PML-IV functions as a negative regulator of telomerase by interacting with TERT

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Accepted 15 April 2009
Journal of Cell Science 122, 2613-2622 Published by The Company of Biologists 2009
doi:10.1242/jcs.048066

Summary

Maintaining proper telomere length requires the presence of the telomerase enzyme. Here we show that telomerase reverse transcriptase (TERT), a catalytic component of telomerase, is recruited to promyelocytic leukemia (PML) nuclear bodies through its interaction with PML-IV. Treatment of interferon-α (IFNα) in H1299 cells resulted in the increase of PML proteins with a concurrent decrease of telomerase activity, as previously reported. PML depletion, however, stimulated telomerase activity that had been inhibited by IFNα with no changes in TERT mRNA levels. Upon treatment with IFNα, exogenous TERT localized to PML nuclear bodies and binding between TERT and PML increased. Immunoprecipitation and immunofluorescence analyses showed that TERT specifically bound to PML-IV. Residues 553-633 of the C-terminal region of PML-IV were required for its interaction with the TERT region spanning residues 1-350 and 595-946. The expression of PML-IV and its deletion mutant, 553-633, suppressed intrinsic telomerase activity in H1299. TERT-mediated immunoprecipitation of PML or the 553-633 fragment demonstrated that these interactions inhibited telomerase activity. H1299 cell lines stably expressing PML-IV displayed decreased telomerase activity with no change of TERT mRNA levels. Accordingly, telomere length of PML-IV stable cell lines was shortened. These results indicate that PML-IV is a negative regulator of telomerase in the post-translational state.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/15/2613/DC1

Key words: Telomerase, TERT, PML, TERC, PML nuclear bodies

Introduction

The gene encoding promyelocytic leukemia (PML) protein was first identified as PML-RARa and RARa-PML fusion proteins as a result of the t(15;17) chromosomal translocation present in acute promyelocytic leukemia (APL) (Melnick and Licht, 1999). PML is a tumor suppressor predominantly expressed in the nucleoplasm, which is tightly linked to the nuclear matrix as discrete nuclear speckles. These punctuate structures are variously known as PML nuclear bodies (PML-NBs), PML oncogenic domains (POD), nuclear domains-10 (ND-10), and Kremer bodies (Bernardi and Pandolfi, 2007). PML-NBs vary in diameter from 0.2 to 1.0 μm, and their size, shape and number change according to cellular stresses and the cell cycle (Dellaire and Bazett-Jones, 2004). The PML C-terminus displays diverse alternatively spliced forms, resulting in the appearance of up to 11 isoforms (Fogal et al., 2000; Pearson and Pelicci, 2001; Zheng et al., 1998). However, the N-terminus is conserved and encodes the RBCC-TRIM motif, which has three cysteine-rich zinc-binding domains, a RING-finger, two B-boxes (B1 and B2) and an α-helical coiled-coil domain (Reymond et al., 2001). A growing number of proteins have been found to interact with PML-NBs, suggesting important cellular functions regulating tumorigenesis, replicative senescence, cellular proliferation, DNA-damage response and antiviral defense (Bernardi and Pandolfi, 2007; Dellaire and Bazett-Jones, 2004). Phenotype analyses of PML-deficient mice have revealed defects in apoptosis induced by tumor necrosis factor (TNF), interferon (IFN) and Fas (Bernardi and Pandolfi, 2003; Takahashi et al., 2004). The fact that these mice thrive while exhibiting sensitivity to cancer-inducing drugs and resistance to γ-irradiation suggests a role for PML as a mediator in the apoptotic process rather than as a major tumor suppressor (Bernardi and Pandolfi, 2003).

The human telomerase holoenzyme is a ribonucleoprotein (RNP) complex composed of two essential core components: telomerase reverse transcriptase (TERT) and telomerase RNA template (hTR or hTERC) (Blackburn, 2000). Telomerase maintains telomere homeostasis by adding tandem hexameric (TTAGGG) repeats at the end of the chromosomes through de novo synthesis, which results in single-stranded 3′-end overhangs (McEachern et al., 2000). This DNA structure associates with various protein subunits including Ku70, Ku80, MRE11, TRF1, TRF2 and POT1, which form D- and T-loops to stabilize the end of chromosome (de Lange, 2005). The telomere stabilizes the chromosome by preventing aberrant recombination and end-to-end fusions, as well as preventing the DNA-repair system from recognizing the chromosomal ends as damaged DNA (Blasco, 2003). However, erosion of telomeres takes place in most normal human somatic cell lines that are defective in expressing TERT while still expressing hTERC (Blasco, 2005; Harley, 2008). The shortening of telomeres is due to defects in end replication; this might eventually limit the numbers of replications resulting in replicative senescence (Harley, 2008). A limited number of human cells, such as germ cells, activated lymphocytes, and several tissue stem cell populations do express telomerase (Harley, 2008). In addition, over 90% of tumor cells express active telomerase,

making this enzyme a notable cancer marker (Blasco, 2003; Harley, 2008). Replicative senescence can be avoided by introducing telomerase to telomerase-deficient cells, which leads to unlimited replication of cells and immortalization (Bodnar et al., 1998; Stampfer et al., 2001). However, the introduction of several oncogenes, such as those encoding SV40 large-T antigen and V-Ha-Ras Harvey rat sarcoma homolog (HRAS), along with telomerase, is usually required for tumorigenic transformation of normal human cells such as epithelial cells and fibroblasts (Beausejour et al., 2003; Masutomi et al., 2003). Over the past 10 years, various cellular proteins regulating telomerase activity have been identified. The levels of telomerase can be regulated at the transcription level by various factors such as p53, RB, Myc and WT1. Telomerase can be also affected by post-translational modification such as phosphorylation by protein kinase C (PKC), Akt, Abl and mitogen-activated protein kinase (MAPK), as well as ubiquitylation by MKRN1 (Kang et al., 1999; Kharbanda et al., 2000; Kim et al., 2005; Liu, 1999; Seimiya et al., 1999).

In this study, we determined that one isoform of PML, PML-IV, has a negative effect on the activity of TERT. We showed that telomerase activity was specifically regulated by this PML isoform. When both proteins were co-expressed within cells, they were co-localized to the PML-NBs. Exogenous TERT was also observed to localize to PML-NBs upon treatment with IFNα. IFNα-dependent repression of TERT activity was significantly relieved when endogenous levels of PML were abrogated, suggesting that telomerase activity can be suppressed by endogenous PML.

