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Identification of ATPases Pontin and Reptin as Telomerase Components Essential for Holoenzyme Assembly

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SUMMARY

Telomerase is a multisubunit ribonucleoprotein (RNP) complex that adds telomere repeats to the ends of chromosomes. Three essential telomerase components have been identified thus far: the telomerase reverse transcriptase (TERT), the telomerase RNA component (TERC), and the TERC-binding protein dyskerin. Few other proteins are known to be required for human telomerase function, limiting our understanding of both telomerase regulation and mechanisms of telomerase action. Here, we identify the ATPases pontin and reptin as telomerase components through affinity purification of TERT from human cells. Pontin interacts directly with both TERT and dyskerin, and the amount of TERT bound to pontin and reptin peaks in S phase, evidence for cell-cycle-dependent regulation of TERT. Depletion of pontin and reptin markedly impairs telomerase RNP accumulation, indicating an essential role in telomerase assembly. These findings reveal an unanticipated requirement for additional enzymes in telomerase biogenesis and suggest alternative approaches for inhibiting telomerase in cancer.

INTRODUCTION

Telomerase is a ribonucleoprotein (RNP) enzyme that adds DNA repeats to telomeres, nucleoprotein structures that cap the ends of chromosomes (Blackburn, 2001). Telomerase comprises a protein subunit, TERT, and an RNA subunit, TERC, which act together to copy the template sequence within TERC to the chromosome end. By synthesizing telomere repeats, telomerase offsets the end replication problem, the inability of DNA polymerase to fully replicate chromosome ends, thus keeping telomeres sufficiently long and stable. In settings of insufficient telomerase, telomeres progressively shorten, ultimately compromising telomere end protection at a subset of chromosome ends. These dysfunctional telomeres impair stem cell self-renewal, leading to profound defects in proliferating tissues of telomerase knockout mice (Lee et al., 1998; Allsopp et al., 2003). In human cancer, telomerase is upregulated where it serves to promote tumor proliferation and survival by supporting the maintenance of functional telomeres. These crucial roles for telomerase in tissue progenitor cells and in developing cancers highlight the need to understand mechanisms of human telomerase regulation and telomerase action.

One means by which telomerase is regulated involves the protein subunits of the telomere itself. Each telomere is bound by a six-member protein complex, shelterin, which remodels the chromosome end into a t loop conformation, prevents the telomere from being recognized as a double-strand break, and protects it from recombination (de Lange, 2005). In addition to these structural roles at the telomere, components of the shelterin complex control the action of telomerase at the 3′ chromosome end. Experiments examining the function of the shelterin components TRF1 and TRF2 through overexpression indicate that these proteins can inhibit the action of telomerase, leading to telomere shortening (Smogorzewska et al., 2000). POT1, a shelterin protein that binds the single-stranded telomere overhang, can both inhibit telomerase at telomere ends (Loayza and De Lange, 2003) and serve as a processivity factor for telomerase, enhancing telomerase-mediated telomere lengthening in vitro (Wang et al., 2007; Xin et al., 2007). Thus, telomere-binding proteins can control the activity of telomerase at chromosome ends, which is important for telomere length homeostasis.

Given the complexity of other polymerases and of other RNPs, it is likely that human telomerase requires multiple associated proteins for proper assembly, regulation, and enzymatic action on its substrate (Collins, 2006). Although few essential telomerase-associated proteins have thus far been identified, several observations suggest the existence of other telomerase components. Biochemical analyses of human telomerase by glycerol gradient sedimentation and gel filtration have shown that

telomerase resides in a large complex of ~1–2 MDa, with known components accounting for only a fraction of this mass (Schnapp et al., 1998; Xin et al., 2007). Although recombinant TERT and TERC can produce telomerase activity in vitro, telomerase reconstitution is facilitated by the presence of a eukaryotic lysate and ATP (Weinrich et al., 1997; Holt et al., 1999; Wenz et al., 2001). Thus, other cellular factors may aid in the assembly of telomerase or assist in its catalytic function.

Analysis of TERC structure and the human disease dyskeratosis congenita led to identification of the third telomerase component discovered thus far, the RNA-binding protein dyskerin (Mitchell et al., 1999a, 1999b). Dyskerin binds the H/ACA motif, a sequence in TERC required for its accumulation, and a sequence present in a subset of small nucleolar RNAs (snoRNAs) involved in RNA modification (Meier, 2005). In patients with the X-linked form of dyskeratosis congenita, mutations in dyskerin cause a significant reduction in TERC levels, diminished telomerase activity, and very short telomeres. Telomere dysfunction presumably underlies the dyskeratosis congenita phenotype, which includes severe defects in multiple self-renewing tissues, including blood, skin, lung, and oral mucosa (Marrone et al., 2005). Consistent with a central role for impaired telomerase function in this disease, autosomal dominant forms of dyskeratosis congenita are linked to mutations in TERT and TERC (Vulliamy et al., 2001; Armanios et al., 2005). Although dyskerin is associated with catalytically active telomerase (Cohen et al., 2007) and is clearly required for TERC accumulation, its precise function in the telomerase RNP remains to be determined (Mitchell et al., 1999b).

The low abundance of telomerase in human cancer cell lines and in mammalian tissues has presented significant challenges in studying telomerase in biochemical terms. As a consequence, our understanding of how human telomerase is assembled and regulated and how it interacts with telomeres remains limited. To identify additional components of the human telomerase complex and to advance our understanding of telomerase regulation and function, we purified TERT complexes from human cells using a dual-affinity chromatography strategy coupled with mass spectrometry. Here, we report the identification of the related ATPases pontin and reptin as components of a telomerase complex. We find that these previously uncharacterized telomerase components interact with both TERT and dyskerin at the endogenous level and are essential for telomerase holoenzyme assembly.

RESULTS

Purification and Mass Spectrometric Identification of Human TERT Complex Components

To characterize the size of the telomerase holoenzyme complex, we performed glycerol gradient sedimentation analyses using extracts from HeLa cells and extracts from HeLa cells stably expressing Flag-TERT from a retroviral promoter. Endogenous telomerase activity, measured by telomerase repeat amplification protocol (TRAP), and endogenous TERC, assayed by northern blot, cosedimented in a size range consistent with previous estimates of 1–2 MDa (Figure 1A) (Schnapp et al., 1998; Xin et al., 2007). Flag-TERT similarly sedimented as a large complex, suggesting that retrovirally expressed TERT associates stably with other factors that have potential relevance for telomerase function. To identify novel protein components of the telomerase complex, we purified TERT protein complexes using a modified TAP tag approach. TERT was fused at its N terminus with a dual-affinity tag consisting of a protein A moiety and three HA epitopes separated by a TEV protease cleavage site (AH3). To determine whether our tagged TERT protein was functional, we expressed AH3-TERT by retroviral transduction in primary human fibroblasts that lack TERT expression and senesce after many population doublings due to progressive telomere shortening. Tagged TERT reconstituted telomerase activity, lengthened telomeres, and immortalized human fibroblasts, indicating that AH3-TERT is fully active at telomeres (see Figure S1 available online).

For telomerase complex purifications, HeLa S3 cells expressing AH3-TERT were grown in suspension cultures. In optimizing conditions for extracting TERT protein, we found that a detergent-based lysis procedure solubilized ~75% of AH3-TERT (referred to here as "S100" extract) and salt extraction of the nuclear pellet liberated the remaining 25% of AH3-TERT (data not shown). To ensure a thorough analysis of TERT-associated proteins, TERT complexes were purified from both S100 extracts (n = 2) and nuclear extracts (n = 2). AH3-TERT was purified on rabbit IgG resin, eluted specifically with TEV protease, captured again with anti-HA resin, then eluted and analyzed by SDS-PAGE (Figures S1E and S1F). After staining with Coomassie blue, protein bands were excised, digested, and analyzed by nanoflow reverse-phase LC-MS/MS (see the Experimental Procedures). Mass spectrometric analysis of the 127 kDa band identified between 23 and 57 unique peptides matching the TERT open reading frame (Figures 1B and 1D). In addition, at least six copurifying polypeptides were enriched in each TERT purification compared to negative controls (Figure 1B).

