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The Pin2/TRF1-Interacting Protein PinX1 Is a Potent Telomerase Inhibitor

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Summary

Telomerase activity is critical for normal and transformed human cells to escape from crisis and is implicated in oncogenesis. Here we describe a novel Pin2/TRF1 binding protein, PinX1 that inhibits telomerase activity and affects tumorigenicity. PinX1 and its small TID domain bind the telomerase catalytic subunit hTERT and potently inhibit its activity. Overexpression of PinX1 or its TID domain inhibits telomerase activity, shortens telomeres, and induces crisis, whereas depletion of endogenous PinX1 increases telomerase activity and elongates telomeres. Depletion of PinX1 also increases tumorigenicity in nude mice, consistent with its chromosome localization at 8p23, a region with frequent loss of heterozygosity in a number of human cancers. Thus, PinX1 is a potent telomerase inhibitor and a putative tumor suppressor.

Introduction

Telomerase plays a crucial role in maintaining telomere length and is implicated in aging and cancer (reviewed by Artandi and DePinho, 2000; McEachern et al., 2000; Shay and Wright, 2001; Stewart and Weinberg, 2000). Telomeres consist of simple DNA repeats and associated proteins. The function of telomeres includes maintaining genomic stability and allowing cells escaping from crisis. Most normal human cells have a defined lifespan (Hayflick and Moorhead, 1961) and do not have telomerase activity (Broccoli et al., 1995; Taylor et al., 1996). Their telomeres undergo shortening following each cell division due to the "end-replication problem" (Cooke and Smith, 1986; Harley et al., 1990; Hastie et al., 1990). When telomere length reaches a critical point, senescence is believed to occur (Cooke and Smith, 1986; Harley et al., 1990; Hastie et al., 1990; Vaziri et al., 1994).

Consistent with these observations, any mechanism that restores and/or maintains telomeres is thought to be important for cellular immortalization and transformation (Artandi and DePinho, 2000; McEachern et al., 2000; Shay and Wright, 2001; Stewart and Weinberg, 2000). The telomeric DNA sequences can be replenished by telomerase, which contains the catalytic subunit hTERT and the RNA component TR (Counter et al., 1997; Greider and Blackburn, 1985, 1989; Lingner et al., 1997; Nakamura et al., 1997). Expression of hTERT prevents telomere erosion and senescence in some human cells (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Kiyono et al., 1998). Telomerase activity is readily detected in most human tumor tissues and immortalized and/or transformed cell lines (Broccoli et al., 1995; Counter et al., 1992; Hahn et al., 1999b; Kim et al., 1994; Taylor et al., 1996; Meyerson et al., 1997). Furthermore, it is critical for cells to escape from crisis and for transforming primary human cells (Bodnar et al., 1998; Girardi et al., 1965; Hahn et al., 1999a; Halvorsen et al., 1999; Vaziri and Benchimol, 1988). Conversely, inhibition of telomerase function shortens telomeres and forces transformed cells to enter crisis (Feng et al., 1995; Hahn et al., 1999b; Herbert et al., 1999; Zhang et al., 1999). Gene knockout experiments also reveals an essential role of telomerase in highly proliferative organs in mice (Blasco et al., 1997; Lee et al., 1998). Finally, hTERT transcription is regulated by oncogenic proteins such as c-Myc and E6 (Wang et al., 1998; Wu et al., 1999; Klingelhutz et al., 1996). These results indicate that telomerase activity is critical for life span of primary and transformed human cells.

Compelling evidence indicates that the ability of telomerase to elongate telomeres is regulated by other factors. In yeast a number of telomere proteins such as RAP1, Taz1, and Cdc13 influence telomere maintenance (Conrad et al., 1990; Cooper et al., 1997; Evans and Lundblad, 1999; Lustig et al., 1990). The helicase Pif1p binds telomere DNA and inhibits telomerase-mediated telomere lengthening in yeast, suggesting that Pif1 may function as a telomerase inhibitor although its effect on telomerase activity has not yet been shown (Zhou et al., 2000a). In mammals, the telomere protein Pin2/TRF1, its interacting proteins, tankyrase and Tin2, and heterogeneous nuclear ribonucleoproteins such as A1 influence telomere length (LaBranche et al., 1998; Kim et al., 1999; Smith et al., 1998; van Steensel and de Lange, 1997). However, since none of these and other telomerase- or telomere-associated proteins directly affects telomerase activity, the in vivo route for regulation of telomerase activity is unknown.

We isolated a telomere binding protein Pin2 as one of three proteins Pin1–3 that are involved in mitotic regulation (Lu et al., 1996). Pin2 is identical to TRF1 with the exception of a 20 amino acid (aa) internal deletion but is more abundant than TRF1 in cells (Shen et al., 1997); TRF1 and Pin2 are likely generated from the same gene *PIN2/TRF1* (Young et al., 1997). For clarity, we shall use Pin2 for the 20 aa deleting isoform and TRF1 for the 20 aa containing isoform (Chong et al., 1995; Lu et al., 1996), but refer to endogenous proteins as Pin2/TRF1. We have shown that Pin2/TRF1 affects mitotic regulation and its levels are altered in human breast tumor samples (Kishi et al., 2001a). Furthermore, Pin2/TRF1 is an ATM kinase substrate important for maintaining the mitotic checkpoint in response to DNA damage and is involved in the mitotic spindle checkpoint (Kishi et al., 2001b; Nakamura et al., 2001). These results indicate that Pin2/TRF1 is involved in mitotic regulation and telomere mainte-

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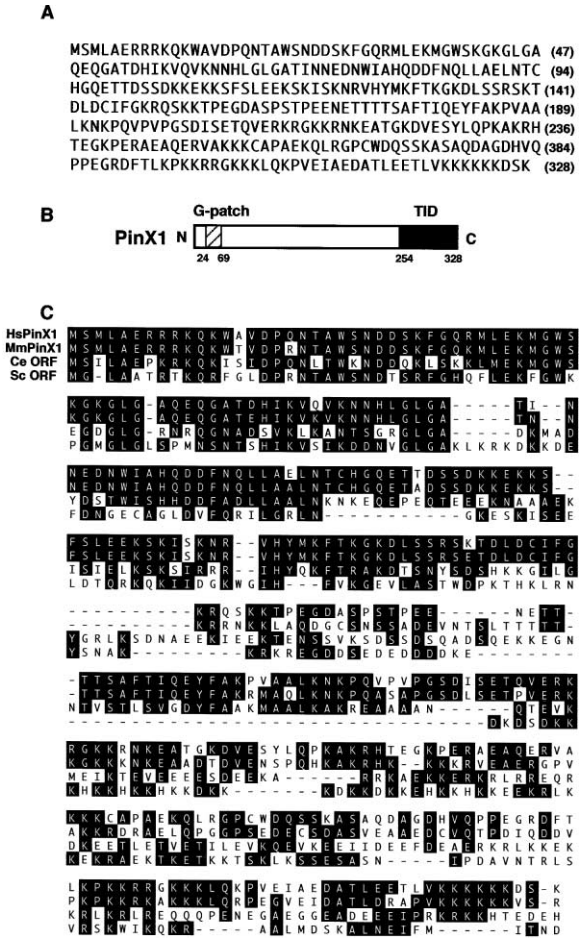


Figure 1. Identification of Human and Mouse *PINX1*Genes

(A and B) Amino acid sequence and domain structure of human PinX1. Human PinX1 contains an N-terminal a Gly-rich region and a C-terminal TID domain (telomerase inhibitory domain).

(C) Sequence alignment of PinX1s from human (Hs) and mouse (Mm) and ORFs present in *Caenorhabditis elegans* (Ce) and *Saccharomyces cerevisiae* (Sc). Black boxes with white letters highlight identical or conserved residues.

nance. To further elucidate Pin2/TRF1 function, we employed a two-hybrid screen and identified six known and four unknown genes (PinX1-4), whose products interact with Pin2. Our results demonstrate that PinX1 is a potent telomerase inhibitor and a putative tumor suppressor.

