

Structure of *Tetrahymena* telomerase-bound CST with Polymerase α -Primase

Yao He^{1,2,4}, He Song^{1,4}, Henry Chan¹, Baocheng Liu¹, Yaqiang Wang¹, Lukas Susac¹,
Z. Hong Zhou^{2,3}, and Juli Feigon^{1,*}

1 Department of Chemistry and Biochemistry, University of California, Los Angeles; Los Angeles, CA 90095-1569, USA

2 Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles; Los Angeles, CA 90095, USA

3 California NanoSystems Institute, University of California, Los Angeles; Los Angeles, CA 90095, USA

4 These authors contributed equally to this work

*Corresponding author. Email: feigon@mbi.ucla.edu

1 Telomeres are the physical ends of linear chromosomes, composed of short repeating
2 sequences (e.g. TTGGGG in *Tetrahymena* for the G-strand) of double-stranded DNA with a
3 single-strand 3'-overhang of the G-strand and, in humans, a group of six proteins called
4 shelterin^{1,2}. Among these, TPP1 and POT1 associate with the 3'-overhang, with POT1 binding
5 the G-strand³ and TPP1 (in complex with TIN2⁴) recruiting telomerase via interaction with
6 telomerase reverse transcriptase (TERT)⁵. The telomere DNA ends are replicated and maintained
7 by telomerase⁶, for the G-strand, and subsequently DNA Polymerase α -Primase^{7,8} (Pol α Prim), for
8 the C-strand⁹. Pol α Prim activity is stimulated by CTC1–STN1–TEN1 (CST)¹⁰⁻¹², but the structural
9 basis of Pol α Prim and CST recruitment to telomere ends remains unknown. Here we report
10 cryo-EM structures of *Tetrahymena* CST in the context of telomerase holoenzyme, both in the
11 absence and presence of Pol α Prim, and of Pol α Prim alone. *Tetrahymena* Ctc1 binds telomerase
12 subunit p50, a TPP1 ortholog, on a flexible Ctc1 binding motif unveiled jointly by cryo-EM and
13 NMR spectroscopy. Pol α Prim polymerase subunit POLA1 binds Ctc1 and Stn1, and its interface
14 with Ctc1 forms an entry port for G-strand DNA to the POLA1 active site. Together, we obtained
15 a snapshot of four key players required for telomeric DNA synthesis in a single active
16 complex—telomerase core ribonucleoprotein (RNP), p50/TPP1, CST and Pol α Prim—that
17 provides unprecedented insights into CST and Pol α Prim recruitment and handoff between G-
18 strand and C-strand synthesis.

19

20 MAIN

21 Synthesis of the G-strand at the ends of telomeres by telomerase is terminated by the heterotrimeric
22 complex CST^{12,13}. Human CST (hCST) is essential for maintaining the telomere C-strand and also plays
23 a role in overcoming genome-wide replication stress^{10,14,15}. Like the shelterin proteins and telomerase,
24 mutations in hCST lead to telomere biology disorders¹⁶ including Coats Plus and dyskeratosis
25 congenita¹⁷. CST small subunits STN1 and TEN1 are structurally homologous to those in replication
26 protein A (RPA)¹⁸, a single-stranded DNA binding protein involved in all aspects of DNA replication and
27 repair, while the large subunit Ctc1(*Tetrahymena*)/CTC1(vertebrate)/Cdc13(yeast) is more diverse¹⁹⁻²³.

28 Structural and biochemical studies of CST proteins have suggested various stoichiometries,
29 oligomerization states, and functions of subunits^{19,20,23}. The only structure of complete CST¹⁹, from
30 human, revealed a decameric architecture of heterotrimers in the presence of single-stranded telomeric
31 (G-strand) DNA (sstDNA). In vertebrates, CST is proposed to inhibit telomerase activity by physical
32 interaction with shelterin proteins TPP1 or POT1 at telomere ends^{12,24,25} and G-strand
33 sequestration^{12,13}, and it promotes C-strand fill-in by association with Pol α Prim²⁶⁻²⁹, but structures of
34 these interactions in any organism are lacking. Pol α Prim is an unusual polymerase containing both
35 primase and DNA polymerase subunits; the primase synthesizes an RNA primer on a DNA template
36 and then hands off the duplex to the polymerase that initiates synthesis of a short DNA duplex^{7,8}.
37 Pol α Prim initiates both the leading and lagging strand synthesis in eukaryotes⁸, and in association with
38 CST has functions in genome-wide DNA repair¹⁵ in addition to its role in C-strand synthesis at telomere
39 ends.

40 *Tetrahymena* telomerase holoenzyme comprises, in addition to its RNP catalytic core of TERT,
41 telomerase RNA (TER) that provides the template for G-strand telomere repeat synthesis, and LARP7
42 assembly protein p65, several proteins that are orthologous to human proteins that only transiently
43 associate with telomerase at telomeres³⁰. These include p50, the structural and functional equivalent of
44 TPP1 that recruits and activates telomerase^{22,31-33}; Teb1, a subunit of a trimeric RPA related complex
45 TEB^{22,34}, that binds the sstDNA³⁵ and together with p50 increases activity and processivity like its
46 ortholog POT1³⁶⁻³⁸; and another trimeric RPA related complex p75–p45–p19 that has been identified as
47 *Tetrahymena* Ctc1–Stn1–Ten1 (*Tt*CST)^{22,23}. The constitutive association of these proteins with
48 telomerase catalytic core makes *Tetrahymena* telomerase an ideal model system for elucidating details
49 of the protein structures and interactions that regulate G-strand and C-strand synthesis^{22,39,40}. Structural
50 and functional studies of *Tetrahymena* telomerase and Pol α Prim described here show how monomeric
51 *Tt*CST binds p50/TPP1 and Pol α Prim on different interfaces to coordinate G-strand termination and C-
52 strand fill-in synthesis, and suggest commonalities with interactions at human telomeres.

53 **Structure of *Tetrahymena* telomerase CST**

54 While cryo-EM studies of *Tetrahymena* telomerase have provided high-resolution structures of the RNP

55 catalytic core, TEB heterotrimer, and p50 OB⁴⁰, the dynamic positioning of *Tt*CST has limited its
56 structure modeling to date. Here, we combined three previously reported datasets of *Tetrahymena*
57 telomerase bound to sstDNA⁴⁰ and conducted focused classification on *Tt*CST followed by refinement
58 of the holoenzyme to obtain a reconstruction with an overall resolution of 3.5 Å (Fig. 1a, Extended Data
59 Fig. 1, Extended Data Table 1). For the model of *Tt*CST, Stn1–Ten1 crystal structures^{22,23} were rigid
60 body fit into the density and manually refined with little change, and Ctc1 was built *de novo* (Fig. 1b,
61 Extended Data Fig. 2). Modeling of the N-terminal domain of Ctc1, which has lower resolution
62 (Extended Data Fig. 1c), was facilitated by using information derived from NMR data on secondary
63 structure elements and inter-β-strand NOEs (see Methods) (Extended Data Fig. 3). Ctc1, whose
64 domain structure was not previously established, comprises three OBs (OB-A, -B, and -C) connected
65 by structured linkers that stabilize the rigid pairwise interactions between the domains (Fig. 1c, d). Ctc1
66 OB-C has a C-shaped cleft and Zn-ribbon motif (Fig. 1c) typical of the C-terminal OB of the large
67 subunit of RPA¹⁸ and related complexes, including mammalian CTC1¹⁹, POT1^{37,38}, and *Tt*Teb1^{35,40}.
68 Ctc1 OB-C forms a heterotrimer with Stn1 and Ten1 OB that is stabilized by an intermolecular three-
69 helix bundle (Fig. 1c, e) and by Ten1–Stn1 and Stn1–Ctc1 OB interactions (Extended Data Fig. 2c-h).
70 The tandem winged helix-turn-helix (WH-WH) domain of Stn1 is connected to OB by a flexible linker
71 and is not visible in the cryo-EM map, consistent with its multi-positioning shown by negative-stain
72 EM²². Overall, this structure of monomeric *Tt*CST strongly suggests its origin from RPA and establishes
73 the domain structure of the least conserved subunit Ctc1.

74 **Flexible interface between Ctc1 and p50**

75 p50 has an N-terminal OB and a C-terminal domain that is invisible in the cryo-EM map (Fig. 1a).
76 p50/TPP1 OB interacts with telomerase on TERT TEN and TRAP domains^{32,33,39-42}, but how p50/TPP1
77 or POT1 interacts with CST is unknown^{24,25}. We find that *Tt*CST is anchored on p50 via Ctc1 OB-A (Fig.
78 1c). In the structure, *Tt*CST is positioned across the top of the TERT ring (Fig. 1b), and stabilized in this
79 predominant conformation by additional interactions between Ctc1 and TERT–TER catalytic core
80 (Extended Data Fig. 2j-l). However, these are not stable interactions, as other conformations resolved
81 by 3D classification show *Tt*CST hinged away from TERT (Fig. 2a, Extended Data Fig. 1a). In the cryo-

82 EM map, a previously uncharacterized density of p50 protrudes from its OB C-terminus into Ctc1 OB-A
83 (Extended Data Fig. 1h). p50 residues 185-208 were built against the density as helix $\alpha 5$ and strand $\beta 7$,
84 the latter of which forms an extended β sheet with $\beta 1$ - $\beta 4$ - $\beta 5'$ of Ctc1 OB-A (Fig. 2b). However, previous
85 biochemical studies showed that p50 C-terminal truncation at residue 213 almost abrogates binding
86 with Ctc1, while truncation at residue 252 showed binding with Ctc1 comparable to full-length protein⁴³.
87 We therefore investigated whether p50 residues between 208-255 might be contributing to the binding
88 interface with Ctc1. We made a series of p50 peptides and monitored their interaction with Ctc1 OB-A
89 by NMR (Extended Data Fig. 4). ¹H-¹⁵N HSQC spectra show that optimal binding requires residues
90 228-250 (Fig. 2c, Extended Data Fig. 4b). This peptide forms a 1:1 complex with Ctc1 OB-A that is in
91 slow exchange on the NMR timescale indicating slow off-rate (Extended Data Fig. 4c). Talos+
92 secondary structure scores, CS-Rosetta modeling, and chemical shift mapping (see Methods) indicate
93 that p50 peptide residues 228-241 form a β -hairpin that interacts with the Ctc1 OB-A β -barrel near $\beta 1$ -
94 $\beta 2$ linker, $\beta 4$, and $\beta 5$ (Fig. 2d, Extended Data Fig. 4e-g). Together, these cryo-EM and NMR data define
95 a Ctc1 binding motif (CBM) adjacent to p50 OB that tightly associates with Ctc1 but allows hinging
96 movement of the entire *Tt*CST complex on p50. Deletion of residues containing this motif results in loss
97 of cell viability⁴³, indicating the importance of *Tt*CST association with telomerase *in vivo*.

98 **Overall structure of Pol α Prim with CST**

99 A defining feature of CST function at telomere ends is its ability to recruit Pol α Prim for C-strand
100 synthesis; however, the interface between Pol α Prim and CST has not been structurally characterized in
101 any organism^{10,26-29,44}. To both verify that p75-p45-p19 is functionally *Tt*CST and define the mechanism
102 of Pol α Prim recruitment, we assembled *Tetrahymena* telomerase-Pol α Prim complex using
103 endogenously expressed telomerase and recombinant Pol α Prim in the presence of sstDNA
104 d(GTTGGG)₁₀, and determined its cryo-EM structure (Fig. 3a, b, Extended Data Fig. 5, 6). We verified
105 that this complex was active for both G-strand synthesis by telomerase and C-strand synthesis by
106 telomerase associated CST-Pol α Prim individually (Extended Data Fig. 5i), and for handoff of G-strand
107 on telomerase to Pol α Prim for C-strand synthesis (Fig. 3c) using direct telomerase and Pol α Prim
108 activity assays. With d(GTTGGG)₁₀ as template, as in the cryo-EM sample, *Tt*CST-Pol α Prim could

109 copy the entire G-strand template (Extended Data Fig. 5i). In comparison, almost no C-strand synthesis
110 is observed with Pol α Prim alone (Extended Data Fig. 5i).

111 In the telomerase holoenzyme, Pol α Prim binds *Tt*CST in the absence or presence of sstDNA
112 (Extended Fig. 5f). Since the entire *Tt*CST–Pol α Prim complex was flexibly positioned relative to p50, as
113 seen for *Tt*CST alone, the *Tt*CST–Pol α Prim and telomerase RNP core–TEB–p50 complexes were
114 processed separately to obtain cryo-EM 3D reconstructions of 4.2 Å and 2.9 Å resolution, respectively
115 (Fig. 3a, Extended Data Fig. 6, Extended Data Table 1). The modeled structures are the first of
116 telomerase associated CST-bound Pol α Prim and the highest resolution structure of telomerase RNP
117 core to date (Fig. 3b, Extended Data Fig. 6g). Modeling of *Tt*CST in the complex by initial rigid body
118 fitting of the structure determined in the absence of Pol α Prim revealed the presence of additional
119 density on Ctc1 OB-B and OB-C that could be fit with the crystal structure of Stn1 WH-WH^{22,23} (Fig. 3b,
120 Extended Data Fig. 6i). Binding of Pol α Prim to *Tt*CST displaces Ctc1 from its stable position across the
121 top of the TERT ring (compare Fig. 3a, b to Fig. 1a, b), and instead POLA1 is positioned near TER loop
122 2. The Stn1 WH-WH binding site on Ctc1 would also be occluded in the stable conformation of *Tt*CST
123 on p50–TERT in the absence of Pol α Prim.

124 Pol α Prim comprises two polymerase (POLA1 and POLA2) and two primase (PRIM1 and PRIM2)
125 subunits^{7,8} (Fig. 3d). The presence of all four subunits in the complex was confirmed by silver-stain
126 SDS-PAGE (Extended Data Fig. 5f) and negative-stain EM 2D classification analysis (Extended Data
127 Fig. 5g). However, only the catalytic POLA1_{core} was well resolved in the cryo-EM map (Fig. 3a, d). The
128 rest of Pol α Prim appears as a fuzzy density connected to POLA1_{core} opposite the interface with Ctc1
129 (Extended Data Fig. 5g). Therefore, we also investigated the cryo-EM structure of Pol α Prim alone, and
130 obtained a 4.0–4.3 Å resolution structure for POLA2–POLA1_{CTD}–PRIM2_N–PRIM1 (Fig. 3e, f, Extended
131 Data Fig. 7, Extended Data Table 1). 2D class averages of Pol α Prim show that POLA2–POLA1_{CTD}–
132 PRIM2_N–PRIM1 forms a platform that holds POLA1_{core} at various positions (Fig. 3e). Initial models of
133 Pol α Prim subunits generated using AlphaFold2 were rigid-body fit into corresponding densities and
134 manually adjusted (see Methods). The structures of the individual subunits are highly similar to those of
135 human Pol α Prim^{45–47} (Extended Data Fig. 8a, b). However, POLA2–POLA1_{CTD} and PRIM1 that are

located on either end of the platform can apparently rotate relative to each other, with PRIM2_N as the pivot (Extended Data Fig. 8b). PRIM2_C, which specifically interacts with and coordinates RNA–DNA duplex translocation from the active site on PRIM1 to the active site on POLA1_{core} (ref⁸), was not observed during cryo-EM data processing, suggesting its dynamic positioning. Such flexible organization of PolαPrim would allow for the large-scale domain movements expected for the switch from RNA primer to C-strand DNA synthesis⁸. Previous structures of human PolαPrim determined by X-ray crystallography and cryo-EM of a crosslinked sample are in an autoinhibited conformation (Extended Data Fig. 8c) with the active site on POLA1_{core} sterically blocked by POLA1_{CTD} and POLA2 for DNA entry^{45,46}. Here, our studies provide the first structures of a PolαPrim compatible with activity, and they establish its direct interactions with *Tt*CST, as described in detail next.

CST interaction with POLA1 and sstDNA

POLA1_{core} comprises an N-terminal domain (NTD) that brackets a catalytically dead exonuclease (Exo), and a C-terminal DNA polymerase that contains palm, fingers, and thumb domains⁸ (Fig. 4a, b). All elements of POLA1_{core} except the tip of the thumb are well defined in the cryo-EM map. *Tt*CST interacts with POLA1_{core} via Ctc1 OB-C and Stn1, and the interface is about 3000 Å² (Fig. 4a-d, Extended Data Fig. 9). On Ctc1 OB-C, the conserved Zn-ribbon motif interacts primarily with conserved Exo β11-β12 hairpin (Fig. 4c, Extended Data Fig. 8d). Ctc1 helix α14 and Stn1 OB β1-β2 and β3-β4 loops form a binding pocket that accommodates POLA1_{core} NTD helix α19 (residues 731-748) with charge complementarity to its side chains (Fig. 4c, Extended Data Fig. 8e-g). Helix α19 is a flexible loop in all other structures of PolαPrim⁴⁵⁻⁴⁷, and apparently becomes structured only on binding Ctc1–Stn1 (Extended Data Fig. 8e), indicating the importance of this interaction for *Tt*CST binding. Behind this interface, the three helices of Stn1 WH2 are inserted into a gap between Ctc1 OB-B and POLA1 Exo (Fig. 4d). The structure shows that POLA1_{core} Exo and NTD form an extensive interface with *Tt*CST involving Ctc1 OB-C, Stn1 OB, and Stn1 WHWH, which is otherwise flexibly tethered to Stn1 OB in the absence of PolαPrim. Structure-based sequence alignment suggests conservation of these regions on POLA1_{core} that interface with *Tt*CST across a wide-range of species (Extended Data Fig. 8g).

Cryo-EM density for ~10 nucleotides of sstDNA is observed on Ctc1 OB-C across the C-shaped

163 binding cleft near the Zn-ribbon (Fig. 4a, c) but side-chain interactions cannot be discerned.
164 Substitution of three residues R395E, Y445A and F473A on the binding surface decreases the K_D for
165 sstDNA binding to purified *Tt*CST by ~3-fold (from 0.18 to 0.52 μ M), as determined by electromobility
166 shift assays (EMSA) with d(GTTGGG)₅ (Fig. 4e, f, Extended Data Fig. 9f-k), verifying the observed
167 DNA binding site. The sstDNA extends 5'→3' into an entry port, formed by POLA1_{core} NTD and Exo and
168 Ctc1 OB-C (Extended Data Fig. 8h), of a highly basic channel that leads to the active site of POLA1 ~40
169 Å away, where the primer–sstDNA duplex would bind (Fig. 4g). Since sstDNA is added in excess
170 during the purification, both telomerase and *Tt*CST can bind separate sstDNA strands. No density for
171 sstDNA is visible on *Tt*CST in the absence of Pol α Prim, perhaps due to dynamics and/or steric
172 occlusion by interaction of Ctc1 with TERT in the predominant conformation (Fig. 1b, Extended Data
173 Fig. 2i-l).

