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Reconstitution of a telomeric replicon organized by CST

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Telomeres, the natural ends of linear chromosomes, are comprised of repeat-sequence DNA and associated proteins[1]. Replication of telomeres allows continued proliferation of human stem cells and immortality of cancer cells[2]. Replication begins with telomerase[3] extending the single-stranded DNA (ssDNA) of the telomeric G-strand [$(TTAGGG)_n$]; the synthesis of the complementary C-strand [$(CCCTAA)_n$] is much less well characterized. The CST (CTC1-STN1-TEN1) protein complex, a DNA Polymerase α-primase accessory factor[4,5], is known to be required for telomere replication in vivo[6,7,8,9], and the molecular analysis presented here reveals key features of its mechanism. We find that CST uses its ssDNA-binding activity to specify the origins for telomeric C-strand synthesis by bound Polα-primase. CST-organized DNA polymerization can copy a telomeric DNA template that folds into G-quadruplex structures, but the suboptimality of this template likely contributes to telomere replication problems observed in vivo. Combining telomerase, a short telomeric ssDNA primer, and CST-Polα-primase gives complete telomeric DNA replication, resulting in the same sort of ssDNA 3'-overhang found naturally on human telomeres. We conclude that the CST complex not only terminates telomerase extension[10,11] and recruits Polα-primase to telomeric ssDNA[4,12,13], but it also orchestrates C-strand synthesis. Because replication of the telomere has features distinct from replication of the rest of the genome, targeting telomere-replication components including CST holds promise for cancer therapeutics.

Biochemical reconstitutions using purified macromolecules have provided detailed understanding of the key steps in the Central Dogma of Molecular Biology, including DNA replication[14,15,16], as well as transcription, pre-mRNA splicing, and translation. The replication of telomeres, the natural ends of eukaryotic chromosomes, is now poised to be reconstituted in an equivalent manner.

The first step of telomere replication, the synthesis of G-strand repeats by telomerase, was first shown in the Tetrahymena system[3] and subsequently in the human system[17]. In these experiments, synthetic oligonucleotides with telomere-like sequences serve as primers for telomerase. Human telomerase extension terminates with the help of CST, which sequesters free primers and prevents re-initiation[10,11].

CST is a heterotrimeric protein complex first identified as an accessory factor for Polα-primase. CST binds single-stranded DNA with some specificity for G-rich sequences[18], which allows it to have special roles in telomere protection and replication[6-9], and at the same time to participate in re-starting stalled replication forks genome-wide[9]. Sequence comparisons indicated that CST is related to RPA (Replication Protein A), and that relationship was confirmed by the recent cryo-EM structure of the human CST complex[19]. The cryo-EM structure identified a portion of the ssDNA binding site within the CTC1 subunit, which allowed designing separation-of-function mutants defective in ssDNA binding[19].

After telomerase extends the G-strand tail at the chromosome end, Polα-primase uses this G-strand DNA as a template for complementary C-strand synthesis. Polα-primase is known to be necessary for telomere replication in vivo[20], and it can use telomeric repeats as a template for C-strand synthesis in vitro[21,22]. The CST complex binds Polα-primase and is thought to recruit it to telomeres[4,12,13]. In fact, even before the heterotrimeric CST complex was identified in yeast, its Cdc13p and Stn1p components had been shown to be required for telomere maintenance[12,13,23,24]. The importance of CST for telomere replication was subsequently extended to plant and mammalian systems[6,7,8,9]. These pioneering studies set the stage for the reconstitution presented here.

CST determines origins of C-strand synthesis

When we express recombinant human CST in HEK-293T cells, the affinity-purified CST also contains the four subunits of Polα-primase[11]. Because Polα-primase is not overexpressed, it represents the naturally occurring enzyme. Co-purification of Polα-primase with CST is consistent with their known binding interaction[4,12,13].

