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Telomerase and telomere-associated proteins: Structural insights into mechanism and evolution

Karen A. Lewis and **Deborah S. Wuttke***
Department of Chemistry and Biochemistry, University of Colorado Boulder, Boulder, Colorado, 80309

Abstract

Recent advances in our structural understanding of telomerase and telomere-associated proteins have contributed significantly to elucidating the molecular mechanisms of telomere maintenance. The structures of telomerase TERT domains have provided valuable insights into how experimentally identified conserved motifs contribute to the telomerase reverse transcriptase reaction. Additionally, structures of telomere-associated proteins in a variety of organisms have revealed that, across evolution, telomere-maintenance mechanisms employ common structural elements. For example, the single-stranded 3′ overhang of telomeric DNA is specifically and tightly bound by an OB-fold in nearly all species, including ciliates (TEBP and Pot1a), fission yeast (SpPot1), budding yeast (Cdc13), and humans (hPOT1). Structures of the yeast Cdc13, Stn1, and Ten1 proteins demonstrated that telomere maintenance is regulated by a complex that bears significant similarity to the RPA heterotrimer. Similarly, proteins that specifically bind double-stranded telomeric DNA in divergent species use homeodomains to execute their functions (human TRF1 and TRF2 and budding yeast *Sc*Rap1). Likewise, the conserved protein Rap1, found in budding yeast, fission yeast, and humans, contains a structural motif that is known to be critical for protein-protein interaction. In addition to revealing the common underlying themes of telomere maintenance, structures have also elucidated the specific mechanisms by which many of these proteins function, including identifying a telomere-specific domain in Stn1 and how the human TRF proteins avoid heterodimerization. In this review, we summarize the high-resolution structures of telomerase and telomere-associated proteins and discuss the emergent common structural themes among these proteins. We also address how these high-resolution structures complement biochemical and cellular studies to enhance our understanding of telomere maintenance and function.

Keywords

telomere; telomerase; shelterin; tRPA; OB fold; TPP1; POT1; RAP1; TRF1; TRF2; TRFH; TERT; TRBD; TEN; RCT; TIN2; Apollo; Sir3; Taz1; Cdc13; Stn1; Ten1; TEBP

*Correspondence: deborah.wuttke@colorado.edu.

Introduction

Telomeres are nucleoprotein complexes required for chromosomal stability. They shield the ends of linear chromosomes from recognition by DNA damage machinery and provide a solution to the end-replication problem through the action of the reverse transcriptase telomerase (de Lange, 2009; Greider and Blackburn, 1987). Protection of the chromosomal end is conferred by essential protein complexes that prevent the severe and lethal consequences of a cellular response to exposed DNA ends, including chromosomal end-to-end fusions and nucleolytic processing (de Lange, 2009). Additionally, the limitations of semiconservative DNA replication result in gradual telomere shortening, limiting the number of cell divisions as short telomeres trigger cellular senescence (Harley et al., 1990; Hayflick, 1979; Lundblad and Szostak, 1989). This limitation can be circumvented by the telomerase-mediated extension of telomeric DNA, as observed in unicellular eukaryotic organisms and proliferative metazoan cells (Bodnar et al., 1998; Lundblad and Szostak, 1989; Yu et al., 1990). Both the end-protection and telomerase activities are tightly controlled (de Lange, 2009), and their dysregulation is associated with several human diseases (Armanios, 2009; Calado and Young, 2009; Garcia et al., 2007).

The sequences of telomere-associated proteins diverge rapidly (Linger and Price, 2009), confounding our ability to identify the unifying themes underlying telomere maintenance. Fortunately, the high-resolution structures of telomere-associated factors have revealed the repeated use of common structural elements with some intriguing elaborations. In particular, some apparently divergent telomere-associated proteins in distantly related species share folds, while others are similar to well-characterized non-telomeric proteins, suggesting the evolution of telomere-specific function. These structures, both through their similarities and their differences, provide direction for biochemical and cellular studies that aim to define the mechanisms of action of telomere-associated factors. In this review, we will discuss the emergent common structural themes in telomere-associated proteins and describe how these high-resolution structures have enhanced our understanding of telomere maintenance and function.

Telomerase reverse transcriptase

The ends of linear chromosomes terminate in G-rich single-stranded 3′ overhangs (Klobutcher et al., 1981; Larrivee et al., 2004; McElligott and Wellinger, 1997; Moyzis et al., 1988; Shampay et al., 1984; Wright et al., 1997). *In vitro*, telomeric single-stranded DNA (ssDNA) readily forms higher order G-quadruplex structures amenable to high-resolution characterization (Burge et al., 2006; Neidle and Parkinson, 2003), although these structures have only been reported *in vivo* at ciliate telomeres (Lipps and Rhodes, 2009; Paeschke et al., 2005). Chromosome ends also form large DNA duplex “t-loops”, where the ssDNA overhang invades the duplex region to form a structure similar to a recombination intermediate, which may represent the higher-order structure *in vivo* (de Lange, 2004; Griffith et al., 1999; Munoz-Jordan et al., 2001), but they have not yet been amenable to high-resolution structural studies *in vitro*.

Telomerase catalyzes the addition of telomeric repeats onto this 3′ overhang using an integrated ribonucleoprotein complex that consists of a reverse transcriptase protein (TERT)

and a large RNA component (TR) which provides the template sequence for the telomeric repeat (Greider and Blackburn, 1989; Lingner et al., 1997). Within the family of reverse transcriptases, telomerase is anomalous in that its RNA component is a constitutive component of the transcriptase with multiple functions (Autexier and Lue, 2006). TERT and TR associate with additional proteins *in vivo* to form a functional holoenzyme (Lendvay et al., 1996; Lingner and Cech, 1996; Venteicher et al., 2009; Witkin and Collins, 2004; Witkin et al., 2007). Although the structure of an intact telomerase holoenzyme has yet to be solved, structures of individual telomerase and TR subdomains have informed our understanding of its organization and the mechanisms of its unique reverse transcriptase activity (Kelleher et al., 2002). Here, we limit our discussion to structures of TERT, as the structures of domains within telomerase RNA have been expertly reviewed elsewhere (Zhang et al., 2011a).

TERTs contain a reverse transcriptase domain (RT) that possesses the canonical RT motifs 1, 2, A, B′, C, D, and E, including the three invariant catalytic aspartate residues (Lingner et al., 1997). Three additional domains fully define a TERT protein: a “telomerase essential N-terminal” domain (TEN); a telomerase RNA binding domain (TRBD); and a C-terminal extension (CTE) (Fig. 1A) (Blackburn, 2005; Bryan and Cech, 1999; Wyatt et al., 2010). Much of our current structural understanding of TERT has come from the *Tetrahymena thermophila* TRBD and TEN domain structures and the structure of the putative TERT from the flour beetle *Tribolium castaneum* (*Tc*TERT). *Tc*TERT is somewhat removed from other known telomerases; it lacks the TEN domain that is present in all other known telomerases, the RNA component has not been identified, and as yet there is no *in vivo* evidence for telomerase-mediated telomere maintenance in this organism (Osanai et al., 2006). However, *Tc*TERT is clearly a reverse transcriptase that contains telomerase-specific sequence motifs (see below), which suggests that, if not an active telomerase, it may be an evolutionary intermediate. *Tc*TERT thus serves as a judicious starting point for understanding the structural framework of telomerase action while we await a high-resolution structure of an intact TERT+TR holoenzyme.

