Telomerase Inhibitor PinX1 Provides a Link between TRF1 and Telomerase to Prevent Telomere Elongation*

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Telomerase maintenance is essential for protecting chromosome ends. Aberrations in telomere length have been implicated in cancer and aging. Telomere elongation by human telomerase is inhibited in cis by the telomeric protein TRF1 and its associated proteins. However, the link between TRF1 and inhibition of telomerase elongation of telomeres remains elusive because TRF1 has no direct effect on telomerase activity. We have previously identified one Pin2/TRF1-interacting protein, PinX1, that has the unique property of directly binding and inhibiting telomerase catalytic activity (Zhou, X. Z., and Lu, K. P. (2001) Cell 107, 347–359). However, nothing is known about the role of the PinX1-TRF1 interaction in the regulation of telomere maintenance. By identifying functional domains and key amino acid residues in PinX1 and TRF1 responsible for the PinX1-TRF1 interaction, we show that the TRF homology domain of TRF1 interacts with a minimal 20-amino acid sequence of PinX1 via hydrophilic and hydrophobic interactions. Significantly, either disrupting this interaction by mutating the critical Leu-291 residue in PinX1 or knocking down endogenous TRF1 by RNAi abolishes the ability of PinX1 to localize to telomeres and to inhibit telomere elongation in cells even though neither has any effect on telomerase activity per se. Thus, the telomerase inhibitor PinX1 is recruited to telomeres by TRF1 and provides a critical link between TRF1 and telomerase inhibition to prevent telomere elongation and help maintain telomere homeostasis.

Telomeres cap the ends of linear chromosomes, which are composed of repetitive sequences and associated proteins that are necessary for telomeric maintenance and regulation (1–5). Telomeric sequences can be extended by telomerase, a unique reverse transcriptase composed of the catalytic subunit TERT and the RNA template TER (6–16). Although TER is constitutively expressed in most cells, TERT is expressed at very low levels or not at all in most normal human cells with the exception of highly proliferative tissues (17–19). Therefore, telomeres shorten during each cell division due to the end replication problem of DNA polymerase as well as postreplication processing by unidentified nucleases or helicases (20–23). Telomere shortening to a critical length and/or the uncapping of the telomere leads to replicative senescence or crisis (24–31).

Telomerase activation has been well documented as one of the necessary factors for cell immortalization and transformation. It is reactivated in most human cancer cells (17, 18, 32, 33) and is not only critical for transforming primary human cells (34) but is also sufficient to allow transformed cells to escape from crisis (35–38). In fact, many oncogenes and tumor suppressors regulate transcription of TERT (39–42). Furthermore, ablation of telomerase shortens telomeres and limits cell proliferation (43–45). It confers resistance to tumorigenesis in mice (46–51), although a lack of telomerase in mice over five consecutive generations leads to oncogenesis especially in the p53 mutant genetic background (43, 45, 49, 52, 53). Although telomerase activation allows telomere extension, it does not lead to unlimited telomere elongation; telomeres are stably maintained within a relatively narrow size distribution in telomerase-positive cells (32, 54, 55), although massive overexpression of TERT and TER can elongate telomeres to beyond physiological lengths (56). These results indicate that the ability of telomerase to elongate telomeres is further regulated under physiological conditions. Preserving optimal telomere length is crucial for cells as excessive telomere attrition can result in genomic instability and chromosome fusions. In yeast, telomere maintenance is regulated by a number of telomere proteins, including RAP1, Pif1, Cdc13, Taz1, and Pot1 (57–67). In humans, telomere surveillance is carried out in cis by telomere-associated proteins, including six major players that form a protective complex termed shelterin (5). At the base of the complex are two telomeric DNA binding factors, TRF1 and TRF2, that act as scaffolds to recruit other proteins to telomeres. Although TRF2 protects telomere integrity possibly by forming T-loop structures, TRF1 maintains telomeres at reasonable lengths (5, 55, 68, 69).