Mass spectrometric analysis of the 50 kDa band, one of the most prominent TERT-associated proteins by SDS-PAGE, revealed that it comprised two independent proteins, the related ATPases pontin and reptin. Pontin and reptin peptides were recovered in each of four independent experiments, spanning more than 50% of the pontin and reptin open reading frames (Figures 1B–1D). To study their role in the telomerase complex, we generated highly specific polyclonal antibodies to pontin and reptin. We found that pontin and reptin participate in large complexes, which overlap the size distributions of both endogenous telomerase and Flag-TERT by glycerol gradient sedimentation (Figure 1A). Analysis of extracts from Flag-TERT cells fractionated by glycerol gradient sedimentation revealed that endogenous pontin and reptin were stably associated with Flag-TERT (Figure 1E). These results show that endogenous pontin and reptin interact with Flag-TERT in a large molecular weight complex. Based on these data, we investigated a potential role for pontin and reptin in telomerase function using biochemical and genetic approaches.

Pontin and Reptin Interact with Endogenous TERT Protein

Pontin and reptin are well-conserved AAA+ ATPases (for *A*TPases *a*ssociated with various cellular *a*ctivities) and have been identified in chromatin remodeling complexes, as

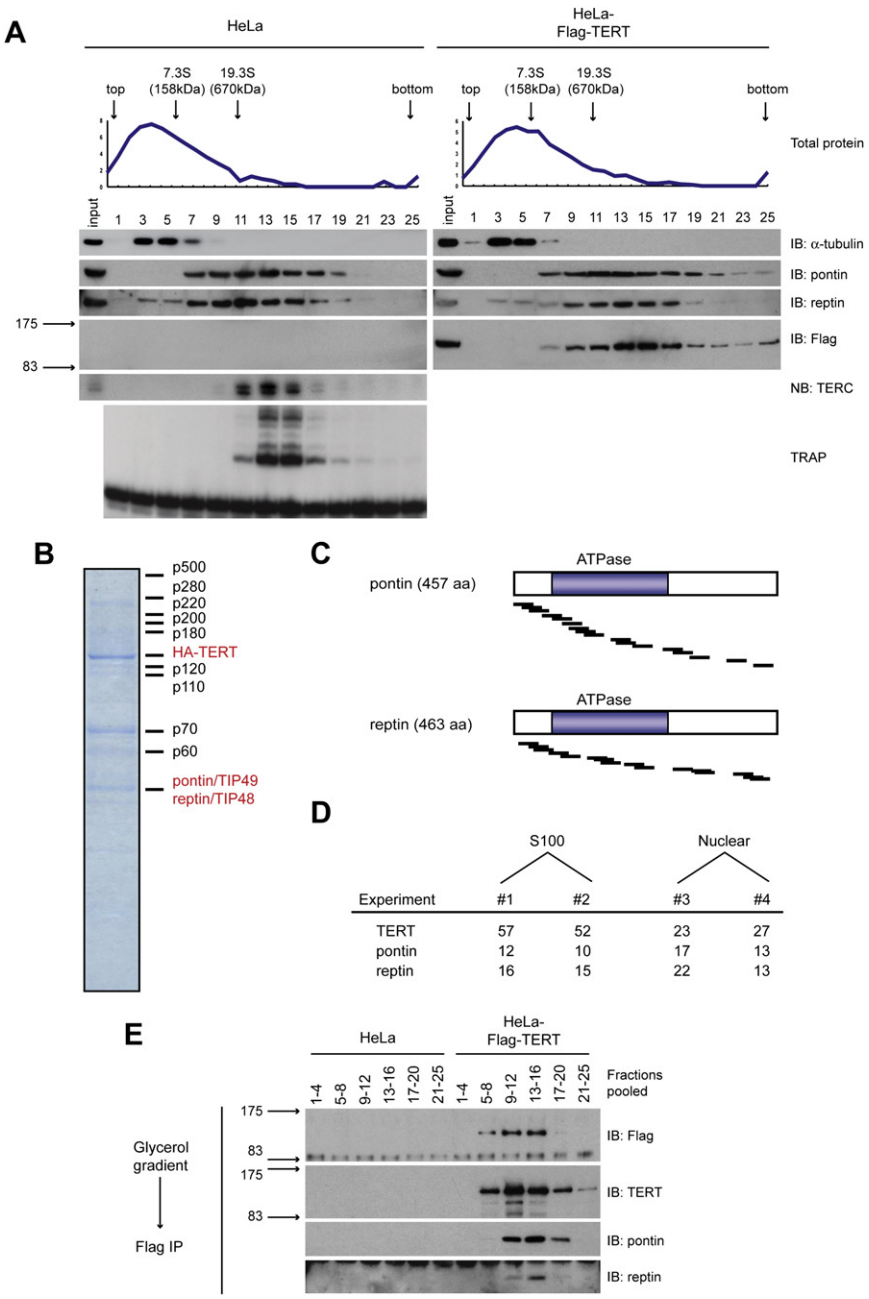

Figure 1. Pontin and Reptin Copurify with TERT through Dual-Affinity Purification and Are Components of a Large TERT Complex

(A) Sedimentation of endogenous telomerase and Flag-TERT complexes. Extracts from HeLa cells (left) and HeLa-Flag-TERT cells (right) were fractionated through 10%–30% glycerol gradients. Total protein across the gradient was measured by Bradford assay (top). IB, immunoblot; NB, northern blot.

(B) Coomassie stain of affinity-purified TERT complexes fractionated by SDS-PAGE.

(C) Diagram of pontin and reptin proteins shows location of unique peptides identified by MS (black bars). Blue boxes denote ATPase domains.

(D) Number of unique peptides obtained by mass spectrometry from four independent TERT purifications.

(E) Immunoprecipitation of Flag-TERT complexes after sedimentation in (A). Adjacent fractions were pooled and immunoprecipitated. Pontin and reptin association with TERT peaked in fractions 13–16.

cofactors for the transcriptional regulators c-*myc* and β-catenin and as proteins that interact with snoRNA complexes (Newman et al., 2000; Gallant, 2007). Pontin and reptin physically interact and are thought to function together, although there is some evidence that pontin and reptin can act independently or in an opposing fashion (Rottbauer et al., 2002; Kim et al., 2005). Pontin-specific shRNA sequences not only depleted pontin but also codepleted reptin (Figure 2A). Similarly, shRNA sequences directed against reptin codepleted pontin, indicating that these proteins mutually depend on their interaction for stability. We also found that pontin and reptin biochemically codepleted one another in cotransfection assays (Figure S2A). Furthermore, HA-TERT interacted with Flag-pontin, but not with Flag-reptin in cotransfection experiments (Figures S2B and S2C). Interestingly, the addition of pontin facilitated interaction between Flag-reptin and HA-TERT by coimmunoprecipitation, indicating that reptin is recruited into a TERT complex through bridging pontin molecules (Figure S2B). Association of endogenous pontin and reptin with Flag-TERT stably expressed in HeLa cells was resistant to treatment with DNase I, ethidium bromide, and RNase A, indicating that these interactions are not mediated by copurifying nucleic acids (Figure S2D). Together, these data show that pontin and reptin are interdependent and are recruited into TERT complexes through an association between TERT and pontin.

We reasoned that endogenous TERT should be detectable in purified complexes of pontin and reptin. However, reliable detection of the endogenous TERT protein by western blot has proven difficult due to both the low abundance of TERT and a dearth of antibodies that recognize TERT protein (Wu et al., 2006). We sought to address these significant technical hurdles by developing quantitative methods for immunoprecipitating pontin and reptin and by enhancing our ability to detect TERT through generation of high-affinity polyclonal antibodies. In attempting to overexpress pontin, we noticed that Flag-pontin underaccumulated relative to the endogenous protein (Figure 2B, compare lanes 1 and 5). However, expression of an shRNA-resistant form of Flag-pontin followed by transduction of an shRNA retrovirus targeting