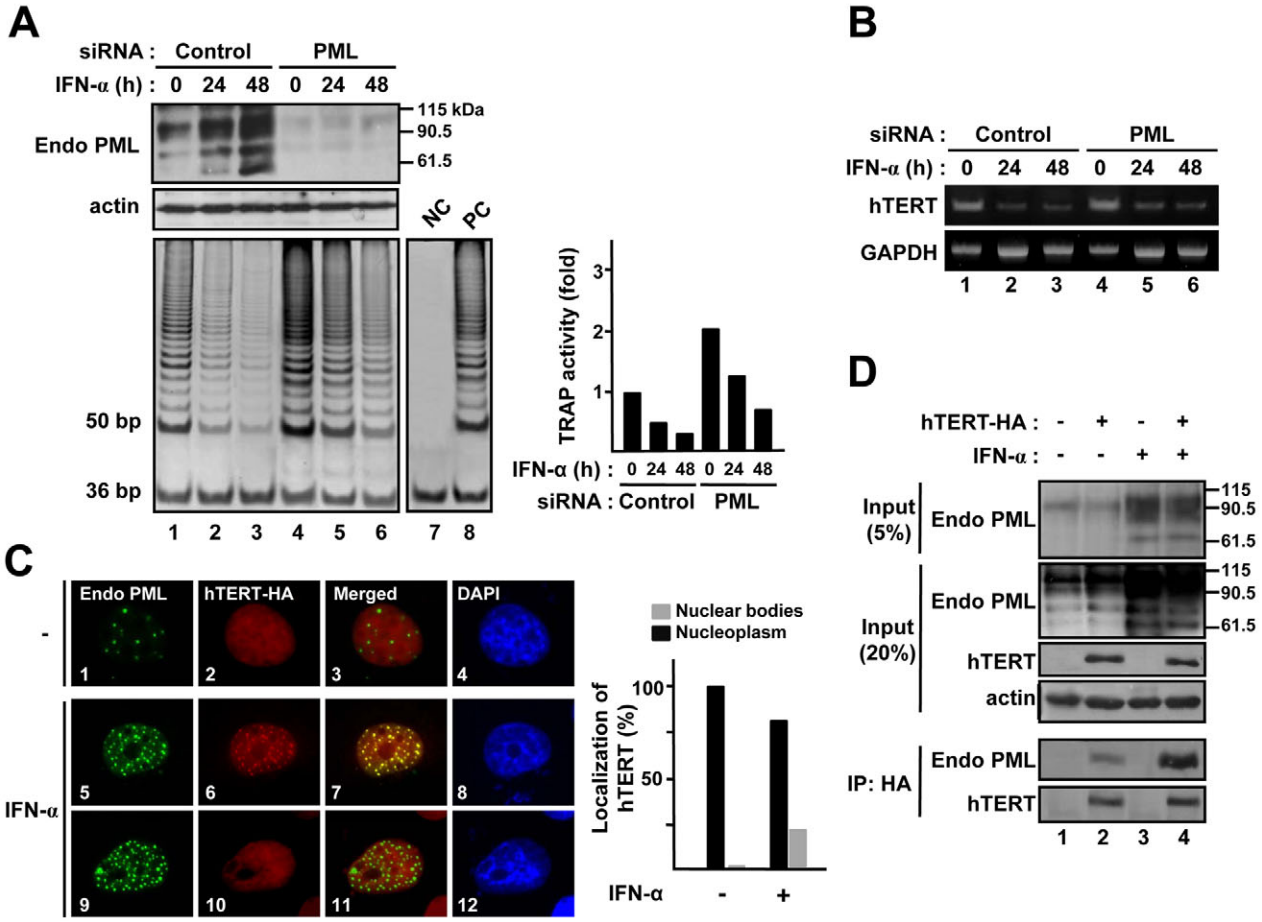

Fig. 1. PML induced by IFNα suppresses intrinsic telomerase activities in H1299 cells. (A) Ablation of endogenous PML under IFNα treatment. H1299 cell lines were treated with 1000 U/ml IFNα in the presence of control or 200 nM PML siRNA. The levels of PML and actin in cell extracts harvested at indicated times were subjected to immunoblotting using anti-PML and anti-actin antibodies. The cell extracts (30 ng) were added for TRAP analyses. NC and PC denote negative control and positive control, respectively. TRAP analysis for NC was performed in the absence of cell extracts. The 36 bp represents the internal TRAP assay standards. The ImageJ (NIH) program was used to measure the relative amounts of bands in the TRAP assays after normalizing to 36 bp band, and are shown in the panel on the right. (B) Detection of the TERT mRNA levels. The mRNA of the cells treated with control or PML siRNA in the presence or absence of IFNα was extracted and tested for the levels of TERT mRNA using RT-PCR. The mRNA levels of GAPDH were analyzed as a control. (C) Co-localization of TERT-HA with PML-NBs. H1299 cells were transfected with plasmid expressing TERT-HA with or without IFNα. Cells were fixed after 24 hours and analyzed by immunofluorescence with anti-HA and anti-PML antibodies followed by Alexa Fluor 594 rabbit (red) and Alexa Fluor 488 mouse (green) secondary antibodies. A fluorescent microscope was used to detect the proteins, with a total of 200 cells counted for each experiment. DAPI was used to visualize the nuclei. Cells displaying colocalization of TERT and PML-NBs were counted and shown as graph on right. Cells were captured with the same exposure times (200 milliseconds for TERT, 800 milliseconds for PML). (D) Immunoprecipitation of TERT-HA with endogenous PML. Plasmid expressing TERT-HA was transfected into untreated or IFNα-treated H1299 cells. Whole cell lysates (WCLs) were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-PML, anti-HA and anti-actin antibodies. Cell lysates (5% or 20%) used for immunoprecipitation were subjected to Western blot as an input.

Results

Depletion of PML suppresses IFNα-mediated inhibition of telomerase activity

IFNα suppresses the transcription of TERT mRNA and stimulates PML expression (Everett and Chelbi-Alix, 2007; Nisole et al., 2005; Xu et al., 2000). To examine a possible interaction between TERT and PML, we tested the effect of PML on telomerase activity in H1299 cells treated with IFNα. This resulted in the increase of endogenous PML within 24 hours (Fig. 1A, upper panel, lanes 1-3), confirming earlier observations (Everett and Chelbi-Alix, 2007). Treatment with PML small-interfering RNA (siRNA) induced depletion of the endogenous PML (Fig. 1A, upper panel, lanes 4-6). The same extracts presented in Fig. 1A were used for telomeric repeat amplification protocol (TRAP) analyses. Interestingly, PML depletion increased telomerase activity ~twofold in the absence of IFNα treatment (Fig. 1A, bottom panel, lanes 1 and 4, graph). These data suggest that the endogenous PML might suppress intrinsic telomerase activity under normal condition. Although treatment of IFNα suppressed telomerase activity under control siRNA treatment, depletion of PML relieved inhibition of telomerase activity, implying a negative regulatory role of PML on telomerase function (Fig. 1A, bottom panel, lanes 2, 3, 5 and 6, graph). RT-PCR showed that mRNA levels of TERT were decreased upon IFNα treatment. However, depletion of PML did not affect mRNA levels of TERT (Fig. 1B). These data suggest that PML might negatively control telomerase activity in the post-translational state.

The suppressive effects of PML and TERT led us to examine the interaction between PML and TERT. Immunofluorescence analyses demonstrated that approximately 24% of cells expressing exogenous TERT-HA co-localized with endogenous PML-NBs upon IFNα treatment (Fig. 1C, panels 5-8, graph). Around 76% of cells had TERT dispersed in the nucleoplasm (60%) or cytoplasm (16%) without any obvious dotted structures within the nucleoplasm (Fig. 1C, panels 9-11). Under normal conditions, fewer than 2% of cells displayed such co-localization (Fig. 1C, panels 1-4) suggesting that increased concentrations of PML were required for co-localization. Immunoprecipitation analyses indicated that endogenous PML and exogenous TERT could interact with each other, possibly through adaptor proteins (Fig. 1D, lanes 1 and 2). When the levels of PML were increased by the treatment of IFNα, about 80% more PML interacted with exogenous TERT (Fig. 1D, lanes 1-4). To clearly identify the levels of endogenous PML levels, we loaded reduced amounts of whole cell extracts (Fig. 1D, first panel). The effect of IFNα-induced PML-NBs on telomerase activity was measured using U2OS, which is an ALT cell line with intact endogenous p53. Since U2OS cells do not have endogenous TERT, we introduced exogenous TERT-HA into the cells. Upon depletion of PML, the suppressive effect of IFNα on telomerase activity was relieved, as previously observed for H1299 cells (supplementary material Fig. S1A). Furthermore, colocalization of overexpressed TERT with PML-NBs under IFNα treatment was detected in up to 30% of the cells (supplementary material Fig. S1B). These results were consistent with the suggestion that the ALT cell line displays similar patterns of PML-NBs and TERT interaction as evident in H1299 cells.

To better understand whether endogenous PMLs could affect the recruitment of endogenous TERT to PML-NBs, we attempted to detect endogenous TERT localization in the absence and presence of IFNα. For this experiment we used an anti-TERT antibody (RCK-hTERT), which detects endogenous TERT in immunofluorescence analyses (Wu et al., 2006). Endogenous TERT colocalized with the endogenous PML-NBs under IFNα treatment in about 30% of H1299 cells (supplementary material Fig. S2A). As a control, RCK-TERT antibodies were tested to see if they could detect exogenous TERT-HA or endogenous TERT in the presence and absence of exogenous PML (supplementary material Fig. S2B,C). Overall, the data indicated that depletion of PML with IFNα treatment resulted in the increase of endogenous telomerase activity in H1299 cells. These results indicate that PML-NBs might be able to suppress telomerase activity, potentially by recruiting TERT as well as other adaptor proteins. Upon induction of PML expression using IFNα treatment, exogenous or endogenous TERT co-localized to

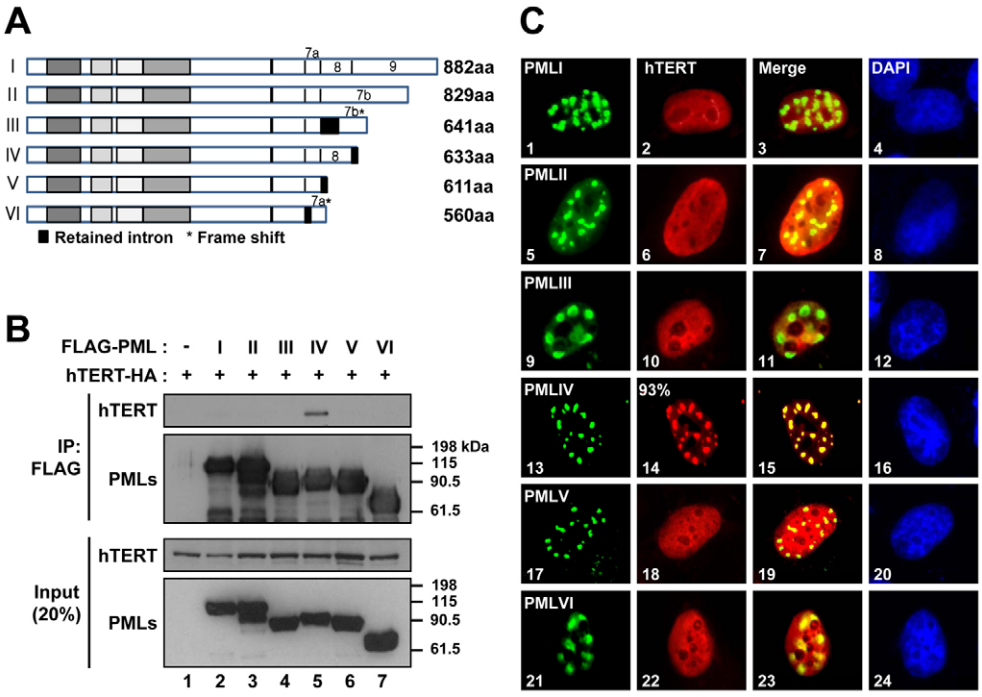

Fig. 2. PML-IV interacts specifically with TERT. (A) Schematic view of the six PML isoforms. (B) The interaction between TERT and PML isoforms. Six FLAG-PML isoforms (I-IV) were transfected into HEK293T cells along with TERT-HA. Whole cell lysates were immunoprecipitated with anti-FLAG antibodies. FLAG immunoprecipitates and 20% input were loaded and immunoblotting was performed using anti-HA and anti-FLAG. (C) Co-localization of TERT with PML-IV. H1299 cells were co-transfected with plasmids expressing TERT-HA and FLAG-PML isoforms. Cells were fixed after 24 hours and analyzed by immunofluorescence using anti-HA and anti-FLAG antibodies followed by Alexa Fluor 594 rabbit (red) and Alexa Fluor 488 mouse (green) secondary antibodies. Nuclei were visualized by DAPI staining.

