TPX2 Promotes the Proliferation and Metastasis of Osteosarcoma Cells Through Stabilizing E2F1

Min Mao

Shanghai General Hospital

Lei Zhou

Shanghai General Hospital

Xinglong Ma

Shanghai General Hospital

Mengkai Yang

Shanghai General Hospital

Gangyang Wang

Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiaotong University

Dongqing Zuo

Shanghai General Hospital

Zhuoying Wang

Shanghai General Hospital

Zongyi Wang

Shanghai General Hospital

Tao Zhang

Shanghai General Hospital

Jing Xu

Shanghai General Hospital

Yingqi Hua

Shanghai General Hospital

Zhengdong Cai (✉ caizhengdong@sjtu.edu.cn)

Shanghai General Hospital

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Abstract

Background: Osteosarcoma (OS) is the most common malignant bone cancer. Recently, more studies have shown that TPX2, a potential oncogene considered to be an important prognostic biomarker, is widely expressed in various tumors. However, until now, little is known about the impacts of TPX2 on the pathogenesis of OS.

Methods: TPX2 expression of in OS cells and tissues was detected by Western blot and immunohistochemical analyses. Various assays were used to analyze OS cell proliferation and metastasis *in vitro* and *in vivo*. The effect of TPX2 on the expression of E2F1 and proteins encoded by E2F1-regulated genes was evaluated by Western blotting. Coimmunoprecipitation, immunofluorescence and *in vitro* ubiquitination assays were employed to explore the molecular mechanisms underlying the effect of TPX2 on stabilizing E2F1 and maintaining E2F1 signaling.

Results: TPX2 is overexpressed in OS and is significantly correlated with the malignant progression of OS. TPX2 probably plays a critical role in the proliferation and metastasis of OS cells *in vitro*. Furthermore, TPX2 regulates E2F1 signaling by affecting the stability of E2F1, thereby affecting the proliferation and metastasis of OS cells. In addition, PSMD14 impacts the degradation and regulates the stability of E2F1. Most importantly, TPX2 regulates the interaction of E2F1 and PSMD14 to influence E2F1 degradation.

Conclusions: Our research not only identifies TPX2 as a promising therapeutic target in cancer but also illuminates its underlying molecular mechanism. We showed that TPX2 promotes the malignant progression of OS by regulating E2F1 signaling.

Background

Osteosarcoma (OS), arising from mesenchymal cells, is the most common malignant bone cancer and occurs mainly in children, adolescents, and young adults[1, 2]. Although clinical treatments for OS including surgical techniques and adjuvant chemotherapy have greatly improved, the prognosis of OS patients still remained unsatisfactory over the last three decades[3, 4]. Metastasis is commonly a critical cause of death in OS patients, and many OS patients experience recurrence due to existing or potential metastasis[5, 6]. On the one hand, due to the sensitivity of conventional imaging techniques, only approximately 20% of OS patients presenting with localized disease have detectable metastatic lesions at diagnosis; however, 40% of patients eventually develop metastasis during the course of OS[7]. On the other hand, because of its heterogeneity and genomic instability, OS has a high possibility of metastasis to the lungs[8, 9]. Therefore, an increasingly popular consensus is that new therapeutic and preventive targets that can effectively inhibit recurrence and metastatic progression must be developed. The identification of new targets for OS, focusing especially on the molecular mechanisms responsible for OS metastasis, is urgently needed and important.

TPX2, also referred to as Xklp2 in previous studies, was first described in *Xenopus laevis* by Heidebrecht et al in 1997 [10]. TPX2 is one of the microtubule-associated proteins regulated by the Ran-GTP gradient, which is critical for spindle assembly regulation at different levels. Ran-GTP is a GTPase that is widely distributed in the nucleus; it can promote the separation of TPX2 from importin- α and importin- β and activate TPX2[11, 12]. After activation in the nucleus, TPX2 can recruit Aurora-A to microtubules and induce the kinase activity of Aurora A during mitosis[13, 14]. Accumulating evidence has demonstrated that TPX2 is associated with numerous processes related to tumor progression, such as cancer cell proliferation, apoptosis, invasion, and migration. In particular, TPX2 regulates multiple signaling processes and exerts various influences on clinical stage and prognosis in various cancers, including hepatocellular carcinoma, lung, colorectal and gallbladder cancers[15–18]. For instance, overexpression of TPX2 plays a direct role in the presence of chromosomal instability in several cancers[19, 20]. However, until now, little is known about the impacts of TPX2 on the pathogenesis of OS. Moreover, more research is needed to fully elucidate the potential mechanism of TPX2 and its downstream pathways in OS.