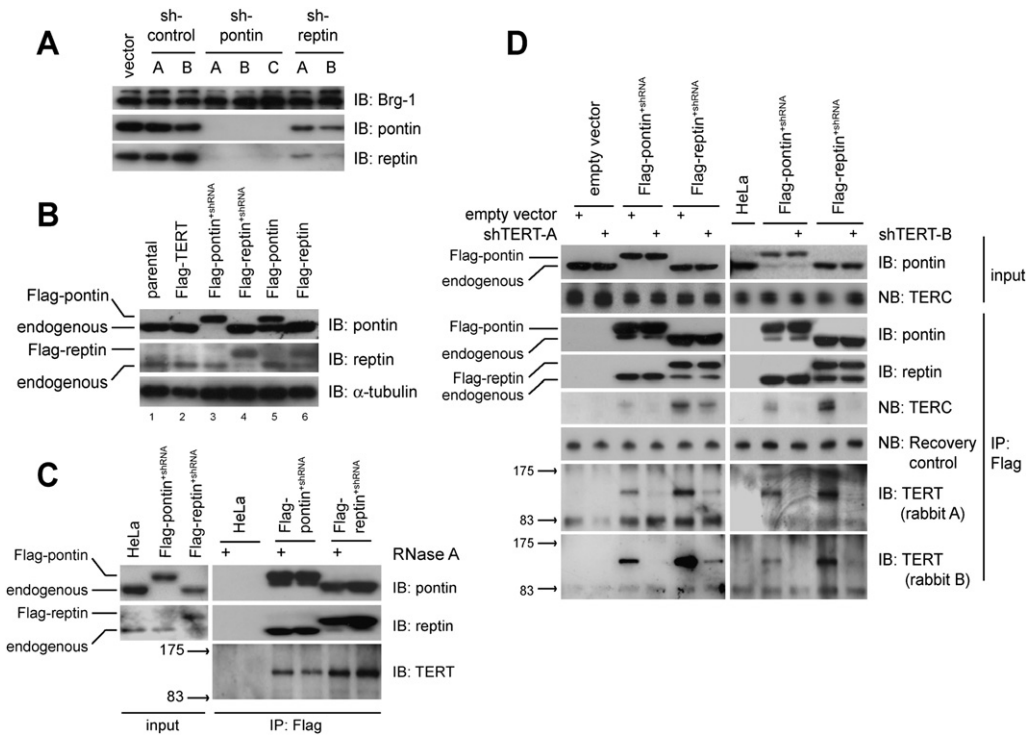

Figure 2. Pontin and Reptin Interact with Endogenous TERT and TERC

(A) Western blot showing genetic codepletion of endogenous pontin and reptin with shRNA specifically targeting either protein. Control shRNA had no effect on pontin or reptin levels. Western for Brg-1 was used as a loading control. Independent shRNAs are denoted as follows: A, B, C.

(B) Flag-pontin expressed by retroviral transduction underaccumulates relative to endogenous levels (lane 5). Serial transduction with shRNA-resistant Flag-pontin followed by shRNA against endogenous pontin results in depletion of endogenous pontin and accumulation of Flag-pontin to endogenous levels by pontin western blot (compare lanes 1 and 3). Cells treated in this manner are referred to as Flag-pontin^{+shRNA} cells. A similar strategy was used to generate Flag-reptin^{+shRNA} cells.

(C) Endogenous TERT is detected in pontin and reptin complexes purified from Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA} cells. Flag immunoprecipitation was followed by western blot with anti-TERT antibodies. RNase A treatment during immunoprecipitation did not reduce TERT association.

(D) Pontin and reptin interact with endogenous TERT and with endogenous TERC in a largely TERT-dependent manner. Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA} cells treated with independent shRNA vectors targeting TERT (A and B) resulted in decreased association of pontin and reptin with TERT and TERC. A recovery control RNA was spiked into each IP-northern blot sample to control for differential nucleic acid extraction.

the endogenous pontin protein resulted in accumulation of Flag-pontin to endogenous levels (Figure 2B, compare lanes 1 and 3). Reptin was substituted with Flag-reptin by an analogous strategy (Figure 2B). We refer to these cell lines as Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA}, respectively. Substituting pontin and reptin with tagged versions allows for quantitative immunoprecipitation using well-characterized monoclonal antibodies directed against the tag, a strategy routinely employed in yeast.

To study the endogenous TERT protein, we generated rabbit polyclonal antibodies directed against a bacterially expressed TERT polypeptide, followed by affinity purification of anti-TERT antibodies on the cognate antigen (see the Experimental Procedures). In extensive testing, these anti-TERT antibodies readily recognized stably expressed TERT by western blot and immunofluorescence, and specifically immunoprecipitated both TERC by northern blot and telomerase activity by TRAP assay (Figure S4). To determine whether endogenous TERT associates with endogenous levels of pontin and reptin, extracts from Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA} cells were immunoprecipitated with anti-Flag resin and assayed by western blot with anti-TERT antibodies. Anti-TERT antibodies reproducibly detected a polypeptide consistent with TERT's molecular mass of 127 kDa in pontin and reptin immunoprecipitates, but not in negative controls (Figure 2C). Anti-TERT immunoreactive bands were significantly diminished by two TERT-specific shRNAs and were recognized by affinity-purified antibodies from two independent rabbits (Figure 2D). As an independent measure of the association of endogenous telomerase with pontin and reptin, we found that TERC was specifically associated with pontin and reptin by immunoprecipitation-northern blot and this interaction was largely TERT dependent (Figure 2D). Together, these data show that pontin and reptin interact with endogenous TERT and TERC, the catalytic core of telomerase.

Essential Role for Pontin and Reptin in TERC Accumulation

Based on these data showing that TERT associates with pontin and reptin at the endogenous level, we investigated how loss of pontin and reptin affected telomerase activity using pontin shRNA that depletes both pontin and reptin. Whole-cell lysates

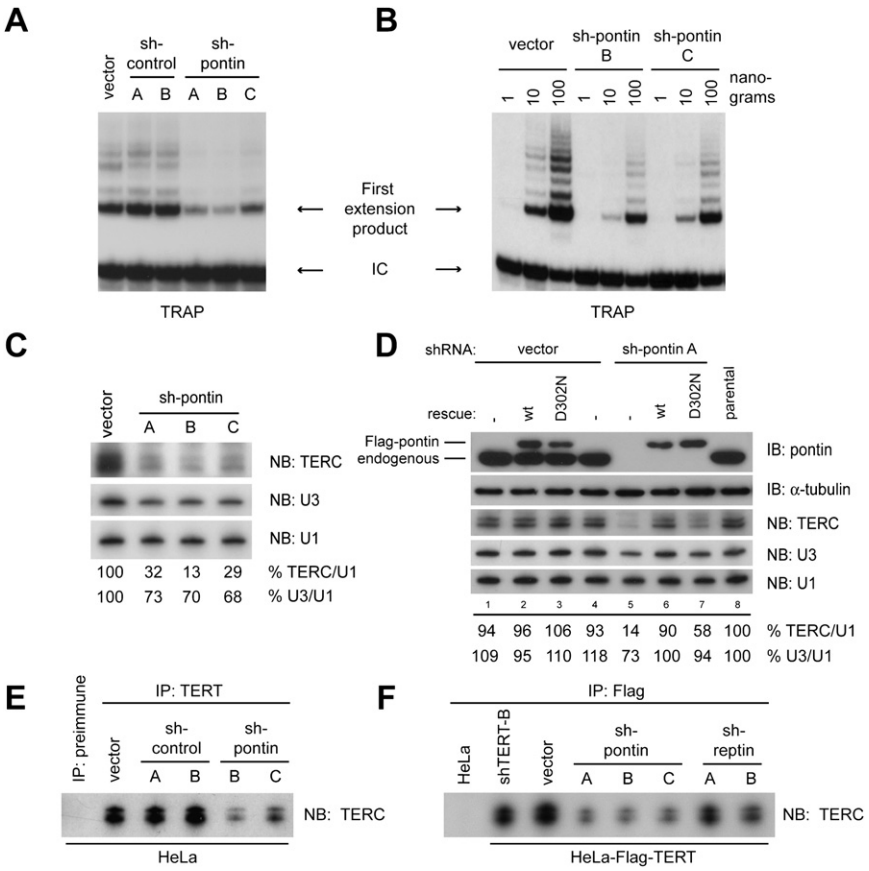

Figure 3. Pontin and Reptin Are Required for Telomerase Activity and for TERC Accumulation

(A) Diminished telomerase activity by pontin shRNA in HeLa cells.

(B) Titration of protein extracts from (A). Telomerase activity is reduced to 10%–20% of wild-type levels by pontin shRNA B or C sequences.

(C) TERC depends on pontin and reptin for accumulation to wild-type levels. Pontin shRNA reduced TERC to ~25% of wild-type levels. Band intensities for TERC and U3 snoRNA were quantified and presented as a fraction of the loading control U1 snRNA.

(D) ATPase activity of pontin is required for maintenance of TERC levels. HeLa cells depleted of pontin using shRNA are rescued by expression of a wild-type Flag-pontin construct, but not the ATPase mutant Flag-pontin^{D302N}. Note that both pontin cDNAs contained silent mutations rendering them insensitive to the pontin A shRNA. Band intensities were quantified as in (C).

