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# Suppression of telomere-binding protein TPP1 resulted in telomere dysfunction and enhanced radiation sensitivity in telomerase-negative osteosarcoma cell line

Weiguang Qiang [a,b], Qinqin Wu [a,c], Fuxiang Zhou [a], Conghua Xie [a], Changping Wu [b,*], Yunfeng Zhou [a,*]

[a] Hubei Cancer Clinical Study Center, Hubei Key Laboratory of Tumor Biological Behaviors, Zhongnan Hospital, Wuhan University, Wuhan, China
[b] Department of Oncology, The Third Affiliated Hospital, Soochow University, Changzhou, China
[c] Department of Radiation Oncology, Changzhou Tumor Hospital, Soochow University, Changzhou, China

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ABSTRACT

Mammalian telomeres are protected by the shelterin complex that contains the six core proteins POT1, TPP1, TIN2, TRF1, TRF2 and RAP1. TPP1, formerly known as TINT1, PTOP, and PIP1, is a key factor that regulates telomerase recruitment and activity. In addition to this, TPP1 is required to mediate the shelterin assembly and stabilize telomere. Previous work has found that TPP1 expression was elevated in radioresistant cells and that overexpression of TPP1 led to radioresistance and telomere lengthening in telomerase-positive cells. However, the exact effects and mechanism of TPP1 on radiosensitivity are yet to be precisely defined in the ALT cells. Here we report on the phenotypes of the conditional deletion of TPP1 from the human osteosarcoma U2OS cells using ALT pathway to extend the telomeres.TPP1 deletion resulted in telomere shortening, increased apoptosis and radiation sensitivity enhancement. Together, our findings show that TPP1 plays a vital role in telomere maintenance and protection and establish an intimate relationship between TPP1, telomere and cellular response to ionizing radiation, but likely has the specific mechanism yet to be defined.



## 1. Introduction

Osteosarcoma is the most common primary malignant bone tumor, primarily affecting the metaphysis of the long tubular bones in children and adolescents. Surgery, multi-agent chemotherapy and radiation are current major therapeutic strategies. However, the prognosis in both localized and metastatic osteosarcoma has been generally poor in the past decades. Innovative approaches are needed to further improve outcome in osteosarcoma patients. It is generally known that osteosarcoma tumors are notoriously radioresistant. Thus, the use of radiotherapy is limited in the treatment of primary osteosarcoma [1]. Currently, conventional photon radiotherapy plays only a minor role in the multi-disciplinary approach which involves surgery and chemotherapy maximizing the tumor under control [2]. Therefore, it is necessary to search radiosensitizing drugs improving the antitumor efficacy of radiotherapy.

Recently, telomeres have emerged as a promising and important factor modulating cellular and organism responses to ionizing radiation (IR). Telomeres are highly ordered DNA–protein structures, consisting of repeated TTAGGG sequences and a protein complex termed telosome/shelterin, which effectively protect the end of linear eukaryotic chromosomes from degradation, end-to-end fusion, and from engaging the DNA damage response [3–5]. Many studies have shown that telomere shortening and dysfunction impair DNA repair and significantly modulates the effects of IR on mice and human cells independently of telomerase activity [6–9]. Our previous research also indicated that there was a significant negative correlation of telomere length and radiosensitivity [10]. Thus, telomeres will likely be useful to optimize the anticancer effects of radiation in future.

Telomere are protected by shelterin, composed of interdependent telomeric core proteins consisting of telomeric-repeat-binding factor 1 (TRF1), TRF2, TRF1-interacting protein 2 (TIN2), the transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1) and the TIN2 and POT1 interacting protein (TPP1), formerly named PTOP/PIP1/TINT1. The shelterin participates in both telomere maintenance and telomere protection. For instance, it prevents the chromosome ends from being seen as DNA damage and initiating the inappropriately process by DNA repair pathways, mostly through inhibition of ATM and ATR.

* Corresponding authors. Fax: +86 519 68870978.
*E-mail addresses:* wcpzlk@163.com (C. Wu), yfzhouwhu@163.com (Y. Zhou).

