



# Akt regulates TPP1 homodimerization and telomere protection

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## Summary

**Telomeres are specialized structures at the ends of eukaryotic chromosomes that are important for maintaining genome stability and integrity. Telomere dysfunction has been linked to aging and cancer development. In mammalian cells, extensive studies have been carried out to illustrate how core telomeric proteins assemble on telomeres to recruit the telomerase and additional factors for telomere maintenance and protection. In comparison, how changes in growth signaling pathways impact telomeres and telomere-binding proteins remains largely unexplored. The phosphatidylinositol 3-kinase (PI3-K)/Akt (also known as PKB) pathway, one of the best characterized growth signaling cascades, regulates a variety of cellular function including cell proliferation, survival, metabolism, and DNA repair, and dysregulation of PI3-K/Akt signaling has been linked to aging and diseases such as cancer and diabetes. In this study, we provide evidence that the Akt signaling pathway plays an important role in telomere protection. Akt inhibition either by chemical inhibitors or small interfering RNAs induced telomere dysfunction. Furthermore, we found that TPP1 could homodimerize through its OB-fold, a process that was dependent on the Akt kinase. Telomere damage and reduced TPP1 dimerization as a result of Akt inhibition was also accompanied by diminished recruitment of TPP1 and POT1 to the telomeres. Our findings highlight a previously unknown link between Akt signaling and telomere protection.**

**Key words:** Akt; telomere protection; TPP1.

## Introduction

Chromosomal ends or telomeres are specialized protein–DNA complexes that ensure chromosome stability and integrity (Palm & de Lange, 2008; Xin *et al.*, 2008; O'Sullivan & Karlseder, 2010). In mammalian cells, the six-protein telosome/shelterin complex (TRF1, TRF2, RAP1, TIN2, TPP1, and POT1) assembles on telomeres and recruits the telomerase as well as

other factors from diverse pathways (e.g., DNA damage response) for telomere maintenance and protection (de Lange, 2005; O'Connor *et al.*, 2006; Nandakumar & Cech, 2013). Telomere dysregulation can lead to the loss of genetic information and genomic instability, cellular senescence, abnormal cell growth and proliferation, premature aging, and cancer (Deng *et al.*, 2008; Donate & Blasco, 2011; Armanios & Blackburn, 2012; Frias *et al.*, 2012). For instance, progressive telomere shortening is directly implicated in replicative senescence, and reactivation of the telomerase represents one of the hallmarks of cancer cells (Shay & Wright, 2011). In addition, mutation and dysfunction of telomerase complex components (e.g., dyskerin) and telosome/shelterin subunits (e.g., TIN2) have been identified in diseases with premature aging phenotypes and predisposition to cancer (Heiss *et al.*, 1998; Savage *et al.*, 2008; Vulliamy *et al.*, 2008; Walne *et al.*, 2008; Zhong *et al.*, 2011; Sasa *et al.*, 2012).

The protein kinase Akt/PKB has also been intimately linked to cancer and aging. Functioning downstream of phosphatidylinositol 3-kinase (PI3-kinase), Akt is frequently activated in human cancers and a prime target for pharmacological intervention (Manning & Cantley, 2007; Carnero, 2010; Kloet & Burgering, 2011; Tzivion & Hay, 2011). Akt regulates a wide array of signaling pathways, including cell growth, survival, proliferation, metabolism, and migration. Its well-studied role in the insulin/IGF-1 pathway among others also connects Akt to aging and longevity (Kloet & Burgering, 2011). Akt has also been shown to suppress DNA damage processing and checkpoint activation in late G2 and promote DNA double-strand break (DSB) repair (Xu *et al.*, 2010; Deng *et al.*, 2011). In addition, several studies point to links between Akt and telomere regulators. For example, Akt was shown to interact with and phosphorylate hTERT and TRF1. Phosphorylation of the hTERT nuclear localization signal appeared critical for hTERT nuclear targeting (Kang *et al.*, 1999; Haendeler *et al.*, 2003; Chung *et al.*, 2012), and Akt may modulate TRF1 levels and telomere length through its interaction with TRF1 (Chen *et al.*, 2009). These studies suggest cross talk between Akt and telomere regulatory pathways and implicate Akt in communicating growth and proliferative signals to the telomeres.

To further study how growth signals in the cytosol are transmitted to the nucleus for the maintenance of telomeres, we investigated telomere status in response to nutritional stress and inhibition of Akt activity. Our data indicate telomere damage as well as disrupted TPP1 and POT1 recruitment under these conditions. Interestingly, we also found that TPP1 could homodimerize through its OB-fold, a process sensitive to starvation and regulated by Akt. These findings suggest that Akt can protect telomeres by regulating TPP1 homodimerization, likely enhancing the association of TPP1 and TPP1–POT1 heterodimer with the telomeres.