E2F1, as a transcription factor, plays a vital role in various biological processes, such as apoptosis, tumorigenesis and metastasis. Early studies showed that E2F1 can act as a protective factor by inducing apoptosis via P53-dependent or P53-independent mechanisms upon exposure to genotoxic drugs[21]. Additionally, recent studies have revealed that aberrant upregulation of E2F1 correlates with malignant phenotypes and poor prognosis in a number of cancers, such as ovarian cancer, hepatocellular carcinoma, esophageal carcinoma, and lung cancer[22–24]. Our previous study demonstrated that E2F1 was upregulated in OS and positively correlated with the proliferation and metastasis of OS cells. In brief, the oncogenic role of E2F1 is regulated by other proteins in OS, and the expression of E2F1 and TPX2 showed a significant positive correlation through analyzing the commonly used OS databases, suggesting that TPX2 probably exerts various influences on these processes.

Herein, in this study, we explored the role and function of TPX2 in the growth and metastasis of OS. First, we found that TPX2 is upregulated in OS and correlates with poor prognosis. Via *in vitro* and *in vivo* assays, we further elucidated that TPX2 significantly enhances the growth and metastasis of OS. More importantly, we showed, for the first time, that TPX2 contributes to OS growth and metastasis by influencing the degradation of E2F1. In addition, we explored the molecular mechanism by which TPX2 affects E2F1 degradation. We elucidated that TPX2 influences the degradation of E2F1 through regulating the interaction of the deubiquitinating enzymes PSMD14 and E2F1.

Methods

Tissue microarray (TMA) and immunohistochemistry

TMA samples and scoring Tissue microarray contained 59 samples from the OS patients in Shanghai General Hospital. All TMA samples were retrieved after the patient consent and Institutional Review Board approve. The slide contained 3 μ m paraffin-embedded tumor tissues were dewaxing and rehydration, then underwent heat-induced antigen retrieval with 10mM citrate buffer (pH 6.0) and incubated with 3% H₂O₂

in methanol to neutralize peroxidase activity. Each slide was incubated overnight with a rabbit TPX2 primary antibody (ab32795) at 4°C followed blocking with 10% goat serum, then were reacted with secondary antibody. The slides were scored by using H-score method.

Expression data sets

A series of the microarray data (Mixed Osteosarcoma-Kuijjer-127-vstilmnhwg6v2) that included gene expression of TPX2 from 88 OS patient biopsies which was obtained from the R2: Genomics Analysis and Visualization Platform (Academic Medical Center, Amsterdam, the Netherlands; <http://r2.amc.nl>). The OS clinical difference was analyzed on basis of high versus low TPX2 expression,

Clinical samples

OS tissue samples and para tumoral tissue samples were collected from the patients in Shanghai General Hospital. No patient had received radiotherapy and chemotherapy prior to surgery. All samples in this study were retrieved after the patient consent and Institutional Review Board approve.

Cell culture and treatment

HEK-293T and human OS cell line 143B, HOS-MNNG, SJSA, MG63, HOS, KHOS and U2OS were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MG63.2 cell was derived from the metastasis of parental MG63, as previously reported. All cell lines were maintained in high glucose DMEM supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin. All the cell lines were cultured in an incubator at 37 °C in 5% CO₂.

Generation of shRNA knockdown and overexpression cell lines

Stable knockdown of TPX2 and PSMD14 was accomplished with Lv-shRNA-GP lentiviral expressing an shRNA against TPX2 and PSMD14 in conjunction with GFP and a puromycin selection marker. (TPX2 shRNA2: CTAATCTTCAGCAAGCTATTG, TPX2 shRNA4: GCTCGAGAAATTGCAACAATA, PSMD14 shRNA1: CAGATTGATCAATGCTAATAT, PSMD14 shRNA4: ACAGCAGAACAAGTCTATATC), Control Lv-shRNA-GP vector with nontargeting shRNA was used as a control. (control shRNA: CAACAAGATGAAGAGCACCAA).

Cell Counting Kit-8 (CCK-8) assay and Colony formation assay

The proliferation of OS cells was determined by CCK-8 assay (Dojindo, Tokyo, Japan). Various OS cells (1×10^3 /ml) were seeded into 96-well plates overnight. After 1, 3, 5, 7 and 9d, absorbance was read at a wavelength of 450 nm.