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Moreover, through the interaction with telomerase, it is involved in the control of telomere length to maintain the stability of telomeres [4,5,11–14]. In the process of the above, TPP1 is identified as a crucial role. For example, TPP1 is a key component in mediating high-order shelterin complex assembly, which is required to stabilize the TRF1–TIN2–TRF2 interaction and promote six-protein complex formation. In addition, TPP1 is able to regulate telomere length by interacting with POT1 and recruiting telomerase to telomeres [15–18].

Our previous study observed that the expression of TPP1 was increased in radioresistant Hep-2R than in more sensitive Hep-2 cell lines. Further research shows that overexpression of TPP1 led to radioresistance and telomere lengthening in HCT116 cells [19,20]. Currently, the amount of studies about TPP1 was limited in telomerase positive tumor cells. However, a subset of tumors and immortal cell lines utilize a telomerase-independent mechanism, termed alternative lengthening of telomeres (ALT), to maintain telomeric DNA [21]. The exact effects and mechanism of TPP1 on radiosensitivity are yet to be precisely defined in the ALT cells. Here we investigate the role of TPP1 in ALT cells and demonstrate that down-regulation of this factor promotes cell apoptosis and significantly increases the radiation sensitivity. Thus, TPP1 is an essential factor for ALT cell survival and it is expected to become one of the radiotherapy sensitization drugs.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

The human osteosarcoma cell U2OS was purchased from the Cell Bank of the Chinese Academy of Science, Shanghai, China. The cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% $CO_2$ at 37 °C (SanYo Electric).

### 2.2. Preparation and transfection of siRNA

We derived the gene sequence of TPP1 from National Center for Biotechnology Information (NCBI) in this study (Gene ID: 65057). The siRNA against TPP1 and a negative control siRNA were designed and chemically synthesized by GenePharma (Shanghai, China). The siRNA sequences were as follows: for TPP1, forward (5′-GUUGCAACCAAGACUUAGATT-3′) and reverse (5′-UCUAAGUCU UGGUUGCAACTT; for GAPDH, forward (5′-GUAUGACAACAGCCU CAAGTT-3′) and reverse (5′-CUUGAGGCUGUUGUCAUACTT-3′). Cells were incubated in medium without serum and antibiotics for 24 h. After mixed gently according to the manufacturer's instructions and incubated for 20 min at room temperature, the transfection mixture of siRNA and Lipofectamine 2000 (Invitrogen) was added into culture plates. After 6 h, the transfection mixture was replaced by medium. Cells were harvested at 48 h after transfection.

### 2.3. Semi-quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The first strand of cDNA was obtained using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). For quantitative analysis of TPP1 mRNA, human GAPDH gene was used as an internal control. DNA primer sequences were designed as follows: sense CCGCAGAGTTCTATCTC-CAGGTG and antisense CATCCAGAAGCTGGGACAGTGA for TPP1, and AGCGTCAAAGGTGGAGGAGT and antisense ATCACTGCCACC-CAGAAGAC for GAPDH. The cycling conditions for all the cDNA included preincubation for 4 min at 94 °C and followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a final extension for 7 min at 72 °C. PCR products were identified using electrophoresis on 1.5% agarose gels containing 0.5% ethidium bromide (EB). Gel images were obtained and the densities of PCR products were quantified using Bio-ID gel analysis software (Vilber Lourmat, France). All experiments were repeated at least three times.

### 2.4. Western blot analysis

We analyzed proteins by Western blotting as described previously [20]. Autoradiographs were recorded onto X-Omat AR film (Eastman Kodak Co). The protein band intensities were analyzed by the Image J analysis program. The primary antibodies used in this study were TPP1 and β-actin (Santa cruz).