## Results

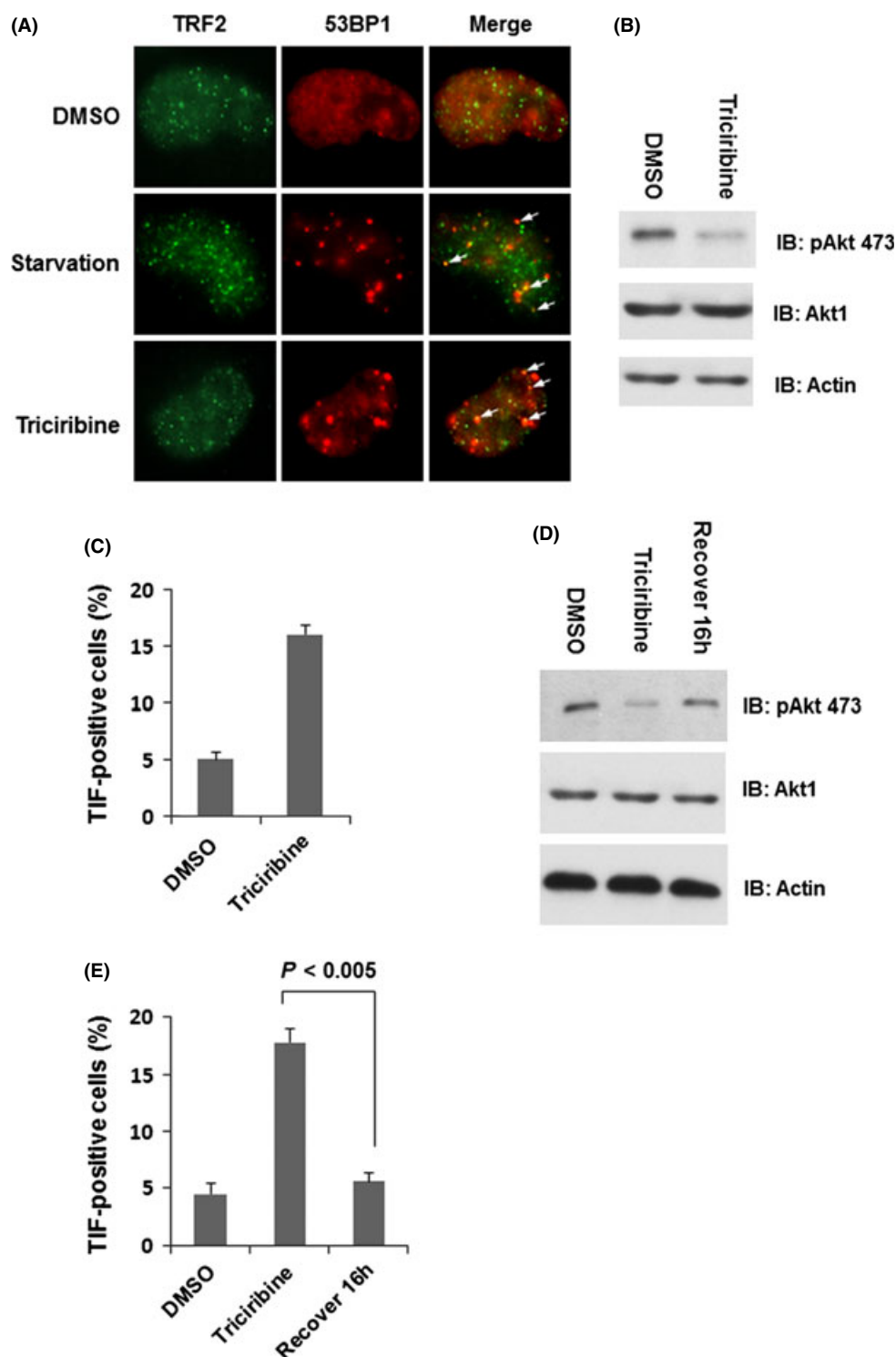
### Akt activity is important for telomere end protection

It is plausible that proliferative stimuli may activate protective mechanisms for telomeres to ensure prolonged growth. Conversely, nutritional stress may prove deleterious for telomeres. Indeed, when human HTC75 cells were grown under starvation conditions (0.01% serum), we noticed increased 53BP1 foci at telomeres that accompanied growth inhibition in these cells (Fig. 1A). These observations suggest that telomeres may be sensitive to changes in nutrients, and that cell growth signaling may play

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Accepted for publication 10 July 2013



**Fig. 1** Akt activity is important for telomere end protection. (A) Telomere dysfunction-induced foci (TIF) analysis of HTC75 cells serum starved (0.01% FBS) for 16 h or treated with triciribine (1  $\mu$ M) for 3 h. Cells were immunostained with anti-53BP1 (red) and TRF2 (green) antibodies. DMSO-treated cells were used as controls. Arrows indicate overlapping foci. (B) Western blot analysis was carried out using HTC75 cells treated with DMSO or triciribine (1  $\mu$ M) for 3 h with the indicated antibodies. (C) Cells from (B) were immunostained as in (A) and the percentage of TIF-positive cells was quantified. Only cells with >4 co-localized foci were scored. Error bars indicate SEM ( $n = 3$ ). (D) HTC75 cells were treated with triciribine for 3 h and then maintained for another 16 h in the absence of triciribine. Western blot analysis was then carried out using the indicated antibodies. (E) Cells from (D) were examined by immunostaining as described in (A) and quantitated. Error bars indicate SEM ( $n = 3$ ).  $P$ -value was determined by the Student  $t$ -test.

a positive role in telomere maintenance. Given the importance of Akt in mediating cellular growth responses, we first determined its role in telomere end protection under growth conditions. We treated cells with triciribine, an Akt specific inhibitor, and analyzed telomere dysfunction-induced foci (TIF) in these cells. As shown in Fig. 1B, treatment with triciribine led to reduced Akt phosphorylation, indicating inhibition of Akt activity and its signaling. Concurrently, we found a ~3-fold increase

in the percentage of TIF-positive cells in triciribine-treated cells (Fig. 1C), supporting the idea that Akt activity is important for telomere protection.

Removing triciribine can reverse its inhibition of Akt and allow cells to resume growth and proliferation. We next examined whether telomere damage as a result of Akt inhibition would persist in cells after the inhibitor was removed. As expected, Akt activity recovered (as indicated

by Akt phosphorylation) following removal of triciribine (Fig. 1D). This recovery coincided with the decrease in TIF formation in these cells (from 17.8% to 5.6%) (Fig. 1E). In fact, we were able to observe fewer telomeric DNA damage foci as early as 3 h after inhibitor removal (data not shown). These results support the notion that Akt plays a role in protecting telomeres, and that telomere dysfunction as a result of Akt inhibition may be a reversible process.

