MOLECULAR AND CELLULAR BIOLOGY, Oct. 2009, p. 5611–5619 Vol. 29, No. 20
0270-7306/09/$08.00+0 doi:10.1128/MCB.00286-09

POT1 Association with TRF2 Regulates Telomere Length▽‡

Megan F. Kendellen,† Katharine S. Barrientos,† and Christopher M. Counter*

Department of Pharmacology and Cancer Biology, Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina

Received 4 March 2009/Returned for modification 26 March 2009/Accepted 24 July 2009

Deleting the OB folds encoding the telomeric single-stranded DNA (ssDNA)-binding activity of the human telomeric protein POT1 induces significant telomere elongation, suggesting that at least one critical aspect of the regulation of telomere length is disrupted by this POT1$^{\Delta OB}$ mutant protein. POT1 is known to associate with two proteins through the protein interaction domain retained in POT1$^{\Delta OB}$—the telomeric double-stranded DNA-binding protein TRF2 and the telomere-associated protein TPP1. We report that introducing a mutation that reduces association of POT1 with TRF2, but not a mutation that reduces the association with TPP1, abrogates the ability of POT1$^{\Delta OB}$ to promote telomere elongation. Mechanistically, expression of POT1$^{\Delta OB}$ reduced the association of TRF2 with POT1, RAP1, and TIN2; however, of these proteins, only ectopic expression of POT1 suppressed the telomere elongation induced by POT1$^{\Delta OB}$. Lastly, replacing endogenous POT1 with a full-length POT1 mutant defective in the association with TRF2 induced telomere elongation. Thus, we conclude that the association of POT1 with both ssDNA and TRF2 is critical for telomere length homeostasis.

Telomeres are DNA-protein complexes that protect the ends of eukaryotic chromosomes from degradation and detection as sites of DNA damage (reviewed in reference 23). Telomeric DNA is composed of tandem arrays of repetitive double-stranded DNA (dsDNA), wherein the G-rich strand extends beyond the C-rich strand. The resultant 3′ single-stranded DNA (ssDNA) overhang can invade the dsDNA, forming a lariat structure termed the t-loop (28).

The regulation of telomere length affects mammalian biology at both the cellular and organismal levels. In normal human somatic cells, telomeres progressively shorten (32, 33) to a critical length before entering a state of permanent growth arrest termed senescence (1, 12, 21, 34, 35). Abnormally short telomeres in humans and mice are associated with various anemias, cirrhosis of the liver, and other disorders due to the premature induction of senescence, particularly in highly proliferative tissues (7, 11, 61). Conversely, de novo elongation of the telomere by the reverse transcriptase telomerase (27, 54) ensures maintenance of telomere length in the germ line and endows cultured cells with an immortal life span (8, 17, 18, 20, 30, 36, 70, 78). Moreover, telomerase activation and subsequent stabilization of telomere length occur in the vast majority of cancer cells (41, 62) and are required for cellular immortalization and the tumorigenic conversion of normal human cells (19, 30, 31, 36, 78).

The dsDNA portion of telomeres is bound directly by two proteins, TRF1 and TRF2 (9, 23). In turn, TRF1 and TRF2 are bridged by the protein TIN2 (23, 43, 76) and bind other telomere-associated proteins such as tankyrase 1 (23, 63, 64) and RAP1 (48, 56). Telomeric ssDNA is bound directly by the protein POT1 (4, 5, 23, 52). POT1 acts in a heterodimer with the protein TPP1; this heterodimer promotes POT1 binding to telomeric ssDNA at least in vitro (72, 73), protects telomeres from being detected as sites of DNA damage (3, 24, 29, 37, 73, 74), and inhibits replication protein A (RPA) localization to telomeres (3). In vitro-transcribed and -translated POT1 has also been shown to associate with recombinant glutathione *S*-transferase–TRF2 (75). Additionally, endogenous TRF2 has been found in some (3), but not all (51), cases to coimmunoprecipitate with exogenous epitope-tagged POT1, although different antibodies were used to detect TRF2. Endogenous POT1 has also been reported to coimmunoprecipitate with endogenous TRF2 (75). Moreover, NAAIRS substitution mutations in POT1 that reduced its ability to coimmunoprecipitate TRF2, but not to coimmunoprecipitate TPP1 or to bind telomeric ssDNA, failed to associate with telomeric chromatin, as assessed by chromatin immunoprecipitation and immunofluorescence studies (3). Taken together, these data suggest that POT1 also associates with TRF2, potentially directly, and this interaction facilitates POT1 association with telomeric chromatin (3, 37, 53, 75). All the aforementioned proteins are found in larger complexes containing other proteins involved in telomere length regulation, DNA damage response, and sister chromatid association (13, 16, 22, 49, 56, 57, 60, 67).

Inhibition of any of the proteins that directly bind telomeric DNA (TRF1, TRF2, or POT1) or many of their interacting partners (TPP1, TIN2, or RAP1) in telomerase-positive human cells results in telomere elongation (10, 38, 43, 47, 51, 56, 66, 69, 71, 74, 77). Such inhibition has also been reported to disrupt telomere protein complexes (57) and induce the mislocalization of other telomere-associated proteins (37, 39, 50, 51, 75, 77). Thus, direct and indirect effects of disrupting multiple protein interactions may underlie such alterations in telomere length.

Of the aforementioned proteins, POT1 is an attractive pro-

* Corresponding author. Mailing address: Department of Pharmacology and Cancer Biology, Department of Radiation Oncology, Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-9890. Fax: (919) 684-8958. E-mail: count004@mc.duke.edu.

† M. F. Kendellen and K. S. Barrientos contributed equally to this work.

‡ Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 3 August 2009.

tein in terms of directly regulating telomere length for the following reasons. First, POT1 homologues in budding and fission yeasts are known to regulate the ability of telomerase to elongate telomeric telomeres (14, 53, 55, 59, 65). Second, human POT1 is the only protein to bind telomeric ssDNA (4, 52), the substrate of telomerase (27, 54), and POT1 can modulate telomerase access to its substrate at least in vitro (40, 45, 52). Third, the POT1-TPP1 heterodimer recruits telomerase to telomeres and promotes its processivity (72, 73). As an entrée to studying how POT1 regulates telomere length in telomerase-positive cells, we note that expression of POT1$^{\Delta OB}$, a mutant lacking the N-terminal OB folds responsible for telomeric ssDNA binding (3, 50, 51), is one of the most potent means of inducing elongation of telomeres (2, 50, 51, 74). This mutant clearly disrupts at least one critical aspect of telomere length regulation. This prompted us to investigate the mechanism by which POT1$^{\Delta OB}$ induces telomere elongation to gain better insight into how telomere length is controlled by telomere-binding proteins.

