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PinX1 is involved in telomerase recruitment and regulates telomerase function by mediating its localization

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ABSTRACT

Telomerase recruitment to telomere is the prerequisite for telomere extension, but the proteins involved in this process are still largely unknown. PinX1 is a telomerase inhibitor and has been implicated in telomere maintenance. Silencing of PinX1 significantly reduced the localization of telomerase to telomere during mid-late S phase, suggesting the involvement of PinX1 in the cell cycle-dependent trafficking of hTERT to telomere. We also revealed that PinX1 mediated the chromosomal localization of hTERT during anaphase. This study revealed the role of PinX1 in telomerase function regulation by mediating its localization inside cells.
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1. Introduction

Telomere is a nucleoprotein structure that caps the ends of the eukaryotic chromosomes. The telomere is bound by six core proteins that involved in the regulation of telomere length and telomere function, called shelterin complex [1,2]. Owing to the "end replication problem", telomere undergoes shortening during cell division [3,4]. When the shortened telomere reaches a critical length, the chromosome ends are sensed as double-stranded breaks and lead to cellular senescence or apoptosis [5–7]. Telomerase is an enzyme responsible for telomere lengthening and maintenance which consists of two core domains, the catalytic subunit hTERT and the RNA template hTR [8,9]. Immortalization of cells is characterized by telomerase activation and telomere length maintenance in which 85% of immortal cancer cell lines express high level of telomerase and maintain short telomere length [10,11]. This suggested the critical role of telomerase reactivation in cellular immortalization and cancer progression. Telomere maintenance is tightly regulated. Although telomerase activity was detectable during entire cell cycle, telomere extension only occurred during S-phase [12,13]. This was explained by the cell-cycle dependent subcellular trafficking of core telomerase components, hTERT and hTR for active telomerase biogenesis and the recruitment process of telomerase to telomere [14,15]. Throughout most of the cell cycle stages, hTR and hTERT are localized in intranuclear sites that distinct from telomere. During S-phase, hTERT and hTR are recruited to subsets of telomeres, which are their substrate; this step is believed to be critical and prerequisite for the telomere extension [14,16,17]. The underlying mechanism of this trafficking and the factors involved are still poorly defined.

PinX1 is a potent telomerase inhibitor that can directly interact with hTERT and hTR [18,19]. PinX1 C-terminal 290–328aa carries the telomerase inhibitory domain (TID) [18,20]. PinX1 or PinX1-C (PinX1 254–328aa) stable expressing HT1080 cell lines induced crisis and shortened telomere. At the same time, stable expression of PinX1 can suppress tumor growth in mice [18]. On the other hand, silencing of PinX1 also induced senescence and telomeres shortening in telomerase positive cells, despite the increased telomerase activity in the cell extract [21]. This suggested that PinX1 although can potently inhibit telomerase activity, it is also a crucial factor in the telomere maintenance process. Therefore to further elucidate the role of PinX1 in telomere maintenance and the factors that mediate telomerase recruitment, we examined if PinX1

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is involved in the cell-cycle dependent trafficking of hTERT to telomere during S-phase and thus the telomerase recruitment process by co-localization assays.

2. Materials and methods

2.1. Cell culture and synchronization

HeLa and HEK293T (ATCC) cells were cultured in Minimal Essential Medium (Gibco, Invitrogen) with 10% fetal calf serum (Gibco, Invitrogen) in 37 °C with 5% CO_2. PinX1 siRNA (Dharmaco) and Plasmid DNA were transfected into cells by RNAiMAX (Invitrogen) and Lipofectamine 2000 (Invitrogen) respectively. Twenty-four hours post-transfection of PinX1 siRNA, the HeLa cells were treated with 1 mM hydroxyurea for further 24 h and synchronized to G1/S phase boundary. The hydroxyurea block was released by the removal of medium with hydroxyurea, followed by one PBS wash and then cultured in fresh medium. Cells were collected at 0, 2, 4, 6 h after releasing from the block for the indicated analysis.