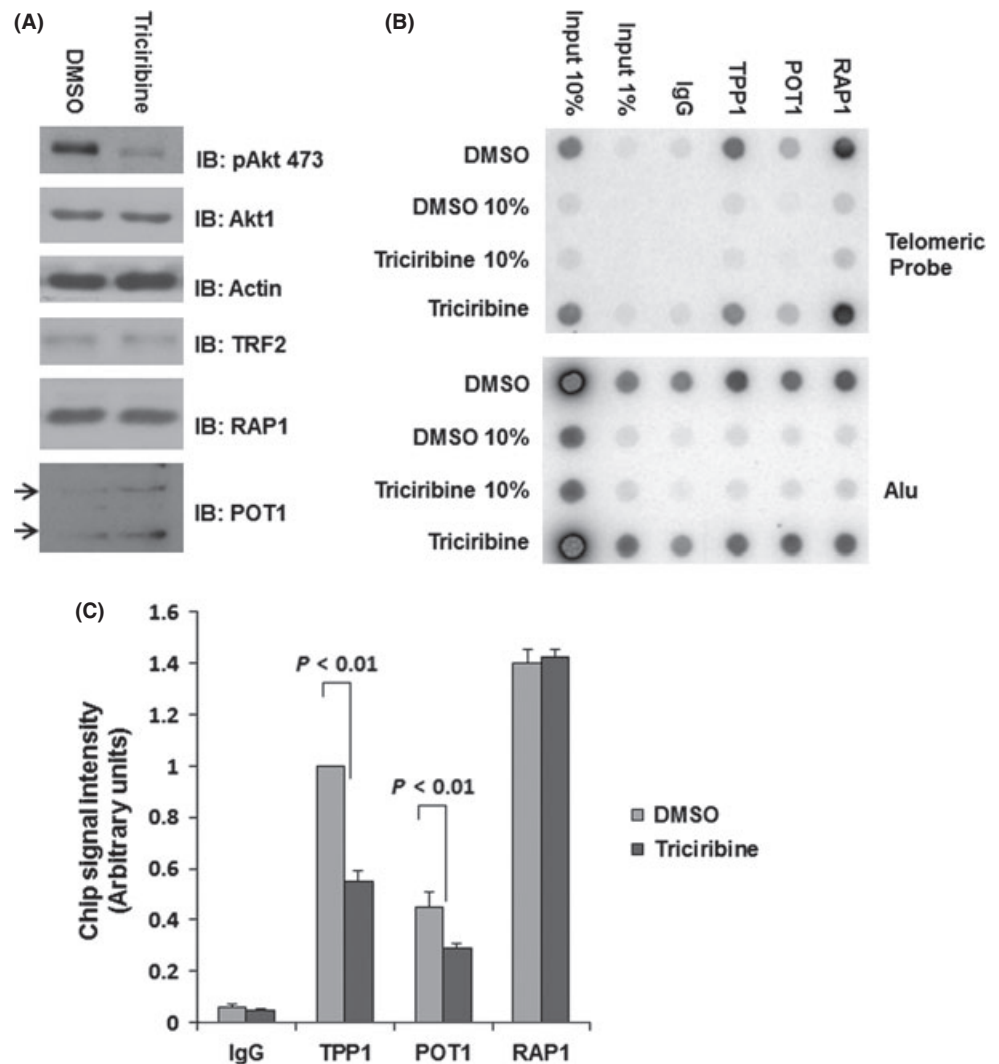
### Akt activity is important for TPP1 and POT1 recruitment to the telomeres

Akt may exert its protective activity through up-regulating total amounts of telomeric proteins; consequently, Akt inhibition may down-regulate telomere protein levels. However, we did not observe any drastic changes after triciribine treatment in the levels of core telomeric proteins that we examined (Fig. 2A). We therefore speculated that Akt might protect telomeres by promoting the telomeric recruitment of the telosome/shelterin complex. When Akt is inhibited, reduced association of telomeric proteins would lead to exposed telomere ends. Loss of telomere end protection as a result of compromised telomeric protein

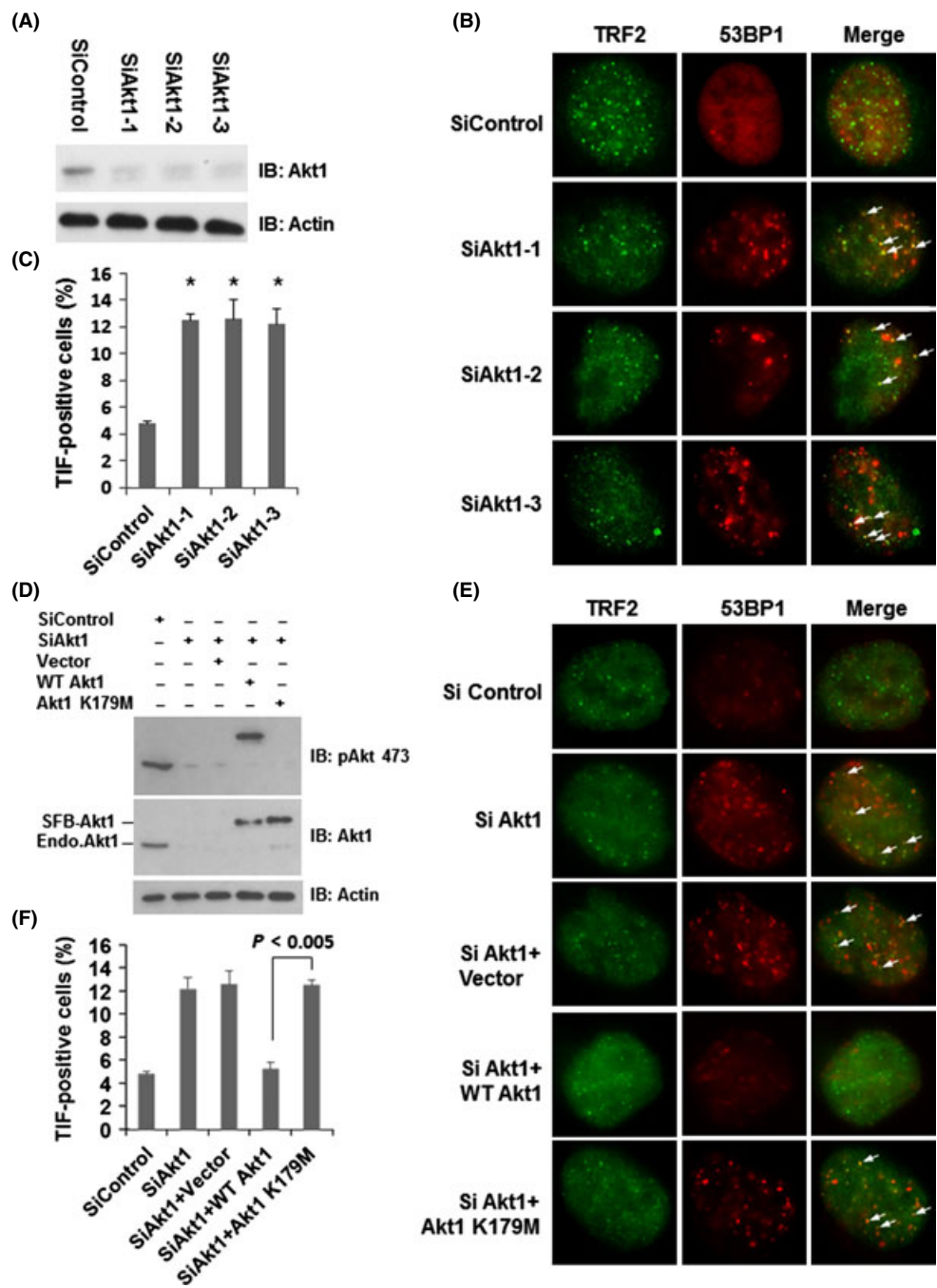
targeting has been well established. For example, we have shown previously that loss of TPP1 can decrease POT1 telomere recruitment and increase telomere damage (Liu *et al.*, 2004). To test our hypothesis, we performed chromatin immunoprecipitation (ChIP) assays to examine association of core telomeric proteins with telomeres in cells treated with triciribine. Of the six core telomeric proteins, TPP1 and POT1 were the only ones that consistently exhibited diminished telomere binding with drug treatment (Fig. 2B and C), lending support to the notion that Akt may positively regulate the concentration of TPP1 and POT1 at telomeres.