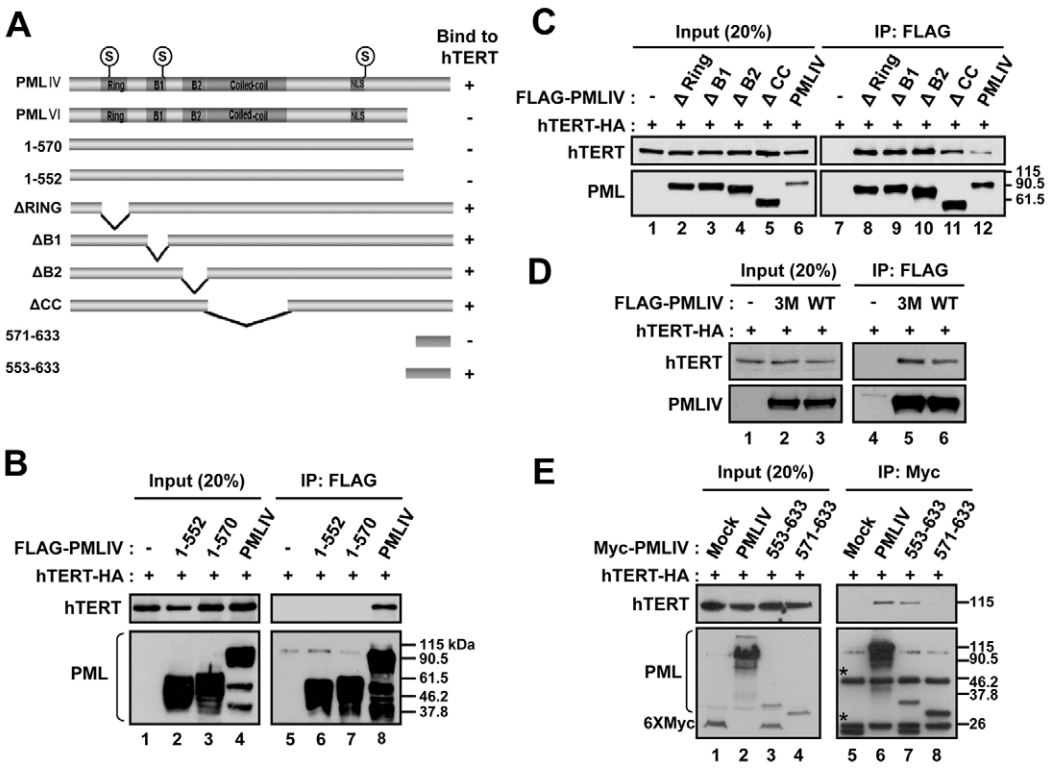

Fig. 3. The C-terminal region of PML-IV is required for TERT recruitment. (A) Schematic representation of various PML mutants showing their functional domains. (B) Interaction between TERT and C-terminal deletion PML mutants. Expression plasmids for TERT-HA and/or FLAG-tagged C-terminal deletion mutants of PML-IV were transfected into HEK293T cells. 24 hours after transfection, whole cell lysates were immunoprecipitated with anti-FLAG antibodies and assayed by immunoblotting with anti-HA and anti-FLAG antibodies. (C) Interaction between TERT and PML mutants. Plasmids expressing wild-type or truncated PML mutants and/or TERT-HA were transfected into HEK293T cells. Whole cell lysates were immunoprecipitated with anti-FLAG antibodies. Immunoblotting was performed with anti-HA and anti-FLAG antibodies. (D) Interaction between PML3M and TERT. Plasmids expressing TERT-HA, FLAG-PML-IV, or FLAG-PML3M were transfected into HEK293T cells. Whole cell lysates were subjected to immunoprecipitation using anti-FLAG antibodies followed by western blotting using anti-HA and anti-FLAG antibodies. (E) The interaction between TERT and the C-terminal fragment of PML-IV. Plasmids expressing TERT-HA, Myc-PML-IV, Myc-553-633 or Myc-571-633 were transfected into HEK293T cells. Whole cell lysates were subjected to immunoprecipitation using anti-Myc antibodies followed by western blotting using anti-HA and anti-Myc antibodies. The asterisks indicate heavy and light chains. pCS3-MT-BX empty vector produces 25 kDa polypeptide consisting of 6×Myc epitope (lane 2). In the immunoprecipitation analysis (B-E), 20% of lysates were used as an input.

endogenous PML-NBs. The effects of IFNα and PML were observed both in telomerase-positive and ALT cell lines.

PML-IV isoform specifically interacts with TERT

As TERT seems to associate with endogenous PML, we next tested which PML is involved with TERT. Seven major PML isotypes have been identified that share the conserved N-terminal region containing the RBCC-TRIM motif, although their C-termini vary because of alternative splicing of the 3′ exon (Fig. 2A) (Jensen et al., 2001). To identify the TERT-interacting PML isoforms, TERT was cotransfected with PML isoforms I-VI in HEK293T cells, and interactions were detected using immunoprecipitation. The results showed that only PML-IV bound to TERT (Fig. 2B, lane 5). Consistent with these data, immunofluorescent analyses confirmed that, of the six isoforms, only PML-IV could co-localize with TERT in H1299 cells (93%) (Fig. 2C, panels 13-16). No co-localization of TERT with other PML isoforms was observed. We further used HepG2, U2OS and HFF cell lines, and tested whether PML-IV could colocalize with TERT (supplementary material Fig. S3). Colocalization of PML-IV and TERT was evident in all three cell lines, suggesting that the interaction between these two proteins might be a general phenomenon. However, we could not discern whether the association of PML-IV and TERT was direct or required other intermediate factors.

The protein interaction data between TERT and PML-IV in Fig. 2 indicate that the C-terminus of PML-IV might be responsible for TERT interaction. Thus, two C-terminus deletion mutants of PML-IV, 1-570 and 1-552, were constructed, and their interaction with TERT was analyzed (Fig. 3A). Furthermore, because of the possibilities of the RBCC-TRIM region affecting the structural conformation of PML-IV, which might affect the interaction between PML-IV and TERT, various RBCC-TRIM deletion mutants were used (Fig. 3A). The results indicated that the region between residues 553 and 633 of PML-IV is required for interaction with TERT, because 1-570 and 1-552 mutant forms of PML were not able to bind to TERT (Fig. 3B). The various BBRC mutants were not evidently influential on their interaction (Fig. 3C). These findings were further supported by immunofluorescence analyses, which showed that 1-570 and 1-552 were not able to recruit TERT (supplementary material Fig. S4A).

SUMOylation of PML has been implicated in development and maturation of PML-NBs, which, in turn, recruit a wide variety of proteins (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001;