174 The sstDNA on Ctc1 OB-C appears positioned for entry into a template (G-strand) binding tunnel
175 on POLA1_{core}, and there is weak density within the tunnel that we attribute to sstDNA (Extended Data
176 Fig. 8i, j), suggesting the possibility that this structure has captured the polymerase mode after handoff
177 from primase. Although no DNA–RNA or DNA–DNA duplex is present in the active site of POLA1, there
178 is unassigned density between POLA1_{core} palm and thumb (Fig. 3a) that fits the dimensions of a G-
179 quadruplex formed by four *Tetrahymena* telomere repeats⁴⁸ (Extended Data Fig. 8j). *Tetrahymena*
180 telomere repeats can form unimolecular G-quadruplexes with 3 or 4 G-quartets in the presence of Na⁺
181 or K⁺, respectively^{48,49}, while G-quadruplexes do not form with Li⁺ in the absence of other cations.
182 Addition of 50 mM of these cations individually to the activity assay buffer for telomerase–Pol α Prim with
183 d(GTTGGG)₁₀ prepared as for the cryo-EM studies decreases C-strand synthesis in the order of
184 increasing G-quadruplex stability, i.e. Li⁺ > Na⁺ > K⁺ ⁵⁰ (Extended Data Fig. 5j). Since the newly
185 synthesized telomeric DNA is single-stranded as it exits onto Teb1⁴⁰, the slow folding kinetics of G-
186 quadruplexes would likely limit the amount of G-quadruplex formed *in vivo* prior to G-strand binding on
187 CST–Pol α Prim. We propose that the apparent G-quadruplex present in the cryo-EM density may have
188 serendipitously trapped Pol α Prim in an incipient inhibited DNA polymerization state. Overall, the
189 structure defines the interface between POLA1 and CST and the pathway of the G-strand from CST to

190 the active site of POLA1, where it provides the template for C-strand synthesis.

191 **Comparison to human CST**

192 While *Tetrahymena* Stn1 and Ten1 have the same domain structure as human STN1 and TEN1,
193 respectively, human CTC1 (hCTC1) is much larger than *Tetrahymena* Ctc1, with seven OBs (OB-A
194 through OB-G)¹⁹ (Fig. 5a, b, Extended Data Fig. 9a) that may have arisen from a gene duplication of
195 RPA70 (e.g. OB-N,A,B,N,A,B,C). A DALI search of Ctc1 against all proteins in the PDB (see Methods)
196 found the highest structural similarity with hCTC1 (Z-score 14.5). For the individual domains, Ctc1 OB-
197 B and OB-C are most similar to hCTC1 OB-F and OB-G, respectively (Extended Data Fig. 9b, c). A
198 cryo-EM study of hCST with bound sstDNA revealed a decameric structure with D5 symmetry¹⁹.
199 Comparing the hCST monomer extracted from the decamer to *Tt*CST shows Ten1/TEN1, Stn1
200 OB/STN1 OB, and Ctc1 OB-A,B,C/CTC1 OB-E,F,G are positioned similarly (Fig. 5a, b). However, Stn1
201 WH-WH, which is only visible in the PolαPrim bound *Tt*CST structure, is positioned on Ctc1 OB-B and
202 OB-C (Fig. 5a, Extended Data Fig. 9d), while in hCST STN1 WH-WH is positioned on OB-E and sticks
203 out from the decamer in what has been called the 'Arm' conformation¹⁹ (Fig. 5b, c). Intriguingly, low
204 resolution cryo-EM densities of monomeric hCST revealed an additional conformation¹⁹, called 'Head',
205 where hSTN1 WH-WH occupies a position apparently close to that observed in *Tt*CST-bound PolαPrim
206 (Fig. 5c). In our structure, Stn1 WH-WH in this position forms part of the interface with PolαPrim (Fig.
207 4d). If hCST binds PolαPrim in a similar manner to *Tt*CST, it could only bind as a monomer since the
208 binding interface would be occluded by intermonomer interactions in the hCST decamer.

209 Different but adjacent binding sites for sstDNA are observed for equivalent regions on hCST
210 decamer¹⁹ vs *Tt*CST (Fig. 5a, b, Extended Data Fig. 9e). For hCST, the four DNA nucleotides visible in
211 the decameric structure interact with hCTC1 OB-F (Extended Data Fig. 9e), two of which also interact
212 with OB-G¹⁹. Comparison of Ctc1 OB-B to CTC1 OB-F shows sequence similarity for the residues on
213 the OB-F binding cleft that interact with the four DNA nucleotides (Extended Data Fig. 9f). To
214 investigate whether there might be a similar DNA interaction on *Tt*Ctc1 OB-B (in addition to the ten we
215 observe on OB-C), we substituted three conserved residues (K303E/K306E/F308A), equivalent to a set
216 shown to decrease K_D for hCST with sstDNA^{13,19}. These substitutions decrease the K_D for d(GTTGGG)₅

217 by ~1.5-fold as assayed by EMSA (Extended Data Fig. 9i-k). Substitution of two conserved aromatic
218 residues (F264A/Y268A) that might contribute to DNA binding through stacking interactions^{13,19}
219 decreases K_D by 1.2-fold (Extended Data Fig. 9i-k). Together, these comparisons suggest hCtc1 C-
220 terminal OB-E, -F, -G may interact with POLA1 and sstDNA in a similar manner to Ctc1 OB-A, -B, -C,
221 and that Pol α Prim interaction could only be accommodated on monomeric CST.

222 **Coordinated synthesis of G and C strands**

223 Co-ordinated synthesis of telomeric G- and C-strands in vertebrates is orchestrated by interactions
224 between telomerase, shelterin proteins TPP1 and POT1, sstDNA, CST, and Pol α Prim, whose
225 molecular details have been largely undefined. Taking advantage of the constitutive association of
226 p50/TPP1, Teb1 (a POT1 orthologue), and *Tt*CST with *Tetrahymena* telomerase along with previous
227 structural studies of telomerase^{22,40} we determined structures and interfaces between all of these
228 components. *Tt*CST Ctc1 C-terminal domain OB-C and Stn1 bind to Pol α Prim POLA1 and Ctc1 N-
229 terminal domain OB-A binds p50/TPP1 (Fig. 5d). p50/TPP1 OB in turn binds to TERT TEN–TRAP
230 domains, constitutively in *Tetrahymena* but transiently to recruit telomerase to telomeres in
231 humans^{32,33,40}. Biochemical studies have shown that hCST binds G-strands released from telomerase
232 during telomere repeat synthesis^{12,13}, and our structures and activity assays verify these results in
233 *Tetrahymena*. Our structures show that *Tt*CST binds p50 on a flexible hinge (CBM), placing it in
234 proximity to where the 3'-end of sstDNA released from the TER template would be. Teb1, that also
235 interacts with p50 and TERT TEN domain, could contribute by initially maintaining a hold on the 5'
236 exiting DNA until it binds *Tt*CST. C-strand synthesis requires that the G-strand be released from
237 telomerase catalytic core, to provide the 3'-end of the G-strand as the template for C-strand synthesis.
238 PRIM2c is proposed to bind the RNA–DNA duplex and hand it off from the primase to the active site of
239 POLA1⁸ (Fig. 5d). Our structure explains how CST enhances the activity of POLA1, by binding the G-
240 strand and feeding it into the entry port for the POLA1 template channel (Fig. 4g). The autoinhibited
241 conformation of Pol α Prim^{45,46} would be occluded in the CST–Pol α Prim complex, possibly explaining
242 how CST could enhance the primase-to-polymerase switch⁴⁴. It is less clear how CST binding could
243 activate PRIM1 for primer synthesis, consistent with proposals for a large conformational switch

244 between priming and polymerization steps⁸. Here we have captured a pre-DNA C-strand polymerization
 245 step in a Pol α Prim complex with *Tetrahymena* telomerase RNP core, CST, p50, and TEB, linking G-
 246 strand and C-strand synthesis in an almost complete telomere-end replicon.

247

248 Main References

- 249 1 de Lange, T. Shelterin-Mediated Telomere Protection. *Annu Rev Genet* **52**, 223-247 (2018).
- 250 2 Lim, C. J. & Cech, T. R. Shaping human telomeres: from shelterin and CST complexes to
 251 telomeric chromatin organization. *Nat Rev Mol Cell Biol* (2021).
- 252 3 Baumann, P. & Cech, T. R. Pot1, the putative telomere end-binding protein in fission yeast and
 253 humans. *Science* **292**, 1171-1175 (2001).
- 254 4 Abreu, E. *et al.* TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. *Mol Cell*
 255 *Biol* **30**, 2971-2982 (2010).
- 256 5 Nandakumar, J. *et al.* The TEL patch of telomere protein TPP1 mediates telomerase recruitment
 257 and processivity. *Nature* **492**, 285-289 (2012).
- 258 6 Greider, C. W. & Blackburn, E. H. Identification of a specific telomere terminal transferase
 259 activity in *Tetrahymena* extracts. *Cell* **43**, 405-413 (1985).
- 260 7 Pellegrini, L. The Pol alpha-primase complex. *Subcell Biochem* **62**, 157-169 (2012).
- 261 8 Baranovskiy, A. G. & Tahirov, T. H. Elaborated Action of the Human Primosome. *Genes (Basel)* **8**
 262 (2017).
- 263 9 Qi, H. & Zakian, V. A. The *Saccharomyces* telomere-binding protein Cdc13p interacts with both
 264 the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein.
 265 *Genes Dev* **14**, 1777-1788 (2000).
- 266 10 Wang, F. *et al.* Human CST has independent functions during telomere duplex replication and C-
 267 strand fill-in. *Cell Rep* **2**, 1096-1103 (2012).
- 268 11 Surovtseva, Y. V. *et al.* Conserved telomere maintenance component 1 interacts with STN1 and
 269 maintains chromosome ends in higher eukaryotes. *Mol Cell* **36**, 207-218 (2009).
- 270 12 Chen, L. Y., Redon, S. & Lingner, J. The human CST complex is a terminator of telomerase
 271 activity. *Nature* **488**, 540-544 (2012).
- 272 13 Zaug, A. J. *et al.* CST does not evict elongating telomerase but prevents initiation by ssDNA
 273 binding. *Nucleic Acids Res* **49**, 11653-11665 (2021).
- 274 14 Stewart, J. A. *et al.* Human CST promotes telomere duplex replication and general replication
 275 restart after fork stalling. *EMBO J* **31**, 3537-3549 (2012).
- 276 15 Mirman, Z. *et al.* 53BP1-RIF1-shieldin counteracts DSB resection through CST- and Polalpha-
 277 dependent fill-in. *Nature* **560**, 112-116 (2018).
- 278 16 Holohan, B., Wright, W. E. & Shay, J. W. Cell biology of disease: Telomeropathies: an emerging
 279 spectrum disorder. *J Cell Biol* **205**, 289-299 (2014).
- 280 17 Chen, L. Y., Majerska, J. & Lingner, J. Molecular basis of telomere syndrome caused by CTC1
 281 mutations. *Genes Dev* **27**, 2099-2108 (2013).
- 282 18 Prakash, A. & Borgstahl, G. E. The structure and function of replication protein A in DNA
 283 replication. *Subcell Biochem* **62**, 171-196 (2012).

284 19 Lim, C. J. *et al.* The structure of human CST reveals a decameric assembly bound to telomeric
285 DNA. *Science* **368**, 1081-1085 (2020).

286 20 Ge, Y. *et al.* Structural insights into telomere protection and homeostasis regulation by yeast
287 CST complex. *Nat Struct Mol Biol* **27**, 752-762 (2020).

288 21 Bryan, C., Rice, C., Harkisheimer, M., Schultz, D. C. & Skordalakes, E. Structure of the human
289 telomeric Stn1-Ten1 capping complex. *PLoS One* **8**, e66756 (2013).

290 22 Jiang, J. *et al.* Structure of Tetrahymena telomerase reveals previously unknown subunits,
291 functions, and interactions. *Science* **350**, aab4070 (2015).

292 23 Wan, B. *et al.* The Tetrahymena telomerase p75-p45-p19 subcomplex is a unique CST complex.
293 *Nat Struct Mol Biol* **22**, 1023-1026 (2015).

294 24 Kratz, K. & de Lange, T. Protection of telomeres 1 proteins POT1a and POT1b can repress ATR
295 signaling by RPA exclusion, but binding to CST limits ATR repression by POT1b. *J Biol Chem* **293**,
296 14384-14392 (2018).

297 25 Wan, M., Qin, J., Songyang, Z. & Liu, D. OB fold-containing protein 1 (OBFC1), a human homolog
298 of yeast Stn1, associates with TPP1 and is implicated in telomere length regulation. *J Biol Chem*
299 **284**, 26725-26731 (2009).

300 26 Kelich, J. M., Papaioannou, H. & Skordalakes, E. Pol alpha-primase dependent nuclear
301 localization of the mammalian CST complex. *Commun Biol* **4**, 349 (2021).

302 27 Ganduri, S. & Lue, N. F. STN1-POLA2 interaction provides a basis for primase-pol alpha
303 stimulation by human STN1. *Nucleic Acids Res* **45**, 9455-9466 (2017).

304 28 Feng, X., Hsu, S. J., Kasbek, C., Chaiken, M. & Price, C. M. CTC1-mediated C-strand fill-in is an
305 essential step in telomere length maintenance. *Nucleic Acids Res* **45**, 4281-4293 (2017).

306 29 Feng, X. *et al.* CTC1-STN1 terminates telomerase while STN1-TEN1 enables C-strand synthesis
307 during telomere replication in colon cancer cells. *Nat Commun* **9**, 2827 (2018).

308 30 Wang, Y., Susac, L. & Feigon, J. Structural Biology of Telomerase. *Cold Spring Harb Perspect Biol*
309 (2019).

310 31 Wang, F. *et al.* The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature*
311 **445**, 506-510 (2007).

312 32 Sekne, Z., Ghanim, G. E., van Roon, A. M. & Nguyen, T. H. D. Structural basis of human
313 telomerase recruitment by TPP1-POT1. *Science* **375**, 1173-1176 (2022).

314 33 Liu, B. *et al.* Structure of active human telomerase with telomere shelterin protein TPP1. *Nature*
315 **604**, 578-583 (2022).

316 34 Upton, H. E., Chan, H., Feigon, J. & Collins, K. Shared Subunits of Tetrahymena Telomerase
317 Holoenzyme and Replication Protein A Have Different Functions in Different Cellular Complexes.
318 *J Biol Chem* **292**, 217-228 (2017).

319 35 Zeng, Z. *et al.* Structural basis for Tetrahymena telomerase processivity factor Teb1 binding to
320 single-stranded telomeric-repeat DNA. *Proc Natl Acad Sci U S A* **108**, 20357-20361 (2011).

321 36 Lei, M., Podell, E. R. & Cech, T. R. Structure of human POT1 bound to telomeric single-stranded
322 DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol* **11**, 1223-1229
323 (2004).

324 37 Chen, C. *et al.* Structural insights into POT1-TPP1 interaction and POT1 C-terminal mutations in
325 human cancer. *Nat Commun* **8**, 14929 (2017).

326 38 Rice, C. *et al.* Structural and functional analysis of the human POT1-TPP1 telomeric complex.
327 *Nat Commun* **8**, 14928 (2017).

328 39 Jiang, J. *et al.* Structure of Telomerase with Telomeric DNA. *Cell* **173**, 1179-1190 e1113 (2018).
329 40 He, Y. *et al.* Structures of telomerase at several steps of telomere repeat synthesis. *Nature* **593**,
330 454-459 (2021).
331 41 Ghanim, G. E. *et al.* Structure of human telomerase holoenzyme with bound telomeric DNA.
332 *Nature* (2021).
333 42 Wang, Y., Gallagher-Jones, M., Sušac, L., Song, H. & Feigon, J. A structurally conserved human
334 and Tetrahymena telomerase catalytic core. *Proceedings of the National Academy of Sciences*,
335 202011684 (2020).
336 43 Hong, K. *et al.* Tetrahymena telomerase holoenzyme assembly, activation, and inhibition by
337 domains of the p50 central hub. *Mol Cell Biol* **33**, 3962-3971 (2013).
338 44 Lue, N. F., Chan, J., Wright, W. E. & Hurwitz, J. The CDC13-STN1-TEN1 complex stimulates Pol
339 alpha activity by promoting RNA priming and primase-to-polymerase switch. *Nat Commun* **5**,
340 5762 (2014).
341 45 Baranovskiy, A. G. *et al.* Mechanism of Concerted RNA-DNA Primer Synthesis by the Human
342 Primosome. *J Biol Chem* **291**, 10006-10020 (2016).
343 46 Kilkenny, M. L. *et al.* Structural basis for the interaction of SARS-CoV-2 virulence factor nsp1
344 with DNA Polymerase α - Primase. *Protein Sci* (2021).
345 47 Coloma, J., Johnson, R. E., Prakash, L., Prakash, S. & Aggarwal, A. K. Human DNA polymerase
346 alpha in binary complex with a DNA:DNA template-primer. *Sci Rep* **6**, 23784 (2016).
347 48 Beseiso, D. *et al.* The first crystal structures of hybrid and parallel four-tetrad intramolecular G-
348 quadruplexes. *Nucleic Acids Res* **50**, 2959-2972 (2022).
349 49 Bryan, T. M. G-Quadruplexes at Telomeres: Friend or Foe? *Molecules* **25** (2020).
350 50 Williamson, J. R., Raghuraman, M. K. & Cech, T. R. Monovalent cation-induced structure of
351 telomeric DNA: the G-quartet model. *Cell* **59**, 871-880 (1989).