### 2.5. Real-time quantitative PCR

Genomic DNA was extracted from cells by standard procedures using TIANamp genomic DNA kit (TIANGEN biotech Co, Ltd.) and stored at −70 °C. Relative telomere length (RTL) was detected by using quantitative real-time polymerase chain reaction (qRT-PCR) described by Cawthon previously [22]. Duplicate PCR reactions using the TaKaRa realtime PCR kit (TaKaRa biotechnology Co, Ltd) according to the manufacturer's instructions. The telomere and single copy gene specific primers used for the experiment were given as below: Tel 1: 5′-GGTTTTTGAGGGTGAGGGTGA GGGTGA GGGTGAGGGT-3′; Tel 2: 5′-TCCCGACTATCCCTATCCCTATCCCTAT CCCTATCCCTA-3′; 36B4u: 5′-CAGCAAGTGGGAAGGTGTAATCC-3′, 36B4d: 5′-CCCATTCTATCATCAACGGGTACAA-3′. The cycling conditions consisted of a preincubation for 5 s at 95 °C and followed by 35 cycles of 95 °C for 15 s, 54 °C for 2 min. Thermal amplification was carried out on Mx3000P (Stratagene) and the results were analyzed using the MXP 3000 analysis program.

### 2.6. Flow cytometry analysis of apoptosis

Apoptosis assay was performed using an annexinV-FITC apoptosis detection kit (Beyotime, China) according to the manufacturer's instruction. Fluorescence was measured using a flow cytometer (Beckman Coulter, Brea, CA) and the data were analyzed with Cell Quest software.

### 2.7. Colony formation assay

The cells were plated into 6-well plates at various densities based on our results of pre-experiments. The next day, cells were irradiated with graded doses (0, 1, 2, 4, 6, 8, 10 Gy), respectively, followed by immediate incubation at 37 °C, 5% $CO_2$ for 14 days. After fixation and staining with 1% w/v crystal violet (Sigma, St. Louis, MO) in dehydrated alcohol, colonies of >50 cells were scored. The data were fit into the single-hit multi-target model, and survival curve of each group were demonstrated by Graphpad prism 5.0 software. Radiobiological parameters, such as D0, Dq and SF2 were calculated according to the survival curves.

### 2.8. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed by ANOVA. Statistical analysis was performed using software SPSS 13.0 and Graphic Prism 5.0. $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. TPP1 expression was suppressed by RNA interference (RNAi)

To verify the inhibition efficiency of TPP1 in U2OS cells, TPP1 expression was detected by RT-PCR and Western blotting. Expression of TPP1 mRNA (Fig. 1A and B) and TPP1 protein (Fig. 1C and D) in TPP1-siRNA group were inhibited compared with negative control group or Mock (untreated cells) group. The results confirmed that TPP1 expression was inhibited by transfection with siRNA targeting TPP1.

### 3.2. Down-regulation of TPP1 shortened telomere length in U2OS cells

RTL in U2OS cells was detected by qRT-PCR. As shown in Fig. 2, RTLs of different groups were 1.55 ± 0.05, 1.64 ± 0.05 and 0.99 ± 0.07, respectively. RTL was shorter in TPP1-siRNA group compared with negative control group and Mock group, showing significant difference ($P < 0.01$), whereas such a difference in negative control group and Mock group was not significant ($P = 0.09$). These data indicated that down-regulation of TPP1 expression could shorten telomere length in U2OS cells.

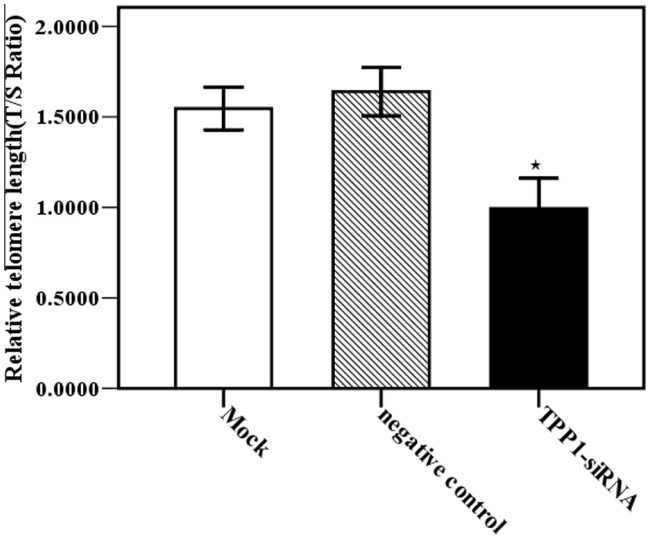


**Fig. 2.** Down-regulation of TPP1 shortened telomere length in U2OS cells. RTL was detected by using qRT-PCR. The Bar graph shows means ± SD from three independent experiments (*$p < 0.05$).