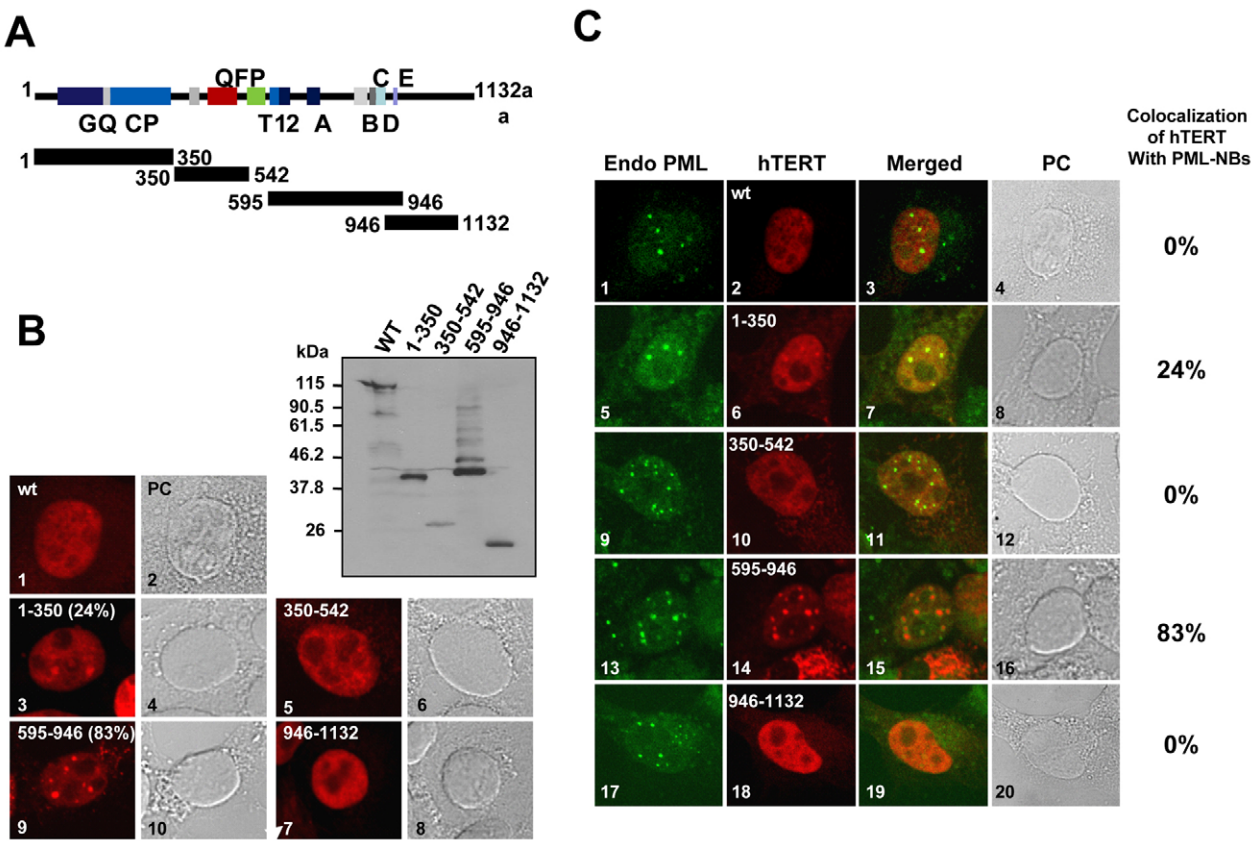

Fig. 4. Deletion fragments of TERT localize to the nucleoplasm in speckles. (A) Schematic representation of the TERT deletion constructs used in this study (Autexier and Lue, 2006; Xia et al., 2000). (B) Subcellular localization of TERT and its deletion constructs. H1299 cells were transfected with plasmids expressing TERT or the TERT fragments. 24 hours after transfection, cells were lysed and immunoblotted using anti-HA-rabbit antibodies (right panel). H1299 cells were transfected with plasmids expressing TERT-HA or its deletion mutants. The expressed proteins were detected with anti-HA antibodies followed by Alexa Fluor 594 rabbit (red) secondary antibodies. (C) Colocalization of TERT or its deletion mutants with PML-NBs. H1299 cells were co-transfected with expression vector for TERT-HA (panels 1-4) or its mutants (panels 5-20). Cells were fixed and detected using anti-HA and anti-PML antibodies followed by Alexa Fluor 594 rabbit (red) and Alexa Fluor 488 mouse (green) secondary antibodies. Transfected cells were analyzed using confocal microscopy. Endo PML, endogenous PML; PC, phase contrast. A total of 100 cells were counted for each experiment.

Zhong et al., 2000). To better understand whether SUMOylation of PML was absolutely required for the interaction, PML3M was used. PML3M cannot be SUMOylated because it has three SUMOylation sites mutated to arginine (K65R, K160R, K490R) (Fogal et al., 2000). Immunoprecipitation and immunofluorescent data suggested that TERT was still able to interact with PML3M, indicating that SUMOylation of PML might not necessarily be required for the recruitment of TERT (Fig. 3D; supplementary material Fig. S1B).

Since the C-terminus of PML-IV binds to TERT, two deletion mutants consisting of residues 571-633 and 553-633 were constructed and tested for their interaction with TERT. Immunoprecipitation data indicated that only 553-633 was able to bind to TERT (Fig. 3E). Collectively, these data suggest that TERT could bind to the C-terminal region of PML-IV between residues 553 and 633. Moreover, SUMOylation was not necessarily required for the interaction between TERT and PML-IV, because TERT was still able to interact with PML3M mutants. However, the detailed effects of PML SUMOylation on the recruitment of TERT on PML-NBs were not clearly defined. Furthermore, the data presented here do not indicate whether other adaptors are required for the interaction between TERT and the C-terminus of PML-IV.

The 1-350 and 595-946 mutants of TERT are required for interaction with PML-IV

Four TERT deletion mutants were constructed to identify the region responsible for the TERT interaction with PML (Fig. 4A). The four deletion mutants were expressed in H1299 cells (Fig. 4B). When their localization within the cells was detected using immunofluorescence and confocal laser microscopy, TERT fragments 1-350 and 595-946 were observed as speckles in the nucleoplasm (Fig. 4B, panels 3, 4, 7 and 8), whereas wild-type TERT and other mutants (350-542 and 946-1132) were evenly diffused in the nucleoplasm (Fig. 4B, panels 1, 2, 5, 6, 9 and 10). Particularly, 83% of H1299 cells expressing the 595-946 fragment displayed prominent multiple dots in the nucleoplasm whereas 24% of cells with the 1-350 fragment showed dotted forms. Similar and consistent results were also obtained in other cell lines such as HepG2 and HEK293T suggesting that the localization of the TERT deletion fragments could be a general phenomenon (data not shown).

The marked nucleoplasmic localization of the TERT fragments 1-350 and 595-946 as speckled structures with sizes similar to PML-NBs prompted us to test whether the deletion mutants were indeed recruited to these structures. Endogenous PML was detected by

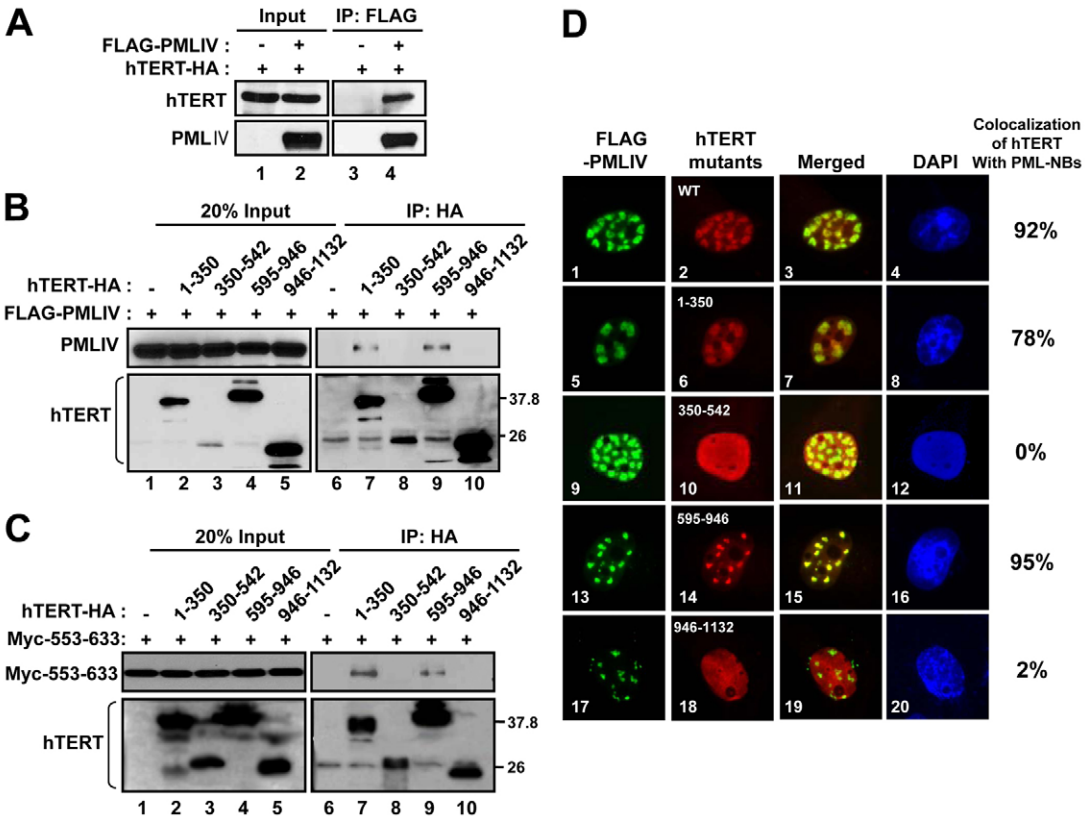

Fig. 5. Two regions of TERT, 1-350 and 595-946, are required for TERT interaction with PML-IV. (A) Interaction between PML-IV and TERT. To detect the interaction between TERT and PML-IV, HEK293T cells were transfected with expression plasmids for TERT-HA and/or FLAG-PML-IV. Immunoprecipitation analyses were carried out as indicated in Fig. 2B. (B) The interaction between TERT fragments and PML-IV. To analyze the binding domain of TERT, HEK293T cells were transfected with expression plasmids for FLAG-PML-IV and/or HA-TERT mutants. Cell lysates were immunoprecipitated as described above using anti-HA antibodies and immunoblotted using anti-HA and anti-FLAG antibodies. (C) Interaction between TERT fragments and PML-IV 553-633 fragment. To analyze the binding domain between TERT and PML-IV, HEK293T cells were transfected with expression plasmids for FLAG-PML-IV (553-633) and/or HA-TERT mutants. Immunoprecipitations were carried out as described above. Immunoblotting was performed using anti-Myc and anti-HA antibodies. (D) Co-localization of TERT or its deletion mutants with PML-IV. H1299 cells were cotransfected with expression plasmids for TERT-HA (panels 1-4) or its mutants (panels 5-20) with FLAG-PML-IV. Cells were fixed and detected using anti-HA and anti-FLAG antibodies followed by Alexa Fluor 594 rabbit (Red) and Alexa Fluor 488 mouse (Green) secondary antibodies. Transfected cells were analyzed as described n Fig. 2C. A total of 100 cells were counted for each experiment. Cells were captured with the same exposure times (200 milliseconds for TERT, 800 milliseconds for PML).

