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Tying up the Ends: Plasticity in the Recognition of ssDNA at Telomeres

Neil R. Lloyd, Thayne H. Dickey[#], Robert A. Hom, and Deborah S. Wuttke^{*}

Department of Chemistry and Biochemistry, 596 UCB, University of Colorado Boulder, Boulder, CO 80309-0596, USA
Department of Chemistry and Biochemistry, 596 UCB, University of Colorado Boulder, Boulder, CO 80309, USA

Abstract

Telomeres terminate nearly exclusively in ssDNA overhangs comprised of the G-rich 3' end. This overhang varies widely in length from species to species, ranging from just a few bases to several hundred nucleotides. These overhangs are not merely a remnant of DNA replication, but rather are the result of complex further processing. Proper management of the telomeric overhang is required both to deter the action of the DNA-damage machinery and to present the ends properly to the replicative enzyme telomerase. This Current Topic review addresses the biochemical and structural features used by the proteins that manage these variable telomeric overhangs. The Pot1 protein tightly binds the single-stranded overhang, preventing DNA damage sensors from binding. Pot1 also orchestrates the access of telomerase to that same substrate. The remarkable plasticity of the binding interface exhibited by the *S. pombe* Pot1 provides mechanistic insight into how these roles may be accomplished, and disease-associated mutations clustered around the DNA-binding interface in the hPOT1 highlight the importance of this function. The budding yeast Cdc13-Stn1-Ten1, a telomeric RPA complex closely associated with telomere function, also interacts with ssDNA in a fashion that allows degenerate sequences to be recognized. A related human complex composed of hCTC1-hSTN1-hTEN1 has recently emerged with links to both telomere maintenance and general DNA replication and also exhibits mutations associated with telomere pathologies. Overall, these sequence-specific ssDNA binders exhibit a range of recognition properties that allow them to perform their unique biological functions.

Introduction

Telomeres are the nucleoprotein caps at the ends of linear chromosomes^{1–9} that buffer against the loss of genomic DNA.^{10–13} This specialized heterochromatin comprises a region of repetitive non-coding DNA that terminates in a conserved single-stranded overhang^{3,13,14} and protein complexes that tightly bind telomeric DNA. These proteins protect the DNA from degradation, prevent the erroneous recognition of the single-stranded overhang as DNA damage, and regulate the extension of telomeres by the reverse-transcriptase

^{*}Corresponding Author: Deborah.wuttke@colorado.edu. Telephone: (303) 492-4576.

Current Address: Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06511, USA

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telomerase.^{15–22} During DNA replication, daughter strands are shortened because DNA polymerase requires RNA primers in lagging strand synthesis that cannot be replaced by DNA at the extreme 5′ ends of the chromosome.²³ Further shortening arises from the replication of the shorter C-rich strand. The loss of telomeric DNA is exacerbated during telomere processing by the action of the Exo1 and Apollo/SNM1B nucleases, which resect the 5′ end to create the overhang at mammalian telomeres. The processing pathway to generate these ends in budding yeast is different, involving the Sae2-MRX exonuclease pathway (^{24–27} and reviewed in²⁸), but in both cases the ends produced by DNA replication are resected. These processing pathways standardize both the 3′ overhang length and the sequence register.²⁹ As a result of these processes, progressive DNA replication leads to the shortening of telomeres until they reach a critically short length and cells undergo senescence.³⁰ Senescent cells are thought to contribute to aging and telomere length is correlated with age, thus telomere length is a potential target for age-related diagnostics and therapeutics.^{30–32}

In stem cells and unicellular organisms, the loss of telomeric DNA is counteracted by the activity of the reverse transcriptase telomerase.^{33,34} Telomerase is a ribonucleoprotein that minimally requires a template/scaffold RNA and a protein subunit that is related to viral reverse transcriptases.^{35,36} Telomerase adds single-stranded telomeric repeats to the 3′ end of chromosomes by partially aligning the template region of the RNA component to the 3′ overhang and catalyzing the addition of dNTPs based on the template sequence.^{37,38} The 5′ strand is subsequently filled in by standard 5′ to 3′ DNA synthesis.³⁹ Telomerase adds DNA to these ends with high nucleotide processivity and unique repeat addition processivity in which a single telomerase molecule can dissociate and realign its template RNA to add multiple telomeric repeats.^{39,40} Approximately 90% of all human cancers overcome the end replication problem by activating telomerase, making this enzyme a potential target for cancer therapeutics.^{41–44}

The telomeric sequence added by human telomerase is GGTTAG, which repeats throughout the 5–15 kb double-stranded and 50–500 nucleotide single-stranded portions of the 3′ strand.^{13,14,38,40} High-throughput sequencing, however, has revealed some variation in telomeric sequence in both primary and immortal human cell lines.⁴⁵ Other species, such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, have a shorter and even more irregular telomeric sequences.^{24,46–49} *S. pombe* telomerase RNA appears to template a GGTTACA repeat, but does so inconsistently, resulting in nucleotide deletions and additions between repeats.^{50,51} Similarly, *S. cerevisiae* telomeres are best described by the sequence (TG)_{1–6}TG_{2–3} with an overhang length that varies from less than 10 to over 70nts.^{52–55} These variable sequences provide a unique and interesting challenge for the proteins that interact with them, coincidentally providing excellent model systems for the study of sequence specificity in DNA-binding proteins.

Shelterin and the Pot1 protein

A six-membered protein complex known as shelterin is roughly conserved from fission yeast to humans and is responsible for capping and protecting the telomere in most eukaryotes (reviewed by Palm and de Lange⁵). Shelterin contains dsDNA-binding proteins (TRF1 and

TRF2 in humans, Taz1 in *S. pombe*), ssDNA-binding proteins (Pot1 in both species), bridging proteins (TIN2 and TPP1 in humans, Rap1, Poz1 and Tpz1 in *S. pombe*), and other associated proteins (RAP1 in humans, Ccq1 in *S. pombe*) (Figure 1). Telomeres are further protected by the formation of t-loops in humans in which the ssDNA overhang loops back on the double-stranded region via a strand invasion mechanism dependent on topological changes induced in telomeric DNA by TRF2.^{56–59} Deletion of components of the shelterin complex has been reported to trigger an increase in the volume occupied by telomeric chromatin as well as an increase in DNA-damage response signaling at telomeres, suggesting that shelterin further excludes DNA damage response machinery by compacting telomeric chromatin.⁶⁰ In *S. pombe* and *S. cerevisiae*, both the duplex region and the overhang of the telomere are much shorter and thus do not appear to form t-loops.