*Tc*TERT forms a ring-like structure with the TRBD making considerable contact with the CTE even though they are separated in primary sequence by the RT domain (Gillis et al., 2008) (Fig. 1A,B). The RT folds into a palm-and-fingers organization reminiscent of other reverse transcriptases (Das and Georgiadis, 2004; Rodgers et al., 1995). The active site is in the palm of the RT, and contains universally catalytic aspartates and a Mg^{2+} ion (Gillis et al., 2008; Mitchell et al., 2010a) (Fig. 1B). The CTE curls around to contact the TRBD and complete the ring (Fig. 1B). A co-crystal of *Tc*TERT and a hybrid RNA/DNA hairpin shows that, analogous to retroviral reverse transcriptases, the nucleic acid docks in the center of the ring, contacting elements from the TRBD, RT, and CTE (Mitchell et al., 2010a). The *Tc*TERT structure also reveals the context of two previously characterized telomerase-specific motifs in the RT palm that affect activity: motif 3 and IFD (Fig. 1A,C). Motif 3 is located between motifs 2 and A in the primary sequence (Xie et al., 2010) (Fig. 1A). In *Tc*TERT, motif 3 forms two helices on the RT surface adjacent to the active site (Fig. 1C). IFD is an insertion between the A and B′ motifs, originally named the “insertion in the fingers domain” based on mapping to the HIV-1 structure (Lue et al., 2003) (Fig. 1A,C).

The *Tc*TERT IFD is part of the solvent-exposed surface on the outside of the RT domain (Fig. 1C), where it likely affects the active site through a helix that interacts with the IFD on one side and with the RNA substrate in the active site on the other (Lue et al., 2003).

The specialized RNA-binding activity of TERT is conferred by the essential and highly conserved TRBD, which uses the telomerase-specific T and CP motifs to recognize essential elements of TR (Bryan et al., 1998; Nakamura et al., 1997; O'Connor et al., 2005). TRBD structures are available in isolation from *T. thermophila* and within the full-length *T. castaneum* TERT. These structures are very similar; both consist of two asymmetrical helical lobes connected by a β-hairpin hinge region (Gillis et al., 2008; Rouda and Skordalakes, 2007) (Fig. 1D). This novel topology places the phylogenetically conserved T and CP motifs adjacent on the hinge surface (Lai et al., 2001; Rouda and Skordalakes, 2007) (Fig. 1D). The details of RNA binding will require a co-crystal structure, although mutagenesis suggests that RNA binding employs hydrophobic interactions within the conserved T and CP motifs. Full understanding of nucleic acid recognition will also require analysis of the structurally uncharacterized N-terminal region of the *T. thermophila* TRBD (residues 195–253), which was found to be required for biochemical activity (Lai et al., 2001) but was not contained in the structurally characterized construct (residues 254–519) (Rouda and Skordalakes, 2007).

Telomerases are additionally distinguished from canonical reverse transcriptases by the TEN domain, which is essential for telomerase activity *in vivo* and *in vitro* (Bryan et al., 2000; Friedman and Cech, 1999; O'Connor et al., 2005; Xia et al., 2000). The TEN domain binds both TR and the telomeric ssDNA substrate, and is critical for the telomerase-specific repeat-addition processivity (RAP) activity (Moriarty et al., 2004; Zaug et al., 2008). The *T. thermophila* TEN domain adopts a novel protein fold, consisting of N-terminal and C-terminal subdomains ending in a C-terminal tail (Jacobs et al., 2006) (Fig. 1E, key tail residues highlighted in red). The structure was used to direct mutagenesis experiments that identified the C-terminal tail (residues 177–191) as essential for RNA binding and weak binding of telomeric ssDNA primers (Jacobs et al., 2006) (Fig. 1E). Protein flexibility may contribute to nucleic acid binding, as deletion of the disordered C-terminal tail of TEN compromises its ability bind RNA (Jacobs et al., 2006).

Telomere-Associated Proteins

Our knowledge of telomere organization is being built from the ground up, focusing on the structural characterization of individual proteins and subcomplexes from a range of organisms (Fig. 2). This review focuses on the structurally characterized protein domains and their complexes of factors whose primary cellular role is linked to telomere maintenance. Metazoan telomeres contain the six-member shelterin complex (Palm and de Lange, 2008) (Fig. 2), comprised of the double-stranded binding proteins TRF1 and TRF2, which bind telomeric dsDNA as homodimers and interact with RAP1 and TIN2. TIN2 interacts with hTPP1, which in turn binds the telomeric ssDNA binding protein, hPOT1 (Fig. 2). Fission yeast also employ a shelterin-like complex, comprised of a single TRF1/2 homolog, Taz1, which interacts with a Rap1 homolog. This Rap1 also interacts with Poz1, which then binds the hTPP1-hPOT1 homologs *Sp*Tpz1-*Sp*Pot1 (de Lange, 2009; Miyoshi et

al., 2008) (Fig. 2). Budding yeast appear to use a distinct mechanism of telomere maintenance through the Rap1/Rif1/Rif2 complex, which binds telomeric dsDNA, and a telomere-specific RPA-like complex containing Cdc13/Stn1/Ten1 (t-RPA), which binds telomeric ssDNA (Shore and Bianchi, 2009) (Fig. 2). Fission yeast and metazoan telomeres also employ an RPA-like complex (Martín et al., 2007; Miyake et al., 2009; Song et al., 2008). While the double-stranded DNA-binding factors in ciliates have not been reported, *O. nova* amitotic macronuclear telomeres employ the heterodimeric complex TEBP (Gottschling and Zakian, 1986), while *T. thermophila* contains a Pot1/TPP1-like complex (Jacob et al., 2007; Linger et al., 2011).

Single-stranded DNA binders and their complexes

High-resolution structures have revealed similarities among the telomere end-protection (TEP) proteins that were not predicted from their primary sequences. TEP proteins universally bind the ssDNA overhang using the oligosaccharide/oligonucleotide/oligopeptide binding (OB) fold, a common Greek key motif in ssDNA and RNA binding proteins (Theobald et al., 2003). Since ssDNA is present throughout the genome during replication, TEP proteins must discriminate between telomere and non-telomere sequence. Some TEPs execute this exquisite specificity while also accommodating degeneracy or variable spacer sequences within the telomeric repeats. All of this is accompanied by very high binding affinities, with unusually tight K_D values often in the low pico- to nanomolar range (Croy and Wuttke, 2006). The high-resolution structures of several TEP domains have provided insight into how OB folds perform these myriad duties simultaneously.

***On*TEBP—**The first high-resolution TEP structures were of the heterodimeric telomere end-binding protein complex (TEBP) from the hypotrichous ciliate *Oxytricha nova* (“*On*”, now called *Sterkiella nova* (Foissner and Berger, 1999)). TEBP consists of two subunits, α and β, which bind tenaciously to *O. nova* macronuclear chromosomal termini as a heterodimeric complex specific for T_4G_4 repeats (Gottschling and Zakian, 1986; Gray et al., 1991; Horvath et al., 1998). TEBPα consists of two OB folds in an N-terminal domain (OB1 and OB2) and a third OB fold as a C-terminal domain (OB3) (Horvath et al., 1998), while TEBPβ is comprised of a single N-terminal OB fold (OB4), a central globular domain, and a lysine-rich unstructured tail (CTD) (Buczek and Horvath, 2006; Horvath et al., 1998) (Fig. 3A,B). This first TEP structure unveiled new mechanisms for OB fold binding and set the benchmark for how this protein family functions. Notably, OB1 and OB2 were found to form a single extended ssDNA-binding surface that cooperates with OB4 to form the complete binding pocket. Additionally, the canonical ligand-binding site in OB3 interacts with the globular domain of TEBPβ (Horvath et al., 1998) (Fig. 3B). This discovery expanded the set of known OB fold ligands to include oligopeptides as well as oligonucleotides and oligosaccharides (Horvath et al., 1998).