### The Akt1 isoform is the predominant player in telomere protection

Of the three Akt isoforms (Akt1-3), Akt1 appears the most widely expressed and the best studied to date. In HTC75 cells, Akt1 is highly expressed (Kim *et al.*, 2001). When we transiently transfected three different siRNAs specific for Akt1 into these cells, all three oligos achieved >80% knockdown efficiency (Fig. 3A). Importantly, depletion of Akt1 by all three siRNAs elevated TIF formation (Fig. 3B and C). In comparison, knocking down Akt2 and Akt3 isoforms had little effect on



**Fig. 2** Telomeric recruitment of TPP1 and POT1 requires Akt activity. (A) HTC75 cells treated with triciribine or mock-treated with DMSO for 3 h were analyzed by Western blotting using the indicated antibodies. Actin was used as loading control. Arrows indicate the two POT1 isoforms. (B) Cells from (A) were examined by telomere chromatin immunoprecipitation (ChIP) using antibodies against TPP1, POT1 and RAP1, followed by dot-blotting with probes against telomere sequences or Alu repeats. Rabbit IgG served as control. (C) Quantification of data from (B) (two independent experiments). ChIP signal intensities were normalized against input DNA. Error bars represent SD. *P*-values were determined by the Student *t*-test.



**Fig. 3** Akt1 participates in telomere protection. (A) HTC75 cells transiently transfected with three different siRNA oligos against Akt1 were examined by Western blotting. Actin was used as loading control. (B) Cells from (A) were examined by immunostaining using anti-53BP1 (red) and TRF2 (green) antibodies. Arrows indicate overlapping foci. (C) Quantification of data from (B). Only cells with >4 co-localized foci were scored. Error bars indicate SEM. ( $n = 3$ ). The symbol \*  $P$ -values ( $P < 0.005$ ) were determined by the Student  $t$ -test. (D) HTC75 cells were transfected with a siRNA oligo against Akt1 (siAkt1-1) in combination with siRNA-resistant SFB-tagged wild-type (WT) Akt1 or kinase-dead Akt1 (Akt1 K179M) and analyzed by Western blotting using the indicated antibodies. Actin was used as loading control. Bands that correspond to exogenous and endogenous (Endo) Akt were indicated. (E) Cells from (D) were examined by immunostaining using anti-53BP1 (red) and TRF2 (green) antibodies. Arrows indicate overlapping foci. (F) Quantification of data from (E). Only cells with >4 co-localized foci were scored. Error bars indicate SEM ( $n = 3$ ).  $P$ -values were determined by the Student  $t$ -test.

TIF formation in these cells (Fig. S1), suggesting that Akt1 is the key Akt isoform that mediates telomere end protection.

To further explore the role of Akt1 activity in telomere protection, we ectopically expressed RNAi-resistant wild-type Akt1 as well as the kinase-dead Akt1 mutant (Akt1 K179M) in Akt1 knockdown cells. Both proteins were expressed at levels comparable to endogenous Akt (Fig. 3D). Furthermore, only endogenous and wild-type Akt could be blotted with the anti-phospho Akt (residue S473) antibody, indicating that Akt1-K179M was indeed inactive. As shown in Fig. 3E and F, expression of wild-type Akt1, but not kinase-dead Akt1, could rescue the TIF phenotype of the Akt1 knockdown cells, further supporting the notion

that Akt1 is critical for telomere protection and underscoring the importance of its kinase activity in this process.

### TPP1 can homodimerize *in vivo*

Extensive studies have demonstrated the importance and function of multiple pair-wise interactions within the telosome/shelterin complex. By binding to multiple telosome subunits, TIN2 acts as the linchpin for telosome/shelterin assembly, bridging double- and single-stranded DNA binding activities (O'Connor *et al.*, 2006; Takai *et al.*, 2011). TIN2–TPP1 interaction ensures TPP1 targeting to the telomeres, and the heterodimer



of TPP1–POT1 in turn helps recruit the telomerase and regulate its activity and processivity (Liu *et al.*, 2004; Wang *et al.*, 2007; Xin *et al.*, 2007; Nandakumar *et al.*, 2012; Zhong *et al.*, 2012; Zhang *et al.*, 2013). The disruption of such interactions can compromise telomere length control and end protection.

Interactions between telomere proteins can be visualized in live cells using the Bi-molecular Fluorescence Complementation (BiFC/PCA) assay (Kim *et al.*, 2009; Lee *et al.*, 2011). In an YFP-based BiFC assay (Fig. 4A), the interaction between two proteins that are tagged respectively with split YFP fragments brings the YFP fragments to close proximity for co-folding and fluorescence complementation (Hu *et al.*, 2002; Wilson *et al.*, 2004). As expected, cells co-expressing the TPP1–TIN2 pair displayed strong YFP-fluorescence complementation (Fig. 4A). Interestingly, we were also able to detect fluorescence in cells stably expressing the TPP1–TPP1 pair, suggesting homodimerization of the TPP1 protein. To confirm TPP1 dimer formation, we also carried out immunoprecipitation experiments using cells co-expressing Myc- and Flag-tagged TPP1. As shown in Fig. 4B, anti-Flag IP could bring down Flag-TPP1 as well as Myc-TPP1, providing further evidence for the ability of TPP1 to homodimerize *in vivo*.