352

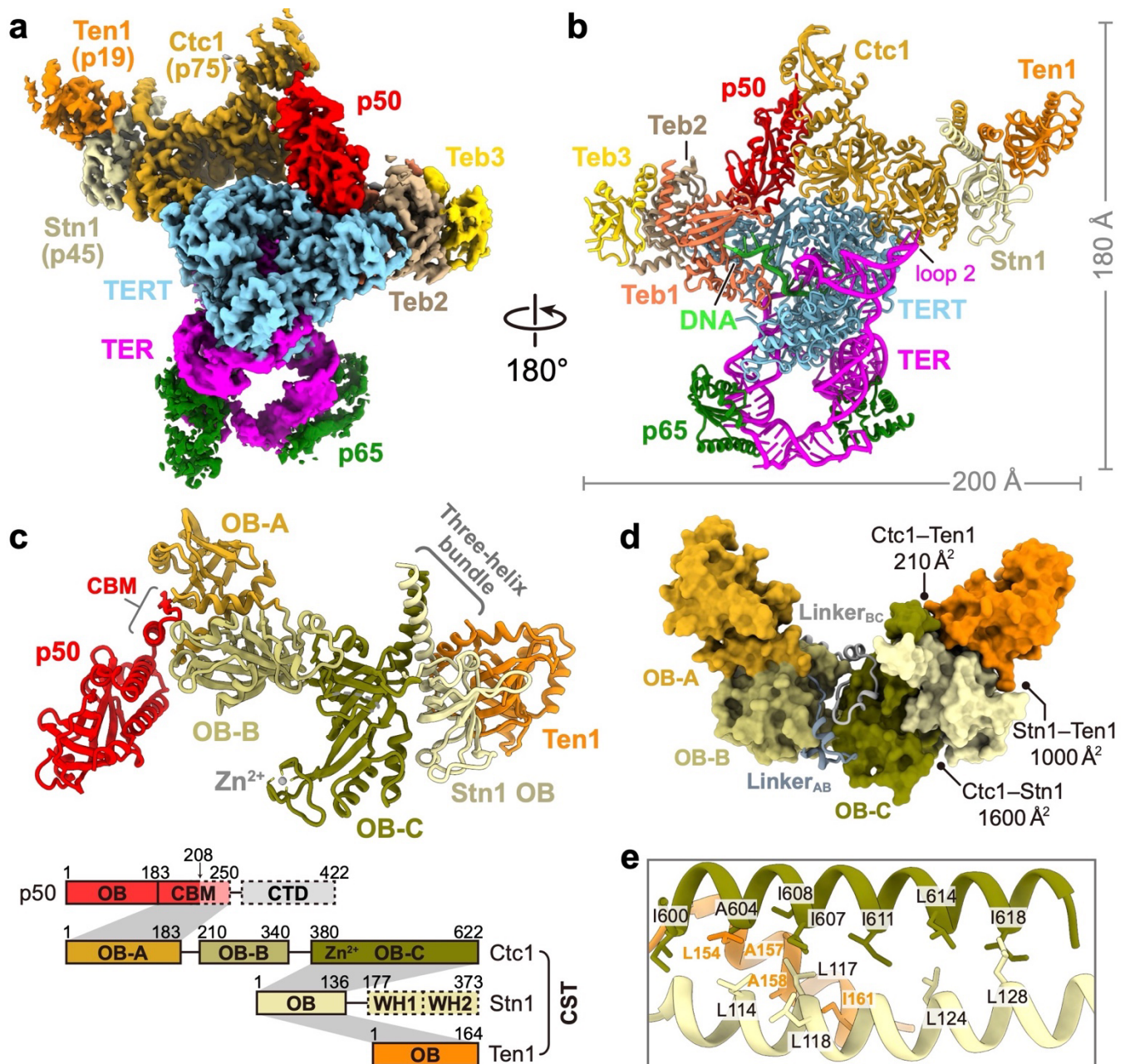


Fig. 1: Cryo-EM structure of *Tt*CST in telomerase holoenzyme. **a**, Cryo-EM map of telomerase holoenzyme. **b**, Ribbon representation of the model of telomerase holoenzyme, viewed after a 180° rotation from **a**. The proteins, TERT and DNA are color-coded as indicated. **c**, Structure and schematic of *Tt*CST with p50. The three OB domains (OB-A, -B, and -C) of Ctc1 are colored individually as indicated. In the schematic, invisible regions in the cryo-EM map are shown as dashed boxes. Intermolecular interactions between proteins are indicated as gray shading. OB, oligonucleotide/oligosaccharide-binding fold domain; WH, winged-helix domain; CBM, CST binding motif; CTD, C-terminal domain; Zn²⁺, Zn-ribbon motif. **d**, Surface representation of *Tt*CST structure. Buried surface areas in the interfaces between *Tt*CST subunits are indicated. The two structured linkers between Ctc1 OB domains are shown as ribbon. **e**, Zoom-in view of the hydrophobic interface of *Tt*CST intermolecular three-helix bundle.

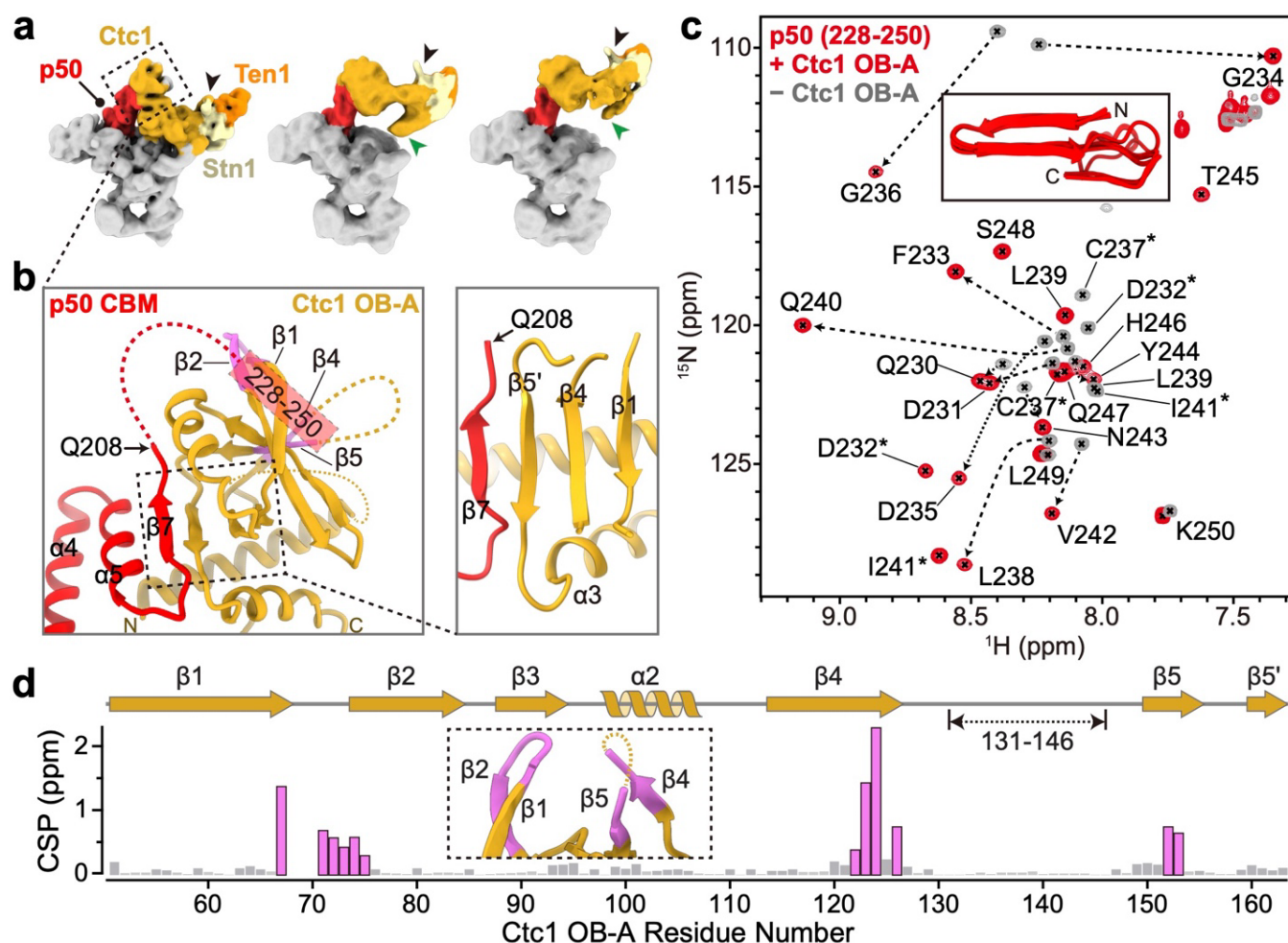


Fig. 2: Interface between *TtCST* and p50. **a**, Cryo-EM maps of telomerase holoenzyme with *TtCST* at different positions. *TtCST* subunits and p50 are colored as indicated. The three-helix bundle and Zn-ribbon motif are labeled with black and green arrows, respectively. Cryo-EM maps are low-pass filtered to similar resolution for comparison. **b**, Interactions between Ctc1 OB-A and p50 CBM. Unmodeled regions in the cryo-EM structure are shown as dashed lines. **c**, ^1H - ^{15}N HSQC spectra of ^{15}N -labelled p50 peptide (residues 228-250) with (red) and without (gray) Ctc1 OB-A. NMR signals from the same residues are connected with dashed arrows or labeled with asterisks. Inset shows the 10 lowest energy CS-Rosetta models of p50 peptide (C α RMSD 1.73 Å) in the presence of Ctc1 OB-A. **d**, Chemical shift perturbation (CSP) index of ^{15}N -labelled Ctc1 OB-A upon binding p50 peptide. Residues with CSP over 0.25 ppm are highlighted in magenta, and their locations on the cryo-EM structure are shown in an inserted panel and on **b**.

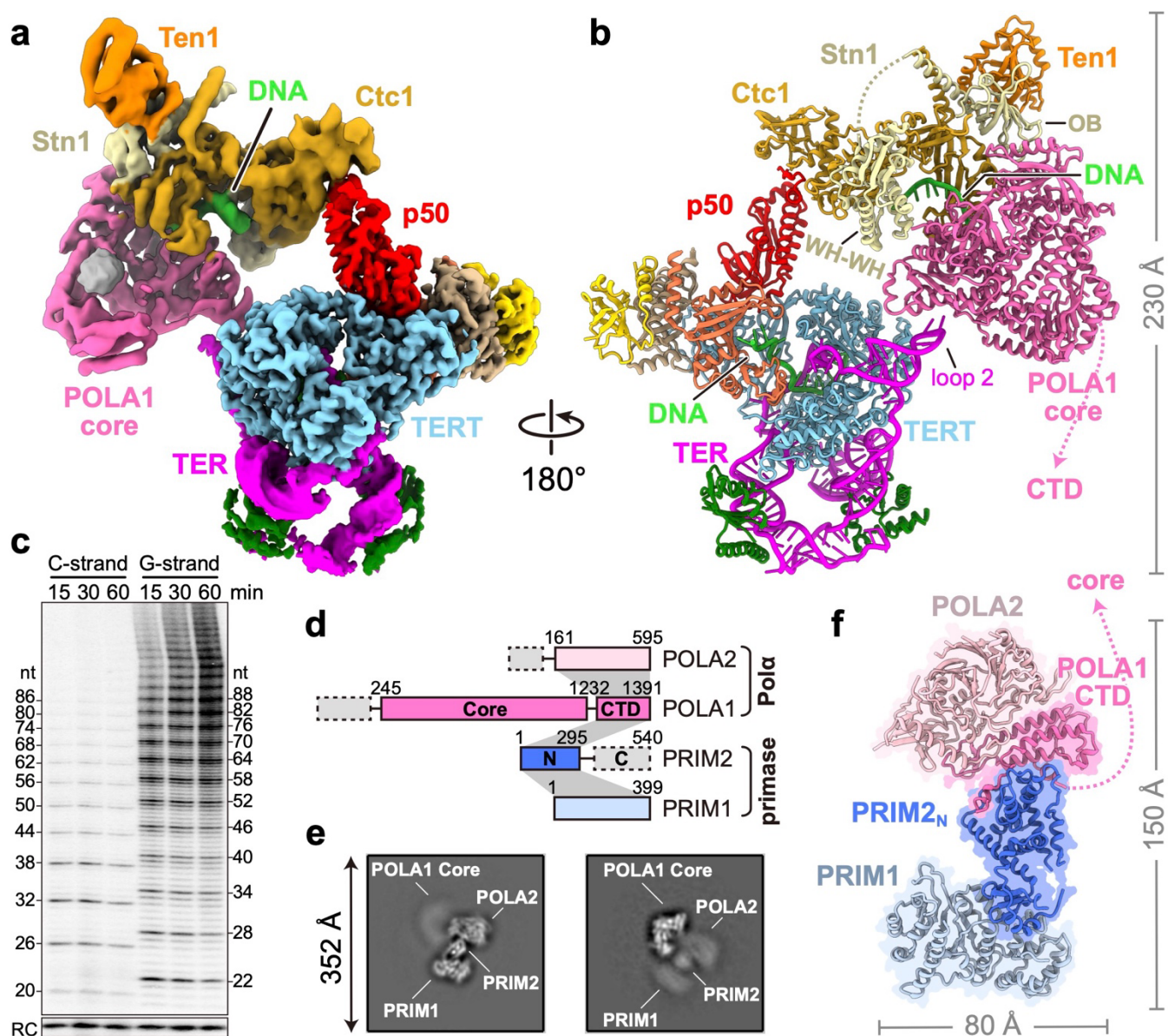


Fig. 3: Structure of *Tetrahymena* telomerase holoenzyme in complex PolαPrim. **a**, Composite map of the complex generated with focused refined cryo-EM maps (Extended Data Fig. 6a). **b**, Atomic model of the complex. The unstructured linker between Stn1 OB and WH-WH domains is shown as dashed line. **c**, Concurrent time courses of G-strand and C-strand synthesis by telomerase-PolαPrim using d(GTTGGG)₃ DNA primers (detailed in Methods). ³²P-dGTP and ³²P-dCTP were used to label the G-strand and C-strand products, respectively. The G-strand products provide the template for C-strand synthesis. RC, recovery control. Supplementary Fig. 1 provides gel source data for all figures. **d**, Structure-based diagram of PolαPrim. Intermolecular interactions between subunits are indicated as gray shading. **e**, Representative 2D-class averages of PolαPrim. **f**, Cryo-EM structure of PolαPrim. The flexible linker between POLA1_{core} and POLA1_{CTD} is shown as dashed line.

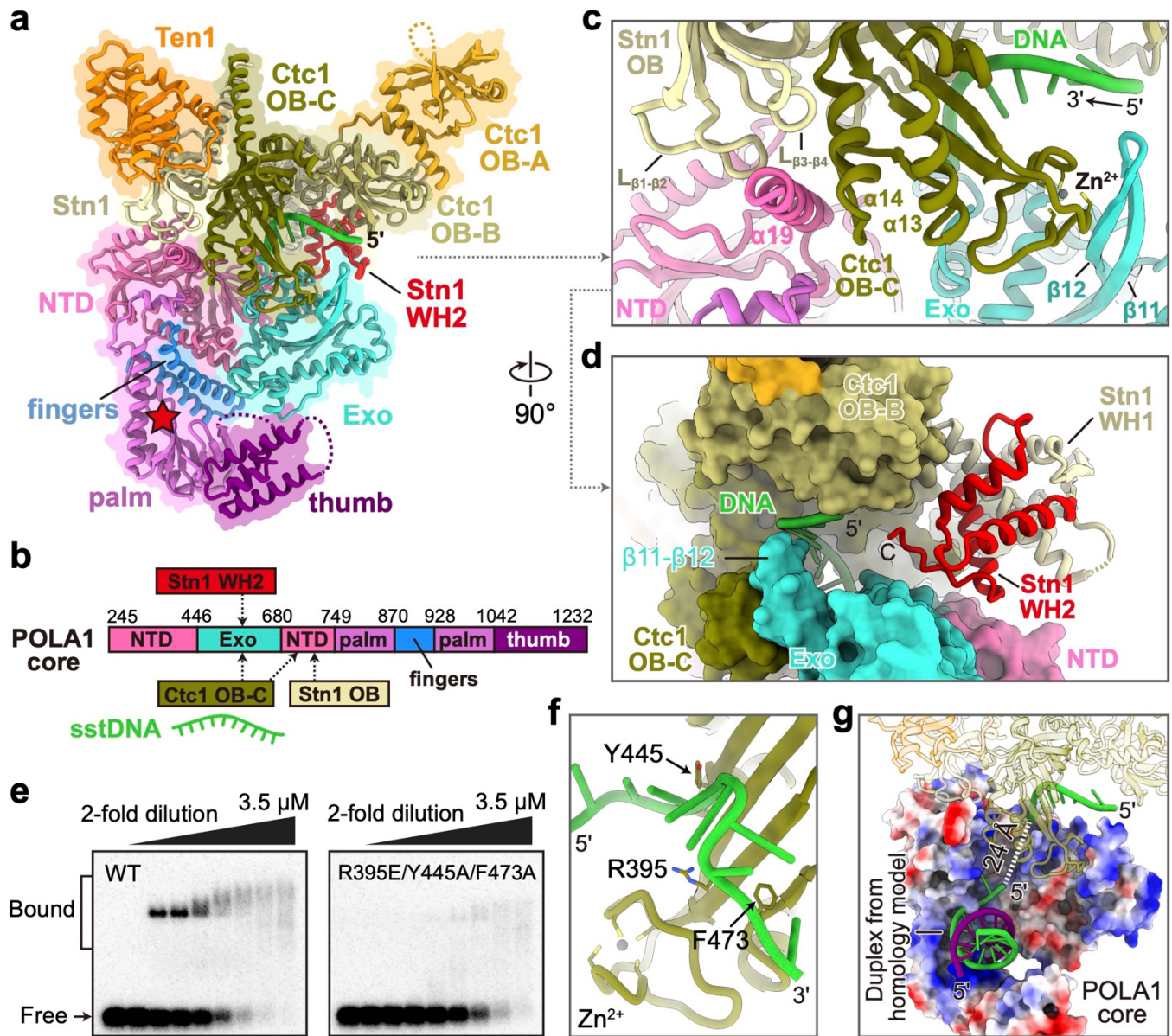


Fig. 4: Interface between *TtCST* and *POLA1*_{core}. **a**, Ribbon representation of *TtCST* and *POLA1*_{core} with individual protein/domain/motif colored as indicated. Location of *POLA1* active site is shown as a red star. NTD, N-terminal domain; Exo, exonuclease domain. **b**, Structure-based diagram of *POLA1*_{core} and interactions with *TtCST*. **c, d**, Zoom-in views of the interface between *POLA1*_{core} and *TtCST* with sstDNA shown from perpendicular directions. **e**, EMSA of d(GTTGGG)₅ DNA binding by wild-type (WT) and Ctc1 mutant *TtCST*. Wedges indicate two-fold dilutions of *TtCST* from 3.5 to 0.03 μM. The first lane of each gel is a *TtCST*-free control. Quantification results of EMSAs were shown in Extended Data Fig. 9j. **f**, sstDNA binding site on Ctc1 OB-C. Sidechains of residues substituted for EMSA are shown as sticks. **g**, Zoom-in view of *POLA1*_{core} (electrostatic surface) with a DNA duplex modeled based on human *POLA1* structure⁴⁷ (PDB 5IUD). The template and product strands in the duplex are colored in green (G-strand) and purple (C-strand), respectively. The path of sstDNA in the channel is shown as dashed line.

