Polo-like Kinase 1 (Plk1) Up-regulates Telomerase Activity by Affecting Human Telomerase Reverse Transcriptase (hTERT) Stability*  

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Background: hTERT plays a central role for telomerase activity and telomeric chromatin maintenance. Results: Plk1 interacts with hTERT and regulates its stability. Overexpression of Plk1 up-regulates the activity of telomerase. Plk1 enhances the chromatin loading of hTERT and inhibits its ubiquitination. Conclusion: Plk1 is a positive regulator of telomerase. Significance: We revealed the mechanism of Plk1 regulating the telomerase activity by stabilizing hTERT protein.

Maintenance of telomere is regulated by active telomerase complex, including telomerase holoenzyme and its associated proteins. The activity of telomerase is precisely controlled in cells, and its dysregulation is one of the hallmarks of cancer. The telomerase catalytic subunit human telomerase reverse transcriptase (hTERT) plays a central role for telomerase activity. In this study, we indentified that Polo-like kinase 1 (Plk1) is a novel telomerase-associated protein. Plk1 can interact with hTERT independently of its kinase activity. More importantly, we found that Plk1 is associated with active telomerase complex. In addition, we demonstrated that knockdown of Plk1 caused the reduction of telomerase activity, whereas overexpression of Plk1 increased telomerase activity. Further analysis showed that overexpression of Plk1 led to a significant increase of hTERT protein by prolonging its half-life but did not affect the level of hTERT mRNA. Furthermore, we found that Plk1 enhanced the chromatin loading of hTERT and inhibited its ubiquitination. This implied that Plk1 affected hTERT stability by inhibiting its ubiquitin-mediated degradation. Collectively, these observations suggested that Plk1 is a positive modulator of telomerase by enhancing the stability of hTERT.

Telomeres are the unique structures that cap the ends of eukaryotic chromosomes to prevent their degradation and fusion and maintain genome stability (1, 2). Due to the end replication problem, telomeres can function as a mitotic clock, shortening progressively during each cell division. Telomeres are maintained by telomerase, and therefore the activity of telomerase determines the life span (3). Telomerase is a ribonucleoprotein enzyme that contains two essential subunits, telomerase reverse transcriptase (TERT) and telomerase RNA, which acts as a template for the synthesis of TTAGGG repeats by TERT and its associated proteins (4, 5). It is well known that TERT is the rate-limiting determinant of telomerase activity. Its activity can be regulated at various levels such as the transcriptional control and post-translational modifications including phosphorylation (6, 7). As telomerase RNA and TERT are the minimal elements for telomerase activity, a number of telomerase-associated proteins are involved in the biogenesis or assembly of active telomerase. For example, the chaperone proteins Hsp90 and p23 can interact with hTERT and are required for the assembly of active telomerase (8, 9). KIP (DNA protein kinase catalytic subunit-interacting protein) is a positive regulator for telomerase activity and telomere length by binding to hTERT (10). The 14-3-3 signaling protein is required for efficient accumulation of hTERT in the nucleus (11). The Ku complex can interact with hTERT and regulate telomerase access to telomeric DNA ends (12). PinX1 binds directly to hTERT and functions as a potent catalytic inhibitor of telomerase (13). In addition, the abundance of hTERT can be regulated through ubiquitin-mediated degradation controlled by E3 ligases such as MKRN1 and HDM2 (14, 15). Based on these studies, telomerase-associated proteins can mediate or regulate the accessibility and function of telomerase on telomeres in different manners.