It has been proposed that TRF1 acts in cis to progressively block telomerase access and telomere elongation as increased numbers of TRF1 molecules bind to telomeres. Consistent

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The abbreviations used are: TRF1, telomeric DNA-binding protein 1; TRF, telomere restriction fragment; TRFH, TRF homology; Pin2, protein interacting with NIMA-2; PinX1, Pin2/TRF1-interacting protein X1; PD, population doubling; TID, TRF1-interacting domain or telomerase-inhibitory domain of PinX1; RFP, red fluorescent protein; TRAP, telomere repeat amplification protocol.
with this idea is that TRF1 and its interacting proteins, including Tankyrase, Tin2, Pot1, TPP1, and Fbx4, have been shown to regulate telomere length (5, 66, 69–85). However, because neither TRF1 nor these associated proteins affect telomerase activity in vitro or in vivo, it is not fully understood how TRF1 performs this crucial function. It has been shown that the TRF1 complex brings Pot1 to the single-stranded DNA 3’ overhang of telomeres to physically block telomerase from accessing telomeres (73, 79). On the other hand, more recent studies indicate that during telomere extension, the Pot1-TPP1 complex does not prevent telomerase from elongating telomeres but, instead, serves rather as a processivity factor for telomerase to promote telomere elongation (84, 85). These results suggest that Pot1 or Pot1 complexes that are recruited by TRF1 to telomere ends have at least two potentially opposing functions; that is, blocking telomerase from accessing telomeres to inhibit telomere elongation and increasing telomerase processivity to elongate telomeres during telomere extension. However, it remains unclear how these two opposite functions are coordinated. Therefore, the link between TRF1 and telomerase inhibition remains elusive.

We isolated TRF1 as Pin2 in the same screen for Pin1 (86, 87). Both Pin1 and TRF1/Pin2 are important for mitotic regulation (87–91), and Pin1 regulates TRF1 function in telomere maintenance and aging (92). We have identified new TRF1-interacting proteins, including PinX1–4, to elucidate important TRF1 functions in telomere maintenance and mitosis (80, 92–94).

Unlike all other TRF1-interacting proteins, PinX1 has the unique property of directly interacting with the telomerase catalytic component, TERT, and potently inhibiting telomerase catalytic activity (93). Furthermore, PinX1 overexpression in human cancer cells shortens telomeres, induces crisis, and inhibits tumorigenicity, whereas PinX1 deletion increases telomerase activity, elongates telomeres, and enhances tumorigenicity. The ability of PinX1 to inhibit telomere elongation has been shown to be highly conserved in other organisms such as yeast and rats (95–98). These results indicate that PinX1 is a potent telomerase inhibitor that inhibits telomere elongation. However, it is not known how PinX1 is recruited to telomeres, where telomere elongation by telomerase action takes place. Similarly, even though PinX1 was identified as a TRF1-interacting protein (93), nothing is known about whether this interaction has any role in telomere regulation.

To address these questions, we first mapped the minimal domains responsible for the PinX1–TRF1 interaction and then identified a single PinX1 point mutation, L291E, that is able to completely abolish the ability of PinX1 to interact with TRF1 but does not affect its ability to bind and inhibit telomerase. Comparing the subcellular localization and telomere function of PinX1 and its TRF1 binding mutant uncovered that TRF1 is required for recruiting PinX1 to telomeres as well as for PinX1 to inhibit telomere elongation in vivo. The biological significance of these structure-function analyses was further confirmed by our demonstration that PinX1 fails to localize to telomeres and to inhibit telomere elongation when endogenous TRF1 is knocked down. These results demonstrate for the first time that the telomerase inhibitor PinX1 is recruited to telomeres by TRF1 and provides a crucial link between TRF1 and inhibition of telomere elongation by telomerase to help maintain telomere homeostasis.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Recombinant Protein Purification**—Truncation and point mutants of PinX1 and TRF1 were generated by PCR mutagenesis procedures and confirmed by sequencing. N-terminal GST-tagged proteins were generated by subcloning cDNAs encoding PinX1, TRF1, or their mutants into a pGEX vector. The resulting fusion proteins were expressed in BL21 *Escherichia coli* cells and purified by glutathione beads, as described (87, 93). Briefly, cells were pelleted and lysed in buffer containing 50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% Triton, 5% glycerol, lysozyme, and protease inhibitors. After sonication, lysates were centrifuged, and glutathione-agarose beads were added to the supernatant followed by incubation at 4 °C for 2 h. The beads were then washed, and protein was eluted with 30 mM glutathione. Proteins were then dialyzed in 10 mM HEPES, pH 7.5, 10 mM NaCl, 1 mM DTT, 0.5 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 5% glycerol, and protease inhibitors before use.