2.2. Flow cytometry

Cells at different time points were harvested and washed once with PBS. Ice cold 70% ethanol was added to the cell pellet while vortexing for fixation. Cells were fixed overnight at −20 °C. Fixed cells were collected and washed once with PBS. PI DNA staining buffer (20 μg/ml propidium iodide, 10 μg/ml RNase in PBS) was added to resuspend the cells and incubated at 37 °C in dark for 30 min. The stage of cell cycle of the stained cells was analyzed by flow cytometry (FACSCanto, BD).

2.3. Immunofluorescence and microscopy

HeLa cells were seeded and cultured on 13 mm circle cover glasses (Thermo-Menzel) in a 24-well plate. The immunofluorescence was performed as described [14] with some modifications. Cells were washed once with warm PBS and then fixed and permeablized by acetone/methanol (1:1) for 10 min at −20 °C. Cells were washed twice by PBS and incubated with 2 M HCl for 20 min at room temperature. Cells were than washed by PBS once and incubated with 0.1 M boric acid for 10 min. After 2 PBS washes, cells were blocked in blocking buffer (5% FBS in PBS) at room temperature for 30 min. Primary antibodies were added to the cover glass and incubated at room temperature for 2 h. The dilutions of primary antibodies used were as follow: mouse IgM Anti-hTERT 1:3000 in 5% FBS (2C4, Abcam), and Rabbit Anti-TRF2 1:250 in 5% FBS (Santa Cruz). Primary antibodies were removed and the cells were washed four times by PBS. Secondary antibodies were added to the cover glass and incubated at room temperature for 2 h.

The dilutions of secondary antibodies used were: Cy2-conjugated AffiniPure Goat Anti-mouse IgM, μ Chain Specific 1:100 in 5% FBS (Jackson ImmunoResearch) and AlexaFluor 594 Goat anti-rabbit 1:500 in 5% FBS and AlexaFluor 488 Goat anti-mouse 1:500 in 5% FBS (Invitrogen). Secondary antibodies were removed and the nuclei were stained by 0.5 μg/ml DAPI for 1 min at room temperature. Cells were washed by PBS for three times and then mounted on the glass slides by mounting medium (Dako). Immunofluorescence images were analyzed on Olympus IX71 research inverted microscope with fluorescence observation. Images were acquired at 40× magnification by cooled CCD camera Olympus DP30BW. Images were merged by OLYMPUS MICRO software. Total 400 cells from four experiments were analyzed at each time point. The percentages of cells with hTERT and TRF2 co-localization signal were calculated in each experiment and the mean percentages were plotted. An unpaired Student's *t*-test was used for analyses of significance between the control and PinX1 silenced experiments.

2.4. Immunoprecipitation

Transfected HEK293T cells were washed once by PBS and 500 μl ice cold IP buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% triton X-100, protease inhibitor cocktail) was added to the cells. Cells were harvested by cell-scraper and subject to sonication for 5 s. Cell lysate was centrifuged at 14000 rpm for 10 min at 4 °C. Two hundred microliters of supernatant was added to 300 μl IP Buffer with 1 μl anti-myc antibody (9B11, cell signaling), another 200 μl supernatant was added to 300 μl IP Buffer for antibody negative control. The tubes were incubated at 4 °C with gentle rocking for 16–18 h. Fifteen microliters of protein A beads (50% slurry, Sigma) were added to each tube and incubated at 4 °C with gentle rocking for 1.5 h. The protein A beads were washed three times by ice cold IP buffer and 30 μl 2× protein dye was added. The samples were heated at 95 °C for 10 min and then analyzed by western blot.

3. Results and discussion

3.1. PinX1 silencing does not affect cell cycle progression in S-phase

To meet our purpose, first of all we need to ensure that silencing of PinX1 does not affect the cell cycle progression through S-phase so that the differences of co-localization at different time points are not due to the differences in cell cycle stage. HeLa cells were synchronized by hydroxyurea at G1/S boundary and the endogenous PinX1 was down-regulated by siRNA transfection. After the synchronization, cells from 0, 2, 4, 6 h after releasing from the hydroxyurea block were collected for PinX1 expression level and cell cycle stage analysis. From the western blot, the endogenous PinX1 level was shown to be successfully down-regulated (Fig. 1a). Upon the hydroxyurea treatment, both the control cells and the PinX1 silenced cells were successfully synchronized into G1/S boundary (Fig 1c). After releasing from the hydroxyurea block, the flow cytometric analysis showed the same rate of peak shift towards right in both control and PinX1 silenced cells, indicated the rate of DNA synthesis is the same (Fig 1c). The level of S-phase marker cyclin A, the increasing expression of G2-phase marker cyclin B1 (Fig 1b) also indicated that the cells successfully entered S-phase and progress through S phase with the same rate in both cells. This data indicated that transient silencing of PinX1 does not affect the cell cycle progression during S-phase.