POT1$^{\Delta OB}$ retains the protein interaction domain of the wild-type protein, which mediates its interaction with both TPP1 and TRF2 (3, 39, 50, 74, 75, 77), and elongates telomeres in a dominant-negative fashion (2, 50, 51, 74). This suggests that one of these protein interactions, when uncoupled from telomeric ssDNA binding, underlies the ability of POT1$^{\Delta OB}$ to elongate telomeres. To determine which protein interaction is most important in POT1-mediated regulation of telomere length, we exploited the POT1$^{\Delta OB}$ mutant by introducing separation-of-function mutations that disrupt association with either TPP1 or TRF2 (3). We found that introducing a mutation that decreases the interaction with TRF2, but not TPP1, abrogated the ability of POT1$^{\Delta OB}$ to promote telomere elongation. Furthermore, expression of POT1$^{\Delta OB}$ reduced the association of TRF2 with its binding partners POT1, RAP1, and TIN2, suggesting that, mechanistically, POT1$^{\Delta OB}$ may act in a dominant-negative manner by disrupting other protein interactions at the telomere. Notably, only ectopic expression of POT1 was able to suppress telomere elongation induced by POT1$^{\Delta OB}$. Finally, replacing endogenous POT1 with either POT1$^{\Delta OB}$ or a mutant defective in associating with TRF2 (POT1$^{\Delta TRF2}$) induced telomere elongation. Thus, we surmise that the association of POT1 with both telomeric ssDNA and TRF2 is critical for regulating telomere length.

MATERIALS AND METHODS

Plasmids. pBabe-puro or pBabe-hygro carrying the FLAG-tagged RNA interference (RNAi)-resistant cDNAs F-POT1, F-POT1$^{\Delta OB}$, F-POT1^{2OB}, F-POT1$^{\Delta TRF2}$, F-POT1$^{\Delta TPP1}$, F-RAP1, and myc-tagged TIN2 (myc-TIN2) and pCI-neo encoding F-POT1 or myc-TIN2 were previously described (3, 56). Specifically, F-POT1 encodes an N-terminal FLAG epitope tag in frame with the cDNA representing the v1 major spliced version (4, 38) of human POT1. F-POT1$^{\Delta OB}$ encodes an N-terminal FLAG epitope tag in frame with the fragment from amino acid 127 to 634 of v1 human POT1. F-POT1^{2OB} encodes an N-terminal FLAG epitope tag in frame with the fragment from amino acid 2 to 350 of v1 human POT1 engineered with a stop codon at amino acid 350. F-POT1$^{\Delta TPP1}$ and F-POT1$^{\Delta TRF2}$ (previously termed F-POT1$^{\Delta TRF2\text{-}2}$) encode an N-terminal FLAG epitope tag in frame with v1 human POT1 containing NAAIRS substitution mutations at S_{317}SGSVS or A_{599}YPWLE, respectively (3). F-RAP1 encodes an N-terminal FLAG epitope-tagged version of the human RAP1 protein, as previously described (56). Myc-TIN2 encodes a myc-tagged version of human TIN2, a kind gift from Judith Campisi. F-POT1$^{\Delta OB\Delta TPP1}$ and F-POT1$^{\Delta OB\Delta TRF2}$ were generated by introducing the aforementioned NAAIRS substitution mutations into F-POT1$^{\Delta OB}$ by site-directed mutagenesis and subcloned into pBABE-puro. Yellow fluorescent protein (YFP)-POT1$^{\Delta OB}$ was generated by subcloning untagged POT1$^{\Delta OB}$ into pEYFP-C1 (Clontech). pSUPER-Retro-puro-POT1 short hairpin RNA (shRNA) was previously described (3, 71).

Cell lines. Using previously described approaches (3, 58), immortal HA1 cells (population doubling [PD] 200) (17) were stably infected with the indicated combinations of retroviruses derived from pBabe-puro carrying no transgene (empty vector) or encoding F-POT1, F-POT1$^{\Delta TPP1}$, F-POT1$^{\Delta TRF2}$, F-POT1$^{\Delta OB}$, F-POT1$^{\Delta OB\Delta TPP1}$, F-POT1$^{\Delta OB\Delta TRF2}$, or F-RAP1 or with retroviruses derived from pBabe-hygro carrying no transgene (empty vector) or encoding F-POT1, F-POT1$^{\Delta OB}$, F-POT1$^{\Delta TPP1}$, F-POT1$^{\Delta TRF2}$, or myc-TIN2. As indicated, some cell lines were subsequently infected with pSUPER-Retro-puro carrying no transgene (empty vector) or POT1 shRNA (71). All cells lines were selected with puromycin (Sigma) or hygromycin B (Sigma) as previously described (2), and the first confluent population of cells following antibiotic selection was defined as PD 0.

Detection of telomeres. Telomere lengths were assessed using ^{32}P-labeled $(CCCTAA)_3$ Southern hybridization of HinFI- and RsaI-digested genomic DNA as previously described (2, 25). Mean telomere lengths were determined using the Telometric program (26). Where indicated, statistical significance was determined using Student's *t* test.

Immunoprecipitation and immunoblotting. Using previously described approaches (2, 3), F-POT1 and F-RAP1 proteins stably expressed in HA1 cells were detected by immunoprecipitation with anti-FLAG M2 agarose affinity resin (Sigma), followed by elution with FLAG-peptide (Sigma) and immunoblotting with an anti-FLAG M2 antibody (1:1,200, Sigma). myc-TIN2 stably expressed in HA1 cells was detected by immunoprecipitation with an anti-myc antibody (Invitrogen), followed by incubation with G-Sepharose beads (Amersham Biosciences) and immunoblotting with the aforementioned anti-myc antibody (1:3,000). Coimmunoprecipitation of endogenous TRF2 with stably expressed F-POT1, F-POT1^{2OB}, or F-POT$^{\Delta OB}$ was detected by immunoprecipitation and elution of the FLAG-tagged proteins as described above, followed by immunoblotting with an anti-TRF2 antibody (1:250; Imgenex). Coimmunoprecipitation of endogenous TRF2 with ectopic F-POT1, F-RAP1, or myc-TIN2 was detected by transient transfection of 293T cells using the FuGENE 6 reagent (Roche) with the plasmid pCI-neo carrying no transgene or encoding F-POT1 or myc-TIN2 or pBABE-puro encoding F-RAP1 in the absence or presence of transiently cotransfected YFP-POT1$^{\Delta OB}$. Forty-eight hours later, FLAG- and myc-tagged proteins were immunoprecipitated as described above, followed by immunoblotting with the aforementioned anti-FLAG M2, anti-myc, and anti-TRF2 antibodies. Protein expression of YFP-POT1$^{\Delta OB}$ was validated by immunoblotting with an anti-green fluorescent protein antibody (1:1,000, Roche).