We prepared ssDNA templates representing telomerase extension products ("*R*xTEL" represents *R* repeats of TTAGGG). When CST-Polα-primase was incubated with 9xTEL and 15xTEL templates, ladders of products with a periodicity of ~6 nt (nucleotides) were synthesized (Fig. 1a). Formation of products was dependent on the CST having Polα-primase bound and dependent on inclusion of both ribo- and deoxyribo-nucleotides in the reaction (Extended Data Fig. 1a). Given that high-affinity binding of CST requires 16 nt (Hom and Wuttke) and that primase would not be expected to act within the portion of the template bound by CST, we did

not expect the entire template to be copied. This expectation was borne out, as each ladder extended to a length ~ 16 nt less than that of the template, consistent with the longest C-strand product initiating ~ 16 nt from the 3' end of the template and running off the 5' end (Fig. 1b). Note that the most prominent product with 9xTEL as template ('repeat 3') initiates within the third telomeric repeat, but a minor longer product ('repeat 4') has a length indicating initiation within the second telomeric repeat. The fact that two telomeric repeats are sufficient to define an origin is confirmed with additional templates discussed below.

The shortest purely telomeric DNA that had robust template activity was 5xTEL; both 5xTEL and 6xTEL gave C-strand products that were 10-11 nt shorter than the template, consistent with initiation in the second telomeric repeat instead of the third (Extended Data Fig. 1b). Thus, RNA priming can start close to the 3'-end of a telomeric DNA template; the site of initiation varies somewhat among templates, perhaps determined by which telomeric repeats form G-quadruplex structures.

The model that C-strand synthesis initiates at each telomeric repeat and continues to the end of the template was supported by two additional tests. First, quantification of the time-course showed that all products increased with the same kinetics (Fig. 1b), indicating that the smaller products were not intermediates that could be chased into the longer products. Second, adding a C-containing 10-nt extension to the 5' end of a template gave 10-nt longer run-off products, but only when dGTP was included in the reaction mix (Extended Data Fig. 1c). When we added an antisense oligonucleotide complementary to the 10-nt tail, C-strand synthesis was 10 nt shorter (Extended Data Fig. 1c), indicating that CST-Polα-primase terminated when it encountered double-stranded DNA.

A striking feature of the C-strand synthesis is the six-nt ladder of products that looks superficially like the six-nt repeats produced by telomerase. Yet our data indicate that the mechanisms are very different. With telomerase, there is a single point of initiation (the 3' end of the primer) and elongation can terminate or pause after each repeat, where telomerase must translocate to continue. With CST-Polα-primase, on the other hand, synthesis initiates at multiple sites, phased by the telomeric repeats, and then extends as far as the end of the template (Fig. 1b).

The apparent high processivity of C-strand synthesis was unexpected for Polα-primase[25]. Multiple rounds of distributive extension involving re-priming by Polα-primase represent an alternative pathway to obtain long extension products. We therefore evaluated processivity by performing reactions at increasing DNA template concentrations, such that any distributive extension would occur on a new template and reduce the product size distribution (Extended Data Fig. 2). These experiments revealed that both re-priming and processive extension were occurring, and the four-repeat products were estimated to be formed by 70% distributive/30% processive extension for both the 9xTEL and 15xTEL templates. The very large excess of templates used in vitro would not pertain in vivo, where it is more likely that the processivity of a single Polα-primase accomplishes C-strand synthesis.

CST can recruit Polα-primase to non-telomeric sequences

If telomeric repeats indeed define origins of priming and replication due to the DNA-binding specificity of CST, then C-strand synthesis should require the DNA-binding activity of CST. We tested this hypothesis with two DNA-binding mutants of the CTC1 subunit of CST, both of which still bind Polα-primase[11]. Each mutant has groups ('g') of two or three amino acids mutated. The g2.1 mutant of CST (32-fold lower affinity for 3xTEL DNA) had < 5% C-strand synthesis activity with multiple templates, while the less-impaired g3.1 mutant (15-fold down) lost about half its activity (Fig. 2a). Thus, CST-DNA binding is important for CST-Polα-primase action.