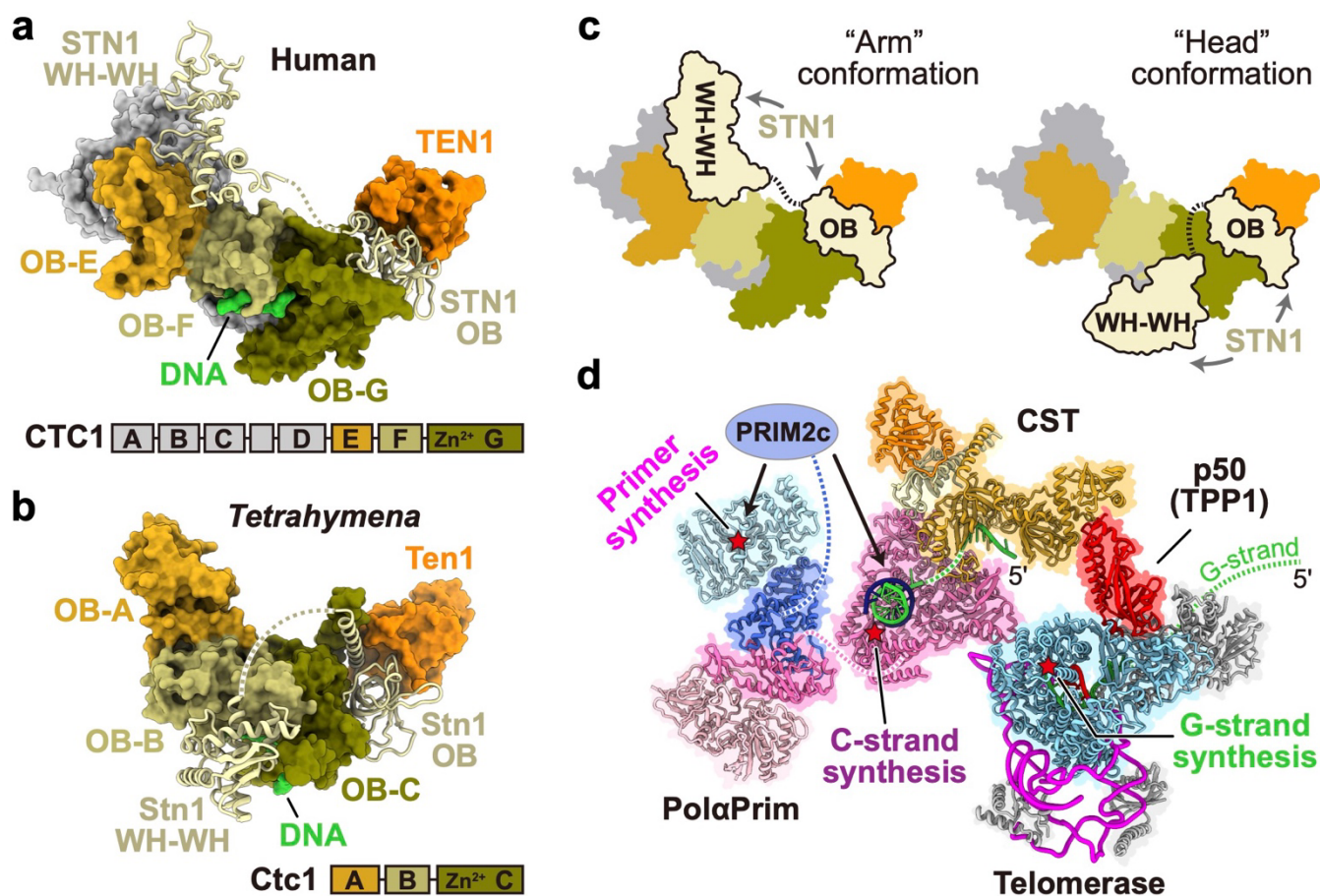


Fig. 5: Structural comparison of *Tt*CST and hCST. **a, b**, Surface representation of *Tt*CST (**a**) and hCST¹⁹ (PDB 6W6W) (**b**) with Stn1/STN1 shown as ribbons. Corresponding subunits/domains are colored the same as indicated. **c**, Cartoon illustrations of hCST with STN1 WH-WH in "arm" and "head" conformations¹⁹. The linker between STN1 OB and WH-WH is shown as dashed line. **d**, Model of *Tetrahymena* telomerase holoenzyme in complex with PolαPrim. The DNA duplex on POLA1_{core} is modeled based on a homology model of human POLA1_{core}(ref⁴⁷) (PDB 5IUD). Position of the POLA2–POLA1_{CTD}–PRIM2_N–PRIM1 platform relative to POLA1_{core} is based on a low-resolution cryo-EM map in Extended Data Fig. 7a. Telomeric DNA G-strand and C-strand are colored green and purple, respectively. PRIM2_C is shown as an oval connecting to PRIM2_N. Active sites on TERT, PRIM1, and POLA1 for the synthesis of G-strand, C-strand primer, and C-strand, respectively, are denoted by red stars. During G-strand and C-strand synthesis, these active sites would be occupied successively for a given G-strand, not simultaneously.

353 **Methods**

354 ***Tetrahymena* PolaPrim cloning and expression.** *Tetrahymena* PolaPrim complexes were expressed
355 using the Bac-to-Bac system (Thermo Fisher Scientific) in *Sf9* cells. Briefly, cDNAs encoding
356 *Tetrahymena* POLA1 (Accession number: Q23AJ0), POLA2 (I7MAE1), PRIM1 (Q24HY6) and PRIM2
357 (Q246C7) were chemically synthesized and purchased from IDT (Integrated DNA Technologies, Inc.).
358 To co-express the POLA1–POLA2 complex, POLA1 and POLA2 cDNAs were cloned into a
359 pFastBacDual vector (Thermo Fisher Scientific), under the polyhedrin promoter and the p10 promoter,
360 respectively. The POLA1 has an N-terminal hexa-histidine-TEV (His₆-TEV) tag, in which TEV is a
361 tobacco etch virus protease cleavage site. To co-express the POLA1–POLA2–PRIM1–PRIM2 complex,
362 PRIM1 and PRIM2 cDNAs were cloned into a separate pFastBacDual vector. The expression vectors
363 were used to make baculoviruses based on the established protocol for Bac-to-Bac system (Thermo
364 Fisher Scientific). *Sf9* cells (2.0×10^6 /ml) were transfected with viruses using a multiplicity of infection
365 (MOI) of 3 at 27°C in SF-900™ II SFM media (Thermo Fisher Scientific). The cells were harvested 48 h
366 after infection and stored at -80°C until purification.

367 ***Tetrahymena* CST–p50 cloning and expression.** *Tetrahymena* CST–p50 complex was expressed in
368 insect cells. Briefly, cDNAs encoding p50 (D2CVN8), Ctc1/p75 (A0PGB2), Stn1/p45 (Q6JXI5) and
369 Ten1/p19 (D2CVN7) were chemically synthesized and purchased from IDT (Integrated DNA
370 Technologies, Inc.). The Ctc1 and p50 cDNAs were cloned into a pFastBacDual vector (Thermo Fisher
371 Scientific), with a His₆-TEV tag fused onto the N-terminal of Ctc1. The Stn1 and Ten1 cDNAs were
372 cloned into a separate pFastBacDual vector for baculoviruses expression. *Sf9* cells (2.1×10^6 /ml) were
373 transfected with viruses using a multiplicity of infection (MOI) of 3 at 27°C in SF-900™ II SFM media
374 (Thermo Fisher Scientific). The cells were harvested 48 h after infection and stored at -80°C until
375 purification.

376 **Purification of *Tetrahymena* PolaPrim and CST–p50 complexes.** The purification steps for both
377 POLA1–POLA2–PRIM1–PRIM2 and p50–Ctc1–Stn1–Ten1 were performed at 4°C using an AKTA
378 chromatography system with prepacked columns (GE Healthcare), following the same protocol. Cells
379 were suspended in buffer A [30 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10% (v/v) glycerol, 1mM

380 dithiothreitol (DTT), and 25 mM imidazole] supplemented with protease inhibitor cocktail (Sigma), lysed
381 by sonication, and centrifuged at 34,000×g for one hour. The supernatant was applied onto a 5-ml
382 HisTrap HP column pre-equilibrated in buffer A. The column was washed with buffer A and the complex
383 was eluted with buffer B [30 mM Tris-HCl (pH 7.5), 1 M NaCl, 10% (v/v) glycerol, 1mM DTT, and 400
384 mM imidazole]. The protein complex was digested overnight with 0.2 mg/ml TEV protease and buffer-
385 exchanged to buffer A. The digest was applied onto a 5-ml HisTrap HP column pre-equilibrated in
386 buffer A. The target complex was isolated in the column flow-through, concentrated to 10 ml, and then
387 applied onto a Superdex 200 gel filtration column pre-equilibrated in buffer C [25 mM Tris-HCl (pH 7.5),
388 150 mM NaCl, and 1mM DTT]. The complex was collected from peak fractions and analyzed by SDS
389 polyacrylamide gel electrophoresis.

390 **NMR sample preparation.** cDNAs of Ctc1 OB-A (residues 1-183) and p50 peptides (Extended Data
391 Fig. 4a) were cloned into the pETduet vector with a His₆-MBP-TEV tag at the N terminus of each
392 construct, and expressed by *Escherichia coli* strain BL21(DE3). ²H, ¹³C, ¹⁵N-labelled Ctc1 OB-A was
393 expressed from M9 minimal media with 0.5 liters of D₂O, 2 g of ¹³C D-glucose, and 0.5 g of ¹⁵N
394 ammonium chloride, while ¹³C, ¹⁵N-labelled p50 peptide was expressed from M9 minimal media with ¹³C
395 D-glucose and of ¹⁵N ammonium chloride. Cultures were grown to mid-log phase at 37°C, induced by
396 the addition of β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and incubated at
397 18°C for an additional 12 h before harvesting by centrifugation. The purification steps for Ctc1 OB-A
398 and p50 peptides were similar to those for *Tetrahymena* PolaPrim described above. Briefly, cells were
399 re-suspended in buffer A, lysed by sonication and centrifuged at 4,500×g for 30 min. The supernatant
400 was loaded onto a 5-ml HisTrap HP column pre-equilibrated in buffer A. The column was washed with
401 buffer A and the protein was eluted with buffer B. The protein was digested overnight with 0.2 mg/ml
402 TEV protease and buffer-exchanged to buffer A. The digest was applied onto a 5-ml HisTrap HP
403 column pre-equilibrated in buffer A. The flow-through was concentrated and further purified on an
404 Superdex 75 gel filtration column pre-equilibrated in buffer C. Fractions containing pure protein were
405 pooled, buffer exchanged into protein NMR buffer [20 mM Tris (pH 7.5), 50 mM NaCl, 1 mM TCEP, 3
406 mM NaN₃, and 8% D₂O], and concentrated to 0.5-0.8 mM for NMR studies.

NMR spectroscopy and data processing. NMR experiments were performed at 298 K on 800 and 600 MHz Bruker spectrometers equipped with HCN cryoprobes. The backbone assignments of Ctc1 OB-A were obtained using the TROSY-type HNCACB, HN(CO)CACB, HNCA, HN(CO)CA, HNCO, and HN(CA)CO spectra collected on an 800 MHz Bruker instrument with ^2H , ^{13}C , ^{15}N -labeled Ctc1 OB-A. Conventional triple resonance backbone assignment experiments (HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA) were used for p50 peptide (228-250) backbone assignments. Spectra were collected and processed using Topspin 4.1, and analyzed with CARA (<http://cara.nmr.ch>) and NMRFAM Sparky⁵¹ to interactively obtain sequence-specific resonance assignments. NOE peak lists were automatically generated by Atnos⁵², and assigned by CYANA 3.98 (ref⁵³). Secondary structure of Ctc1 OB-A and p50 peptide (228-250) were predicted by software TALOS+ (ref⁵⁴). Models of p50 peptide (228-250) were predicted by CS-Rosetta⁵⁵ using chemical shift data. C α RMSD of the 10 lowest energy models is 1.73 Å, suggesting a high confidence of the prediction. To investigate the interaction between Ctc1 OB-A and p50 peptides, ^1H - ^{15}N HSQC spectra of titration of unlabeled p50 peptides with labeled Ctc1 OB-A and unlabeled Ctc1 OB-A with labeled p50 peptides were obtained, respectively. Chemical shift mapping was analyzed by comparing the apo and bound-form HSQC spectra. The chemical shift perturbation value for each residue was calculated as $CSP = \sqrt{((\Delta\delta_{HN})^2 + 0.14 \times (\Delta\delta_N)^2)/2}$ (ref⁵⁶). The backbone assignments of ^2H , ^{13}C , ^{15}N -labeled Ctc1 OB-A in the presence of unlabeled p50 peptide (228-250) were obtained using the same TROSY-type spectra as listed above, while the backbone assignment of ^{13}C , ^{15}N -labeled p50 peptide (228-250) in the presence of unlabeled Ctc1 OB-A were obtained using conventional triple resonance spectra.

Telomerase sample preparation. *Tetrahymena* telomerase holoenzyme was expressed and purified as described previously^{40,57}. To prepare the telomerase–Pol α Prim complex sample, 0.5 μM of purified POLA1–POLA2–PRIM1–PRIM2 was incubated with telomerase holoenzyme at the anti-Flag M2 affinity gel (Sigma) step overnight at 4°C, in the presence of excess d(GTTGGG)₁₀ primer. Excess DNA and Pol α Prim were removed with wash buffer [20 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM MgCl₂, 1 mM TCEP, 10% (v/v) glycerol, 0.1% (v/v) IGEPAL CA-630] and the final product was eluted using a small volume (30-50 μL) of elution buffer [20 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM MgCl₂, 1 mM TCEP,

434 and 0.1% (v/v) IGEPAL CA-630] supplemented with 1 mg/mL 3× FLAG peptide.

435 **Cryo-EM specimen preparation and data collection.** For telomerase–PolαPrim complex, 3 μL of the
436 purified sample was applied to glow-discharged lacey carbon grids with a supporting ultrathin carbon
437 film (Ted Pella). The grids were then blotted with filter paper and flash-frozen in liquid ethane using an
438 FEI Vitrobot Mark IV at 10°C and 100% humidity. Cryo-EM grids of PolαPrim were prepared similarly
439 with Quantifoil 200 mesh R2/1 grids. Cryo-EM grids were loaded into a ThermoFisher Titan Krios
440 electron microscope operated at 300 kV for automated data collection using SerialEM⁵⁸. Movies of
441 dose-fractionated frames were acquired with a Gatan K3 direct electron detector in super-resolution
442 mode at a pixel size of 0.55 Å on the sample level. A Gatan Imaging Filter (GIF) was inserted between
443 the electron microscope and the K3 camera and operated at zero-loss mode with the slit width of 20
444 eV. The microscope was carefully aligned prior to each imaging session and parallel beam was
445 optimized using coma-free alignment in SerialEM. The total dose rate on the sample was set to ~55
446 electrons/Å², which was fractionated into 50 frames with 0.06 s exposure time for each frame. For
447 telomerase–PolαPrim, 36,716 movies were collected in two separate imaging sessions with the same
448 batch of cryo-EM grids. For PolαPrim, 7,120 movies were collected in a single imaging sessions.

449 **Cryo-EM data processing.** Cryo-EM data processing workflows are outlined in Extended Data Fig. 1,
450 6 and 7 for the structure determination of *Tt*CST in telomerase, PolαPrim-bound *Tt*CST in telomerase,
451 and PolαPrim alone, respectively. All steps described below were performed with RELION 3.1 (ref⁵⁹)
452 unless otherwise indicated.

453 To determine *Tt*CST structure, telomerase particles selected from three published datasets (the
454 T3D2, T4D4 and T5D5 datasets as detailed in ref⁴⁰) were combined, resulting in over 2.5 million good
455 particles (Extended Data Fig. 1a). Refinement of these particles without a mask generated a
456 reconstruction with only weak density for *Tt*CST, which confirms the multiple orientation of *Tt*CST
457 relative to the rest of telomerase holoenzyme²². To separate particles with *Tt*CST at different positions,
458 an alignment-free 3D classification was performed using a spherical mask covering the *Tt*CST region
459 (mask1). Particles from classes with *Tt*CST at similar positions were grouped together and refined,
460 resulting in three reconstructions (P1, P2 and P3 in Extended Data Fig. 1a), among which P1 has the

best density and the largest number of particles. To improve the overall density of P1, we performed another round of 3D classification with local angular search (RELION options: --sigma_ang 8 --healpix_order 4). A soft mask (mask2) was used in this step to exclude the flexible p65. 259,330 particles from the best class were selected for 3D refinement, following by refinement of contrast transfer function (CTF) parameters and Bayesian polishing in RELION. The resulting “shiny” particles were refined to 3.5 Å resolution for the entire telomerase holoenzyme including *Tt*CST (Extended Data Fig. 1b-d). An additional focused 3D classification step was conducted to improve the local resolution of Ctc1 OB-A. The resulting 78,471 particles were refined to 3.8 Å resolution using mask2.

For the newly collected telomerase–PolaPrim and PolaPrim datasets, dose-fractionated frames of each movie were 2x binned (pixel size of 1.1 Å), aligned for the correction of beam-induced drift, and dose weighted using RELION’s implementation of UCSF MotionCor2 (ref⁶⁰). CTF parameters, including defocus and astigmatism, of each dose-weighted micrograph were determined by CTFFIND4 (ref⁶¹) within RELION.