Various OS Cells were seeded in six-well plates at a density of 500 cells per well. The medium was changed with fresh medium for approximately 14 days until the cells grew into visible colonies. Colonies

were fixed with 4% paraformaldehyde and then stained with crystal violet for 15 min at room temperature. The colonies that consisted of 450 cells were counted.

Wound healing assay and Transwell migration/invasion assay

Various OS cells were seeded into six-well plates, then a wound was created by a 100 µl pipette tip when growing into full confluence. After 24h, cells were fixed with 4% paraformaldehyde, and photos of migration were obtained by an inverted microscope (Olympus, Tokyo, Japan).

Cells were starved with serum-free medium for 24 h and seeded at a density of 5×10^4 cells in each transwell insert with or without 100 µl matrigel. In all, 500 µl of growth medium was placed in each bottom well. Invaded cells in the lower side of the insert were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet after 24 h. Invaded cells were counted by an inverted microscope.

Western blot assay

Western blot assay was performed according to the standard protocol. Primary antibodies: TPX2 (#12245, Cell Signaling), E2F1(ab112580,abcam) , PSMD14(#4197,Cell Signaling),MMP9(#13667, Cell Signaling), MMP13(#94808,Cell Signaling), Survivin(#2808,Cell Signaling),Ub(#3936,Cell Signaling),Flag(#14793, Cell Signaling),His-tag(#12698, Cell Signaling). GAPDH(#5174, Cell Signaling) served as loading control.

RNA extraction and qPCR analysis

RNA samples from cells were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 1µg total RNA was reverse transcribed using a Reverse Transcription Kit (TaKaRa Inc., RR036A, JAPAN). The primer sequences were E2F1: Forward 5'-CCATCCCAGGAGGTCACTT-3' Reverse 5'-TGAGGTCCCCAAAGTCACA-3';Actin:Forward 5'-CCGTGAAAAGATGACCCAGATC-3', Reverse 5'-CACAGCCTGGATGGCTACGT -3'.

Immunofluorescence

The cells were fixed for 10 min at room temperature with 2% paraformaldehyde and permeabilized with 0.1% Triton-X100 for 15 min. After blocking with 5% BSA buffer, the cells were incubated with specific primary antibodies overnight at 4 °C. After washing by PBS, the cells were incubated with secondary antibodies, PSMD14-anti-rabbit and E2F1-anti-mouse, and both antibodies were dilute in blocking buffer and incubated at room temperature for 1 h. The cells were then washed by PBS, and counterstained with DAPI to stain nuclei.

Co-immunoprecipitation

The total protein of the cells was collected with an IP lysis buffer (Beyotime Institute of Biotechnology, P0013). 5mg of total protein was incubated with 2 µg of the specific primary antibody for 3 h at 4 °C, and

then incubated with Protein A-agarose suspension (Life Technologies) for 3 h at 4 °C. A total of 100 ml of Protein A-agarose was then added to each immunoprecipitation mixture, and the incubation was continued overnight at 4 °C on a rocking platform. After extensive washing, the beads were carried out by western blot to detect the potential interacting proteins.

Tumor growth and metastasis in xenografts

All animal experiments were performed under the protocols approved by the Shanghai General Hospital Institutional Animal Care and Use Committee. Six-week-old female BABL/c nude mice were obtained from SLRC laboratory Animal (Shanghai, China).

In vivo tumor growth assays, a total of 1×10^6 control and TPX2-sh 143B cells were injected subcutaneously. After 35 days, all mice were killed, their tumors were removed, fixed, paraffin-embedded, and sectioned.

In vivo tumor metastasis assays by conducting orthotopic xenograft tumor model, a total of 1×10^6 control and TPX2-sh 143B cells were injected into the medullary cavity of tibia of each mouse. After 35 days, all mice were killed. The posterior limb with tumors and lungs were finely excised for further study. Tumor weight was measured and lung metastasis nodules numbers were calculated using a dissecting microscope.

The tumor size was calculated using the formula $[\text{length} \times (\text{width})^2]/2$. Tumor tissues were snap frozen in liquid nitrogen for western blotting. Another independent animal experiment was performed to determine survival curve.

Statistical analysis

All data are expressed as the means $\pm$ SD, and vertical error bars denote the SD in the figures. Univariate and multivariate Cox proportional hazard regression models were used to analyze independent prognostic factors. An independent student's t-test was used to compare two groups. All experiments were performed at least three times except for the experiments using the animal models. P value ≤ 0.05 was considered statistically significant.