Independent evidence for the importance of CST-DNA binding for Polα-primase action came from testing poly(dT), which is used classically as a template for Polα-primase but does not bind CST[18]. Pol(dT) showed extremely low activity with CST-Polα-primase (see dT72 in Fig. 2b). According to our model, in which TEL repeats act as origins of replication for CST-Polα-primase, adding a CST-binding sequence to the 3' ends of poly(dT) templates should increase their utilization. Adding a 3xTEL sequence increased activity by 10 to 15-fold (range of 4 independent experiments; e.g., 3xTEL-dT72 and 3xTEL-dT54 in Fig. 2b). Thus, CST can recruit Polα-primase to non-telomeric sequences and greatly increase their template activity.

We next asked if fewer than three TEL repeats might suffice for an origin of replication. We found 2xTEL to be just as good an origin as 3xTEL (Fig. 2b). Because the TEL sequence begins with two T's, 2xTEL provides just 10 nt prior to the poly(dT) template. In contrast, 1xTEL was completely inactive as an origin, as indicated by the 1xTEL-dT72 and 1xTEL-dT54 templates giving the same faint signal as the dT72 control. The longest products formed with 2xTEL-dT18 and 3xTEL-dT18 were ~24 and ~30 nt, respectively (Fig. 2b), indicating that CST-Polα-primase initiated primer synthesis in the second TEL repeat.

Initiation with an RNA primer

Because the CST-directed C-strand synthesis had several unique features, we tested whether it still retained the hallmarks of a Polα-primase reaction. Classically, the primase subunit initiates with ATP, synthesizes a 6 – 10 nt RNA primer, and then hands the primer to Polα, which extends it with deoxynucleotides[21,26]. Four experiments showed that the C-strands initiated with an RNA primer. First, C-strand synthesis required ribonucleotides in addition to deoxyribonucleotides (Extended Data Fig. 1a). Second, with γ-^{32}P-ATP as the only label, products were formed on the 9xTEL and 15xTEL templates similar to those labeled with α-^{32}P-dCTP (Extended Data Fig. 3a). Because the 5'-terminal label is retained on the products, synthesis must initiate with ATP. Third, NaOH treatment to hydrolyze RNA shifted the ladders of products down by ~8 nt, indicating the approximate length of the RNA primer; treatment with RNase A caused a slightly smaller shift, as expected given its sequence specificity for pyrimidines (Extended Data Fig. 3b). Finally, reactions in the absence of deoxynucleotides allowed direct visualization of ~8-9 nt RNA primers (Extended Data Fig. 4). The 4xTEL template, which was too short to support DNA synthesis, was able to initiate RNA primer synthesis, while the 3xTEL template was not (Extended Data Fig. 4).

Problematic G-quadruplexes

Given that difficulties in replicating telomeres in vivo have been ascribed in part to G-quadruplex (GQ) structure formation, we were surprised that the TEL templates functioned so well in K^+-containing reaction buffers, known to stabilize GQs. To test if removing GQ-formation ability would affect C-strand synthesis, we retained 3xTEL as an origin of replication and then mutated the remaining TEL repeats from TTAGGG to TGAGTG; breaking up the consecutive G's prevents GQ formation[27], while the mutant sequence can still bind CST[18]. As shown in Fig. 3a, preventing GQ formation in the template greatly increased C-strand synthesis for both the 9x and 15x templates (7.0 $\pm$ 1 fold increase, mean $\pm$ range of values, n = 4). The products still have a hexameric repeat pattern, because the mutant sequence provides a CST binding site every six nt. Under slightly different conditions where the only salt was 100 mM KCl, the No-GQ templates resulted in an even greater increase in activity: 10.5 $\pm$ 1.0-fold for 9xTEL-NoGQ and 12.9 $\pm$ 0.7-fold for 15xTEL-NoGQ (means $\pm$ ranges for n = 2, compare lane 12 to 15 and lane 18 to 21 in Fig. 3b).