The TEBPαβ heterodimer and ssDNA co-fold into a stable complex, with the ssDNA forming a loop within a groove formed by the N-terminal OB folds of TEBPα and the globular domain of TEBPβ (Fig. 3B). The nucleotide bases are generally buried, with the 3′ end completely solvent inaccessible, and make extensive contacts with amino acid side chains through a chemically diverse range of interactions, including aromatic stacking,

hydrophobic interactions, hydrogen-bonding, and electrostatic interactions, with electrostatics contributing little to the thermodynamics of binding (Horvath et al., 1998) (Fig. 3C). The occlusion of the bases and the 3′ end established a physical basis for sequence-specific binding and end protection (Horvath et al., 1998). The number and diversity of nucleotide-protein contacts provides a mechanism for the exquisite specificity of TEP proteins for their cognate ligands. As TEBPα can also bind ssDNA independently, a model emerged in which TEBPα coats ssDNA with a terminal TEBPβ binding event to form a stable complex that caps the very end of the chromosome (Classen et al., 2001; Peersen et al., 2002).

Structures of TEBPαβ bound to a panel of noncognate ligands showed that *On*TEBPαβ accommodates sequence variation using modest changes in side chain conformation and dramatic shifts in nucleic acid binding register to retain key specificity contacts (Theobald and Schultz, 2003). The ligand rearrangement explains why profound nucleotide sequence alterations caused less than a 10-fold change in affinity (Theobald and Schultz, 2003). Such nucleotide shuffling may be a primary mechanism by which TEP proteins bind variable 3′ overhangs; evidence for similar ligand accommodation is present in fission and budding yeast, although those mechanisms are currently structurally undefined.

Pot1 and hTPP1—Weak sequence identity between TEBPα OB1 and an uncharacterized *S. pombe* protein led to the identification of Pot1 (protection of telomere-1) in fission yeast and humans (Baumann and Cech, 2001). Pot1 binds telomeric ssDNA as part of shelterin, and is conserved in eukaryotes from *S. pombe* (*Sp*Pot1) to humans (hPOT1) (Croy and Wuttke, 2006). Deletion of Pot1 from either *S. pombe* or vertebrate cells is catastrophic, resulting in telomeric instability, chromosomal end-to-end fusions, and cell death (Baumann and Price, 2010). Like TEBPα, Pot1 is comprised of an N-terminal DNA-binding domain (DBD) and a C-terminal protein-protein interaction domain (Fig. 4A). Also like TEBPα, Pot1 binds an OB-fold containing partner protein (hTPP1/*Sp*Tpz1) (Liu et al., 2004; Miyoshi et al., 2008; Ye et al., 2004).

Human hPOT1 and hTPP1: The hPOT1-DBD comprises residues 1–340, and has been co-crystallized with ssDNA (Lei et al., 2004; Nandakumar et al., 2010) (Fig. 4B, dark green and dark gray, respectively). The DBD is comprised of tandem OB folds (OB1 and OB2). OB1 was predicted by a variety of sequence alignments, but OB2 was only weakly predicted using a profile-based sequence analysis (Theobald and Wuttke, 2004). The DNA adopts an extended conformation and makes specific contacts with both OB folds, where hydrogen bonds between amino acid side chains and nucleotide bases provide a structural basis for the biochemically observed nucleotide specificity preference for the 5′ end of the ligand and the terminal 3′ guanine (Lei et al., 2004) (Fig. 4B).

hPOT1 is localized to the larger multimeric shelterin complex through the interaction of its C-terminal domain with a central domain in hTPP1 (Hockemeyer et al., 2007; Xin et al., 2007; Ye et al., 2004). hTPP1 also contains an N-terminal OB fold that bears striking resemblance to that of TEBPβ (residues 90–250) and an as-yet uncharacterized C-terminal hPOT1-interaction domain (Wang et al., 2007). hTPP1 additionally enhances hPOT1 affinity by 10-fold (Gray et al., 1991; Wang et al., 2007), and also refines hPOT1

discrimination against ribonucleic acids (Nandakumar et al., 2010). Although the isolated domains of hPOT1/hTPP1 closely correspond structurally to those of TEBPαβ, the two complexes exhibit somewhat different biochemical behavior (Fig. 4C). The DNA-binding domains of TEBPα and hPOT1 alone exhibit modest preference for the nature of the 3′ base (Classen et al., 2003; Lei et al., 2004), and the structures reveal a partially buried 3′OH. In contrast, TEBPαβ completely buries the 3′G (Horvath et al., 1998). While no structure is yet available of the hPOT1/hTPP1 complex, biochemically the 3′ end requirement is relaxed (Wang et al., 2007). Both the TEBPα and TEBPαβ complexes inhibit telomerase activity, presumably through steric occlusion of the 3′ end (Froelich-Ammon et al., 1998). Similarly, when localized to the 3′ end, hPOT1 alone inhibits human telomerase *in vitro* (Lei et al., 2005). However, when bound coincidentally with hPOT1, hTPP1 triggers recovery of telomerase activity, behaving as a telomerase processivity factor by decreasing the rate of primer dissociation (Abreu et al., 2010; Latrick and Cech, 2010; Tejera et al., 2010; Wang et al., 2007; Xin et al., 2007). The structural similarity and functional divergence of hTPP1 and TEBPβ is a prime example of the species-specific adaptations that are characteristic of telomere maintenance complexes throughout phylogeny.

***S. pombe* Pot1:** Like hPOT1, *S. pombe* Pot1 is also comprised of a DBD (1–389) and a C-terminal domain (Croy et al., 2006) (Fig. 4A). Although sequence homology is limited to the N-terminal OB fold, protein threading models and biochemical data strongly suggest that the DBD, like hPOT1, contains two tandem OB folds, *Sp*Pot1pN and *Sp*Pot1pC (Croy et al., 2006; Croy et al., 2009). Only *Sp*Pot1pN has been structurally characterized to date (Fig. 4B), and as the first Pot1 structure to be elucidated, it provided initial insight into how Pot1 specifically recognizes ssDNA (Lei et al., 2003). *Sp*Pot1pN superpositions well on hPOT1-OB1, with the central ligand bases overlaying tightly with more divergence at the 3′ and 5′ ends. Stacking interactions between aromatic amino acid on the protein and bases of the ssDNA are largely conserved (Fig. 4B). Solution dynamics analysis of the free and bound states of *Sp*Pot1pN suggests that specificity is achieved by a conformational selection mechanism where residues involved in forming the specific contacts experience dynamics that are quenched upon binding (Croy and Wuttke, 2009; Croy et al., 2008).

In contrast to TEBPα and hPOT1, the *Sp*Pot1 OB folds exhibit independent DNA-binding activities, allowing for characterization of how these domains work in concert to perform sequence-specific recognition of DNA. *Sp*Pot1pN binds a single *S. pombe* telomeric repeat, d(GGTTAC), while *Sp*Pot1pC minimally binds to one and a half repeats (Croy et al., 2009; Lei et al., 2002). These binding activities are decoupled in the intact *Sp*Pot1-DBD, which binds the combined two-and-a-half repeat ssDNA with low picomolar affinity (Croy et al., 2009). Interestingly, *Sp*Pot1-DBD can also bind ssDNA of two repeats with identical affinity but with different specificities and tolerance for substitution as the longer ligand (Altschuler et al., 2011). These biochemical observations suggest that *Sp*Pot1 is capable of remarkable conformational plasticity and ligand accommodation. Supporting this hypothesis, structures of *Sp*Pot1pN complexed with non-cognate ligands identified novel ligand conformations that nonetheless bound with similar thermodynamic parameters (Croy et al., 2008). As the core *S. pombe* telomere sequence repeats are often separated by a variable number of nucleotides (Trujillo et al., 2005), the ability to accommodate alternate

telomeric sequences with minimal thermodynamic impact may be an essential element of *Sp*Pot1 function.