Results

TPX2 is an independent prognostic factor in OS patients

To investigate the correlation between TPX2 and OS progression, we first tested and evaluated the expression level of TPX2 in 59 human primary tumor specimens by immunohistochemistry (IHC) via the H-score method. 33 of the specimens had high TPX2 expression, and 26 had low TPX2 expression. Representative images of TPX2 staining patterns are shown in Fig 1A. We further analyzed the correlation

between TPX2 expression and the clinical features of OS patients, which are summarized in Table 1. TPX2 overexpression was significantly associated with OS metastasis. Consistent with this finding, analysis of a preexisting RNA microarray database of 88 pretreatment high-grade OS primary tumor biopsies (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) indicated that low TPX2 mRNA expression correlated with metastasis-free survival (Fig.1B). Furthermore, Western blot (WB) analysis showed that OS tissues had significantly higher TPX2 protein levels than paratumor tissues in the five paired samples (Fig.1C). In the next step, we evaluated TPX2 expression in 8 OS cell lines (Fig.1D). Endogenous TPX2 was overexpressed in 6 of these OS cell lines: MG63.2, HOS-MNNG, MG63, 143B, KHOS, and SJSA. Therefore, these results collectively indicated that TPX2 expression significantly correlated with the malignant progression of OS.

TPX2 contributes to the proliferation, migration, and invasion of OS cells

To investigate the potential biological functions of TPX2 in OS, we generated 143B TPX2-sh cells and SJSA TPX2-sh cells, along with TPX2-overexpressing U2OS cells. As shown in Fig 2A, the knockdown and overexpression efficiencies of TPX2 were determined via Western blot analysis. First, cell proliferation was examined by a CCK-8 assay, and the results indicated that knockdown of TPX2 slowed proliferation compared with that of control OS cells (Fig.2B) and that the proliferation of TPX2-overexpressing U2OS cells was prominently promoted compared to that of control OS cells. Furthermore, in the colony formation assay, knockdown of TPX2 significantly suppressed the colony-forming ability of OS cells, and overexpression of TPX2 promoted the colony-forming ability of U2OS cells (Fig.2C and D). Then, the influence of TPX2 on the migration and invasion of OS cells was further evaluated by wound healing and Transwell assays. The wound healing assay showed that the migration ability of 143B and SJSA cells was significantly inhibited when TPX2 was knocked down (Fig.3A) and that the migration ability of TPX2-overexpressing U2OS cells was promoted (Fig.3B). Consistent with the wound healing assay results, we further found that knockdown of TPX2 significantly decreased cell migration and invasion (Fig.3C and D). In contrast, overexpression of TPX2 dramatically increased cell migration and invasion (Fig.3E and F). Taken together, our results reveal that TPX2 probably plays a critical role in the proliferation and metastasis of OS cells *in vitro*.

TPX2 regulates and stabilizes E2F1 signaling

According to our previous study, E2F1, a critical transcription factor, is overexpressed in OS tissue. Moreover, E2F1 upregulation has been reported to correlate with malignant phenotypes and poor prognosis in a number of cancers. Then, we found that E2F1 expression is positively correlated with TPX2 expression in databases. To reveal the underlying signaling pathways regulated by TPX2 in OS, we next investigated the effects of TPX2 inhibition on E2F1 and its regulated genes, including MMP9, MMP13 and Survivin. TPX2 knockdown in 143B and SJSA cells resulted in decreased E2F1, MMP9, MMP13 and Survivin expression levels compared with those in control cells (Fig.4A). Conversely, TPX2 overexpression in U2OS cells elevated E2F1, MMP9, MMP13 and Survivin expression levels (Fig.4B). These findings suggested that TPX2 can activate E2F1 signaling in OS. Furthermore, RT-PCR

