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A Dynamic Molecular Link between the Telomere Length Regulator TRF1 and the Chromosome End Protector TRF2

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Summary

Background: Human telomeres are coated by the telomere repeat binding proteins TRF1 and TRF2, which are believed to function independently to regulate telomere length and protect chromosome ends, respectively.
Results: Here, we show that TRF1 and TRF2 are linked via TIN2, a previously identified TRF1-interacting protein, and its novel binding partner TINT1. TINT1 localized to telomeres via TIN2, where it functioned as a negative regulator of telomerase-mediated telomere elongation. TIN2 associated with TINT1, and TRF1 or TRF2 throughout the cell cycle, revealing a partially redundant unit in telomeric chromatin that may provide flexibility in telomere length control. Indeed, when TRF1 was removed from telomeres by overexpression of the positive telomere length regulator tankyrase 1, the TIN2/TINT1 complex remained on telomeres via an increased association with TRF2.
Conclusions: Our findings suggest a dynamic cross talk between TRF1 and TRF2 and provide a molecular mechanism for telomere length homeostasis by TRF2 in the absence of TRF1.

Introduction

Telomere integrity is essential for growth of normal human cells, and telomere length maintenance plays a role in aging and cancer [1, 2]. Mammalian telomeres consist of tandem arrays of TTAGGG repeats and associated proteins that function to protect and replicate the telomeric DNA. Telomere length maintenance depends upon telomerase, a reverse transcriptase that adds telomeric repeats to chromosome ends [3, 4]. Most normal human cells lack sufficient levels of telomerase to maintain telomere length, hence telomeres shorten over time and result in replicative senescence [5, 6]. By contrast, in most human tumor cells, telomerase is highly expressed [7], and telomere length is maintained at a constant length setting [8, 9]. In addition to telomerase, telomere length maintenance depends upon proteins that act in *cis* at individual chromosome ends to control recruitment or access to telomerase [10].

Mammalian telomeres are bound by the double-strand DNA binding proteins TRF1 and TRF2 [11–13]. These proteins are related in their primary structure; both contain a carboxy terminal Myb-type DNA binding motif and an internal dimerization domain required for homodimerization [12, 14]. The proteins do not form heterodimers [12]. Although related, the two proteins have very different functions at telomeres. TRF1 functions as a negative regulator of telomere length, acting in *cis* to control access of telomerase [9, 15], whereas TRF2 serves a protective function, preventing telomeres from being viewed as double-strand breaks [16, 17].

According to the protein-counting model for telomere length regulation [10, 18], long telomeres recruit more TRF1, blocking access to telomerase. Conversely, short telomeres recruit less TRF1, permitting access to telomerase. A number of TRF1 binding partners, which can influence this negative feed back loop, have been identified. Tankyrase 1, a member of the poly(ADP-ribose) polymerase (PARP) family of enzymes [19], binds to and ADP-ribosylates TRF1 in vitro, inhibiting its ability to bind telomeric DNA [20]. Overexpression of tankyrase 1 in the nucleus induces loss (and subsequent ubiquitination and degradation) of TRF1 from telomeres, as well as promotes telomerase-dependent telomere elongation [21–23]. Thus, tankyrase 1 mimics a TRF1 dominant negative allele [9]; both strip TRF1 off telomeres, allowing access to telomerase.

Another TRF1 binding protein, TIN2, has a novel primary structure with no known domains [24]. Overexpression of the carboxy terminal TRF1 binding domain of TIN2, TIN2-13, results in telomerase-dependent telomere elongation [24]. Unlike nuclear tankyrase 1 [21] and dominant negative TRF1 [9], TIN2-13 overexpression does not induce loss of TRF1 from telomeres [24], suggesting that the deleted TIN2 amino terminal domain might normally relay a negative signal from TRF1 to telomerase. Consistent with this notion, knockdown of TIN2 expression resulted in telomere elongation [25].

POT1, a highly conserved protein, was initially identified based on its homology to ciliate proteins [26]. POT1 binds to the 3′ G-rich overhang at chromosome ends [26, 27] via an oligosaccharide/olignucleotide binding (OB) domain. In addition, POT1 binds along the double-strand length of the telomere by association with the TRF1 complex [28]. Overexpression of a POT1 allele lacking the DNA binding domain, POT1$^{\Delta OB}$, induced dramatic telomere elongation [28, 29]. Again, as for TIN2-13, telomere elongation occurred even though TRF1 remained on telomeres [28], suggesting that the deleted OB domain of POT1 might normally relay a negative signal from TRF1 to telomerase by binding the 3′ overhang and blocking telomerase access [28].

Even though these studies establish TRF1 as the link between telomeric DNA and proteins that regulate telomerase access, a number of observations raise the possibility that TRF2 could play a similar role. First, when TRF1 is removed from telomeres (via a dominant negative TRF1 allele or nuclear tankyrase 1), telomeres lengthen but eventually stabilize at a new length setting [9, 21, 22]. Thus, negative regulation of telomere length occurs even in the absence of TRF1, indicating an alternate or redundant telomere length regulation pathway perhaps through TRF2. Second, overexpression of a

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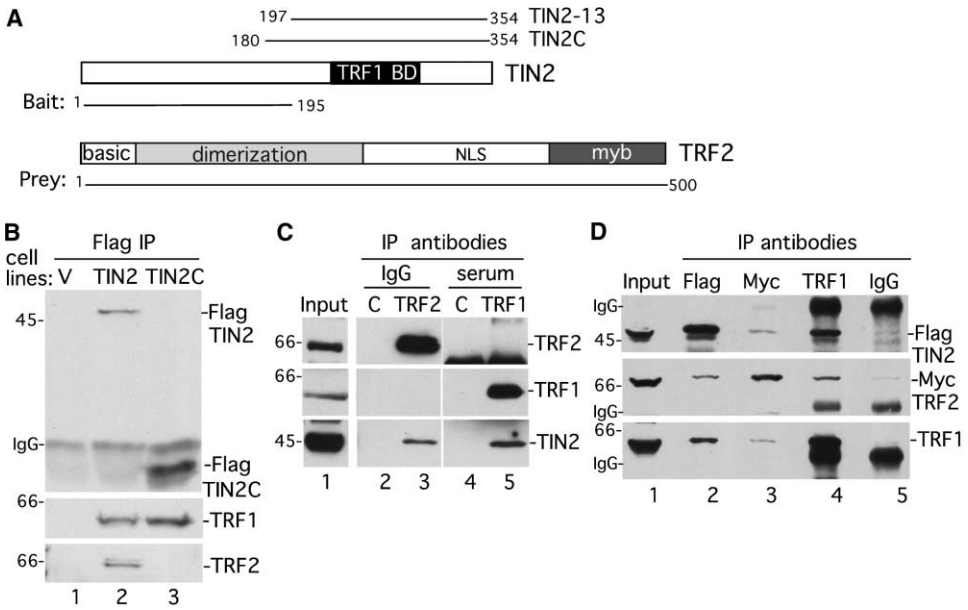

Figure 1. TIN2 Binds TRF2 In Vivo

(A) Schematic representation of TIN2 and TRF2. TRF1BD, TRF1 binding domain; NLS, nuclear localization signal; myb, myb-type DNA binding motif. TIN2-13 indicates the amino terminally deleted allele of TIN2 that induces telomere elongation. TIN2C indicates the carboxy terminal domain expressed in HTC75 cells. Bait indicates the clone used in the two-hybrid screen. Prey indicates the single full-length isolate of TRF2.

