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Original Article

Nuclear localization signal of TGF-β-inducible nuclear protein 1 mediates functions of TINP1 in suppression of the p53 signaling pathway and promotion of cell proliferation

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Abstract: Transforming growth factor (TGF)-β-inducible nuclear protein 1 (TINP1) functions to regulate cell proliferation. This study used bioinformatics techniques to analyze TINP1 cDNA and assessed the role of the nuclear localization signal 2 (NLS2) in the regulation of TINP1-induced cell proliferation. An NLS2-truncated TINP1 DNA fragment was generated and subcloned into the pcDB vector. Hela cells were transfected with pEGFP-N1-TINP1-ΔNLS2 and cellular localization of TINP1-ΔNLS2 was analyzed by fluorescence microscopy. HEK293T cells were co-transfected with luciferase reporter plasmids for proliferation-related genes. Expression of proliferation-related genes was also assessed by PCR and Western blot. The effect of TINP1-ΔNLS2 on proliferation was assessed by cell viability assay. TINP1-ΔNLS2 was found to not affect the nuclear localization of TINP1, but significantly enhanced expression of T-bet, NF-κB, AP-1, WNT, C/EBP and p53 ($P<0.05$) more strongly than TINP1. TINP1-ΔNLS2 transfection induced HeLa and HCT116 cell proliferation more weakly than TINP1. TINP1-ΔNLS2 transfection reduced levels of p53 and p21 mRNA and protein less strongly than TINP1, whereas TINP1-ΔNLS2 transfection substantially reduced expression of cyclin E mRNA and protein. In conclusion, TINP1-ΔNLS2 did not affect nuclear localization of TINP1, and possessed a weaker capacity to induce proliferation and modulate gene expression than TINP1, suggesting that NLS2 is required for TINP1 suppression of p53 signaling and TINP1-induced proliferation.

Keywords: *TINP1*, proliferation, p53 pathway, protein domain function

Introduction

Cellular proliferation is strictly regulated through both spatial and temporal steps in DNA replication [1]. Abnormal proliferation contributes to the development and progression of multiple human diseases. For example, dysregulation of cell cycle progression is a hallmark of cancer cells [2]. Several genes have been implicated in the regulation of cell cycle progression including cyclin-dependent kinases (CDKs), CDKs inhibitors, and E3 ubiquitin ligases [3], but many others remain undefined, and the precise molecular mechanisms underpinning spatial and temporal regulation of cell cycle progression in the dynamic cellular microenvironment remain to be fully characterized. Targeting of the cell cycle-regulatory machinery, such as CDKs, was previously reported to be a promising strategy in development of anticancer drugs [3]. Furthermore, elucidation of the mechanisms underlying proliferation and the cell cycle may improve our understanding of multiple physiological and pathological processes involved in hemostasis and human disease.

The transforming growth factor (TGF)-β-inducible nuclear protein 1 (TINP1) is a homologue of Saccharomyces cerevisiae NSA2, which plays an important role in regulation of Saccharomyces cerevisiae proliferation [4]. TINP1

is localized on chromosome 5q13, and abnormalities in this chromosome are frequently observed in hairy cell leukemia (HCL) [5], implicating TINP1 in HCL development. TINP1 is expressed in various cells, including epithelia, endothelia, fibroblasts, and monocytes. A high level of TINP1 expression is also observed in normal tissues, such as the brain, heart, testicles, and muscles (Li et al., 2013). However, to date, the role of TINP1 in the development and progression of human cancers remains largely unknown. Silencing of TINP1 expression using TINP1 siRNA has been previously reported to reduce cell viability and cause cell cycle arrest in the S phase, while ectopic overexpression of TINP1 protein promotes proliferation of several human cell lines [6]. At the gene level, TINP1 siRNA-induced cell cycle arrest is accompanied by upregulation of p53 and p21 expression [6]. Thus, in this study, we further investigated the role of TINP1 and its nuclear localization signals (NLSs) in cell cycle regulation. Bioinformatic analysis revealed that the gene encoding TINP1, NSA2, contains two NLSs, NLS1 (amino acids 1 to 52) and NLS2 (amino acids 131 to 154) [7-9]. Thus, we investigated the influence of TINP1 mutations on cell cycle regulation and constructed an NLS2-deficient truncated TINP1 mutant (TINP1-ΔNLS2) to assess the role of NLS2 in TINP1 activity, and regulation of proliferation and cell cycle progression in various cancer cell lines. The results of this study could aid elucidation of the role of TINP1 in cell cycle progression and targeting of TINP1 that may represent a novel strategy for treatment of human cancers.