Pot1 is the sole protein in shelterin that exhibits autonomous ssDNA-binding activity and is critical for end protection.^{20,61} Disruptions of human POT1 (hPOT1), mouse Pot1a, or chicken Pot1 result in activation of the Rad3-related (ATR) DNA-damage response pathways, chromosomal fusion, and cell death, likely through a failure to exclude the ATR damage sensor RPA.^{62–64} Loss of just the ssDNA-binding activity of hPOT1, however, leads to rapid and extensive telomere elongation.⁶⁵ Furthermore, knockdown of hPOT1 also disrupts the terminal sequence of the 5' strand, suggesting that hPOT1 sets the register for end resection.⁶⁶

Challenges facing telomere end-protection proteins

The telomere end-protection proteins, such as Pot1, must overcome several challenges to accomplish their vital functions. First, these proteins must bind telomeres tenaciously to prevent degradation by nucleases as well as occlude telomeric structures from recognition by damage response pathways. Moreover, because these proteins can displace proteins that sense DNA damage, they must have limited binding activity to non-telomeric sequence so as to not interfere with proper recognition of *bona fide* DNA damage and subsequent repair.⁶⁷ Furthermore, non-specific binding to other regions of the genome would overshadow the limited binding sites present at telomeres⁶⁸ and leave telomeres inadequately protected. These activities are achieved through the combination of unique biochemical properties and the association of Pot1 with the shelterin complex. Seemingly counter to this need for specificity, however, sequence variation at telomeres necessitates that telomere binding proteins somehow also accommodate some level of non-specificity.^{45,49} TPP1 in humans and Tpz1 in *S. pombe* in part aid to resolve these challenges by bridging Pot1 to the dsDNA binding components of the Shelterin complex through an interaction between TPP1 and the C-terminal domain of Pot1 to increase the avidity of Pot1 to telomeres.^{20,21} Additionally, the evolution of Pot1's recognition features for ssDNA addresses these functional challenges in a remarkable manner.

Structures of the Pot1 proteins reveal how binding affinity and specificity are achieved

Pot1, and telomere end-proteins in general, use a common structural topology known as the OB fold to recognize ssDNA. OB-folds are multifunctional domains found throughout biology and are frequently implicated in the recognition of disordered linear polymers, most commonly ssDNA and ssRNA.^{69,70} The structural framework is a simple 5-stranded β -barrel

elaborated with loops and helical elements to form a virtual platform for polymer recognition whose properties can be tailored to the desired specificity and affinity through a variety of mechanisms. The ligand can bind to a single OB fold, multiple independently binding OB folds, an extended binding interface across several OB folds in tandem, or through homo/hetero-oligomerization.

The N-terminal portion of Pot1 contains a dual OB-fold that confers full DNA-binding activity while the C-terminal half (predicted to be an OB-fold) interacts with the shelterin component TPP1 in humans/Tip1 in *S. pombe* (Figure 2).^{20,21,71} Structures of both the complete human DNA-binding domain (DBD) and the 2 OB folds that together comprise the *S. pombe* DBD have been solved.^{72–74} hPOT1 adopts an elongated structure comprised of these 2 OB folds that are closely linked together by a short 9 amino acid linker such that the two domains functionally bind a 10-nt telomeric ssDNA ligand as one contiguous unit with an extensive domain/domain interface (Figure 3A). hOB1, the N-terminal OB fold, binds the first 6-nt (TTAGGG) with strong specificity, especially for nucleotides 2–5.⁷³ hOB2, the C-terminal OB fold of the pair, binds to the final 4-nt (TTAG) with less specificity than hOB1 except for the terminal G10.⁷³ Consistent with the specificity data, hOB1 forms over two-thirds of the hydrogen-bonding interactions between the ligand and the protein (22 out of 31 total).⁷³ At the interface between the two, the phosphodiester bond of T7 kinks 90° to shift into the binding interface of hOB2.⁷³

Recent genome wide association studies (GWAS) have found several mutations in hPOT1, associated with chronic lymphocytic leukemia, familial glioma, and several other cancers types as well as the rare familial disorder Coat's plus.^{75–80} In CLL, Pot1 is one of the most frequently mutated genes with 3.5% of CLL cases containing somatic mutations in Pot1. Strikingly, most of the disease-associated point mutations occur at residues contacting DNA in the crystal structure of hPOT1-DBD (Figure 3B). Some of these mutations appear to disrupt ssDNA-binding *in vitro*,^{75,79} deprotect telomeres, and trigger telomere lengthening and oncogenic fusions.⁷⁵ However, this phenotype is not observed for all of the DBD mutations or the C-terminal domain, suggesting some mutations may exercise their influence through other pathways.^{78,80,81} *In vivo*, deletion of the DNA-binding domain of hPOT1 results in telomere elongation, supporting a role in negative length regulation.⁶⁵ Conversely, some of these mutations lead to telomere shortening, currently ascribed to a loss of interaction with another ssDNA binding complex of hCTC1-hSTN1-hTEN1 and suppression of appropriate lagging strand synthesis.⁸⁰ This differential impact speaks to the complexity of processing at the telomere and the myriad roles hPOT1 plays.

S. pombe Pot1 is a functional homologue of hPOT1 and shares a similar domain organization.^{21,72–74} This includes an N-terminal DNA-binding domain (Pot1-DBD) composed of two OB-folds (Pot1pN and Pot1pC).^{72–74,82} Biochemical experiments already suggest a difference in mechanism of action between the homologs. Pot1pN and Pot1pC can be separated and retain biochemical activity individually, in contrast to hOB1 and hOB2 which appear to function only as a tightly packed unit.^{73,74,82–84} This is likely in part due to the expanded linker between Pot1pN and Pot1pC, composed of 25 proteolytically labile residues as opposed to only 5 disordered residues between hOB1 and hOB2.^{74,84} Thus Pot1pN and Pot1pC appear to be flexibly tethered subdomains in contrast to the more tightly

packed arrangement between hOB1 and hOB2.^{72–74,84} Curiously, *Sp*Pot1 also binds DNA with an affinity three orders of magnitude stronger than hPOT1 (low pM vs. low nM).^{73,82,83,85} An outstanding question is how these differences are related to their respective roles at telomeres.