### 3.3. Silencing of TPP1 induced cell apoptosis in U2OS cells

The percentage of apoptotic cells were assessed by Annexin V-FITC, followed by flow cytometric analysis. It was observed that the percentage of apoptotic cells of each group was 8.18 ± 0.21%, 8.59 ± 0.38% and 13.67 ± 0.85%, respectively (Fig. 3). TPP1-siRNA group increased the percentage of cells undergoing apoptosis compared to negative control group or Mock group ($P < 0.05$). The result suggested that the absence of TPP1 could cause U2OS cells apoptosis.

### 3.4. Knockdown of TPP1 resulted in increased radiosensitivity

To study whether TPP1 affected cell radiosensitivity, changes of cell survival were evaluated by clonogenic assay following 0–10 Gy

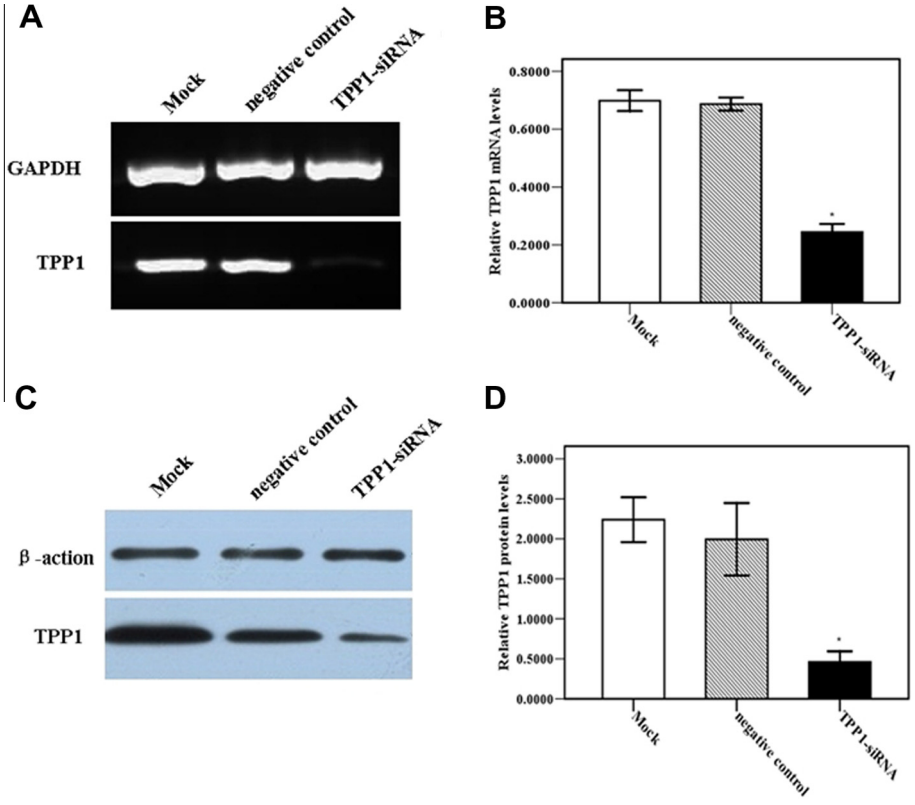


**Fig. 1.** Effect of knockdown of TPP1 on the mRNA and protein expression levels in U2OS cells. (A) mRNA expression levels of TPP1 was detected by RT-PCR. (B) RT-PCR data of TPP1 mRNA level following the knockdown compared with negative control group and Mock group. (C) TPP1 protein expression levels in TPP1 siRNA-infected and control cells was detected by Western blotting. (D) The bar chart showed the semiquantitative analysis of TPP1 protein expression. Data represent means ± SD. *$p < 0.05$ was considered significant.