RPA-like complexes—An additional widely conserved complex also contributes to telomere function. This complex, first discovered in *S. cerevisiae*, is composed of three essential proteins Cdc13, Stn1, and Ten1 and impacts several aspects of telomere maintenance (Grandin et al., 2001; Grandin et al., 1997; Nugent et al., 1996) (Fig. 5). Cdc13 positively regulates telomere length by recruiting telomerase (Bianchi et al., 2004; Nugent et al., 1996; Pennock et al., 2001), and all three proteins are also genetically implicated in negative length regulation (Chandra et al., 2001; Grandin et al., 2001; Grandin et al., 1997; Qi and Zakian, 2000). Cdc13 specifically binds yeast telomeric ssDNA with 300 pM affinity through its DBD, which is a single OB fold with no sequence similarity to TEBP or Pot1 proteins (Anderson et al., 2002; Nugent et al., 1996) (Fig. 5A). The isolated DBD binds more tightly, with 3 pM affinity, using an unusually long, structured loop between β2 and β3 ($L_{2–3}$) to extend the binding interface (Eldridge and Wuttke, 2008; Mitton-Fry et al., 2002; Mitton-Fry et al., 2004). As seen in TEBP and Pot1, aromatic stacking and hydrophobic interactions mediate recognition of nucleotide bases and electrostatics stabilizing the backbone phosphates (Anderson et al., 2003; Mitton-Fry et al., 2002). As observed in the Pot1 proteins, the most critical protein-DNA contacts are located in the 5′ end of the ligand, where these contacts define the highly sequence-specific binding behavior of Cdc13 (Anderson et al., 2003; Eldridge et al., 2006) (Fig. 5A).

Exciting insights into the function of this complex came from the proposal that Cdc13, Stn1, and Ten1 form a telomere-specific RPA-like heterotrimer (t-RPA) (Gao et al., 2007) (Fig. 5), based on sequence analysis of Cdc13, Stn1, and Ten1 and *in vivo* domain swapping (Gao et al., 2007; Theobald and Wuttke, 2004). This was a paradigm-shifting idea, as RPA nonspecifically binds to ssDNA throughout the genome but Cdc13 specifically recognizes telomeric ssDNA. Structural data have been central to refining this hypothesis (Fig. 5). Like RPA70, Cdc13 has an OB fold in the extreme N-terminus (N-OB), but unlike RPA70, this domain contains a long helix that mediates dimerization (Mitchell et al., 2010b; Sun et al., 2011) (Fig. 5A). The N-OB has been alternately proposed to bind either ssDNA or DNA polymerase α (Mitchell et al., 2010b; Sun et al., 2011). In addition to the N-OB and DBD domains, two additional OB folds flanking the DBD have been predicted (Sun et al., 2011; Theobald and Wuttke, 2004). The demonstrated presence of four OB folds in Cdc13 would conclusively establish a domain organization analogous to RPA70 (Fig. 5A). However, several elaborations confer the unique telomere functions of Cdc13, namely that the DBD is a single OB fold with specificity for telomeric DNA, the observation of dimerization, and the presence of a telomerase regulatory domain (RD) within the N-terminus (Fig. 5A).

Structures of Stn1 and Ten1 domains revealed that they closely mimic their RPA counterparts (Fig. 5B,C). The N-terminal domains of Stn1 (Stn1-N) from the divergent yeasts *S. pombe* and *Candida tropicalis* are OB folds that superimpose on the RPA32 OB fold (Sun et al., 2009) (Fig. 5B). The C-terminal domain (Stn1-C) from *S. cerevisiae* is comprised of tandem winged helix-turn-helix (wHTH) motifs (Gelinas et al., 2009; Sun et al., 2009). The N-terminal wHTH superimposes on the lone wHTH of RPA32 (Fig. 5B), while *in vivo* studies identified a telomere-specific function for the C-terminal wHTH in

negative telomere length regulation (Gelinas et al., 2009). Like RPA14, both the *S. pombe* and *C. tropicalis* Ten1proteins are single OB folds (Gelinas et al., 2009; Sun et al., 2009) (Fig. 5C). Furthermore, RPA32/14 and Stn1-N/Ten1 form stable heterodimers *in vitro* (Bochkarev et al., 1999; Gao et al., 2007; Sun et al., 2009). The presence of multiple OB folds in Cdc13 and the remarkable structural identity between Stn1/Ten1 and RPA32/14 strongly support the hypothesis that Cdc13, Stn1, and Ten1 are RPA-like proteins (Fig. 5).

Although the RPA-like telomere proteins have been studied most extensively in budding yeast, a heterotrimeric complex composed of the well-conserved Stn1 and Ten1 proteins and a less conserved DNA-binding large subunit contributes to telomere function in a diverse set of species, including fission yeast, plants, and mammals (Casteel et al., 2009; Martín et al., 2007; Miyake et al., 2009; Surovtseva et al., 2009). In fission yeast, *stn1*-Δ and *ten1*-Δ strains exhibit the same phenotype as a Pot1 knockout, with rapid telomere degradation accompanied by high levels of inviability (Martín et al., 2007). Knockout of a Stn1 homolog in Arabidopsis, AtStn1, also exhibits a telomere shortening phenotype and severe morphological defects (Song et al., 2008). However, the rapid divergence of the large subunit has confounded the analysis of this complex. Even Cdc13 proteins from other yeast do not retain the level of telomere-specific DNA binding observed for *S. cerevisiae* Cdc13 (Mandell et al., 2011). Structural studies will clearly play a major role in the refinement of these models, including high-resolution structures of multimeric complexes and structure-directed *in vivo* mutagenesis.

Double-stranded DNA binders and their complexes

A separate set of factors specifically binds double-stranded telomeric DNA, where they function in chromosomal protection and telomere-length regulation (de Lange, 2009; Shore and Bianchi, 2009) (Fig. 2). These proteins share common folds despite considerable sequence variability and differences in domain topology. As observed in the ssDNA-binding proteins, dsDNA-protein interactions and protein-protein interactions at the telomere in evolutionarily divergent species employ similar structural solutions to execute telomere-specific functions.

Sc*Rap1—S. cerevisiae* telomeric dsDNA is bound by *Sc*Rap1, which interacts with the proteins Rif1 and Rif2 to regulate telomere length (Shore and Bianchi, 2009). *Sc*Rap1 also mediates gene silencing both at the telomere and at mating-type loci through interactions with Sir3 and Sir4 (Moretti and Shore, 2001). The DNA-binding domain (DBD; residues 361–596), the BRCT domain (residues 6–102), and the C-terminal protein-protein interaction domain (CTD; residues 672–827) have been structurally characterized, revealing the physical bases for these interactions and providing functional insights (Fig. 6A).

The structure of *Sc*Rap1-DBD was seminal for the field, and demonstrated that DNA binding activity is carried out by two homeodomains, each comprised of an N-terminal arm and a Myb-like three-helical bundle that includes a recognition helix, followed by a C-terminal tail (Konig et al., 1996) (Fig. 6B). The N-terminal arms of each homeodomain make specific contacts within the minor groove and nonspecific contacts on the backbone of the dsDNA, and the recognition helix lies within the major groove. The tandem

homeodomains wrap around the dsDNA with a separation of 8 basepairs (Fig. 6B). The C-terminal tail contacts major groove bases and the recognition helix of the first homeodomain as it wraps back around the DNA to fully enclose the substrate (Konig et al., 1996). Specificity is mediated through direct and water-mediated hydrogen-bond contacts between the recognition helix and nucleotide bases of both strands.

The C-terminal protein-protein interaction domain of *Sc*Rap1 (RCT, for Rap1 C-terminus) adopts an entirely novel helical topology, in which the N-terminal helices form a highly hydrophobic cleft (Feeser and Wolberger, 2008) (Fig. 7A). A recent crystal structure of *Sc*Rap1-RCT complexed with a Sir3 peptide showed the peptide buried in the hydrophobic cleft (Chen et al., 2011). Recently, the structure of the *Sc*Rap1 BRCT domain was found to contain less secondary structure and more flexible loops than observed in canonical BRCT domains, but the physiological relevance of the more flexible structure has yet to be elucidated (Zhang et al., 2011b).