Materials and methods

Cell lines and culture

Human cervical cancer (HeLa), human colorectal cancer (HCT116), and human embryonic kidney (HEK) 293T cell lines were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml, Sigma Chemicals, St Louis, MO, USA), and streptomycin (100 μg/ml, Sigma Chemicals) in a humidified incubator with 5% CO_2 at 37°C.

Construction of NLS2-truncated mutant TINP1 (TINP1-ΔNLS2)

An NLS2-truncated *TINP1* DNA fragment was generated by PCR amplification of the template pcDB-TINP1 and two mutation-specific primers (5'-CAG GGA GAA ACA TTT GTT GGA GAT-3' and 5'-ATC TCC AAC AAA TGT TTC TCC CTG-3'), and subcloned into the pcDB vector. After amplification and DNA sequence confirmation, this vector was identified as TINP1-ΔNLS2 and used in the following experiments.

Fluorescent microscopic detection

Hela cells were seeded into cell couture dishes, transfected with pEGFP-N1-TINP1-ΔNLS2, and after 48 h fluorescence microscopy was used to analyze cellular localization of TINP1-ΔNLS2.

Dual-luciferase reporter assay

HEK293T cells were resuspended at density of 1.1×10^6 cells/ml in DMEM containing 10% FBS, and 100 μL of cell solution was seeded in each well of a 96-well plate. After 18 to 24 h culture at 37°C with 5% CO_2 cells had reached 40% to 60% confluency, and were co-transfected with one of six luciferase reporter plasmids for proliferation-related genes including pNF-κB-LUC, pWNT-LUC, pTP53-LUC, pC/EBP-LUC, pT-bet-LUC, and pAP-1-LUC (50 ng each) with 5 ng of pRL-TK-LUC and 50 ng of TINPI1, TINP1-ΔNLS2, or control plasmid using the Vigofect transfection reagent (Vigorous Biotechnology, Beijing, China). Cells transfected with an empty vector served as a negative control, while vector-only served as a transfection control. All transfections were performed in triplicate and repeated at least once. After 24 h incubation, luciferase activity was assessed using a Promega luciferase kit (Madison, WI, USA) and luminescence was measured using a GENios Pro™ multifunctional plate reader. The relative luminescence of each experimental condition was normalized to the value of cells transfected with the empty vector and expressed as the mean ± SEM.

Cell counting kit-8 (CCK-8) assay

Cells were resuspended at 2×10^3 cells/mL and 100 μL of cell solution was seeded into each well of a 96-well plate. After 18 to 24 h culture when reached 40% to 60% confluency, cells