### The TPP1 OB-fold can mediate TPP1 homodimerization

Previous studies have demonstrated that TPP1 utilizes multiple domains for its interaction with other proteins. When we tested the N-terminal OB-fold deletion mutant of TPP1 (TPP1-ΔOB), we found that it lost the ability to homodimerize (Fig. 4C), indicating that the requirement of the OB-fold for TPP1–TPP1 interaction.

To further confirm the role of OB-fold in mediating TPP1 homodimerization, we carried out *in vitro* GST pull-down assays using bacterially expressed GST or maltose-binding protein (MBP)-tagged TPP1 OB-fold proteins. As controls, MBP-tagged FAM118B (a membrane protein) and GST-tagged Ras-binding domain (RBD) of Raf-1 were also included. As shown in Fig. 4D, GST-TPP1 OB was able to precipitate MBP-tagged TPP1 OB, indicating that the OB-fold is sufficient for mediating TPP1 homodimerization.

We reasoned that Akt might regulate telomeres through core telomere-binding proteins such as TPP1. To test this idea, we ectopically expressed full-length and OB-fold deletion mutant of TPP1 in Akt1 knockdown cells (Fig. 4E) and assessed TIF formation. As shown in Fig. 4F and G, expression of wild-type TPP1 in Akt1-depleted cells led to a reduction of TIFs to levels comparable to control cells, supporting the idea that Akt1 can function through TPP1 to regulate telomeres. In addition, deletion of the OB-fold abolished the rescue ability of TPP1, again confirming the importance of OB-fold and suggesting that OB-fold-dependent dimerization of TPP1 might be important for telomere protection.

### TPP1 homodimerization depends on Akt1 activity

Growth arrest signals often set off cascades of cellular events that lead from inhibition of PI 3-kinase/Akt to dysfunction at the telomeres, perhaps by altering the concentration (or composition) of telomere-bound proteins and protein complexes. Such alterations can be revealed using the BiFC assay, offering clues to the dynamics of the telosome/shelterin complex. To this end, we compared YFP-fluorescence complementation signals in HTC75 cells co-expressing YFPn-tagged TPP1 with YFPc-tagged TPP1 or TIN2, under serum starvation conditions or with triciribine. As shown in Fig. 4A, the percentage of YFP + TPP1–TPP1 co-expressing cells was considerably lower than TPP1–TIN2 co-expressing

cells. However, only the interaction between TPP1 and TPP1, not TPP1 and TIN2, was sensitive to serum starvation or triciribine treatment (Fig. 5A). In support of this observation, co-immunoprecipitation of Myc-TPP1 and TPP1-Flag was also sensitive to triciribine (Fig. 5B), suggesting that Akt is important for regulating TPP1 homodimerization. In fact, we found Akt1 could interact with TPP1. As shown in Fig. 5B, Myc-TPP1 could co-immunoprecipitate with SFB-Akt1. When Akt1 knockdown cells were examined, TPP1–TPP1 BiFC complementation was abolished with all three siRNAs (Fig. 5C and D).

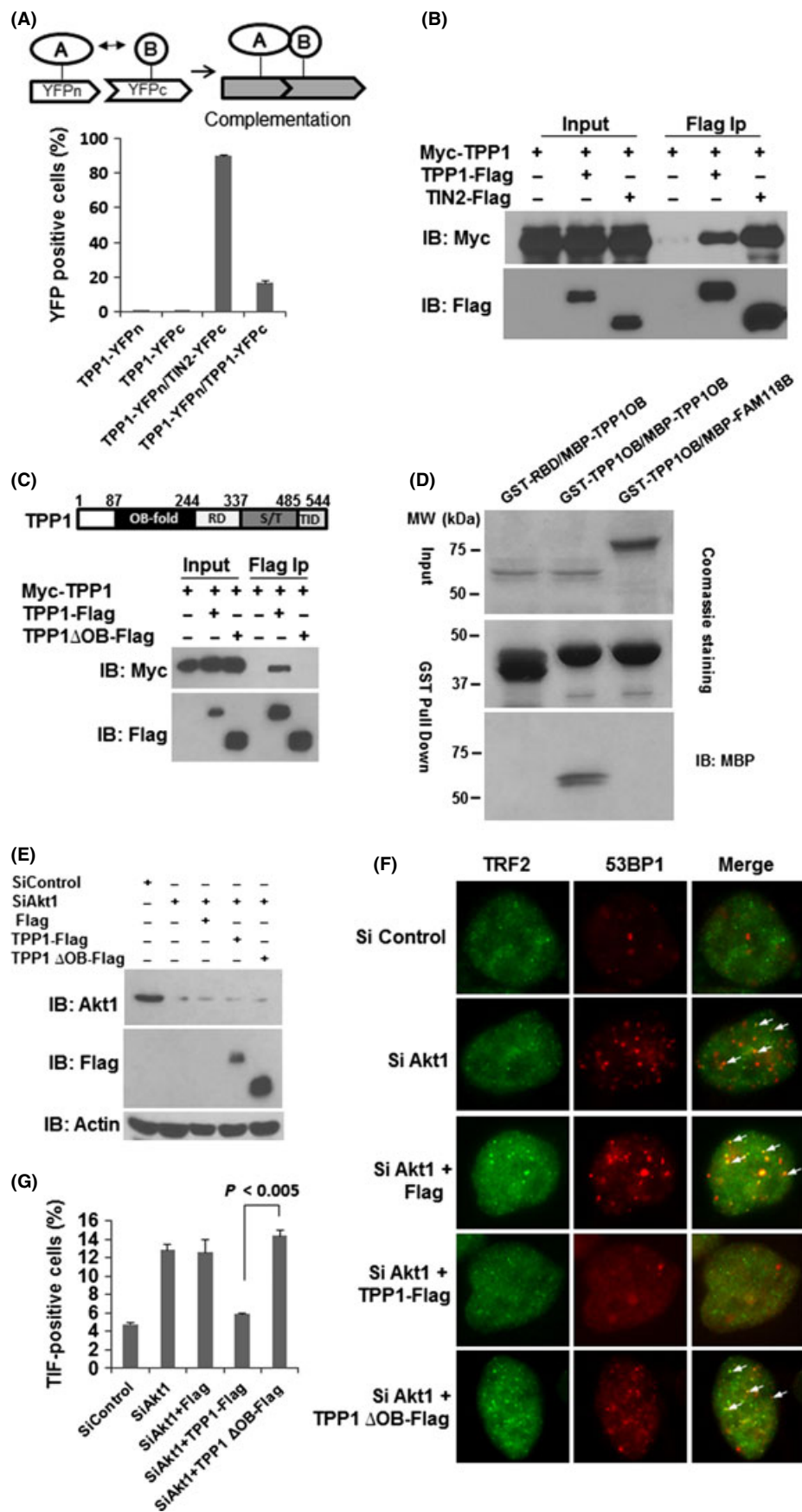
To further investigate the requirement of Akt1 kinase activity in regulating TPP1 dimer formation, we transiently introduced SFB-tagged wild-type and kinase-dead Akt1 into cells already co-expressing differently tagged TPP1 proteins and carried out BiFC assays (Fig. 5E). Consistent with the observation that Akt inhibition could block the formation of TPP1 dimers, rescue expression of wild-type Akt1 was able to restore TPP1 homodimerization to levels similar to control cells (Fig. 5F). In fact, in control knockdown cells, ectopic expression of Akt1 enhanced TPP1 dimer formation as well. These data support the notion that Akt1 may participate in telomere protection through regulating TPP1 homodimerization. Importantly, an active Akt1 kinase is required in this process, because the kinase-dead mutant Akt1 K179M failed to rescue TPP1–TPP1 interaction in the BiFC assay (Fig. 5F). Collectively, these results suggest that TPP1 homodimerization may promote telosome/shelterin integrity and increase TPP1 concentration at telomeres, a process regulated by cell growth signaling pathways via Akt activation. As a result, perturbations in Akt signaling pathway may impact telomere protection through changes in TPP1 recruitment at the telomeres.