Reverse transcription-PCR (RT-PCR). Endogenous POT1, or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a control, was reverse-transcribed and PCR amplified as previously described (3).

RESULTS

Association with TRF2 is required for POT1$^{\Delta OB}$ to induce telomere elongation. POT1 binds telomeric ssDNA (4, 44, 52), the substrate of telomerase (27, 52), and can inhibit the access of telomerase to telomeric ssDNA at least in vitro (40, 45, 52). Moreover, expression of a POT1 mutant lacking OB folds, which mediate binding of telomeric ssDNA (POT1$^{\Delta OB}$), is one of the most potent means of inducing telomere elongation (2, 50, 51, 74). Thus, we investigated how POT1$^{\Delta OB}$ promotes telomere elongation to understand how POT1 normally regulates telomere length. Given that POT1$^{\Delta OB}$ acts in a dominant-negative fashion (2, 50, 51, 74) and retains the protein interaction domain through which it associates with both TPP1 and TRF2 (3, 39, 50, 74, 75, 77), we hypothesized that one of these protein interactions, when uncoupled from telomeric ssDNA binding, may mediate the telomere elongation observed in cells overexpressing POT1$^{\Delta OB}$.

To determine if the protein interaction of POT1 with either TPP1 or TRF2 is required for the telomere elongation induced by POT1$^{\Delta OB}$, separation-of-function mutations previously shown to reduce the interaction of POT1 with TPP1 or TRF2 (3) were introduced into an N-terminal FLAG-tagged version

of POT1$^{\Delta OB}$, yielding the mutants F-POT1$^{\Delta OB\Delta TPP1}$ and F-POT1$^{\Delta OB\Delta TRF2}$, respectively (see Fig. S1 in the supplemental material). F-POT1$^{\Delta OB\Delta TPP1}$ and F-POT1$^{\Delta OB\Delta TRF2}$ were verified to coimmunoprecipitate TRF2 but not TPP1, or TPP1 but not TRF2, respectively (data not shown). We note the caveat that coimmunoprecipitation does not differentiate between direct and indirect binding, and hence we cannot discount the possibility that these mutations disrupt other protein interactions that are indirectly manifested as a loss in the association with TPP1 or TRF2. Empty vector as a negative control, F-POT1$^{\Delta OB}$ as a positive control, F-POT1$^{\Delta OB\Delta TPP1}$, or F-POT1$^{\Delta OB\Delta TRF2}$ was stably expressed in the telomerase-positive, immortal simian virus 40-transformed human embryonic kidney cell line HA1. These cells were chosen because they maintain short telomeres in a telomerase-dependent fashion over many cell divisions (17), and hence perturbations that result in telomere elongation are easily detected. Appropriate transgene expression was confirmed by immunoprecipitation and immunoblotting with an anti-FLAG antibody (Fig. 1A). Genomic DNA from the resultant cell lines was isolated at regular intervals and digested with restriction enzymes to liberate the repetitive telomeric DNA, which was detected by Southern hybridization with a ^{32}P-labeled telomeric probe.

Negative control vector cells maintained a mean telomere length of ~3.6 kb over the observed period of 40 PDs, whereas the telomeres of positive control cells expressing F-POT1$^{\Delta OB}$ were elongated to ~6.0 kb over the same number of PDs (Fig. 1B). We note a slight decrease in telomere length in cells expressing F-POT1$^{\Delta OB}$ at PD 20, which was lost by PD 40, perhaps reflecting a selective pressure in the polyclonal population over time. This transient decrease in telomere length was not observed in all cases (e.g., compare Fig. 1B and 4B). Telomeres of cells expressing F-POT1$^{\Delta OB\Delta TPP1}$ elongated to a length similar to that observed in positive control F-POT1$^{\Delta OB}$-expressing cells, suggesting that the interaction with TPP1 does not underlie the ability of F-POT1$^{\Delta OB}$ to induce telomere elongation. Conversely, the telomeres of cells expressing F-POT1$^{\Delta OB\Delta TRF2}$ maintained a mean telomere length of ~3.9 kb, just slightly longer than vector control cells (Fig. 1B). This supports the notion that association with TRF2 is critical to the telomere phenotype induced by POT1$^{\Delta OB}$. These results were reproducible, as similar telomere dynamics were observed in two additional independently derived sets of cell lines (Fig. 1B). In summary, the association of F-POT1$^{\Delta OB}$ with TRF2, but not with TPP1, promotes telomere elongation.

POT1$^{\Delta OB}$ disrupts TRF2 protein interactions. Since the association of POT1$^{\Delta OB}$ with TRF2 was required to promote telomere elongation (Fig. 1B) and since TRF2 interacts with most proteins through its homodimerization domain (15, 25a, 43, 46, 48, 68, 76), we reasoned that ectopic POT1$^{\Delta OB}$ might act to elongate telomeres by displacing other proteins from endogenous TRF2. To test this hypothesis, we first determined whether F-POT1$^{\Delta OB}$ associated with TRF2. Specifically, stably expressed empty vector or a truncation mutant of F-POT1 that encodes only the telomeric ssDNA-binding activity (F-POT1^{2OB}) (3, 52) as negative controls, full-length FLAG-tagged POT1 (F-POT1) as a positive control, or F-POT1$^{\Delta OB}$ was immunoprecipitated by virtue of the FLAG epitope and immunoblotted with an anti-TRF2 antibody to detect coimmunoprecipitated endogenous TRF2. As expected (3, 75), TRF2 coimmunoprecipitated with F-POT1 in positive control cells but not with empty vector or F-POT1^{2OB} in negative control cells (Fig. 2A). F-POT1$^{\Delta OB}$ coimmunoprecipitated with TRF2, indicating that F-POT1$^{\Delta OB}$ readily associated with endogenous TRF2. Of note, the interaction of endogenous TRF2 and F-POT1$^{\Delta OB}$ was more robust than that between endogenous TRF2 and full-length F-POT1 (Fig. 2A).