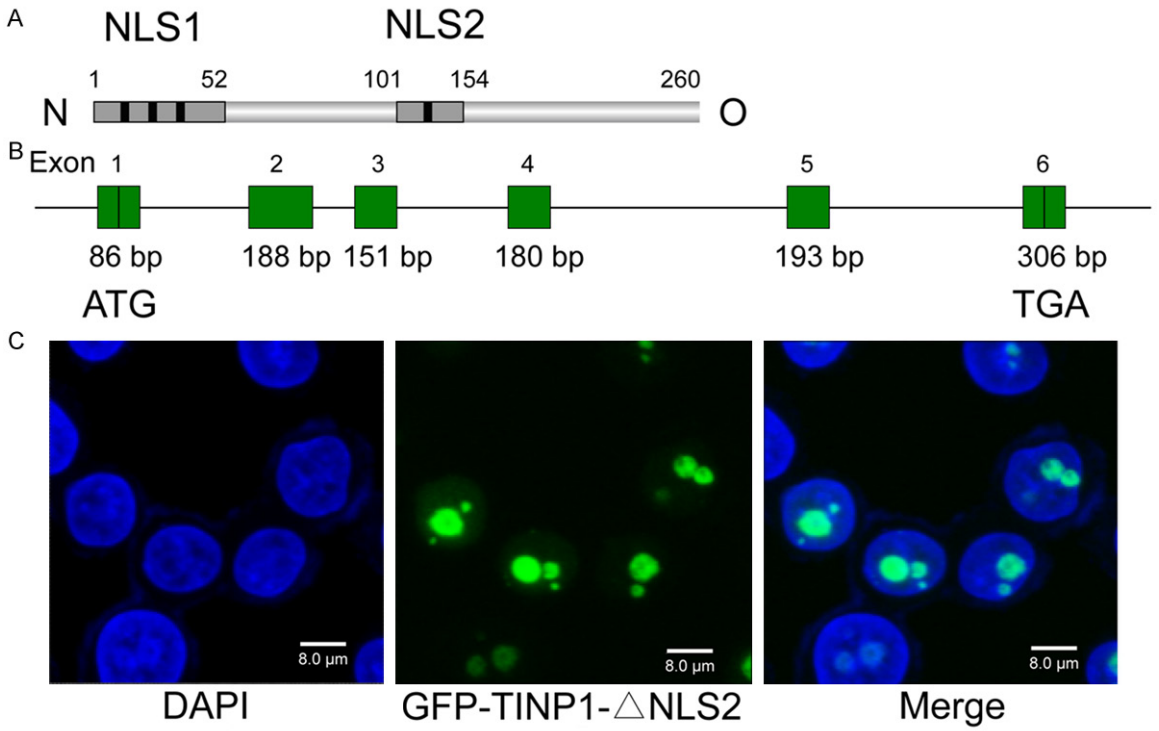

Figure 1. *TINP1* structure and bioinformatic analysis of *TINP1* nuclear localization signal. A. Bioinformatic analysis of *TINP1* nuclear localization signal using the predict NLS tool on the cNLS Mapper website. B. TINP1 cDNA and structure. *TNP1* cDNA was retrieved from GenBank database (The accession number: NM_014886). Color boxes is exon, Black belt shows the nls1 and nls2 positioning. C. Fluorescent microscopy. The data showed that TINP1-ΔNLS2 construct-induced expression of TINP1 mutated protein was localized in HeLa cell nuclei under an inverted fluorescent microscope.

were transfected in triplicate with 20 ng of empty vector (a negative control), H-Ras vector (a positive control), TINP1, or TINP1-ΔNLS2 using the VigoFect transfection reagent. After 96 h culture of cells, 10 μL CCK-8 reagent (Shanghai Fanke Industry and Developing Co., Ltd., Shanghai, China) was added to each well and the cells were further incubated for 2 h. Subsequently, the absorbance of each well was measured using a GENios Pro™ multifunctional plate reader. Cell viability was reported as the percentage of control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were seeded into 6-well plates at a density of 3×10^5 cells per well. After 18 h culture, they were transfected with 3 ng empty vector (a negative control), p53 (a positive control), TINP1, or TINP1-ΔNLS2 vector using the VigoFect transfection reagent for 24 h. After that, total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and reversely transcribed to cDNA using the Quant Reverse Transcriptase kit (TianGen BioTech Co., Ltd., Beijing, China). PCR amplification was performed using specific primers for each gene, as previously described (6). The PCR products were then separated in 1.5% agarose gel and semi-quantified relative to *mRNA* levels of the internal control GAPDH. The two mutation-specific PCR primers were 5'-CTG CTG GAC AGA GAG GGA CAA-3' and 5'-TTT CTC CCT GGG CAC GTA CTT-3'. The plasmid expressing GFP was cotransfected and GFP expression was detected by confocal microscopy to quantify transfection. The transfection efficiency (the number of cells fluorescing green fluorescence/the total cell number) was approximately 40%.

