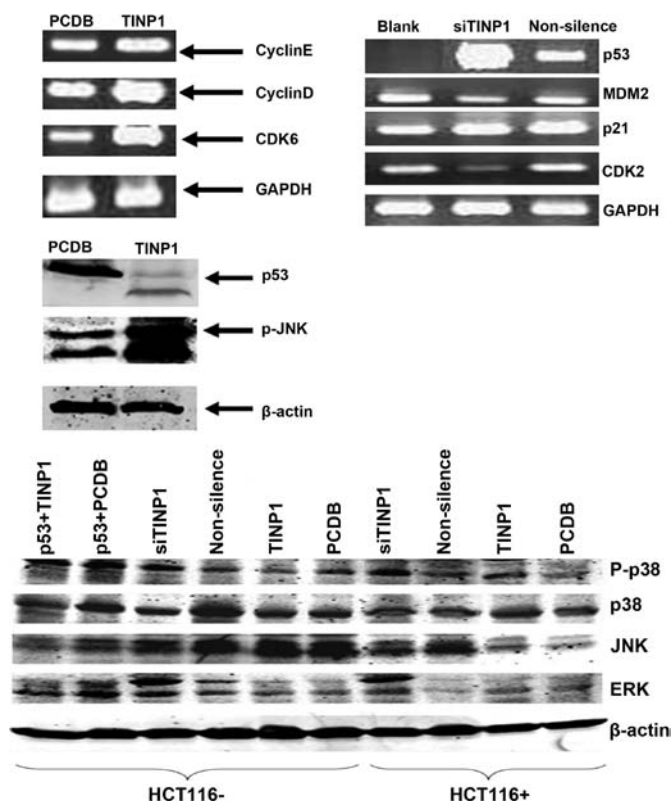


Figure 6. Effect of endogenous *TINP1* expression on the regulation of p53 expression and CDK2 activation.

**Effect of *TINP1* overexpression on signaling pathways.** To explore the role of *TINP1* in signaling pathway regulation, we performed a dual-luciferase reporter assay to investigate six signaling pathways (including NF- $\kappa$ B, WNT, p53, C/EBP, T-bet and AP1). The data showed that *TINP1* did not significantly alter the activities of these six signaling pathways (Fig. 3). However, *TINP1* significantly promoted pRL-TK-LUC activity in the p53 pathway (Fig. 4). Next, we determined the expression of these p53-related genes and found that *TINP1* significantly inhibited p53 mRNA levels, silenced p21 mRNA expression in HCT-116 cells, and induced cyclin D1 and CDK6 expression (Figs. 5 and 6). By contrast, *TINP1* siRNA induced p53 and p21 expression but reduced the expression of MDM2 and CDK2 mRNA (Fig. 5).

## Discussion

In the present study, we investigated the effects of *TINP1* knockdown and overexpression in the regulation of cell growth and gene expression in various cell lines. We found that *TINP1* was expressed in multiple cell lines and that knockdown of *TINP1* expression reduced HeLa cell proliferation. By contrast, the overexpression of *TINP1* promoted cell proliferation in various cell lines, which was associated with the inhibition of p21 and p53 expression to promote the S phase of the cell cycle. However, *TINP1* overexpression did not affect the activities of five different signaling pathways (NF- $\kappa$ B, WNT, C/EBP, AP1 and T-bet). These data suggest that *TINP1* plays a role in cell growth by regulating the expression of p53 and p21-related genes. Future studies will further investigate how *TINP1* affects p53 and p21 gene expression so

that targeting *TINP1* expression in tumor cells may be used in clinical settings as a novel strategy to treat cancer.

The data from the present study clearly show that *TINP1* overexpression significantly promoted cell proliferation and advanced the cells to the S phase of the cell cycle, whereas *TINP1* knockdown reduced HeLa cell viability. These data confirmed a previous study that indicated a proliferative role of *TINP1* in cells, since the human *TINP1* gene is a homolog of the *NSA2* gene in *Saccharomyces cerevisiae*. The *NSA2* gene plays a role in the growth of *Saccharomyces cerevisiae* (2). Furthermore, the SymAtlas online application revealed that the human *TINP1* gene is expressed in multiple tissues (2). Indeed, our present study demonstrated that *TINP1* was expressed in multiple cell lines. Since cell proliferation (i.e., growth) is involved in multiple gene signaling pathways, we assessed the role of *TINP1* in the activation of six different gene pathways. Our data showed that *TINP1* does not alter the luciferase activities of NF- $\kappa$ B, WNT, C/EBP, AP1 or T-bet genes. However, further study showed that the overexpression of *TINP1* inhibited the expression of p53 and p21 mRNA but induced cyclin D1 and CDK6 expression, whereas knockdown of *TINP1* induced p53 and p21 expression. Our present study linked *TINP1* and the p53 gene pathway as promoting cell cycle progression. Further studies will investigate how *TINP1* inhibits p53 and p21 expression and will also examine *TINP1* as a novel target in cancer therapy.

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