Two datasets of telomerase–PolaPrim, one for each data collection session, were initially processed separately (Extended Data Fig. 6a). Particles picked from 2,000 representative micrographs using template-free auto-picking in RELION were screened by 2D classification, and the best particles were selected to train a particle detection model in Topaz⁶² for subsequent neural-network based particle picking for all micrographs. After several rounds of 2D and 3D classifications as detailed in Extended Data Fig. 6a, good particles selected from two datasets were combined, resulting in over 1.6 million particles. Refinement of these particles without using any mask generated a reconstruction with only weak density for *Tt*CST–PolaPrim, suggesting that *Tt*CST–PolaPrim also has multiple orientations relative to the rest of telomerase holoenzyme, including telomerase core RNP, TEB heterotrimer and p50, as previously observed for the *Tt*CST dataset (Extended Data Fig. 1a). Therefore, these two parts were processed separately in the following steps. For telomerase core RNP–TEB–p50, a soft mask was used to exclude the dynamic *Tt*CST–PolaPrim during 3D refinement, which resulted in a 3.1 Å resolution reconstruction. After an additional round of focused classification with local angular search in 8° (RELION options: --sigma_ang 8 --healpix_order 4), 539,078 particles from the best class were

488 selected and refined to 2.9 Å resolution (Extended Data Fig. 6b, c). For *Tt*CST–PolαPrim, three rounds
489 of alignment-free 3D classification with an optimized regularization parameter (RELION option: --
490 tau2_fudge 16) were performed in parallel using a spherical mask covering the *Tt*CST–PolαPrim region
491 (mask2). 427,158 particles from classes with interpretable *Tt*CST–PolαPrim densities were combined
492 after removing duplicates. Refinement of these particles generated a reconstruction with clear *Tt*CST–
493 PolαPrim density. Then, we shifted the center of each particle to *Tt*CST–PolαPrim and performed signal
494 subtraction using mask4 to only keep the signal from *Tt*CST–PolαPrim. After two rounds of 3D
495 classification using mask4, 142,912 particles were selected and refined using the same mask, which
496 resulted in a 4.2 Å resolution reconstruction for *Tt*CST–PolαPrim (Extended Data Fig. 6b, d). These
497 particles were back projected to original particles without signal-subtraction and refined to 4.4 Å
498 resolution for the entire complex including both telomerase and *Tt*CST–PolαPrim.

499 For the PolαPrim dataset (Extended Data Fig. 7), particle picking was conducted using Topaz⁶² in
500 a similar way as described above for the PolαPrim bound telomerase datasets. After two rounds of 2D
501 classification, 1.3 million particles were selected and refined using an initial model generated by
502 cryoSPARC⁶³. The resulting cryo-EM map has a “head” that is the catalytic core domain of POLA1 and
503 a bow-tie-shaped “body” that contains POLA2, PRIM1, PRIM2_N and the C-terminal domain of POLA1.
504 The “head” and the “body” have multiple orientations relative to each other as indicated by their low-
505 resolution densities in the 3D reconstruction and the 2D classification results (Extended Data Fig. 7b).
506 Focused refinement of the “head” didn't work well due to its small size, but focused refinement of the
507 “body” generated a map with clear secondary structure features. After one round of 3D classification
508 using the same mask for the “body”, 264,498 particles were selected and refined to 4.5 Å resolution.
509 During the 3D classification step, a notable hinge movement was observed within the “body”. Due to
510 that, we further refined the two halves of the “body” individually and obtained a 4.0 Å resolution
511 reconstruction for POLA2–POLA1_{CTD}–PRIM2_N and a 4.3 Å resolution reconstruction for PRIM2_N–PRIM1
512 (Extended Data Fig. 7c-e).

513 All cryo-EM maps were sharpened with a negative B-factor and low-pass filtered to the stated
514 resolution using the *relion_postprocess* program in RELION. Local resolution evaluations were

determined by ResMap⁶⁴ with two independently refined half-maps. Directional resolution anisotropy analyses were performed using 3DFSC⁶⁵. Data collection and processing statistics are given in Extended Data Table 1.

Model building and refinement. For the modeling of *Tt*CST, two maps were generated from the 3.5 Å resolution cryo-EM reconstruction: an unsharpened map with the best density continuity was used for backbone tracing and secondary structure assignment, and a sharpened map with the best high-resolution features was used to place Cα and sidechain of individual residues. Crystal structures of *Tetrahymena* Ten1–Stn1-OB^{22,23} (PDB 5DOI and 5DFM) and cryo-EM structure of *Tetrahymena* telomerase RNP–TEB–p50⁴⁰ (PDB 7LMA) were initially rigid-body fitted into the maps using UCSF Chimera⁶⁶, and refined manually in COOT⁶⁷. Density of Ctc1 was traced from its signature C-terminal α helix, and models of OB-C and OB-B were built *de novo* against the density in COOT. Visible densities of amino acid residues with bulky side chains, such as Phe, Tyr and Trp were used as guidance for sequence assignment (Extended Data Fig. 1g). For Ctc1 OB-A, an initial model was built against the cryo-EM density and refined manually with structure information obtained from NMR (Extended Data Fig. 3). Briefly, secondary structure information obtained from TALOS+ (ref⁵⁴) was used to define the boundaries of β strands within the OB (Extended Data Fig. 3d), and 105 inter-β-strand NOE restraints were used to refine the relative position of the β strands (Extended Data Fig. 3e). Lastly, p50 residues 184-208 following the C-terminus of the previous p50 OB model⁴⁰ (PDB 7LMA) were built into the cryo-EM map adjacent to Ctc1 OB-A (Extended Data Fig. 1h).

For the modeling of *Tt*CST–PolαPrim, the *Tt*CST model obtained as described above, crystal structures of Stn1 WH-WH^{22,23} (PDB 5DFN and 5DOK), and a computed model of POLA1 core generated using AlphaFold2 (ref⁶⁸) were initially rigid-body fitted into the 4.2 Å resolution map (Extended Data Fig. 6d) using UCSF Chimera⁶⁶, and refined manually in COOT⁶⁷ (Extended Data Fig. 6h-j). A segment of sstDNA was built manually against the density in the C-shape cleft of Ctc1 OB-C. The previously reported cryo-EM structure of *Tetrahymena* telomerase RNP–TEB–p50⁴⁰ (PDB 7LMA) was refined against the 2.9 Å resolution map (Extended Data Fig. 6c).

For the modeling of PolαPrim platform (POLA2–POLA1_{CTD}–PRIM2_N–PRIM1), a composite cryo-

EM density map was generated using the “combine focused maps” function in Phenix⁶⁹ with two focused refined maps (Extended Data Fig. 7a, e). Computed models of individual subunits generated using AlphaFold2 (ref⁶⁸) were rigid-body fitted into the composite map and refined manually in COOT⁶⁷ (Extended Data Fig. 7g).

All models were refined using Phenix⁶⁹ in real space with secondary structure, Ramachandran, and rotamer restraints. Refinement statistics of the models are summarized in Extended Data Table 1. Model vs map FSC validations were shown in Extended Data Fig. 1f, 6f and 7f. Structural similarity analyses were conducted using the Dali server⁷⁰. Sequence alignment results were presented using Jalview⁷¹. All figures presenting the model were prepared using UCSF ChimeraX⁷².

Telomerase–PolaPrim activity assays. The direct telomerase activity assays were carried out as previously described⁴⁰ in 20 µl solutions containing 50 mM Tris-HCl pH 8.0, 2 mM Mg²⁺, 1 mM spermidine, 2 mM TCEP, 200 µM dTTP, 3 µM dGTP, 5 µCi dGTP [α -³²P] (Perkin-Elmer, 6000 Ci/mmol), 20 nM telomerase-PolaPrim and 1 µM d(GTTGGG)₃ or d(GTTGGG)₁₀ primer as indicated. The C-strand synthesis assay was carried out in 20 µl solutions containing 50 mM Tris-HCl pH 8.0, 2 mM Mg²⁺, 1 mM spermidine, 2 mM TCEP, 500 µM dATP, 3 µM dCTP, 5 µCi dCTP [α -³²P] (Perkin-Elmer, 3000 Ci/mmol), 0.2 mM ATP, 0.2 mM CTP, 1 µM d(GTTGGG)₁₀ primer and 20 nM telomerase-PolaPrim or PolaPrim as indicated. Reactions were performed at 30 °C for 60 min and stopped with quench buffer [10 mM Tris-HCl pH 8.0 and 10 mM EDTA].

The complete telomere replication reactions were carried out in 20 µl solutions containing 50 mM Tris-HCl pH 8.0, 2 mM Mg²⁺, 1 mM spermidine, 2 mM TCEP, 2 µM dTTP, 3 µM dGTP, 500 µM dATP, 3 µM dCTP, 0.2 mM ATP, 0.2 mM CTP, 1 µM d(GTTGGG)₃ primer and 20 nM telomerase-PolaPrim. Either additional 5 µCi dGTP [α -³²P] was supplemented to visualize the G-strand synthesis or 5 µCi dCTP [α -³²P] to visualize the corresponding C-strand synthesis. The reactions were performed at 30 °C and stopped with quench buffer at indicated times. All products were phenol–chloroform-extracted and ethanol-precipitated together with a 15 nt ³²P-end-labelled DNA oligonucleotide as a recovery control and resolved on a 10% denaturing polyacrylamide gel. The gels were dried and exposed to a phosphor imaging screen and scanned on an Amersham Typhoon scanner (GE Lifesciences).

Electrophoretic mobility shift assay (EMSA). EMSAs were conducted following a previously reported method¹³. *TtCST* and its mutants were expressed and purified from insect cells as described above. For each binding reaction, 0.5 nM ³²P-labeled primer (for Extended Data Fig. 9h) or 0.5 nM ³²P-labeled primer plus 20 nM unlabeled primer (for Fig. 4e and Extended Data Fig. 9i) was incubated with or without *TtCST* in 10 µl EMSA buffer [20 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 2 mM MgCl₂, 5% (v/v) glycerol, 50 µg/ml BSA, 1µM random hexamer (Invitrogen), 0.05% (v/v) Tween-20, and 1 mM TCEP] for 1 hour on ice before being loaded onto a 0.5× TBE, 0.7% SeaKem® LE agarose (Lonza) gel. The gels were run at 6.8 v/cm in 0.5× TBE buffer for 40 mins and then vacuum dried onto a Hybond-N+ membrane (Cytiva) with 2 pieces of 3MM chromatography paper (Whatman™). The gels were exposed to a phosphor imaging screen overnight. The final images were obtained by scanning the screen on an Amersham Typhoon scanner (GE Lifesciences) and then quantified with QuantityOne (Bio-rad). The dissociation constant (K_D) was calculated by fitting the Hill equation using the fraction of protein bound primer θ . $\theta = P_f^n / (P_f^n + K_D^n)$, $P_f = P_t - (P_t + D_t + K_D - \sqrt{(P_t + D_t + K_D)^2 - 4 * P_t * D_t}) / 2$, where P_f is the unbound protein concentration, P_t is the total protein concentration, D_t is the total DNA concentration, and n is the Hill coefficient. K_D numbers obtained using the simplified Hill equation ($P_f \approx P_t$) were 2-5% higher.

Statistics and reproducibility. POLA1–POLA2 and its complex with telomerase were successfully purified three times. POLA1–POLA2–PRIM1–PRIM2 and its complex with telomerase were successfully purified more than ten times. *TtCST* were successfully purified more than three times for WT and each Ctc1 variant. SEC profiles and protein gels shown in Extended Data Fig. 5a, 5c, 5e, 5f, and 9g present representative results. EMSAs of d(GTTGGG)₅ DNA binding by WT and Ctc1 mutant *TtCST* shown in Fig. 4e and Extended Data Fig. 9i were successfully repeated three times. EMSAs of variant sstDNA binding by WT *TtCST* shown in Extended Data Fig. 9h were successfully repeated two times. Activity assay results shown in Fig. 3c and Extended Data Fig. 5i-j were successfully repeated three times for each condition.

Methods References

596 13 Zaug, A. J. *et al.* CST does not evict elongating telomerase but prevents initiation by ssDNA
597 binding. *Nucleic Acids Res* **49**, 11653-11665 (2021).

598 19 Lim, C. J. *et al.* The structure of human CST reveals a decameric assembly bound to telomeric
599 DNA. *Science* **368**, 1081-1085 (2020).

600 22 Jiang, J. *et al.* Structure of Tetrahymena telomerase reveals previously unknown subunits,
601 functions, and interactions. *Science* **350**, aab4070 (2015).

602 23 Wan, B. *et al.* The Tetrahymena telomerase p75-p45-p19 subcomplex is a unique CST complex.
603 *Nat Struct Mol Biol* **22**, 1023-1026 (2015).

604 40 He, Y. *et al.* Structures of telomerase at several steps of telomere repeat synthesis. *Nature* **593**,
605 454-459 (2021).

606 43 Hong, K. *et al.* Tetrahymena telomerase holoenzyme assembly, activation, and inhibition by
607 domains of the p50 central hub. *Mol Cell Biol* **33**, 3962-3971 (2013).

608 45 Baranovskiy, A. G. *et al.* Mechanism of Concerted RNA-DNA Primer Synthesis by the Human
609 Primosome. *J Biol Chem* **291**, 10006-10020 (2016).

610 46 Kilkenny, M. L. *et al.* Structural basis for the interaction of SARS-CoV-2 virulence factor nsp1
611 with DNA Polymerase α - Primase. *Protein Sci* (2021).

612 47 Coloma, J., Johnson, R. E., Prakash, L., Prakash, S. & Aggarwal, A. K. Human DNA polymerase
613 alpha in binary complex with a DNA:DNA template-primer. *Sci Rep* **6**, 23784 (2016).

614 48 Beseiso, D. *et al.* The first crystal structures of hybrid and parallel four-tetrad intramolecular G-
615 quadruplexes. *Nucleic Acids Res* **50**, 2959-2972 (2022).

616 51 Lee, W., Tonelli, M. & Markley, J. L. NMRFAM-SPARKY: enhanced software for biomolecular
617 NMR spectroscopy. *Bioinformatics* **31**, 1325-1327 (2015).

618 52 Herrmann, T., Guntert, P. & Wuthrich, K. Protein NMR structure determination with automated
619 NOE-identification in the NOESY spectra using the new software ATNOS. *J Biomol NMR* **24**, 171-
620 189 (2002).

621 53 Guntert, P. & Buchner, L. Combined automated NOE assignment and structure calculation with
622 CYANA. *J Biomol NMR* **62**, 453-471 (2015).

623 54 Shen, Y., Delaglio, F., Cornilescu, G. & Bax, A. TALOS+: a hybrid method for predicting protein
624 backbone torsion angles from NMR chemical shifts. *J Biomol NMR* **44**, 213-223 (2009).

625 55 Shen, Y. *et al.* Consistent blind protein structure generation from NMR chemical shift data. *Proc*
626 *Natl Acad Sci U S A* **105**, 4685-4690 (2008).

627 56 Williamson, M. P. Using chemical shift perturbation to characterise ligand binding. *Progress in*
628 *Nuclear Magnetic Resonance Spectroscopy* **73**, 1-16 (2013).

629 57 Jiang, J. *et al.* The architecture of Tetrahymena telomerase holoenzyme. *Nature* **496**, 187-192
630 (2013).

631 58 Mastronarde, D. N. Automated electron microscope tomography using robust prediction of
632 specimen movements. *J Struct Biol* **152**, 36-51 (2005).

633 59 Zivanov, J. *et al.* New tools for automated high-resolution cryo-EM structure determination in
634 RELION-3. *Elife* **7** (2018).

635 60 Zheng, S. Q. *et al.* MotionCor2: anisotropic correction of beam-induced motion for improved
636 cryo-electron microscopy. *Nat Methods* **14**, 331-332 (2017).

637 61 Rohou, A. & Grigorieff, N. CTFFIND4: Fast and accurate defocus estimation from electron
638 micrographs. *J Struct Biol* **192**, 216-221 (2015).

639 62 Bepler, T. *et al.* Positive-unlabeled convolutional neural networks for particle picking in cryo-
640 electron micrographs. *Nat Methods* **16**, 1153-1160 (2019).
641 63 Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid
642 unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296 (2017).
643 64 Kucukelbir, A., Sigworth, F. J. & Tagare, H. D. Quantifying the local resolution of cryo-EM density
644 maps. *Nat Methods* **11**, 63-65 (2014).
645 65 Tan, Y. Z. *et al.* Addressing preferred specimen orientation in single-particle cryo-EM through
646 tilting. *Nat Methods* **14**, 793-796 (2017).
647 66 Pettersen, E. F. *et al.* UCSF Chimera--a visualization system for exploratory research and
648 analysis. *J Comput Chem* **25**, 1605-1612 (2004).
649 67 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta*
650 *Crystallogr D Biol Crystallogr* **66**, 486-501 (2010).
651 68 Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-
652 589 (2021).
653 69 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular
654 structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221 (2010).
655 70 Holm, L. Using Dali for Protein Structure Comparison. *Structural Bioinformatics: Methods and*
656 *Protocols* **2112**, 29-42 (2020).
657 71 Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. & Barton, G. J. Jalview Version 2--a
658 multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191
659 (2009).
660 72 Goddard, T. D. *et al.* UCSF ChimeraX: Meeting modern challenges in visualization and analysis.
661 *Protein Sci* **27**, 14-25 (2018).
662 73 Baranovskiy, A. G. *et al.* Structural basis for inhibition of DNA replication by aphidicolin. *Nucleic*
663 *Acids Res* **42**, 14013-14021 (2014).
664 74 Perera, R. L. *et al.* Mechanism for priming DNA synthesis by yeast DNA polymerase alpha. *Elife*
665 **2**, e00482 (2013).