immunofluorescent antibodies to determine the co-localization of PML-NBs with the TERT fragments. As expected, 22% and 81% of cells expressing fragments 1-350 and 595-946 , respectively, co-localized to the endogenous PML-NBs (Fig. 4C, panels 5-8 and 13-16). However, the other two deletion mutants and TERT were diffused throughout the nucleoplasm without any co-localization with PML-NBs (Fig. 4C, panels 1-4, 9-12 and 17-20). The recruitment of the 1-350 and 595-946 fragments to PML-NBs was also observed in HepG2 and HEK293T cells indicating that this co-localization was not cell-type specific (data not shown).

Since TERT fragments 1-350 and 595-946 were shown to localize with PML-NBs, we further tested whether these two deletion fragments could interact with PML-IV using immunoprecipitation analyses. The results suggest that both fragments were able to interact with overexpressed PML-IV, whereas the other deletion fragments were not interactive (Fig. 5A,B), confirming the data shown in Fig. 4. Since the 553-633 fragment of PML-IV bound to TERT, we tested the binding of TERT fragments with this region. The results indicated that the 1-350 and 595-946 deletion mutants of TERT were able to bind to the PML-IV 553-633 fragment (Fig. 5C). It is conceivable that adaptor proteins mediate the interactions between these domains. These interaction data were further supported by immunofluorescence results, which showed that 92%, 78% and 95% of the cells expressing wild-type TERT, 1-350 fragment, and the 595-946 fragment, respectively, co-localized with overexpressed PML-IV (Fig. 5D). Collectively, these results indicate that two regions of TERT, 1-350 and 595-946, could spontaneously co-localize to PML-NBs. In addition, these two regions were able to interact with PML-IV C-terminus from residues 553-633, possibly through adaptor proteins.

PML-IV negatively regulates telomerase activity

To establish whether PML-IV could have a regulatory effect on TERT, we transiently expressed PML-IV in H1299 cells and used TRAP assays to determine intrinsic telomerase activity. The expression of increasing amounts of PML-IV induced significant reduction in telomerase activity in H1299 cells compared with controls (Fig. 6A, lanes 1-6). The same results were obtained in HEK293T cells (data not shown). Furthermore, when we used U2OS and HFF to analyze the effects of PML-IV on telomerase activity, we observed that PML-IV was able to suppress the telomerase activity as shown in H1299 cells (supplementary material Fig.

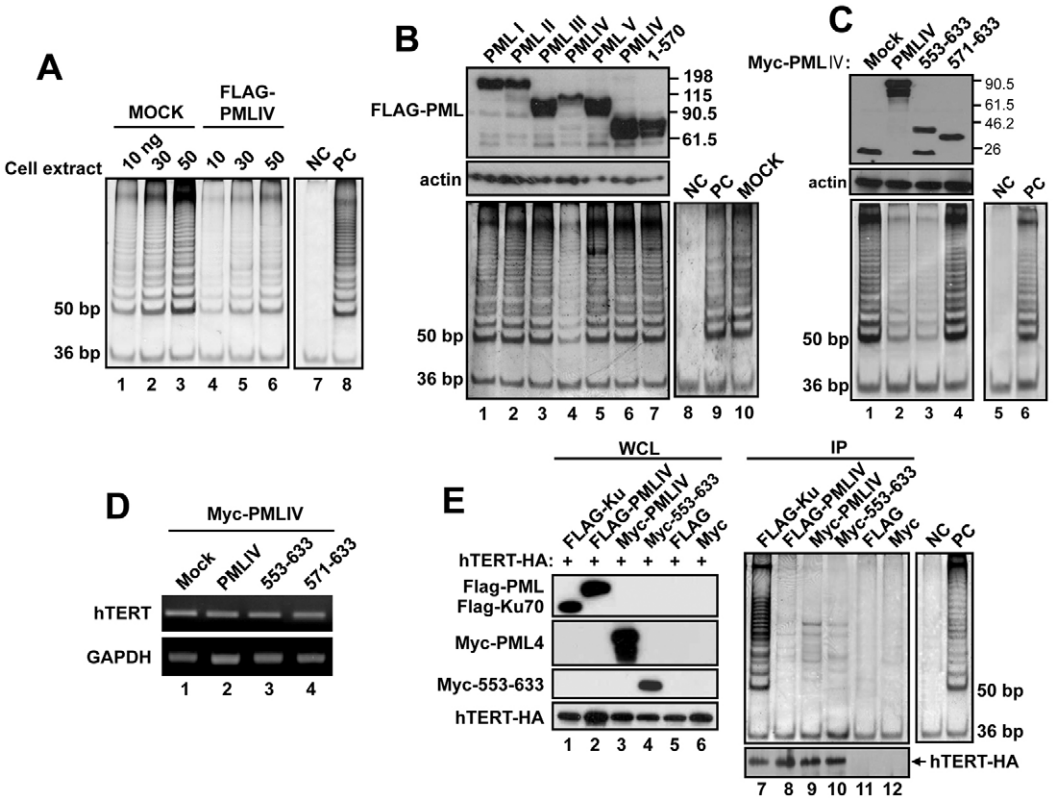

Fig. 6. PML-IV negatively regulates telomerase activity in H1299 cells. (A) The effect of PML-IV on telomerase activity. H1299 cells were transfected with either empty vector or the plasmids expressing FLAG-PML-IV. Increasing concentrations of cell extracts (10, 30 and 50 ng) were added for TRAP analyses as described in Fig. 1A. (B) The effects of PML isoforms on telomerase activities. H1299 cells were transfected with the empty plasmid or the vectors expressing PML I-IV (upper panel). The extracts from the transfected cells (30 ng) were tested for telomerase activities and subjected to immunoblotting using anti-FLAG and anti-actin antibodies. (C) The effects of PML C-terminal fragment on telomerase activities. H1299 cells were transfected with the vectors expressing 6×Myc epitope (control vector), Myc-PML-IV, Myc-553-633 or Myc-571-633 (upper panel). The extracts from the transfected cells (30 ng) were tested for telomerase activity and subjected to immunoblotting using anti-Myc and anti-actin antibodies. (D) Detection of the TERT mRNA levels. H1299 cells transfected with expression vectors for Myc-PML-IV, Myc-553-633 or Myc-571-633. After RNA extraction from transfected cells, mRNA levels of TERT were measured by RT-PCR. The mRNA levels of GAPDH were analyzed as a control. (E) Telomerase activity of the immunoprecipitated TERT by PML-IV and its 553-633 deletion mutant. Plasmids expressing FLAG-Ku70, FLAG-PML-IV, Myc-PML-IV, Myc-553-633, or empty vectors were transfected into HEK293T cells with expression vector for TERT-HA. Whole cell lysates were subjected to immunoblotting with anti-HA, anti-FLAG and anti-Myc antibodies (lanes 1-6). The lysates were immunoprecipitated with anti-FLAG or anti-Myc antibodies and assayed by immunoblotting with anti-HA antibodies (lanes 7-12) to detect TERT. 5% of the immunoprecipitated sample was analyzed for telomerase activity using a TRAP assay.

5A,B). Analyses of other PML isomers demonstrated that the inhibitory effects on the telomere-lengthening activities of telomerase were only limited by PML-IV (Fig. 6B, lane 5). These results were consistent with the immunoprecipitation and immunofluorescence data in Fig. 2, which showed that only PML-IV was capable of interacting with TERT. Furthermore, PML fragment 553-633, which binds to TERT, could inhibit the intrinsic telomerase activity upon overexpression in H1299 cells (Fig. 6C). The data suggest that the C-terminus of PML might be an actual motif responsible for the inhibition of telomerase activity. RT-PCT analyses showed that PML-IV or the 553-633 mutant did not have any effects on mRNA levels of the endogenous TERT (Fig. 6D).