TRF1, TRF2 and human Rap1—Human TRF1 and TRF2 bind to telomeric dsDNA as homodimers, with each monomer containing a single dsDNA-binding homeodomain that is similar to those that comprise *Sc*Rap1-DBD (Bianchi et al., 1999) (Fig. 6A,C). The free and bound forms of TRF1-DBD and TRF2-DBD were independently solved by both solution NMR and x-ray crystallography (Court et al., 2005; Hanaoka et al., 2005; Nishikawa et al., 1998; Nishikawa et al., 2001). Specificity of binding is achieved primarily by the DNA recognition helix, H3, which lies in the major groove of the DNA where it makes numerous sequence-specific contacts with the telomeric dsDNA. Additionally, the N-terminal arms become more rigid in the bound state as they make specific contacts in the minor groove, similar to the specific contacts made between the *Sc*Rap1 loops and its cognate DNA (Court et al., 2005; Nishikawa et al., 2001) (Fig. 6C).

Homodimerization of the TRF proteins is mediated by their TRF homology domains (TRFH) (Fig. 6A). TRF1-TRFH and TRF2-TRFH share 27% sequence identity, and the crystal structures showed highly similar tertiary structures between the two homodimers (Fairall et al., 2001) (Fig. 6D). Despite their structural similarity, TRF1 and TRF2 do not heterodimerize, due to incompatible hydrophobic networks that form the dimerization interface (Fairall et al., 2001) (Fig. 6E). A superposition of the α1 helices from the TRF1 and TRF2 homodimers shows the incompatibility between sidechains (Fig. 6E).

While the high-resolution structure of the full shelterin complex has yet to be achieved, insights into higher order protein assembly have been obtained from structures of shelterin domains complexed with peptides derived from binding partners. For example, the TRFH homodimers harbor interfaces for protein-protein interactions that stabilize shelterin and bind shelterin-interacting factors (Chen et al., 2008). Both TRF1 and TRF2 bind a TIN2-derived peptide; TRF2 also binds a peptide from the nuclease Apollo, which is associated with telomere protection during S phase (van Overbeek and de Lange, 2006). These peptides share a common binding interface, in a groove at the base of the homodimer horseshoe, and interact through a conserved hydrophobic surface (Chen et al., 2008) (Fig. 6F). This peptide-based approach has also been employed to understand the mechanism of interaction between Rap1 proteins and their potential binding partners (Fig. 2). Although the Rap1 DBD is not

conserved, the C-terminal domains of the *S. pombe* and human Rap1 proteins share significant structural homology with the C-terminal domain of *S. cerevisiae* Rap1 (Fig. 7A, B), and also bind α-helices from their respective interaction partners in a helical cleft (Chen et al., 2011) (Fig. 7C). The hRAP1 C-terminal domain (RAP1-RCT) (residues 303–399) binds to a peptide derived from the intervening sequence between the TRFH and DBD domains of TRF2. *Sp*Rap1-RCT (residues 639–693) binds a peptide derived from the *S. pombe* TRF-like protein Taz1 (Fig. 8C). As the shelterin complex is reconstituted, it will be exciting to see how many more structural and mechanistic similarities exist between species that were not detectable by sequence homology.

Conclusion

High-resolution structures have been instrumental in informing telomere function and guiding biological studies aimed at elucidating the underlying mechanisms of telomere maintenance. The three-dimensional structures of several sets of telomerase and telomere-associated proteins revealed that they often display similar topologies in the absence of discernible sequence relationship, while variations on these shared protein folds highlight the unique ways in which organisms address their species-specific functions. With the structural picture of telomere function coming into focus, the next challenges will be the study of larger and larger complexes, as well as to probe the alteration in their state as a function of outside triggers, such as phosphorylation. The next frontier is to understand how regulation of the telomere is achieved, and how interactions between DNA, RNA, and proteins work dynamically together to perform telomere biogenesis, regulation, and maintenance.

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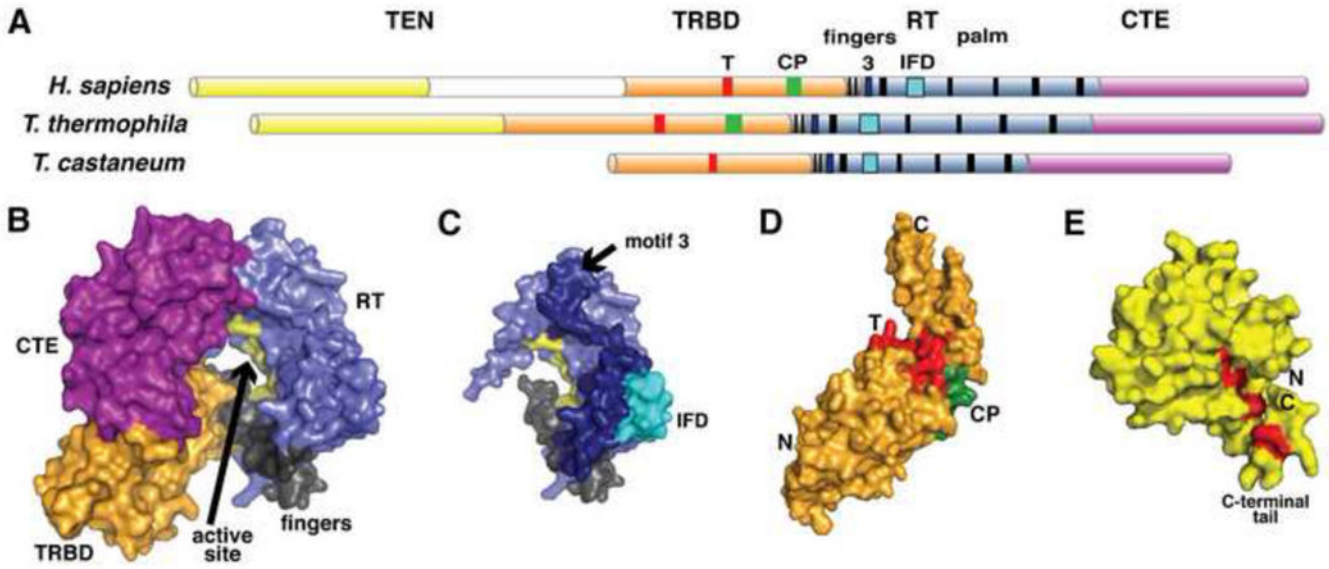

Figure 1. Telomerase enzymes are telomere-specific reverse transcriptases

(A) Telomerase domain topology (TEN, yellow; TRBD, orange; fingers, gray; RT, blue; CTE, magenta) with conserved motifs (T, green; CP, red; IFD, cyan; and left to right in black, motifs 1, 2, 3, and the canonical RT motifs A, B′, C, D, E, and F, black). (B) Surface representation of *T. castaneum* TERT. Domains are labeled and colored as in (A), with the active site residues in yellow (PDB: 3DU6). (C) The *T. castaneum* RT domain is shown in light blue, showing the locations of the telomerase-specific motifs 3 (dark blue) and IFD (cyan). As in (B), the active site residues are in yellow. (D) The *T. thermophila* TRBD domain is shown as a surface representation, with the T motif (red) and CP motif (green) highlighted (PDB: 2R4G). (E) The *T. thermophila* TEN domain is also shown as a surface (PDB: 2B2A). Mutations that alter nucleotide binding are shown as red sticks and red surface lining the pocket formed by the C-terminal tail, which is labeled. All structures were modeled using the PyMol Molecular Graphics System, Version 1.3 Schrödinger, LLC (Schrodinger, 2010).