Results

Identification of *PINX1*

To identify Pin2/TRF1-interacting proteins, we screened a human HeLa cell yeast two-hybrid cDNA library using Pin2 as bait (Lu et al., 1996). Out of 10^8 clones screened, we obtained 274 strongly positive clones and identified six known genes and four unknown genes (PinX1-4). The known genes included Pin2 (2 clones), nm23-H1 (11), and Tin2 (5). The fact that Pin2/TRF1 forms dimers (Bianchi et al., 1997; Shen et al., 1997) and interacts with Tin2 and nm23-H1 (Kim et al., 1999; Nosaka et al., 1998) validates our screen. We here describe PinX1.

Out of three *PINX1* cDNA clones, the longest one encodes a novel 328 aa protein (Figure 1A), which contains no known domain structure except an N-terminal Gly-rich patch (Figure 1B) (Aravind and Koonin, 1999). The mouse *PINX1* cDNA cloned by PCR encoded a protein ~74% identical to the human protein (Figure 1C). A GenBank database search revealed the presence of PinX1 ORFs in other eukaryotic cells, including yeast and *C. elegans*, which have an overall ~50% similarity to the human protein (Figure 1C), suggesting a conservation of PinX1 proteins.

To verify that the *PINX1* cDNA encodes a full-length protein, we first performed Northern analysis and a single 1.9 kb *PINX1* mRNA transcript was detected in all 16 human adult tissues examined (Supplemental Figure S1A at http://www.cell.com/cgi/content/full/107/3/347/DC1). We next raised anti-PinX1 antibodies, which recognized a single 45 kDa protein in HeLa cell extracts both in immunoprecipitation and immunoblot analyses (Supplemental Figure S1B; Figures 4A and 4B). This 45 kDa protein was shown to be PinX1 by expression of HA-tagged PinX1 (~50 kDa), followed by immunoblotting analysis with anti-PinX1 or anti-HA antibodies (Supplemental Figures S1C and S1D; Figure 4A). These results indicate that *PINX1* encodes a 45 kDa protein in cells.

PinX1 Interacts with Pin2 In Vivo and In Vitro

To confirm the PinX1 and Pin2 interaction, we first performed coimmunoprecipitation and colocalization experiments. When cells were cotransfected with PinX1 and Pin2 expression constructs and subjected to immunoprecipitation, Pin2 was detected in anti-PinX1 immunoprecipitates, but not in the preimmune control (Figure 2A), indicating that PinX1 interacts with Pin2/TRF1 in cells. When cells were cotransfected with GFP-PinX1 and RFP-Pin2 and their subcellular localization examined directly, PinX1 colocalized with Pin2 in the nucleus, especially in nucleoli and at telomere speckles (Figure 2B) (Chong et al., 1995; Kishi et al., 2001b; Shen et al., 1997). Thus, PinX1 and Pin2/TRF1 coimmunoprecipitate and colocalize in cells.

We further determined their in vitro interaction using GST pulldown experiments (Lu et al., 1999b; Shen et al., 1998). GST-Pin2, but not GST, precipitated HA-PinX1 from cell extracts (Figure 2C). Conversely, PinX1, but not GST, precipitated in vitro synthesized [^{35}S]-Pin2 (Figure 2D). These results indicate that PinX1 interacts with Pin2 in vitro. To map the region in PinX1 that interacts with Pin2, we expressed different PinX1 fragments in HeLa cells as GFP fusion proteins (Figure 2E), and subjected them to GST pulldown experiments. GST-Pin2 bound PinX1, its C-terminal 142–328 and 254–328 fragments, but not its N-terminal 142 fragment (Figure 2F), indicating that Pin2 binds the C-terminal 74 aa of PinX1. We referred the N-terminal 142 and C-terminal 74 aa fragments of PinX1 as PinX1-N and PinX1-C, respectively (Figure 2E).

Overexpression of PinX1 or PinX1-C in HT1080 Cells Induces Crisis

Pin2/TRF1, tankyrase, and Tin2 regulate telomere length in telomerase-positive fibrosarcoma cell line HT1080 (Kim et al., 1999; Smith et al., 1998; van Steensel and de Lange, 1997). To examine whether PinX1 affects telomere maintenance, we tried to establish HT1080 cell lines stably expressing PinX1, PinX1-N, or -C. To deplete

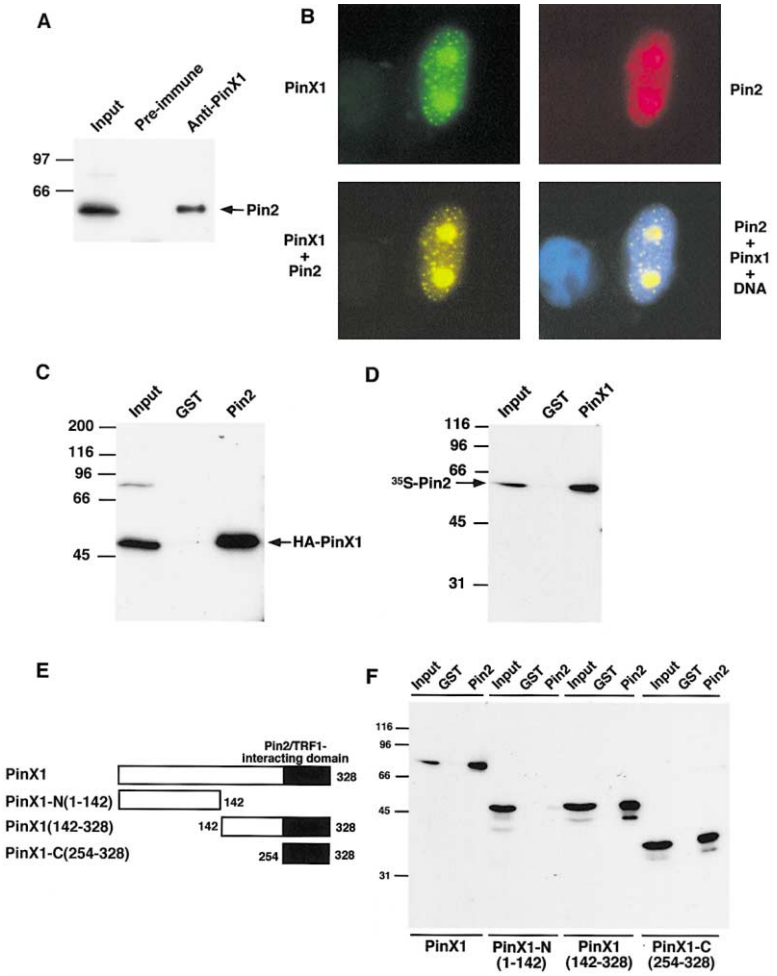


Figure 2. Interaction between PinX1 and Pin2/TRF1 In Vivo and In Vitro

(A) Coimmunoprecipitation of PinX1 and Pin2/TRF1. HeLa cells were cotransfected with PinX1 and Pin2 expression constructs and then subjected to immunoprecipitation with anti-PinX1 or preimmune sera, followed by immunoblotting with anti-Pin2 antibodies.

(B) Colocalization of PinX1 with Pin2/TRF1 in cells. HeLa cells were cotransfected with expression constructs of GFP-PinX1 and RFP-Pin2 and subjected to fluorescence microscopy.

(C and D) Interaction of PinX1 with Pin2/TRF1 in vitro. GST or GST-Pin2 beads were incubated with cell extracts containing HA-PinX1 (C) or GST or GST-PinX1 beads with synthesized ^{35}S-Pin2 (D), following by detecting bound HA-PinX1 by immunoblot or ^{35}S-Pin2 by autoradiography.