Polo-like kinase 1 (Plk1) is a Ser/Thr kinase in eukaryotes and plays a critical role in various aspects of mitotic events such as G2/M transition, spindle formation, chromosome congression and segregation, and cytokinesis (16–19). The overexpression of Plk1 has also been observed in various human tumors, implying a close correlation between Plk1 expression and carcinogenesis (20). Plk1 consists of a highly conserved N-terminal...
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kinase domain and two relatively divergent C-terminal Polo box domains that function as a phosphoserine/phosphothreonine-binding module (21). The phosphorylation of Thr-210 in the T-loop (or activation loop) of Plk1 is crucial for its activation, and the activated Plk1 can further phosphorylate multiple substrate proteins (20). Plk1 can phosphorylate cyclin B and Cdc25C and lead to Cdk1 activation for mitotic entry through phosphorylation of two critical regulators of Cdk1 (cyclin B and Cdc25C) during the onset of mitosis (22). Plk1 also plays critical roles in DNA replication and DNA damage processes through the phosphorylation of Hbo1 (23). In addition, it was found that Plk1 can also phosphorylate TNKS1 to facilitate mitotic spindle assembly and promote telomeric chromatin maintenance (24).

In this study, we demonstrated that Plk1 is a positive regulator of telomerase. Plk1 can interact with hTERT and prolong its half-life by enhancing its chromatin loading and inhibiting its ubiquitination. Consistently, we found that Plk1 promotes telomerase activity. These data suggested that Plk1 is involved in telomeric chromatin maintenance through direct interaction with hTERT.

Experimental Procedures

Cell Culture and Synchronization—HEK293T, HeLa, and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), and H1299 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C.

U2OS cells were cultured in Dulbecco’s modified Eagle’s medium for 8 h, and then incubated with 2 mM thymidine for another 16 h.

Cell synchronization was achieved by double thymidine block. In brief, HeLa cells were incubated for 16 h in complete medium with 2 mM thymidine, released in fresh medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C with 5% CO2. The full-length hTERT was cloned into pET41b (Novagen). FLAG-hTERT(1–1050) expression plasmid was made by cloning hTERT into the pET41b (Novagen). The full-length hTERT was cloned into pET41b (Novagen). FLAG-hTERT(1–1050) expression plasmid was made by cloning hTERT into the pET41b (Novagen). The full-length hTERT was cloned into pET41b (Novagen). FLAG-hTERT(1–1050) expression plasmid was made by cloning hTERT into the pET41b (Novagen).

The immunoprecipitates were dissolved in SDS loading buffer, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. For the GST pulldown assay, GST-hTERT was purified from BL21 and immobilized on Sepharose 4B-glutathione beads (GE Healthcare). GST-hTERT or GST as a negative control was incubated with His-Plk1 purified from BL21 at 4°C for 1 h. The bound proteins were dissolved in SDS loading buffer, separated by SDS-PAGE, and immunoblotted with Plk1 antibody.

Western Blotting—The cells were harvested and lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100) with protease inhibitor (Roche Applied Science). The cell extracts were subjected to 8% SDS-PAGE, then transferred onto PVDF membrane (Millipore) followed by blocking with Tris-buffered saline/Tween 20 (TBST) containing 5% skim milk for 1 h at room temperature, and then probed with the indicated primary antibody at 4 °C overnight. After three washes, membranes were incubated with 1:5000 diluted HRP-conjugated secondary antibodies for 1 h at room temperature. The immunoblots were detected by ECL (Engreen Biosystem Co. Ltd.) according to the manufacturer’s protocol.

RNA Interference—HeLa cells were transfected with Plk1-specific small interfering RNA (siRNA) or control siRNA (50 nM) (Ribobio) for 72 h using Lipofectamine 2000 (Invitrogen). Two sets of Plk1 siRNAs were synthesized corresponding to the following cDNA sequences: 5'-CAACCAAAAGTGGAAATGAAATG-3' (siPlk1-1); 5'-CCTTAAATATTCCGCATATT-3' (siPlk1-2).

Flow Cytometry Analysis—Cells were harvested at the indicated time after transfection, fixed in ethanol, and stained with propidium iodide. Flow cytometry analysis was performed with a FACSscan instrument.