**GST Pulldown, Immunoprecipitation, and Immunoblot Analyses**—GST pulldown and immunoprecipitation assays were performed as described (93). Relevant proteins were expressed in vitro using a TNT Quick Coupled Transcription/Translation System (Promega) in the presence of [35S]Met or in human cell lines by transient or stable transfection. Proteins were then incubated at 4 °C for 1 h with 1 μM GST fusion proteins in lysis buffer. Protein A/G beads or glutathione-agarose beads were then added followed by additional incubation for 1 h at 4 °C. Beads were then collected by centrifugation, washed multiple times, and boiled in SDS sample buffer. Samples were then separated by electrophoresis on acrylamide gels and transferred by semidry transfer to PVDF membranes for immunoblot analysis.

**Protein Localization and Fluorescence Microscopy**—The subcellular localization of proteins was determined as described previously (87, 93). HT1080 cells seeded on glass coverslips were transiently transfected with GFP-PinX1 and RFP-TRF1. At 16 h post-transfection, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Nonidet P-40/PBS, and DAPI-stained. Coverslips were mounted using Fluormount-G (SouthernBiotech) and visualized by fluorescence microscopy using a Zeiss microscope.

**TRF1 Knockdown Cell Lines or Pools**—Two different TRF1 RNAi sequences and vectors were used. The TRF1 RNAi retroviral construct (CGCTTGCCAGTTGAGAACGATA) and control vector pSM2C were purchased from Open Biosystems followed by generating TRF1 RNAi retroviral stable cell lines as described (80). Plasmids were transfected into Pheonix packaging cells, and viral supernatants were harvested 48 h after transfection followed by infection of HT1080 cells. After selection with antibiotics and limiting dilution, multiple independent cell lines were isolated and checked for protein expression by immunoblotting analysis with anti-TRF1 antibodies. For generating stable TRF1 RNAi lentiviral cell pools, PinX1 stable or vector cell lines were infected with lentivi-
ruses produced from the TRF1 shRNA lentiviral vector containing sense sequence CCCCTGATGCACAGTTTGAAA or the pLKO.1 control vector (Open Biosystems). Lentiviral production and infection was performed as suggested by the RNAi Consortium. Briefly, 293T cells were transfected with the pLKO.1 shRNA or control construct along with CMV-D8.9 viral packaging vector and CMV-VSVG viral envelope vector. Media was replaced 18 h post-transfection, and viral supernatants were harvested after an additional 24 h. Target cells were infected with viral supernatant and subsequently selected with 2 \mu g/ml puromycin, as described (99). Knockdown of TRF1 protein levels was confirmed by immunoblotting analysis.

Stable Cells Overexpressing PinX1 or Its Mutants—HA epitope-tagged PinX1 or mutants were stably transfected into HT1080 fibrosarcoma cells in multiple independent experiments using the vector as a control followed by protein expression confirmation using immunoblotting analysis with anti-HA antibodies (Covance) as described (93). Cells were seeded at 5 \times 10^5 cells per 10-cm culture dish, then passaged and collected every fourth day for telomerase activity and telomere length studies.
Telomerase Activity and Telomere Length Assays—Telomerase activity was assayed using the TRAPeze Telomerase detection kit (Millipore) according to manufacturer’s instructions and as described (93). Briefly, cells were lysed with CHAPS lysis buffer and centrifuged, and the telomerase-containing supernatant was collected. To assay the effects of purified PinX1 proteins on telomerase activity, recombinant proteins were incubated with cell lysates containing telomerase for 10 min at 4 °C before setting up reactions for telomere extension by PCR. Telomerase products were separated on 10% non-denaturing polyacrylamide gels, stained with SYBR Green I Nucleic Acid Stain (Molecular Probes), and visualized.