Pot1pN has significant sequence identity to its human counterpart, hOB1, and, as expected, the protein structures are quite similar (alignment shown in Figure 4).^{72,73,86,87} This similarity is also evident in the specificity profiles of both domains in which binding is strongly disrupted when the individual nucleotides at positions 2–5 of either ligand are substituted with the complementary base.⁸³ Notably, four of these nucleotides overlay well in both structures and occupy nearly identical binding pockets (Figure 5A). The DNA-binding surfaces of both proteins participate in extensive hydrogen bonding with the Watson-Crick face of the DNA ligand and form several protein DNA stacking interactions (two in Pot1pN and three in hOB1). Additional specificity in Pot1pN appears to be achieved through intramolecular hydrogen bonding and stacking interactions within the DNA ligand itself between the bases of the nucleotides 1–4 (Figure 6A).

Pot1pC and hOB2 lack sequence identity and exhibit differing biochemical behavior, confounding direct extrapolation between them.^{73,74,83,84,88} However, a structural comparison of the two domains reveals the mechanistic basis for their divergent behaviors. Despite their sequence divergence, the overall structures of Pot1pC and hOB2 are strikingly similar and they are easily identified as structural homologues by computational algorithms.⁸⁹ However, the clear structural differences between them have had a profound impact on their respective recognition of ssDNA. While hOB2 interacts with only four nucleotides in the structure of hPOT1-DBD bound to DNA, Pot1pC alone binds a minimal 9-nt ligand roughly across the canonical ligand-binding interface of the OB fold (Figure 5B).⁷⁴ Interestingly, the 9-nt ligand is bent $\sim 90^\circ$ as it traverses the surface. When compared to the path of human ssDNA along hOB2,⁷³ it becomes apparent that a substantially different region of the OB-fold barrel is used for ligand binding, which results in a stunning lack of ligand overlap between the two structures. Indeed, the binding pocket for only one nucleotide overlaps between these 2 domains (Figure 5B). Surprisingly, these dramatic differences in ssDNA-binding activity stem from the reorientation of a single loop connecting strands 2 and 3, which allows the proteins to take advantage of completely different binding surfaces. The path of ssDNA in Pot1pC suggests also that the OB-OB domain interface observed in the human structure is not achievable in *S. pombe*; arrangement of the *S. pombe* N and C OB folds in the human packing geometry leaves a 23 Å gap in the path of ssDNA.⁹⁰

A remarkably plastic interface confers non-specificity

One of the most surprising features of the Pot1pC/9mer structure is the large number (22) of apparently sequence-specific H-bonds between both the Watson-Crick and Hoogsteen faces and the surface of the protein. This recognition interface is composed of several stacking interactions and a set of base-mediated H-bonds that largely resemble those that confer specificity in the N-terminal domain (Figure 6A, B).⁷² Canonically, sequence-specific recognition is thought to occur through the readout of a pattern of H-bond donor and

acceptor atoms characteristic of a nucleotide sequence. Conversely, non-specific nucleic acid recognition is believed to be achieved by stacking/hydrophobic interactions and/or interactions with the phosphate backbone.^{91–94} Thus, the specificity at position 2, for example, would typically be ascribed to the presence of three direct H-bonds between the base and the protein. In the Pot1pC/ssDNA interface, though, the presence of those H-bonding interactions does not predict specificity, for example, examination of the interactions at position 1 reveals 4 direct H-bonds that confer no specificity.⁸⁴ Base-mediated H-bonds such as the ones observed here are frequently assigned roles in conferring specificity, and nothing about the chemical nature of the interface suggests a biochemical difference of specificity relative to Pot1pN. Thus, the cognate structure alone cannot be used to predict the biochemical specificity of the interface.

Fortuitously, structures of complexes containing non-telomeric (non-cognate) sequences provided insight to understanding how this seemingly specific interface accommodates other sequences.⁷⁴ Despite having similar affinities, study of this series of complexes revealed unanticipated structural changes at the protein/nucleic acid interface. These range from a local reorientation of a base to wholesale reorganization of the interface. For example, only local reorientation at the site of substitution is observed following base alterations at positions 3, 5 and 6.⁷⁴ These modest rearrangements do not substantially impact the positioning of the base but neatly compensate for lost H-bonds by forming new ones (Figure 7A).⁷⁴ The overall protein backbone is largely unaffected as well, with only minor changes in protein structure distal to the interface.⁷⁴ Some base substitutions lead to more pronounced local changes in both DNA and protein conformation. For example, substitution from guanine to cytosine at position 8 results in a 180° rotation to the base coupled with a rearrangement of the $\beta 2$ – $\beta 3$ loop (L23).⁷⁴ As expected, the substitution of cytosine disrupts a suite of H-bonds, but the base's rearrangement creates an equally intricate network of H-bonds with almost completely new intra- and intermolecular partners (Figure 7B).⁷⁴ Although these adjustments are all proximal to the site of base substitution, these structures suggest conformational plasticity within both the protein surface and the DNA that allows the interface to adapt structurally and thermodynamically to the base changes.⁷⁴ While some non-cognate ligands can be accommodated by these local (although significant) adjustments to the interface, others lead to even larger changes, with a global reorganization of the complex.⁷⁴ Substitution of the base at positions 2 and 4 leads to a second binding mode.⁷⁴ For example, substitution at position 4 triggers a repositioning of the base, presumably because the large A cannot fit in the pocket previously occupied by a T (Figure 7C).⁷⁴ As a result, the base rotates ~90° around the phosphodiester backbone, flipping it out of the original binding pocket into the largely unoccupied space below. This reorientation is stabilized by a stacking interaction with Arg68, and leads to a “chain reaction” of molecular events both 5' and 3' of the site of substitution, causing a complete reorganization of the interface marked by an overall 3.05 Å ligand RMSD compared to the cognate ligand structure.⁷⁴ For comparison, excluding the flexible L23, hOB2 and Pot1pC have a 1.9 Å RMSD for 125 α -carbons (out of 139).^{73,74} All in all, Pot1pC has at its disposal several structural elements to accommodate sequence heterogeneity, including ligand and protein flexibility (particularly in loop regions), an enlarged binding cleft, and a complex network of H-bonding interactions.