analysis showed that TPX2 knockdown in 143B cells did not significantly influence the mRNA level of E2F1 (Fig.4C). Therefore, we confirmed that TPX2 affected the protein level but not the mRNA level of E2F1. The degradation of intracellular proteins depends mainly on two pathways: the lysosomal and ubiquitin-proteasome pathways. Approximately 80% of proteins are degraded by the ubiquitin-proteasome pathway. Thus, we investigated whether ubiquitination may play a crucial role in the regulation of gene expression. To determine whether TPX2 affects mainly the stability of the E2F1 protein in OS cells, we evaluated the effect of TPX2 knockdown on E2F1 protein degradation using a cycloheximide (CHX) chase assay. As expected, TPX2 knockdown in 143B cells significantly decreased the protein half-life of E2F1 compared with that in control cells (Fig.4D). Furthermore, TPX2-mediated E2F1 degradation was rescued by treatment with the proteasome inhibitor MG132 (Fig.4E). Then, *in vitro* ubiquitination experiments were used to determine the ubiquitination level of E2F1 after TPX2 knockdown. Remarkably, our results showed that the ubiquitination level of E2F1 increased after TPX2 knockdown (Fig.4F). In summary, these results collectively indicate that TPX2 regulates E2F1 signaling by affecting the ubiquitination level of E2F1 in OS cells.

The deubiquitinating enzyme PSMD14 is responsible for the protein stability of E2F1 in OS cells

The ubiquitination state of proteins is reported to be regulated through homeostasis of the ubiquitination and deubiquitination systems. Recent reports indicate that the deubiquitination enzyme PSMD14 can affect the ubiquitination state of E2F1 in liver cancer. Therefore, to further explore whether PSMD14 can also affect the ubiquitination state of E2F1 in OS, we next investigated the effects of PSMD14 on the protein level of E2F1 and the levels of proteins encoded by E2F1-regulated genes, including MMP9, MMP13 and Survivin. PSMD14 knockdown in 143B and SJSA cells resulted in decreased E2F1, MMP9, MMP13 and Survivin expression levels compared with those in control cells (Fig.5A). Conversely, PSMD14 overexpression in U2OS cells elevated E2F1, MMP9, MMP13 and Survivin expression levels (Fig.5B). RT-PCR analysis showed that PSMD14 knockdown in 143B cells did not significantly influence the mRNA level of E2F1 (Fig.5C). Next, to verify whether PSMD14 stabilizes the E2F1 protein, we measured the effects of PSMD14 knockdown on degradation of E2F1 protein using the cycloheximide chase assay. Our results showed that PSMD14 knockdown in 143B cells significantly decreased the protein half-life of E2F1 compared with that in control cells (Fig.5D). In summary, these data collectively suggest that PSMD14 can stabilize E2F1 in OS cells.

TPX2 regulates the interaction of E2F1 and PSMD14 to influence the degradation of E2F1

We have shown that TPX2 influences the degradation of E2F1, and the deubiquitinating enzyme PSMD14 can also affect the ubiquitination of E2F1 in OS. Therefore, to explore the correlation between TPX2 expression and E2F1 degradation, we perform co-immunoprecipitation analyses to determine whether PSMD14 may interact with E2F1. E2F1 was co-immunoprecipitated with PSMD14 in HEK293T cells (Fig.6A). In addition, the interaction between endogenous E2F1 and PSMD14 was demonstrated by co-immunoprecipitation in 143B cells (Fig.6B). Furthermore, to elucidate whether E2F1 and PSMD14 are co-

localized in cells, we examined the subcellular localization of E2F1 and PSMD14 by immunofluorescence staining. Cytoplasmic co-localization of E2F1 and PSMD14 was clearly verified (Fig.6C). Therefore, we hypothesize that TPX2 specifically regulates the interaction of E2F1 and PSMD14, which further affects the degradation of E2F1 in OS cells. To further verify this hypothesis, we next investigated the effects of TPX2 on the protein level of PSMD14. Our results showed that TPX2 knockdown in 143B cells did not influence the protein level of PSMD14 (Fig.6D). Then, co-immunoprecipitation is conducted to detect the interaction of E2F1 and PSMD14 after downregulation of TPX2 in OS cells. Our results indicate that interaction of E2F1 and PSMD14 decrease after the interference of TPX2 in 143B cells (Fig.6E). Collectively, these data demonstrate that TPX2 regulates the interaction of E2F1 and PSMD14 to influence the degradation of E2F1, thereby affecting the proliferation and metastasis of OS cells.