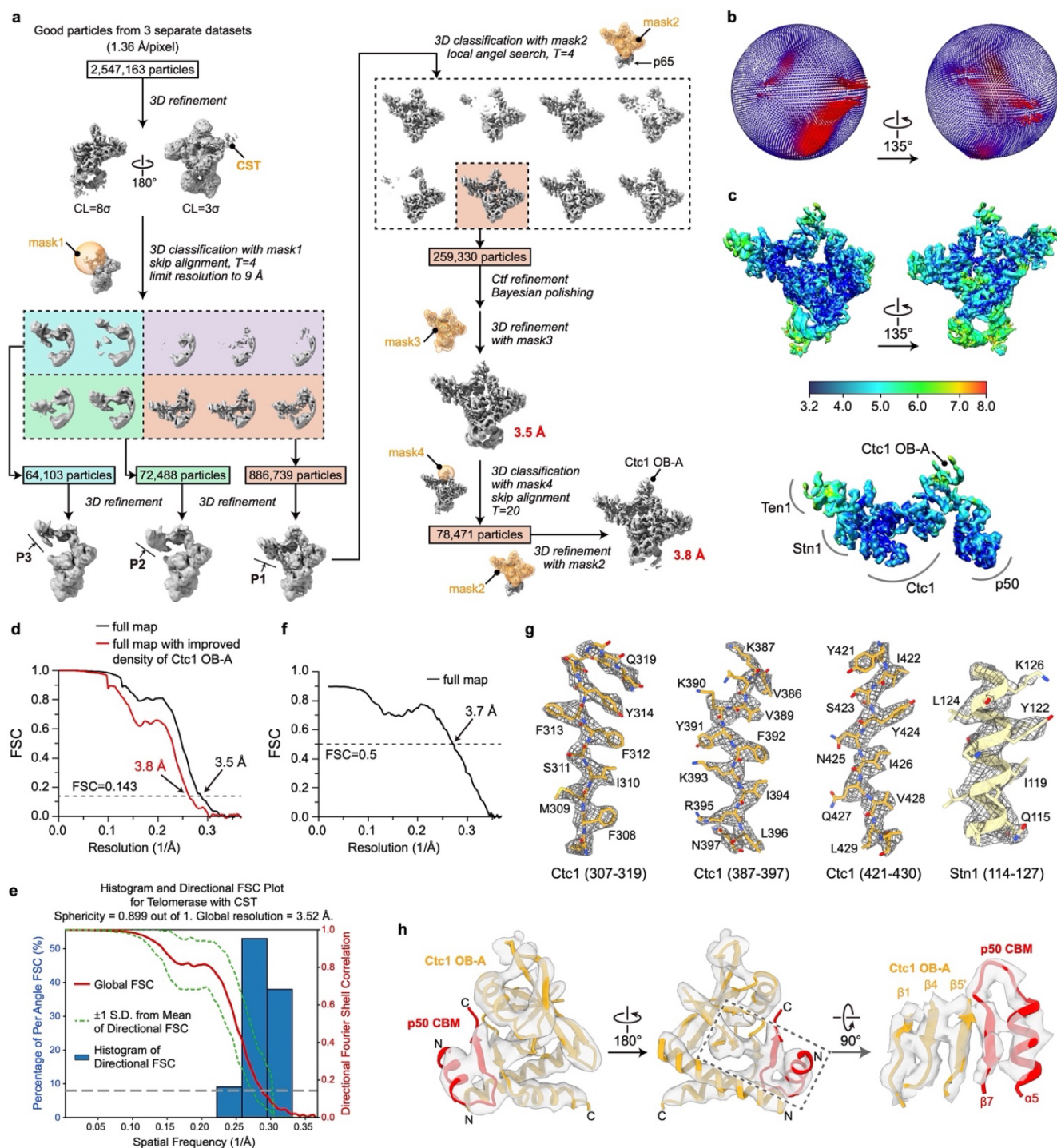
666 **Data availability** Cryo-EM density maps have been deposited in the Electron Microscopy Data Bank
667 under accession numbers EMD-26863 (telomerase with CST), EMD-26864 (telomerase with CST–
668 PolαPrim), EMD-26865 (telomerase), EMD-26866 (CST–PolαPrim), EMD-26867 (PolαPrim platform),
669 EMD-26868 (POLA2–POLA1_{CTD}–PRIM2_N), and EMD-26869 (PRIM2_N–PRIM1). The atomic models
670 have been deposited in the Protein Data Bank under accession codes 7UY5 (telomerase with CST),
671 7UY6 (telomerase), 7UY7 (CST–PolαPrim), and 7UY8 (PolαPrim platform). Backbone chemical shifts
672 have been deposited in BMRB under accession codes 51441 (Ctc1 OB-A), 51442 (Ctc1 OB-A with p50
673 peptide 228-250), and 51443 (p50 peptide 228-250).

674 **Acknowledgements** This work was supported by grants from NIH R35GM131901 and NSF
675 MCB2016540 to J.F. and NIH R01GM071940 to Z.H.Z. We acknowledge use of instruments at the

676 Electron Imaging Center for Nanomachines supported by UCLA and instrumentation grants from NIH
677 (1S10OD018111 and U24GM116792) and NSF (DBI-1338135 and DMR-1548924). The UCLA-DOE
678 NMR core facility is supported in part by US Department of Energy grant DE-AC02-06CH11357 and
679 NIH instrumentation grants S10OD016336 and S10OD025073.

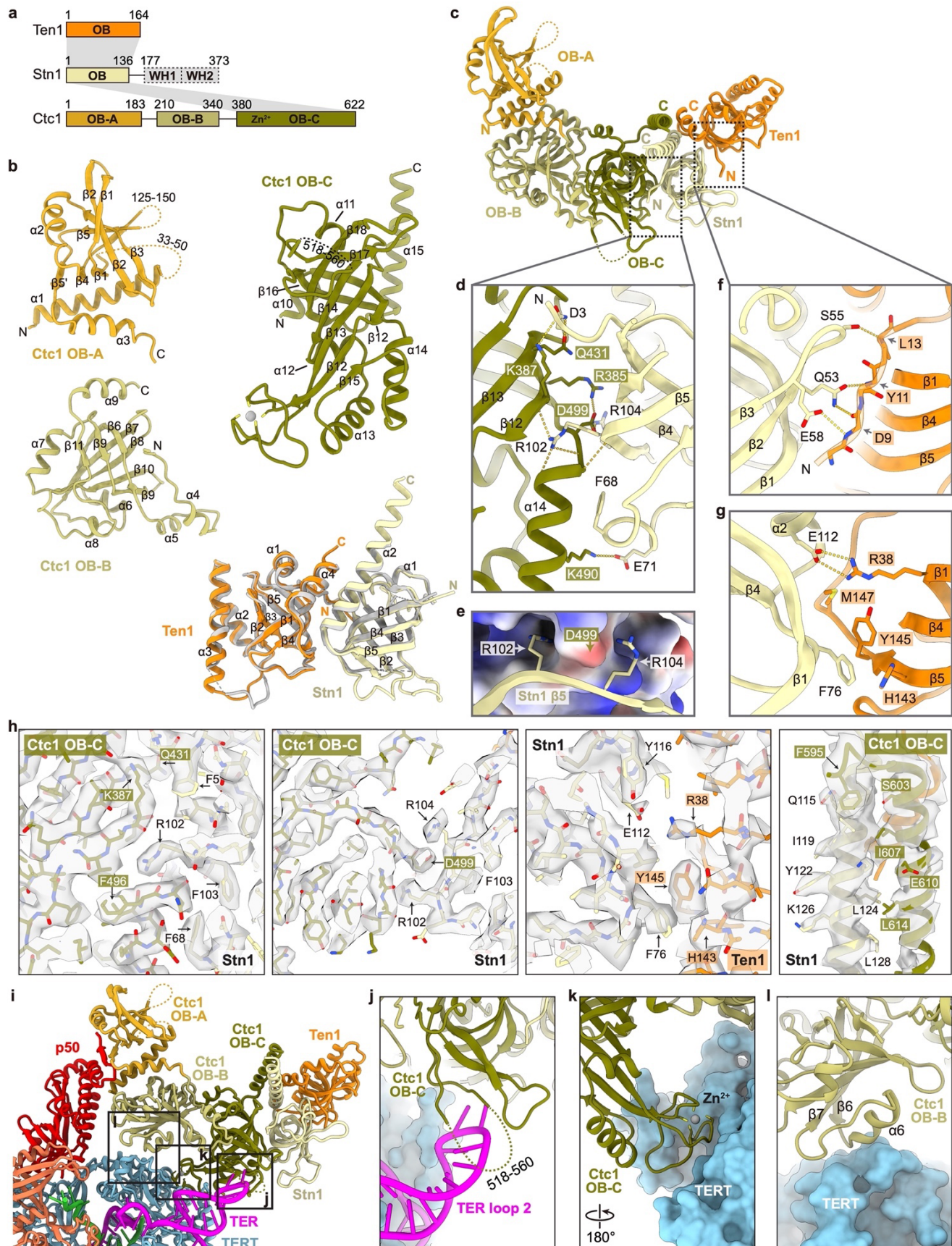
680 **Author Contributions** Y.H. and H.S. prepared and checked EM samples; Y.H. collected and analyzed
681 cryo-EM data; Y.H. and H.S. built the models; H.C. expressed and purified samples for NMR analysis;
682 H.C., Y.W., and L.S. collected and analyzed NMR data; H.S. and B.L. conducted activity assays; B.L.
683 and Y.H. conducted EMSA assays; Z.H.Z. supervised cryo-EM data collection and processing; J.F.
684 supervised all aspects of the project; Y.H. and J.F. made figures and wrote the manuscript, with input
685 from H.S., Y.W. and B.L.

686 **Competing interests:** Authors declare that they have no competing interests.

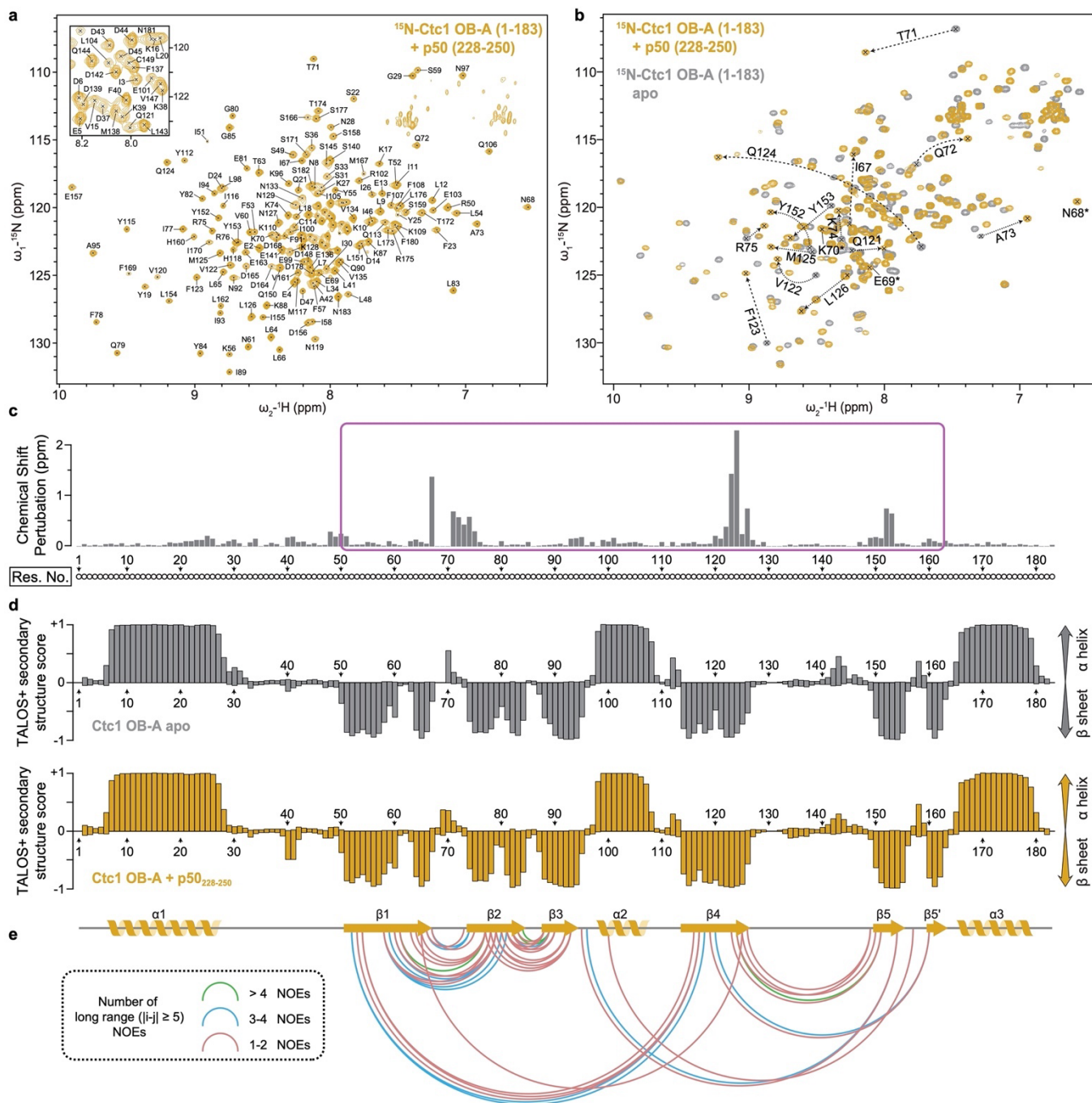


Extended Data Fig. 1: Cryo-EM data processing workflow of *Tt*CST in telomerase holoenzyme and evaluations of the final reconstructions. **a**, Data processing workflow (detailed in Methods). **b**, Euler angle distributions of particles used for the final 3.5 Å resolution reconstruction. **c**, Local resolution evaluation of the 3.5 Å resolution reconstruction shown for overall map (upper) and for the *Tt*CST–p50 region (lower). **d**, Plot of the Fourier shell correlation (FSC) as a function of the spatial frequency demonstrating the resolutions of final reconstructions. **e**, 3D FSC analysis⁶⁵ of the 3.5 Å resolution reconstruction. Shown are the global FSC (red line), the spread of directional resolution

values (area encompassed by the green dotted lines) and the histogram of directional resolutions evenly sampled over the 3D FSC (blue bars). A sphericity (0.5 threshold) of 0.899 indicates almost isotropic angular distribution of resolution (a value of 1 stands for completely isotropic angular distribution). **f**, FSC coefficient as a function of spatial frequency between model and cryo-EM density map. **g**, Representative cryo-EM densities (gray and mesh) encasing the related atomic models (colored sticks and ribbons). **h**, Superimposition of cryo-EM densities (low-pass filtered to 5 Å) and model of Ctc1 OB-A in complex with p50 CBM.

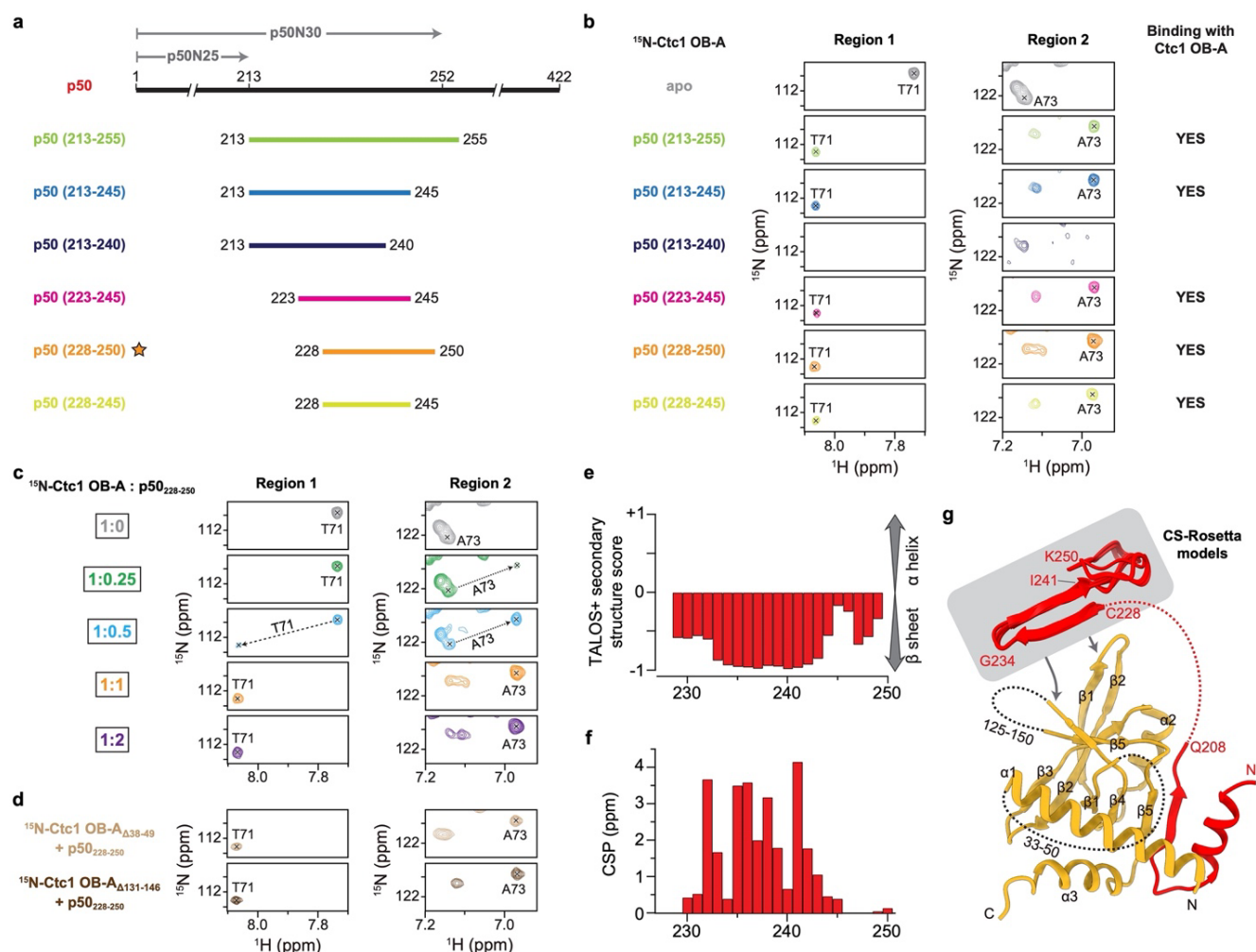


Extended Data Fig. 2: Structural details of *Tt*CST. **a**, Domain organization of *Tt*CST subunits. Invisible regions in the cryo-EM map are shown as dashed boxes. Intermolecular interactions are indicated as gray shading. **b**, Ribbon diagrams of *Tt*CST subunits/domains with secondary structure elements labelled. Unmodeled regions are shown as dashed lines. Crystal structure of Ten1–Stn1 OB²³ (PDB 5DOI) is shown in gray and overlaid with the cryo-EM structure for comparison. **c**, Ribbon representation of *Tt*CST structure with individual OB domain colored as indicated. **d**, Zoom-in view of the interface between Ctc1 OB-C and Stn1 OB. Salt bridge and hydrogen-bonding interactions are shown as dashed yellow lines. **e**, Two arginine sidechains on Stn1 OB (ribbon) clamp D499 on Ctc1 OB-C (electrostatic surface). **f, g**, Detailed interactions between Stn1 OB and Ten1. **h**, Close-up views of the cryo-EM densities of the interfaces between CST subunits (Fig. 1e, Extended Data Fig. 2d-g). **i**, Overall view of the interface between *Tt*CST and TERT–TER. **j-l**, Zoom-in views of interactions between *Tt*CST and TERT–TER as indicated in dashed boxes in **h**. These interactions stabilize *Tt*CST in the predominant conformation.