Given that POT1$^{\Delta OB}$ associates more strongly than full-length POT1 with endogenous TRF2 (Fig. 2A), we next explored whether POT1$^{\Delta OB}$ reduced the binding of telomeric proteins to TRF2. TRF2 binds the proteins POT1 (75), RAP1 (47, 48, 56), and TIN2 (42, 76). All three of these proteins are

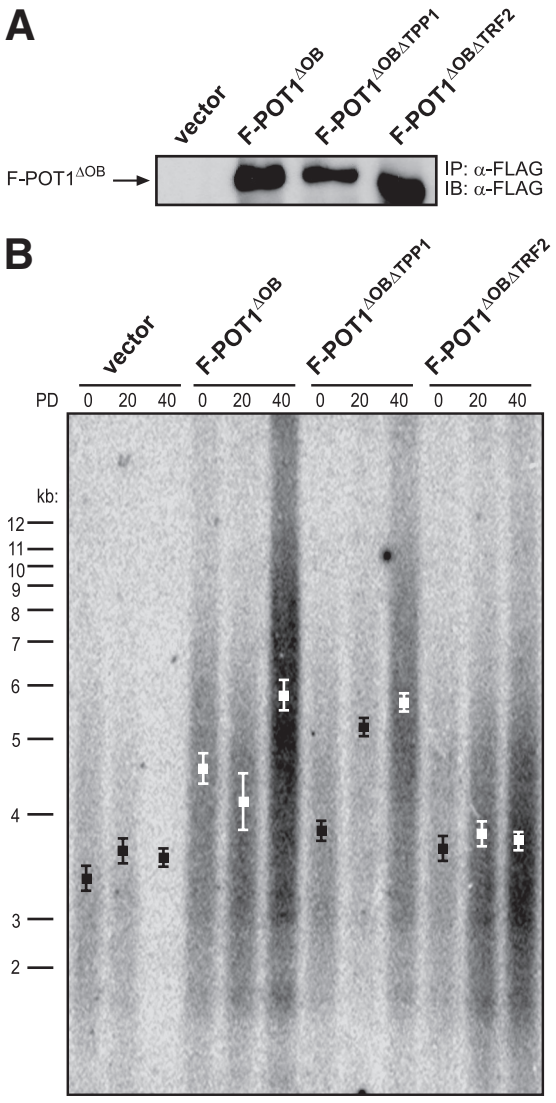

FIG. 1. Association with TRF2 is required for POT1$^{\Delta OB}$ to induce telomere elongation. (A) Detection of the indicated FLAG-tagged POT1 (F-POT1) proteins stably expressed in HA1 cells by immunoprecipitation (IP) and immunoblotting (IB) with an anti-FLAG antibody. (B) Telomere length, as assessed by Southern hybridization with a ^{32}P-labeled telomeric probe, of HA1 cells stably expressing the indicated transgenes at PDs 0, 20, and 40. Boxes indicate mean telomere length ± standard deviation calculated from three independently derived sets of cell lines.

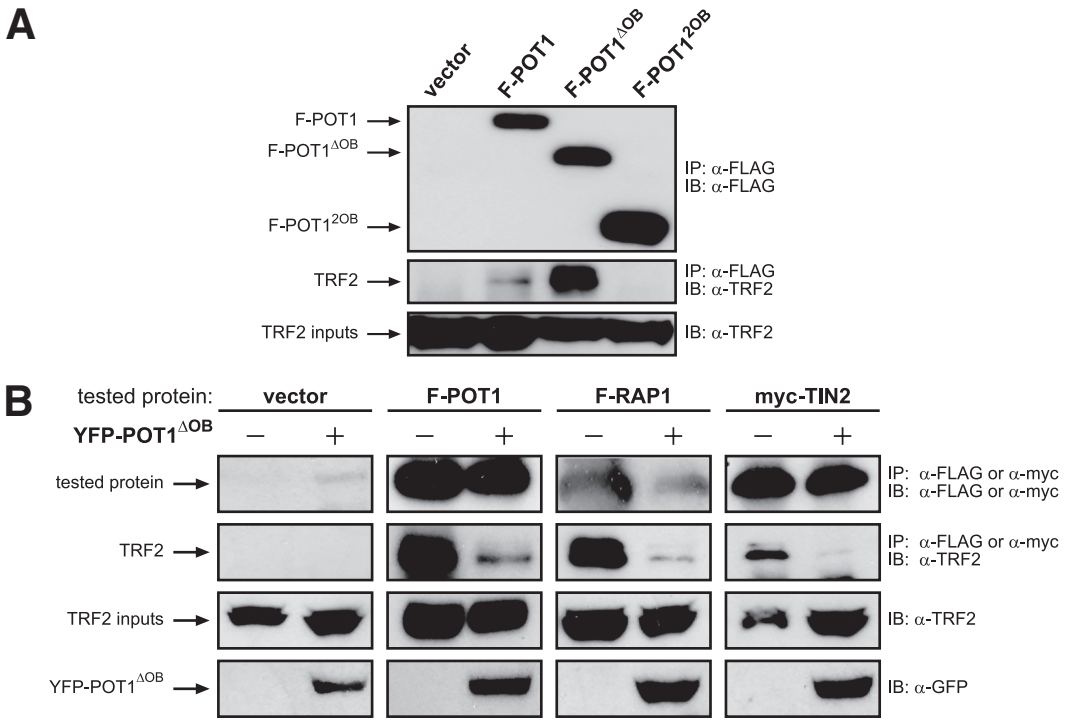

FIG. 2. POT1$^{\Delta OB}$ disrupts TRF2 protein interactions. (A) Immunoprecipitation (IP) of stably expressed FLAG-tagged POT1 (F-POT1) proteins followed by immunoblotting (IB) to detect whether endogenous TRF2 coimmunoprecipitated with indicated F-POT1 proteins in 293T cells. (B) Immunoprecipitation of the indicated transiently expressed FLAG- or myc-tagged proteins followed by immunoblotting to detect whether endogenous TRF2 coimmunoprecipitated with empty vector, F-POT1, F-RAP1, or myc-TIN2 in the absence (−) or presence (+) of transiently coexpressed YFP-tagged POT1$^{\Delta OB}$ (YFP-POT1$^{\Delta OB}$) in 293T cells.