(E) Endogenous TERT-TERC association is compromised following pontin and reptin depletion. Immunoprecipitation using anti-TERT antibodies in HeLa cells treated with shRNA to pontin reveal decreased TERT-TERC association relative to control shRNA vectors.

(F) The experiment in (E) was repeated in HeLa-Flag-TERT cells using anti-Flag resin to immunoprecipitate Flag-TERT.

prepared from HeLa cells transduced with shRNA vectors targeting pontin were analyzed for telomerase activity by TRAP assay. Treatment with pontin shRNA severely diminished telomerase activity to 10%–20% of wild-type levels (Figures 3A and 3B). To understand why telomerase activity was so significantly reduced following pontin and reptin depletion, we assessed TERC levels by northern blot. Strikingly, three different pontin shRNA sequences markedly reduced TERC RNA to ~25% of the level in negative controls (Figure 3C). Pontin and reptin were previously shown to be required for accumulation of the human U3 snoRNA (Watkins et al., 2004). As a control, we confirmed that pontin and reptin depletion reduced U3 snoRNA levels but did not affect the amount of U1, a small nuclear RNA (snRNA) involved in mRNA splicing (Figure 3C).

To further address the specificity of the pontin knockdown effect on TERC, we asked whether TERC levels were rescued by retroviral transduction with an shRNA-resistant Flag-pontin. Pontin knockdown had no effect on TERC levels in HeLa cells expressing shRNA-resistant Flag-pontin (Figure 3D, lane 6), demonstrating that loss of TERC in cells treated with pontin shRNA is not due to off-target effects. Correspondingly, we found that U3 levels were also restored. The ability to rescue the defect in TERC levels allowed us to ask whether pontin's ATPase activity is required for TERC accumulation. TERC levels were significantly reduced in HeLa cells expressing shRNA-resistant Flag-pontin^{D302N} following treatment with pontin shRNA, indicating that pontin ATPase activity is required to maintain wild-type levels of TERC (Figure 3D, lane 7). To understand whether pontin depletion affects only a pool of TERC molecules that are free of TERT or whether the TERC associated with TERT is also reduced, we measured the amount of TERC bound by TERT in cells treated with pontin shRNA. Immunoprecipitates of endogenous TERT in HeLa cells or ectopically expressed TERT in HeLa-Flag-TERT cells contained significantly less TERC in cells treated with pontin shRNA compared to negative controls (Figures 3E and 3F). Together, these results show that pontin and reptin are essential for telomerase activity and for TERC accumulation through a mechanism that requires ATPase function.

Pontin and Reptin Interact with Dyskerin, Forming a Complex Required for Telomerase RNP Assembly

The loss of TERC that occurs with depletion of pontin and reptin was particularly striking because it is reminiscent of the reduction in TERC levels seen in those dyskeratosis congenita patients with mutations in dyskerin. Dyskerin binds TERC at its 3′ H/ACA motif, a structural element required for TERC stability. With mutation or inactivation of dyskerin, TERC levels diminish, resulting in decreased telomerase activity (Mitchell et al., 1999b). The similar requirements for pontin, reptin, and dyskerin in maintenance of TERC levels suggested a functional relationship among these proteins. To determine whether dyskerin interacts with pontin or reptin, we first performed cotransfection assays. HA-dyskerin bound both Flag-pontin and Flag-reptin, but not the negative control Flag-BAF57 in cotransfection assays (Figure 4A).

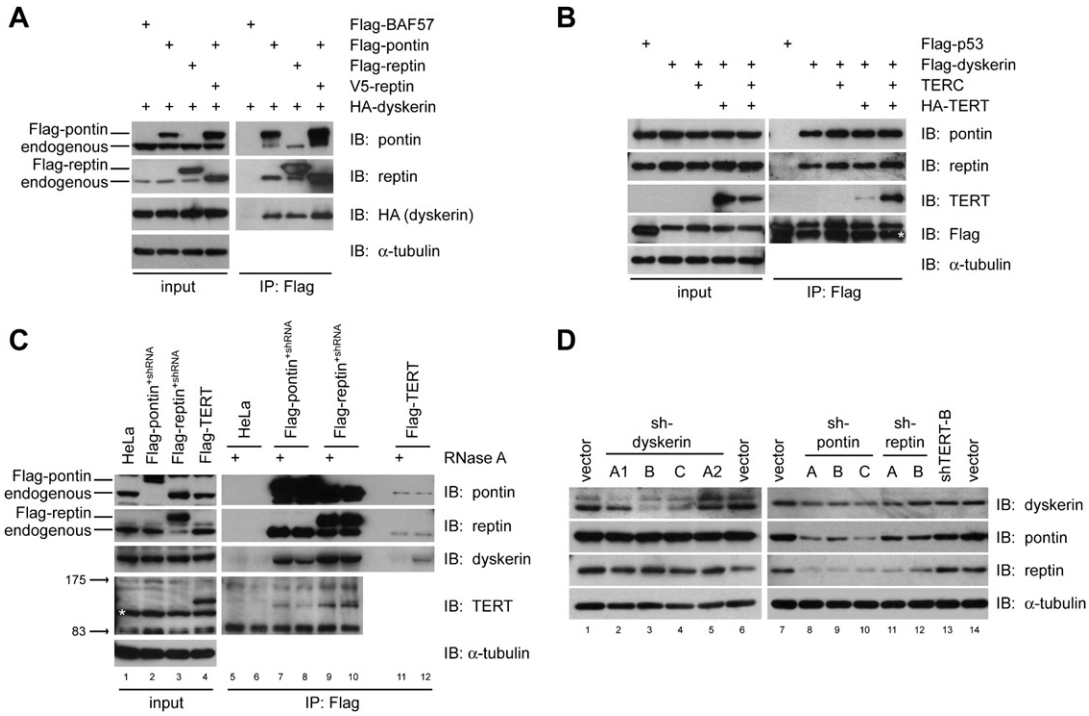

Figure 4. Pontin and Reptin Interact with Dyskerin and Are Required for Dyskerin Accumulation

(A) HA-dyskerin associates with either Flag-pontin or Flag-reptin by anti-Flag immunoprecipitation from cotransfected cells. Flag-BAF57 was used as a negative control.

(B) Flag-dyskerin coimmunoprecipitates endogenous pontin and reptin in transfected 293T cells. HA-TERT interacts weakly with Flag-dyskerin, but shows enhanced binding by coexpression of TERC. Flag-p53 serves as a negative control. The white asterisk indicates mouse IgG heavy chain.

(C) Dyskerin, pontin, and reptin interact at the endogenous level in an RNase A-insensitive manner. Anti-Flag immunoprecipitation in Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA} cells readily recovers endogenous dyskerin and TERT. Extracts from HeLa cells stably expressing Flag-TERT were immunoprecipitated with anti-Flag resin in parallel. RNase A treatment significantly reduced the amount of dyskerin associated with Flag-TERT but did not alter the amount of dyskerin bound to pontin or reptin. Asterisk indicates a nonspecific band detected by western blot of whole-cell lysate with anti-TERT antibodies.

(D) Suppression of pontin or reptin with shRNA impairs dyskerin accumulation (right panels), but shRNA vectors targeting dyskerin affect steady-state levels of neither pontin nor reptin (left panels).

Furthermore, when transfected alone, Flag-dyskerin efficiently complexed with endogenous pontin and reptin (Figure 4B). To understand the interaction between dyskerin and telomerase, TERC and/or TERT was coexpressed with Flag-dyskerin. Neither exogenous TERT nor exogenous TERC altered the amount of pontin or reptin associated with Flag-dyskerin. Coexpression of HA-TERT with Flag-dyskerin resulted in a minor amount of HA-TERT in Flag-dyskerin immunoprecipitates. However, coexpression of TERC and TERT dramatically enhanced the amount of TERT associated with dyskerin, consistent with recruitment of TERT into dyskerin complexes through their common interaction with TERC (Figure 4B) (Mitchell et al., 1999b). Consistent with these findings, immunoprecipitation of stably expressed Flag-TERT pulled down endogenous dyskerin in an RNase A-sensitive manner (Figure 4C, lanes 11 and 12).