These structures in total revealed a sophisticated mechanism of conformational malleability by which Pot1pC is able to accommodate heterogeneous ssDNA ligands with little to no change in the overall thermodynamics of binding. This plasticity is likely shared by other proteins that are either fully non-specific such as RPA⁹⁵ or require graded specificity, such as t-RPA (see below). It is an open question as to what biophysical features of the protein and ligand, for example, types of amino acids at the interface or dynamic properties, facilitate this type of malleable recognition. Moreover, this raises the questions of what makes an interface biochemically specific for an inherently flexible ligand and which type of interface is in fact harder to evolve.

How do the subdomains work together?

Our structural understanding of the *S. pombe* Pot1-DBD is derived from studies of the individual subdomains, primarily because the full DBD proved intractable to high-resolution structural characterization. This caveat raises the question of how many of its characteristics can be explained through the action of the two subdomains in isolation. A reasonable first measure is to compare the biochemical features of the individual domains to those of the intact DBD (and full-length protein). The full Pot1-DBD shows much of the same specificity trends as the individual domains but has reduced specificity at A5 and C6 for Pot1pN and G2 for Pot1pC (Figure 7D).^{83,88} However, the absolute specificity for the Pot1pC sequence is dramatically reduced such that complete substitution of the Pot1pC 9mer sequence results in less than a 2-fold change in binding.⁸³ The full-DBD can also bind a 12mer ligand comprised of two 6mer repeats whereas Pot1pC exhibits no observed binding to a 6mer sequence.⁸⁸

While the structure of the homologous hPOT1 has been solved, the disparate DNA-binding surfaces of Pot1pC and hOB2 make homology modeling unreliable. As noted above, simply docking the *S. pombe* DNA-bound structures in the relative hOB1/hOB2 orientation seen in the crystal structure creates a physically impossible path for the ssDNA to adopt. While it is possible that the DNA completely rearranges in the full DBD relative to the conformation adopted in the individual domains, the similarity of the biochemical features between the two suggests the DBD is more like the individual domain structures than not. The more likely scenario is that the long flexible linker that connects Pot1pN and Pot1pC allows for a domain/domain reorientation that differs considerably from that observed in the human homologue.

Solution NMR strategies provide a complementary tool to x-ray crystallography to probe the overall conformation of the *S. pombe* Pot1-DBD complex. Comparison of the full assigned spectra of the Pot1pN+6mer and the Pot1pC+9mer complexes to that of the Pot1-DBD +15mer allows for high-resolution mapping of regions of difference.⁸⁴ Overall, the notion that the whole equals the sum of the parts holds true. The vast majority of assigned residues coincide precisely in chemical shift between the Pot1-DBD and its constituent subdomains, suggesting a large degree of structural similarity.⁸⁴ Mapping of the few residues that are shifted pinpoints a potential Pot1pN/Pot1pC interface that is indeed rotated significantly away from the orientation in the human structure.)⁸⁴ Interestingly, deletion of the majority of the linker did not lead to any change in affinity for telomeric substrate, indicating that this

altered conformation can be accommodated with a relatively short (only 4 amino acid) linker sequence.⁸⁴ Furthermore, perturbation of the putative contact residues within this interface also leads to minimal (less than 2-fold) changes in ssDNA binding affinity.⁹⁰ Together, these data support a model where the Pot1pN and Pot1pC subdomains are relatively structurally independent, lacking in a precise, stable protein/protein interface and acting merely as weakly associated partners in binding.

Why this evolutionary divergence and what does it suggest regarding telomere maintenance in general? It is quite common to identify proteins that have relatively similar structures in the absence of identifiable sequence relationship, as structure is generally more conserved than sequence. However, we are unaware of any examples of structurally homologous domains that bind the same ligand via a completely novel interface. The marked differences between hPOT1 and *Sp*Pot1 DBDs may have evolved to accommodate the unusual and specific needs of the telomeres in each species: hPOT1 only needs to recognize a relatively invariant repeat while *Sp*Pot1 must accommodate degenerate sequences and likely does in part via domain-domain rearrangement. Other potential reasons include differences in shelterin, need for t-loop assembly, and/or differences in the length of the overhang. Conversely, it may be that these two homologues represent the range of conformations needed to be accessed at different points in the process of telomere maintenance.

How might the structural and biochemical similarities and differences of the Pot1 proteins tie into telomerase regulation?

In vitro, Pot1 inhibits telomerase activity by sequestering the 3' ssDNA overhang that telomerase requires as a substrate, presumably through a simple competition event, suggesting that its intrinsic nature is to restrict access of telomerase to the overhang.^{17,18} This is consistent with the observation that deleting a DNA-binding OB fold in hPOT1 leads to significantly longer and more heterogeneous telomeres.⁶⁵ *In vitro* addition of hPOT1's direct binding partner within the shelterin complex, TPP1, however, ameliorates this inhibitory effect and significantly increases the repeat addition processivity of telomerase,^{19,20,96} by slowing primer dissociation and aiding translocation, perhaps by increasing the dynamic sliding of Pot1 on DNA.^{96,97} TPP1 (or Tpz1 in *S. pombe*) has no significant DNA-binding ability of its own but somewhat alters the *in vitro* DNA-binding properties of Pot1.¹⁹ In addition to tethering hPOT1 to the shelterin complex, hTPP1 also recruits telomerase to telomeres *in vivo* through, incidentally, yet another OB fold.^{98–101}

It remains unclear if there is active regulation of hPOT1 binding to ssDNA, or if telomerase simply competes with hPOT1 for access to the 3' end. The bias in end sequence provides some insight- 40% of 3' overhangs in telomerase active human cells terminate in the sequence 5'-GGTTAG-3'.²⁹ Based on the crystal structure of hPOT1 bound to ssDNA, this sequence should be bound and fully sequestered from telomerase.⁷³ While different terminal sequences are extendable to some extent, full human telomerase activity requires an unprotected overhang of at least eight nucleotides.¹⁸ Aside from the structural considerations, there are kinetic features to consider as well. As is typical of tight binding interactions, hPOT1/TPP1 dissociates slowly from ssDNA, with a half-life of nearly 30 minutes *in vitro*, pointing to the need for active regulation of POT1 binding.¹⁹ Less is known

about *S. pombe* proteins, but a similar mechanism of telomerase recruitment is proposed via a Pot1-Tpz1-Ccq1 complex and *S. pombe* Pot1 has a ssDNA-bound half-life of approximately one hour.^{21,22} These common features point to a shared requirement for active regulation to allow telomerase access.⁸³