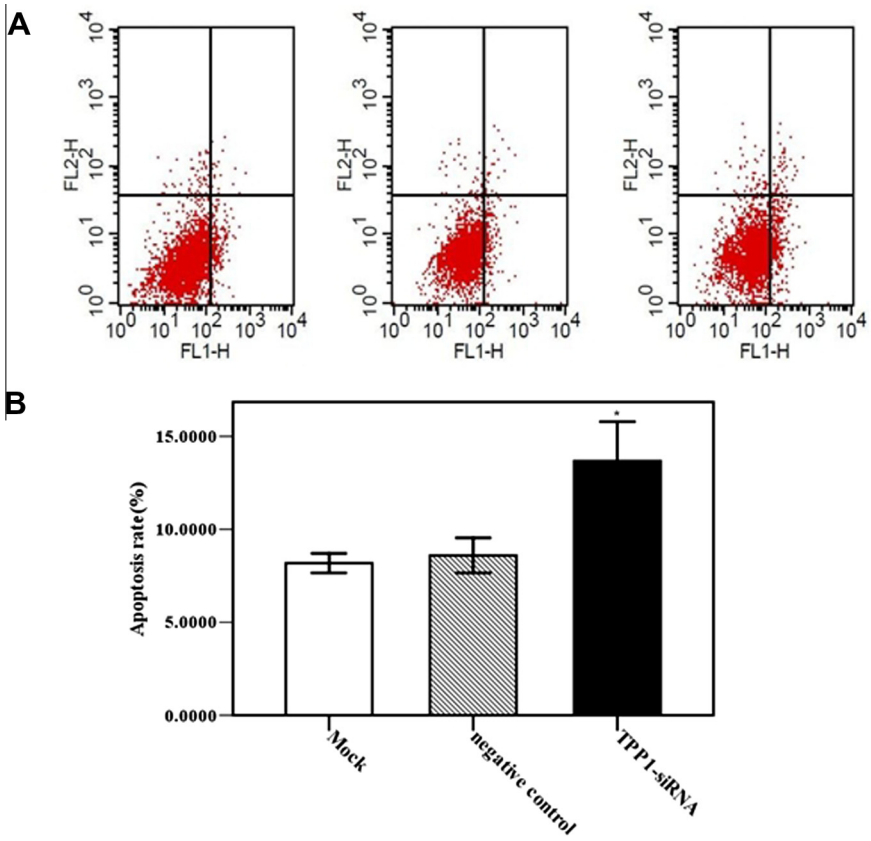


**Fig. 3.** Silencing of TPP1 induced cell apoptosis in U2OS cells. (A) The level of apoptosis in U2OS cells were assessed by Annexin V-FITC, followed by flow cytometric analysis. Data shown are representative of three independent experiments. (B) Statistic results of apoptosis assays are expressed as means ± SD from three independent experiments (*$p < 0.05$).

radiation, and the results are shown in Fig. 4. Transfection of U2OS cells with TPP1 siNRA significantly decreased cell survival after radiation exposure (SERsf2 1.33). These data showed that the knockdown of TPP1 proteins expression levels resulted in an increase in the radiosensitivity of U2OS cells.