Cell Fractionation—Nuclear and cytoplasmic proteins were obtained by treating the cells with the Nuclear-Cytosol Extraction kit (Applygen Technologies Inc.) according to the manufacturer’s instructions. To obtain chromatin-enriched fractions, the cells were resuspended in Buffer I (20 mM HEPES buffer (pH 7.9), 25% glycerol, 5 mM MgCl2, 0.2% Nonidet P-40, 1 mM DTT, 150 mM KCl, protease inhibitor) on ice for 15 min and centrifuged at 1000 × g for 5 min at 4 °C. The supernatants (the 150 mM KCl fractions) containing soluble cytoplasmic and nucleoplasmonic proteins were collected. The pellets were resuspended in Buffer II (same as Buffer I but containing 420 mM KCl) on ice for 15 min and centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was collected as the 420 mM KCl fraction, which contained proteins tightly bound to chromatin. The pellets were sonicated in SDS loading buffer.

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Telomerase Repeat Amplification Protocol (TRAP) Assay—The cells were lysed in lysis buffer and subjected to TRAP assay. 2 μg of protein of cell lysates were mixed with 50 μM dNTPs and 80 ng/μl TS primer (5’-AATCCGTGCAGACAGTT-3’) in TRAP buffer (20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 μg/μlEGTA, 0.1 mg/ml BSA) and incubated for 30 min at 30 °C and then for 10 min at 94 °C. The mixture was subsequently subjected to real-time PCR in the presence of ACX primer (5’-GCCGTGTTACCTTTGCTTACC-3’) (40 ng/μl) and SYBR Green Premix reagent (Toyobo). The PCR was carried out as follows: 10-min incubation at 94 °C and 40 cycles of amplification.

RT-PCR for hTERT—Total RNAs were prepared using TRIzol (Invitrogen). PCR was performed according to the manufacturer’s instructions (Invitrogen). Primers for hTERT were: forward, 5’-GCCGATTGTGAAATGACTACG-3’; reverse, 5’-GCTCGTAGTTGACAGCTCGGAA-3’. Primers for β-actin were: forward, 5’-GTAAAGGTGACAGTCCGGTT-3’; reverse, 5’-GAAAGTGGGTGCTTTTAAAGGA-3’.