(B) TRF2 is coimmunoprecipitated by TIN2 not TIN2C. Stable HTC75 cell lines expressing vector (V), FlagTIN2, or FlagTIN2C were lysed and immunoprecipitated (IP) with anti-Flag beads. Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-Flag, anti-TRF1 415, or anti-TRF2.

(C) Endogenous TRF2 and TIN2 coimmunoprecipitate. HeLaI.2.11 cells were lysed and immunoprecipitated (IP) with IgG (anti-Flag as control [C] or anti-TRF2) or with serum (normal serum as control [C] or anti-TRF1). Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-TRF2, anti-TRF1 415, or anti-TIN2 701. Input indicates 4% of input extract.

(D) Evidence for a ternary complex of TRF1, TRF2, and TIN2. Stable HTC75 cells expressing FlagTIN2 were cotransfected with plasmids encoding MycTRF2 and TRF1. Cell lysates were immunoprecipitated (IP) with anti-Flag beads, anti-Myc beads, anti-TRF1 415, or IgG. Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-TIN2 701, anti-Myc, or anti-TRF1 415. Input indicates 4% of input extract.

number of alleles of RAP1 (a TRF2 binding protein) in HTC75 cells induces telomerase-dependent telomere elongation [30–32].

Here, we provide a molecular connection between TRF1 and TRF2 and potentially for telomere length maintenance as well. We show that TIN2, along with an associated novel telomere length regulator TINT1, binds to both TRF1 and TRF2. Our studies indicate a dynamic cross talk between TRF1 and TRF2; when TRF1 is removed from telomeres, association of TIN2 and TINT1 with TRF2 is increased. Increased binding of the TIN2/TINT1 complex to TRF2 could act as a signal to communicate changes in TRF1 levels and to ultimately establish a new telomere length setting in the absence of TRF1.

Results

TIN2 Binds TRF2

Previous work indicated that overexpression of an amino terminally deleted form of TIN2, TIN2-13, (Figure 1A) resulted in dramatic telomere lengthening [24], raising the possibility that the deleted amino terminal domain of TIN2 might normally interact with proteins to negatively regulate telomere length. A two-hybrid screen using this domain as bait resulted in identification of two proteins: the previously characterized telomeric protein, TRF2 (Figure 1A) and a novel uncharacterized protein (hypothetical protein 24432; GenBank accession # BC016904), which we named TINT1 for *T*IN2 *int*eracting protein *1* (Figure 2A).

Because TIN2 was previously characterized as a TRF1-interacting protein, we sought to substantiate the TIN2-TRF2 interaction by immunoprecipitation analysis. Whole cell extracts from stable HTC75 cell lines expressing Flag-epitope-tagged full-length TIN2 or an amino terminally deleted form (TIN2C) (Figure 1A) were immunoprecipitated with anti-Flag antibody and analyzed by immunoblot. As shown in Figure 1B, both TRF1 and TRF2 coimmunoprecipitated with full-length TIN2 (lane 2). TRF2 did not coimmunoprecipitate with TIN2C (lane 3), indicating that the amino terminal domain of TIN2 was required for the TRF2 interaction. Interestingly, TRF2 showed a positive interaction with TIN2C in a two-hybrid assay (data not shown). However, because this interaction was not substantiated by coimmunoprecipitation analysis (Figure 1B, lane 3), its physiological relevance is unclear. To determine if the endogenous proteins were complexed in cells, immunoprecipitation analysis was performed on HeLaI.2.11 cells. As shown in Figure 1C, endogenous TIN2 coimmunoprecipitated with endogenous TRF2 (lane 3) or TRF1 (lane 5).

The observation that TIN2 contained distinct binding

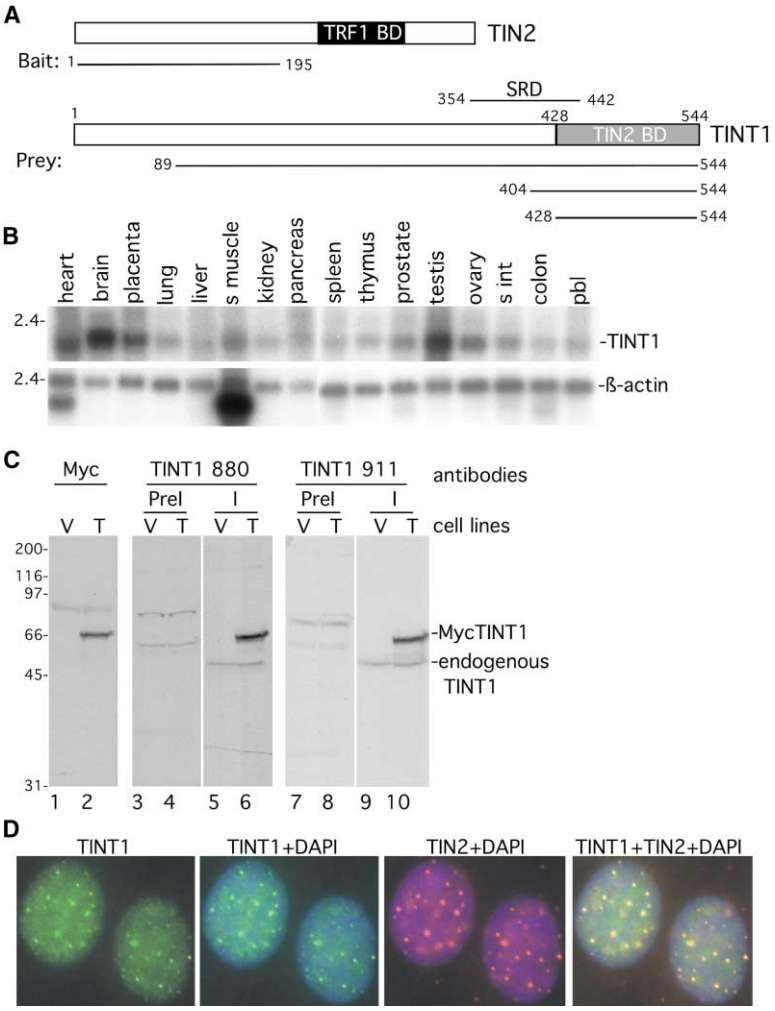

Figure 2. Identification, Expression, and Subcellular Localization of TINT1, a Novel TIN2-Interacting Protein

(A) Schematic representation of TIN2 and TINT1. TRF1BD, TRF1 binding domain; TIN2BD, TIN2 binding domain; SRD, serine rich domain. Bait indicates the clone used in the two-hybrid screen. Prey indicates three unique isolates of TINT1.

(B) Northern blot of polyadenylated RNAs from the indicated human tissues (Clontech) probed with a TINT1 cDNA or β-actin.

(C) Characterization of anti-TINT1 antibodies. Whole cell extracts from HTC75 cells expressing vector (V) or MycTINT1 (T) were analyzed by immunoblotting with anti-Myc, anti-TINT1 880 (raised against a TINT1 peptide), or anti-TINT1 911 (raised against a TINT1-GST fusion protein) antibodies. PreI, preimmune serum; I, immune affinity purified IgG.

(D) Localization of TINT1 to the nucleoplasm and to telomeres. Immunofluorescence analysis of paraformaldehyde-fixed HeLaI.2.11 cells stained with anti-TINT1 880 (green) and anti-TIN2 701 (red). DAPI staining of the DNA is blue.

sites for TRF2 (in its amino terminus) and TRF1 (in its carboxy terminus) raised the possibility of a ternary complex containing all three proteins. However, as shown in Figure 1C, TRF1 did not coimmunoprecipitate with TRF2 (lane 3), nor did TRF2 coimmunoprecipitate with TRF1 (lane 5), suggesting that TRF1 and TRF2 were not in a ternary complex with TIN2. Indeed, a ternary complex was only observed under conditions where all three proteins (TRF1, TRF2, and TIN2) were overexpressed (Figure 1D and data not shown), suggesting that (at least under our immunoprecipitation conditions), a ternary complex is either unstable or rare.