known to negatively regulate telomere length (38, 43, 47, 50, 56, 71, 74, 77), which is consistent with POT1$^{\Delta OB}$ potentially disrupting their function. Thus, F-POT1, FLAG-tagged RAP1 (F-RAP1), myc-tagged TIN2 (myc-TIN2), or an empty vector as a negative control was transiently expressed in the absence or presence of transiently coexpressed YFP-tagged POT1$^{\Delta OB}$ (YFP-POT1$^{\Delta OB}$). Expression of YFP-POT1$^{\Delta OB}$ was confirmed by immunoblotting using an anti-green fluorescent protein antibody (Fig. 2B). F-POT1, F-RAP1, or myc-TIN2 was immunoprecipitated by virtue of the FLAG or myc epitopes, as appropriate, followed by immunoblotting with an anti-TRF2 antibody to assess the relative amount of coimmunoprecipitated endogenous TRF2. As previously reported (3, 42, 56, 57, 75), while empty vector failed to do so, ectopic F-POT1, F-RAP1, and myc-TIN2 all coimmunoprecipitated with TRF2. Importantly, the amount of endogenous TRF2 which coimmunoprecipitated with each of these three binding partners (F-POT1, F-RAP1, and myc-TIN2) was decreased upon expression of YFP-POT1$^{\Delta OB}$ (Fig. 2B). Notably, previous reports demonstrated that expression of POT1$^{\Delta OB}$ does not result in the loss of endogenous TRF2 from telomeres, as assessed by chromatin immunoprecipitation (51), suggesting that the reduction in the interaction of endogenous TRF2 with POT1, RAP1, and TIN2 is not due to mislocalization of TRF2. Thus, we conclude that POT1$^{\Delta OB}$ robustly associates with endogenous TRF2 and disrupts its interaction with POT1, RAP1, and TIN2.

POT1 suppresses telomere elongation induced by POT1$^{\Delta OB}$. We next determined which of the TRF2 protein interactions disrupted by POT1$^{\Delta OB}$ were critical in the regulation of telomere length. Specifically, we reasoned that if POT1$^{\Delta OB}$ induced telomere elongation by disrupting the interaction of TRF2 with POT1, RAP1, or TIN2, such elongation may be suppressed by overexpressing the essential displaced protein. To test this hypothesis, telomere length was determined at regular intervals by Southern hybridization in immortal HA1 cells stably expressing F-POT1, F-RAP1, or myc-TIN2 in the absence or presence of stably expressed F-POT1$^{\Delta OB}$. Expression of the ectopic proteins was assessed by immunoprecipitation and immunoblotting using an anti-FLAG or anti-myc antibody, as appropriate (Fig. 1A and 3A).

As expected (2, 43, 47, 50, 51, 56), overexpression of F-POT1, F-RAP1, or myc-TIN2 alone induced subtle changes in telomere length, whereas the mean telomere length in cells expressing F-POT1$^{\Delta OB}$ was increased to ~7 kb over 40 PDs of observation (Fig. 3B). Coexpression of F-RAP1 or myc-TIN2 failed to suppress the telomere elongation induced by F-POT1$^{\Delta OB}$, as evidenced by the fact that telomeres of cells coexpressing F-RAP1 or myc-TIN2 with F-POT1$^{\Delta OB}$ reached mean lengths of ~7 to 8 kb, similar to those of control cells expressing F-POT1$^{\Delta OB}$ alone (Fig. 3C). Thus, simply overexpressing any TRF2-interacting protein does not suppress POT1$^{\Delta OB}$-mediated elongation of telomeres. Importantly, expression of F-POT1 did suppress the telomere elongation in-

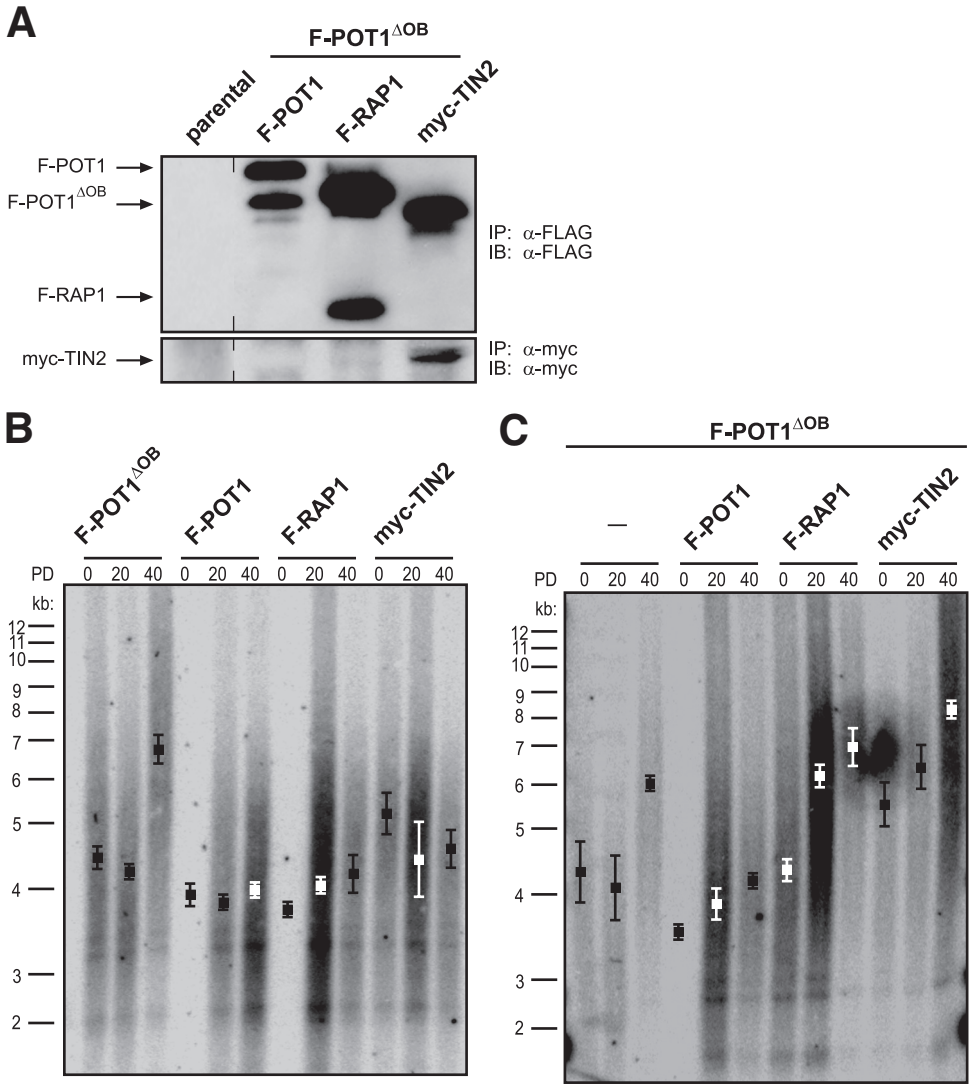

FIG. 3. POT1 suppresses telomere elongation induced by POT1$^{\Delta OB}$. (A) Detection of the indicated FLAG- or myc-tagged proteins stably expressed in HA1 cells by immunoprecipitation (IP) and immunoblotting (IB) with an anti-FLAG or anti-myc antibody as indicated. Vertical lines denote where the image has been cut. (B and C) Telomere length, as assessed by Southern hybridization with a ^{32}P-labeled telomeric probe, of HA1 cells stably expressing the indicated transgenes at PDs 0, 20, and 40. Boxes indicate mean telomere length ± standard deviation calculated from two independently derived sets of cell lines.