Results

Plk1 Interacts with hTERT and Is Present in Active Telomerase Complex—Previous studies have shown that telomerase activity is cell cycle-related. The maximum telomerase activity was detected in the S phase with barely detectable levels observed at the G₂/M phase (25). However, the mechanism underlying this correlation is unclear. To investigate whether cell cycle-related kinases (Cdks and Plk1) are involved in the regulation of telomerase activity, we first examined whether they can interact with hTERT. The co-immunoprecipitation data showed that ectopically expressed Cdk2 and Plk1 can interact with FLAG-hTERT in 293T cells (Fig. 1A). The polyclonal antibody against hTERT was generated by immunizing rabbits with a GST-hTERT(1051–1132) fusion protein. To test the specificity of hTERT antibody, we also generated the expression plasmid pCMV FLAG-hTERT(1–1050), which encodes truncated hTERT without antigen epitope recognized by both the in-house and Abcam hTERT antibodies. We transfected FLAG-hTERT or FLAG-hTERT(1–1050) expression plasmids into the hTERT-negative cell line U2OS (26). The cell lysates were harvested for immunoblotting. The results indicated that both in-house and Abcam antibodies can recognize endogenous hTERT protein in hTERT-positive cells (293T, HeLa, and H1299 cells) and full-length FLAG-hTERT expressed in U2OS but not antigen epitope-truncated hTERT (Fig. 1B). Then we transfected U2OS or 293T cells with pCMV FLAG-Plk1 and performed the co-immunoprecipitation experiment. The data showed that hTERT can be specifically detected in 293T cells but not U2OS cells (Fig. 1C). To further confirm the interaction for the endogenous proteins, HeLa cell lysates were immunoprecipitated with hTERT antibody followed by immunoblotting with Cdk2 or Plk1 antibodies. The results indicated that hTERT could interact with Plk1 but not Cdk2 (Fig. 1D). To verify the reliability of the above result, we performed the co-immunoprecipitation experiment with U2OS as a negative control and pCMV FLAG-hTERT-transfected U2OS as a positive control and obtained consistent results (Fig. 1E). To examine whether Plk1 interacts with hTERT directly, we performed a GST pulldown assay with purified GST-hTERT and His-Plk1. The data indicated that Plk1 can interact with hTERT directly (Fig. 1F). To identify which region of Plk1 is required for the interaction with hTERT, we generated FLAG-Plk1 full length, FLAG-Plk1(1–330), and FLAG-Plk1(330–603) expression plasmids (Fig. 1G) and transfected them into 293T cells. The cell lysates were immunoprecipitated with FLAG antibody and subjected to immunoblotting with hTERT antibody. The result showed that the kinase domain of Plk1 at its N terminus is responsible for its binding with hTERT (Fig. 1H). Next, we examined whether the kinase activity of Plk1 was involved in its interaction with hTERT. FLAG-Plk1, constitutively active mutant FLAG-Plk1-TD, or kinase-deficient mutant FLAG-Plk1-KD were expressed in 293T cells respectively and then immunoprecipitated with FLAG antibody followed by immunoblotting with hTERT antibody. The result indicated that Plk1 can interact with hTERT independently of its kinase activity (Fig. 1I). Because Plk1 can interact with hTERT, we wondered whether Plk1 was associated with active telomerase. Therefore, we transfected myc-Plk1 into 293T cells. Then the cell lysates were harvested, immunoprecipitated with myc antibody, and subjected to TRAP assay. The result showed that telomerase activity was detected in the immunoprecipitates from cells expressing myc-Plk1 but not that from control cells (Fig. 1J). Next, 293T cells were transfected with Plk1 antibody or normal mouse IgG as a control and subjected to TRAP assay. Consistently, the results showed endogenous Plk1 present in active telomerase complex and that telomerase activity in the Plk1 immunoprecipitates increases in a dose-dependent manner (Fig. 1K). Taken together, these results indicate that Plk1 can interact with hTERT independently of its kinase activity and be associated with active telomerase complex.

Plk1 Positively Regulates the Activity of Telomerase—To understand the functional significance of the interaction between hTERT and Plk1, we took an RNA interference approach to determine whether knockdown of Plk1 affects telomerase activity. As shown in Fig. 2A, the Plk1 siRNAs (siPlk1-1 and siPlk1-2) can knock down endogenous Plk1 efficiently in HeLa cells. The cell lysates were subjected to a telomerase activity assay. The data showed that knockdown of Plk1 decreased telomerase activity significantly (Fig. 2B). Flow cytometry analysis indicated that knockdown of Plk1 does not affect the cell cycle progression in HeLa cells (Fig. 2C). Also, we generated siRNA-resistant myc-Plk1 expression plasmid pCMV myc-Plk1R and transfected it or wild type Plk1 with Plk1 siRNA into HeLa cells. As shown in Fig. 2D, myc-Plk1R is resistant to siPlk1. Then we analyzed telomerase activity in transfected cells by TRAP assay. Consistently, we found that only myc-Plk1R can rescue the inhibitory effect on telomerase activity caused by Plk1 knockdown (Fig. 2E). Then we transfected myc-Plk1, myc-Plk1-TD, or myc-Plk1-KD expression plasmids into H1299 cells (Fig. 2F) and examined telomerase activity. In the TRAP assay, we observed that Plk1 can increase telomerase activity independently of its kinase activity (Fig. 2G) and that overexpression of myc-Plk1, myc-Plk1-TD, or myc-Plk1-KD plasmids did not affect the cell cycle progression in H1299 cells.
significant (Fig. 2H). The results suggested that Plk1 positively regulates the activity of telomerase.