TIN2 Binds a Novel Telomeric Protein, TINT1

We next turned to characterization of the second TIN2 interacting protein, TINT1 (Figure 2A). Analysis of its primary structure showed no homology to previously characterized proteins in the database. A search for known protein motifs uncovered a serine rich domain (SRD) between amino acids 354 and 442. The TIN2 binding domain (TIN2 BD), based on the smallest TINT1 clone isolated in the two-hybrid screen, mapped between amino acids 428 and 544 (Figure 2A). Northern blot analysis indicated that TINT1 was ubiquitously expressed in a variety of human tissues (Figure 2B), similar to other human telomeric proteins [12, 13, 24].

To further characterize TINT1, rabbit polyclonal antibodies were generated against a synthetic peptide of TINT1 amino acids 466–496 (rabbit anti-TINT1 880) or against recombinant glutathione S-transferase (GST) fusion protein containing TINT1 amino acids 89–544 (rabbit anti-TINT1 911). Immunoblot analysis indicated that the affinity purified TINT1 antibodies specifically detected exogenously expressed Myc-epitope-tagged TINT1, MycTINT1 (Figure 2C, lanes 6 and 10), as well as an endogenous protein (Figure 2C, lanes 5, 6, 9, and 10) which migrated at approximately 58 kDa, consistent with the predicted molecular weight of TINT1. Immunofluorescence analysis of HeLaI.2.11 cells with the anti-peptide antibody TINT1 880 revealed a diffuse nuclear and punctate telomeric staining pattern, which costained with TIN2 (Figure 2D). Anti-TINT1 911 gave a similar telomeric staining pattern.

TINT1 Is a Negative Regulator of Telomere Length

To assess the role of TINT1 in telomere length regulation, several different Myc-epitope-tagged alleles of TINT1 were created (Figure 3A) and used for generating stable

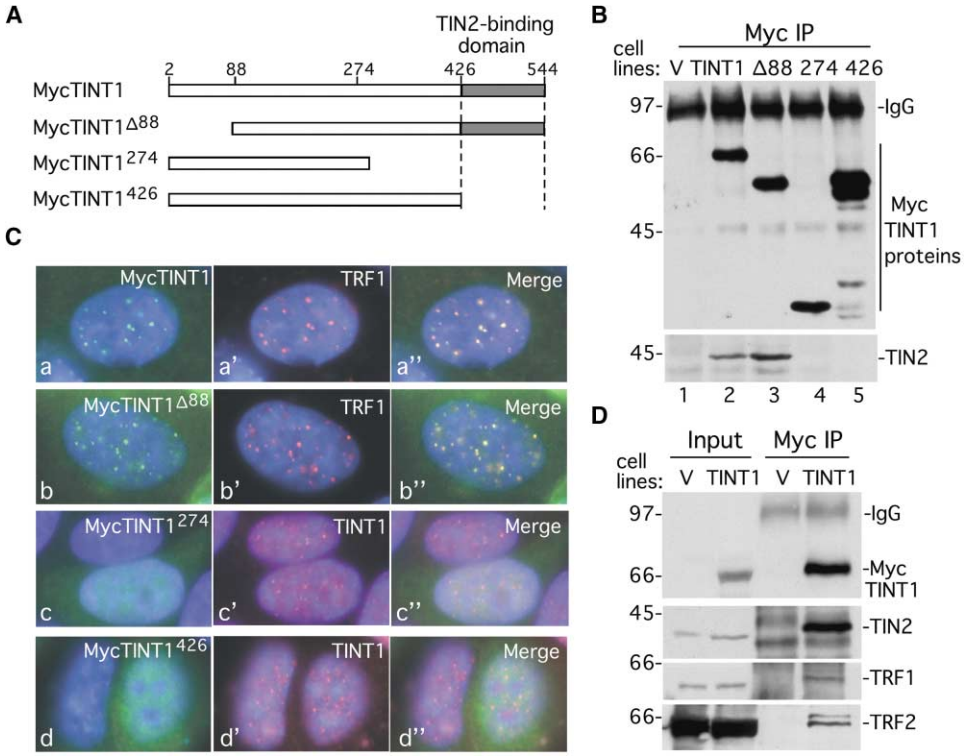

Figure 3. The Carboxy Terminal Domain of TINT1 Is Required for Interaction with TIN2 and for Localization to Telomeres

(A) Schematic diagram of Myc-epitope-tagged TINT1 alleles.

(B) Immunoprecipitation analysis of TINT1 alleles. Stable HTC75 cell lines expressing vector (V), MycTINT1, MycTINT1$^{\Delta 88}$, MycTINT1^{274}, or MycTINT1^{426} were lysed, and immunoprecipitated (IP) with anti-Myc beads. Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-Myc or anti-TIN2 701 antibodies.

(C) Subcellular localization of TINT1 alleles. Immunofluorescence analysis of paraformaldehyde-fixed HeLaI.2.11 cells after transfection for 24 hr with MycTINT1 (C_a), MycTINT1$^{\Delta 88}$ (C_b), MycTINT1^{274} (C_c), or MycTINT1^{426} (C_d). Cells were stained with anti-Myc 9E10 (C_a–C_d) (green) and anti-TRF1 415 ($C_{a'}$ and $C_{b'}$) or anti-TINT1 880 ($C_{c'}$ and $C_{d'}$) (red). DAPI staining of the DNA is blue.

(D) TINT1 coimmunoprecipitates TRF1 and TRF2. Stable HTC75 cell lines expressing vector (V) or MycTINT1 (TINT1) were lysed and immunoprecipitated (IP) with anti-Myc beads. Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-TINT1 880, anti-TIN2 701, anti-TRF1 415, or anti-TRF2 antibodies. Input indicates 4% of input extract.

HTC75 cell lines. TINT1 alleles containing the TIN2 binding domain (MycTINT1 and MycTINT1$^{\Delta 88}$) (Figure 3A) coimmunoprecipitated TIN2 (Figure 3B, lanes 2 and 3) and localized to telomeres (as evidenced by the punctate nuclear staining pattern and colocalization with TRF1) (Figures 3C_a and 3C_b). By contrast, TINT1 alleles lacking the TIN2 binding domain (MycTINT1^{274} and MycTINT1^{426}) (Figure 3A) did not coimmunoprecipitate TIN2 (Figure 3B, lanes 4 and 5), and showed a diffuse nucleoplasmic staining pattern (Figure 3C_c and 3C_d). Together, these data confirm that the carboxy terminal domain of TINT1 (amino acids 427–544) is required for binding to TIN2 and further suggest that the TIN2 interaction is required for localization of TINT1 to telomeres. To determine if TINT1 was complexed with TRF1 or TRF2, MycTINT1 immunoprecipitates were analyzed by immunoblot. As shown in Figure 3D, MycTINT1 coimmunoprecipitated TIN2, TRF1, and TRF2, indicating that the TIN2/TINT1 complex is bound to both TRF1 and TRF2.