Protein extraction and Western blot

Cytoplasmic and nuclear extracts were prepared and the concentration of solubilized pro-

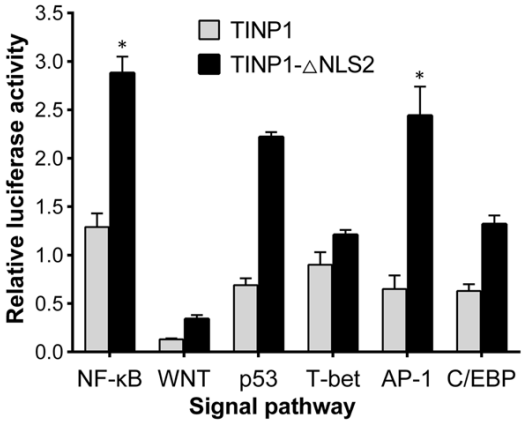

Figure 2. Effect of TINP1-ΔNLS2 on regulation of signaling pathway gene activities. HEK293T cells were transfected with pNF-κb-LUC, pWNT-LUC, pP53-LUC, pC/EBP-LUC, pT-bet-LUC, or pAP-1-LUC plus pRL-TK-LUC and TINPI1, TINP1-ΔNLS2, or control vector using Vigofect transfection reagent. After 24 h, the dual luciferase reporter activity was assessed. There are statistical significance, and mark with *Presented data represents the mean of triplicate experiments, which were repeated at least twice with similar results. *P<0.05.

tein was determined according to the manufacturer's protocol (Bio-Rad, CA, USA). For whole cell lysis, transfected cells were lysed on ice for 20 min using a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 1% DTT, and 1% protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cell lysates were then centrifuged at 12,000 g for 10 min at 4°C and the supernatants were then stored at -80°C. To isolate nuclear protein, the cells were washed with ice-cold PBS three times and nuclear extracts were prepared following the manufacturer's protocol (Bio-Rad). The cytoplasmic and nuclear extracts were then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were then blocked in 5% nonfat milk in Tris-based saline-Tween 20 (TBS-T) at room temperature for 2 h, followed by incubation with an antibody against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p65 (Santa Cruz Biotechnology), β-actin (Santa Cruz Biotechnology), or PARP (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. On the next day, the membranes were washed with TBS-T thrice and further incubated with horseradish peroxidase-coupled secondary goat anti-rabbit or anti-mouse immunoglobulin G (Santa Cruz Biotechnology). The protein bands were then detected using the Immune-Star Western Chemiluminescence Kit (Bio-Rad) according to the manufacturer's instructions.

Statistical analysis

The data were expressed as means ± standard deviation with different groups compared using analysis of variance (ANOVA), and Bonferroni test. $P<0.05$ was considered to indicate statistical significance. All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA).

Results

Activity of the nuclear localization signal domains in TINP1

TINP1 encodes a 260-amino-acid protein, but, to date, the precise cellular localization of TINP1 remains unknown. We performed bioinformatic analysis and found two potential NLSs in TINP1 (**Figure 1**), NLS1 (amino acids 1 to 52) and NLS2 (amino acids 131 to 154). We then constructed a mutated TINP1 containing a truncated NLS2 to assess whether this putative NLS influences the effect of TINP1 on cell cycle control. We found that TINP1-ΔNLS2 TINP1 mutated protein was still localized in cell nuclei. However, in comparison to cells transfected with wild-type TINP1, the TINP1-ΔNLS2 mutation slightly enhanced the activity of dual-luciferase reporters for T-bet, WNT, C/EBP and p53, and substantially increased the activity of a dual-luciferase reporters for NF-κB, and AP-1 (**Figure 2**). These results suggest that NLS2 in TINP1 regulated activity of several transcription factors, particularly NF-κB, and AP-1.