3.2. PinX1 down-regulation reduced hTERT localization to telomere as well as the association with TRF2

The telomerase localization to telomere was evaluated by the co-localization of hTERT and TRF2. TRF2 is a well-known double-stranded telomere binding protein for detecting telomere localization in previous studies [22,23], as 65–80% of telomeres possess immunodetectable TRF1/TRF2 [24]. Immunofluorescence experiment was carried out by detecting endogenous hTERT and TRF2 in HeLa cells. hTERT and TRF2 were localized as their characteristic punctate spots within the nucleus in both control HeLa cells and PinX1 silenced HeLa cells (Fig. 2a) which were consistent with the previous studies on their localization pattern [23,14]. As aforementioned, the telomerase recruitment process occurred during S-phase, therefore we investigated the percentage of hTERT and

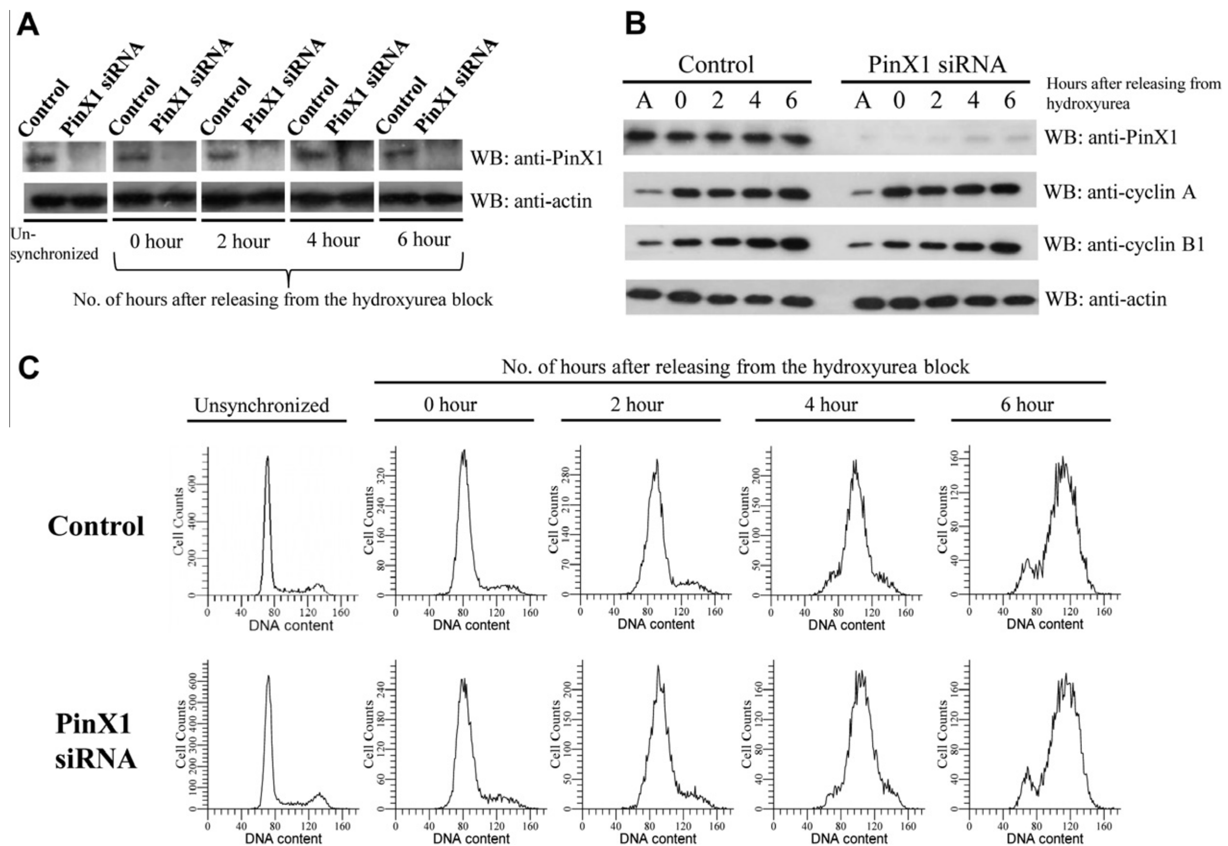

Fig. 1. Silencing of PinX1 does not affect the cell cycle progression through S phase. PinX1 was silenced by transfecting PinX1 siRNA. Cells were then synchronized by hydroxyurea, released and collected at indicated time points. (A) Western blot analysis showing the endogenous PinX1 expression level in control and PinX1 siRNA transfected cells at different time points. (B) Western blot showing the expression profile of cyclin A (S-phase marker) and cyclin B1 (G2 phase marker) during S-phase progression in control and PinX1 siRNA transfected cells. "A" represents asynchronous cells. (C) Control and PinX1 siRNA transfected cells from different time points were stained with propidium iodide and subjected to flow cytometric analysis.

TRF2 co-localization at 2 h (early S-phase), 4 h (mid-S phase) and 6 h (late S-phase) after releasing from the hydroxyurea block (Fig 2b). The percentage of cells with hTERT and TRF2 co-localization of both un-transfected control and PinX1 silenced cells at different time points were calculated by counting around 400 cells from four experiments. For the control cells, there was an increase in co-localization signal of hTERT and TRF2 when