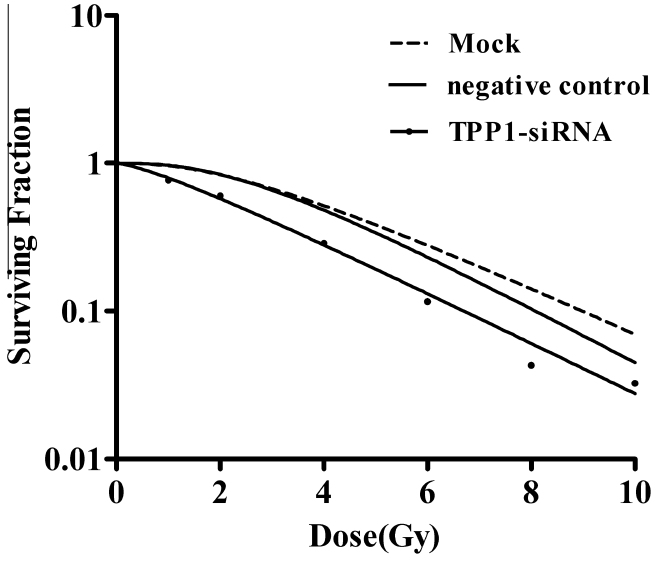


**Fig. 4.** Effects of down-regulated TPP1 expression on U2OS cells survival after irradiation. The cells in different groups were irradiation with graded doses (0, 2, 4, 6, 8, 10 Gy), respectively. U2OS cell survival was determined using colony formation assay and then survival curves of each group were analyzed according to Graphpad prism 5.0 software.

## 4. Discussion

The involvement of TPP1 and the other telomere end-binding proteins in telomere maintenance and protection has been extensively studied and drawn a lot of valuable conclusions in telomerase-positive tumor cell lines. However, the role of TPP1 in ALT cells has not been researched in depth. For this reason, we took some approaches to determine the telomere length and radiosensitivity phenotype of reduced TPP1 levels in human osteosarcoma U2OS cells, an ALT cell line.

Real-time quantitative PCR experiments clearly show that RNAi-mediated TPP1 down-regulation results in the shortening of relative telomere length, thus implicating a role in the process of maintaining telomere length in ALT cells. Previously, the telomere lengthen upon shRNA-mediated depletion of the TPP1 in human HTC75 cells [23], whereas TPP1 deletion resulted telomere shortening in embryonic fibroblasts (MEFs) and mice [24]. Recently, cell biological studies have confirmed that TPP1 recruits telomerase to chromosome ends and stimulate telomerase processivity due to the interdependence among the telomere end-binding proteins [25–28]. Taken together, we speculate that TPP1 and associated proteins serve a dual function in maintaining telomere length. Furthermore, the TEL patch, a small patch of amino acids on the surface of TPP1, facilitates binding of telomerase to TPP1 and is required for telomerase recruitment and processivity stimulation [29]. The find highlights this domain as a potential drug targets. Our most recent research also suggested that TPP1 overexpression could increase telomere length in telomerase-positive tumor cells [19]. However, Alternative lengthening of telomere (ALT) tumors elongate telomeres DNA by a telomerase-independent mechanism, which involves DNA repair and

recombination processes, but the molecular details are currently poorly understood. One hallmark of the ALT mechanism is the presence of ALT associated promyelocytic leukemia (PML) bodies, referred to as APBs containing telomere-related proteins and telomeric DNA [30]. Interestingly, de novo assembly of APBs induces telomeric extension through a repair mechanism and an increase of the telomere length [31]. The present study show that four of six shelterin components, TRF1, TRF2, TIN2 and RAP1 were required for APB formation, and hence for ALT-mediated telomere lengthening. Knockdown of any of these proteins except RAP1 resulted in decrease of the other proteins [32]. Thus, it seems that maintaining the integrity of telomere is important for the formation of APBs as well as for telomere maintenance in ALT-positive cells. It has been suggested that TPP1 plays a critical role in high-order telomeric complex assembly. Not only TIN2 but also TPP1 are required to bridge the TRF1 and TRF2 subcomplexes. Specifically, TPP1 helps to stabilize the TRF1–TIN2–TRF2 interaction. In addition, loss of TPP1 in vivo is accompanied by reduced levels of TIN2 [18,33]. Consequently, we conclude that deletion of TPP1 may inhibit the formation of APBs through affecting TIN2 and the other APBs associated proteins at telomere, and then results in the shortening of telomere length in ALT-positive cells. However, it is not excluded that knockdown or inactivation of TPP1 causes the disruption of telomere maintenance and loss of end-protection, eventually induces telomere shortening.