Telomere restriction fragment (TRF) length at various population doublings was measured, as described previously (93). Genomic DNA was isolated by phenol extraction and digested with HinfI and RsaI restriction enzymes at 37 °C overnight. DNA concentrations were determined by fluorometry before samples were separated on 0.8% agarose gels. Gels were dried at room temperature, and in-gel hybridization was performed with a (TTAGGG)3 telomeric DNA probe. Average telomere restriction fragment length was calculated by quantifying the hybridization signals using ImageQuant.

RESULTS

Multiple Charged Residues and Leu-291 in PinX1 Are Essential for the PinX1-TRF1 Interaction—Although PinX1 was originally identified as a TRF1-interacting protein (93), nothing is known about whether this interaction is important for telomere regulation. To address this question, we started by searching for the domains responsible for the TRF1-PinX1 interaction by generating systematic truncations of TRF1 (Fig. 1A) and PinX1 (Fig. 1D) followed by various binding assays, performed as previously described (93). GST-PinX1 bound to GFP-TRF1 fragments encompassing residues 1–439 and 1–336 but not 322–439, which were expressed in mammalian cells (Fig. 1B). Conversely, GST-TRF1 fragments encompassing residues 1–439, 1–336, and 1–220, but not 1–130, bound to PinX1 that was synthesized by in vitro transcription and translation (Fig. 1C). These results indicate that the PinX1 binding domain is located in the TRF homology (TRFH) domain of TRF1, as shown for several other TRF1-binding proteins (71, 80, 100).

Upon further examination of the TRF1 binding domain in PinX1, we confirmed our previous findings that the TRF1 binding domain is located at the C-terminal 75 amino acids of PinX1 (Fig. 1, D and E), which we previously named TID for the TRF1-interacting domain and telomerase-inhibitory domain (93).

FIGURE 2. TRF1 binding and telomere localization of a large panel of PinX1 mutants. A, TRF1 binding and telomere localization of single point mutants or quadruple Ala substitution mutants (K292A, K294A, K295A, and R297A) in different PinX1 fragments were summarized, respectively. B and C, examples of TRF1 binding of selected PinX1 mutants are shown. The amino acid residues of PinX1 truncation mutants are from 254 to 328 (TID), from 291 to 328 (C37), and from 291 to 310 (C20). D, examples of subcellular localization of TID or TID4A with TRF1 are shown. The localization of these mutant proteins were determined by co-transfecting HT1080 cells overnight with vectors expressing RFP-tagged TRF1 and GFP-tagged wild-type full-length PinX1 or its TID with or without mutations followed by subjecting them to fluorescence microscopy (D). Yellow arrows point to colocalization of TRF1 and TID at telomeres.

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indicate that the 20-amino acid fragment (C20) from residue 291 to 310 of PinX1 is both necessary and sufficient to mediate the interaction with TRF1.