duced by F-POT1$^{\Delta OB}$. Specifically, the telomeres of cells coexpressing F-POT1 and F-POT1$^{\Delta OB}$ reached a mean length of ~4.1 kb, ~3 kb shorter than that observed in cells coexpressing F-POT1$^{\Delta OB}$ and F-RAP1 or myc-TIN2 (Fig. 3C), similar to telomere lengths in cells expressing empty vector (Fig. 1B). These results were reproducible, as similar telomere dynamics were observed in an additional independently derived set of cell lines (Fig. 3B and C). Since F-POT1$^{\Delta OB}$ disrupts the association of TRF2 with F-POT1, F-RAP1, and myc-TIN2 (Fig. 2B), but of these proteins only expression of F-POT1 suppressed the telomere elongation induced by F-POT1$^{\Delta OB}$ (Fig. 3C), we surmise that disruption of the specific association between POT1 and TRF2 underlies the ability of POT1$^{\Delta OB}$ to induce telomere elongation.

POT1 association with telomeric ssDNA and TRF2 regulates telomere length. We have demonstrated that POT1$^{\Delta OB}$ disrupts the association of TRF2 with POT1 and induces telomere elongation in a dominant-negative fashion (Fig. 1B, 2B, and 3C). We thus tested whether disrupting the interaction of POT1 and TRF2 through a different means, specifically by expressing the POT1$^{\Delta TRF2}$ mutant defective in associating with TRF2, would similarly induce telomere elongation. To this end, immortal HA1 cells were engineered to stably express empty vector, F-POT1, F-POT1$^{\Delta TPP1}$ or F-POT1$^{\Delta OB}$ as controls or F-POT1$^{\Delta TRF2}$ (see Fig. S1 in the supplemental material). Expression of the ectopic proteins was assessed by immunoprecipitation and immunoblotting using an anti-FLAG antibody (Fig. 4A), and telomere length in these cells was determined at regular intervals by Southern hybridization. Vector control cells maintained a mean telomere length of ~3.8 kb, and telomeres in cells stably expressing F-POT1$^{\Delta OB}$ exhibited extensive elongation to a final length in excess of 12

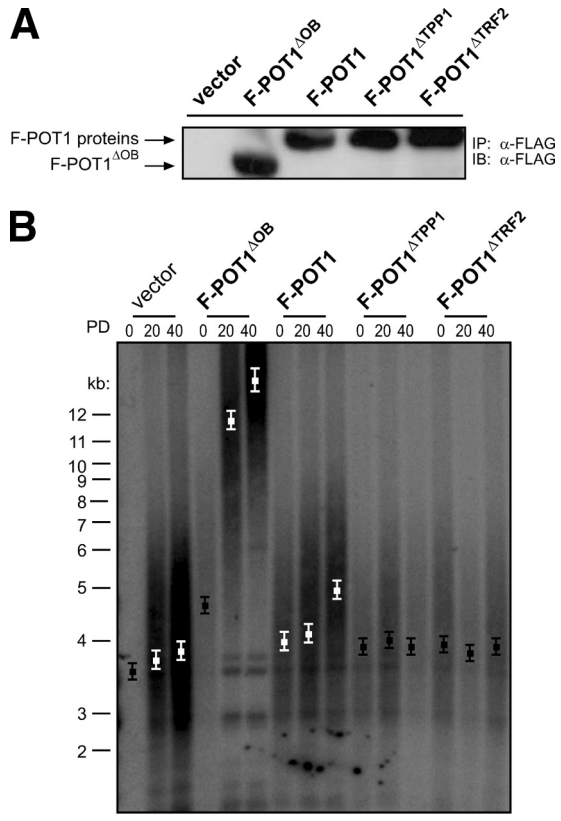

FIG. 4. POT1$^{\Delta OB}$, but not POT1$^{\Delta TRF2}$, acts in a dominant-negative fashion to elongate telomeres. (A) Detection of the indicated FLAG-tagged POT1 (F-POT1) proteins stably expressed in HA1 cells by immunoprecipitation (IP) and immunoblotting (IB) with an anti-FLAG antibody. (B) Telomere length, as assessed by Southern hybridization with a ^{32}P-labeled telomeric probe, of HA1 cells stably expressing the indicated proteins at PDs 0, 20, and 40. Boxes indicate mean telomere length ± standard deviation calculated from three independently derived sets of cell lines.

kb (Fig. 4B). As expected (2, 50), expression of F-POT1 resulted in a small elongation of telomeres to a final mean length of ~5 kb. Notably, telomeres in HA1 cells stably expressing F-POT1$^{\Delta TPP1}$ or F-POT1$^{\Delta TRF2}$ maintained a mean length of ~3.9 kb, similar to those in vector control cells (Fig. 4B). Such results were reproducible, as similar telomere dynamics were observed in two additional independently derived sets of cell lines (Fig. 4B). Thus, unlike POT1$^{\Delta OB}$, POT1$^{\Delta TRF2}$ does not act in a dominant-negative fashion to promote telomere elongation.

One caveat for the POT1$^{\Delta TRF2}$ mutant is that it does not efficiently colocalize with telomeric chromatin, as assessed by immunofluorescence and chromatin immunoprecipitation (3). However, expression of POT1$^{\Delta TRF2}$ reduces the number of telomere dysfunction-induced foci and the amount of the ssDNA-binding complex RPA localizing to telomeres upon knockdown of endogenous POT1 (3). These data suggest that POT1$^{\Delta TRF2}$ associates with telomeres, but at a reduced capacity. Thus, perhaps POT1$^{\Delta TRF2}$ fails to displace enough endogenous POT1 from critical telomeric binding sites to affect telomere length in a dominant-negative fashion, potentially because the endogenous POT1 protein is bound more strongly or localized more efficiently to telomeres through its association with endogenous TRF2. We therefore tested whether replacing endogenous POT1 with POT1$^{\Delta TRF2}$ could induce telomere elongation. Specifically, immortal HA1 cells were engineered to stably express empty vector as a negative control, RNAi-resistant F-POT1 as a positive control, RNAi-resistant F-POT1$^{\Delta OB}$, F-POT1$^{\Delta TPP1}$, or F-POT1$^{\Delta TRF2}$. These cells were subsequently stably infected with retrovirus encoding either empty vector as a negative control or POT1 shRNA to reduce endogenous POT1 expression (3, 71). Ectopic wild-type or mutant F-POT1 expression was confirmed by immunoprecipitation and immunoblotting with an anti-FLAG antibody (Fig. 5A), and appropriate knockdown of endogenous POT1 was validated by RT-PCR (Fig. 5B). Telomere length in the resultant cell lines was then assessed by Southern hybridization at regular intervals over 40 PDs.