Silencing TPX2 reduces tumor growth and lung metastasis development in vivo

To further identify whether the biological functions of TPX2 in OS have potential clinical relevance, a subcutaneous xenograft model was established. We used 143B control cells and TPX2-sh cells. The mean tumor volume in the 143B TPX2-sh group was much smaller than that in the control group, as the mean tumor weight in the 143B TPX2-sh group was lower than that in the control group (Fig.7A). Furthermore, to investigate TPX2's effect on metastasis *in vivo*, we further constructed an orthotopic xenograft model by injecting control or TPX2-sh 143B cells into the cavity of the tibia. Consistent with the results in the subcutaneous xenograft model, silencing TPX2 obviously inhibited the proliferation of OS cells in situ (Fig.7B). More importantly, the mean lung weight in the TPX2-sh group was significantly decreased compared to that in the control group because of the metastatic burden. Moreover, few lung metastatic nodules were observed in the TPX2-sh group, and the mean number of lung metastatic nodules in the TPX2-sh group was obviously decreased compared to that in the control group *in vivo* (Fig.7C and D). In addition, total protein was extracted from the tumor specimens from the nude mice, and Western blot analysis provided substantial evidence of the relative changes in the expression levels of MMP9, MMP13, and Survivin along with TPX2 (Fig.7F). We also found that silencing TPX2 significantly inhibited the expression of E2F1, as determined by IHC (Fig.7E). Collectively, these data indicate that TPX2 suppresses the proliferation and metastasis of OS cells *in vivo*.

Discussion

The majority of OS-related deaths are the result of tumor metastasis, but the precise molecular mechanisms that promote the metastatic process are still poor elucidated. Therefore, it is crucial to investigate the molecular mechanisms of tumor metastasis to develop novel therapeutic targets for OS. Recently, emerging evidence has indicated that TPX2 promotes tumorigenesis and tumor progression in various types of cancers[17, 25, 26]. However, to date, the role of TPX2 in the malignant progression of OS has not been reported. In our current study, we observed that TPX2 was significantly overexpressed in both OS tissues and OS cell lines. By combining IHC and analysis of preexisting RNA microarray databases, we further determined that high expression of TPX2 is positively correlated with metastasis

and malignant progression in OS. Taken together, these results suggest that overexpression of TPX2 is an important oncogenic factor and plays vital roles in the development of OS.

TPX2, a microtubule-associated protein, has been reported to be a candidate oncogene in other cancers, including prostate cancer, breast cancer, and bladder cancer, suggesting that it is involved in the development of many cancers. Although overexpression of TPX2 has been reported in many human cancers, to our knowledge, we are the first to describe TPX2 as a growth and metastasis promoter in OS. Therefore, we conducted gain- and loss-of-function studies *in vitro* to elucidate the role of TPX2 in the growth and metastatic ability of OS. Functional studies showed that TPX2 enhances the migration and invasion capacities of OS cells. Moreover, our studies on TPX2 functional loss induced by shRNA-mediated knockdown in OS cell models indicated that downregulation of TPX2 significantly inhibited OS cell growth and metastasis. A recent report demonstrated that TPX2 promotes the development of breast cancer by regulating PI3k/AKT/P21 signaling[27]. To date, however, the precise molecular mechanisms by which TPX2 promotes OS cell growth and metastasis are not fully understood.

E2F1 signaling is well recognized and well-studied. Recent reports have shown that E2F1 can regulate a variety of biological processes, including the cell cycle, apoptosis, cell differentiation and DNA damage repair[28–31]. In addition, accumulating evidence shows that E2F1 signaling promotes tumorigenesis in different types of cancer, including lung cancer, breast cancer, prostate cancer and colorectal carcinoma. For example, E2F1 signaling was reported to promote the progression of lung cancer by regulating MMP9, MMP14 and MMP15[32]. In our study, we elucidated the mechanism by which TPX2 regulates E2F1 signaling in OS. We first found that downregulation of TPX2 decreases E2F1 protein expression and the expression of proteins encoded by E2F1-regulated genes, including Survivin, MMP9 and MMP13, in OS cells. The correlation among TPX2, E2F1, and proteins encoded by E2F1-regulated genes demonstrated a deeper connection between TPX2 and E2F1 signaling. More importantly, we found that TPX2 affects the protein level but not the mRNA level of E2F1. We hypothesize that TPX2 functions as a tumor-promoting protein, which may correlate with the protein stability of E2F1. The degradation of intracellular proteins depends mainly on two pathways: the lysosomal and ubiquitin-proteasome pathways[33]. To verify the main pathway through which E2F1 is degraded, we applied the lysosomal inhibitor CHX and the proteasome inhibitor MG132 to determine the protein half-life of E2F1 in OS cells. Collectively, the results confirmed that TPX2 can indeed regulate E2F1 signaling by affecting the ubiquitination of E2F1 in OS cells.