The stable HTC75 cell lines expressing MycTINT1 alleles were carried for 144 population doublings (PD) and subjected to telomere length analysis. As shown in Figures 4A and 4B, overexpression of MycTINT1 resulted in a progressive telomere shortening, indicating TINT1 as a negative regulator of telomere length. Cells overexpressing MycTINT1$^{\Delta 88}$ or MycTINT1^{274} also displayed telomere shortening, but here, telomere length stabilized and eventually slightly lengthened. In contrast to the other three alleles, overexpression of MycTINT1^{426} resulted in progressive telomere elongation. To determine if MycTINT^{426} was acting as a dominant negative allele (i.e., promoting telomere lengthening by inhibiting endogenous TINT1), TINT1^{426}-overexpressing cells were costained with anti-TINT1 880 anti-peptide antibody (which detects endogenous TINT1 but not exogenous MycTINT1^{426}). As shown in Figure 3$C_{d'}$, endogenous TINT1 was unaffected, indicating that MycTINT1^{426} does not promote telomere elongation by removing endogenous TINT1 from telomeres.

To determine if the aforementioned TINT1 telomere length effects were dependent on telomerase, TINT1 alleles were expressed in human primary WI38 cells. As shown in Figure 4C, overexpression of TINT1 and TINT1^{274} did not increase the rate of telomere shortening compared to a vector control. Conversely, TINT1^{426} did not induce telomere lengthening. These results indicate

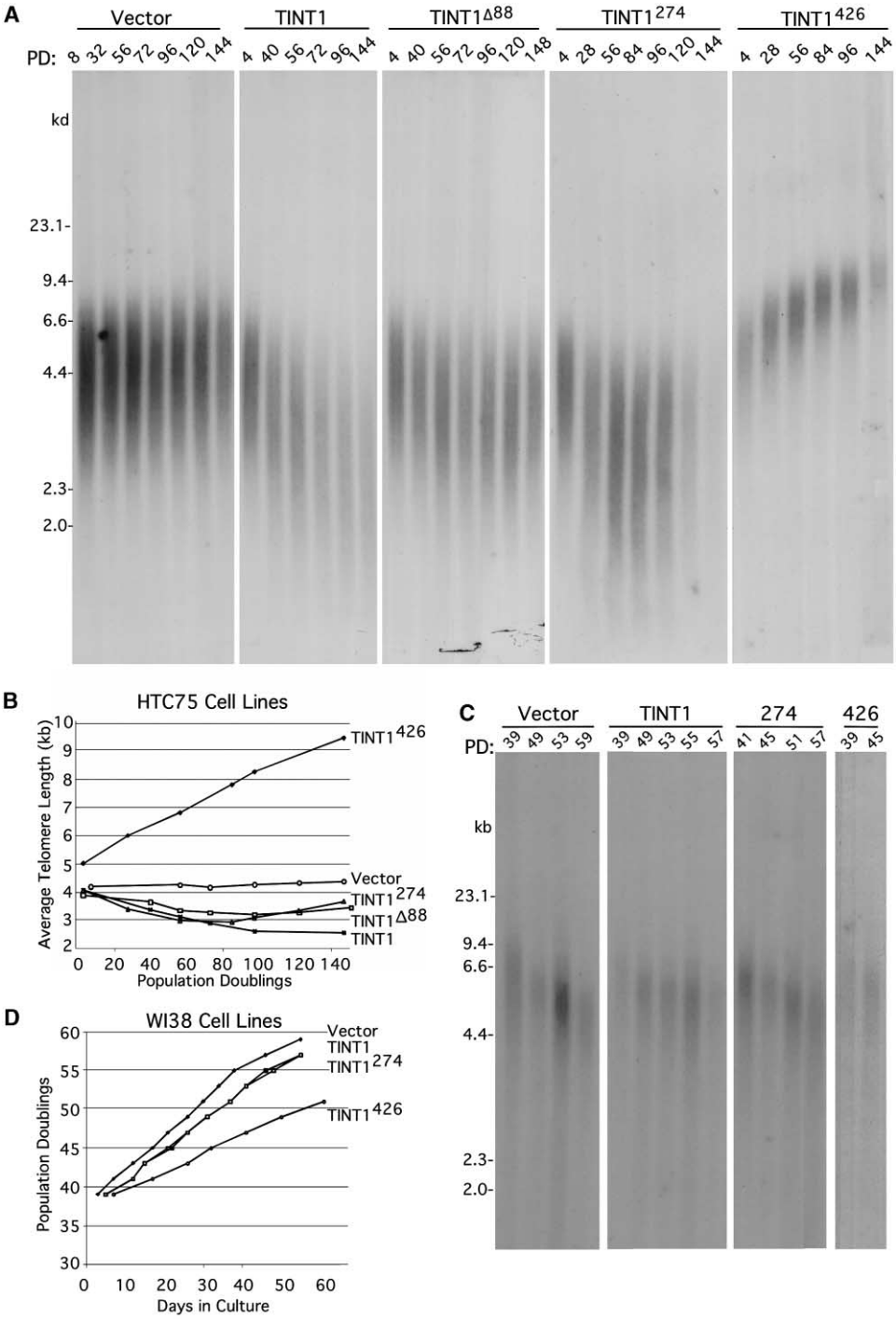

Figure 4. TINT1 Regulates Telomere Length

(A) Analysis of telomere restriction fragments isolated from stable HTC75 cell lines expressing vector, MycTINT1, MycTINT1$^{\Delta 88}$, MycTINT1^{274}, or MycTINT1^{426} at the indicated population doublings (PD) and fractionated on agarose gels. Native gels were hybridized to a ^{32}P end-labeled $(CCCTAA)_4$ oligonucleotide probe.

(B) Graphical representation of telomere length changes in the stable HTC75 cell lines expressing the indicated alleles. Plots represent the mean telomere length values derived from the Southern blots in (A).

(C) Analysis of telomere restriction fragments isolated from stable WI38 cell lines expressing vector, MycTINT1, MycTINT1^{274}, or MycTINT1^{426} at the indicated population doublings (PD) and fractionated on agarose gels. Native gels were hybridized to a ^{32}P end-labeled $(CCCTAA)_4$ oligonucleotide probe.

(D) Graphical representation of the effect of TINT1 overexpression on WI38 cell growth. Cell growth was monitored and plotted as population doublings versus days in culture.

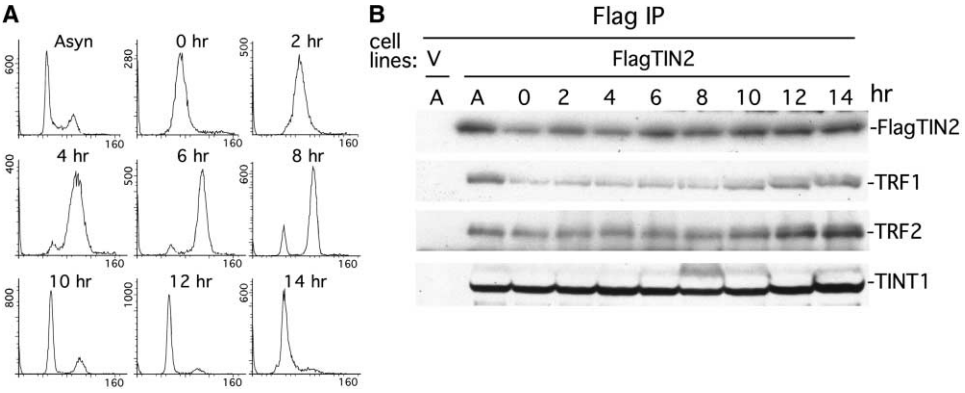

Figure 5. TIN2 Is Complexed with TINT1, TRF1, and TRF2 across the Cell Cycle

(A) FACS analysis of synchronized FlagTIN2 HTC75 cells. *y* axis, cell numbers; *x* axis, relative DNA content based on propidium iodide staining. Asyn, asynchronous; cells were released for 0–14 hr from a double-thymidine block.