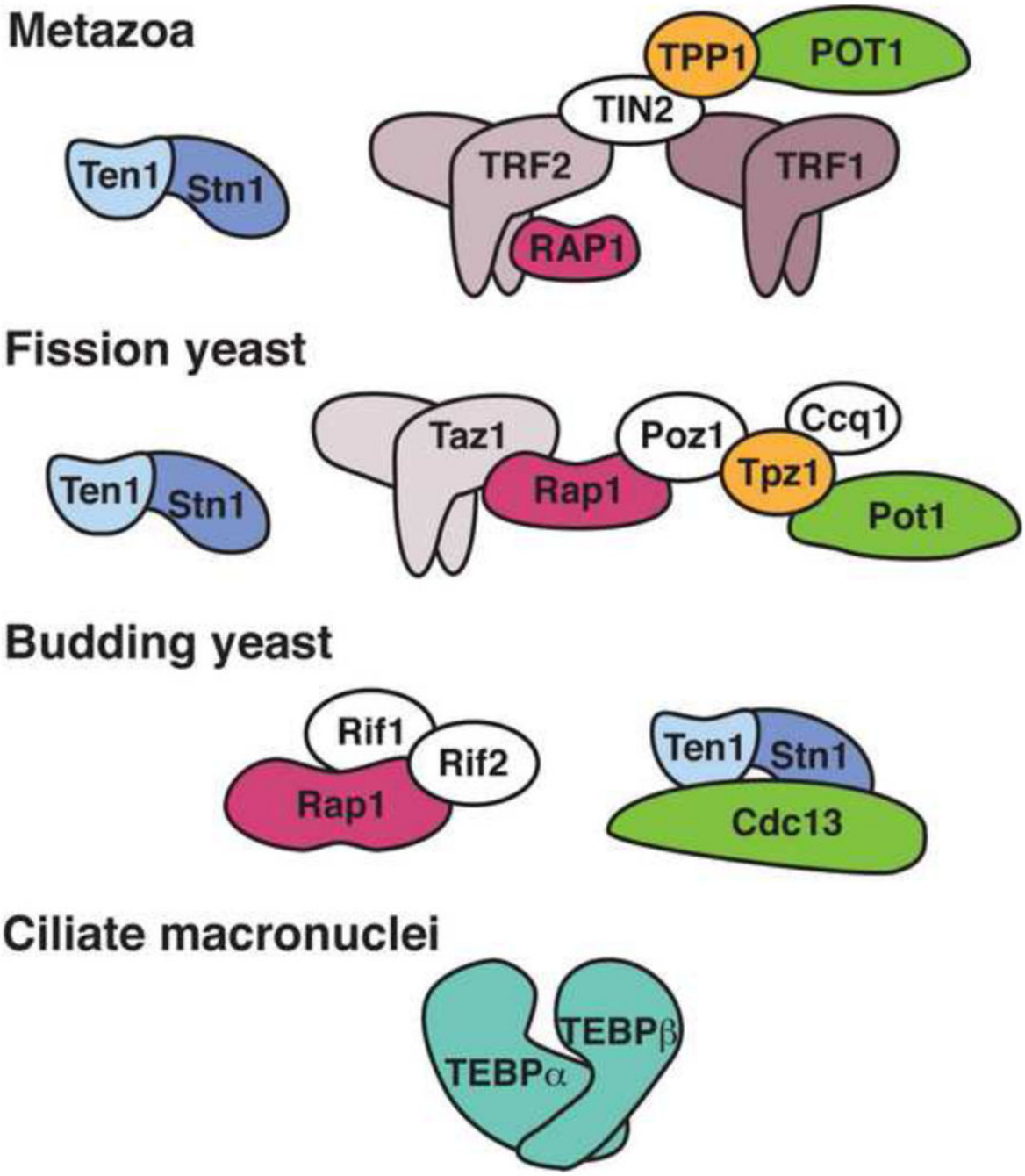

Figure 2. Telomere-associated protein complexes in different species

The proteins that are shown in color indicate that high-resolution structural data are available for either that protein or a close homolog. Metazoans: shelterin complex of TRF1, TRF2, RAP1, TIN2, hTPP1, and hPOT1; RPA-like proteins Stn1 and Ten1. Fission yeast: shelterin-like complex of Taz1, Rap1, Poz1, Tpz1, Ccq1, and Pot1; also present are Stn1 and Ten1. Budding yeast: dsDNA-binding complex of Rap1, Rif1, and Rif2; ssDNA-binding complex of Cdc13, Stn1, and Ten1. Ciliate macronuclei: TEBPα/β.

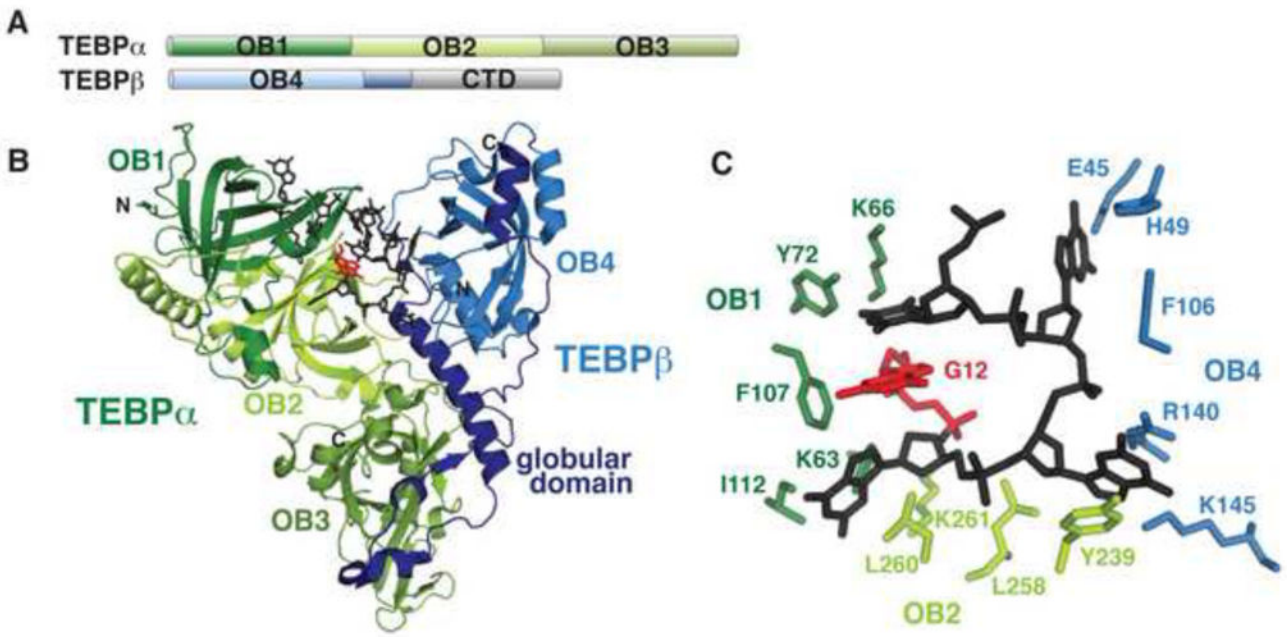

Figure 3. *On*TEBP proteins bind ciliate telomeric ssDNA

(A) Domain topology of *O. nova* TEBP proteins. Four OB folds are present, as well as a structurally uncharacterized C-terminal domain (CTD) in TEBPβ. (B) TEBPα OB1-3 (green) forms a complex with TEBPβ (blue) to bind ssDNA ligand (black; PDB: 2I0Q). The TEBPβ globular domain that is bound by OB3 is in dark blue. The 3′ base, G12, is colored red and is fully buried in the groove between the protein subunits. (C) OB1, OB2, and OB4 make critical contacts with the 3′ loop of the ssDNA ligand to sequester the bases from solvent.