**Plk1 Affects the Stability of hTERT Protein**—To explore the mechanism of Plk1 regulating telomerase activity, we first examined whether knockdown of Plk1 affects the level of hTERT protein. The immunoblotting data indicated that knockdown of Plk1 caused the reduction of hTERT protein level in HeLa cells (Fig. 3, A and B). To know whether knockdown of Plk1 influences the transcription of hTERT, we analyzed the level of hTERT mRNA by real time PCR. The data showed that knockdown of Plk1 did not affect the transcription of hTERT (Fig. 3C). Although telomerase activity peaks at S phase (27), we wondered whether knockdown of Plk1 influences the level of hTERT throughout the cell cycle. We transfected HeLa cells with siPlk1 and synchronized the cells at different phases. The cell lysates were harvested and subjected to immunoblotting with hTERT antibody (Abcam) or normal rabbit serum as the control followed by immunoblotting with Plk1 and Cdk2 antibodies. E, U2OS cells were transfected with FLAG-hTERT expression plasmids or empty vector as the control. The U2OS and HeLa cell lysates (TCL) were immunoprecipitated with hTERT antibody (Abcam) or normal rabbit serum as the control followed by immunoblotting with Plk1 antibody.

F, GST pulldown assay. Purified His-Plk1 was incubated with immobilized GST or GST-hTERT, respectively. The bound protein was detected by immunoblotting with Plk1 antibody. CBB, Coomassie Brilliant Blue staining. G, scheme of Plk1 deletion mutants. H and I, 293T cells were transfected with pCMV2 FLAG-Plk1 or the indicated Plk1 deletion mutants (H) or with pCMV2 FLAG-Plk1, pCMV2 FLAG-Plk1-TD, or pCMV2 FLAG-Plk1-KD expression plasmids (I). The total cell lysates (TCL) were immunoprecipitated (IP) with FLAG antibody and immunoblotted (IB) with hTERT antibody. J, 293T cells were transfected with pCMV myc-Plk1 or empty vector as the control. The total cell lysates were immunoprecipitated with myc antibody. The immunoprecipitates were subjected to TRAP assay, and the relative telomerase activity was quantified by real time PCR (left) and native PAGE (right). LB, lysis buffer as the negative control. K, different amounts of 293T cell lysates were immunoprecipitated with Plk1 antibody or normal mouse IgG as a control. The immunoprecipitates were subjected to TRAP assay, and the relative telomerase activity was quantified by real time PCR.

Error bars represent S.D.
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Plk1 positively regulates the activity of telomerase. A–C, HeLa cells were transfected with control siRNA or Plk1 siRNA (siPlk1-1 and siPlk1-2) for 72 h. The cell lysates were subjected to immunoblotting with indicated antibodies (A), quantitative TRAP to measure telomerase activity (B), and flow cytometry analysis (C). D and E, HeLa cells were transfected with myc-Plk1WT or myc-Plk1KD expression plasmids together with Plk1 siRNA. The cell lysates were harvested for immunoblotting with the indicated antibodies (D) and quantitative TRAP to measure telomerase activity (E). F–H, H1299 cells were transfected with pCMV myc-Plk1, pCMV myc-Plk1-TD, or pCMV myc-Plk1-KD expression plasmids or with empty vector as the control. The cell lysates were subjected to immunoblotting with the indicated antibodies (F), quantitative TRAP (G), and flow cytometry analysis (H). Data represent the average of three independent experiments. Error bars represent S.D. *p < 0.05.