(E and F) Pin2/TRF1-interacting domain in PinX1. HeLa cells transfected with various GFP-PinX1 mutants (E) were subjected to immunoblotting analysis with anti-GFP antibodies directly (Input) or first precipitated by GST or GST-Pin2 beads (F).

endogenous protein, we expressed the *PINX1* cDNA in an antisense orientation (PinX1^{AS}). Although several clones expressing PinX1-N were obtained initially, they all lost expression during expansion. However, multiple cell lines were established that stably expressed PinX1, PinX1-C, PinX1^{AS}, or the vector (Figures 3A and 3B). Importantly, levels of endogenous PinX1 protein were decreased ~3 fold in PinX1^{AS}-expressing clones (Figure 3B). Therefore, we can manipulate PinX1 levels in these stable HT1080 clones.

Cells expressing PinX1^{AS} and the control vector grew normally with no detectable difference in growth rate or morphology over 60 population doublings (PD) (Figures 3C, 3D, and 4). Initially, all PinX1 or PinX1-C stable cell lines grew at the same rates as those of control vector cells (Figures 3C and 3D). However, after 20–30 PD, the growth of PinX1-expressing cells slowed down, as indicated by cell number, BrdU incorporation and cell cycle analysis (Figures 3C, inset, 4A, and 4B). Notably, on the third day after subculture when control cells reached confluency, PinX1-expressing cells were only ~62% of the control (Figure 3C, inset). Interestingly, a fraction of cells exhibited increased size and a flattened morphology and also stained positively for the senescence-associated β-galactosidase (SA-β-Gal) (Figure 4D) (Dimri et al., 1995). However, other PinX1-expressing cells were able to continue to divide and reach the confluency on the fourth day (Figure 3C, inset), which explains no obvious effect on the overall growth rate (Figures 3C and 3D). These results indicate that PinX1 overexpression induces a fraction of cells into crisis.

The most striking phenotype was observed in PinX1-C-expressing cells. All three PinX1-C-expressing cell lines tested underwent crisis characterized by an overall reduction in growth rate (Figures 3C and 3D). Incorporation of BrdU was significantly reduced and cells with the G2/M DNA content increased (Figures 4A and 4B). In some instances, reduced growth appeared to be due to an increased rate of cell death. Cells in this death phase were rounded and loosely attached to culture flasks, ~40% of which contained a sub-G1 DNA content (Figure 4C), a characteristic of apoptosis. However, in most cases, the cells exhibited increased size and a flattened morphology with elongated cellular processes and they were stained positive for the SA-β-Gal (Figure 4D). These results suggest that overexpression of PinX1-C forces transformed cells into crisis.

PinX1 Partially and PinX1-C Completely Inhibit Telomerase Activity in Cells

Inhibition of telomerase forces transformed cells into crisis and telomerase activity is sufficient for transformed cells to escape from crisis (Feng et al., 1995; Hahn et al., 1999b; Halvorsen et al., 1999; Herbert et al., 1999; Zhang et al., 1999). Our findings that expression of Pinx1 or PinX1-C induces crisis suggest that PinX1-C and

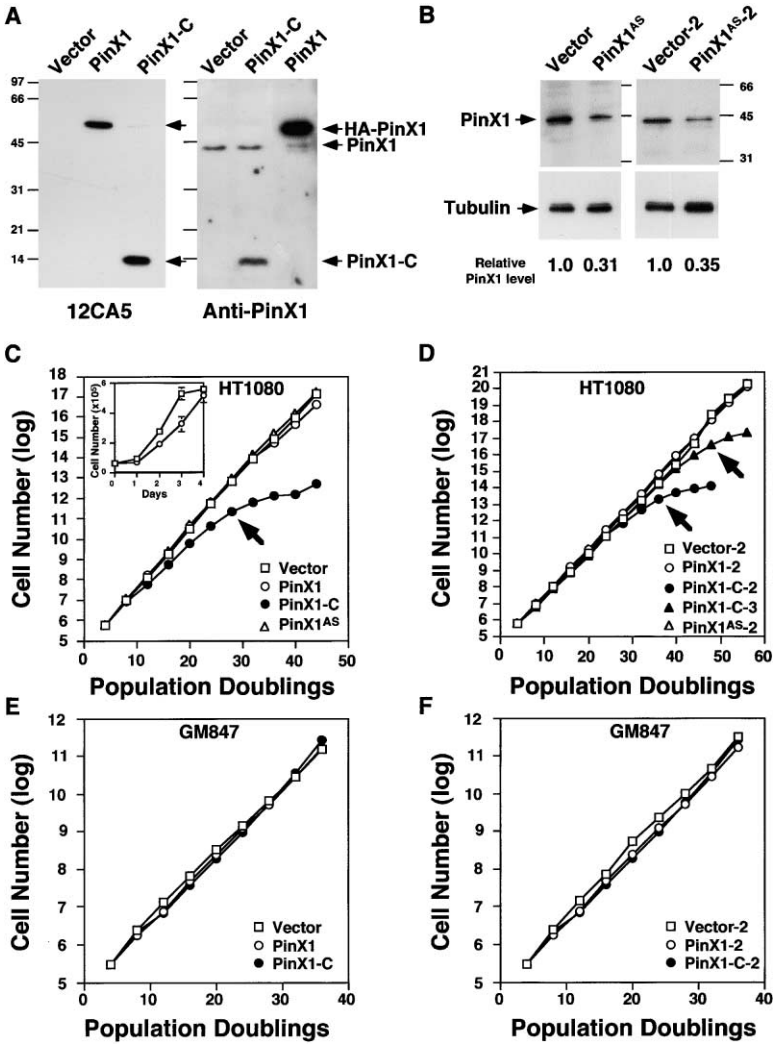


Figure 3. Growth Curves of Stable Cell Lines Expressing PinX1, PinX1-C, or PinX1^{AS}

(A and B) Establishment of stable HT1080 cell lines expressing PinX1 or PinX1-C or PinX1^{AS}. HT1080 cells were stably transfected with the control vector (vector) or a vector expressing HA-PinX1 or HA-PinX1-C (A) or PinX1^{AS} (B), followed by detecting protein expression using immunoblot.

(C–F) Growth curves of stable lines. The stable HT1080 (C and D) or GM847 (E and F) cell lines were maintained continuously in culture, splitting on every fourth day and seeding at the same number of cells at each subculture. Arrows point to PinX1-C-expressing cells that entered crisis. (Inset in C) The cell number was counted every day after subculture to show the slower growth rate of PinX1-expressing cells at 28 PD.

PinX1 might affect telomerase activity. To examine this possibility, we compared telomerase activity in stable HT1080 cell lines.

Telomerase activity, as assayed by the standard TRAP assay, was readily detected in vector HT1080 cell extracts, but not in the extracts that were heat inactivated or pretreated with RNase (Figure 5A, data not shown) (Kim et al., 1999; van Steensel and de Lange, 1997). Importantly, telomerase activity in PinX1-stable cells was significantly reduced (Figure 5), indicating an inhibition of telomerase activity. Most strikingly, telomerase activity was almost not detectable in cells expressing PinX1-C (Figure 5). Similar inhibitions on telomerase activity were also observed in other independent cell lines examined (data not shown). These results demonstrate that PinX1 partially and PinX1-C almost completely inhibit telomerase activity in cells. This difference in inhibiting cellular telomerase activity correlates with their ability to induce crisis (Figures 3 and 4).

Depletion of Endogenous PinX1 Increases Telomerase Activity In Vivo

The above results demonstrate that PinX1 inhibits telomerase activity in vivo, suggesting that PinX1 may be a negative regulator of telomerase. If so, depletion of endogenous PinX1 would result in an increase in cellular telomerase activity. To test this possibility, we assayed telomerase activity in HT1080 cell lines that stably expressed PinX1^{AS} (Figure 3B). Telomerase activity in several PinX1^{AS} stable cell lines was significantly higher than that in the vector-transfected cells (Figure 5). These results indicate that depletion of endogenous PinX1 results in an increase in telomerase activity in cells, further supporting that overexpression of PinX1 inhibits telomerase activity.