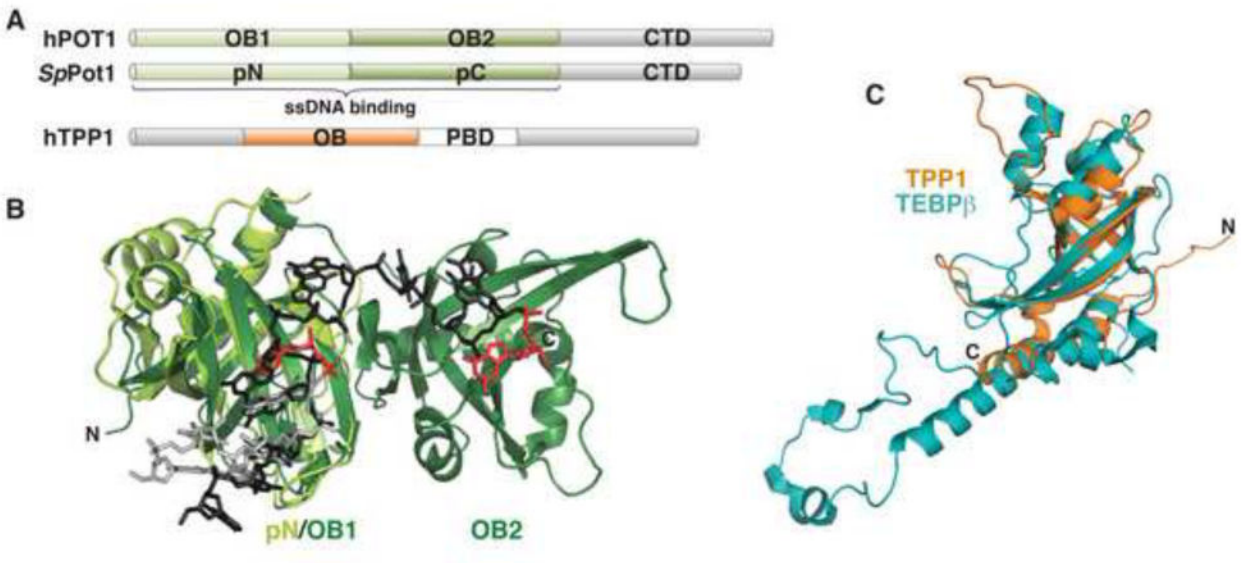

Figure 4. Pot1 and hTPP1

(A) Domain topology of hPOT1, *Sp*Pot1, and hTPP1 proteins. Following the tandem OB folds that comprise the DNA-binding domain, Pot1 contains a C-terminal protein-protein interaction domain (CTD), and hTPP1 contains a central Pot1-binding domain (PBD). (B) Superposition of *Sp*Pot1pN (bright green; PDB: 1QZH) on hPOT1-DBD (dark green; rmsd = 1.09 Å; PDB: 3KJP). ssDNA ligands are modeled as sticks, with 6mer bound to *Sp*Pot1pN (light gray) and 12mer bound to hPOT1-DBD (dark gray). The 3′ bases of both ligands are shown in red. (C) hTPP1-OB (orange; PDB: 2I46) superimposed on TEBPβ (teal; PDB: 2I0Q; rmsd =2.0 Å).

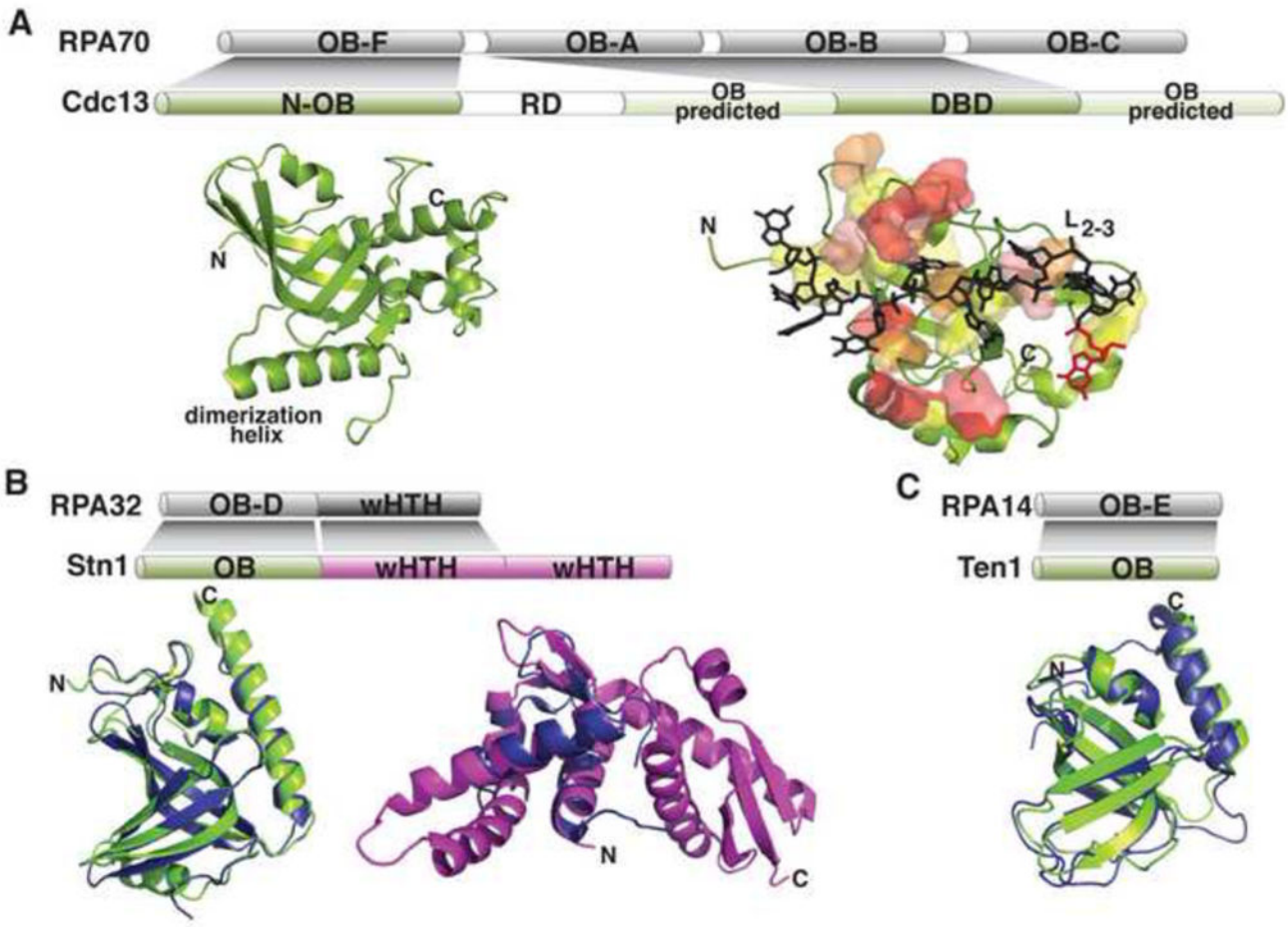

Figure 5. Budding yeast Cdc13, Stn1, and Ten1

(A) Cdc13 domain topology and subdomain structures compared to RPA70. N-OB is in green, with the dimerization helix labeled (PDB: 3NWS). The DBD is shown as a cartoon, with superimposed transparent surfaces of the residues involved in DNA binding in increasing thermodynamic contribution from yellow to red (PDB: 1S40). The 11mer ligand is shown as sticks (gray) with the 3′ base in red. (B) Stn1 domain topology and structures compared to RPA32. *Left*, the *S. pombe* Stn1-N (green) is superimposed on RPA32-N (blue; rmsd = 1.6Å; PDB: 3KF6 and 1QUQ, respectively); *right*, the *S. cerevisiae* Stn1-C (magenta) is superimposed on RPA32-C (blue; rmsd = 1.9Å; PDB: 3K10 and 1DPU, respectively). (C) Ten1 domain topology and structure compared to RPA14. The *S. pombe* Ten1 (green) is superimposed on RPA14 (blue; rmsd =1.96Å; PDB 3K0X and 1QUQ, respectively).