The data showed that overexpression of Plk1 increased the half-life of endogenous hTERT (Fig. 4, B and C). Furthermore, we found that Plk1 can prolong the half-life of endogenous hTERT in a dose-dependent manner (Fig. 4, D and E). These data suggested that Plk1 increases the stability of hTERT protein, which may in turn facilitate the telomerase activity. Because the nuclear localization of hTERT is required for telomerase activity to elongate telomeric DNA in vivo, we wondered whether Plk1 could influence the nuclear localization of hTERT. Therefore, we compared the amount of hTERT protein in cytoplasmic and nuclear fractions in Plk1-overexpressing cells and control cells by immunoblotting analysis. As shown in Fig. 4F, the overexpression of Plk1 led to a significant increase of FLAG-hTERT in the nucleus as well as an obvious decrease of FLAG-hTERT in the cytoplasm in a dose-dependent manner. Then, we determined whether Plk1 could affect the subcellular localization of endogenous hTERT. The data showed that hTERT in the nucleus was higher in Plk1-overexpressing cells than that in control cells (Fig. 4, G and H). We then compared the amount of hTERT in Plk1 knockdown cells and control cells. Consistently, the data showed that knockdown of Plk1 led to a decrease of hTERT in the nucleus (Fig. 4, I and J). To further...
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**Figure 3.** Plk1 influences the level of hTERT protein. A–C, HeLa cells were transfected with Plk1 siRNA or control siRNA for 72 h. The cell lysates were harvested for immunoblotting with the indicated antibodies (A). The protein level was quantified. The results represent the average of three independent experiments (B). The total RNA was extracted for real time PCR with hTERT primers. The relative amount of hTERT mRNA was calculated (C). D and E, HeLa cells were transfected with control siRNA or Plk1 siRNA. After 24 h, the cells were synchronized at G1/S phase by double thymidine treatment and then released for different times, asy, asynchronized cells. The cells were harvested for immunoblotting with the indicated antibodies. Proliferating cell nuclear antigen (PCNA) and cyclin B were used as the indicators for S and G2/M phases, respectively (D). The amount of hTERT protein was quantified, and the results represent the average of two independent experiments (E). F and G, 293T cells were transfected with pCMV2 FLAG-Plk1 at different concentrations (0.5 and 1 μg) or empty vector as the control for 48 h. The cell lysates were harvested for immunoblotting with hTERT antibody (F). The protein level was quantified. The results represent the average of three independent experiments (G). H, 293T cells were transfected with pCMV2 FLAG-Plk1 or empty vector as the control. The total RNA was extracted and subjected to real-time PCR. I and J, 293T cells were transfected with pCMV2 FLAG-Plk1, pCMV2 FLAG-Plk1-TD, or pCMV2 FLAG-Plk1-KD expression plasmids for 48 h. The cell lysates were subjected to immunoblotting with hTERT antibody (I). The protein level was quantified. The results represent the average of three independent experiments (J). Error bars represent S.D. *p < 0.05; **p < 0.01.

investigate which fraction of hTERT in the nucleus was affected by Plk1, we extracted the cells with different concentrations of KCl and collected each fraction for immunoblotting analysis as described in a previous report (28). We observed that chromatin-bound hTERT (in 420 mM KCl fraction) was obviously decreased in Plk1 siRNA-transfected HeLa cells compared with that in control cells (Fig. 4, K and L). The result suggested that Plk1 could prolong the half-life of hTERT protein and increase the amount of chromatin-bound hTERT.

**Plk1 Decelerates the Ubiquitination of hTERT**—It has been reported that the half-life of hTERT protein is generally controlled by the ubiquitin-mediated degradation in which MKRN1 and HDM2 have been identified as an E3 ubiquitin ligase for hTERT in mammalian cells (14, 15). To know whether Plk1 affects the ubiquitination of hTERT, we transfected 293T cells with FLAG-hTERT-, myc-Plk1-, and HA-ubiquitin-expressing plasmids as indicated and then treated the cells with or without MG132. The cell lysates were immunoprecipitated with FLAG antibody and subjected to immunoblotting with HA antibody. As shown in Fig. 5A, the ubiquitinated hTERT was reduced in Plk1-overexpressing cells compared with that in control cells (lane 3 versus lane 5 and lane 4 versus lane 6) in both MG132-un-treated and -treated cells. We performed a similar experiment by transfecting 293T cells with different amounts of Plk1 expression plasmids. The data indicated that Plk1 inhibited the ubiquitination of FLAG-hTERT in a dose-dependent manner (Fig. 5B). Taken together, we propose that Plk1 prolongs the half-life of hTERT by inhibiting its ubiquitination-mediated degradation. In conclusion, Plk1 can interact with hTERT and prevent its ubiquitination, in turn enhancing hTERT stability and telomerase activity.