the cells migrated from early to mid S-phase (Fig 2b), indicating that the amount of telomere localized telomerase is increased, which is consistent with the previous observation that the localization of hTERT to telomere is peaked at mid-S phase [14]. For the PinX1 silenced population, the percentage of cells with co-localization signal remains more or less the same or increased only slightly during S-phase progression (Fig 2b). At the same time, the percentages of hTERT localization to TRF2 in PinX1 silenced cells were significantly lower than the control cells in mid-late S-phase (Fig 2b), indicating that less hTERT can be recruited to the telomere in the absent of PinX1 during mid-late S phase. This reduction in telomere recruitment of hTERT is not due to the reduction in endogenous hTERT and TRF2 expression level (Fig 2c). Until now, we have not succeeded in establishing a telomere-FISH protocol that is compatible with the hTERT immnoufluorescence staining and thus we cannot investigate the co-localization signal from the telomere probe and hTERT. Since not all telomeric DNA possess immunodetectable TRF2 [24], the percentage of hTERT/TRF2 co-localization we showed may not reflect the actual percentage of hTERT localized on telomere inside the cell. But this limitation does not affect the comparison between the control and PinX1 silenced experiments as well as the conclusion in our experiment.

To further confirm our observation, the association between TRF2 and hTERT was examined. TRF2 is one of the shelterin complex proteins on telomere [1] and it forms complex with Pot1 at the ds-ss telomere junction [25]. The association between TRF2 and hTERT can reflect the amount of telomerase loading to telomere. Myc-tagged TRF2 and hTERT were transiently co-transfected into HEK293T and the myc-immunoprecipitate was captured and the amount of hTERT was analyzed by western blot. Silencing of PinX1 reduced the association of hTERT in the myc-TRF2 immunoprecipitate (Fig 2d) indicating that silencing of PinX1 disrupted the loading process of telomerase to telomere.