The deubiquitinating enzyme PSMD14 has been reported to be closely related to the ubiquitination and degradation of E2F1 in HCC cells[34]. To confirm the precise molecular mechanism by which TPX2 stabilizes E2F1 expression in OS cells, we further clarified that PSMD14 can promote the degradation of E2F1 in OS cells. Furthermore, in this study, we found that PSMD14 interacts with E2F1, thus affecting the ubiquitination of E2F1 in OS cells. We next sought to determine how the role of PSMD14 in TPX2 regulate the degradation of E2F1 in OS cells. Most importantly, we found that the interaction of E2F1 and PSMD14 decreased after knockdown of TPX2 in OS cells. Taken together, our results demonstrate that TPX2 can regulate the interaction of E2F1 and PSMD14, thereby ultimately influencing the degradation of

E2F1 and promoting the E2F1-regulated gene expression required for the malignant progression of OS. However, more research still needed to be continued, such as exploring the specific molecular mechanism by which TPX2 regulate the interaction of E2F1 and PSMD14.

Conclusions

In summary, our research demonstrates that TPX2 plays an important role in the growth and metastasis of OS. Moreover, our research not only identifies TPX2 as a promising therapeutic target in cancer but also illuminates its underlying molecular mechanism. We show that TPX2 promotes the malignant progression of OS by regulating E2F1 signaling. Therefore, our collective data provide a new perspective on the development of OS and identify a novel anticancer therapeutic approach via targeting TPX2.

Abbreviations

OS, osteosarcoma; TMA, tissue microarray; CCK-8, Cell Counting Kit-8; IHC, immunohistochemistry; CHX, cycloheximide

Declarations

Ethics approval and consent to participate

All animal experiments were performed under the protocols approved by the Shanghai General Hospital Institutional Animal Care and Use Committee.

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Availability of data and materials

The data used and analyzed during this study are available from the corresponding author on request.

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Authors' contributions

Min Mao, and Tao Zhang designed the experiments; Min Mao, Lei Zhou and Xinglong Ma performed most experiments; Gangyang Wang, Dongqing Zuo, Zongyi Wang and Jing Xu performed

parts of the experiments and analyzed the data. Min Mao wrote the manuscript. Yingqi Hua and Zhengdong Cai conceived and directed the project. All authors read and approved the final manuscript.

Consent for publication

The authors agree for publication.

Competing interests

The authors declare no conflict of interest.

Authors' information

1. Min Mao

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: maomin0708@163.com

Telephone: +86-15601894847

2. Lei Zhou

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: zl8160@qq.com

Telephone: +86-15900927749

3. Xinglong Ma

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: 752185579@qq.com

Telephone: +86-18918209041

4. Mengkai Yang

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: yangmengkai@88.com

Telephone: +86-15800807285

5. Gangyang Wang

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: gangyang_wang@163.com

Telephone: +86-15216637389

6. Dongqing Zuo

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: dongqing.zuo1@shgh.cn

Telephone: +86-15021094847

7. Zhuoying Wang

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: wyz135@163.com

Telephone: +86-13701710769

8. Zongyi Wang

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Email: wzy1992@sjtu.edu.cn

Telephone: +86-18301921772

9. Tao Zhang

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;
Email: zhangtaoabc@2008.sina.com
Telephone: +86-15216709440
10. Jing Xu

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;
Email: piao_hay@163.com
Telephone: +86-13816884368
Corresponding author:

1. Yingqi Hua

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;
Email: yhua@shsmu.edu.cn
Telephone: +86-13817651474

2. Zhengdong Cai

Affiliation: Department of Orthopaedics, Shanghai Bone Tumor Institute, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Poster address: 100 Haining Road Shanghai; Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;
Email: caizhengdong@sjtu.edu.cn
Telephone: +86-13901832868

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Correlation between the expression of TPX2 in OS tissue and metastasis in OS patients. A. Immunohistochemical staining was used to detect high and low TPX2 protein expression levels in OS

tissues. B. Data analysis (88 patients with OS) with the R2 database and Kaplan-Meier analysis of metastasis-free survival in OS patients stratified by the TPX2 expression level. C. Western blot analysis was used to determine the protein level of TPX2 in paired OS tissues and adjacent normal tissues from five patients (T: OS tissue, N: normal tissue). D. Western blot analysis was used to determine the protein level of TPX2 in various OS cell lines.