To validate the inhibitory effect of GQs for the unmutated TEL templates, we relied on the cation specificity of GQ formation. GQ folding is highly stabilized by K^+, which fits in the central cavity of an intramolecular quadruplex and stabilizes the partial negative charges on the carbonyl oxygens of the four guanines in each G-quartet[28,29]. GQs are much less stabilized by Na^+ [30] and not stabilized by Li^+. We first determined if changing the cation would affect the intrinsic activity of CST-Polα-primase by using the 3xTEL-dT72 template, which cannot form GQs. Li^+ reduced activity by 3.4 $\pm$ 0.4-fold relative to K^+ (mean $\pm$ range, n = 2) (Fig. 3b). A similar decrease was seen with the 9xTEL-NoGQ and 15xTEL-NoGQ templates (3.8 $\pm$ 1.0 fold, n = 4). In stark contrast, the GQ-forming templates (9xTEL, 15xTEL, 25xTEL and 30xTEL) showed a 2.9-fold increase in C-strand synthesis in Li^+ relative to K^+ (see Fig. 3c for statistical details). Correcting for the 3.4-fold lower activity of CST-Polα-primase in Li^+ relative to K^+, we found that substituting Li^+ for K^+ resulted in a 10-fold increase in activity for the GQ-forming templates. Substituting Na+ for K+ made no difference for the NoGQ templates, but gave a 3.8-fold increase in C-strand synthesis for the GQ-forming templates (Fig. 3c). Thus, CST-Polα-primase copies telomeric templates in spite of substantial inhibition by GQs.

Complete telomere replication

Having studied the ability of CST-Polα-primase to synthesize C-strands when provided with synthetic telomeric templates, we attempted to reconstitute G-strand and C-strand synthesis in a single reaction. The mixtures contained 3xTEL as a primer for telomerase; importantly, this primer is too short to give any reaction products with CST-Polα-primase (Extended Data Fig. 1a and 1b), so CST-Polα-primase will be able to make C-strands only if telomerase extends the 3xTEL to produce longer templates.

As shown in Fig. 4a, a robust ladder of C-strand products was formed even when telomerase and CST-Polα-primase were added simultaneously ("0 min") and increased when the telomerase reaction was given a head start (10, 30 or 60 min). The reactions were dependent upon addition of CST-Polα-primase, and when the CST was stripped of Polα-primase ("WT – PP") there was little reaction. Furthermore, the DNA-binding-defective g2.1 mutant had

little activity, while the binding-competent g4.1 control mutant had restored activity. Thus, coupled G-strand and C-strand synthesis have been reconstituted in a reaction containing only telomerase, a 3xTEL DNA primer, CST-Polα-primase, ribonucleotides and deoxynucleotides.

Discussion

The telomeric ssDNA overhang is a primitive replicon in that its replication does not involve a replication fork and requires only a limited number of macromolecules. As shown here, only two enzymatic components are required: the telomerase ribonucleoprotein complex and CST-Polα-primase. Furthermore, any pair of TTAGGG repeats can act as an "origin of replication" or, more accurately, an origin of RNA priming and C-strand DNA synthesis. Note that the reaction products resemble a natural telomere, with double-stranded telomeric repeats and – because the CST-binding site itself is not copied – a 3'-overhang of the G-strand. The shortest 3'-overhangs produced in this system are only about 2 telomeric repeats, and they would either need to be extended further by telomerase or subjected to C-strand resection to give the 100 – 300 nt G-overhangs seen in vivo[31].

This is a minimum replication system, in that only the ssDNA portion of the telomere is replicated, the RNA primer is not removed, and nucleosomes and the shelterin complex are not included. The present work provides a basic framework upon which addition of these other components could be explored.

Previously, CST was known to have two functions in telomere replication: termination of telomerase extension of the G-strand[10,11], and recruitment of Polα-primase to begin replication of the C-strand[4,12,13]. We now find that CST has a third role: specifying the start sites of C-strand synthesis by binding to telomeric ssDNA in a conformation where bound Polα-primase can access the DNA template. Remarkably, the mutants of CST that are defective in ssDNA binding have reduced ability to deposit their Polα-primase, and replication is substantially impaired. Thus, the reconstituted replication system now makes it clear why the partnership between CST and Polα-primase is so important to telomere replication. The fact that CST binds well to the telomeric repeat sequence allows it to position primase for initiation and then move along the telomeric DNA template with Polα.