In the previous studies, it was shown that PinX1 silencing can reduce the telomerase activity detected in the Pot1 telomeric protein components [21]. Besides, an anti-cancer drug, anthracyclines worked by disrupting telomere maintenance and leaded to telomere dysfunction through the down-regulation of PinX1 [26]. All these implicated that PinX1 does not act solely as a telomerase inhibitor, but it is also involved in the telomerase recruitment process. Our data provided direct evidence on the involvement of PinX1 in the cell-cycle dependent trafficking of hTERT to telomere and thus its importance in telomerase recruitment process. PinX1 may help this process through the bridging between the shelterin complex and telomerase as silencing of PinX1 reduced the association between hTERT and TRF2 (Fig 2d) as well as Pot1 [21]. This explains why depletion of PinX1 caused telomere shortening even with enhanced telomerase activity. To further study the role of PinX1 in telomerase recruitment process, characterization of interaction between PinX1 and the shelterin proteins will be a good target in the future. PinX1 may take part

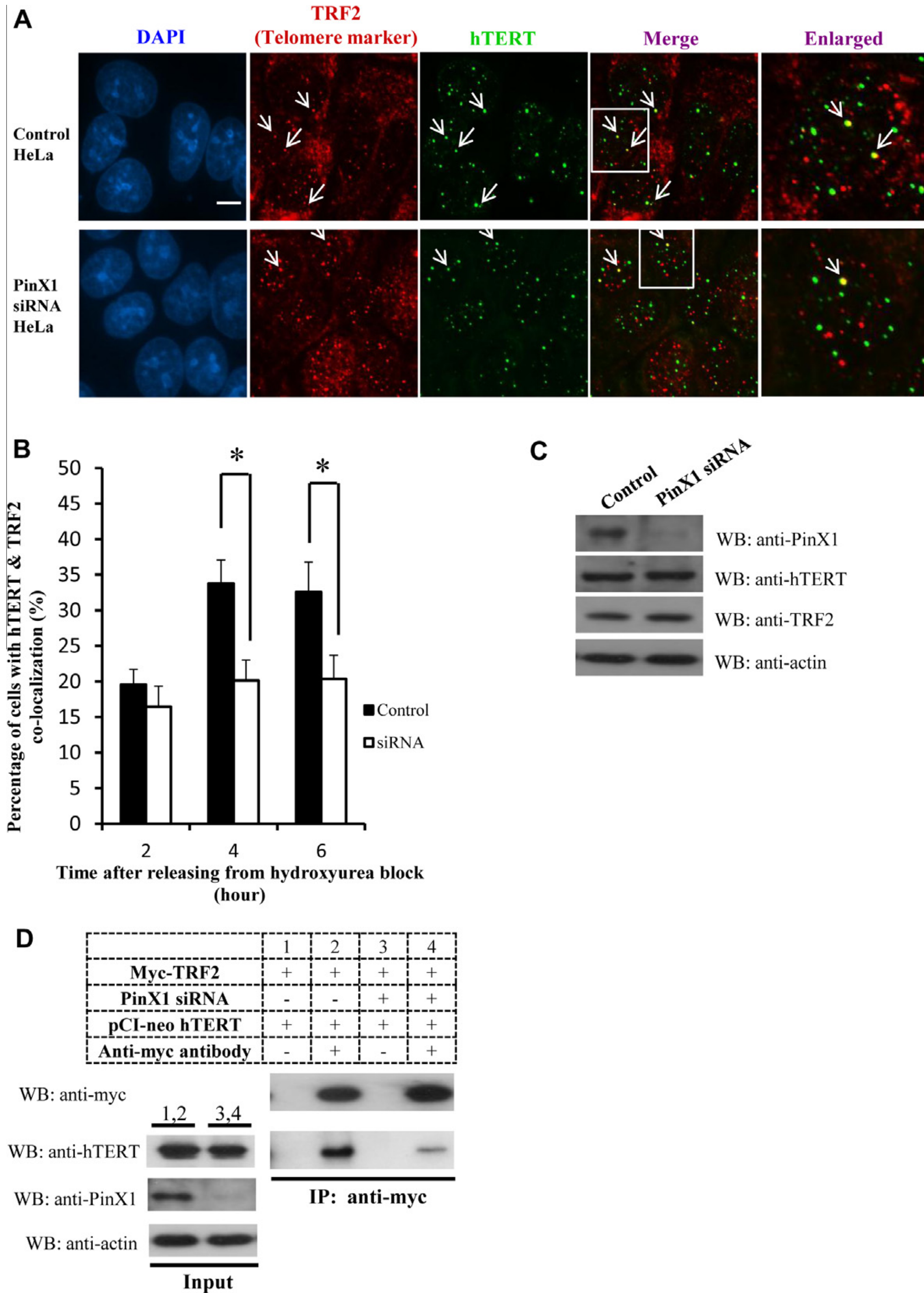

Fig. 2. PinX1 silencing disrupts the cell cycle dependent trafficking of hTERT to telomere. (A) Representative images showing the immunofluorescence staining of endogenous hTERT and TRF2. The blue panel shows the DAPI staining for the location of nucleus. The red and green panels show the staining of TRF2 and hTERT respectively. The merge panel shows the merged images from hTERT and TRF2 staining. The white arrows indicate the co-localization signal of hTERT and TRF2.The enlarged panel showed magnified images of the white boxes indicated. Bar, 10 μM. (B) Bar chart showing the percentage of cells with hTERT and TRF2 co-localization in both control (black bars) and PinX1 siRNA transfected (white bars) cell at 2 (early S-phase), 4 (mid S-phase), 6 (late S-phase) hours after releasing from hydroxyurea block. The results represent the mean percentages ± standard deviations of ~400 cells from four experiments (*, $P < 0.01$). (C) Western blot analysis showing the endogenous expression level of hTERT and TRF2 in control and PinX1 siRNA transfected cells. (D) Immunoprecipitation showing the association of hTERT with myc-TRF2 immunoprecipitate in control and PinX1 siRNA transfected cells.