Manipulation of Cellular PinX1 Function Influences Telomere Length in HT1080 Cells

To confirm that PinX1 regulates cellular telomerase activity, we measured telomere restriction fragment (TRF) length in stable HT1080 cells lines. Vector control cells maintained relatively short telomeres, with average TRF length being ~4.0 kb (Figures 6A and 6C) (Kim et al., 1999; van Steensel and de Lange, 1997). Importantly, the same TRF length was also detected in cells stably expressing PinX1 at early PDs, but TRF length was slowly and progressively shortened at the late PD (>20–24 PD) (Figures 6A and 6C). The loss of telomeric

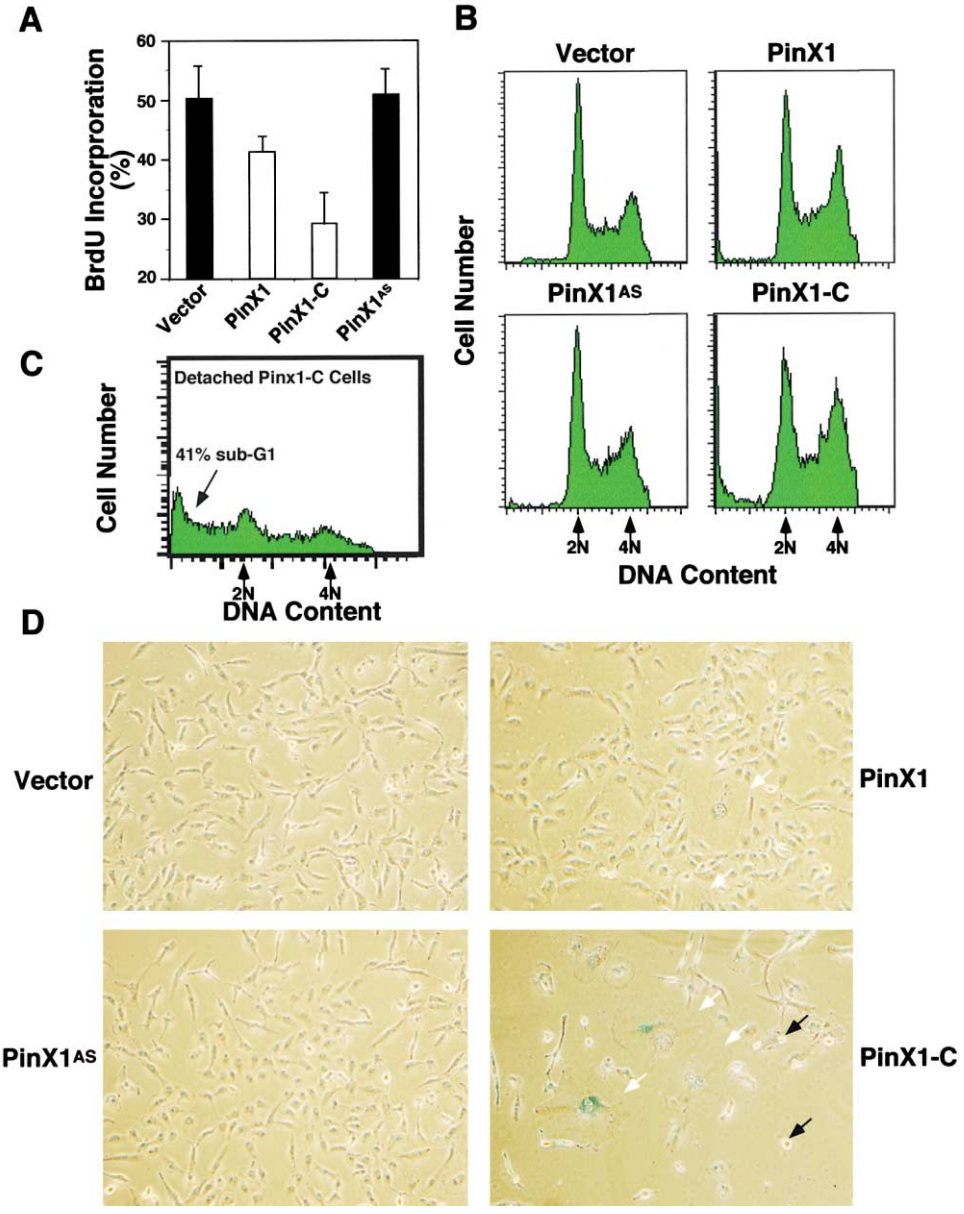


Figure 4. Overexpression of PinX1 or PinX1-C Induces Crisis

(A) Reduced DNA synthesis. Stable cell lines at 28 PD (Figure 3C) were labeled with BrdU, followed by determining BrdU incorporation using flow cytometry.

(B and C) Cell cycle arrest and apoptosis. Attached cells at PD28 were harvested and stained with propidium iodide, followed by flow cytometry (B). A fraction of PinX1-C expressing stable cells were rounded, which were collected and their DNA content was analyzed (C).

(D) Senescence-like morphologies. Cells at 36 PD (Figure 3C) were fixed and then subjected to senescence-associated β-galactosidase (SA-β-gal) staining, followed by microscopy.

sequence was evident from the shortening of TRF length and from a reduction in the TTAGGG hybridization signal (Figures 6A and 6C). However, more rapid and pronounced telomere shortening was observed in cells stably expressing PinX1-C, even occurring at 4 PD (Figures 6B and 6C). This is expected because 4 PD cells would have passaged for ~24 PD given that 0 PD was defined when single cell clones had expanded to the number sufficient for characterization. In these PinX1-C-expressing cells, telomeres continued to shorten, with TRF length reaching the minimal length (~2.5 kb) at about 20 PD (Figures 6B, and 6C). Similar results were also obtained in other independent cell lines (Supplemental Figure S2 at http://www.cell.com/cgi/content/full/107/3/387/DC1). Significantly, telomere shortening is correlated with the crisis phenotype (Figures 3C, 3D, 6A–6C, and Supplemental Figure S2). In contrast, telomeres were significantly elongated and more uniform in the length in cells expressing PinX1^{AS}, with an increase in TRF length to ~7 kb and in the hybridization signal at 4 PD (actually ~24 PD) (Figures 6B and 6C, and Supplemental Figure S2). These results indicate that whereas

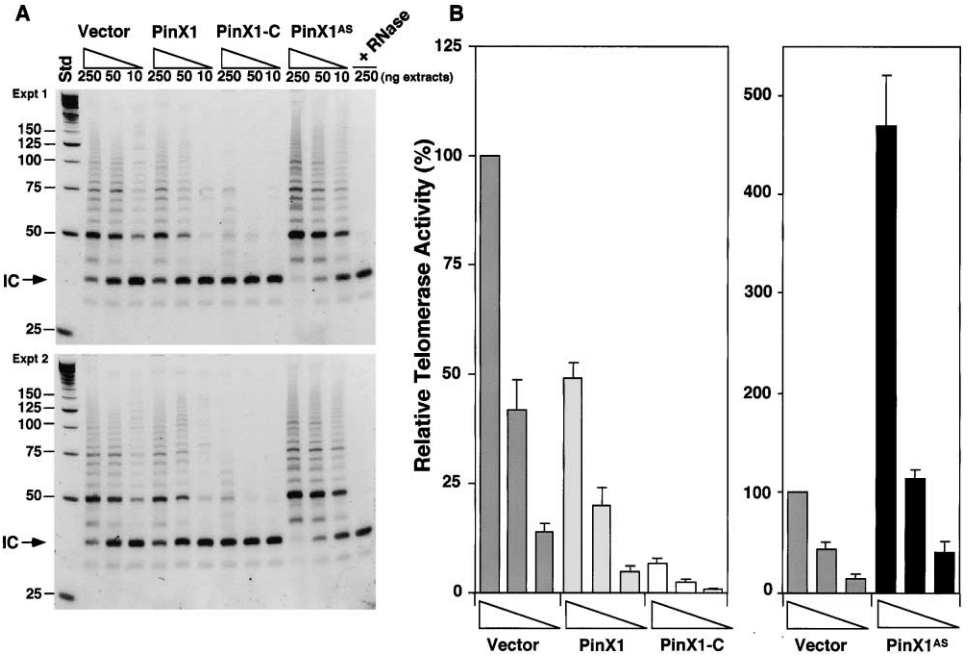


Figure 5. Overexpression of PinX1 and PinX1-C Inhibits, Whereas Depletion Increases, Telomerase Activity