Effect of TINP1-ΔNLS2 on tumor cell proliferation

We previously reported that TINP1 expression promoted proliferation of various human cell lines [6]. Thus, we assessed whether TINP1-ΔNLS2 plays a role in TINP1-mediated regulation of proliferation. In comparison to transfection with wild type TINP1, in HeLa or HCT116 cells TINP1-ΔNLS2 transfection reduced cell viability (**Figure 3**). These results suggest that NLS2 is required for TINP1-mediated promotion of proliferation.

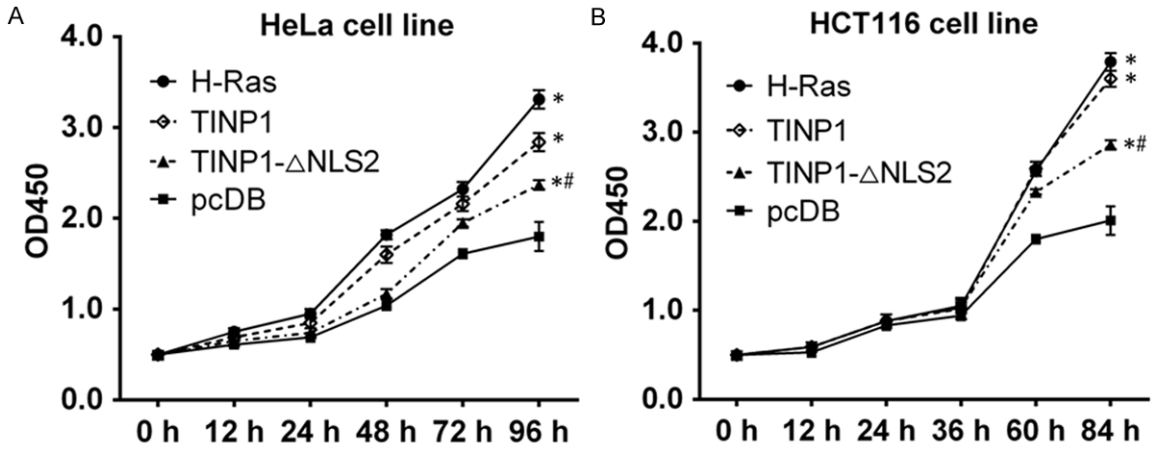

Figure 3. Effect of TINP1-ΔNLS2 on cell proliferation. HeLa and HCT116 cells were transfected with empty vector (negative control), H-Ras plasmid (positive control), TINP1, or TINP1-ΔNLS2 for the indicated duration. Proliferation was assessed by CCK-8 assay. (A) Hela cells and (B) HCT116 cells. P<0.05 pcDB, TINP1, or TINP1-⊿NLS2 vs. H-Ras; #P<0.05 TINP1-⊿NLS2 vs. TINP1.