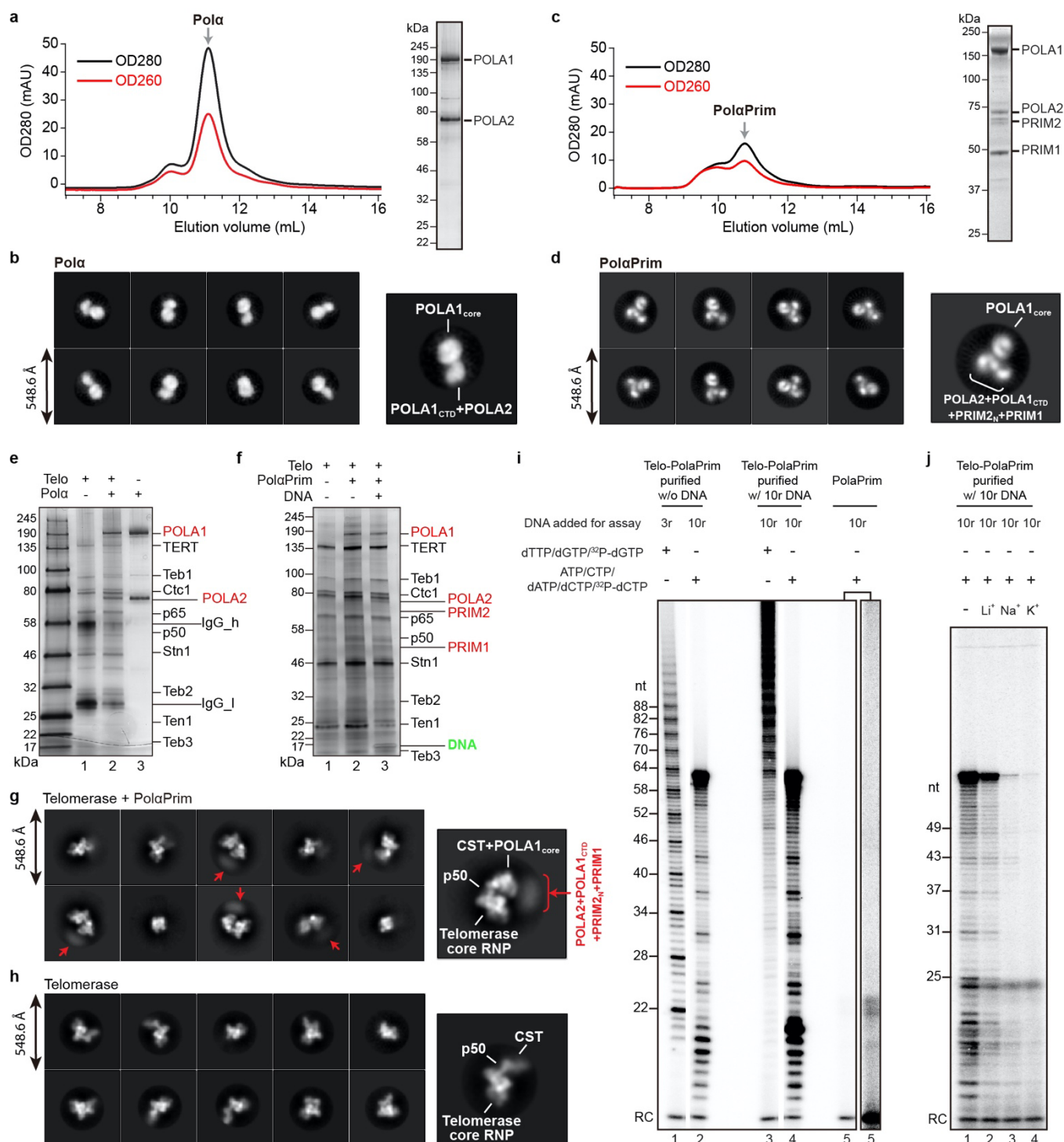


Extended Data Fig. 3: NMR spectra and structural study of Ctc1 OB-A with p50 peptide. **a**, Assigned ^1H - ^{15}N HSQC spectrum of ^{15}N -labelled Ctc1 OB-A (residues 1-183) in the presence of unlabeled p50 peptide (residues 228-250). Inset shows the expanded central region of the spectrum. **b**, Superimposed ^1H - ^{15}N HSQC spectra of ^{15}N -labelled Ctc1 OB-A in the presence (yellow) and absence (gray) of unlabeled p50 peptide. Signals from the same residues with chemical shift differences of >0.25 ppm are connected by dashed arrows. Signals from residues 68-70 that only appear in the presence of p50 peptide are labeled with asterisks. **c**, Chemical shift perturbation (CSP) index of ^{15}N -labelled Ctc1 OB-A upon binding p50 peptide. Magenta box indicates the region that is shown in Fig. 2d. **d**, Chemical-shift-based secondary structure score of Ctc1 OB-A in the absence (gray) and

presence (yellow) of p50 peptide. The scores are determined using TALOS+ (ref⁵⁴). Top and bottom edges of each bar represent the probabilities of each residue assigned to be α helix and β sheet, respectively. The secondary structure of Ctc1 OB-A observed in the cryo-EM structure is shown below for comparison. **e**, Plot of long range (greater than 5 residues) ^1H - ^1H NOE restraints observed within Ctc1 OB-A. Residues with pairwise NOE restraint(s) are connected by a link. Links are color coded as indicated based on the number of NOE restraints between the two connected residues.

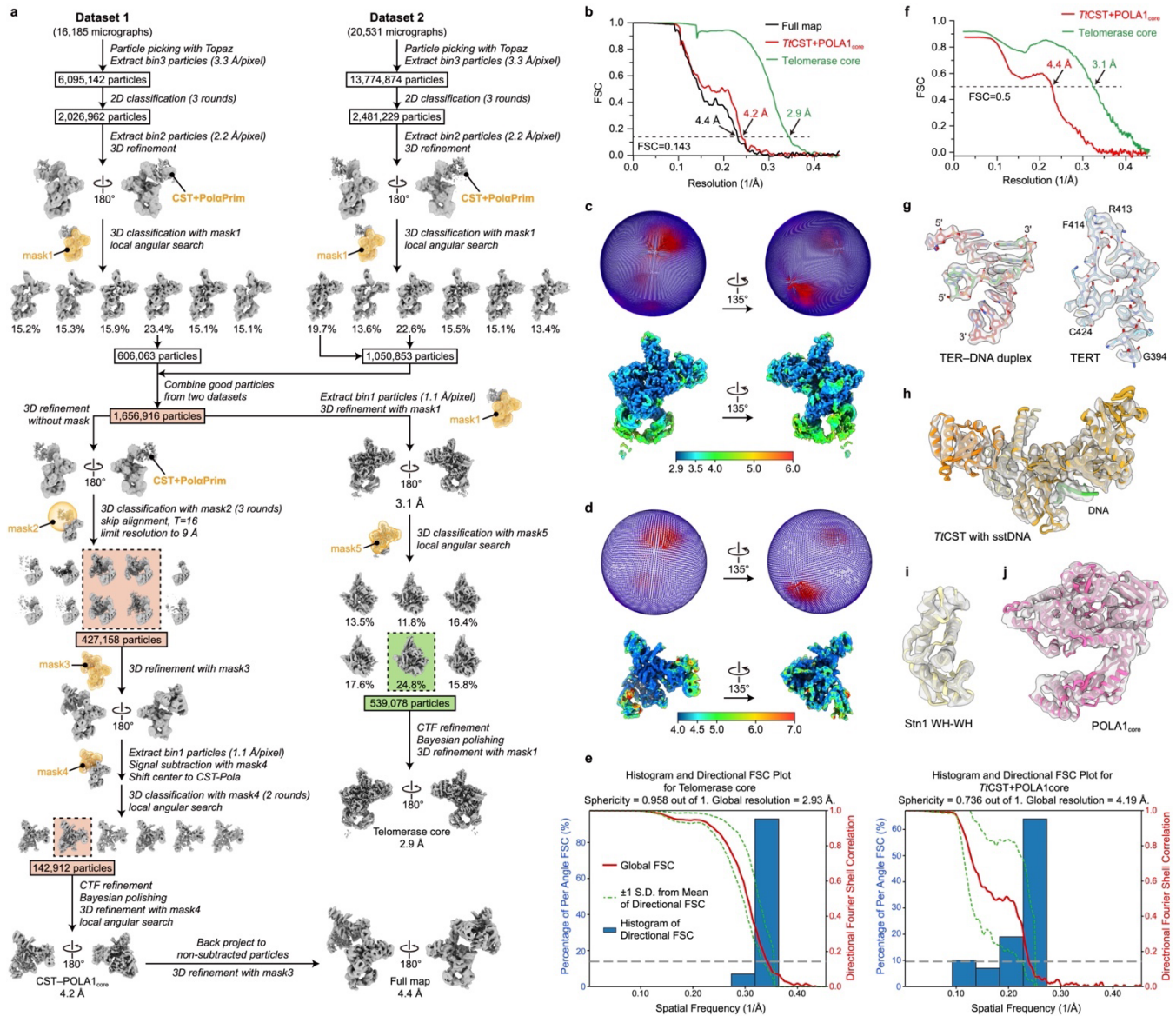


Extended Data Fig. 4: Identifying the "invisible" interface between Ctc1 OB-A and p50 peptide using NMR methods. **a**, Schematic diagram of p50 and constructs of p50 peptide. The N-terminal 30 kDa and 25 kDa fragments of p50 are labeled as p50N30 and p50N25, respectively. Previous biochemical study showed that p50N30 could bind Ctc1, whereas p50N25 could not⁴³. The cryo-EM structure of p50 ends at residue 208 (Fig. 2b). Based on these facts, a series of p50 peptides in the range of residues 213-255 were designed to explore additional interface between p50 and Ctc1 OB-A that are "invisible" in the cryo-EM structure. **b**, NMR binding study of p50 peptides with Ctc1 OB-A. Two regions of ¹H-¹⁵N HSQC spectra of ¹⁵N-labelled Ctc1 OB-A in the absence (apo) and presence of unlabeled p50 peptides were shown. Chemical shifts of T71 and A73 were chosen to illustrate the binding process in this and the following panels **c** and **d**. p50₂₂₈₋₂₅₀ peptide is determined to be the optimal construct and was used for other NMR studies presented in this paper. **c**, Titration series of p50 peptide into ¹⁵N-labelled Ctc1 OB-A. The binding is in the slow exchange regime and saturated at 1:1 stoichiometry. **d**, Truncations of two unstructured loops (residues 38-49 and 131-146) of Ctc1 OB-A individually have no effect on its binding with p50 peptide. **e**, Secondary structure score of p50₂₂₈₋₂₅₀ in the presence of Ctc1 OB-A. **f**, CSP index of p50 peptide upon binding Ctc1 OB-A. ¹H-¹⁵N HSQC spectra shown in Fig. 2c were used for the CSP calculation. **g**, Model of the interactions between Ctc1 OB-A and p50. CS-Rosetta models of p50₂₂₈₋₂₅₀ are shown in gray box with arrows pointing to the binding surface on Ctc1 OB-A. Unstructured linkers are shown as dashed lines.



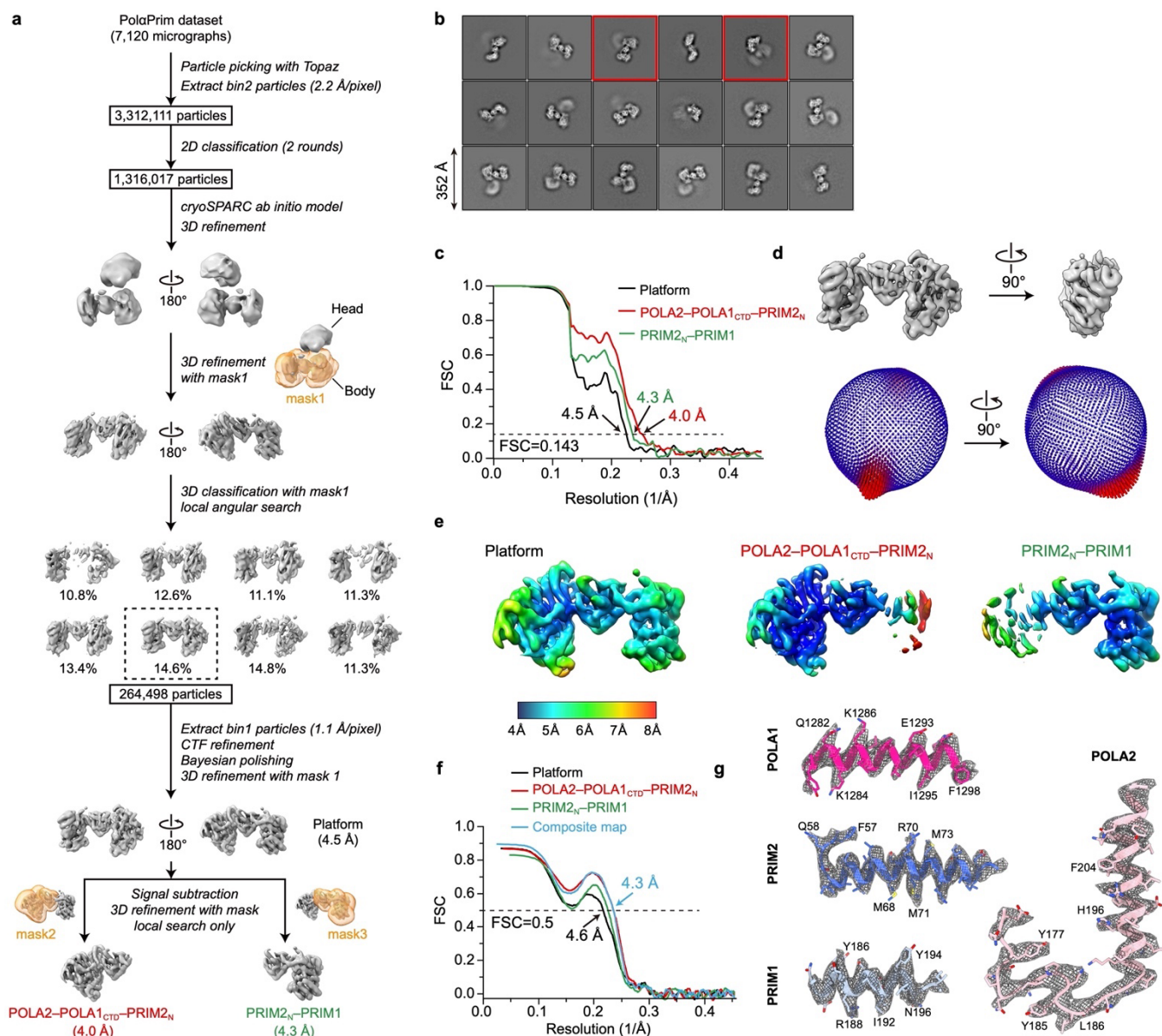
Extended Data Fig. 5: Characterization of purified *Tetrahymena* PolαPrim samples and their assembly with telomerase holoenzyme. **a**, Size-exclusion chromatography (SEC) profile (left) and SDS-PAGE gel (right) of Polα. **b**, Representative 2D-class averages of Polα particles obtained from negative-stain EM images. **c**, SEC profile (left) and SDS-PAGE gel (right) of PolαPrim. **d**, Representative 2D-class averages of PolαPrim particles obtained from negative-stain EM images. **e**, Silver-stained SDS-PAGE gel of affinity purified telomerase–Polα (lane 2) shows that Polα can bind

telomerase in the absence of Primase. Telomerase (lane 1) and Pol α (lane 3) samples were loaded on the same gel for comparison. **f**, Silver-stained SDS-PAGE gel of affinity purified telomerase–Pol α Prim samples shows assembly of the complex with or without sstDNA. **g**, Representative 2D-class averages of affinity purified telomerase–Pol α Prim obtained from negative-stain EM images. Densities are assigned based on the cryo-EM structure (Fig. 3a) obtained with the same batch of sample. Smeared densities (red arrows) are observed near POLA1_{core} in several classes, so we were able to assign them to the Pol α Prim platform, which comprises POLA2, POLA1_{CTD}, PRIM2_N, and PRIM1. **h**, Representative 2D-class averages of telomerase particles shown for comparison with **g**. **i**, Activity assays of telomerase-Pol α Prim (lanes 1-4) and Pol α Prim alone (lane 5). Direct telomerase activity assays were conducted for G-strand synthesis alone in the presence of dTTP and dGTP (lanes 1 and 3). Pol α Prim activity assays were conducted for C-strand synthesis alone in the presence of ATP, CTP, dATP and dCTP (lanes 2, 4 and 5). ³²P-dGTP and ³²P-dCTP were used to label the G-strand and C-strand products, respectively. A longer exposure is shown for lane 5 so that products can be seen. RC, recovery control. All lanes are from a single gel. **j**, Activity assays of C-strand synthesis (lane 1) relative to that in 50 mM LiCl (lane 2), 50 mM NaCl (lane 3), or 50 mM KCl (lane 4). For lanes 2-4, the DNA templates were incubated in assay buffer containing 50 mM of the indicated cations on ice for 30 min before the reaction.