Stable HT1080 cell lines were harvested at 4 PD and telomerase-containing fractions prepared, followed by subjecting different amounts of proteins to the TRAP assay. Telomerase products were stained with SYBR green (A) and semiquantified, with the average and standard deviation from four experiments being present in (B). RNase was included in one assay. Arrows point to the 36 bp internal control (IC) for PCR amplification.

depletion of endogenous PinX1 elongates telomeres, overexpression of PinX1 and PinX1-C shortens telomeres, with PinX1-C being much more potent than full-length protein.

PinX1 and PinX1-C Do Not Induce Telomere Shortening or Crisis in Telomerase-Negative Cells

To examine whether the effects of PinX1 on telomeres and cell growth depend on telomerase activity, we stably expressed PinX1 and PinX1-C in telomerase-negative GM847 cells, which maintain very long but heterogeneous telomeres by an alternative (ALT) mechanism (Bryan et al., 1995). TRF length in these cells is not affected by dominant-negative telomerase (Hahn et al., 1999b), but rapidly shortened by fusion with other cell types, which is detected as soon as single cells expand to the number sufficient for measuring TRF length ($\sim$20 PD, defined as 0 PD here) (Perrem et al., 1999). However, even after 32 PD, telomeres in PinX1- or PinX1-C-expressing GM847 cells exhibited patterns similar to those in vector control cells, the characteristic of ALT cells (Figure 6D). In addition, these cells did not enter crisis (Figures 3E and 3F). These results indicate that neither PinX1 nor PinX1-C induces telomere shortening or crisis in these telomerase-negative cells and further support that the ability of PinX1 and PinX1-C to shorten telomeres and induce crisis is via inhibiting telomerase.

PinX1 and PinX1-C Bind hTERT and Directly Inhibit Its Activity

The above results that PinX1-C almost completely inhibits and PinX1 reduces telomerase activity, whereas depletion of PinX1 increases telomerase activity in cells, suggest that PinX1 and PinX1-C function as catalytic inhibitors of telomerase. To test this possibility, we examined whether they interact with hTERT and whether they directly inhibit telomerase activity in vitro.

To examine the in vivo interaction, we first transiently transfected HT1080 cell lines stably expressing PinX1 or PinX1-C with HA-hTERT and then subjected them to immunoprecipitation. The purified anti-PinX1, but not preimmune IgG, immunoprecipitated HA-hTERT from PinX1 and PinX1-C-expressing cells (Figure 7A), indicating that PinX1 or PinX1-C forms stable complexes with hTERT in cells. We next used GST pulldown experiments to examine their in vitro interaction. GST-PinX1, but not GST, precipitated hTERT produced either in cells as an HA-tagged protein or a GFP fusion protein by transient transfection or synthesized by in vitro transcription and translation (Figures 7C and 7D, data not shown). It appeared that both N- and C-terminal domains of PinX1 precipitated hTERT (Figures 7C and 7D). These results indicate that PinX1 and its TID domain associate with hTERT both in cells and in vitro.

To examine the effects on telomerase activity, telomerase-containing cell extracts were incubated with various GST-PinX1 proteins for 10 min before the TRAP assay. Both GST-PinX1 and GST-PinX1-C potently inhibited telomerase activity in a concentration-dependent manner (Figures 7E–7H), whereas neither GST nor GST-PinX1-N had any significant effect (Figures 7E and 7H). Furthermore, His-PinX1 also potently inhibited telomerase (Figure 7H). Although at higher concentrations, PinX1 and PinX1-C could also inhibit Taq polymerase

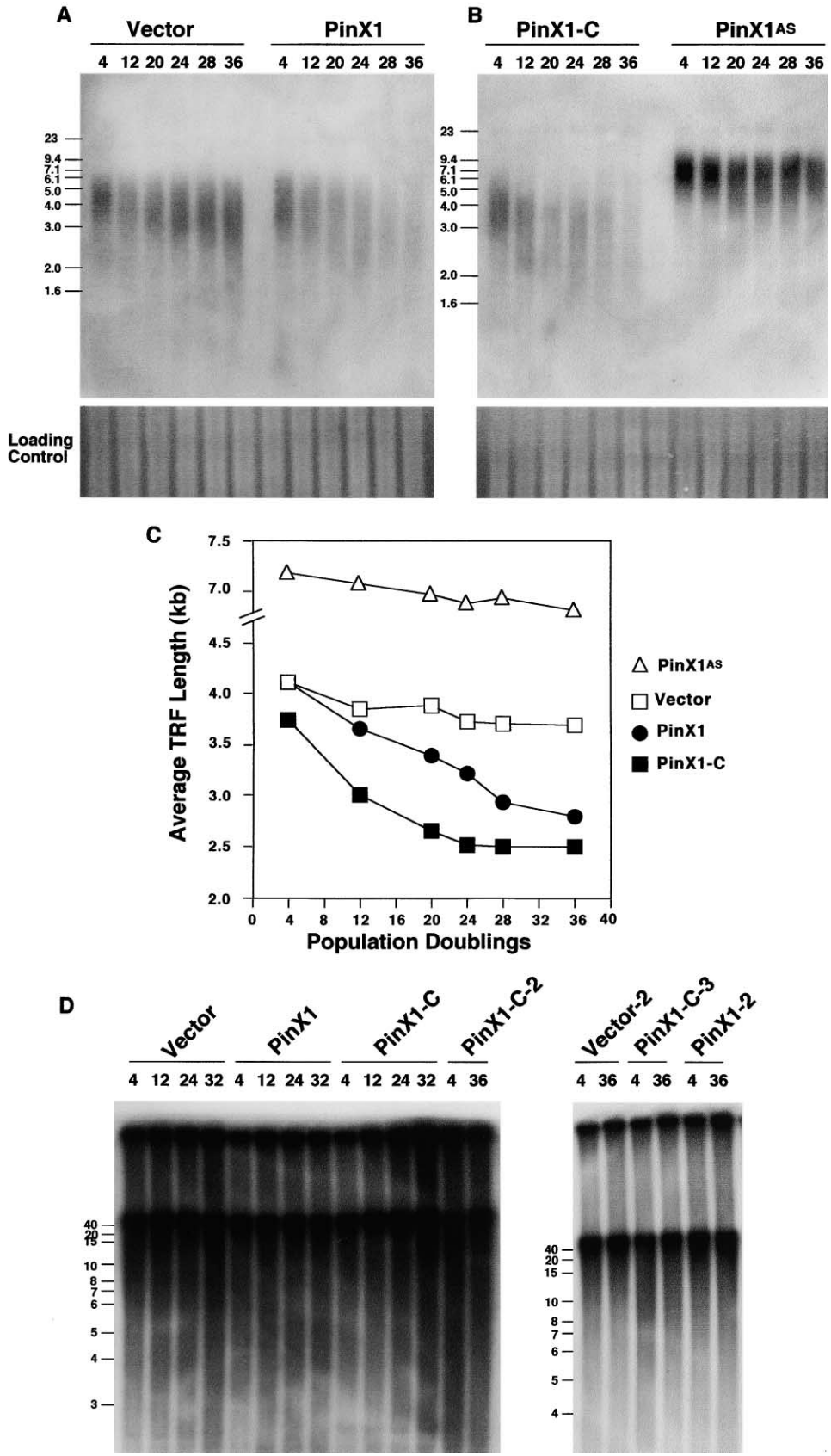


Figure 6. PinX1 Influences Telomere Length in Telomerase-Positive, but not in Telomerase-Negative Cells

Stable cell lines of HT1080 cells (A–C) or GM847 cells (D) were harvested at various PDs and genomic DNA was digested with HinfI and RsaI, followed by Southern blot analysis using a TTAGGG repeat as a probe (A, B, and D). Prior to hybridization, the gels were stained with ethidium bromide to insure equal loading of genomic DNA (lower panels). Average TRF length versus PD number in HT1080 cells was quantified using ImageQuant and presented in (C).