(B) Immunoprecipitation of FlagTIN2 across the cell cycle. Stable HTC75 cell lines expressing vector (V) or FlagTIN2 from the indicated times after release from a double-thymidine block or from asynchronous cultures (A) were lysed, and immunoprecipitated (IP) with anti-Flag beads. Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-TIN2 701, anti-TRF1 415, anti-TRF2, and anti-TINT1 880 antibodies.

that the telomere length effects induced by TINT1 alleles depend upon telomerase, consistent with TINT1 affecting the *cis*-acting telomerase-dependent pathway of telomere length control. Interestingly, although expression of TINT1 alleles had no effect on the growth rate of HTC75 cells (data not shown), it did influence the growth of WI38 cells. As shown in Figure 4D, the WI38-TINT1 cell lines (in particular TINT1^{426}) grew more slowly than the vector control, indicating that TINT1 overexpression can influence the growth rate of human primary cells.

TIN2, TINT1, TRF1, and TRF2 Are Complexed Across the Cell Cycle

Our studies thus far indicate that TIN2 is bound to three telomeric proteins, TRF1, TRF2, and TINT1. To determine if TIN2 interaction with these three proteins varied during the cell cycle, FlagTIN2 cells were arrested in S phase by a double thymidine block. After release from arrest, cells progressed synchronously through S and G2/M, as shown by FACS analysis (Figure 5A). Immunoblot analysis of FlagTIN2 immunoprecipitates, from either asynchronous or cell cycle staged extracts, showed coimmunoprecipitation of TRF1, TRF2, and TINT1 (Figure 5B), indicating that these proteins are complexed throughout the cell cycle.

The TIN2/TINT1 Complex Associates with TRF2 and Telomeres in the Absence of TRF1

Our results suggest a partially redundant unit at telomeres of TIN2 and TINT1 bound to TRF1 or TRF2. If true, the prediction is that TIN2 and TINT1 could remain associated with telomeres via TRF2 even in the absence of TRF1. To address this question, we determined the fate of TIN2 and TINT1 when TRF1 was removed from telomeres by tankyrase 1 overexpression. For this, we used a Flag-epitope-tagged allele of tankyrase 1, which contained a nuclear localization signal, FN-tankyrase 1 [21]. As shown in Figure 6A and previously shown in [21], overexpression of tankyrase 1 in the nucleus removed TRF1 from telomeres. This is a very robust reaction in which the majority of cells overexpressing tankyrase 1 show a complete loss of telomeric TRF1. For example, out of 200 tankyrase-1-overexpressing cells, 95% showed a complete loss of TRF1, and 5% were unaffected. By contrast, in most tankyrase-1-overexpressing cells, some TIN2 remained on telomeres (Figures 6A$_b$ and 6A$_{b'}$). Only 7% of the transfected cells showed a complete loss of TIN2, 76% showed a partial loss (i.e., some TIN2 remained on telomeres), and 17% were unaffected. Similarly, costaining of tankyrase-1-overexpressing cells with TINT1 showed that TINT1 remained associated with telomeres in the absence of TRF1 (Figures 6A$_c$ and 6A$_{c'}$). Finally, we used triple immunofluorescence analysis to visualize TRF1 and TIN2 or TINT1 in the same tankyrase-1-overexpressing cell. As shown in Figure 6B, in cells overexpressing tankyrase 1 (Figure 6B$_a$), TRF1 was removed from telomeres (Figure 6B$_{a'}$), whereas in the same cells, TIN2 remained bound (Figure 6B$_{a''}$). Similar results were obtained for TINT1 (Figures 6B$_b$, 6$_{b'}$, and 6$_{b''}$).

The association between the TIN2/TINT1 complex and TRF2 in the absence of TRF1 was confirmed by immunoprecipitation analysis. Here, we took advantage of a previously generated HTC75 cell line, FN30, which expresses a doxycyclin-inducible allele of FN-tankyrase 1 [21]. As shown in Figure 6C, lane 2, overexpression of tankyrase 1 induced degradation of TRF1 but had no effect on TRF2 or TINT1. However, we did observe a small but reproducible increase in TIN2 levels. Immunoprecipitation analysis indicated an increased association between TRF2 and the TIN2/TINT1 complex in the absence of TRF1: TIN2 coimmunoprecipitated increased levels of TRF2, TRF2 coimmunoprecipitated increased levels of TIN2 and TINT1, and TINT1 coimmunoprecipitated increased levels of TRF2 (Figure 6C, lane 2). The increased association is not the result of telomere length changes as it occurs after short-term induction of tan-

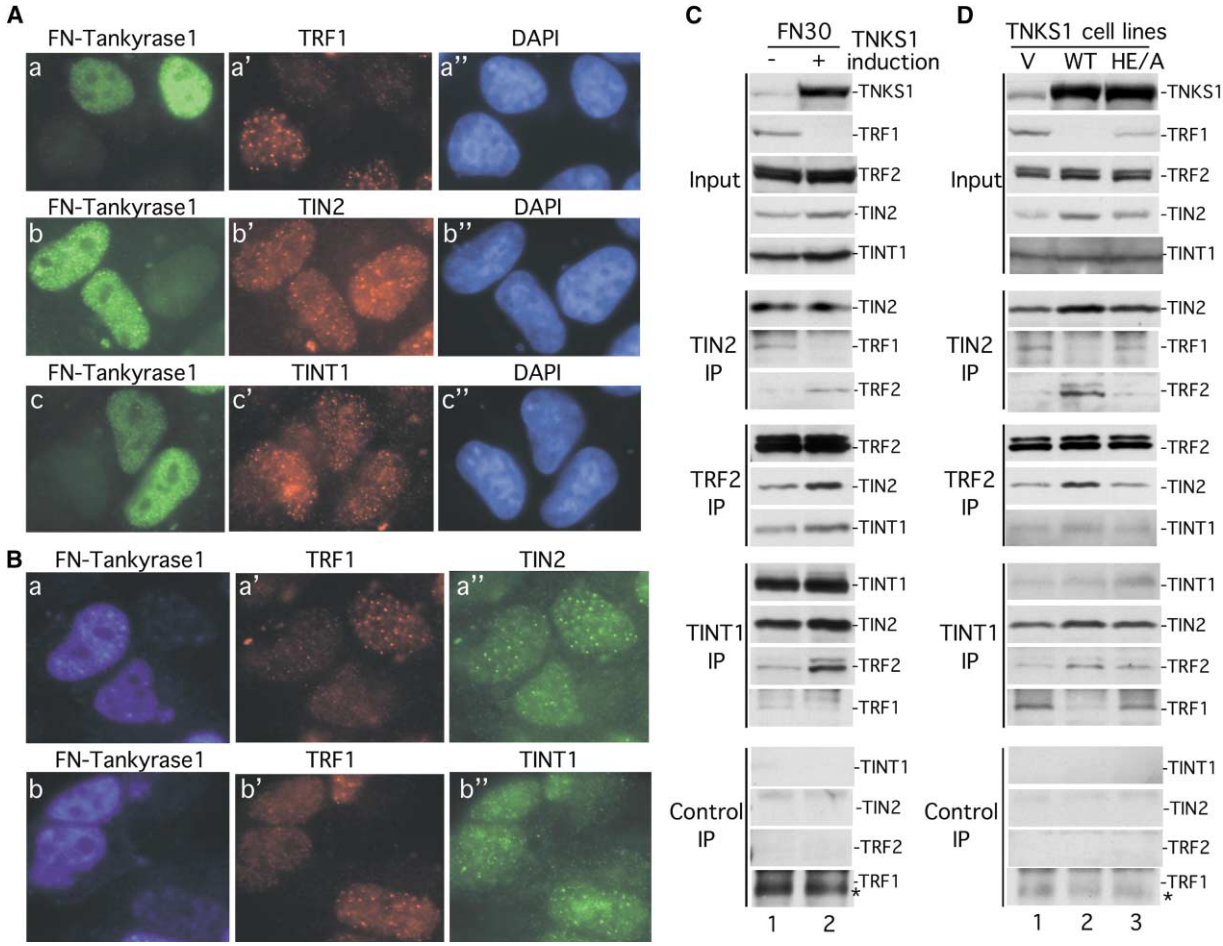

Figure 6. TIN2 and TINT1 Remain Associated with TRF2 at Telomeres in the Absence of TRF1