**Discussion**

Telomerase activity is modulated at various steps including telomerase RNA biosynthesis, transcription, and protein modification of TERT and telomerase holoenzyme formation. Here we report that the protein kinase Plk1 functions as a positive regulator on telomerase activity. We found that Plk1 interacts with hTERT and associates with the active telomerase complex. Phosphorylation is an important protein modification of TERT. Several lines of evidence have suggested that phosphorylation of hTERT plays an important role in its activity. Akt and PKC have been shown to phosphorylate hTERT and enhance telomerase activity (29, 30). In contrast, Dyrk2 could phosphorylate TERT, promote its degradation, and subsequently inhibit the telomerase activity (31). Tyrosine kinase c-Abl inhibits telomerase activity by phosphorylating hTERT (32). However, we did not observe that Plk1 can phosphorylate hTERT by *in vitro* kinase assay (data not shown). Based on the fact that Plk1, Plk1-TD, and Plk1-KD can bind with hTERT at similar intensities and have comparable effects on telomerase activity, we propose that Plk1 can regulate hTERT independently of its kinase activ-
FIGURE 4. Plk1 can prolong the half-life of hTERT protein. A, 293T cells were transfected with pCMV FLAG-hTERT and pCMV myc-Plk1 or empty vector as the control for 36 h. Then the cells were treated with 100 μg/ml cycloheximide (CHX) for the indicated time. The cell lysates were harvested for immunoblotting withFLAG antibody. B and C, 293T cells were transfected with pCMV2 FLAG-Plk1 or empty vector as the control and then treated with cycloheximide. The cell lysates were harvested and subjected to immunoblotting (B). The protein level was quantified. The results represent the average of three independent experiments (C). D and E, 293T cells were transfected with different amounts of pCMV2 FLAG-Plk1 (1 and 2 μg, respectively) or empty vector as the control and then treated with cycloheximide. The cell lysates were harvested and subjected to immunoblotting (D). The amount of hTERT protein was quantified, and the results represent the average of three independent experiments (E). F, 293T cells were transfected with pCMV FLAG-hTERT accompanied with different concentrations of pCMV myc-Plk1 (1, 2, and 3 μg, respectively). The total cell lysates and cytoplasmic (Cyto) and nuclear (Nuc) fractions were prepared as described under “Experimental Procedures” and subjected to immunoblotting with the indicated antibodies. G and H, 293T cells were transfected with pCMV2 FLAG-Plk1 or empty vector as the control. The total cell lysates and cytoplasmic (C) and nuclear (N) fractions were collected for immunoblotting with hTERT antibody. The relative amount of hTERT protein was calculated. The results represent the average of two independent experiments shown in H. I and J, HeLa cells were transfected with control siRNA or siPlk1. The cell lysates were extracted with lysis buffer containing 150 mM KCl and centrifuged. The supernatants were collected as the 150 mM KCl fraction. The pellets were then extracted with lysis buffer containing 420 mM KCl and centrifuged. The supernatants were collected as the 420 mM KCl fraction. Then the pellets were resuspended in loading buffer and sonicated as the pellet fraction. The total cell lysates were reconstituted by mixing the three fractions proportionally. All fractions were subjected to immunoblotting with the indicated antibodies. β-Tubulin, lamin B, and MCM3 were used as controls for different fractions. The relative amount of hTERT is shown in L. Error bars represent S.D., *p < 0.05.