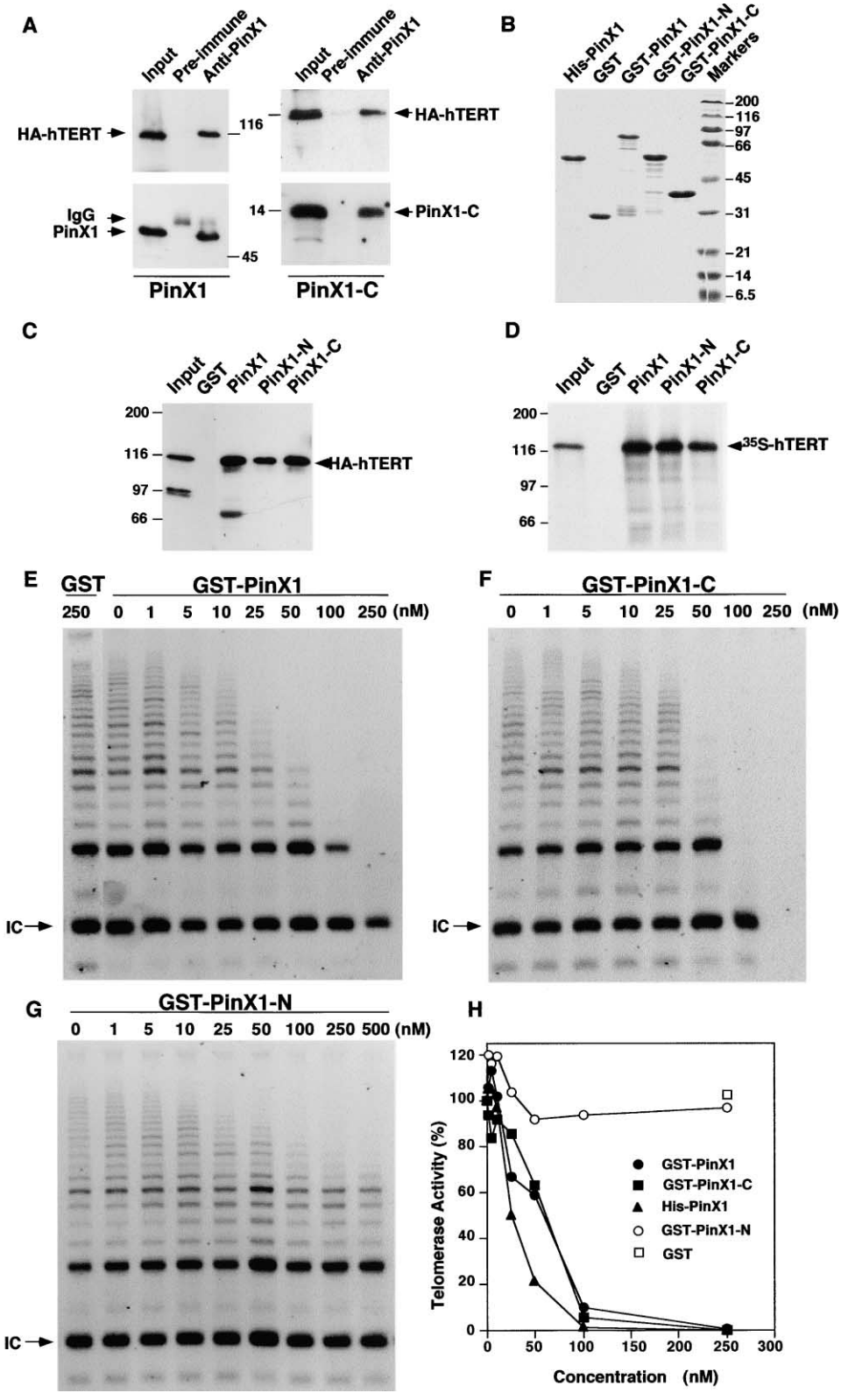
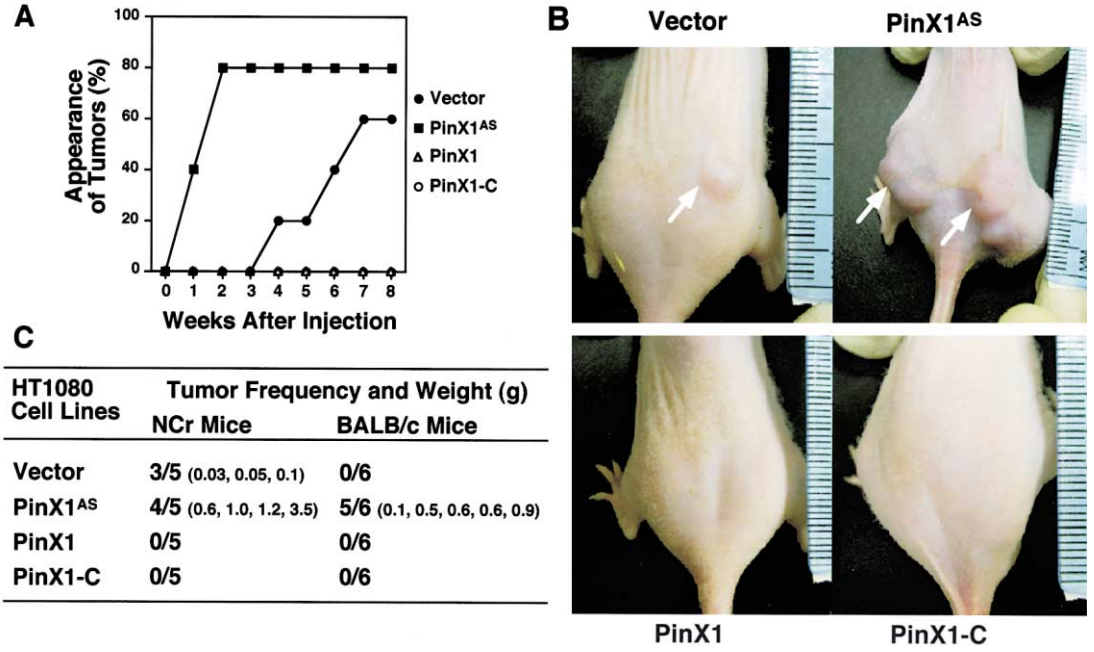


Figure 7. PinX1 and Pinx1-C Bind hTERT In Vivo and In Vitro, and Inhibit its Activity In Vitro

(A) In vivo interaction. PinX1 or PinX1-C stable HT1080 cells were transfected with HA-hTERT and immunoprecipitated with purified anti-PinX1 antibodies or preimmune, followed by immunoblot with anti-HA (Top) or -PinX1 antibodies (Bottom).