(A and B) Immunofluorescence analysis of tankyrase-1-overexpressing cells. HeLaI.2.11 cells were paraformaldehyde-fixed following 18 hr transfection with FN-Tankyrase 1. For double immunofluorescence, (A) cells were stained with anti-Flag (A_a, A_b, and A_c) (green) and anti-TRF1 415 ($A_{a'}$), anti-TIN2 701 ($A_{b'}$), or anti-TINT1 911 ($A_{c'}$) (red). DAPI staining of the DNA is blue. For triple immunofluorescence, (B) cells were stained with anti-ECS/Flag (B_a and B_b) (blue), anti-TRF1 415 ($B_{a'}$ and $B_{b'}$) (red), and anti-TIN2 701 ($B_{a'}$) or anti-TINT1 911 ($B_{b'}$) (green).

(C and D) Immunoprecipitation analysis of tankyrase 1 cell lines. (C) FN30 (HTC75 cells expressing inducible FN-tankyrase 1) grown in the absence or presence of doxycylin or (D) HTC75 cells constitutively expressing a vector control pLPC (V), FN-tankyrase 1 wild-type (WT), or PARP-dead (HE/A) were lysed and immunoprecipitated (IP) with anti-TIN2 701, anti-TRF2, anti-TINT 880 antibodies, or control (anti-rabbit IgG or mouse serum). Proteins were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-tankyrase 1 465, anti-TRF1 415, anti-TRF2, anti-TIN2 701, or anti-TINT1 911 (indicated to the right of each blot). The asterisk indicates a nonspecific band observed in control immunoprecipitates blotted with anti-TRF1 415 antibody. Input indicates 4% of input extract.

kyrase 1 before any significant telomere elongation occurs [21].

Identical results were obtained with a different set of HTC75 stable cell lines, which constitutively express wild-type (WT) or catalytically PARP-dead (HE/A) FN-tankyrase 1 [22]. As shown in Figure 6D, overexpression of tankyrase 1 WT (lane 2), but not tankyrase 1 HE/A (lane 3) or vector control (lane 1), induced degradation of TRF1 but not TRF2, TINT1, or TIN2. Immunoprecipitation analysis indicated increased association between TRF2 and the TIN2/TINT1 complex in tankyrase 1 WT cells (in which TRF1 was degraded) (Figure 6D, lane 2) but not in the vector control (Figure 6D, lane 1) or PARP-dead HE/A cells (Figure 6D, lane 3). Together, the immunofluorescence analysis and coimmunoprecipitation data presented in Figure 6 demonstrate that TIN2 and TINT1 can remain associated with TRF2 at telomeres in the absence of TRF1, and the data further suggest that association between the TIN2/TINT1 complex and TRF2 is increased in cells that overexpress tankyrase 1 and contain reduced levels of TRF1.

Discussion

Mammalian telomeric chromatin consists of tandem arrays of TTAGGG repeats bound to the double-strand DNA binding proteins TRF1 and TRF2. These two proteins do not interact with each other directly; they have each previously been thought to associate with a unique set of proteins to execute discrete functions at telomeres. Our studies show that they do, in fact, associate with a common set of telomere length regulators TIN2 and TINT1, suggesting a molecular mechanism for cross

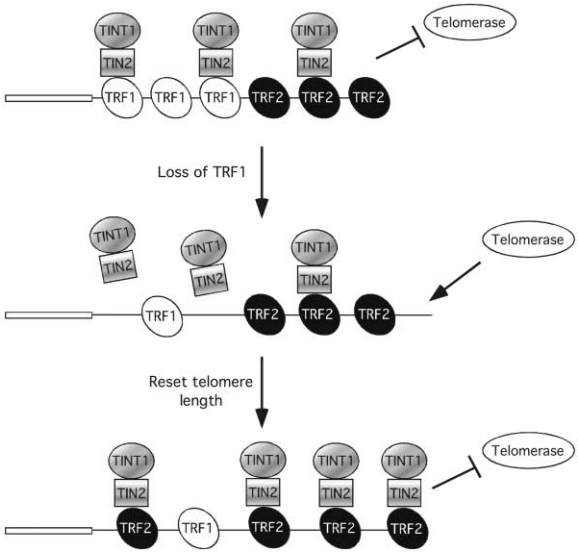

Figure 7. A Hypothetical Model for Telomere Length Regulation by TRF2 in the Absence of TRF1

TINT1, a negative regulator of telomere length, associates with TRF1 or TRF2 via TIN2. When TRF1 is removed from telomeres, the TIN2/TINT1 complex can remain on telomeres via an increased association with TRF2. Eventually, TRF2 can recruit enough TINT1 to block access to telomerase and establish a new telomere length setting.

talk between TRF1 and TRF2 and for a flexibility or redundancy in telomere length regulation.

TIN2 Binds TRF1 and TRF2

Our data indicate that TIN2 contains, in addition to the previously reported TRF1 binding site in its carboxy terminus [24], a TRF2 binding site in its amino terminus. We demonstrate by coimmunoprecipitation analysis of endogenous proteins that TIN2 is complexed with either TRF1 or TRF2 (Figure 1C). Under our immunoprecipitation conditions, only a small fraction of the total TIN2 in the cell is complexed to TRF1 or TRF2. However, this is likely to be an underestimate of the amount in vivo. Indeed, when cells are fractionated into nuclear and cytosolic extracts by hypotonic dounce lysis, whereas TRF1 and TRF2 fractionate exclusively with nuclei, as much as half of the TIN2 leaks out into the cytosol (W.C., S.S., unpublished data), indicating a highly unstable association. Similarly, although we could immunoprecipitate a ternary complex of TIN2, TRF1, and TRF2, it appeared to be minor, and it required simultaneous overexpression of all three proteins (Figure 1D). Again, such a ternary complex may be more prevalent in vivo but fall apart upon cell lysis. It will be important in the future to determine the stability and characteristics of these complexes in vivo. This may be achieved through a combination of in vivo crosslinking, subsequent fractionation, and gradient sedimentation.

TINT1, a Novel Telomere Length Regulator

Overexpression of full-length TINT1 in HTC75 cells resulted in telomere shortening of approximately 11 bp per PD (Figures 4A and 4B), similar to the rate previously shown for TRF1 overexpression (3–10 bp per PD) [9]. Moreover, like TRF1 [15, 33], TINT1 had no effect on telomere shortening in telomerase-negative primary cells (Figure 4C), suggesting that TINT1 acts in *cis* at telomeres to block access of telomerase. While this paper was under review, TINT1 was independently identified as PTOP [34] or PIP1 [35] by mass spectrometric analysis of TIN2 immunoprecipitates, where it was found that in addition to TIN2, TINT1 (PTOP/PIP1) also bound to POT1. Knockdown of TINT1 (PTOP/PIP1) expression resulted in telomere elongation [35], consistent with a role as a negative regulator of telomere length.