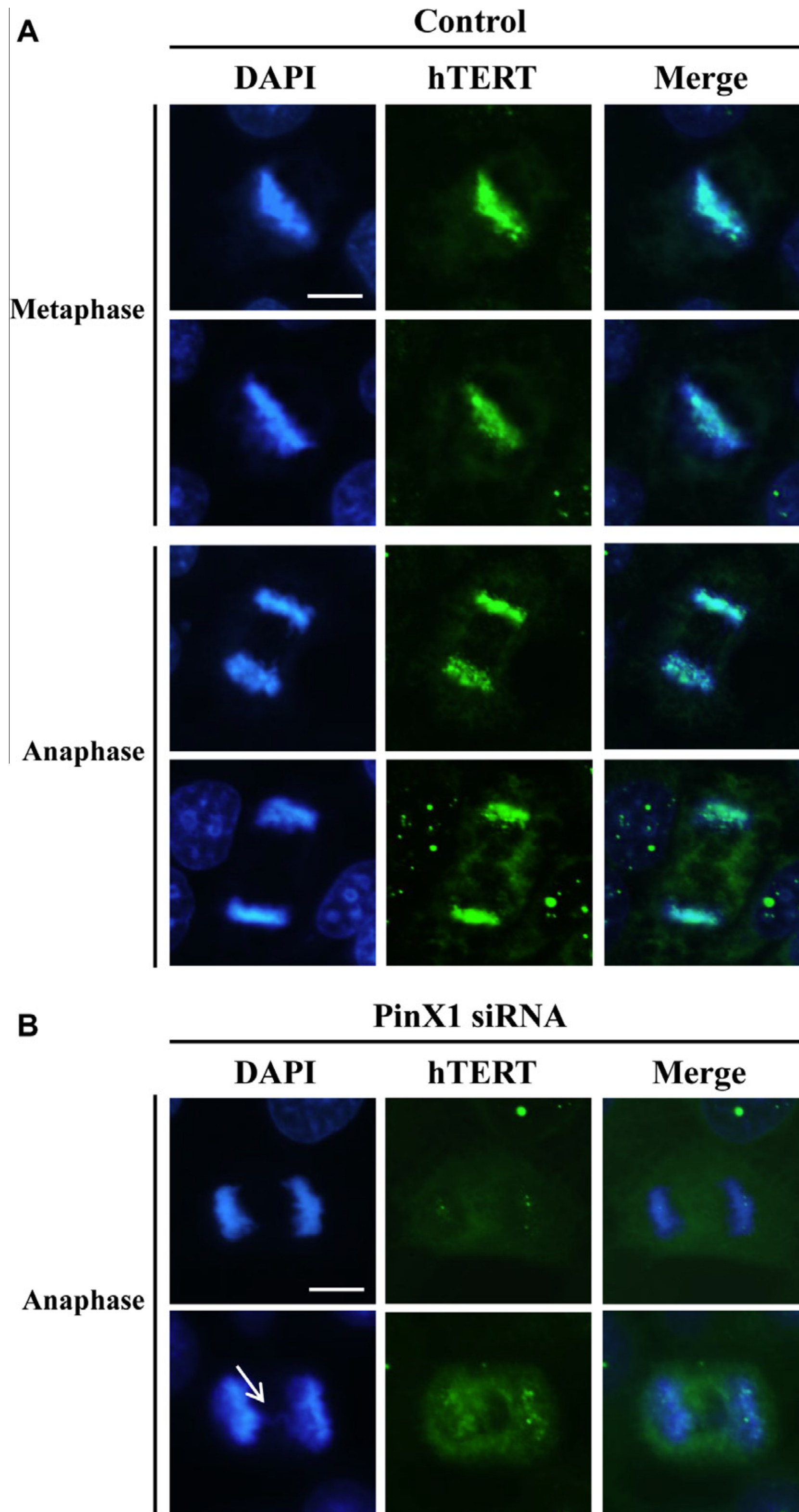

Fig. 3. hTERT localizes with condensed chromosomal DNA during mitosis and PinX1 mediates hTERT localization during anaphase. (A) Immunostaining of endogenous hTERT during metaphase and anaphase in HeLa cells. The blue and green panels are for chromosomal DNA and hTERT respectively. The merge panel shows the merged images of DAPI and hTERT. Bar, 10 μM. (B) Immunostaining of endogenous hTERT at anaphase in PinX1 silenced HeLa cells. The white arrow indicates the chromatin bridge during anaphase in PinX1 depleted cell. Bar, 10 μM.

in bridging the telomerase and shelterin complex, therefore it is worthwhile to investigate if the disruption of PinX1/shelterin complex interaction will lead to aberrant in telomerase recruitment. This will help to reveal the underlying mechanism of the recruitment process, which is crucial for telomere maintenance and cancer cell survival.

3.3. PinX1 mediates the hTERT chromosomal localization during anaphase phase

We have also examined the hTERT localization during mitosis. From the immunofluorescence staining of endogenous hTERT in the mitotic cells, hTERT was found to be co-localized with the condensed chromosomes during metaphase and anaphase (Fig 3a). On the other hand, in PinX1 silenced cells, large portion of cells showed diffuse hTERT pattern during anaphase instead of localizing with the condensed chromosomes (Fig 3b). This indicated that PinX1 mediated the chromosomal localization of hTERT at anaphase. PinX1 has been suggested to be involved in chromosome segregation process during mitosis. Silencing of PinX1 increased the micronuclei-containing cells which are probably