(B). Commassie-stained SDS gel containing various recombinant PinX1 proteins.

(C and D) In vitro interaction. Glutathione beads containing GST or different GST-PinX1 proteins were incubated with cell extracts containing HA-hTERT (C) or with ^{35}S-hTERT synthesized by in vitro transcription and translation (D). After wash, the bound proteins were separated on



C

HT1080 Cell Lines	Tumor Frequency and Weight (g)	
	NCr Mice	BALB/c Mice
Vector	3/5 (0.03, 0.05, 0.1)	0/6
PinX1^{AS}	4/5 (0.6, 1.0, 1.2, 3.5)	5/6 (0.1, 0.5, 0.6, 0.6, 0.9)
PinX1	0/5	0/6
PinX1-C	0/5	0/6

Figure 8. Depletion of Endogenous PinX1 Increases Tumorigenicity in Nude Mice

HT1080 cell lines stably expressing indicated PinX1 constructs or the vector were harvested at 8 PD and injected subcutaneously into NCr outbred (A–C) or BALB/c inbred (C) athymic nude mice. Mice were monitored for the visual appearance of tumors at injection sites (A and B) and tumors were harvested and weighed 2 months after injection (C).

(Figures 7E and 7F), they had no effect at all when expressed in cells (Figure 5), demonstrating the specificity of telomerase inhibition by PinX1 and PinX1-C. These results indicate that PinX1 is a potent telomerase inhibitor and the inhibitory domain is located at its C-terminal 74 aa, which is designated the telomerase inhibitory domain (TID).

PinX1 Depletion Increases Tumorigenicity in Mice

The human *PINX1* gene localizes to chromosome 8p23 based on the human genome sequence (http://www.ncbi.nlm.nih.gov/genemap99/map.cgi?CHR=8) (Deloukas et al., 1998). Loss of heterozygosity (LOH) at 8p23 occurs at a high frequency in a number of human cancers (Baffa et al., 2000; Bockmuhl et al., 2001; Emi et al., 1992; Gustafson et al., 1996; Ishwad et al., 1999; Muscheck et al., 2000; Perinchery et al., 1999; Sun et al., 1999), suggesting that PinX1 may be a potential tumor suppressor. To explore this possibility, we examined whether depletion of PinX1 function increases tumorigenicity of HT1080 cells in mice. In order to detect the increase in tumorigenicity, we injected a relatively low number of cells at 8 PD (1×10^6 cells/per site). At 2 months after injection into NCr outbred nude mice, tumors were visually identified at 60% of sites that were injected with control vector cells, although no tumor was observed at sites injected with cells expressing PinX1 or PinX1-C (Figure 8). These results are consistent with the findings that PinX1 and PinX1-C induced crisis (Figures 3 and 4). However, the most striking phenotype was observed with cells expressing PinX1^{AS}. As compared with control vector cells, PinX1^{AS}-expressing cells produced tumors at a higher frequency and also without latency after injection (Figure 8A). The tumors grew more rapidly, with average tumor weight being ~26 times bigger than that of the controls (Figures 8B and 8C). Similar results were also obtained when injected into BALB/c inbred nude mice; five out of six mice injected with PinX1^{AS}-expressing cells produced tumors, whereas no tumor was detected in mice injected with PinX1 or PinX1-C-expressing cells or control cells (Figure 8C). These results indicate that whereas PinX1 and PinX1-C inhibit tumor growth, depletion of PinX1 increases tumorigenicity in nude mice and support that PinX1 is a putative tumor suppressor.

Discussion

Here we describe characterization of PinX1, a novel protein interacting with Pin2/TRF1. Overexpression of PinX1 and its TID inhibited telomerase activity, shortened telomeres and induced crisis with TID being more potent than the full-length protein, whereas depletion of endogenous PinX1 significantly increased telomerase activity and lengthened telomeres. Significantly, both PinX1 and its TID domain bound hTERT in vivo and in

SDS-containing gels, followed by detecting HA-hTERT by immunoblot with anti-HA antibody or ^{35}S-hTERT by autoradiography.
(E–H) Potent inhibition of telomerase by PinX1 and PinX1-C. Different concentrations of GST or GST-PinX1 (E and H), GST-PinX1-C (F and H), PinX1-N protein (G and H) or His-PinX1 (H) were incubated with telomerase extracts for 10 min before the TRAP assay. Arrows point to the 36 bp internal control (IC) for PCR amplification.

vitro, and also potently inhibited its activity in vitro. In Contrast, neither PinX1 nor its TID induced telomere shortening or crisis in telomerase-negative cells. Therefore, distinct from other proteins that modulate telomere length without affecting telomerase activity per se, PinX1 represents a novel class of proteins that can regulate telomerase activity directly. Interestingly, human *PINX1* is located at 8p23, a region with frequent loss of heterozygosity in a number of human tumors. Moreover, depletion of endogenous PinX1 in HT1080 cells increased their tumorigenicity, whereas overexpression of PinX1 or PinX1-C inhibits their ability to form tumors in mice. These results suggest that PinX1 is a putative tumor suppressor.

Is PinX1 an Endogenous Telomerase Inhibitor?

The following results (Table S1 at http://www.cell.com/cgi/content/full/107/3/347/DC1) support that PinX1 functions as an endogenous telomerase inhibitor. First, expressed PinX1 is a nuclear protein that is localized at the nucleolus and at telomeres, the substrate for telomerase (Greider and Blackburn, 1985). Second, PinX1 and PinX1-C form stable complexes with hTERT both in vivo and in vitro. Third, PinX1 and its small TID domain directly inhibits telomerase activity with a high potency in vitro, indicating that the domain in PinX1 responsible for the telomerase inhibition is located at its small C-terminal TID domain. Fourth, when overexpressed in telomerase-positive HT1080 cells, PinX1 significantly inhibits cellular telomerase activity and leads to progressive telomere shortening. Fifth, the TID domain of PinX1 almost completely inhibits telomerase activity and is much more potent than PinX1 in shortening telomeres. Sixth, although PinX1 and PinX1-C initially have no effect on cell growth rate, they eventually induce crisis, with their ability to affect cell growth being correlated with their ability to inhibit telomerase activity and to shorten telomeres. After crisis, some cells eventually recover and continue to divide with reactivation of telomerase and elongation of telomeres (data not shown). Seventh, neither PinX1 nor its TID induces telomere shortening or crisis in telomerase-negative cells. Eighth, depletion of endogenous PinX1 increases telomerase activity and telomere length in vivo, indicating a critical role for the Pinx1 concentration in regulating telomerase activity in vivo. Finally, depletion of endogenous PinX1 increased tumorigenicity of HT1080 cells, whereas overexpression of PinX1 or PinX1-C inhibits their ability to form tumors in nude mice. These results indicate that PinX1 is a potent telomerase inhibitor and provides a novel and important in vivo route for regulation of telomerase activity.

How Does PinX1 Inhibit Telomerase Activity?

We do not yet know how PinX1 inhibits telomerase activity. Both PinX1 and TID domain bind hTERT and inhibit its activity in vitro and in vivo, indicating that the TID domain is sufficient to potently inhibit telomerase. It is likely that the TID domain may interact with hTERT and inhibit its activity. If this is the case, it would be of great interest to determine why full-length PinX1 protein appears less active in inhibiting telomerase activity than the TID domain in cells, although they have similar potency in vitro. Because they are expressed at similar levels, a likely possibility is that the full-length protein may be subjected to endogenous regulation such as posttranslational modifications to reduce its inhibitory activity. Alternatively, proteins that interact with PinX1 in cells may reduce the ability of its TID domain to bind and inhibit telomerase activity. One such protein could be Pin2/TRF1, which binds PinX1 and colocalizes PinX1 in cells, although the biological significance of this interaction remains to be determined. The presence of PinX1 homologous genes in other eukaryotes such as yeast suggests its conservation. Interestingly, hTERT is also rather conserved, especially in the catalytic site. If the yeast PinX1 would function like the human protein, PinX1 might provide a general mechanism for regulating telomerase activity. In addition, since disruption of the yeast PinX1 ORF appears to be lethal, it may have other yet unidentified function.