We showed that TINT1 associated with TIN2 (and localized to telomeres) via its carboxy terminal amino acids 427–544 (Figures 3B and 3C). Overexpression of an allele lacking this domain, TINT1^{426}, promoted telomere elongation (Figures 4A and 4B). TINT1^{426} did not localize to telomeres but rather exhibited a diffuse nucleoplasmic stain (Figure 3C$_d$). It will be important to determine how TINT1^{426} exerts its effect at telomeres. The finding that its overexpression had no effect on localization of endogenous TINT1 (Figure 3C$_{d'}$) makes a dominant negative effect unlikely. Of the two alleles lacking the TIN2 binding domain (TINT1^{274} and TINT1^{426}), only TINT1^{426} induced telomere elongation, implicating amino acids 275 to 426 in telomere elongation. Interestingly, amino acids 244–337 of TINT1 (PTOP/PIP1) [34] were identified as the POT1 recruiting domain, and this domain alone was shown to induce telomere elongation [34]. Thus, based on these findings, TINT1^{426} could act by stripping POT1 off telomeres and allowing access to telomerase. Although TINT1^{426} had no effect on the growth rate of telomerase positive HTC75 cells, it did dramatically slow the growth rate of primary WI38 cells. It is interesting to speculate that this same activity of TINT1^{426} (removal of POT1 from telomeres) could have a differential effect on cells, depending on their telomerase status.

Two other alleles, TINT1$^{\Delta 88}$ and TINT1^{274}, promoted telomere shortening but not to the same extent as full-length TINT1 (Figures 4A and 4B). The observation that full-length TINT1 promotes a more dramatic shortening than TINT1$^{\Delta 88}$ suggests that the amino terminal 88 amino acids could play some role in telomere length regulation. Similarly, the finding that TINT1^{274} can influence telomere length (despite the fact that it lacks both the POT1- and TIN2-interacting domains) suggests that the amino terminal 274 amino acids could interact with other protein(s) that might influence telomere length.

The TIN2/TINT1 Complex Associates with TRF1 or TRF2

Our immunoprecipitation and immunofluorescence analyses indicate that the TIN2/TINT1 complex associates with TRF1 or TRF2 at telomeres. This association occurs throughout the cell cycle, indicating these complexes as persistent units of telomeric chromatin (Figure 5). Moreover, we demonstrate that association of the complex with TRF1 or TRF2 is dynamic; in cells overexpressing tankyrase 1, there is an increased association between the TIN2/TINT1 complex and TRF2 (Figures 6C and 6D). We propose that this increased association of the TIN2/TINT1 complex with TRF2 may serve to regulate telomere length in the absence of TRF1.

Conclusions

We consider our results in terms of a hypothetical model shown in Figure 7. Normally, TRF1 and its associated factors, TIN2 and TINT1, negatively regulate telomere length by blocking access to telomerase. When TRF1 is removed from telomeres (via a dominant negative TRF1 allele or nuclear tankyrase 1), telomeres lengthen but eventually stabilize at a new length setting [9, 21, 22]. How can telomere length be reset without the negative regulation of TRF1 and its associated proteins? One possibility is that the freed TIN2/TINT1 complex could associate with TRF2 to block access to telomerase and eventually establish a new telomere length setting. The recent demonstration of TINT1 (PTOP/PIP1) as a POT1 binding protein [34, 35] suggests a mechanism for how the TIN2/TINT1 complex could block access to telomerase (i.e., by recruiting POT1 to the 3′ overhang). POT1 was found to associate with TRF1 by coimmunoprecipitation [28], presumably through the TIN2/TINT1 complex. Whether POT1 associates with TRF2 in this same way remains to be determined.

The scenario described above provides a mechanism for telomere length homeostasis in the absence of TRF1 under conditions in which a telomeric protein (such as dominant negative TRF1 or nuclear tankyrase 1) is overexpressed. What role might this shared complex between TRF1 and TRF2 serve under normal steady state conditions? One possibility could be communication between TRF1 and TRF2. For example, during the course of the cell cycle, TRF1 may be released from telomeres transiently, perhaps to allow access to telomerase or passage of the replication fork. The freed TIN2/TINT1 complex could associate with TRF2, signaling changes in TRF1. The TIN2/TINT1 complex could also uniquely affect TRF2's protective function, perhaps contributing to modulations in the protective complex that may occur during telomere elongation or replication. Finally, the TIN2/TINT1 complex may play a general role in the organization of telomeric chromatin, coordinating both TRF1 and TRF2 along with telomeric DNA into a higher-order chromatin structure. Here, redundancy would allow the telomeric chromatin structure to be maintained despite changes to either TRF1 or TRF2.

Experimental Procedures

Plasmids

The LexA-TIN2 two-hybrid bait consisted of human TIN2 cDNA amino acids 1 to 195 cloned into the pBTM116 vector [36]. FN-tankyrase 1 [21] consisted of an amino terminal Flag epitope tag followed by a nuclear localization signal and human tankyrase 1 amino acids 2–1327 cloned into the pcDNA3 expression vector (Invitrogen). The human TIN2 retroviral constructs consisted of an amino terminal Flag epitope tag followed by TIN2 amino acids 2 to 354 (TIN2) or amino acids 180 to 354 (TIN2C) cloned into pBABEpuro (a gift from S. Lowe). The human TINT1 retroviral constructs contained an amino terminal Myc epitope tag followed by amino acids 2–544 (TINT1), 89–544 (TINT1$^{\Delta 88}$), 2–274 (TINT1^{274}), or 2–426 (TINT1^{426}) cloned into the retroviral vector pLPCX (Clontech). TINT1^{274} and TINT1^{426} contain the following additional amino acids (IAAASAKHR; from the polylinker sequence in the pLPCX vector upstream of the stop codon) at their carboxy termini. Amino terminal GST fusion proteins consisted of TIN2 amino acids 1 to 354 (GST-TIN2) or TINT1 amino acids 89–544 (GST-TINT$^{\Delta 88}$) cloned into the pGEX-5X-1 vector (Amersham Biosciences).

Yeast Two-Hybrid Screen

A two-hybrid screen was performed with the yeast reporter strain L40 [37] with a HeLa cDNA library (Clontech) and the LexA-TIN2 bait plasmid according to Clontech Matchmaker protocol. Of 15 positive clones, 11 contained TINT1 cDNA of varying sizes as indicated in Figure 2A. One contained full-length TRF2 cDNA.

Whole Cell Extracts

Whole cell extracts from HTC75 cells stably expressing a vector pLPC or MycTINT1 were prepared as described [22].

Immunoprecipitations

Cells were lysed (per one 15-cm-diameter dish) in 0.5 ml TNE buffer (10 mM Tris [pH7.8], 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, and protease inhibitor cocktail [Sigma]) on ice for 1 hr, then pelleted at 8,000 × g for 10 min. Supernatants were precleared with normal mouse or rabbit serum or rabbit immunoglobulin (IgG) and protein G-Sepharose rotating at 4°C for 30 min. Nonspecific antibody complexes and protein aggregates were removed by centrifugation, and the supernatant was used for immunoprecipitation analysis or fractionated directly on SDS-PAGE (indicated as input, approximately 4% of the amount used in the immunoprecipitation). Supernatants were incubated with 0.35–1.0 μg of rabbit IgG, mouse anti-Flag (Sigma), rabbit anti-TRF1 415, rabbit anti-TIN2 701, rabbit anti-TINT1 880, or mouse anti-TRF2 (Imgenex) IgG, or with .5–1 μl normal mouse or mouse anti-TRF1 serum at 4°C with rocking for 2.5 hr. Antigen-antibody complexes were collected on protein G beads at 4°C with rocking for 30 min. In the case of rabbit anti-Myc-agarose bead conjugates (Sigma) or mouse anti-Flag-agarose bead conjugates (Sigma), following the preclear, the supernatant was incubated with 35 μl of beads at 4°C with rocking for 3 hr. Protein G antibody complexes or antibody-agarose bead conjugates were then washed three times with 1.0 ml TNE buffer and suspended in Laemmli buffer. Samples were either boiled or not boiled (to prevent the IgG from comigrating with TRF1 and TINT1), fractionated on a 10% SDS-PAGE gel, and processed for immunoblotting as described below.