The C20 fragment of PinX1 contains several positively charged residues (Fig. 1F) that are often involved in protein-protein interactions, suggesting they might be important for interacting with TRF1. To test this possibility, we generated PinX1 mutants replacing four positively charged residues with Ala either individually (K292A, K294A, K295A, or R297A) or altogether (4A) (Fig. 1F) and assayed their ability to interact with TRF1. Although all PinX1 mutants containing a single Ala substitution at these four residues still bound to TRF1 (Figs. 1G and 2A), all 4A mutants in various PinX1 truncation fragments completely failed to interact with TRF1 (Fig. 1F) and assayed their ability to interact with TRF1. All four wild-type residues were involved in the hydrophobic interaction with TRF1, whereas quadruple mutants (K292A, K294A, K295A, and R297A) in the hydrophilic interaction are required, although we could not completely rule out the possibility that PinX1 quadruple mutant could simply be misfolded. Taken together, these analyses indicate that the 20-amino acid sequence (residues 291–297) of PinX1 localized to telomeres, although they remained localized to nucleoli (Figs. 3C and 4A). These results indicate that the telomere colocalization is mediated by the TRF1-PinX1 interaction.

To understand whether this telomere colocalization is mediated by the TRF1-PinX1 interaction, we determined the subcellular localization of a large number of PinX1 mutants containing mutations that disrupt its hydrophilic or hydrophobic interaction with TRF1. All the PinX1 or TID mutants that bound to TRF1 localized to telomeres when co-expressed with TRF1 in cells (Fig. 2A). In contrast, all TRF1 or TID mutants that failed to bind TRF1, including PinX1L291E and PinX1L291E, were unable to localize to telomeres, although they remained localized to nucleoli (Figs. 2, A and D, 3, C and E). These results indicate that telomere localization of PinX1 is mediated by its direct interaction with TRF1.
Leu-291 in PinX1 Is Not Essential for PinX1 to Interact with TERT and Inhibit Telomerase Activity—To further determine the importance of the TRF1-PinX1 interaction and, specifically, the recruitment of PinX1 to telomeres for PinX1 and TID to inhibit telomere elongation, we required TRF1 binding mutants of PinX1 and TID that are fully active in inhibiting telomerase activity. Therefore, we examined whether the TRF1 binding mutations in PinX1 or TID affected their ability to bind TERT or inhibit telomerase activity. Although the quadruple Ala substitution of the charged residues (4A mutation) reduced the ability of PinX1 and TID to bind to TERT and to inhibit telomerase activity (data not shown), the L291E point mutation had no effect on the ability of PinX1 or TID to bind or inhibit telomerase (Fig. 4). Compared with PinX1, the C-terminal TID had reduced binding to TERT (Fig. 4A), likely due to the fact that the N-terminal domain of PinX1 also contributes to TERT binding, as shown before (93). More importantly, PinX1L291E and TIDL291E bound to TERT at similar levels as their wild-type counterparts (Fig. 4, B–E). Furthermore, PinX1L291E and TIDL291E inhibited telomerase activity as potently as, if not more than, their wild-type counterparts when detected using the TRAP assay in vitro (Fig. 4, B–E). These results indicate that the failure of PinX1L291E and TIDL291E to localize to telomeres is due to their inability to interact with TRF1. Hence, the TRF1-PinX1 interaction is required for PinX1 to localize to telomeres.

The TRF1-PinX1 Interaction Is Required for PinX1 or TID to Efficiently Inhibit Telomere Elongation in Human Cells—The above results indicate that the L291E mutation completely disrupts the ability of PinX1 or TID to interact with TERT and that TRF1 binding is important for PinX1 and TID to inhibit telomerase activity.

FIGURE 4. The L291E point mutation in PinX1 does not affect the ability of PinX1 or its TID to interact TERT and inhibit telomerase activity. A, the L291E mutation does not affect the ability of PinX1 or TID to interact with human TERT. Glutathione beads bound to control GST, GST-PinX1, -PinX1L291E, -TID (D) or -TIDL291E were incubated with 35S-hTERT synthesized by in vitro transcription and translation. After washing, bound 35S-hTERT was separated on SDS-containing gels followed by autoradiography. B–E, the L291E mutation does not affect the ability of PinX1 to inhibit telomerase activity. Different concentrations of GST or GST-PinX1 (B), -PinX1L291E (C), -TID (D), or -TIDL291E (E) were incubated with telomerase extracts for 10 min before the TRAP assay. Buffer alone or boiled lysates (B. Lys) were used in some assays as controls. Arrows point to the internal control (IC) for PCR amplification.
PinX1 but does not affect the ability of PinX1 or TID to interact with TERT and to inhibit telomerase activity. This allows us to specifically study the role of PinX1 recruitment to telomeres and understand the spatial significance of the protein while retaining its telomerase inhibitory function.