The inhibitory effects of PML-IV and its 553-633 deletion mutant were further pursued by immunoprecipitation followed by TRAP analyses. Ku70/80 interacts with telomerase by associating with TERT (Chai et al., 2002). Using FLAG-Ku70 as a control, FLAG-PML-IV, Myc-PML-IV or Myc-tagged 553-633 was transiently transfected into HEK293T cells with TERT (Fig. 6E). The immunoprecipitation analyses showed that all these proteins bound to similar amounts of TERT (Fig. 6E). However, TRAP analyses of the immunoprecipitates showed that TERT bound to PML-IV or 553-633 displayed barely any telomerase activity, whereas TERT bound to Ku70 maintained telomerase activity (Fig. 6E). These data suggest that the interaction between TERT and PML-IV or fragment 553-633 could lead to suppressive effects on the telomerase activity.

Overall, PML-IV was able to suppress intrinsic telomerase activity in H1299, HEK293T, U2OS and HTT cells. Other PML isomers did not have an affect on the telomerase activity in H1299 cells. Furthermore, the 553-633 fragment of PML alone was able to suppress intrinsic telomerase activity. The suppression of telomerase activity was not due to the inhibition of transcription, because mRNA expression of TERT was maintained in the presence of PML-IV or the 553-633 mutant. Finally, immunoprecipitation followed by TRAP assays indicate that binding of PML-IV or the 553-633 fragment to TERT might lead to the suppression of telomerase activity, possibly mediated by adaptor proteins.

Stable cell lines expressing PML-IV induce telomere shortening

To investigate the long-term effects of overexpressed PML-IV on the function of TERT, H1299 cell lines that stably expressed FLAG-

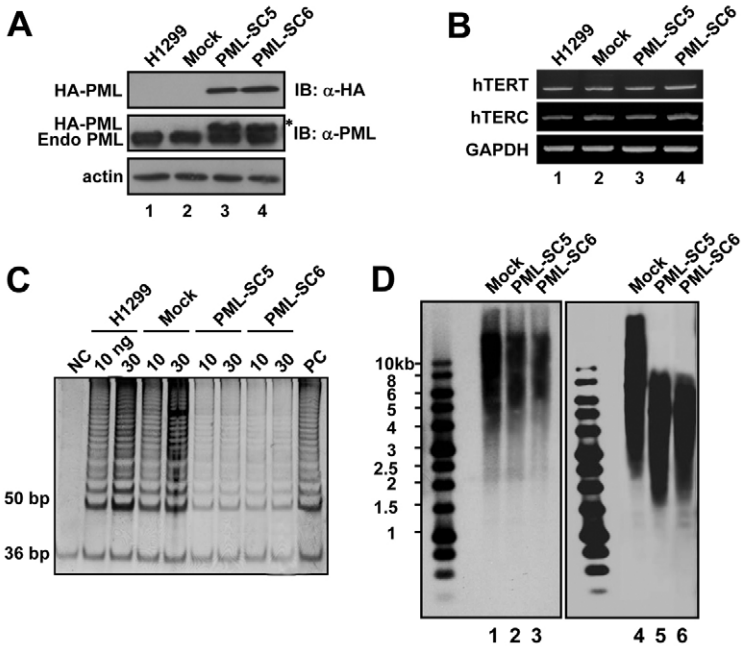

Fig. 7. H1299 stable cell lines overexpressing PML-IV suppress TERT activity. (A) FLAG-PML-IV expression in PML stable cell lines. PML-IV, expressed in PML-IV-stable cell lines, was detected using anti-PML or anti-HA antibodies. H1299 cell lines stably transfected with pCMV-Tag2B (mock) were also tested using the same antibodies. The asterisk indicates stably overexpressed FLAG-PML-IV. (B) Detection of the TERT mRNA and hTERC levels in the stable cell lines. The RNA of the stable cell lines was extracted and tested for the TERT mRNA or hTERC using RT-PCR as described in Fig. 1. The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were tested as a control. (C) Telomerase activity of the PML-IV-stable cell lines. The extracts of H1299, mock, or stable cell lines were tested for telomerase activity as described in Fig. 5. (D) Detection of telomere length in stable cell lines. The telomere length of the cell lines used above was determined by TRF analyses. The mock cell line was used as a control.

PML-IV (H1299-PMLSC5 and H1299-PMLSC6) were generated (Fig. 7A). The amount of stably expressed HA-PML-IV was about 80-90% of the endogenous PML (Fig. 7A). We used the H1299 cell line because it lacks p53, which can be stimulated by PML and can function as a negative transcriptional regulator of TERT (Pearson and Pelicci, 2001; Xu et al., 2000). When the stable cell lines were tested for the expression of TERT mRNA or hTERC after 60 cell divisions, no changes were observed (Fig. 7B). These results further confirmed that the transcription levels of TERT or hTERC were not regulated by PML-IV. TRAP analyses of the cell lines stably expressing PML-IV showed that telomerase activity was constitutively suppressed (Fig. 7C). These data, along with the data presented in Figs 6 and 7, indicate that PML-IV could negatively regulate telomerase activity, possibly with TERT association. To confirm these data, the stable cell lines were cultured for 60 cell divisions (Fig. 7D, lanes 4-6) and then telomere length was measured and compared with that in cells after only seven cell divisions (Fig. 7D, lanes 1-3). The results indicate that the lengths of telomeres were significantly shortened in both the stable cell lines expressing PML-IV, whereas the control did not display a shortening of the telomeres (Fig. 7D). The stable PML cell lines displayed a 40-50% reduction in the growth rate after 50-60 cell divisions (data not shown). This retarded growth rate could be due to a reduction in TERT activity and/or the overexpression of PML, both of which can affect cellular growth rates of cancer cells (Pearson and Pelicci, 2001; Zhang et al., 1999). Overall, these results suggest that constitutively overexpressed PML-IV leads to the shortening of telomeres, confirming the negative regulatory role of PML-IV on telomerase.

Discussion

Telomere dysfunction in the vast majority of human somatic cells is an obligate mechanism that ensures the prevention of cancer formation by inducing replicative senescence (Harley, 2008). Telomere erosion, typically evident as double-strand breaks in DNA, induces activation of p53 and Rb (retinoblastoma) in human cells (Sharpless and DePinho, 2004). Thus, it seems that the mechanism of telomere shortening is intricately interwoven with the essential mechanisms of tumor-suppressor proteins. PML-NBs are considered to be important components in the process of cellular senescence and apoptotic responses (Bernardi and Pandolfi, 2003; Bernardi and Pandolfi, 2007; Pearson and Pelicci, 2001; Takahashi et al., 2004). In particular, PML-NBs associate with p53 and Rb, resulting in cellular apoptosis and senescence. As the overexpression of PML in certain cell lines induces premature senescence as a result of upregulation of p53, which is also a transcriptional suppressor of TERT, it is a plausible hypothesis that PML induction could lead to telomerase inactivation through indirect pathways (Bischof et al., 2002; Xu et al., 2000).

In this study, we observed that PML-IV could have a negative effect on the function of telomerase and, possibly, induce telomere erosion. Interestingly, the 595-946 and 1-350 fragments of TERT could be recruited to PML-NBs spontaneously, possibly through other adaptor proteins recruited to PML-IV. These observations suggest that TERT has an intrinsic motif allowing it to be co-localized with PML-NBs. Furthermore, it seems that hTERC, an RNA component of telomerase, is not required for this process, because the deletion fragments of TERT that localized to the PML-NBs are not required for the association of TERT with hTERC (Bryan et al., 2000). When expressed alone in various cell types, TERT is dispersed throughout the nucleoplasm rather than recruited to the PML-NBs. However, IFNα treatment resulted in the co-localization of TERT with PML-NBs. Thus, telomerase seems to have little or no affinity towards PML-NBs under normal conditions. It might be that the PML binding motifs 1-350 and 595-946 could be masked by other factors or modifications, negatively affecting TERT from associating with PML-NBs. Analysis of these processes could reveal new regulatory pathways for TERT and PML-NB association.

PML is subject to SUMOylation, which is required for maturation of the PML-NBs. SUMOylated PML-NBs induce the recruitment of PML-NB-associated proteins such as sp100, Daxx and SUMO1 (Lallemand-Breitenbach et al., 2001; Zhong et al., 2000). However, a SUMOylation-deficient PML mutant is able to interact with and/or

recruit several proteins, including CBP, p53, Hdm2 (MDM2), PIN1, HDAC7 and HCMV IE1, suggesting that unSUMOylated primary PML-NBs still have an important biological function (Bischof et al., 2002; Gao et al., 2008; Kang et al., 2006; Lallemand-Breitenbach et al., 2001; Reineke et al., 2008; Wei et al., 2003). Indeed, SUMOylation of PML is dispensable for premature senescence induced by p53, because PML3M can induce growth arrest and senescence (Bischof et al., 2002). The process of PML SUMOylation does not seem to be a requirement for the recruitment of TERT to PML-NBs. The capability of PML3M to bind to TERT suggests that unSUMOylated PML-IV itself can still interact with telomerase. However, proteins recruited to SUMOylated PML-NBs could potentially display some regulatory effects on TERT recruited to PML-NBs. At this point, it is not clear what other regulatory pathways might affect the activity of TERT, although previous studies suggest several possibilities. The C-terminus of PML-IV, which is required for TERT interaction, overlaps with the motif necessary for its interactions with p53 and Hdm2 (Fogal et al., 2000; Guo et al., 2000; Wei et al., 2003). PML-NBs contribute to the post-translational modification of p53, including phosphorylation, acetylation and SUMOylation carried out by HIPK2, CHK2, CBP and PIAS, respectively (Bernardi and Pandolfi, 2003; Takahashi et al., 2004). These processes stabilize p53 by preventing Hdm2-dependent degradation and/or stimulate the transcriptional activity of p53, leading to cellular senescence and apoptosis. Since the binding site within TERT that associates with PML-IV corresponds to the binding site for p53, it is possible that the aforementioned p53-modifying enzymes regulate TERT. We cannot exclude the possibility that the various stress signals that determine the state of PML could also affect the interaction between TERT and other PML-associating enzymes.