To understand the nature of this complex at the endogenous level, we used Flag-pontin^{+shRNA} cells and Flag-reptin^{+shRNA} cells to immunoprecipitate pontin and reptin complexes (Figure 4C). Endogenous dyskerin was readily detected in pontin and reptin complexes, indicating that dyskerin interacts with both pontin and reptin at the endogenous level. We further noted that the pontin-reptin-dyskerin association was not sensitive to RNase A treatment, indicating that these contacts are not mediated by copurifying TERC. Thus, TERC connects TERT to dyskerin, while pontin and reptin bind both TERT and dyskerin through protein-protein contacts.

To study the potential interdependence of dyskerin with pontin and reptin, we depleted each protein using RNA interference and assessed protein levels by western blot. Dyskerin was depleted to varying degrees with three independent shRNA sequences, but no reciprocal effect on levels of either pontin or reptin was detected (Figure 4D, lanes 3 and 4). In contrast, knockdown of pontin and reptin led to a significant reduction in steady-state dyskerin levels (Figure 4D, lanes 8–10). Thus, dyskerin depends in part on pontin and reptin for expression at wild-type levels, highlighting the critical importance of pontin and reptin in dyskerin function.

Pontin Interacts Directly with Dyskerin and TERT

Our data show that pontin and reptin associate with both known protein constituents of telomerase, TERT and dyskerin. To better characterize the nature of these interactions, we mapped the domain of TERT that mediates the interaction with pontin and reptin. We assessed binding of endogenous pontin and reptin by

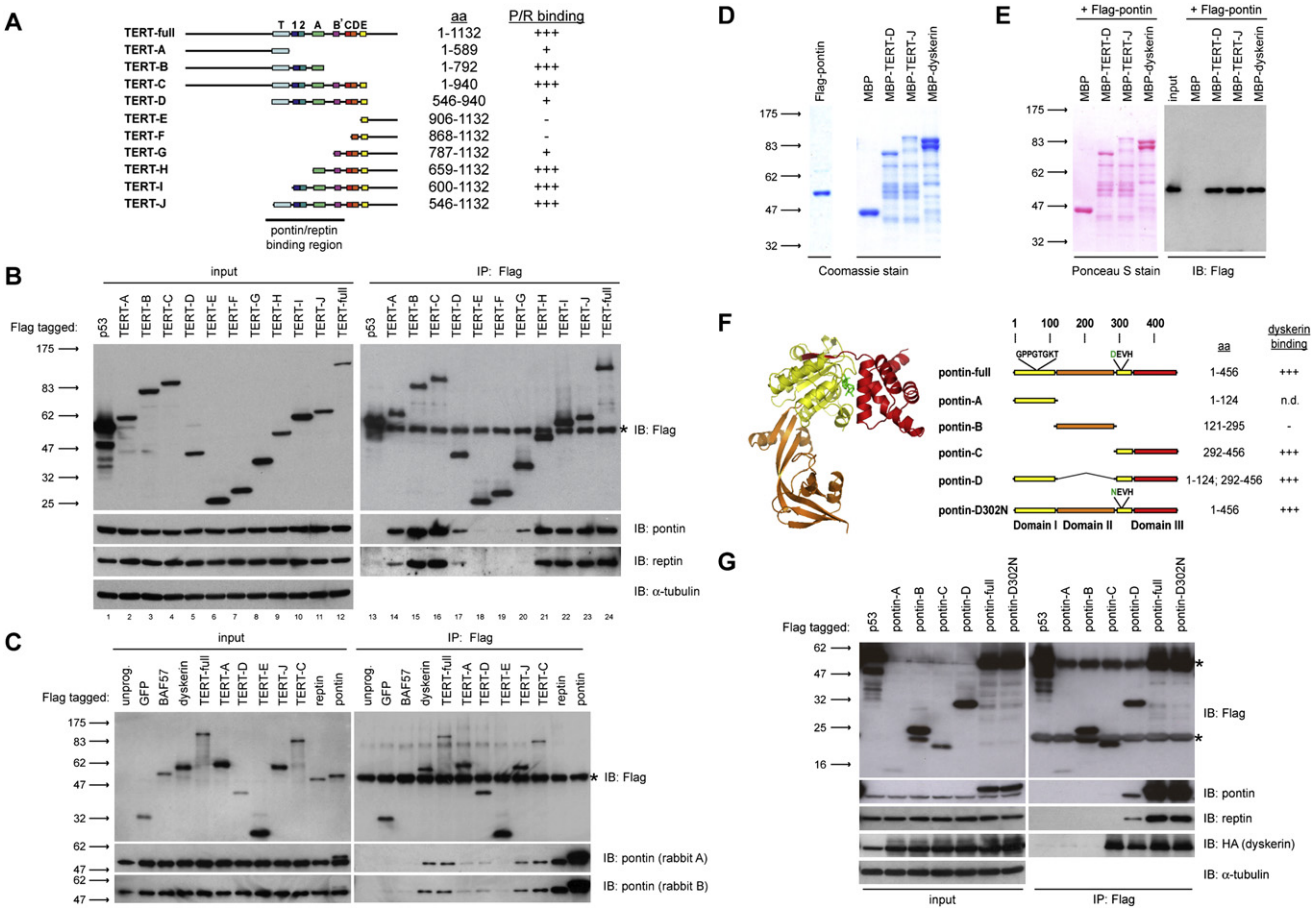

Figure 5. Pontin Interacts Directly with Dyskerin and TERT

(A) Illustration of TERT fragments used for binding assays. Results from (B) are scored in the right column as "P/R binding."

(B) Transfection assays in 293T show that Flag-tagged fragments of TERT incorporating the central reverse transcriptase domains efficiently coimmunoprecipitate endogenous pontin and reptin. Flag-p53 was used as a negative control.

(C) Rabbit reticulocyte lysate transcription-translation of Flag-tagged dyskerin, TERT, and TERT fragments coimmunoprecipitate rabbit pontin intrinsic to the lysate.

(D) Coomassie-stained gels of bacterially expressed and purified Flag-pontin, MBP-TERT fragments, and MBP-dyskerin purified for direct binding assay in (E).

(E) Direct binding assay between Flag-pontin and MBP-tagged proteins. MBP-tagged proteins were immobilized with amylose resin and incubated with Flag-pontin, washed, and analyzed by western blot with anti-Flag antibodies. Ponceau S stain shows relative amount of MBP-tagged proteins.

(F) Illustration of pontin fragments used for binding assays. Ribbon diagram for a pontin monomer (PDB 2C9O; Matias et al., 2006) guided the design for our pontin fragments. Results from (G) are scored in the right column as "dyskerin binding."

(G) Cotransfection of HA-dyskerin with Flag-pontin fragments in 293T cells shows that the C-terminal fragment of pontin (pontin-C) is sufficient to coimmunoprecipitate HA-dyskerin. Flag-p53 was used as a negative control.

immunoprecipitating a series of Flag-tagged amino-terminal or carboxy-terminal deletion fragments of TERT in 293T cells (Figures 5A and 5B). Although fragments containing the C-terminal domains of TERT did not coimmunoprecipitate pontin and reptin, extending these fragments into the RT domain conferred pontin- and reptin-binding ability (Figure 5B, lanes 18–23). Similarly, extending the N-terminal fragment into the RT domain enabled a more efficient interaction with pontin and reptin (Figure 5B, lanes 14–16). Together, these results implicate the central RT domain in binding pontin and reptin.

Recombinant TERT has been extensively studied using rabbit reticulocyte lysates (RRL) as an expression system. Remarkably, we found that Flag-TERT as well as Flag-dyskerin expressed in RRL coimmunoprecipitated rabbit pontin intrinsic to the lysate, whereas negative controls Flag-GFP and Flag-BAF57 did not (Figure 5C). Expression of a subset of the deletion mutants of TERT in RRL showed the same requirement for the RT domain in binding pontin seen in transfected cells (Figures 5B and 5C).

To study interactions in the absence of other eukaryotic proteins, we expressed MBP-dyskerin, two MBP-TERT fragments, and Flag-$(His)_6$-pontin in bacteria (Figure 5D). Purified Flag-$(His)_6$-pontin was incubated with immobilized MBP-dyskerin or MBP-TERT in a pull-down assay, and bound pontin was assayed by Flag western blot. Recombinant pontin bound recombinant MBP-dyskerin and both MBP-TERT proteins, indicating that pontin interacts directly with both dyskerin and TERT

(Figure 5E). Furthermore, the fact that pontin bound MBP-TERT-D provides additional evidence that the RT domain of TERT mediates the interaction with pontin.