Several lines of data on the ssDNA-binding preferences of *Sp*Pot1 suggest that Pot1 can bind ssDNA in alternative modes, predominantly through malleability in the recognition of ssDNA by the less-specific Pot1pC domain. The first observation is that, in addition to the 15mer binding mode described above (that is closely related to the “sum of the parts” idea), *Sp*Pot1 binds a simple 12mer sequence that comprises 2 repeats of the core telomere sequence – GGTTAC GGTTAC. This clearly must adopt a different conformation than the 6+9 mode described above. At high concentrations of Pot1-DBD, the protein binds the 12mer ligand as a dimer, suggesting that the Pot1pN of each monomer binds its core specific sequence (GGTTAC, as described above).¹⁰² This suggests that the avidity of Pot1pC for the remaining 3′ 6mer is too modest to out compete a second binding event at high concentrations. Indeed, Pot1pC binding of a 6mer in isolation is in the mM range.⁹⁰ At lower, more physiologically relevant, concentrations of Pot1-DBD, the dimer is not observed, and gel shift suggests a distinct, as yet structurally uncharacterized, conformation. Preliminary NMR data suggest that the mode of interaction with the Pot1pC part of the DBD is entirely disrupted relative to that present in the 15mer complex.⁹⁰ While the precise structural details are elusive, this new conformation has distinct biochemical features relative to the 15mer binding mode, most prominently a 3′ end that is more accessible to other end-binding factors.

The ability to observe this second binding mode by gel shift allowed the screening of protein mutants able to induce a similar conformation.⁸⁴ In an effort to rationally induce such a conformational change, a panel of mutations was engineered near the binding site of the 3′ end of the DNA. In Pot1pC, the 3′ end of the oligo forms an interleaved aromatic stack, similar to four teeth of a zipper, with W27 and Y28 (W223 and Y224 in full-length DBD) (Figure 6B). Mutation of Y224 in the context of Pot1pC has a drastic effect on binding affinity, however, mutation of Y224 in the context of Pot1-DBD has no effect on affinity. This curious disconnect can be explained through the observation that Pot1-DBD containing this mutation adopts the alternate 12mer binding mode, suggested by the characteristic gel shift. This mode is also induced when alterations in DNA sequence are made at the 3′ end at positions 13 or 15, the bases that stack with Y224, or at high salt conditions that disrupt this more electrostatically driven binding mode.

Despite these distinct biochemical and structural features, the 12mer and 15mer binding modes have similar affinities at physiological salt concentrations.⁸³ This argues that both 1:1 binding modes have to be considered when evaluating biological function. Access to the 3′ overhang at the telomere is an essential step in regulating telomerase activity. *In vitro* telomerase extension is inhibited by the presence of Pot1 and can be restored when the Pot1 binding site is moved away from the 3′ end.¹⁸ The potential ability of another protein to engage the 3′ end in the 12mer, but not 15mer, binding mode suggests that this binding mode does not completely sequester the 3′ end and may represent an extendible telomeric state. Does this happen in hPOT1? As noted above, the specificity for the 5′ end of the

oligonucleotide substrate is shared, and the localization of Pot1 to the telomere via its interaction with TPP1 means it has the flexibility to perhaps shift modes to ones with weaker affinity. The role of this plasticity in these events is an exciting frontier in telomerase regulation.

The role of a telomere-specific RPA complex

Budding yeast telomeres are maintained by a heterotrimeric complex

In *Saccharomyces cerevisiae*, the heterotrimeric protein complex comprised of Cdc13, Stn1, and Ten1 has been shown to be integral to telomere maintenance. Cdc13 is a critical central regulator of telomere functions, and plays a pivotal role in both positively and negatively regulating telomere length.^{103–110} Deletion of full-length Cdc13, Stn1, or Ten1 is lethal due to the accumulation of excessive quantities of ssDNA, which leads to activation of the DNA damage response followed by cell cycle arrest.^{103,111–113}

Our understanding of this complex has been informed by numerous structural studies. Cdc13 is comprised of five domains, with an N-terminal OB-fold that mediates Cdc13 homodimerization and interaction with pol α , an unstructured recruitment domain for the telomerase subunit Est1, a middle OB-fold, a DBD OB-fold, and a C-terminal OB-fold that participates in the formation of the Cdc13-Stn1-Ten1 heterotrimer (Figure 8).^{114–119} Likewise, Stn1 contains an N-terminal OB-fold and Ten1 comprises an OB-fold, the structures of which were solved from *Schizosaccharomyces pombe* and *Candida tropicalis*, respectively.^{120,121} These structures additionally revealed the tandem winged helix-turn-helix domains of Stn1.^{120,121}

Thus far, characterization of the recognition of ssDNA by the intact complex has been hampered by the lack of a well-behaved, recombinant complex. As Stn1/Ten1 bind ssDNA only weakly on their own,¹²² the Cdc13 protein serves as a good stand-in for the activity of the complex and the ability of *S. cerevisiae* Cdc13 to recognize ssDNA has been well studied.^{116,117,123–128} While Cdc13 exists as a homodimer *in vitro*,¹¹⁸ disruption of the dimer with a single point mutation at L91R does not impact ssDNA-binding activity, as the monomeric form of Cdc13 binds the 11-nt sequence with similarly tight affinity.¹²⁸ Interestingly, Cdc13 binds longer strands of telomeric ssDNA with modest positive cooperativity. This activity is independent of its ability to dimerize, suggesting instead that the binding of Cdc13 to a G-rich strand alters the conformation of the nucleic acid such that it is more conducive for binding a second Cdc13 protein.¹²⁸