Figure 2

TPX2 promotes the proliferation of OS cells in vitro. A. Western blot analysis was used to determine the expression level of TPX2 after knockdown and overexpression of TPX2 in OS cells. B. A CCK-8 assay was used to detect the effect of TPX2 expression on cell proliferation in OS cell lines. C. A plate colony formation assay was used to detect the effect of TPX2 knockdown on cell proliferation in 143B and SJSA cells. D. A plate colony formation assay was used to detect the effect of TPX2 overexpression on cell proliferation in U2OS cells. (***) $P < 0.05$

Figure 3

TPX2 promotes the metastasis of OS cells in vitro. A. The effect of TPX2 inhibition on the wound healing rate was assessed by a wound healing assay in 143B and SJSA cells. B. The effect of TPX2 overexpression on the wound healing rate was assessed by a wound healing assay in U2OS cells. C. The effect of TPX2 inhibition on cell migration was assessed by a Transwell migration assay in 143B and SJSA cells. D. The effect of TPX2 overexpression on cell migration was assessed by a Transwell migration assay in U2OS OS cells. E. The effect of TPX2 inhibition on cell invasion was assessed by a Transwell invasion assay in 143B and SJSA cells. F. The effect of TPX2 overexpression on cell invasion was assessed by a Transwell invasion assay in U2OS cells. (***) $P < 0.05$

Figure 4

TPX2 regulates and stabilizes E2F1 signaling. A. Western blot analysis was conducted to detect the effect of TPX2 expression on the protein level of E2F1 in OS cells. B. Western blot analysis was conducted to detect the effect of TPX2 expression on the levels of E2F1-regulated proteins in OS cells. C. RT-PCR was used to detect the effect of TPX2 expression on the mRNA level of E2F1 in OS cells (ns: nonsignificant). D. The protein synthesis inhibitor CHX was used to treat control and TPX2-sh 143B cells,

and the half-life of the E2F1 protein was then determined by Western blotting (concentration of CHX: 100 $\mu\text{g}/\text{mL}$). E. The proteasome inhibitor MG132 was used to treat control and TPX2-shRNA 143B cells. F. Western blot analysis was conducted to detect the ubiquitination level of E2F1 after inhibition of TPX2 expression in 143B cells.

Figure 5

The deubiquitinating enzyme PSMD14 is responsible for the protein stability of E2F1 in OS cells. A. Western blot analysis was conducted to detect the effect of PSMD14 expression on the protein level of E2F1 in OS cells. B. Western blot analysis was conducted to detect the effect of PSMD14 expression on

the levels of E2F1-regulated proteins in OS cells. C. RT-PCR was used to detect the effect of PSMD14 expression on the mRNA level of E2F1 in OS cells (ns: nonsignificant). D. The protein synthesis inhibitor CHX was used to treat control and PSMD14-sh 143B cells, and the half-life of the E2F1 protein was then determined by Western blotting (concentration of CHX: 100 μ g/mL).

Figure 6

TPX2 regulates the interaction of E2F1 and PSMD14 to influence the degradation of E2F1. A. Detection of the interaction between exogenous PSMD14 and E2F1 by immunoprecipitation (HEK-293T cells were transiently transfected with both the PSMD14-Flag plasmid and E2F1-His plasmid). B. The interaction between endogenous PSMD14 and E2F1 in 143B cells was detected by immunoprecipitation. C. The

localization and protein levels of E2F1 and PSMD14 in 143B cells were evaluated by immunofluorescence staining (red scale bar: 100 μ m). D. Western blot analysis was conducted to detect the effect of TPX2 expression on the protein level of PSMD14 in 143B cells. E. The levels of the PSDM14 and E2F1 protein complex in 143B and 143B-TPX2-sh cells were determined by immunoprecipitation.

Figure 7

Silencing TPX2 reduces tumor growth and the development of lung metastases in vivo. A. The different tumor weights in the subcutaneous xenograft model in nude mice. B. The different tumor weights in the orthotopic xenograft model in nude mice. The gross appearance and HE staining of lungs in the different groups of orthotopic xenografts from mice. D. Lung weights of the lung and numbers of lung nodules in the different groups of orthotopic xenografts from mice. E. Immunohistochemical staining was used to assess the protein levels of TPX2 and E2F1 in the different groups of subcutaneous xenografts from nude mice. F. Western blot analysis was conducted to determine the protein levels of Survivin, MMP9 and MMP13 in the different groups of subcutaneous xenografts from nude mice (Scramble: control group, TPX2-sh1: TPX2 inhibition group, * $P < 0.05$, red scale bar: 200 μm).

Supplementary Files

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- [Table1.tif](#)