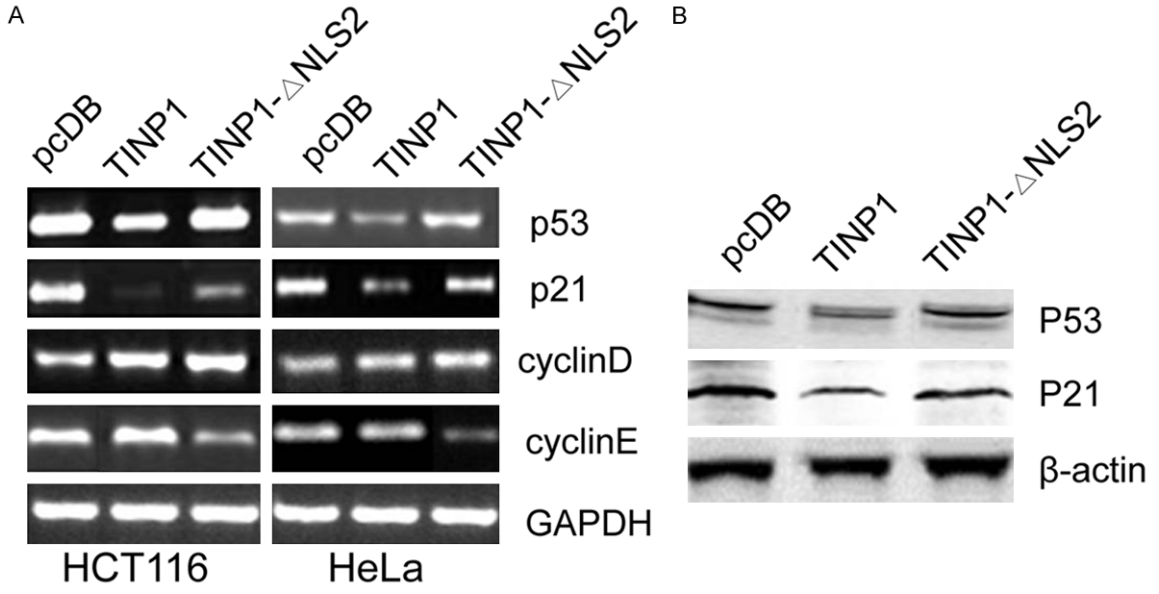

Figure 4. Effect of TINP1-ΔNLS2 on expression of proliferation-related genes. HEK293T (A) and HeLa (B) cells were transfected with pcDB (negative control), TINP1, or TINP1-ΔNLS2 vector. After 24 h total RNA was isolated and expression of proliferation-related genes was assessed by PCR and Western blot. (A) PCR. GAPDH was used as an internal control. (B) Western blot. b-actin was used as a loading control. The experiments were repeated at least twice with similar results.

We then performed cell cycle analysis and found that the percentage of cells in the S phase of cell cycle was higher after TINP1 or TINP1-ΔNLS2 transfection than in PCDB transfected cells. The percentage of cells in S phase was increased from 31±1.4% to 50±4.2% by TINP1 transfection, whereas the percentage of S phase cells was increased from 31.0±1.4 to 40.5±0.71% by TINP1-ΔNLS2 transfection, indicating that TINP1-ΔNLS2 weakly induced proliferation (**Figures 4**, **5**).

Effect of TINP1-ΔNLS2 on expression of cell proliferation-related genes

Cell cycle progression is mainly controlled by CDKs and regulated by p53 and p21. We thus