The ability of *S. cerevisiae* Cdc13 to recognize telomere ends is conferred through the action of a single OB-fold that tightly binds an 11-nt sequence of GTGTGGGTGTG with low picomolar affinity, identical to that of the full-length protein.^{116,123,124,126,128} The solution structure of this complex revealed that the ssDNA binds by stretching out across a large binding surface to accommodate the extended 11-nt sequence (Figure 9).^{116,117} Residues that interact with the DNA are predominantly aromatic or basic. Like the dual domain Pot1 protein, the Cdc13-DBD recognizes the 5' end of the telomeric oligonucleotide specifically and has limited sequence specificity for the 3' end.¹²⁶ The critical binding residues of Y522, Y580, I633 and R635 make contact with the 5' region of the DNA, and mutating

these residues to alanine causes the greatest reduction in binding affinity to DNA.¹²⁵ The large 30 amino acid L23 of Cdc13-DBD plays an essential role in making contact with the last 5 nucleotides on the 3' end of the 11-nt sequence and acts as an extension of the binding interface to accommodate the longer telomeric sequence.^{116,117} Thus, the L23 appears to replace the analogous function of the second OB fold in Pot1. DNA sequence specificity was probed with oligonucleotides containing single position substitutions to a pool of the alternate bases, demonstrating that the 5'-GNGT positions make the most significant contributions to sequence specificity.¹²⁶ Analogous to Pot1, which binds the first 6-nt with strong specificity, this 5'-end recognition allows Cdc13 to accommodate the degenerate telomeric sequence found in *S. cerevisiae*. Positions T2, G7, and G9 are recognized with reduced specificity and base substitutions at other positions have little to no impact on affinity. However, the 3'-end still contributes to ligand affinity as shortening it results in weakened binding.^{123,124}

Like Pot1, Cdc13 has also been shown to inhibit telomerase activity *in vitro* by preventing telomerase access to the 3' ssDNA overhangs.¹²⁹ This mechanism of inhibition has been supported by the observation that DNA-binding defective point-mutation of Y522A restores telomerase activity.¹²⁹ Unlike hPOT1,¹⁸ telomerase activity cannot be reinitiated when an extra 6-nts are protruding from the Cdc13-ssDNA binding site. Inhibition of telomerase activity can still occur up to 17-nts past the Cdc13 binding site, showing that the mechanisms in which Cdc13 represses telomerase are mechanistically different than those seen for hPOT1.

The t-RPA complex

The domain structures and their organization within the Cdc13-Stn1-Ten1 complex revealed a striking similarity to the known crystal structures of the non-sequence specific ssDNA recognition protein replication protein A (RPA) heterotrimer,^{116,117,120,121} fulfilling a prediction made based on early observations that Cdc13-Stn1-Ten1 formed a heterotrimer complex and the predicted OB-domain organization strongly resembled those seen for RPA.^{95,122,130,131} These structural and biochemical similarities between both heterotrimer complexes have led to the proposal that Cdc13-Stn1-Ten1 is a telomere-specific homologue of the RPA heterotrimer that is known as t-RPA.¹²²

The most significant functional, biochemical and structural parallels can be drawn between Stn1-Ten1 and RPA32-RPA14. The domain organization is identical, with the exception that the *S. cerevisiae* Stn1 C-terminal domain was found to contain two WTH domains versus the single WTH domain in RPA32 (Figure 8).^{120,121,132} Several missense mutations of the solvent exposed residues of this second WTH motif showed negative regulation of telomere length,¹²⁰ but how this domain performs this function still remains unclear. The single WTH domain of RPA32 interacts with several DNA damage response proteins,¹³³⁻¹³⁶ and raises the question of whether the WTH domains of Stn1 also interact with as yet unidentified other proteins. The OB-folds of Stn1-Ten1 in *S. pombe* and *C. tropicalis* are similar to those from RPA32-RPA14, with some differences in loop regions and the C-terminal α -helices (Figure 10A,B).^{120,121} RPA32 and RPA14 can modulate ssDNA binding

by the core DBD of RPA70.¹²¹ However, it is still unknown whether Stn1-Ten1 can modulate Cdc13 ssDNA binding in a similar fashion as RPA32 and RPA14.

The overall domain architecture of Cdc13 and RPA70 are broadly similar, with each containing four OB-folds, but distinctive differences in structure and DNA-binding activity likely give rise to the telomere specific functions of t-RPA. The N-terminal OB-folds of RPA70 and Cdc13 share little similarity with one another, and there is no evidence that RPA homodimerizes.^{114,137,138} In contrast to the single DNA-binding OB-fold in Cdc13, both middle OB-folds of RPA70 can bind ssDNA, but individually they bind with only low micromolar binding affinities.^{139,140} The two OB-folds together make up RPA70-DBD, which binds a minimal 8-nt ssDNA with 50 nM binding affinity and no sequence specificity.¹⁴⁰ The complete RPA heterotrimer binds longer ssDNA even more tightly than RPA70-DBD with low nanomolar binding affinity. This improved ssDNA binding activity is predicted to be a result of the involvement of additional OB folds in the complex.^{95,138,139} It is possible that yeast Stn1 and Ten1 similarly extend the binding site of Cdc13 for ssDNA, as seen for the RPA heterotrimer, but *S. cerevisiae* Stn1 and Ten1 are refractory to recombinant expression and purification, precluding their addition to *in vitro* experiments on the well-studied *S. cerevisiae* Cdc13. Testing these hypotheses in related species is confounded by the divergent activities of homologous proteins. While *S. cerevisiae* Cdc13 strongly discriminates against non-telomeric DNA, several Cdc13 proteins in the *Candida* clade do not exhibit this preference for telomeric ssDNA,¹⁴¹ although this may be highly species dependent.^{119,142} This evolutionary divergence suggests that distant forms of Cdc13 may not be telomere specific.¹⁴³

The human hCTC1-hSTN1-hTEN1 shares a common domain architecture with t-RPA

In contrast to the yeast proteins, which were identified through their roles at telomeres, the human hSTN1 protein was first identified as an accessory factor (AAF44) for DNA polymerase α -primase.¹⁴⁴ Secondary structure predication programs suggested that AAF44 contained an N-terminal OB-fold¹⁴⁵ and shortly thereafter it was discovered that hTEN1 and the larger hCTC1 protein associated with hSTN1 to form a heterotrimeric complex now called CST.^{146–148} CST appears to not be required for telomere end protection, but instead has a role in telomere replication in addition to non-telomeric functions,^{147,149–151} Available evidence from several species suggests that a key role of this complex at telomeres is to stimulate C-strand fill in activity after telomerase extension. This is likely achieved through the ability of the complex to interact with polymerase α -primase.^{144,148,152} Analogously, polymerase α in yeast has also been observed to interact with Cdc13 and Stn1.^{114,153–155} Depletion of the complex is not embryonic lethal in mice, but CTC1 null mice do exhibit telomere loss and die prematurely due to bone marrow failure as a result of defects in telomere replication.¹⁵⁶ Human cells lacking CST have either shorter telomeres or complete telomere loss depending on which component of CST is depleted.^{27,146,147,157} In addition to its role in C-strand fill in, CST has been proposed to play a role in regulating telomerase activity by preventing telomerase access to extended 3'-telomeric ends and repressing telomerase activity through an interaction with hPOT1-TPP1.^{145,158} Other studies have focused on a genome-wide role for CST in rescuing stalled replication forks following replication stress.¹⁴⁹ Knockdown of hSTN1 was shown to affect the rate of DNA replication

by decreasing origin firing for replication forks after recovering from hydroxyurea insults, while overexpression of CST was found to increase origin firing after hydroxyurea treatment.^{148–150} Moreover, the CST complex has also been studied *Arabidopsis thaliana*, where knockdown of the complex is also not lethal, but leads to telomere dysfunction and genome instability.^{146,159–161} Further analysis is still needed to decipher how CST plays a role in both telomere replication and DNA replication, but these analogies to the yeast t-RPA suggest it also acts as telomere-specific RPA complex.