Another factor that should be accounted for in future studies is the participation of TERT in function(s) other than telomere elongation. Since the discovery that TERT-HA, which lacks ability to elongate telomeres, is able to induce cellular immortalization, a novel function of TERT other than telomere lengthening has been suggested (Counter et al., 1998). Data from other studies support this suggestion. For example, the anti-proliferative effect of TGFβ can be overcome by telomerase overexpression (Stampfer et al., 2001). Animals expressing telomerase are also more labile to the production of tumors (Artandi et al., 2002; Gonzalez-Suarez et al., 2001). The depletion of TERT renders cells more susceptible to apoptotic cell death, whereas its overexpression enhances survival rates of mice against N-methyl-D-aspartic acid (Lee et al., 2008; Massard et al., 2006). The multi-functional nature of TERT requires further study.

Although the stable PML cell lines displayed a retarded growth rate after 60 cell divisions, they kept growing without cell cycle arrest or cell death. Since the H1299 cell line does not possess p53, which is the main protein activated by PML, it seems likely that the presence of p53 and its related pathway might have synergistic effects on telomere erosion-mediated cellular senescence and cell cycle arrest (Cosme-Blanco et al., 2007; Feldser and Greider, 2007). It would be interesting to observe the effects of PML-linked p53 pathways on TERT and telomerase activities to further clarify the detailed mechanisms of senescence induced by telomere erosion.

In conclusion, we suggest that telomerase activity and telomere homeostasis can be negatively affected by the presence of PML-IV. It would be interesting to explore the regulatory roles of other PML-associated proteins on telomerase function, which could open new avenues of investigation concerning the regulatory pathways of telomerase.

Materials and Methods

Construction of plasmids

Plasmids pEXP-FLAG-PML-IV and pEXP-FLAG-PML-IV3M have been previously described (Kang et al., 2006). They were used as templates to construct pCMV-Tag2B-FLAG-PML-IV and pCMV-Tag2B-FLAG-PML-IV3M. pCI-FLAG-PMLI, -II, -III, -IV, -V, and -VI isoforms were a generous gift from Keith Leppard (Warwick University, Warwick, UK). pCMV-Tag2B-PML-IV and the PML-IV truncated mutants ΔRING, ΔB1, ΔB2 and ΔCC were generated by subcloning into the pCMV-Tag2B vector between the *Bam*HI and *Eco*RI sites. The PML-IV C-terminal deletion mutants 1-552 and 1-570 were constructed by subcloning 1-552 and 1-570 into the pCMV-Tag2B vector between the *Bam*HI and *Eco*RI sites. pCS3-MT-BX-PML-IV, 553-633 and 571-633 were subcloned into the pCS3-MT-BX vector containing 6×Myc epitope tag between the *Eco*RI and *Sal*I sites. pcDNA3-HA/TERT residues 1-350, 350-542, 595-946 and 946-1132 were amplified by PCR and were inserted between the *Eco*RI and *Xba*I sites into the pcDNA3-HA vector (Invitrogen, Carlsbad, CA).

Antibodies and chemicals

Anti-PML (PG-M3), anti-HA mouse (F-7), anti-Myc (9E10) and anti-HA rabbit (Y-11) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG (M2) and β-actin antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St Louis, MO). Alexa Fluor 488 anti-mouse and Alexa Fluor 594 anti-rabbit antibodies were obtained from Invitrogen. Anti-TERT rabbit antibody (600-401-252) was purchased from Rockland (Gilbertsville, PA).

Cell culture and transfection

Cells of the human lung carcinoma cell line H1299, kidney cell line HEK293T, osteosarcoma cell line U2OS, human hepatocellular carcinoma cell line HepG2 and human foreskin fibroblast (HFF) were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY) and 1% penicillin-streptomycin (Invitrogen) in 5% CO_2 at 37°C. Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) and WelfectEx (Welgene, Daegu, Korea), according to the manufacturer's instructions. For the transfection of HFF cells, Microporator (NanoEnTek, Seoul, South Korea) was used according to the manufacturer's instructions. PML knockdown was carried out with using the siRNA 5′-AGATGCAGCTGTATCCAAG-3′. siRNA duplexes were synthesized by Qiagen-Xeragon (Germantown, MD). Control siRNA was purchased from Qiagen-Xeragon. siRNA transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol. H1299 cells were transfected with 200 nM siRNA. 72 hours after transfection, cells were harvested and used for the TRAP assay and western blot analysis. To establish a PML-IV stable cell line, pCMV-Tag2B-PML-IV was transiently transfected into H1299 cells using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Several independent single clones were selected in 500 μg/ml G418 and checked for protein expression by immunoblotting or immunofluorescence staining with anti-FLAG antibodies. For IFNα-stimulated induction of PML, exponentially growing cells were treated with 1000 U/ml IFNα (interferon α-2b; Schering-Plough, Kenilworth, NJ) for 24 hours.

Biochemical analyses

For immunofluorescence staining, cells were plated in 12-well plates with coverslips. 24 hours after transfection, the cells were fixed with 4% paraformaldehyde for 10 minutes at 25°C and then washed and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. After blocking with 5% bovine serum albumin (Santa Cruz Biotechnology) in PBS for 30 minutes, cells were incubated overnight with the specific primary antibodies at 25°C followed by Alexa Fluor 488 anti-mouse or Alexa Fluor 594 anti-rabbit antibodies for 1 hour at 25°C. The cells were stained with DAPI for 5 minutes and analyzed by confocal or immunofluorescent microscopy using LSM510 Axioskop 2 and 5203 Axiophot II microscopes, respectively (Carl Zeiss Vision, San Diego, CA). Immunoprecipitation and western blot analysis was carried out as previously described (Oh et al., 2006), as was TRAP analysis (Kim and Wu, 1997). Briefly, the transfected cells were washed with PBS and lysed with 1× CHAPS lysis buffer (Chemicon, Billerica, MA) containing RNase inhibitor on ice for 30 minutes. After centrifugation at 15,000 ***g*** for 10 minutes, the supernatant protein concentration was determined and then incubated at 30°C for 20 minutes to extend telomeres. The extended products were amplified by PCR using TS and ACX primers for 33 cycles (denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension 72°C for 60 seconds). NT and TSNT primers were added as internal controls. The PCR products were resolved by 12.5% non-denaturing polyacrylamide gel electrophoresis in 0.5× TBE. Silver staining was carried out as previously described (Dalla Torre et al., 2002). Positive control was purchased from Chemicon International (Temicula, CA). For terminal restriction fragment (TRF) analysis, telomere length was analyzed using the TRF assay. Briefly, genomic DNA was extracted from H1299 cells and digested with *Rsa*I and *Hin*dI. Digested genomic DNA was separated on a 0.8% agarose gel and then transferred to a nylon membrane. The blot was hybridized with a telomere-specific probe labeled with [α-^{32}P]dCTP using the Rediprime II random prime labeling system (RPN1633; GE Healthcare, Buckinghamshire, UK). Telomeres were detected by exposing the blot to X-ray film. RT-PCR analysis was carried out as previously

described (Oh et al., 2006). The primers used for RT-PCR were as follows: TERT mRNA, 5′-GAACTTGCGGAAGACAGTGG-3′ (sense), 5′-ATGCGTGAAACCT-GTACGCCT-3′ (antisense); hTERC, 5′-GAAGAGGAACGGAGCGAGTC-3′ (sense), 5′-AAAAAGCGGAAGACGGGAG-3′ (antisense); GAPDH, 5′-GAAGGTGAAG-GTCGGAGTC-3′ (sense), 5′-GAAGATGGTGATGGGATTTC-3′ (antisense).

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-312-C00294), the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (R01-2007-000-20327-02) and a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0820110).

References

Artandi, S. E., Alson, S., Tietze, M. K., Sharpless, N. E., Ye, S., Greenberg, R. A., Castrillon, D. H., Horner, J. W., Weiler, S. R., Carrasco, R. D. et al. (2002). Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proc. Natl. Acad. Sci. USA* **99**, 8191-8196.

Autexier, C. and Lue, N. F. (2006). The structure and function of telomerase reverse transcriptase. *Annu. Rev. Biochem.* **75**, 493-517.

Beausejour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P. and Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* **22**, 4212-4222.

Bernardi, R. and Pandolfi, P. P. (2003). Role of PML and the PML-nuclear body in the control of programmed cell death. *Oncogene* **22**, 9048-9057.

Bernardi, R. and Pandolfi, P. P. (2007). Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat. Rev. Mol. Cell Biol.* **8**, 1006-1016.

Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P. G. and Dejean, A. (2002). Deconstructing PML-induced premature senescence. *EMBO J.* **21**, 3358-3369.

Blackburn, E. H. (2000). The end of the (DNA) line. *Nat. Struct. Biol.* **7**, 847-850.

Blasco, M. A. (2003). Telomeres and cancer: a tale with many endings. *Curr. Opin. Genet. Dev.* **13**, 70-76.

Blasco, M. A. (2005). Mice with bad ends: mouse models for the study of telomeres and telomerase in cancer and aging. *EMBO J.* **24**, 1095-1103.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.

Bryan, T. M., Goodrich, K. J. and Cech, T. R. (2000). Telomerase RNA bound by protein motifs specific to telomerase reverse transcriptase. *Mol. Cell* **6**, 493-499.

Chai, W., Ford, L. P., Lenertz, L., Wright, W. E. and Shay, J. W. (2002). Human Ku70/80 associates physically with telomerase through interaction with hTERT. *J. Biol. Chem.* **277**, 47242-47247.

Cosme-Blanco, W., Shen, M. F., Lazar, A. J., Pathak, S., Lozano, G., Multani, A. S. and Chang, S. (2007). Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence. *EMBO Rep.* **8**, 497-503.

Counter, C. M., Hahn, W. C., Wei, W., Caddle, S. D., Beijersbergen, R. L., Lansdorp, P. M., Sedivy, J. M. and Weinberg, R. A. (1998). Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. *Proc. Natl. Acad. Sci. USA* **95**, 14723-14728.

Dalla Torre, C. A., Maciel, R. M., Pinheiro, N. A., Andrade, J. A., De Toledo, S. R., Villa, L. L. and Cerutti, J. M. (2002). TRAP-silver staining, a highly sensitive assay for measuring telomerase activity in tumor tissue and cell lines. *Braz. J. Med. Biol. Res.* **35**, 65-68.

de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19**, 2100-2110.

Dellaire, G. and Bazett-Jones, D. P. (2004). PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. *BioEssays* **26**, 963-977.

Everett, R. D. and Chelbi-Alix, M. K. (2007). PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* **89**, 819-830.

Feldser, D. M. and Greider, C. W. (2007). Short telomeres limit tumor progression *in vivo* by inducing senescence. *Cancer Cell* **11**, 461-469.

Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P. P., Will, H., Schneider, C. and Del Sal, G. (2000). Regulation of p53 activity in nuclear bodies by a specific PML isoform. *EMBO J.* **19**, 6185-6195.

Gao, C., Cheng, X., Lam, M., Liu, Y., Liu, Q., Chang, K. S. and Kao, H. Y. (2008). Signal-dependent regulation of transcription by histone deacetylase 7 (HDAC7) involves recruitment to PML nuclear bodies. *Mol. Biol. Cell* **19**, 3020-3027.

Gonzalez-Suarez, E., Samper, E., Ramirez, A., Flores, J. M., Martin-Caballero, J., Jorcano, J. L. and Blasco, M. A. (2001). Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. *EMBO J.* **20**, 2619-2630.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W. and Pandolfi, P. P. (2000). The function of PML in p53-dependent apoptosis. *Nat. Cell Biol.* **2**, 730-736.

Harley, C. B. (2008). Telomerase and cancer therapeutics. *Nat. Rev. Cancer* **8**, 167-179.

Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., 3rd and Maul, G. G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J. Cell Biol.* **147**, 221-234.

Jensen, K., Shiels, C. and Freemont, P. S. (2001). PML protein isoforms and the RBCC/TRIM motif. *Oncogene* **20**, 7223-7233.

Kang, H., Kim, E. T., Lee, H. R., Park, J. J., Go, Y. Y., Choi, C. Y. and Ahn, J. H. (2006). Inhibition of SUMO-independent PML oligomerization by the human cytomegalovirus IE1 protein. *J. Gen. Virol.* **87**, 2181-2190.

Kang, S. S., Kwon, T., Kwon, D. Y. and Do, S. I. (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.* **274**, 13085-13090.

Kharbanda, S., Kumar, V., Dhar, S., Pandey, P., Chen, C., Majumder, P., Yuan, Z. M., Whang, Y., Strauss, W., Pandita, T. K. et al. (2000). Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase. *Curr. Biol.* **10**, 568-575.

Kim, J. H., Park, S. M., Kang, M. R., Oh, S. Y., Lee, T. H., Muller, M. T. and Chung, I. K. (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. *Genes Dev.* **19**, 776-781.

Kim, N. W. and Wu, F. (1997). Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.* **25**, 2595-2597.

Lallemand-Breitenbach, V., Zhu, J., Puvion, F., Koken, M., Honore, N., Doubeikovsky, A., Duprez, E., Pandolfi, P. P., Puvion, E., Freemont, P. et al. (2001). Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. *J. Exp. Med.* **193**, 1361-1371.

Lee, J., Sung, Y. H., Cheong, C., Choi, Y. S., Jeon, H. K., Sun, W., Hahn, W. C., Ishikawa, F. and Lee, H. W. (2008). TERT promotes cellular and organismal survival independently of telomerase activity. *Oncogene* **27**, 3754-3760.

Liu, J. P. (1999). Studies of the molecular mechanisms in the regulation of telomerase activity. *FASEB J.* **13**, 2091-2104.

Massard, C., Zermati, Y., Pauleau, A. L., Larochette, N., Metivier, D., Sabatier, L., Kroemer, G. and Soria, J. C. (2006). hTERT: a novel endogenous inhibitor of the mitochondrial cell death pathway. *Oncogene* **25**, 4505-4514.

Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A. et al. (2003). Telomerase maintains telomere structure in normal human cells. *Cell* **114**, 241-253.

McEachern, M. J., Krauskopf, A. and Blackburn, E. H. (2000). Telomeres and their control. *Annu. Rev. Genet.* **34**, 331-358.

Melnick, A. and Licht, J. D. (1999). Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* **93**, 3167-3215.

Nisole, S., Stoye, J. P. and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat. Rev. Microbiol.* **3**, 799-808.

Oh, W., Lee, E. W., Sung, Y. H., Yang, M. R., Ghim, J., Lee, H. W. and Song, J. (2006). Jab1 induces the cytoplasmic localization and degradation of p53 in coordination with Hdm2. *J. Biol. Chem.* **281**, 17457-17465.

Pearson, M. and Pelicci, P. G. (2001). PML interaction with p53 and its role in apoptosis and replicative senescence. *Oncogene* **20**, 7250-7256.

Reineke, E. L., Lam, M., Liu, Q., Liu, Y., Stanya, K. J., Chang, K. S., Means, A. R. and Kao, H. Y. (2008). Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. *Mol. Cell. Biol.* **28**, 997-1006.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S. et al. (2001). The tripartite motif family identifies cell compartments. *EMBO J.* **20**, 2140-2151.

Seimiya, H., Tanji, M., Oh-hara, T., Tomida, A., Naasani, I. and Tsuruo, T. (1999). Hypoxia up-regulates telomerase activity via mitogen-activated protein kinase signaling in human solid tumor cells. *Biochem. Biophys. Res. Commun.* **260**, 365-370.

Sharpless, N. E. and DePinho, R. A. (2004). Telomeres, stem cells, senescence, and cancer. *J. Clin. Invest.* **113**, 160-168.

Stampfer, M. R., Garbe, J., Levine, G., Lichtsteiner, S., Vasserot, A. P. and Yaswen, P. (2001). Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(-) human mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* **98**, 4498-4503.

Takahashi, Y., Lallemand-Breitenbach, V., Zhu, J. and de The, H. (2004). PML nuclear bodies and apoptosis. *Oncogene* **23**, 2819-2824.

Wei, X., Yu, Z. K., Ramalingam, A., Grossman, S. R., Yu, J. H., Bloch, D. B. and Maki, C. G. (2003). Physical and functional interactions between PML and MDM2. *J. Biol. Chem.* **278**, 29288-29297.

Wu, Y. L., Dudognon, C., Nguyen, E., Hillion, J., Pendino, F., Tarkanyi, I., Aradi, J., Lanotte, M., Tong, J. H., Chen, G. Q. et al. (2006). Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. *J. Cell Sci.* **119**, 2797-2806.

Xia, J., Peng, Y., Mian, I. S. and Lue, N. F. (2000). Identification of functionally important domains in the N-terminal region of telomerase reverse transcriptase. *Mol. Cell. Biol.* **20**, 5196-5207.

Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G. and Pisa, P. (2000). Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. *Oncogene* **19**, 5123-5133.

Zhang, X., Mar, V., Zhou, W., Harrington, L. and Robinson, M. O. (1999). Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.* **13**, 2388-2399.

Zheng, P., Guo, Y., Niu, Q., Levy, D. E., Dyck, J. A., Lu, S., Sheiman, L. A. and Liu, Y. (1998). Proto-oncogene PML controls genes devoted to MHC class I antigen presentation. *Nature* **396**, 373-376.

Zhong, S., Muller, S., Ronchetti, S., Freemont, P. S., Dejean, A. and Pandolfi, P. P. (2000). Role of SUMO-1-modified PML in nuclear body formation. *Blood* **95**, 2748-2752.