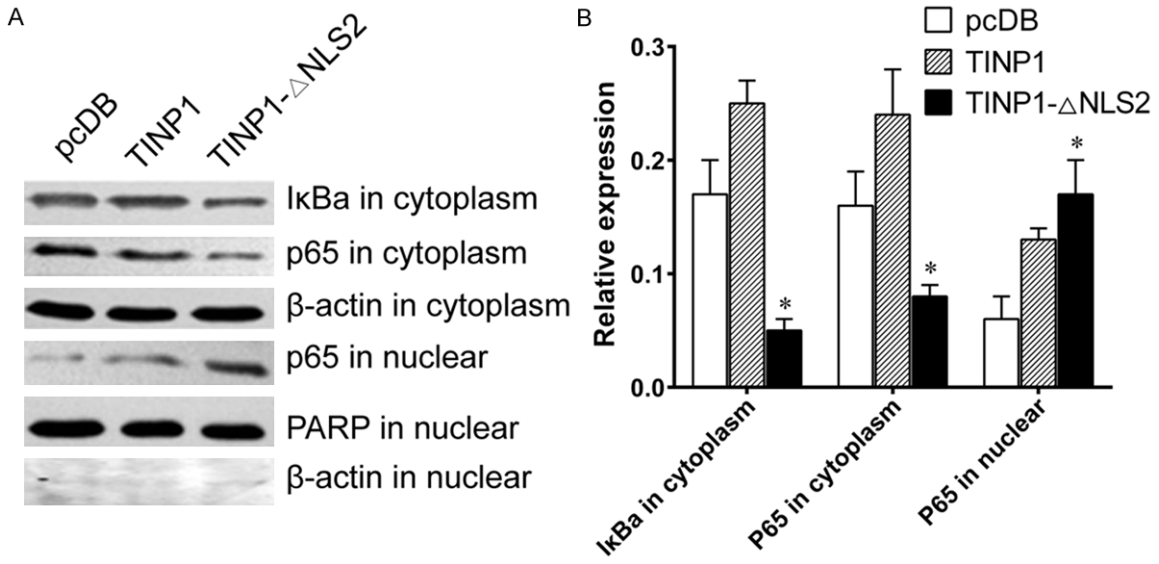

Figure 5. Effect of TINP1-ΔNLS2 on NF-κB activation. (A) IκBα and p65 expression was analyzed using Western blot of the cytoplasm or nuclear extracts. The cytoplasmic and nuclear loading controls were β-actin and PARP, respectively. Presented data represents the mean of triplicate experiments, which were repeated at least twice with similar results. (B) Quantified data of (A). *P<0.05.

assessed levels of proliferation-related genes in after TINP1-ΔNLS2 transfection. Wild type TINP1 protein dramatically reduced the levels of p53 and p21 mRNA and slightly increased levels of cyclin D mRNA but not cyclin E mRNA. However, TINP1-ΔNLS2 protein did not alter the level of p53 mRNA, but reduced the levels of cyclin E mRNA in HCT116 cells. TINP1-ΔNLS2 transfection also reduced the level of p21 mRNA, albeit more weakly than TINP1 (**Figure 4A**). Similar changes in p53, p21, cyclin E, cyclin D mRNA levels were also observed in HeLa cells after transfection of TINP1-ΔNLS2 (**Figure 4B**). TINP1 reduced protein levels of p53 and p21 in HeLa cells, and TINP1-ΔNLS2 also reduced expression of p53 and p21 proteins, but more weakly than TINP1 (**Figure 4B**). Taken together, these data indicate that NLS2 is required for TINP1-mediated suppression of p53 and cyclin E expression, and thus influences the capacity of TINP1 to regulate cell cycle progression.

We also assessed NF-κB activation by Western blot. The resting NF-κB complex consists of P50, P65, and IκB kinase. Activation of NF-κB and degradation of the IκBα protein results in dissociation of NF-κB from IκBα and thereby allows nuclear translocation of the p50/p65 dimer [10]. After transfection of PCDB, TINP1, or TINP1-ΔNLS2 construct, analysis of the cytoplasmic and nuclear extracts of macrophages revealed both IκBα degradation and translocation of p65 into the nuclei (**Figure 5**). However, TINP1 induced less degradation and nuclear translocation than TINP1-ΔNLS2. These results indicate that NLS2 plays a role in inhibition of NF-κB transcriptional activity.

Discussion

Cellular proliferation plays an important role in homeostasis and human disease and is tightly regulated by different proteins. In this study, we investigated the role of TINP1 in regulation of gene expression and proliferation. We found that TINP1 contains two NLS domains and deletion of the second NLS (NLS2) promoted NF-κB, and AP-1 activity compared to wild type TINP1. Ectopic overexpression of TINP1-ΔNLS2 reduced HeLa and HCT116 viability more substantially than wild type TINP1. TINP1-ΔNLS2 expression also slightly reduced levels of p53 and p21 mRNA and protein compared to TINP1. However, TINP1-ΔNLS2 didn't alter TINP1 nuclear localization. Our findings suggest that NLS2 is required for TINP1-mediated suppression of the p53 signaling pathway and promotion of proliferation. Thus, future work will assess whether targeting TINP1 represents a novel strategy for the control of human cancer.