Knockdown of endogenous POT1 in vector control cells increased the mean length of telomeres in these cells by ~3.0 kb, and this effect was rescued by overexpression of F-POT1 (Fig. 5C). Such telomere elongation was not rescued by overexpression of F-POT1$^{\Delta OB}$. In fact, cells in which endogenous POT1 was knocked down and replaced by F-POT1$^{\Delta OB}$ displayed longer telomeres than cells in which F-POT1$^{\Delta OB}$ was expressed in the presence of endogenous POT1 (~10.5 kb) (Fig. 5C). Such data highlight the critical role of the ssDNA-binding activity of POT1 in regulating telomere length. These results were reproducible, as similar telomere dynamics were observed in an independently derived set of cell lines (Fig. 5C). F-POT1$^{\Delta OB}$ did not induce the robust telomere elongation previously observed (Fig. 5C versus 1B, 3B, 3C, and 4B). This is due to the lower expression of F-POT1$^{\Delta OB}$ from the pBabe-hygro-F-POT1$^{\Delta OB}$ plasmid (used in this experiment to accommodate puromycin selection for the POT1shRNA vector) than from the pBabe-puro-F-POT1$^{\Delta OB}$ plasmid (used in previous experiments). Indeed, HA1 cells stably infected with pBabe-hygro-F-POT1$^{\Delta OB}$ express lower levels of F-POT1$^{\Delta OB}$ and more subtle elongation of telomeres than the same cells stably infected with Babe-puro-F-POT1$^{\Delta OB}$ (see Fig. S2 in the supplemental material). Nevertheless, F-POT1$^{\Delta OB}$ expressed from the pBabe-hygro vector induced longer telomeres compared to vector control cells (Fig. 5C; also see Fig. S2 in the supplemental material).

In regard to the ability of POT1 to associate with TRF2, cells expressing F-POT1$^{\Delta TPP1}$ or F-POT1$^{\Delta TRF2}$ in the presence of endogenous POT1 maintained an approximately constant mean telomere length of ~3 to 4 kb, similar to vector control cells (Fig. 1B and 5C). Knocking down endogenous POT1 with shRNA and replacing this with F-POT1$^{\Delta TPP1}$ induced only a small ~1.4-kb increase in mean telomere length, to a final length of ~5 kb. Conversely, replacement of endogenous POT1 with F-POT1$^{\Delta TRF2}$ resulted in greater elongation of ~3.9 kb, to a final mean telomere length of ~8 kb (Fig. 5C), suggesting that loss of an association with TRF2 is more detrimental in regard to the regulation of telomere length. Given that in the presence of endogenous POT1 the overexpression of F-POT1$^{\Delta TPP1}$ resulted in slightly smaller telomeres than overexpression of F-POT1$^{\Delta TRF2}$, it is notable that the difference between mean telomere lengths induced by expressing these mutants in the absence of endogenous POT1 is greater (3.0 kb; $P = 0.003$) than the difference in mean telomere