To understand which region of pontin mediates interaction with dyskerin, we expressed pontin domains based on the X-ray crystal structure of pontin (Matias et al., 2006). Domains I and III interact to form the hexamer ring, whereas domain II projects downward from the plane of the ring. Expression of Flag-tagged pontin fragments (Figure 5F) with HA-dyskerin in 293T cells revealed that pontin-C and pontin-D fragments bound HA-dyskerin like full-length pontin. In contrast, pontin-B comprising domain II did not bind HA-dyskerin (Figure 5G). Together, these data show that pontin directly interacts with dyskerin and with TERT's RT domain, and suggest that the interaction surface of pontin required for binding dyskerin resides in its C-terminal domain, the region containing domain III and the Walker B motif.

Dynamic Regulation of TERT during the Cell Cycle: The TERT-Pontin-Reptin Complex Peaks in S Phase

Although telomerase lengthens short telomeres preferentially during S phase of the cell cycle in yeast (Marcand et al., 2000), and human telomerase localizes to telomeres during S phase (Jady et al., 2006; Tomlinson et al., 2006), it is unclear what mechanisms ensure that human telomerase acts on telomere ends in S phase. To determine whether pontin and reptin contribute to cell-cycle control of endogenous telomerase, we synchronized Flag-reptin^{+shRNA} cells at the G1/S transition using double thymidine blockade. Cells were released and harvested at 2 hr intervals, and synchrony was monitored by measuring DNA content by propidium iodide staining and flow cytometry (Figure 6A).

Immunoprecipitation of Flag-reptin showed that neither the total reptin pool nor the amount of pontin or dyskerin bound to reptin varied through the cell cycle. In marked contrast, the amount of TERT associated with reptin peaked in S phase. The amount of TERT bound to reptin was 3-fold higher in S phase than in G2, M, or G1 (Figure 6B). To control for potential confounding effects from thymidine blockade, we repeated the synchronization experiment and carried the HeLa-Flag-reptin^{+shRNA} cells through two cell cycles. We found that the association of reptin with endogenous TERT peaked in consecutive S phases, closely matching the pattern of expression of the S phase marker PCNA (Figures 6C and 6D). Endogenous TERT also preferentially associated with pontin in Flag-pontin^{+shRNA} cells, and the S phase-specific interaction among TERT, pontin, and reptin was also seen in experiments employing a thymidine-aphidicolin protocol for synchronization (Figure S5). These data provide evidence for dynamic regulation of telomerase during the cell cycle and indicate that TERT's association with pontin and reptin peaks in S phase, which may reflect cell-cycle regulation of total TERT protein and/or assembly of telomerase in the phase of the cell cycle during which it must act on telomeres.

TERT Exists in at Least Two Complexes

Our observations that (1) pontin and reptin are required for telomerase biogenesis and (2) the TERT-pontin-reptin complex peaks during each S phase led us to hypothesize that TERT complexes are dynamic in nature and that pontin and reptin may be involved in cyclical telomerase assembly. We asked to what extent pontin and reptin associate with "active" telomerase particles as measured using the TRAP assay. Flag antibody immunoprecipitation of lysates from Flag-pontin^{+shRNA}, Flag-reptin^{+shRNA}, or Flag-dyskerin^{+shRNA} cells, in which pontin, reptin, or dyskerin was replaced by a Flag-tagged version at the endogenous level, quantitatively depleted each Flag-tagged protein from the lysate (Figures 7A and 7B). TRAP assays performed on lysates pre- and postimmunodepletion demonstrated that, whereas immunoprecipitation of dyskerin or overexpressed Flag-TERT depleted TRAP activity from the lysate, immunoprecipitation of pontin and reptin did not reduce the overall level of TRAP activity in the extract (Figure 7C). Accordingly, TRAP assays performed on the anti-Flag immunoprecipitates showed that dyskerin and Flag-TERT brought down robust TRAP activity, whereas pontin and reptin were associated with a small but reproducible amount of activity (Figure 7D). Remarkably, however, analysis of the immunoprecipitates by western blot for endogenous TERT showed that pontin and reptin were associated with at least as much TERT protein as that bound by dyskerin (Figure 7E). Therefore, our data indicate that pontin and reptin associate with a significant population of TERT molecules that do not yield high-level TRAP activity. This marked discordance between TERT protein and catalytic activity in vitro suggests specific models for understanding how telomerase is assembled in human cancer cells (see the Discussion). Together, these data establish pontin and reptin as both TERT-interacting proteins and dyskerin-interacting proteins and show that pontin and reptin are required for assembly of a core telomerase complex, including TERT, TERC, and dyskerin.

DISCUSSION

Through biochemical purification of human TERT complexes, we have identified the ATPases pontin and reptin as essential telomerase components. Pontin interacts with both known protein constituents of telomerase, dyskerin and TERT. Importantly, the association of pontin and reptin with dyskerin and TERT occurs at the endogenous level in human cells. Furthermore, the TERT-pontin-reptin complex is cell-cycle regulated and peaks during each S phase. In loss-of-function experiments, we find that pontin and reptin are critical for telomerase activity and for accumulation of TERC and dyskerin. Finally, pontin and reptin coimmunoprecipitate a substantial pool of TERT protein that yields only low enzymatic activity as detected by standard TRAP assay. Together, these data identify two additional enzymes required for telomerase assembly and reveal a previously unappreciated complexity in telomerase biogenesis.

Multiple TERT Protein Complexes in Human Cancer Cells: A Model for Telomerase Assembly

Our data indicate that pontin and reptin serve two related roles in telomerase RNP assembly. Through their direct interaction with dyskerin, pontin and reptin are required for assembling a TERC-containing RNP (Figure 7F, bottom). Together with its associated proteins GAR1, NOP10, and NHP2, dyskerin directly binds TERC at its 3′ H/ACA motif (Mitchell et al., 1999b; Dragon et al., 2000; Pogacic et al., 2000; Fu and Collins, 2007). Deletion of the H/ACA sequence or mutation of dyskerin leads to reduced