We have successfully cloned human *TINP1* (GenBank accession number: NM_014886). *TINP1* contains a ribosomal_S8e conserved domain (amino acids 1 to 237) [8]. Ribosomal_S8e is a highly conserved domain in eukaryotes and is involved in RNA-protein interactions that are closely associated with mRNA translation and protein synthesis [8, 11]. A BLAST search also indicated human *TINP1* is homologous to *Saccharomyces cerevisiae Nsa2*. It should be noted Nsa2 has been reported to play an important role in *S. cerevisiae* growth [4]. TINP1 exhibits similar functions to those of *Nsa2,* significantly promoting growth of human cells [12]. However, the molecular mechanism by which TINP1 elicits these effects remains to be determined. By searching mRNA expression profiles at SymAtlas (www.http://symatlas.gnf.org/), we found *TINP1* is expressed in multiple human tissues [13], underlining the significance of this gene and its functions. We previously found that the level of *TINP1* expression in lymphocytes differs from that in cancer cells, and that overexpression of TINP1 promoted proliferation of several human cells, including tumor cells [14]. Moreover, TINP1 was reported to inhibit p53 expression.

Different transcription factors (TF) can modulate transcription of target genes, and in various tumor cells many TFs such as NF-κB, AP-1 and CEBP are activated, and regulate expression of a series of proliferative genes, causing tumor progression [15]. Therefore, TFs play an important role in the signaling cascades involved in tumorigenesis. For example, AP-1 is an important TF that acts downstream of MAPKs. Phosphorylation of MAPK family proteins induced by external stimulation can lead to activation of nuclear transcription factors and cause changes in expression of downstream genes. Various factors have been shown to stimulate nuclear transcription factors including NF-κB and AP-1 by phosphorylating MAPKs, thus MAPK phosphorylation plays an important role in tumorigenesis [16]. Our results indicate that TINP1 contains two NLS sequences, NLS1 (amino acids 1 to 52) and NLS2 (amino acids 131 to 154). As a nuclear protein, TINP1 could regulate DNA transcription and metabolism. The TINP1 NLS2 sequence resembles a typical NLS [17], while TINP1 NLS1 does not. Thus, we mutated NLS2 to produce TINP1-ΔNLS2. Mutant TINP1-ΔNLS2 enhanced TINP1 induction of NF-κB and AP-1, indicating that mutation of TINP1 either blocked TINP1 translocation to the nuclei, or reduced the capacity of TINP1 to bind target proteins or genes. Both NF-κB and AP-1 are important transcription factors that regulate transcription of numerous genes essential for proliferation and survival. Thus our results suggest that NLS2 is required for TINP1 to regulate proliferation. However, in this study we did not assess the molecular mechanism by which TINP1 influences NF-κB and AP-1 expression. We also found that mutation of NLS2 reduced TINP1-mediated suppression of p53 and p21 expression, suggesting that NLS2 may be required for suppression of the p53 signaling pathway by TINP1, which promotes proliferation. Ectopic overexpression of TINP1-ΔNLS2 also downregulated cyclin E expression, which plays an important role in the transition and progression of the G1 and S phases of the cell cycle.

Since development and progression of multiple diseases is associated with enhanced proliferation, therapeutic interventions targeting proliferation represent a logical strategy for therapy of these diseases. Multiple genes are involved in the promotion or inhibition of tumor proliferation, and tumor proliferation may be effectively blocked by interventions targeting signal transduction pathways. Our results suggest that TINP1 may represent an effective target for anticancer drugs.

However, further work will be required to confirm the results of this proof-of-principle study. For example, the mechanism by which TINP1 expression is regulated, the mechanism by which TINP1 is translocated into the nuclei, and which segments of the TINP1 protein are responsible for binding cellular targets remain to be investigated.

Acknowledgements

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Disclosure of conflict of interest

None.

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