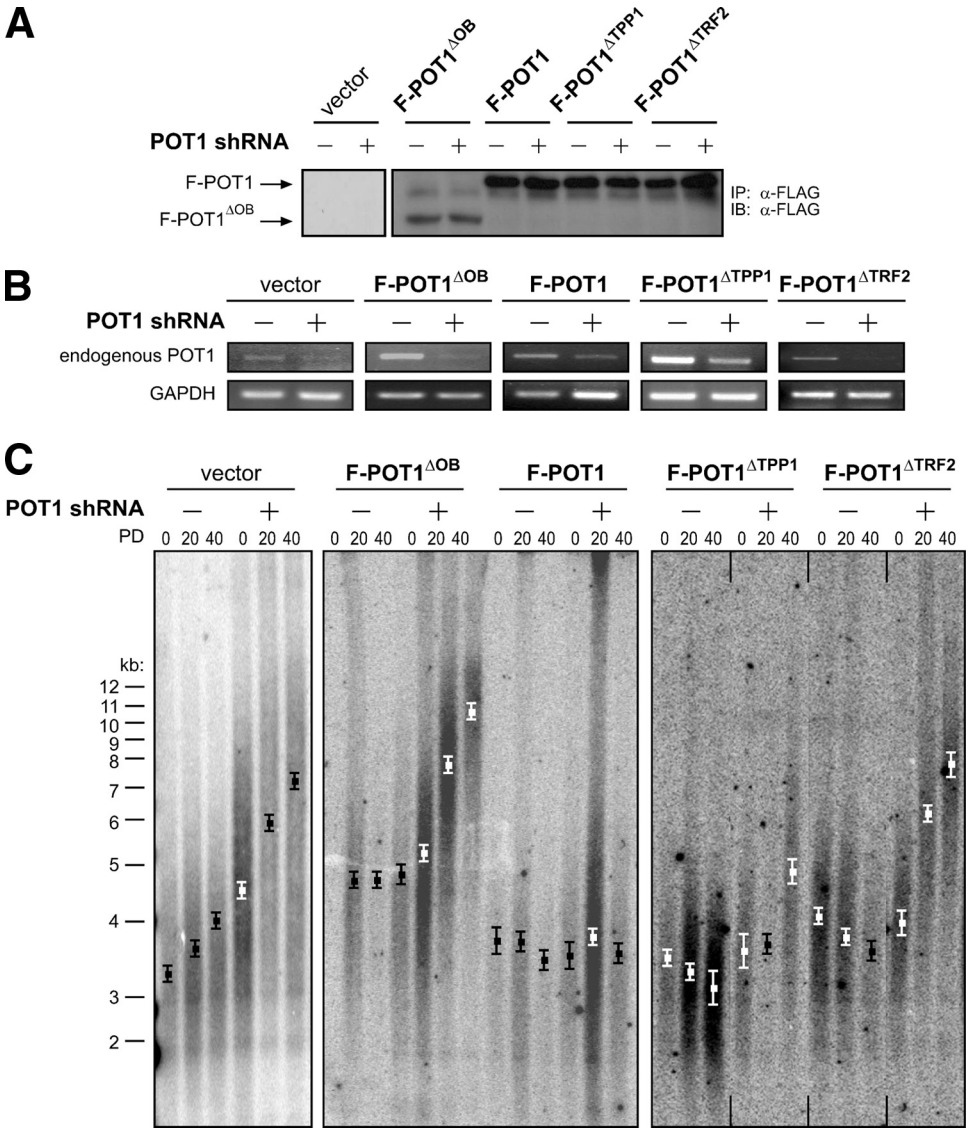

FIG. 5. POT1 association with telomeric ssDNA and TRF2 regulates telomere length. (A) Detection of the indicated RNAi-resistant FLAG-tagged POT1 (F-POT1) proteins stably expressed in HA1 cells by immunoprecipitation (IP) and immunoblotting (IB) with an anti-FLAG antibody. (B) Detection of endogenous POT1 mRNA, or GAPDH mRNA as a loading control, by RT-PCR in the indicated HA1 cells. (C) Telomere length, as assessed by Southern hybridization with a ^{32}P-labeled telomeric probe, of HA1 cells stably expressing the indicated F-POT1 proteins in the absence (−) or presence (+) of POT1 shRNA at PDs 0, 20, and 40. Boxes indicate mean telomere length ± standard deviation calculated from two independently derived sets of cell lines. Vertical lines denote where the image has been cut.

lengths observed due to their expression in the presence of endogenous POT1 (0.4 kb; $P = 0.099$). These results were reproducible, as similar telomere dynamics were observed in an independently derived set of cell lines (Fig. 5C). Thus, we conclude that inhibiting the ability of POT1 to associate with TRF2 induced telomere elongation, consistent with the notion that an interaction between POT1 and TRF2 is required to regulate telomere length.

DISCUSSION

We propose that POT1 suppresses telomere elongation when simultaneously bound to telomeric ssDNA and TRF2. This notion is supported by the following: (i) expression of a POT1 mutant that is unable to bind ssDNA (POT1$^{\Delta OB}$) induced telomere elongation (2, 50, 51, 74) (Fig. 1B, 3B, 3C, and 4B), an effect that was enhanced if endogenous POT1 was replaced with POT1$^{\Delta OB}$ (Fig. 5C); (ii) telomere elongation induced by POT1$^{\Delta OB}$ in a dominant-negative fashion was abrogated by introduction of a mutation that reduced association with TRF2 (Fig. 1B); (iii) POT1$^{\Delta OB}$ associated with TRF2 and displaced POT1, RAP1, and TIN2 (Fig. 2B), but of these proteins, only ectopically expressed POT1 suppressed telomere elongation induced by POT1$^{\Delta OB}$ (Fig. 3C); and (iv) replacing endogenous POT1 with a mutant defective in association with TRF2 resulted in telomere elongation (Fig. 5C).

POT1 associates with both TPP1 and TRF2, yet we find that it is primarily the latter interaction that affects telomere length.

This does not discount a role for TPP1 in POT1 function. Binding to TPP1 enhances the association of POT1 with telomeric ssDNA, at least in vitro (72, 73), and binding to TPP1, but not TRF2, prevents telomere uncapping, as assessed by telomere dysfunction-induced foci and RPA localization at telomeres (3, 24, 29, 37, 73). With regard to telomere length, mutations that inhibit the ability of POT1$^{\Delta OB}$ to promote telomere elongation can reduce TPP1 binding (50), although these mutations may also reduce the association with TRF2, given that one such mutation resides in the region disrupted in POT1$^{\Delta OB\Delta TRF2}$ (3). Knockdown of POT1 and replacement with POT1$^{\Delta TPP1}$ also caused a small increase in telomere length, although this was much less than that observed when POT1 was replaced with POT1$^{\Delta TRF2}$ (Fig. 5C). In sum, an interaction with TPP1 may have some influence on the ability of POT1 to regulate telomere length, although this association appears to play a more prominent role in the capping function of POT1. Overall, POT1 appears to perform different functions at telomeres through specific proteins: TPP1 to cap telomeres and TRF2 to regulate telomere length.

Mechanistically, it remains to be resolved how POT1 regulates telomere length through its association with telomeric ssDNA and TRF2. One possibility is that the connection of telomeric ssDNA (via the OB folds of POT1) with telomeric dsDNA (via association with telomeric dsDNA by TRF2) may promote the formation of higher-order chromatin structures that physically block access of telomerase. At face value, the finding that POT1$^{\Delta TRF2}$ did not promote telomere elongation in a dominant-negative fashion seems inconsistent with this model. However, the lack of dominant-negative activity may simply reflect a reduction in the ability of POT1$^{\Delta TRF2}$ to associate with telomere chromatin (3), and moreover, POT1$^{\Delta TRF2}$ does induce telomere elongation in the absence of endogenous POT1. The idea of POT1-TRF2 regulating telomere length is also supported by the finding that fusing POT1 to the TRF2 paralog, TRF1, both abolishes the telomere elongation observed when POT1 and TRF1 are expressed in *trans* and induces the in vitro formation of t-loops (25), structures found on telomeres (28) that could inhibit access of telomerase to telomere ends (6, 23). Additionally, Pot1 can be recruited to telomeres via an association with telomeric dsDNA-binding protein Taz1 in fission yeast. Moreover, it has been suggested that at long telomeres Pot1 associates with the Taz1 complex and this inhibits telomerase by stabilizing a closed conformation of telomeres, while at short telomeres this protein mediated-association of telomeric ssDNA and dsDNA decreases and exposes telomeres to elongation by telomerase (53). POT1 and TRF2 are found together in larger protein complexes at the telomere (49, 57). Reducing the amount of one of the proteins in such complexes, TRF1, has been reported to decrease POT1 association with telomeric chromatin (51), and knockdown of many telomeric proteins results in telomere elongation (10, 38, 43, 47, 51, 56, 66, 69, 71, 74, 77). Thus, regulation of telomere length by POT1 may yet involve other proteins in addition to TRF2. Nevertheless, TRF2 plays a dominant role in the ability of POT1 to regulate telomere length. In summary, we demonstrate that the interaction of POT1 with telomeric ssDNA and TRF2 suppresses telomere elongation, indicating a critical role for these interactions in telomere length homeostasis.

ACKNOWLEDGMENTS

We thank J. Campisi and Z. Songyang for constructs encoding TIN2 and RAP1, respectively, and members of the Counter lab for helpful discussions.

This research is supported by NIH grant CA082481 and the Edward Spiegel Fund of the Lymphoma Foundation.

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