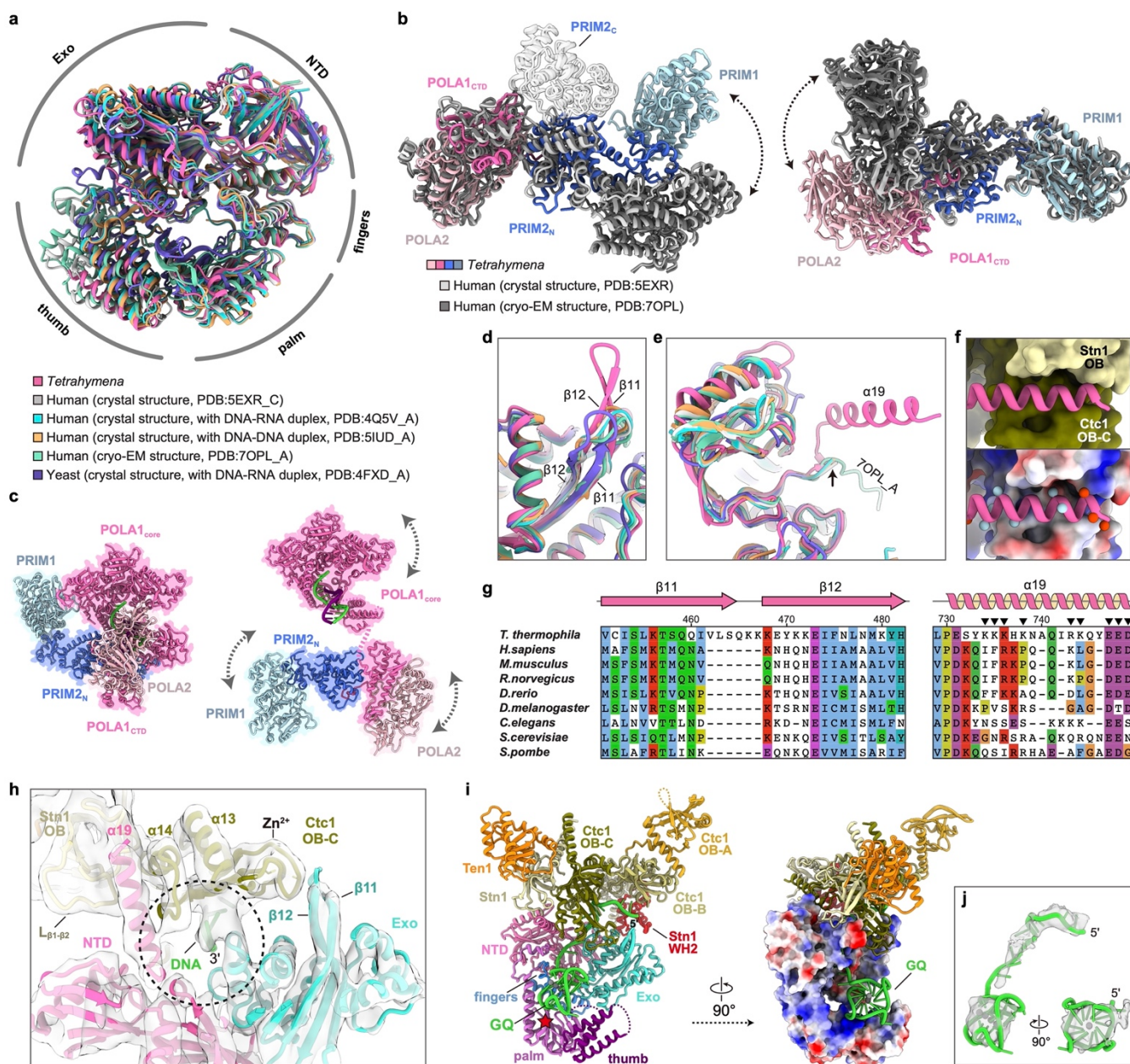


Extended Data Fig. 6: Cryo-EM structure determination of *Tetrahymena* telomerase-PolαPrim complex. **a**, Data processing workflow (detailed in Methods). **b**, Resolution of final reconstructions determined by gold-standard FSC at the 0.143 criterion. **c**, Particle distribution (upper) and local resolution evaluation (lower) of the 2.9 Å resolution reconstruction of telomerase core. **d**, Particle distribution (upper) and local resolution evaluation (lower) of the 4.2 Å resolution reconstruction of TtCST-POLA1_{core}. **e**, 3D FSC analysis⁶⁵ of the 2.9 Å resolution reconstruction of telomerase core (left) and the 4.2 Å resolution reconstruction of TtCST-POLA1_{core} (right). For each reconstruction, the global FSC (red line), the spread of directional resolution values (area encompassed by the green dotted lines) and the histogram of directional resolutions evenly sampled over the 3D FSC (blue bars) are shown. A sphericity of 0.958 was determined for telomerase core (left), indicating almost isotropic angular distribution of resolution. A sphericity of 0.736 was determined for TtCST-POLA1_{core} (right), suggesting slightly anisotropic angular distribution of resolution. **f**, FSC curves for refined models versus the corresponding cryo-EM density maps. **g-j**, Representative cryo-EM densities (transparency

surface) encasing the related atomic models (color sticks and ribbons) for telomerase RNP core (**g**), CST (**h**), Stn1 WH-WH (**i**) and POLA1_{core} (**j**).

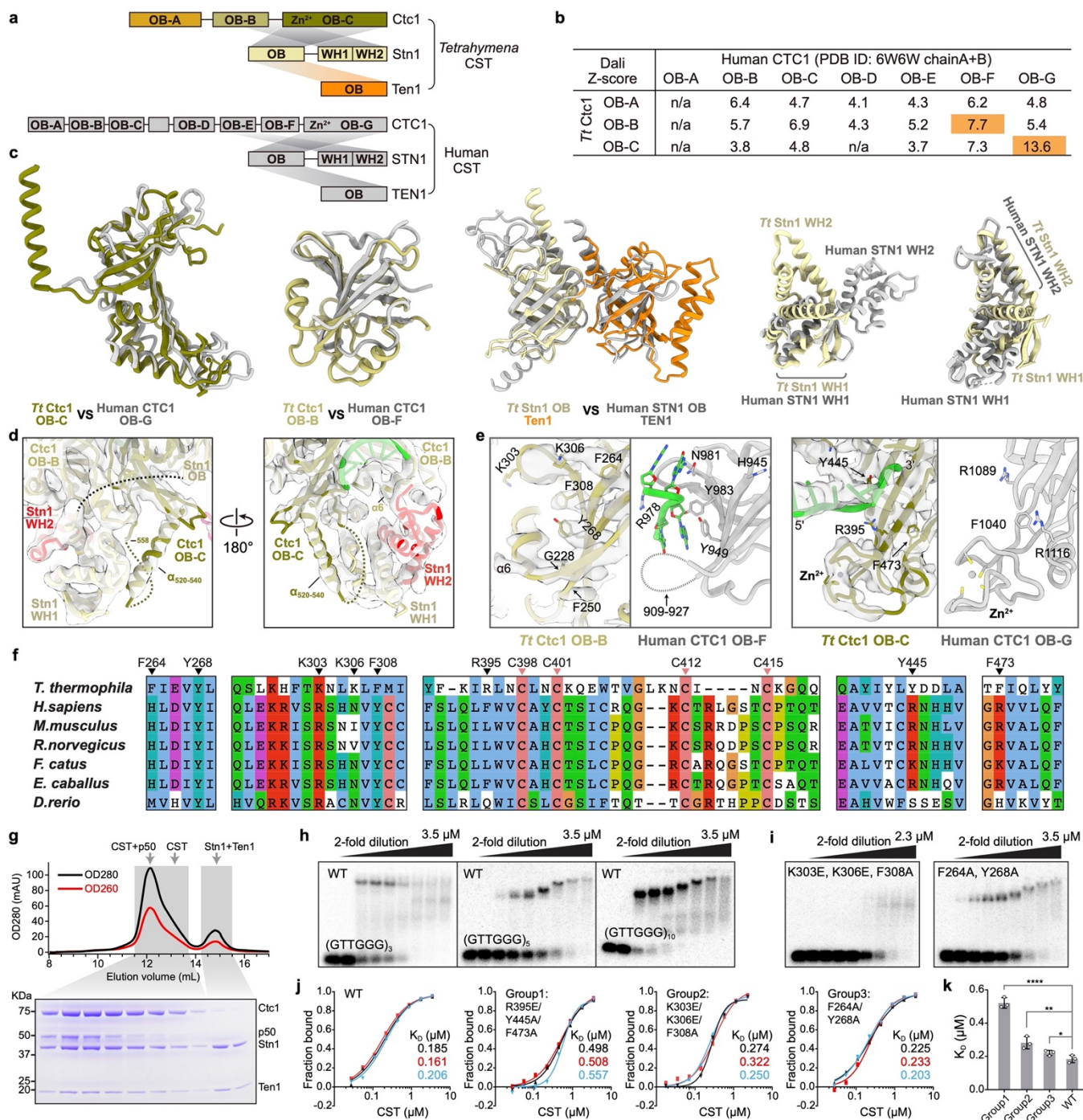


Extended Data Fig. 7: Cryo-EM structure determination of PolaPrim. **a**, Data processing workflow (detailed in Methods). **b**, Representative 2D-class averages of PolaPrim particles obtained from cryo-EM images. The two classes shown in Fig. 3e are labeled with red boxes. **c**, Resolution of final reconstructions determined by gold-standard FSC at the 0.143 criterion. **d**, Euler angle distributions of particles used for the final reconstructions. **e**, Local resolution evaluations of the final reconstructions. **f**, FSC coefficients as a function of spatial frequency between model and cryo-EM density maps. The composite map is generated using Phenix⁶⁹ with two focused refined maps (detailed in Methods). For the full map and the composite map, the complete model was used to calculate the FSCs. For the two focused refined maps, only corresponding regions of the model were used to calculate the FSCs. **g**, Representative cryo-EM densities (gray mesh) encasing the related atomic models (colored sticks and ribbons).



Extended Data Fig. 8: Structural conservation of *Tetrahymena* PolαPrim. **a**, Superposition of *Tetrahymena* (*Tt*), human and yeast POLA1_{core} structures^{45-47,73,74} shown in an overall view. **b**, Structural comparison of PolαPrim platform of *Tt* and human^{45,46}. The structures were superposed based on POLA2–POLA1_{CTD} (left) or PRIM1 (right). Arrows indicate dynamics of the unaligned regions. PRIM2_C in human structures are shown as transparent ribbons. PRIM2_C is not observed in the *Tt* structure. **c**, Structures of PolαPrim in an autoinhibited conformation (left, modeled based on PDB 5EXR⁴⁵) and an active conformation (right, modeled based on a low-resolution cryo-EM map in Extended Data Fig. 7a). The DNA-DNA duplexes on POLA1_{core} were modeled based on PDB 5IUD⁴⁷. In the autoinhibited conformation (left), the active site on POLA1_{core} is sterically blocked by POLA1_{CTD} and POLA2 for DNA entry. In the active conformation (right), dynamics of subunits are indicated with arrows. **d-e**, Superposition of *Tt*, human and yeast POLA1_{core} structures for the regions that are on the

interface with *Tt*CST. Conserved domains/motifs are labeled as indicated. The β 11- β 12 hairpin in *Tt* POLA1_{core} is longer than those in human and yeast (**d**). The α 19 is structured only in *Tt* POLA1 when binding *Tt*CST (**e**). **f**, Zoom-in views of the interface between *Tt* POLA1_{core} α 19 (ribbon) and *Tt*CST (surface/electrostatic surface). In the lower panel, locations of positively and negatively charged residues on α 19 are indicated using blue and red balls, respectively. **g**, Sequence conservation analysis of the β 11- β 12 hairpin and α 19 of POLA1. Charged residues on α 19 are indicated with black arrows. **h**, Zoom-in view of the interface between POLA1_{core} and *Tt*CST with sstDNA. Cryo-EM densities are shown as transparent surface. The template entry port formed by POLA1_{core} NTD and Exo and Ctc1 OB-C is indicated by a cycle. **i**, Path of sstDNA in the cryo-EM structure of *Tt* telomerase–Pol α Prim. The sstDNA binds in the C-shape cleft of Ctc1 OB-C with its 5' side, while its 3' side passes through the template entry port to the active site of POLA1_{core} (left). A G-quadruplex (GQ) formed by four *Tt* telomere repeats (modeled based on PDB 7JKU⁴⁸) is observed on a positively charged DNA binding surface of POLA1_{core} between the palm and thumb (right). **j**, Superimposition of the GQ structure and cryo-EM density. Weak density of sstDNA can be observed connecting the sstDNA on Ctc1 OB-C to the GQ.



Extended Data Fig. 9: Comparison of *Tt*CST and hCST. a, Domain diagrams of *Tt*CST and hCST. **b**, Structural homology analysis of individual OB domains of *Tt*Ctc1 (OB-A to -C) and human CTC1 (OB-A to -G) using the Dali sever⁷⁰. Based on the resulted pairwise Z-scores, *Tt*Ctc1 OB-B and OB-C are identified as homologs of human CTC1 OB-F and OB-G, respectively. **c**, Structural comparison of individual domains from *Tt*CST (color) with corresponding domains from hCST (gray). Structures of WH-WH domains of *Tt* Stn1 and human STN1 were superposed based on WH1 or WH2 domain. The relative orientation of the two WH domains is different between *Tt* and human. **d**, The interface between Stn1 WH-WH and Ctc1 in the cryo-EM structure of *Tt* telomerase-PolαPrim. Cryo-EM densities are

shown as transparent surfaces. An previously unstructured loop of Ctc1 OB-C (Extended Data Fig. 2b) partially forms an α helix ($\alpha_{520-540}$) and contributes to the interface with Stn1 WH-WH. **e**, Comparison of DNA binding sites on *TtCtc1* (color) and human CTC1 (gray). Conserved residues located on the DNA binding interface are shown as sticks. Cryo-EM densities of *TtCtc1* are shown as transparent surfaces. In the decameric structure of hCST¹⁹ (PDB 6W6W), sstDNA primarily binds on CTC1 OB-F. However, in *TtCST*, the equivalent sstDNA binding site on OB-B is partially occluded by a helix (α_6) that is part of an unstructured loop in hCTC1 OB-F. The helix α_6 abuts TERT in *TtCST* without Pol α Prim (Extended Data Fig. 2k) and Stn1 WH2 when Pol α Prim is bound (as shown in **d**). **f**, Sequence conservation analysis of Ctc1 residues on the DNA binding interface. Residues shown in **e** are indicated with black arrows. Conserved cysteines in the Zn-ribbon motifs are indicated with pink arrows. **g**, SEC profile and SDS-PAGE gel of *TtCST*-p50 co-expressed in Sf9 cells. Gel samples are from the peak fractions of the SEC profile as indicated. **h**, EMSA of purified wild-type *TtCST* with d(GTTGGG)_n, where n = 3, 5 or 10. **i**, Substitutions of *TtCtc1* OB-B conserved residues K303E/K306E/F308A and F264A/Y268A substantially decrease d(GTTGGG)₅ binding, as indicated by EMSAs. These results suggest that the binding site on *TtCtc1* OB-B might be accessible to sstDNA in free *TtCST* where neither TERT nor Stn1 WH-WH stabilize helix α_6 . Wedges indicate two-fold dilution of *TtCST*. The first lane of each gel is a *TtCST*-free control. **j**, Quantifications of fraction of bound DNA for all the independent EMSA experiments with *TtCST* WT and variants as indicated (n=3 biological replicates). K_D values were determined as described in Methods. **k**, Effect of *TtCST* residue substitutions on d(GTTGGG)₅ binding. Data are mean $\pm$ s.d. from n=3 biological replicates shown in **j**. **P*=0.04, ***P*=0.009, *****P*<0.0001; one-tailed unpaired t-tests.

Extended Data Table. 1: Cryo-EM data collection, refinement and validation statistics.

	Telomerase with CST (EMD-26863) (PDB 7UY5)	Telomerase with CST- PolaPrim (EMD-26864)	Telomerase (EMD-26865) (PDB 7UY6)	CST-PolaPrim (EMD-26866) (PDB 7UY7)	PolaPrim platform (EMD-26867) (PDB 7UY8)	POLA2- POLA1 _{CTD} - PRIM2 _N (EMD-26868)	PRIM2 _N -PRIM1 (EMD-26869)
Data collection and processing							
Magnification	105,000	81,000	81,000	81,000	81,000	81,000	81,000
Voltage (kV)	300	300	300	300	300	300	300
Electron exposure (e-/Å ²)	48	55	55	55	55	55	55
Defocus range (µm)	-0.8 – -4.0	-0.8 – -4.0	-0.8 – -4.0	-0.8 – -4.0	-0.8 – -4.0	-0.8 – -4.0	-0.8 – -4.0
Pixel size (Å)	1.36	1.1	1.1	1.1	1.1	1.1	1.1
Symmetry imposed	C1	C1	C1	C1	C1	C1	C1
Initial particle images (no.)	2,547,163	19,870,016	19,870,016	19,870,016	3,312,111	3,312,111	3,312,111
Particle images after class2d (no.)	n/a	4,508,191	4,508,191	4,508,191	1,316,017	1,316,017	1,316,017
Final particle images (no.)	259,330	142,912	539,078	142,912	264,498	264,498	264,498
Map resolution (Å)	3.5	4.4	2.9	4.2	4.5	4.0	4.3
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	3.2 – 6.0	4.0 – 7.0	2.9 – 5.0	4.0 – 7.0	4.0 – 7.0	4.0 – 6.0	4.0 – 6.0
Refinement							
Initial model used (PDB code)	7LMA, 5DOI, 5DFM		7LMA	7UY5, 5DFN, 5DOK, AlphaFold2	AlphaFold2		
Model resolution (Å)	3.7		3.1	4.4	4.3		
FSC threshold	0.5		0.5	0.5	0.5		
Map sharpening B factor (Å ²)	-130		-80	-100	-100		
Model composition							
Non-hydrogen atoms	25,772		18,746	15,161	9,552		
Protein residues	2,690		1,847	1,808	1,161		
RNA/DNA Nucleotides	170		170	10	0		
Ligands	2		1	1	1		
B factors (Å ²)							
Protein	21.8		47.5	124.3	74.4		
RNA/DNA/Ligands	82.6		147.8	150.5	180.8		
R.m.s. deviations							
Bond lengths (Å)	0.002		0.002	0.002	0.003		
Bond angles (°)	0.455		0.442	0.557	0.636		
Validation							
MolProbity score	1.54		1.19	1.91	1.87		
Clashscore	7.03		4.00	13.86	8.69		
Poor rotamers (%)	0.04		0.00	0.18	0.00		
Ramachandran plot							
Favored (%)	97.17		98.30	96.17	93.92		
Allowed (%)	2.83		1.70	3.83	6.08		
Disallowed (%)	0.00		0.00	0.00	0.00		