Therefore, we stably transfected telomerase-positive human HT1080 cells with the construct expressing HA-PinX1 or the L291E mutants and confirmed protein expression using immunoblotting with anti-HA antibody (Fig. 5A). These stable cells were continuously passaged in culture and periodically collected to assay telomere restriction fragments lengths at various population doublings (PDs) (55, 71, 93). As shown previously (93), overexpression of PinX1 led to gradual and progressive telomere shortening, which was detected by increased mobility of telomere restriction fragments and a decrease in the intensity of the hybridization signal in multiple independent experiments (Fig. 5B, data not shown). In contrast, there was no obvious change in the mobility of telomere restriction fragments or in the intensity of the hybridization signal in cells stably overexpressing the L291E mutant at various PDs (Fig. 5B, data not shown). This difference was not due to DNA loading as shown by staining gels with ethidium bromide before hybridization (Fig. 5C). These results indicate that the L291E mutation abolishes the ability of PinX1 to induce telomere shortening.

To further confirm these findings, we also generated stable human cells expressing HA-TID or the HA-TIDL291E mutants (Fig. 6A) because the TID induces telomere shortening more potently than the full-length protein and abruptly causes cellular senescence by PD 16 (93). Indeed, stable overexpression of TID was more potent than the full-length protein in inducing telomere shortening (Figs. 6B and 5B). However TIDL291E completely failed to induce obvious telomere shortening in multiple independent experiments (Fig. 6B, data not shown), which was not due to DNA loading (Fig. 6C). Furthermore, as shown previously (93), TID-overexpressing cells experienced growth arrest and exhibited increased cell size, flattened cell morphology, and positive staining for the senescence-associated β-galactosidase and could not grow beyond PD 16 (Fig. 6E). In sharp contrast, this phenotype was not observed in vector or TIDL291E cell lines, which could be continually passaged to PD 32 and which did not exhibit any growth impairment or senescence (Fig. 6, D and F). Taken together, these results indicate that although the L291E point mutation does not affect the ability of PinX1 or TID to inhibit telomerase activity, it completely abolishes the ability of PinX1 or TID to interact with TRF1, to localize to telomeres, and to induce telomere shortening and cell senescence.

**TRF1 Knockdown Completely Abolishes the Ability of PinX1 to Localize to Telomeres and Inhibit Telomere Elongation in Human Cells**—The above structure-function analyses suggest that TRF1 might be essential for recruiting PinX1 to telomeres to negatively regulate telomere elongation. To examine this possibility further, we knocked down endogenous TRF1 in cells using RNAi and then determined its effects on the ability of PinX1 to localize to telomeres and to induce telomere shortening. Localization of PinX1 in a TRF1 knockdown setting was accomplished by stably infecting HT1080 cells with TRF1-specific RNAi or control retroviruses followed by examining the subcellular localization of PinX1 in the presence or absence of re-expressed TRF1. In contrast to
control cells, TRF1 protein was not detectable in TRF1 RNAi-stable cells (Fig. 7A). These TRF1-depleted cells were then co-transfected with GFP-PinX1 and either RFP-TRF1 or the RFP-TRF1-(1–336) mutant that is able to bind PinX1 (Fig. 1, A and B) but unable to localize to telomeres due to the lack of a DNA binding Myb domain (55, 87, 89, 90, 103). If telomeric localization of PinX1 is dependent on TRF1 recruitment, we would expect the mutant RFP-TRF1-(1–336) to be unable to guide GFP-PinX1 to telomeres. As expected, GFP-PinX1 failed to localize to telomeres in TRF1-depleted cells that did not express RFP-TRF1 and instead remained in nucleoli (Fig. 7B, white arrows). More importantly, however, telomere localization of GFP-PinX1 was re-established in TRF1-depleted cells upon re-expression of RFP-TRF1 (Fig. 7B, yellow arrows) but not its mutant TRF1-(1–336) (Fig. 7C). Together with the findings that disrupting the TRF1-PinX1 interaction abolishes PinX1 localization to telomeres (Fig. 2A), these results further emphasize that the presence of TRF1 is necessary for targeting PinX1 to telomeres.