## Discussion

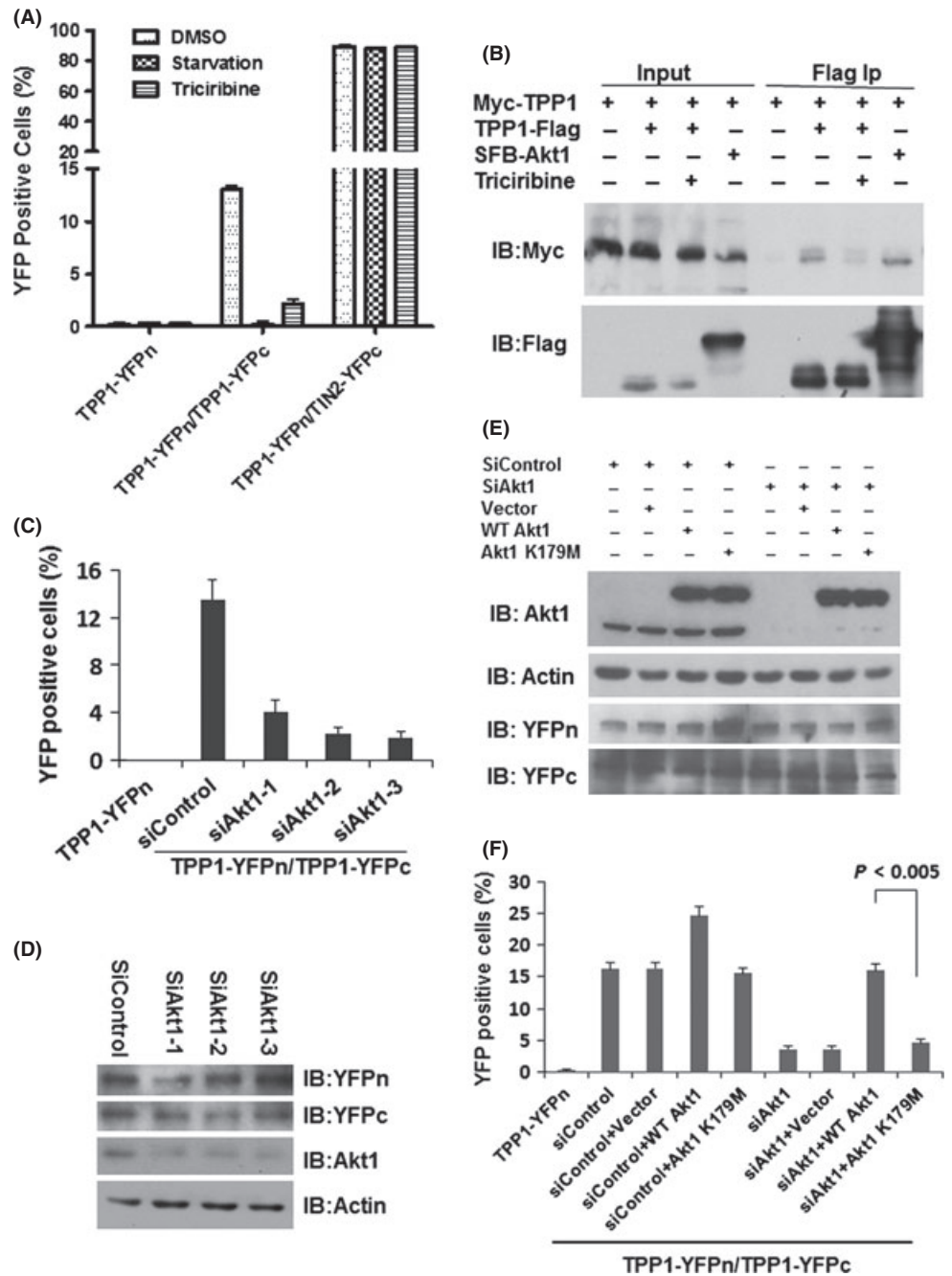
In this study, we linked growth signaling pathways mediated by Akt to telomere maintenance. We provide evidence that Akt may directly regulate telomere protection by promoting homodimerization of TPP1. This is the first study to report TPP1 dimer formation. It is likely that increased TPP1 homodimerization would increase the amount of TPP1 at telomeres, which in turn would also enhance POT1 binding on single-stranded telomere DNA, augmenting telomere protection and maintenance. Indeed, when Akt signaling was inhibited, we observed reduced targeting of both TPP1 and POT1 to telomeres, which was accompanied by increased TIF formation.