TPP1 as an integral component of the shelterin complex, maintains telomere integrity and protects telomeres from eliciting a DNA-damage response (DDR). Both MEFs and mice deleted for TPP1 show induction of telomere damage foci (TIFs) and cell cycle arrest [4,24,34]. And that an inability to repair DNA damage and/or prolonged checkpoint activation can lead to apoptosis [35]. Thus, when too few or none of TPP1 is at a telomere, DDR would become unrestrained, leading to chromosomal end fusions, cell-cycle arrest and apoptosis. In this report, we have found that silencing of TPP1 induced cell apoptosis in U2OS cells. Interestingly, recent studies have found that deletion of TPP1 resulted in triggering excessive apoptosis in zebra fish embryos and mice as well [15,24]. These data suggested that the involvement of apoptosis in TPP1 deficiency is conserved across different species. Given the important function of telomeric proteins in maintaining genome stability, we considered that the defects in TPP1 could contribute to telomere dysfunction and impairment of homologous recombination leading to cell growth arrest and apoptosis in ALT cells. Furthermore, according to our research, it is not exclude that down-regulation of TPP1 shortened telomere to a critical length, eventually induced apoptosis. The great majority of ALT cell lines are p53 deficient, and there is only one, U2OS, that is known to express wild-type p53 and Rb, but lacking p16. Previous studies have shown that apoptosis is the primary mechanism causing TPP1-deficient mice pleiotropic phenotype with perinatal lethality, and that these phenotypes and cellular apoptosis are rescued by p53 suppression [24,36]. So we reason that the apoptosis induced by genetic instability associated with TPP1 deletion is dependent upon the p53 pathway. However, the detail mechanism is still unknown and expected to further study.

Many and varied mechanisms of resistance to ionizing radiation treatment and radiation sensitivity are proposed. Among them, an inverse relationship between telomere length and radiosensitivity has been recently advanced. We observed that overexpression of TPP1 led to radioresistance and telomere lengthening in telomerase-positive cells [19]. In this report, we have found that knockdown of TPP1 resulted in shortened telomere length and increased radiosensitivity in ALT cells as well, which indicates the importance of TPP1 in mediating interaction between telomere and radiosensitivity. However, telomere length shortening is observed in radioresistant B-CLL cells [37]. Another study showed that radiosensitivity is independent of telomere length in mouse lymphoma cell lines [38]. We suspect that this is associated with rapid lymphocyte proliferation and significant cell division. Interestingly, long but dysfunctional telomeres in acute myeloid leukaemia (AML) cells correlates with chromosomal radiosensitivity [39]. This implicates telomere function in addition to telomere length as a determinant of chromosomal radiosensitivity. The radiosensitivity of telomere dysfunctional cells correlated with the formation of DNA double strand breaks (DSBs) rearrangements, persistent chromosomal breaks and cytogenetic profiles characterized by complex chromosomal aberrations and massive fragmentation [8]. TPP1, as one of telomere binding proteins, plays a central role in the formation of the shelterin protein bridge linking the duplex part to the 3-overhang of telomeric DNA and its loss is thus expected to dissociate an essential capping structure, which leads to telomere dysfunction [18]. Collectively, these findings indicate that down-regulation of TPP1 enhance radiation sensitivity of ALT cells due to telomere shortening and dysfunction.

In summary, our results indicated that TPP1 depletion induced apoptosis and enhanced radiation sensitivity in U2OS cells because of telomere shortening and dysfunction. It is worth noting that alternative lengthening of telomeres pathway is present following telomerase inhibition [40]. Additionally, both the alternative lengthening of telomeres mechanism and the telomerase mechanism can coexist over a prolonged period in human cancer cells [41]. These findings suggest that the use of telomerase or ALT inhibitors alone for treatment of some tumors may activate another mechanism for telomere elongation which leads to treatment failure. Our studies have shown that TPP1 depletion enhanced radiation sensitivity of telomerase-positive cells and was also effective on ALT cells. The detailed molecular mechanisms of this action of TPP1 in ALT cells remain unclear, but it represents thus an attractive target for developing new drug strategies to combat cancer.

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