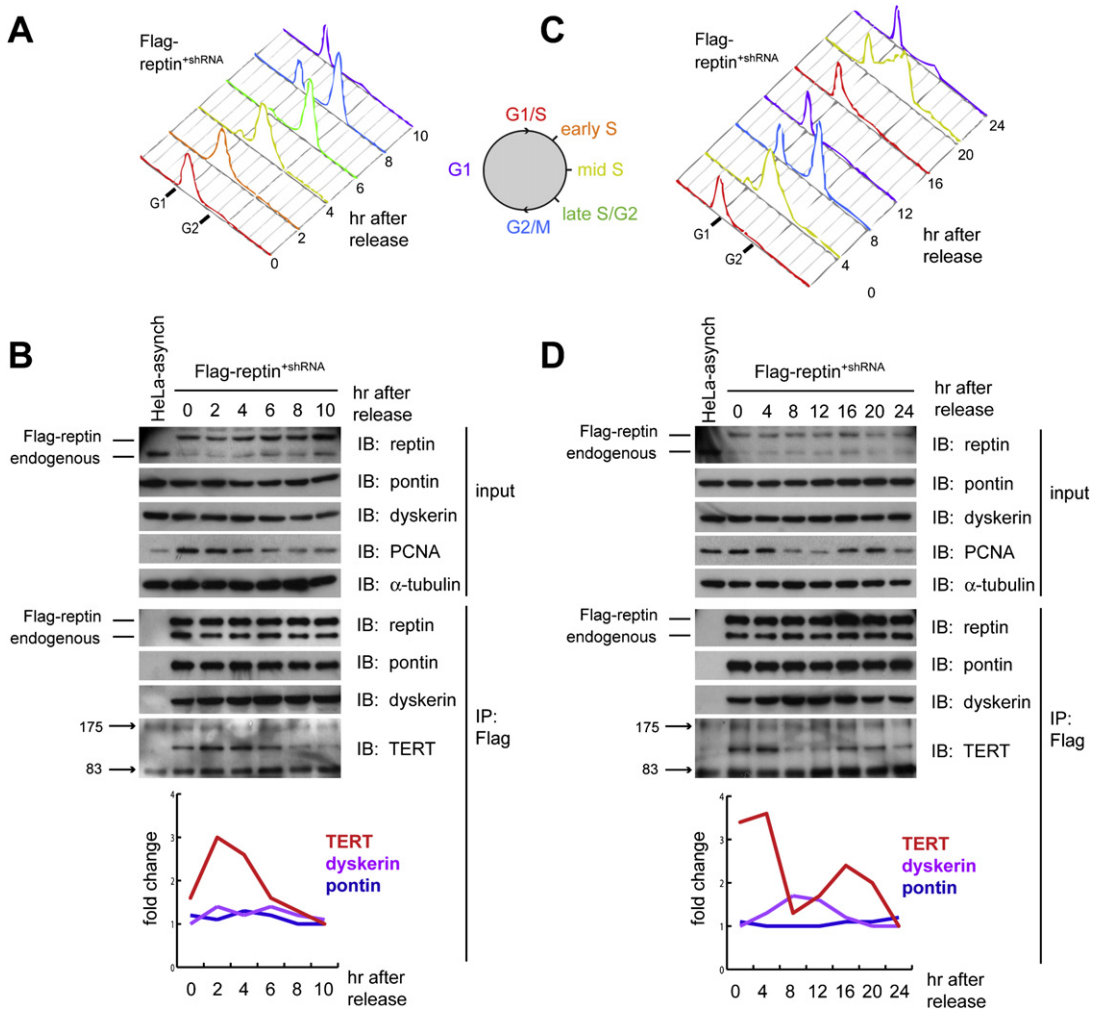

Figure 6. Pontin and Reptin Interact with TERT in S Phase

(A) Flow cytometry analysis of DNA content in HeLa-Flag-reptin^{+shRNA} cells released from double thymidine blockade over a 10 hr time course. Cells were released from the second block and harvested at 2 hr intervals, and a portion of cells were fixed and stained with propidium iodide to monitor synchrony. The 0 hr time point corresponds to unreleased cells.

(B) Coimmunoprecipitation of reptin with TERT and dyskerin over a 10 hr time course. The association of reptin with pontin and dyskerin is constant across the cell cycle, whereas associated TERT peaks in S phase. Band intensities were quantified and displayed as fold change in the accompanying graph. Western blot for PCNA was used as an independent marker of S phase.

(C) Flow cytometry analysis of DNA content in HeLa-Flag-reptin^{+shRNA} cells synchronized using double thymidine blockade as in (A), except cells were harvested at 4 hr intervals over a 24 hr time course.

(D) Coimmunoprecipitation of reptin with pontin, dyskerin, and TERT though a 24 hr time course following synchronization shown in (C). Band intensities were analyzed as in (B). Note that the amount of TERT in the reptin complex increases during the second S phase with kinetics similar to PCNA.

TERC levels. Diminished levels of TERC in patients with dyskerin mutations account for the reduced telomerase activity and markedly shorter telomeres seen in the X-linked form of dyskeratosis congenita (Mitchell et al., 1999b). Our data showing that pontin and reptin interact with dyskerin indicate that these proteins work together to assemble and stabilize the TERC RNP and this step requires pontin's ATPase activity. The loss of TERC seen with depletion of pontin, reptin, or dyskerin strongly supports our biochemical experiments showing interactions among these proteins and indicate that intact function of a pontin/reptin/dyskerin module is required for assembly of a TERC-dyskerin RNP.

In addition to this function, pontin and reptin interact with TERT, suggesting that pontin and reptin also serve to assemble or remodel a telomerase complex containing TERT (Figure 7F, top). This process may occur in a stepwise fashion in which pontin and reptin facilitate assembly of TERT with a TERC-dyskerin RNP, or remodel this maturing telomerase complex. The low catalytic activity of purified TERT-pontin-reptin complexes suggests that they may represent a pretelomerase complex that requires additional factors or remodeling for conversion to a mature telomerase complex. After assembly, pontin and reptin may dissociate from a mature TERT-TERC-dyskerin complex, which appears to comprise the majority of in vitro telomerase activity

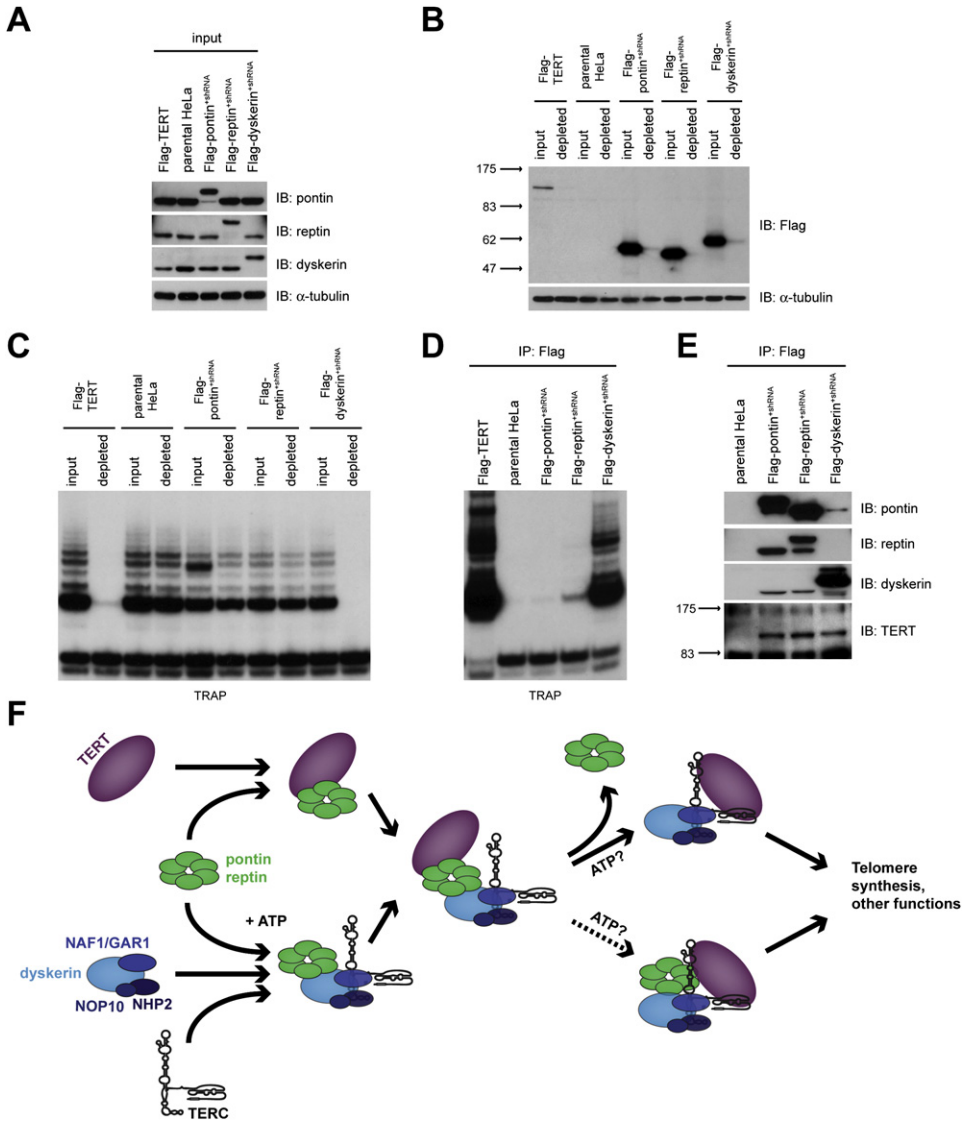

Figure 7. TERT Exists in Multiple Telomerase Complexes

(A) Western blot analysis of whole-cell lysates prepared from "replacement" cell lines for pontin, reptin, and dyskerin. Retrovirally introduced Flag-tagged pontin, reptin, or dyskerin accumulates to endogenous levels upon depletion of the respective endogenous proteins with shRNA. Note that Flag-pontin, -reptin, and -dyskerin coding sequences contain silent mutations rendering them insensitive to the shRNA vectors.

(B) Whole-cell lysates in (A) were depleted of the Flag-tagged protein using anti-Flag resin. Depletion was assessed by western blot with anti-Flag antibodies.

(C) TRAP assay of extracts in (B) shows that immunoprecipitation of Flag-TERT and Flag-dyskerin, but not Flag-pontin or Flag-reptin, depletes telomerase activity.

(D) TRAP assay on immunoprecipitates from (C). Reptin coimmunoprecipitates a small but reproducible amount of telomerase activity.

(E) TERT associates similarly with pontin/reptin and dyskerin by immunoprecipitation-western blot. Isolated pontin and reptin complexes have low TRAP activity but substantial TERT protein (shown in [D]).