To determine whether TRF1 knockdown affects the ability of PinX1 to inhibit telomere elongation, HT1080 cells stably expressing PinX1 or the vector control, which were previously established (93), were infected with lentiviruses expressing a different TRF1 RNAi sequence or control vector followed by...
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TRF1 knockdown has no effect on the ability of PinX1 to inhibit telomerase activity but abolishes the ability of PinX1 to localize to telomeres and to inhibit telomere elongation (Fig. 8), phenotypes that are almost the same as those induced by disrupting the TRF1-PinX1 interaction by the L291E PinX1 mutation (Figs. 5 and 6). Taken together, this study has not only identified residues important for PinX1 and TRF1 interactions but also uncovered the essential role of this interaction for recruiting PinX1 to telomeres and to inhibit telomere elongation in vivo.

DISCUSSION

Although PinX1 was originally identified as a TRF1-interacting protein, nothing is known about the biological function of this interaction. In this report we first performed extensive structure-function analysis to define the PinX1-TRF1 interaction and then used this information to dissect the biological function of the PinX1-TRF1 interaction in regulating telomere maintenance. We found that the TRFH domain of TRF1 specifically recognizes a 20-amino acid sequence (residues 291–310) of PinX1 by both hydrophobic as well as hydrophilic interactions (Fig. 1). Moreover, a single PinX1 point mutation in the hydrophobic interaction or the quadruple mutations in the hydrophilic interaction completely disrupts the ability of PinX1 or its TID to bind to TRF1 and to localize to telomeres in cells (Figs. 2 and 3). This indicates that the TRF1-PinX1 interaction is essential for recruiting PinX1 to telomeres. More importantly, the L291E point mutation in PinX1 completely abolishes its ability to interact with TRF1 (Figs. 2C and 3, A and B) but does not affect its telomerase inhibitory activity (Fig. 4). This allowed us to demonstrate that disrupting the TRF1-PinX1 interaction alone is sufficient to severely impair the ability of PinX1 or its TID to inhibit telomere elongation in human cells (Figs. 5 and 6). These structure-function analyses demonstrate that the TRF1-PinX1 interaction is required not only for targeting PinX1 to telomeres but also for PinX1 to prevent telomere elongation in cells.

To confirm these findings, we knocked down endogenous TRF1 in cells using RNAi to determine its impact on the ability of PinX1 to localize to telomeres and to inhibit telomere elongation. Indeed, TRF1 knockdown does not affect the ability of PinX1 to inhibit telomerase activity but completely abolishes the ability of PinX1 to localize to telomeres and to inhibit telomere elongation (Figs. 7 and 8), phenotypes that are similar to those induced by disrupting the TRF1-PinX1 interaction by the L291E mutation (Figs. 5 and 6). These functional studies demonstrate for the first time the essential role of the TRF1-PinX1 interaction for recruiting PinX1 to telomeres and to inhibit telomere elongation in cells.