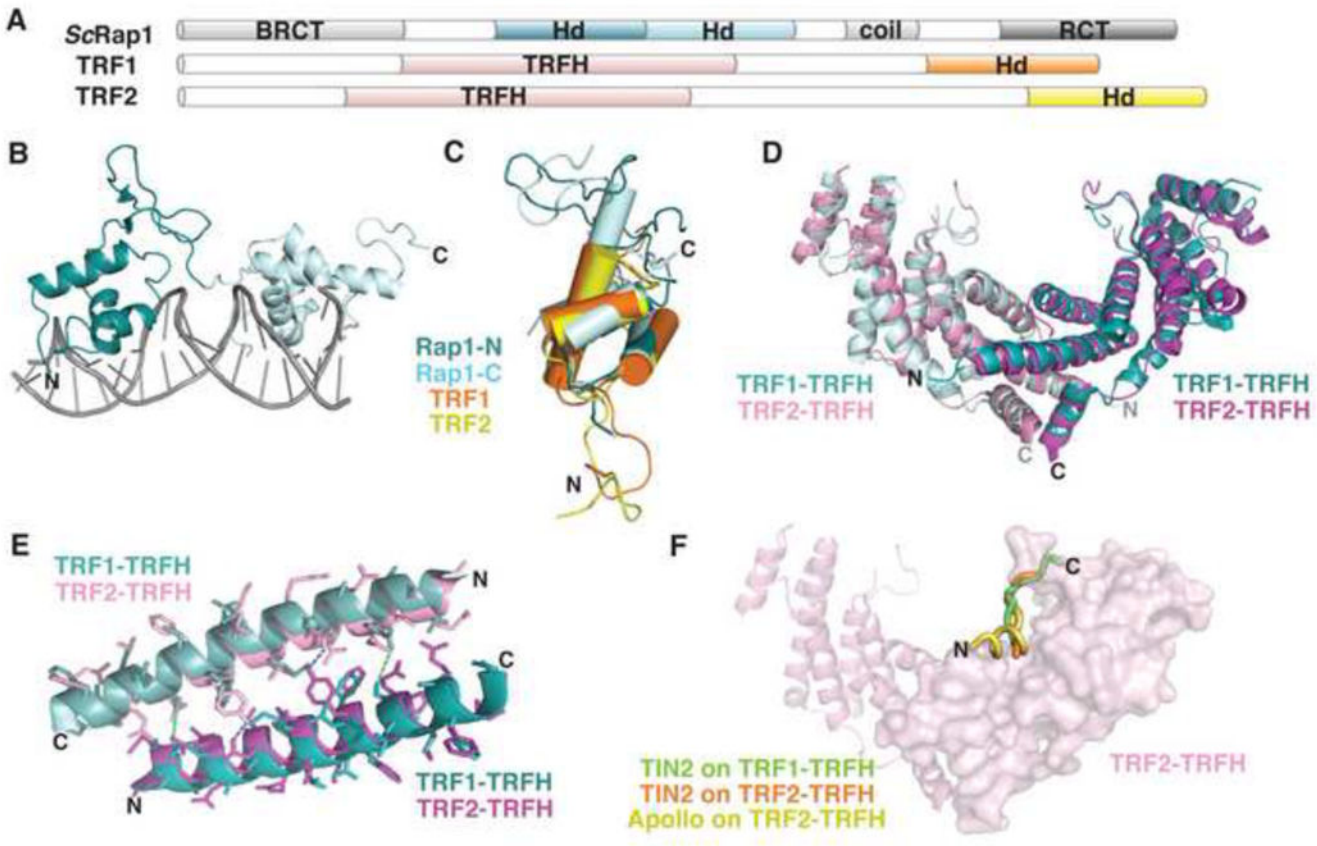

Figure 6. Domains of telomeric dsDNA binding proteins

(A) Domain topology of TRF1, TRF2, and *Sc*Rap1. (B) *Sc*Rap1 binds to double-stranded telomeric DNA with two homeodomains (N-terminal, teal; C-terminal, light blue; PDB: 1IGN). (C) Overlay of the homeodomains from *Sc*Rap1 (*Sc*Rap1-N, teal; *Sc*Rap1-C, light blue; PDB: 1IGN), TRF1-DBD (orange; PDB: 1ITY), and TRF2-DBD (yellow; PDB: 1VF9). (D) Superposition of the TRF1-TRFH homodimer (aqua and light blue; PDB: 1H6O) with the TRF2-TRFH homodimer (pink and magenta; PDB: 1H6P; rmsd =1.23Å). (E) Superposition of the α1 helices that comprise part of the homodimerization interface. TRF1, teal and light blue; TRF2, pink and magenta. Dashes indicate hydrogen bonding between monomers (TRF1, green; TRF2, blue). (F) Superposition of three peptide ligands on the structure of the TRF2-TRFH homodimer. TIN2 peptide bound to TRF1, green (PDB: 3BQO); TIN2 peptide bound to TRF2, orange (PDB: 3BU8); Apollo peptide bound to TRF2, yellow (PDB: 3BUA). For simplicity, only one peptide binding site is shown on the surface of one monomer in the homodimer.

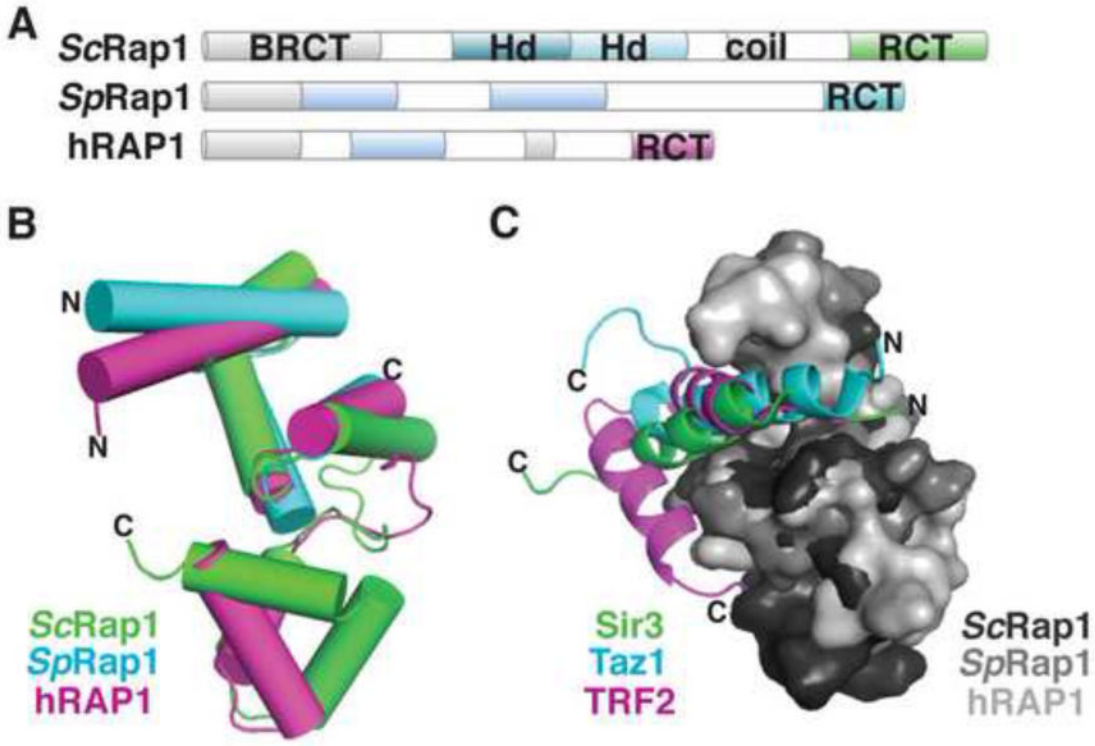

Figure 7. Protein-protein interactions

(A) Domain topology of Rap1 proteins. (B) Cartoon overlay of the Rap1 C-terminal domains (RCT) from *S. cerevisiae* (green; PDB: 3OWT), *S. pombe* (cyan; PDB: 2L3N), and human (magenta; PDB: 3K6G). (C) Surface overlay of the RCTs from *S. cerevisiae* (dark gray), *S. pombe* (medium gray), and human (light gray), shown with their respective peptide ligands shown as cartoons: Sir3 (green), Taz1 (cyan), and TRF2 (magenta).