Consistent with these proposed roles, mutations in hCTC1 are associated with several severe rare human diseases such as dyskeratosis congenita and Coats plus syndrome.^{162–167} Dyskeratosis congenita is a disorder where mortality is typically caused by bone marrow failure, pulmonary fibrosis, and cancer due to telomere dysfunction.^{9,168} Coats plus syndrome is a condition characterized by congenital anomalies ranging from retinopathy, intracranial cysts, skeletal abnormalities, and gastrointestinal bleeding. For both diseases, patients with hCTC1 mutations had either shortened telomeres or no change in their telomere lengths. hCTC1 mutations are severe, as patients tend to die by the age of 30.^{163,165,167,168}

Recognition of ssDNA by the human CST complex

The CST complex shows all the hallmarks of being a versatile ssDNA binder. Available binding data show that CST can bind both telomeric and non-telomeric sequences, implying a modest specificity consistent with coincident telomeric and genome-wide roles.^{146,147,158,169} The complex interacts with human telomeric repeats of TTAGGG with a low nanomolar binding affinity.^{146,147,158,170} The exact minimal length of telomeric ssDNA CST recognizes is not precisely known, but likely lies between 12 to 18nts.^{147,158} Increasing the number of TTAGGG repeats up to 10 fold (60nts) does not appear to change the binding affinity for CST to telomeric DNA. Interestingly, CST binds 30nt or longer G-rich non-telomeric sequences with similar binding efficiencies to those observed with the same length of telomeric sequences, suggesting alternate modes of recognition.¹⁴⁷ For non-G rich ssDNA sequences, the CST heterotrimer can bind when the length of ssDNA exceeded 36-nts with a binding affinity that is ~17-fold weaker when compared to G-rich telomere sequence.¹⁵⁸ While binding to shorter G-rich sequences for CST is much weaker, RPA in the same study was still capable of binding the same shorter 20-nt G-rich sequence, indicating that mechanisms by which CST recognizes ssDNA likely differ from those found in RPA.¹⁴⁷ It appears that CST prefers G-rich sequences and longer strands of ssDNA, but it is still not clear whether there are any specific sequence requirements for CST recognition of ssDNA or if CST can equivalently bind non-specifically to ssDNA.

The structures of human hSTN1 and hTEN1 reveal that they are indeed structural homologs to the Stn1-Ten1 complexes of *S. pombe* and *C. tropicalis* (Figure 10A, B). Stn1-Ten1 form a stable heterodimer that can non-specifically interact to ssDNA with modest low micromolar affinity.¹⁷¹ Both proteins appear to contain a putative DNA binding pocket, but the 1000-fold difference in affinity between the CST and the Stn1-Ten1 complex for ssDNA suggests that hCTC1, like Cdc13 in *S. cerevisiae*, is the main DNA-binding component of the complex. Both human Stn1-Ten1 and RPA32-RPA14 share a degree of structural

similarity, suggesting that the OB-folds of Stn1-Ten1 may contribute to a longer binding mode with hCTC1 as seen for RPA.^{138,139,172} This would support the observations that CST appears to bind ssDNA in a length dependent manner.

While these Stn1 and Ten1 proteins share domain architecture with their yeast counterparts, secondary structure predictions for determining the domain architecture of hCTC1 have been challenging due to its low sequence homology and the well-known difficulty in predicting OB-folds from primary sequences.^{70,173} Three potential OB-folds (Figure 8) of hCTC1 can be reliably predicted using protein threading programs,^{174,175} but it is possible that several other OB-folds exist within the hCTC1 protein as seen with Cdc13 and RPA70. At this time no individual domains of hCTC1 have been structurally characterized, thus the predictions remain speculative.

To assist in assigning a function to these domains, several hCTC1 disease-associated mutants have been screened for their ability to interact with ssDNA and participate in heterotrimer formation to identify the domains associated with these activities.¹⁷⁶ The hCTC1 point mutants V665G, R975G, and R987W appear to reduce hCTC1 association with telomeric ssDNA¹⁷⁰ while the hCTC1 L1142H and 1196-7 mutations disrupt heterotrimer formation with Stn1-Ten1. These data suggest that a hCTC1 C-terminal OB-fold is involved in heterotrimer formation, as seen with Cdc13 and RPA70, and that the DBD may lie somewhere in the range of residues 650–1000, likely spanning one or more OB folds.

Initial studies of the CST complex have yielded a wealth of information. Biochemically characterizing the individual domains, especially a hCTC1-DBD, would greatly enhance our understanding of the molecular mechanisms required for ssDNA recognition by hCTC1. The preference for G-rich sequences suggests that CST may be involved in reconciling secondary structures that are known to form in such sequences.¹⁷⁷ While the sequence specificity of hCTC1-DBD is unclear, binding studies suggests that hCTC1 can accommodate both telomeric and non-telomeric sequence; CST may be another ssDNA binding protein with a plastic interface. Further investigation of CST ssDNA binding will improve our understanding of the importance of CST in telomere and DNA replication.

Concluding Remarks

Recent observations of telomere disease associated mutations in hPOT1 and hCTC1 have highlighted the importance of further understanding the mechanistic details underlying their roles at telomeres and opened up an exciting new frontier in telomere biology. How does binding plasticity play a role in telomere biology? Do alternative Pot1 binding modes play a role in allowing telomerase access to the single-stranded overhang? What roles in telomere biology does the RPA-like hCTC1-hSTN1-hTEN1 complex fulfill in mammals? We anticipate further studies to address these emerging questions.