Given the unique and potent ability of PinX1 to inhibit telomerase activity and to induce telomere elongation in cells (93, 95), our results suggest that the TRF1-PinX1 interaction affects the loading of PinX1 onto telomeres to prevent telomere elongation. This may provide an additional level of telomerase regulation in conjunction to the physical occlusion from telomeres by Pot1 (73, 79) by supplying a link between TRF1 and telomerase inhibition that contributes toward
maintaining telomere homeostasis. TRF1 binds along the duplex part of the telomere and functions as a measuring device to assess telomere length (5, 55, 69, 102). For telomere length homeostasis to be effective, the information about the length of the telomere needs to be relayed from TRF1 to telomerase via other proteins as TRF1 does not directly affect telomerase activity (5, 55). Our data indicate that the telomerase inhibitor PinX1 might be recruited by TRF1 to a telomere to stop telomerase action as the telomere is being elongated and reaches a certain threshold (Fig. 9). The two unique features of PinX1 that allow it to relay information about telomere length to telomerase are that it is a potent telomerase catalytic inhibitor and that its accumulation on telomeres depends on its specific interaction with TRF1. In this model (Fig. 9), when telomerase extends a telomere to a certain length, the elongated telomere binds more TRF1, which might in turn recruit more PinX1 to the telomere. Once concentrated locally on telomeres at a high concentration, which might be important because endogenous levels of TERT and PinX1 are rather low,5 simple mass-action might allow PinX1 to more effectively stop telomerase from adding more repeats. Conversely, when a telomere is shortened after each cell division due to the end replication problem (104–106), the shortened telomere contains less TRF1, which might recruit fewer PinX1 or none at all to the telomere. Therefore, the telomere might have a greater chance of being elongated

5 X. Z. Zhou, unpublished data.
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A telomere shortens due to DNA replication
Fewer TRF1 and PinX1 bind to the telomere

FIGURE 9. A model for PinX1 as a link between TRF1 and telomerase inhibition. As telomeres are elongated by telomerase, increased TRF1 molecules bind, recruiting more PinX1 for telomerase inhibition. Once telomerase activity is inhibited, telomeres begin to shorten with each round of DNA replication, resulting in fewer TRF1 binding sites, less recruitment of PinX1 to inhibit telomerase, and therefore, re-entry of telomerase. This leads to telomere elongation and the start of another round of this cycle.

due to telomerase being inhibited less or even not at all. This negative feedback mechanism might help maintain telomeres at a similar median length. Consistent with this model are the current findings that either disrupting the TRF1-PinX1 interaction by mutating the critical Leu-291 residue in PinX1 or knocking down endogenous TRF1 abolishes the ability of PinX1 to localize to telomeres and to inhibit telomere elongation in cells even though neither effects its ability to bind and inhibit telomerase activity. This model can also explain the previous findings that depleting either endogenous PinX1 (93) or endogenous TRF1 (80) or preventing TRF1 from binding to telomeres (55, 69, 70, 72) can all lead to telomere elongation. In contrast, overexpression of PinX1 (93) or TRF1 (55, 80) results in telomere erosion. Moreover, all these effects of TRF1 or PinX1 depend on the presence of telomerase activity in cells (55, 93). Because many other TRF1-interacting proteins have been shown to regulate telomere length (5, 66, 69–85, 93), further experiments are needed to determine how these different TRF1-interacting proteins are coordinated to regulate telomere homeostasis. Nevertheless, our results demonstrate that TRF1 recruits the telomerase inhibitor PinX1 to telomeres to prevent telomere elongation, which may help maintain telomere homeostasis by providing an important link between TRF1 and telomerase inhibition.

In summary, we have shown that TRF1 recognizes PinX1 through both hydrophobic as well as hydrophilic interactions and that this interaction is required for recruiting PinX1 to telomeres and also for PinX1 to prevent telomere elongation, as demonstrated either by mutating the critical Leu-291 residue in PinX1 to disrupt the TRF1-PinX1 interaction or by using RNA interference to knock down endogenous TRF1. These results indicate that the TRF1-mediated recruitment of PinX1 to telomeres helps maintain telomere homeostasis by providing a critical link between TRF1 and telomerase inhibition. Future studies aimed at understanding the structural details of how PinX1 inhibits telomerase action at telomeres, the relationship between PinX1 and other TRF1-interacting proteins, and the importance of PinX1-mediated telomerase regulation in pathological conditions such as cancer should help elucidate the molecular mechanisms of telomere maintenance and the pathological significance of telomerase deregulation.

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