Could PinX1 Be a Tumor Suppressor?

The chromosomal location of the PinX1 gene, 8p23 near the microsatellite maker D8S277, marks it as a candidate tumor suppressor. This region has been extensively investigated due to its frequent loss of heterozygosity (LOH) in a number of human cancers, including liver, breast, prostate, colorectal, lung, head, and neck (Baffa et al., 2000; Bockmuhl et al., 2001; Emi et al., 1992; Gustafson et al., 1996; Ishwad et al., 1999; Liao et al., 2000; Muscheck et al., 2000; Perinchery et al., 1999; Sun et al., 1999). For example, in human hepatocellular carcinomas, about 40%–50% of tumors exhibit LOH near the maker D8S277 and molecular analysis suggests the presence of tumor suppressor genes on chromosome 8p23. Indeed, the N-terminal 113 residues of PinX1 are almost identical to a small 174 amino acid protein deduced by a putative liver tumor suppressor gene cloned by PCR (Liao et al., 2000), which was reported while we were preparing our manuscript. This putative cDNA contains a premature stop at 113 residue of PinX1 due to an internal deletion. Although it is unknown whether this small protein is expressed in vivo, RT-PCR analysis using primers common for the *PINX1* gene and the putative liver gene has shown that the transcript is downregulated or absent in about 42% of hepatocellular carcinoma cell lines (Liao et al., 2000). Furthermore, our preliminary immunostaining analysis revealed that PinX1 expression is significantly reduced or is not detectable in many human tumor samples including liver, prostate, colon, and lung carcinoma (X.Z.Z and K.P.L. unpublished data). These results suggest that expression of the *PINX1* gene may be reduced or absent in many human cancer tissues.

The significance of reduced expression of PinX1 in tumors is substantiated by our findings that depletion of PinX1 increases tumorigenicity of HT1080 cells, whereas overexpression of PinX1 or PinX1-C inhibits their ability to produce tumors in nude mice. Activation of telomerase is a common and critical event for cellular transformation and this activation is important for transformed cells to continue cell division. The fact that PinX1 inhibits telomerase suggests that inactivation or downregulation of PinX1 may contribute to activation of telomerase in cancer cells. In addition, PinX1 might have

other as yet unidentified functions that contribute to its tumor suppressing function. Nevertheless, our results suggest that PinX1 might be one of the long sought tumor suppressor genes located at chromosome 8p23. We are performing experiments including detecting *PINX1* mutations in cancer and disrupting the PinX1 gene in mice to examine the possibility. Given its ability to inhibit telomerase, induce crisis, and inhibit tumor formation in nude mice, PinX1 or its TID might be useful for cancer therapy.

Experimental Procedures

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was carried out, as described (Lu et al., 1996). Briefly, the Pin2 cDNA was fused to the GAL4 DNA binding domain and transformed into the Y190 yeast strain to establish stable transformants. The stable strains were transformed again with a HeLa cDNA library fused to the GAL4 activation domain. The GenBank accession numbers for human and mouse *PINX1* cDNAs are AY029161 and AF421879, respectively.

Expression and Purification of Recombinant Proteins

To generate N-terminally GST- or His-PinX1 fusion proteins, cDNAs encoding PinX1 and its mutants were subcloned into a pGEX or pET28a vector, respectively, and the resulting fusion proteins expressed and purified by glutathione or Ni^{2+}-NTA agarose column, as described (Lu et al., 1999b; Zhou et al., 2000b). The same method is used to purify various GST-Pin2 proteins.

Production of Anti-PinX1 Antibodies and Immunostaining

To raise antibodies against PinX1, rabbits were immunized with GST-PinX1 and anti-PinX1 antibodies were affinity purified using a GST-PinX1 column, as described (Lu et al., 1996, 1999a). Immunostaining using affinity-purified PinX1 antibodies or anti-HA antibody (12CA5) was performed, as described (Lu and Hunter, 1995).

GST Pulldown, Immunoprecipitation, Immunoblot, and Northern Blot Analysis

GST pulldown, immunoprecipitation and immunoblotting analysis were performed, as described (Lu et al., 1999b; Shen et al., 1997, 1998). Briefly, relevant proteins were transiently or stably expressed in cells, or translated in vitro using the TNT kit (Promega) in the presence of [^{35}S]-Met, followed by lysis or dilution in a buffer. The cellular supernatants were incubated with primary antibodies, or with 1 μM GST fusion proteins for 2 hr at 4°C and 15 μl of protein A beads or glutathione agarose beads were then added, followed by further incubation for 1–1.5 hr at 4°C. The beads were washed extensively and subjected to immunoblotting analysis. Membranes containing RNAs isolated from different human tissues (Clontech) were hybridized to the middle segment of the PinX1 coding sequence and then stripped, followed by reprobing with GAPDH.

Establishment, Growth Rate, and Phenotypic Analysis of Stable Cell Lines

Various PinX1 constructs were stably transfected into HT1080 or GM847 cell line, with the vector as a control. Multiple independent single clones were isolated and checked for protein expression by immunoblotting analysis with anti-HA or anti-PinX1 antibodies. To monitor growth property and morphology of cell lines, they were continuously maintained in culture, splitting on every fourth day and seeding at the concentration of 6×10^5 cells per 10 cm culture dish. Cell growth curves were determined by counting the cell number at each subculture. Cell proliferation was also assayed by incubation with BrdU for 30 min and incorporation of BrdU into cells was determined according to the manufacture's protocol (Pharmingen). To detect phenotype of the rounded and loosely attached cells in PinX1-C-expressing cells, they were harvested by aspiration and subjected to flow cytometry analysis (Becton-Dickinson) (Kishi et al., 2001b; Lu and Hunter, 1995). To stain for SA-β-Gal, cells grown were washed and then fixed and SA-β-gal (pH 6.0) was detected, as reported (Dimri et al., 1995).

Telomerase and TRF Length Assays

For assaying telomerase activity in cells, cells were lysed in a lysis buffer and telomerase-containing fraction was prepared, followed by assaying the telomerase activity using the TRAP-eze telomerase detection kit (Intergen), according to the manufacturer's protocol. To examine the effects on telomerase activity in vitro, various PinX1 proteins were incubated with telomerase for 10 min at 4°C before subjecting to telomerase extension. Telomerase products were separated on 10% polyacrylamide gels, which were stained with SYBR Green. Telomerase activity was semiquantified by normalizing the band intensities of the characteristic 6 bp telomerase-specific ladder to that of the 36 bp internal standard using NIH image software (Kim et al., 1994). TRF length was measured, as described (Counter et al., 1992; Harley et al., 1990). Briefly, genomic DNA was isolated and digested with HinfI and RsaI, separated on 0.7% agarose gels (2 μg/lane). The gels were dried and hybridized with a telomeric DNA probe and average TRF length was calculated by quantifying the hybridization signals using ImageQuant.

Tumorigenicity Assay

Stable HT1080 cell lines at 8 PD were harvested and washed twice in cold serum-free DMEM. Cells were resuspended in 0.25 ml of cold serum-free DMEM, and the suspension was mixed with an equal volume of cold Matrigel (Becton Dickinson). The cell suspension was injected subcutaneously (0.5 ml per site, 1×10^6 cells at 8 PD) into 5- to 6-week-old NCr athymic nude mice (Taconic). Mice were observed weekly for the visual appearance of tumors at injection sites and tumors were harvested and weighed 8 weeks after injection. The cells were also injected into BALB/c athymic nude mice (Taconic) using the same procedure without Matrigel.

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