Immunoblotting

Proteins were electrophoretically transferred to nitrocellulose and blocked in 5% milk in PBS containing 0.1% Tween 20. Blots were incubated with the following primary antibodies: rabbit anti-TRF1 415 (0.36 μg/ml), rabbit anti-tankyrase1 465 (1.8 μg/ml), mouse monoclonal anti-TRF2 (2.0 μg/ml) (Imgenex), rabbit anti-Myc (0.8 μg/ml) (Santa Cruz Biotechnologies), mouse anti-Myc (2 μg/ml) (Upstate Biotechnology), mouse monoclonal anti-Flag M2 (4.4 μg/ml) (Sigma), rabbit anti-TIN2 701 (0.5 μg/ml), rabbit anti-TINT1 880 (0.4 μg/ml), or rabbit anti-TINT1 911 (1.9 μg/ml) followed by horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham) (1:2500). Bound antibody was detected with Super Signal West Pico (Pierce) except in the case of TRF2 immunoblots in which West Dura or West Femto Substrate (Pierce) were used.

Transient Transfections

Transfections were performed with Lipofectamine 2000 regeant (Invitrogen). FlagTIN2 HTC75 cells were cotransfected with pcDNA3hTRF1 (full-length untagged TRF1) and pcDNA3hTRF2 (full-length amino terminally Myc-epitope-tagged TRF2) for 18 hr and processed for immunoprecipitation as described above. HeLaI.2.11 cells [16] were transfected with pcDNA3-FN-tankyrase1.WT for 18 hr or MycTINT1, MycTINT1$^{\Delta 88}$, MycTINT1^{274}, or MycTINT1^{426} for 24 hr and processed for immunofluorescence analysis as described below.

Indirect Immunofluorescence

Cells were processed for immunofluorescence as described previously [22] with rabbit anti-TRF1 415 (0.36 μg/mL; [22]), mouse monoclonal anti-Flag M2 (0.9 μg/mL; Sigma), rabbit anti-TIN2 701 (0.5 μg/ml), mouse anti-TIN2 (5 μg/ml) (Imgenex), rabbit anti-TINT1 880 (0.4μg/ml), rabbit anti-TINT1 911 (1.9μg/ml), goat anti-ECS/Flag (2μg/ml; Bethyl Laboratories), mouse anti-TRF1 serum [20] (1:5000), or mouse anti-Myc 9E10 supernatant (1:50) as primary antibodies. Primary antibodies were detected with fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated donkey anti-

rabbit or anti-mouse antibodies (1:100) (Jackson Laboratories) and for triple immunofluorescence, with coumarin AMCA conjugated donkey anti-goat antibody (1:100) (Jackson Laboratories). DNA was stained with 4,6-diamino-2-phenylindole (DAPI) (0.2 μg/ml) where applicable. Images were acquired on a Zeiss Axioplan 2 microscope with a Photometrix SenSyn camera. Photographs were processed and merged using IPLab software.

Retroviruses and Cell Lines.

Amphotropic retroviruses were generated by transfection of pBABE-puro, pBABE-Flag-TIN2FL, pBABE-Flag-TIN2C, pLPC, pLPCX-Myc-TINT1FL, pLPCX-MycTINT1$^{\Delta 88}$, pLPCX-MycTINT1^{274}, or pLPCX-MycTINT1^{426} into phoenix amphotropic cells with calcium phosphate precipitation. HTC75 cells (an HT1080-derived clonal cell line [9]) or WI38 cells (human primary fibroblasts at population doubling [PD] 35; American Type Culture Collection) were essentially infected as described [38]. On day 1, 10 cm dishes containing 2×10^6 cells were retrovirally infected. On day 2, infected cells were selected with 2 μg/ml puromycin. On day 3, cells were subcultured 1:2 and upon confluence, (day 4 for HTC75 or day 5 to 6 for WI38) were designated PD 0 (HTC75) or PD 37 (WI38). Cells were grown in DMEM supplemented with 10% calf serum (HTC75) or 20% fetal calf serum (WI38) (Hyclone) and were continuously selected in puromycin.

FN30 is a HTC75-derived clonal cell line that stably expresses the doxycyclin-inducible FN-tankyrase 1.WT [21]. Cells were grown in parallel with or without doxycyclin (Sigma; 100 ng/ml) for approximately 1 to 2 weeks prior to harvest for immunoprecipitation analysis. FN-tankyrase 1.WT, FN-tankyrase1.HE/A or vector (pLPC) are HTC75-derived cell lines that stably express the indicated alleles [22]. Cells were harvested for immunoprecipitation analysis at PD 60–80.

Telomere Length Analysis

Genomic DNA was isolated as described [39] and cleaved with *Hin*f I and *Rsa* I. Approximately 1 μg of DNA was fractionated on 0.7% agarose gels, and telomeric restriction fragments were detected by in-gel hybridization to a ^{32}P end-labeled $(CCCTAA)_4$ oligonucleotide probe as described [40]. The mean length of telomeric restriction fragments was determined by TELO, a macro for NIH Image written by the Research Computing Department at Fox Chase Cancer Center (http://www.fccc.edu), by using scanned images of autoradiograms.

Generation of Antibodies

Anti-TIN2 antibody 701 was raised against a GST-TIN2C *E. coli*-derived fusion protein containing N-terminal GST and TIN2 amino acids 180–354 (TIN2C). Immune serum was passed through GST-coupled Sepharose 4B to remove the anti-GST fraction and the flow-through applied to GST-TIN2C coupled Sepharose 4B. The bound fraction was eluted with 0.1M glycine (pH 2.3). Anti-TINT1 antibody 911 was raised against a GST-TINT1$^{\Delta 88}$ *E. coli*-derived fusion protein containing N-terminal GST and TINT1 amino acids 89–544 (TINT1$^{\Delta 88}$). Immune serum was passed through GST-coupled Sepharose 4B to remove the anti-GST fraction and the flow-through applied to GST- TINT1$^{\Delta 88}$ coupled Sepharose 4B. The bound fraction was eluted with 4 M $MgCl_2$. Anti-TINT1 antibody 880 was raised and affinity purified against a peptide containing TINT1 amino acids 466 to 496 (within the TIN2 interacting domain).

Northern Blots

Northern blots containing polyadenylated RNAs from adult human tissues (Clontech) were probed with a DNA probe corresponding to amino acids 89 to 544 of TINT1 or β-actin (Clontech).

Cell Cycle Synchronization

Exponentially growing FlagTIN2 HTC75 cells were treated with 2 mM thymidine for 14 hr, washed with PBS three times, released into fresh medium for 11 hr, treated again with 2 mM thymidine for 14 hr, washed with PBS three times, and released into fresh medium for 0 to14 hr. Synchronization of the cell cycle was verified by FACS analysis. Cells were collected by trypsinization, resuspended in PBS containing 2 mM EDTA, and fixed in cold 70% ethanol. Cells were stained with propidium iodide (50 μg/ml) and analyzed with a Becton-Dickinson FACSCAN and Modfit 3.0 software.

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Accession Numbers

The GenBank accession number for the TINT1 sequence reported in this paper is BC016904.