Compared to its other cellular functions, the precise role of Akt in telomere protection is less well understood. Here, we provide additional evidence that indicates a direct role of Akt in protecting chromosomal ends – positively regulating the concentration of TPP1 and POT1 at the telomeres and promoting telosome/shelterin assembly. In addition to TPP1, Akt may also regulate the assembly and function of the telosome/shelterin through other telosome/shelterin subunits, for example TRF1 (Chen *et al.*, 2009). Moreover, using epitope-tagged hTERT, it was shown that Akt phosphorylation of the nuclear localization signal of hTERT was critical for hTERT nuclear targeting (Kang *et al.*, 1999; Chung *et al.*, 2012). These findings suggest that alternative pathways are utilized to integrate signaling cascades that can connect extracellular stimuli to nuclear events, for telomere maintenance in response to cell growth and proliferation.

The three isoforms of Akt (Akt1–3) are encoded by separate genes and share extensive amino acid sequence identity (especially in the kinase domain), with virtually indistinguishable *in vitro* substrate specificities (Walker *et al.*, 1998). Growing evidence points to distinct function of the three Akt isoforms, likely due to their cell/tissue-specific expression and subcellular localization patterns. This possibility is also



**Fig. 4** TPP1 can homodimerize through its OB-fold. (A) HTC75 cells co-expressing YFPn-tagged TPP1 with YFPc-tagged TPP1 or TIN2 were examined by BiFC assays. The percentage of cells displaying fluorescence complementation was quantitated by flow cytometry. Error bars indicate standard error (n = 3). (B) 293T cells transiently co-expressing Myc-tagged TPP1 with Flag-tagged TPP1 or TIN2 were immunoprecipitated with anti-Flag antibodies. The immunoprecipitates were Western-blotted as indicated. (C) 293T cells transiently co-expressing Myc-tagged TPP1 with Flag-tagged TPP1 or TPP1 OB-fold deletion mutant (TPP1ΔOB) were immunoprecipitated with anti-Flag antibodies. The immunoprecipitates were Western-blotted as indicated. (D) Bacterially purified GST-tagged TPP1 OB-fold only mutant (TPP1 OB) was incubated with maltose-binding protein (MBP)-tagged TPP1 OB for GST pull-down assays. The precipitates were resolved by SDS-PAGE and visualized by Coomassie staining or Western blotting. GST-tagged Raf-1 Ras-binding domain (RBD) and MBP-tagged FAM118B were used as controls. (E) HTC75 cells were transfected with siRNA oligos against Akt1 (siAKT1-1) in combination with Flag-tagged wild-type TPP1 or TPP1 OB-fold deletion mutant (TPP1ΔOB) and then analyzed by Western blotting using the indicated antibodies. Actin was used as loading control. (F) Cells from (E) were examined by immunostaining using anti-53BP1 (red) and TRF2 (green) antibodies. Arrows indicate overlapping foci. (G) Quantification of data from (F). Only cells with >4 co-localized foci were scored. Error bars indicate SEM (n = 3). P-values were determined by the Student t-test.



**Fig. 5** Akt1 can regulate TPP1 homodimerization. (A) HTC75 cells co-expressing YFPn-tagged TPP1 with YFPc-tagged TPP1 or TIN2 were either serum-starved (0.01% FBS) or treated with triciribine, and then examined by BiFC assays. Error bars indicate standard error ( $n = 3$ ). (B) 293T cells transiently co-expressing TPP1-Flag with Myc-TPP1 were treated with DMSO or triciribine. Cells co-expressing SFB-Akt1 and Myc-TPP1 were also examined. Cell lysates were immunoprecipitated with anti-Flag antibodies and blotted as indicated. (C) HTC75 cells co-expressing YFPn-TPP1 with YFPc-TPP1 were transiently transfected with siRNA oligos against Akt1 and analyzed in BiFC assays. Error bars indicate standard errors ( $n = 3$ ). (D) Cells from (C) were examined by Western blotting for Akt1 knockdown efficiency. Actin was used as loading control. (E) HTC75 cells co-expressing YFPn-TPP1 and YFPc-TPP1 were transiently co-transfected with control oligos or siRNA oligos against Akt1 in combination with siRNA-resistant SFB-tagged wild-type (WT) Akt1 or kinase-dead Akt1 (Akt1 K179M). The cells were then examined by Western blotting using the indicated antibodies. Actin was used as a loading control. (F) Cells from (E) were analyzed in BiFC assays.  $P$ -values were determined by the Student  $t$ -test.

supported by our findings that inhibition of Akt2 and Akt3 did not appear to affect telomere protection. It is also possible that due to its high expression in the cells we tested, Akt1 may be the predominant isoform for many cellular processes including telomere protection.

Our study revealed a novel link between telomere maintenance and the PI3-K/Akt pathway. Under normal growth conditions, the activity of Akt ensures telomere integrity and protection that support cell growth and prevent cellular senescence. Conversely, unstable telomeres as a result of attenuated Akt activity from nutritional stress may facilitate growth arrest and checkpoint response. Understanding how such checks and balances may be compromised in human cells will facilitate our quest for uncovering mechanisms of oncogenesis and aging and identifying new treatment and therapeutics.

## Experimental procedures

### Vectors, siRNAs, and Bi-molecular fluorescence complementation assay

cDNAs encoding various human wild-type or mutant Akt1, TPP1, and TIN2 were cloned into pBabe- or pCL-based retroviral vectors. The TPP1 OB-fold deletion mutant was generated by deleting amino acids 1–240. TPP1 OB contains residues 87–240. TPP1 OB-fold alone (residues 87–251) was tagged with either GST or MBP for bacterial expression. Human Raf-1 Ras-binding domain (RBD, residues 1–149) and full-length FAM118B were respectively tagged with GST and MBP for bacterial expression.