(F) Model for telomerase assembly facilitated by pontin and reptin. Pontin and reptin are required for accumulation of a TERC- and dyskerin-containing RNP through steps that require ATPase function (bottom). Pontin and reptin also bind TERT (top) and may help to bring together or remodel a nascent TERT-TERC-dyskerin complex through a stepwise process. Other factors and/or ATP hydrolysis may convert the complex into the enzymatically active, TRAP-positive telomerase complex. At this point, pontin and reptin may dissociate from telomerase or remain associated with a telomerase complex that has low activity in vitro. See the Discussion for further details.

because dyskerin immunoprecipitation depleted TRAP activity from extracts. Alternatively, pontin and reptin may remain associated with a telomerase holoenzyme capable of acting on telomeres in vivo but unable to generate high-level catalytic activity on simple oligonucleotide subtrates in vitro (Figure 7).

The low catalytic activity of pontin-reptin complexes likely explains why they were not identified in a recent telomerase purification strategy that exploited the ability of telomerase to bind and extend an oligonucleotide substrate as a purification step (Cohen et al., 2007). Our data indicate that TERT protein exists

in at least two different complexes. The TERT-TERC-dyskerin complex has high telomerase catalytic activity, whereas the TERT-pontin-reptin complex exhibits much lower enzymatic activity. Although direct comparison is limited by potential variables such as efficiency of extraction, it is striking that the amounts of endogenous TERT associated with pontin and dyskerin are comparable, indicating that the TERT-pontin-reptin complex is not a rare, transient intermediate. Its abundance relative to the TERT-TERC-dyskerin complex may reflect the fact that the TERT-pontin-reptin complex requires significant time for assembly or is a target for regulation. Consistent with this idea, the association of TERT with pontin and reptin is remarkably cell-cycle dependent, appearing in S phase and diminishing in G2, M, and G1 phases. The S phase dependence of the association between TERT and pontin/reptin suggests that the telomerase complex may be assembled de novo during each S phase. Alternatively, or in addition, pontin and reptin may direct TERT into complexes with other molecules that could explain how TERT activates quiescent epidermal stem cells (Sarin et al., 2005; Flores et al., 2005).

A Critical Role for Assembly in Dynamic Macromolecular Complexes

Pontin and reptin are associated with diverse nuclear multisubunit complexes, including chromatin remodeling complexes, transcription factors, and small nucleolar ribonucleoproteins (snoRNPs). In yeast, pontin/reptin orthologs Rvb1p/Rvb2p are members of the 12-subunit Ino80 chromatin remodeling complex (Shen et al., 2000). When Rvb2p is depleted, Ino80 complexes lack not only Rvb1p and Rvb2p but also another core subunit, Arp5p, and these "incomplete" complexes can no longer remodel nucleosomes in vitro (Jonsson et al., 2004). In mammals, pontin and reptin were identified as components of the box C/D class of snoRNPs, which catalyze ribose methylation of target ribosomal and spliceosomal RNAs. Pontin and reptin have affinity for box C/D scaffolds (Newman et al., 2000), associate with "maturing" U3 snoRNPs, and are required for U3 snoRNA accumulation, as we confirmed here (Watkins et al., 2004). Thus, pontin and reptin may serve to assemble diverse complexes by interacting with components specific to each complex, as it does with dyskerin and TERT in the telomerase RNP.

A role for pontin and reptin in telomerase RNP assembly is consistent with the function of other AAA+ ATPases in dynamic nuclear complexes. For example, the complex that forms at replication origins comprises 14 proteins, ten of which are AAA+ ATPases. During each G1 phase, these proteins assemble the "prereplication complex" through sequential ATP binding and hydrolysis events, resulting in a licensed origin prepared for DNA replication in S phase (Bell and Dutta, 2002). Stepwise assembly is particularly relevant for RNP complexes, as illustrated by the example of snRNAs, which require the heptameric Sm proteins for efficient assembly (Yong et al., 2004). Similarly, TERC also requires Sm proteins for assembly in yeast and in humans (Seto et al., 1999; Fu and Collins, 2006). Interestingly, complex assembly has emerged as an important theme in the regulation of telomerase in the ciliate *Tetrahymena*, which requires a La-related protein that binds the telomerase RNA component for efficient assembly and activity (Witkin and Collins, 2004; Prathapam et al., 2005). Thus, the need for an ordered, stepwise assembly of telomerase is likely conserved across species, although the specific proteins that mediate this dynamic process may differ when comparing species separated by large evolutionary distances.

Telomerase and Disease

Telomerase is intimately associated with human cancer and therefore has been considered a potential target for anticancer therapy. However, few high-affinity inhibitors of telomerase have thus far been identified, perhaps reflecting difficulty in targeting the enzyme's reverse transcriptase function. Our data suggest that inhibitors of pontin and reptin may act as highly effective therapeutic drugs targeting telomerase. Inhibiting pontin or reptin catalytic function would mimic the effects of shRNA in impairing telomerase RNP biogenesis, thereby circumventing the empirical difficulties in targeting the active site of the TERT. Although dyskeratosis congenita has been linked to mutations in dyskerin, TERC, or TERT, the causal mutation has yet to be identified in many families (Vulliamy et al., 2006). Our data suggest that mutations in pontin or reptin may also be found in patients with dyskeratosis congenita, aplastic anemia, or pulmonary fibrosis. The essential roles for pontin and reptin in telomerase assembly suggest important future studies for these ATPases in human health and disease.

EXPERIMENTAL PROCEDURES

TERT Complex Purification and Mass Spectrometry

A detailed description of the purification procedure is included in the Supplemental Data. In brief, lysates from HeLa S3 cells expressing AH3-TERT were bound to rabbit IgG resin (Sigma), washed extensively, and eluted overnight with TEV protease. The next day, eluants were precleared with mouse IgG resin and then bound again to 12CA5 anti-HA conjugated resin. After extensive washing, the anti-HA resin was eluted and precipitated for mass spectrometry. All steps were performed at 4°C. Protein identification using mass spectrometry is detailed in the Supplemental Data.

Small-Scale Cell Culture, Transfections, and Transductions

Asynchronous adherent HeLa S3 cultures were grown in DMEM/5% newborn calf serum/1% penicillin-streptomycin (PS); 293T and U2OS cells were grown in DMEM/10% bovine growth serum/1% PS. Details for cell synchronization and for all constructs are provided in the Supplemental Data. Retroviruses were generated by cotransfecting plasmids encoding RSV(Gag+Pol), VSV-G, and the retroviral expression or shRNA plasmids (Dickins et al., 2005) into 293T cells using calcium phosphate precipitation. To generate Flag-pontin^{+shRNA} and Flag-reptin^{+shRNA} cell lines, HeLa S3 cells were first transduced with shRNA-resistant Flag-pontin or Flag-reptin in the pMGIB vector, selected with blasticidin S, transduced with the LMP shRNA vector, and finally selected with puromycin. Transient transfections into 293T cells were done using standard calcium phosphate precipitation, and cells were harvested after 60–72 hr for coimmunoprecipitation analysis.

Coimmunoprecipitations, Western Blots, and Northern Blots

Cells were lysed in NP40 buffer (25 mM HEPES-KOH, 150 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.5% NP40, and 5 mM 2ME [pH 7.5] supplemented with protease inhibitors) for 15–30 min on ice. Extracts clarified by centrifugation at 16,000 × g for 10 min were quantified by Bradford assay and immunoprecipitated with 10–15 μl M2 anti-Flag resin (Sigma) for 1–2 hr at 4°C. Where indicated, RNase A, ethidium bromide, or DNase I was included during the incubation at 0.1 mg/ml. Resins were then washed five times for 10 min each with 1 ml NP40 buffer, boiled in Laemmli sample buffer, and fractionated by SDS-PAGE. Primary antibody concentrations for western blots and details of

polyclonal antibody generation are given in the Supplemental Data. For northern blots, one-half the resin (into which a recovery control RNA was spiked to control for differential recovery in subsequent steps) was extracted with phenol:chloroform:isoamyl alcohol and precipitated with glycogen carrier. RNA pellets were boiled in formamide loading buffer, loaded onto 5% polyacrylamide-8 M urea gels, transferred to Hybond N+ (Amersham), and hybridized with α-^{32}P-dCTP-labeled full-length hTR, U3, or U1 probe in Ultrahyb (Ambion). For total RNA analysis, 1 μg of Trizol (Invitrogen)-extracted RNA was used. Methods for glycerol gradient sedimentation, TRAP assays, immunofluorescence, and telomere length analysis are detailed in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, five figures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/132/6/945/DC1/.

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