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ABBREVIATIONS

ATR	Ataxia telangiectasia and Rad3 related protein
SNM1B	SNM1 Homolog B
Sae2	Sporulation in the Absence of spo Eleven
MRX	Mre11/Rad50/Xrs2
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
Taz1	tafazzin
Pot1/POT1	protection of telomeres 1
TIN2	TERF1-interacting nuclear factor 2
TPP1	TIN2 and Pot1 interacting protein
Rap1/RAP1	Repressor/Activator site binding Protein
Poz1	Pot1-associated protein
Tpz1	TPP1 homolog
Ccq1	Coiled-coil quantitatively enriched protein 1
Cdc13	cell division control protein 13
Stn1/STN1	suppressor of cdc13
Ten1/TEN1	telomeric pathways with Stn1
L23	β 2– β 3 loop
CTC1	CTS Telomere Maintenance Complex Component 1
hPOT1	human protection of telomeres 1
RPA	replication protein A
OB-fold	oligonucleotide/oligosaccharide binding-fold
DBD	DNA binding domain
hOB1	N-terminal OB-fold of hPOT1-DBD
hOB2	C-terminal OB-fold of hPOT1-DBD

Pot1pC	C-terminal OB-fold of SpPot1-DBD
Pot1pN	N-terminal OB-fold of SpPot1-DBD
SpPot1	<i>Schizosaccharomyces pombe</i> Pot1
NMR	nuclear magnetic resonance
GWAS	genome wide association study
CLL	chronic lymphocytic leukemia
ssDNA	single-stranded DNA
dsDNA	double-stranded DNA
dNTPs	deoxyribose nucleoside triphosphates
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>

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Figure 1.
The Shelterin Complex. Schematic diagram of the human shelterin complex.

Figure 2. Domains of Pot1. Schematic domain map of Pot1 proteins with homologous domains color coded and the predicted C-terminal OB-folds shaded with a gray-black gradient.

Figure 3.

Disease mutations in the DNA-binding domain of hPOT1 **A)** Crystal structure of hPOT1-DNA complex with DNA omitted for clarity (IXJV).⁷³ OB1 is in magenta and OB2 is light brown. **B)** hPOT1 with DNA ligand shown. The portion of the ligand bound by OB1 (OB1- 6mer) is yellow and the portion bound by OB2 (OB2- 4mer) is orange. GWAS mutations near the DNA binding interface are shown in cyan for CLL associated mutations, green for glioma associated mutations, and blue for mutations associated with other types of cancer.

Figure 4.

An alignment of Pot1 DNA-binding domains using the MUSCLE algorithm in the program SeaView.^{86,87} The boxed region in $\beta 1$ of Pot1pC shows the conservation of the aromatic residues that anchor the 3' end of the DNA in the *S. pombe* binding mode.

Figure 5.

The structural similarities and differences of hPOT1 and *sp*Pot1 **A)** Crystal structures of hPOT1 OB1 (IXJV)⁷³ overlaid with Pot1pN (1QZH)⁷² OB1 is shown in magenta and Pot1pN is shown in blue. The 6mer ligand bound by OB1 (OB1- DNA) is shown in yellow and the 6mer ligand bound by Pot1pN (Pot1pN- 6mer) is shown in cyan. **B)** Crystal structures of hPOT1 OB2 (IXJV)⁷³ overlaid with Pot1pC (4HIK)⁷⁴ OB2 is shown in light brown and Pot1pC is shown in green. The 4mer ligand bound by OB2 (OB2- DNA) is shown in orange and the 9mer ligand bound by Pot1pC (Pot1pC- 9mer) is shown in purple.

Figure 6.

The hydrogen bond networks for *sp*Pot1 shown for **A)** Pot1pN (1QZH)⁷² nucleotides 1–4, **B)** Pot1pN nucleotides 5–6, **C)** Pot1pC (4HIK)⁷⁴ nucleotides 1–3, and **D)** Pot1pC nucleotides 7–9. Pot1pN is in blue and Pot1pN- 6mer is in cyan. Pot1pC is in green and Pot1pC- 9mer is in purple. Water molecules are shown in yellow and hydrogen bonds are indicated by the dashed red lines.

Figure 7. Plastic accommodation of the DNA ligand for *sp*Pot1pC.⁷⁴ **A)** Non- cognate DNA ligands, T2A (4HIM), A5T (4HJ5), and C6G (4HJ7) in cyan with substituted bases highlighted in red overlay with the cognate Pot1pC- 9mer in purple and the cognate Pot1pC protein structure in green (4HIK). Non-cognate protein structures are omitted for clarity. **B)** Structure of Pot1pC bound to T4A (4HIO) non-cognate ligand (non- cognate- Pot1pC protein, T4A gray with A4 highlighted in red) overlay with cognate bound Pot1pC (cognate- Pot1pC protein green, Pot1pC- 9mer DNA purple). **C)** Compensatory hydrogen bond network for non-cognate G8C structure (4HJ8) shown. G8C bound Pot1pC in dark gray, G8C ligand in white. Cognate 9mer bound Pot1pC in green and cognate Pot1pC- 9mer in purple. **D)** Nucleotide specificity profile for *sp*Pot1-DNA binding domain in which single nucleotide positions of the cognate 15mer sequence (GGTTACGGTTACGGT) are individually substituted with the complementary base.⁸³

Figure 8.
Domains of the RPA-like complexes. Schematic domain map of Cdc13-Stn1-Ten1, RPA, and CST complexes with homologous domains between complexes color coded or gray for domains without common homology. Predicted OB-folded are shaded with a gray-black gradient.

Figure 9. Structure of Cdc13. The structure of DNA-bound Cdc13 is shown with Cdc13 shown in red, the DNA- ligand shown in blue, and the $\beta 2$ - $\beta 3$ loop (L23) colored in green.

Figure 10.

Structural overlays of RPA32 vs. Stn1 and RPA14 vs. Ten1. **A)** RPA32 (1QUQ)¹³² is overlaid with *C. tropicalis* Stn1-Ten1 (3KF8),¹²¹ *S. pombe* (3KF6),¹²¹ and human hSTN1 (4JQF).¹⁷¹ **B)** RPA14 (1QUQ)¹³² overlaid with *C. tropicalis* Ten1 (3KF8)¹²¹, *S. pombe* Ten1 (3K0X)¹²⁰, and human hTEN1 (4JOI).¹⁷¹