Cells expressing protein pairs respectively tagged with YFPc (residues 156–239 of YFP) and YFPn (residues 1–155 of Venus YFP) were analyzed by flow cytometry as previously described (Lee *et al.*, 2011). 293T cells were used for transient expression and retroviral packaging. HTC75 cells were used for stable protein expression.

siRNAs against Akt1, Akt2, and Akt3 were purchased from Sigma:

siAkt1-1: SASI\_Hs01\_00105952; siAkt1-2 SASI\_Hs01\_00105953; and siAkt1-3 SASI\_Hs01\_00105954.

siAkt2-1: SASI\_Hs01\_00035055; siAkt2-2 SASI\_Hs01\_00035057.

siAkt3-1: SASI\_Hs01\_00122808; siAkt3-2: SASI\_Hs01\_00122809; siAkt3-3: SASI\_Hs01\_00122810.

For RNAi-resistant expression of Akt1 constructs, we used siAkt1-1 that targets the 3'UTR of Akt1 mRNA to knockdown Akt1.

## RT-qPCR

Total RNAs were isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed using an ABI StepOnePlus real-time PCR system and SYBR Green Master Mix (Invitrogen, Carlsbad, CA, USA).

## Telomere dysfunction-induced foci assays

Telomere dysfunction-induced foci assays were performed essentially as described previously (Kim *et al.*, 2009). Briefly, cells grown overnight on cover slips were first permeabilized with 0.5% TritonX-100 in 1× phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and then permeabilized again with 0.5% TritonX-100 plus 300 mM sucrose. The cells were subsequently co-immunostained with anti-53BP1 (Novus, NB100-304) and anti-TRF2 (Calbiochem, OP129) antibodies, and visualized under a Nikon TE200 fluorescence microscope.

## Co-immunoprecipitation and Western blotting

To prepare cell lysates for co-immunoprecipitation, cells were lysed in NETN (20 mM Tris (pH8.0), 1 mM EDTA, 100 mM NaCl, 0.5% NP-40) buffer supplemented with protease inhibitor cocktails (Sigma, St. Louis, MO, USA) and subjected to centrifuge at 14 000 g for 30 min. After centrifugation, the supernatant was used for immunoprecipitation with anti-Flag M2 affinity resins (Sigma) on ice for 1 h. After four washes with NETN buffer, the proteins were eluted with Flag peptide (200 µg/ml), resolved by SDS-PAGE, and Western-blotted.

The antibodies used are the following: polyclonal anti-Flag (Sigma, F7425), anti-Flag M2 (Sigma, A8592), polyclonal anti-GFP (Abcam, ab290, Cambridge, MA, USA), monoclonal anti-GFP (N-term) (EPITO-MICS, 1533-1, Burlingame, CA, USA), anti-hTRF2 (CalBiochem, OP129, Billerica, MA, USA), anti-Akt (Cell Signaling Technology, 40D4, Danvers, MA, USA), anti-phospho Akt (Ser473) (Cell Signaling Technology, 9271), polyclonal anti-RAP1 (Bethyl Laboratories, Montgomery, TX, USA), anti-POT1 (Novus Biologicals, NB500, Littleton, CO, USA), anti-MBP (Millipore, 05-499, Billerica, MA, USA), anti-Myc (Santa Cruz Biotechnology, sc-40, Santa Cruz, CA, USA), and anti-actin (Sigma, A3853).

## ChIP

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Liang *et al.*, 2008). Briefly, cells were fixed in 1% formaldehyde and lysed in 1% Triton X-100, 150 mM NaCl, and 2 mM EDTA, and 50 mM HEPES (pH7.5). Sonicated lysates were incubated with

the appropriate antibodies (3 µg), and the DNA was eluted in 0.5%SDS and 0.1 M NaHCO<sub>3</sub> and purified with the QIAquick PCR purification kit (Qiagen). The DNA was slot-blotted onto a Hybond N+ membrane and hybridized with a telomeric G-rich probe (5'-TTAGGGTTAGGGT TAGGGT-3') or Alu probe (5'-GGCCGGGCGCGGTGGCTCACGCCTG TAATCCCAGCA-3'). The signals were quantitated using a PhosphorImager. The antibodies used for ChIP are the following: rabbit polyclonal POT1 (N-terminal) (O'Connor *et al.*, 2006), rabbit polyclonal TPP1 (467) (Liu *et al.*, 2004), polyclonal anti-RAP1 (Bethyl Laboratories), and rabbit IgG (Santa Cruz, SC2027).

## In vitro binding assays

GST-tagged TPP1 OB-fold and Raf-1 RBD proteins, and MBP-tagged TPP1 OB-fold and FAM118B proteins were purified from *Escherichia coli* BL21 cells. GST-TPP1 OB was first immobilized on glutathione-Sepharose 4B beads and then incubated with MBP-TPP1 OB for 2 h at 4 °C. The beads were subsequently washed and used for SDS-PAGE followed by Coomassie staining and Western blotting analysis.

## Acknowledgments

We thank Drs. Hyeung Kim, Jun Xu, and Ka-wing Fong for technical help. This study was supported by the National Basic Research Program (973 Program) (2010CB945401 and 2012CB911201), National Natural Science Foundation (91019020 and 91213302), Specialized Research Fund for the Doctoral Program of Higher Education (20100171110028), Introduced Innovative R&D Team of Guangdong Province (201001Y010 4687244), and Changjiang Scholars Programme of China. We would also like to acknowledge the support of NCI CA133249, NIGMS GM095599, the Welch Foundation Q-1673, and the GRSA Shared Resource at the Dan L. Duncan Cancer Center (P30CA125123). The project described was also supported in part by BCM IDRC (5P30HD024064) from the Eunice Kennedy Shriver National Institute of Child Health & Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute of Child Health & Human Development or the National Institutes of Health.

## Author contributions

D.L., J.C., and Z.S. designed the experiments. X.H. performed the experiments. Y.Z. and Y.L. prepared the samples for GST *in vitro* pull-down assays. W.L. prepared samples for RT-qPCR. X.H., D.L., and Z.S. wrote the manuscript.

## Authors' conflict of interest statement

The authors declare no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Fig. S1** Depletion of Akt2 or Akt3 did not affect telomere protection.