caused by the aberrant chromosome segregation in the prior mitosis. Also, delayed onset of anaphase and increased chromosome bridge phenotype were observed in PinX1 depleted cells [27]. This chromosome bridge phenotype was also observed in our experiment (Fig 3b, indicated by the white arrow). Our data revealed the possibility that the chromosome segregation error caused by PinX1 depletion may also due to the disruption in hTERT localization, although further characterization is needed to dissect between the direct effect of PinX1 on this process and the importance of hTERT localization in anaphase. Up to date, little is known about the role of hTERT in mitotic cancer cells. Therefore it will be interesting to investigate the mitotic phenotype of hTERT silenced cancer cells and compared to that of PinX1 silenced phenotype. This will help to dissect the effect of PinX1 and hTERT and provide more information about the function of hTERT in mitotic cancer cell. Our data also explicated the role of PinX1 in mediating telomerase localization besides its reported role in telomerase inhibition.

To conclude, our study explains the dual role of PinX1 in telomerase regulation by acting as telomerase inhibitor as well as aiding the telomerase recruitment process. Taken together that PinX1 is involved in the cell cycle dependent telomerase-telomere recruitment process and it mediates the hTERT chromosomal localization at anaphase, we revealed that PinX1 does not only affect the telomerase activity, it also regulates telomerase function by mediating the localization of hTERT at different stages of cell cycle.

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