FIGURE 5. Plk1 decreases the ubiquitination of hTERT. A, 293T cells were co-transfected with pCMV HA-ubiquitin (ub)-, pCMV FLAG-hTERT-, and pCMV myc-Plk1-expressing plasmids for 24 h and then treated with or without 5 μM MG132 for 16 h. The total cell lysates were harvested, immunoprecipitated (IP) with FLAG antibody, and immunoblotted (IB) with HA antibody. B, 293T cells were co-transfected with pCMV HA-ubiquitin, pCMV FLAG-hTERT, and different amounts of pCMV myc-Plk1 (0.5, 1, and 1.5 μg, respectively) followed by treatment with 5 μM MG132. The total cell lysates were immunoprecipitated with FLAG antibody and immunoblotted with HA antibody.
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In some cases, Plk1 only phosphorylates the substrate when it is primed by phosphorylation by other kinase(s); for example, Plk1 phosphorylates PTP1B, which requires a priming phosphorylation by Cdk1 at Ser-386 (33), and phosphorylation of Cdc25B at Ser-50 by Cdk1 serves as a docking site for Plk1 (34). Therefore, we still cannot rule out the possibility that Plk1 could phosphorylate hTERT as it was phosphorylated by other kinase(s) in vivo.

Telomere surveillance is carried out by six telomere-associated proteins that form a protective complex termed shelterin (35). One component of shelterin is telomere repeat-binding factor 1, which acts as scaffolds to recruit other proteins to telomeres. Telomere repeat-binding factor 1 also functions as a negative regulator to control telomere length by controlling the access of telomerase to telomeres or recruiting the XPF nuclease at telomeres (36–38). It has been reported that telomerase inhibitor PinX1 is recruited to telomeres by telomere repeat-binding factor 1 and provides a crucial link between telomere repeat-binding factor 1 and inhibition of telomere elongation by telomerase to help maintain telomere homeostasis (39). Interestingly, it has been found that Plk1 negatively regulates the stability of PinX1 by phosphorylation, implying that Plk1 may be involved in the regulation of telomerase activity by governing the turnover of PinX1 (40). Here we reported that Plk1 up-regulates telomerase activity through interacting with hTERT in a way that is independent of its kinase activity. It seems that Plk1 can regulate the telomerase activity in a different manner. However, whether there is cross-talk between the different pathways in which Plk1 is involved in regulating telomerase activity needs further study.

Ubiquitin is a signal protein for proteasome-mediated degradation in cells, and the ubiquitin modification of hTERT in the cytoplasm also contributes to its degradation, which further affects telomere length homeostasis (14, 15). It is known that the localization of proteins determines whether they can be ubiquitinated or not (41). In this study, we showed that the overexpression of Plk1 led to a significant increase of hTERT in the nucleus and bound to chromatin as well as the total amount of hTERT protein in cells (Fig. 4, K and L). Furthermore, we found that the ubiquitinated hTERT was reduced in Plk1-overexpressing cells. Based on these results, we propose that Plk1 may facilitate TERT localization in the nucleus to prevent its nuclear export and ubiquitin-mediated degradation. Ubiquitin E3 ligases HDM2 and MKRN1 have been found to mediate ubiquitin-dependent proteasomal degradation of hTERT (14, 15), but how E3 ligases are regulated and coordinated is not fully understood. Notably, we found that Plk1 led to reduced ubiquitination and proteasomal degradation of hTERT. We propose that Plk1 may have an impact on the function of ubiquitin E3 ligases or interfere with the interaction between hTERT and E3 ligases. The exact molecular mechanism by which Plk1 regulates hTERT protein stability needs to be further elucidated.

In summary, we have identified Plk1 as a novel hTERT-associated protein and a positive modulator of telomerase activity. These findings are helpful to a better understanding of the various roles of Plk1 and the multiple regulation pattern of hTERT. Our results also have considerable impact on the understanding of immortalization of cells through the enhancement of the effect of Plk1 on telomerase activity.

Author Contributions—Y. H. and X. Y. designed the study and wrote the paper. Y. H. and L. S. performed and analyzed the experiments. N. L. provided technical assistance and contributed to the preparation of the figures. Q. W. and L. J. designed and constructed vectors for expression of mutant proteins. X. T. conceived and coordinated the study. All authors analyzed the results and approved the final version of the manuscript.

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