Numerous studies have found that telomeric DNA presents difficulties for replication, including problems with replication fork stalling (reviewed in ref.[32]). Replication of both the ds and ssDNA regions of telomeres could contribute to these difficulties. GQ structure formation has been suggested as one potential contributor, but this is difficult to test in vivo. Now, with our reconstituted biochemical system, we show that GQ formation in the G-strand template is in fact a barrier to telomeric C-strand synthesis; preventing GQ formation either by mutating the telomeric repeat sequence or by replacing K^+ with Li^+ greatly improves C-strand synthesis. What allows the telomeric repeats to serve as a template is their tight binding to CST; this results in the telomeric DNA being a much better template than poly(dT), which has the advantage of being unstructured but has little affinity for CST.

This work also has implications for the mechanism by which CST restarts stalled replication forks genome-wide. We find that even short G-rich sequences can bind CST sufficiently to promote Polα-primase action, and we suggest that most stalled replication forks would present such a sequence. Indeed, if a replication fork stalled because of GQ structure formation, it would necessarily have at least four G tracts, and thus would recruit CST-Polα-primase in the same manner we have observed in the case of telomeric ssDNA.

Because telomere maintenance is required for cancer cell immortality, telomere end-replication components may represent targets with specificity for tumor cells over normal cells, especially for short-telomere cancers. In support of this idea, the three genes for the CST components rose to the very top in CRISPR-Cas9 screens for genes important for growth of cancer cell lines with short telomeres [33]. Based on the work presented here, the ssDNA-binding site in the CTC1 subunit[19] or Polα-primase-binding sites seem particularly attractive targets for interfering with telomere end-replication.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://XXXXX.

Methods

Oligonucleotides

DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and resuspended in water. Their concentrations were then measured by determining UV spectra (NanoDrop One, Thermo Fisher Scientific, Waltham, MA) using extinction coefficients provided by IDT. DNA oligonucleotides are named for the number of consecutive telomeric repeats [e.g., 9xTEL is $(TTAGGG)_9$] or mutated telomeric repeats [e.g., 9xTEL-NoGQ is $(TGAGTG)_9$]. Chimeric template sequences are named in the 5'-to-3' polarity with respect to C-strand synthesis, which is therefore 3'-to-5' with respect to the template [e.g., 3xTEL-dT72 is a 90-nt oligo with the sequence 3'-GGGATTGGGATTGGGATT-T_{72}-5'].

Expression and purification of proteins in human cultured cells

CTC1 cDNA (MGC: 133331) with an N-terminal 3xFLAG tag, STN1 cDNA (MGC: 2472) with an N-terminal Myc tag, and TEN1 cDNA (MGC: 54300) with an N-terminal HA tag were each cloned into the pcDNA mammalian expression vector (V79020, Thermo Fisher Scientific). The three plasmids were transfected into HEK239T cells (CRL-1573, ATCC, Manassas, VA) at 1:1:1 molar ratio using lipofectamine 2000 (11668019, Thermo Fisher Scientific). The cells were further grown in DMEM medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum for 24 h after transfection (typically three-fold expansion) and then harvested. Protein purification proceeded as described[11] and involved successive anti-FLAG and anti-HA immunopurifications. Purity of CST complexes was verified with SDS-PAGE using a silver

staining kit (24612, Thermo Fisher Scientific). To prepare CST stripped of Polα-primase, the NaCl concentration in the wash and elution buffers was increased from 150 mM to 300 mM. The presence of CST subunits and Polα-primase subunits in the purified complexes was determined by western blotting using the antibodies listed[11]. CST protein concentrations were determined by western blot analysis with anti-STN1 antibody (NBP2-01006, Novus Biologicals, Centennial, CO) using a serial dilution of the HEK-cell CST preparation and a standard curve obtained by serial dilution of an insect cell-purified CST standard (See ref. [11] for further discussion.)

C-strand synthesis reactions

Unless indicated otherwise, the following were the standard reaction conditions. HEK cell CST (25 – 50 nM) and ssDNA templates (50 – 100 nM) were incubated at 30°C for 1 h in 50 mM Tris-HCl pH 8.0, 50 mM KCl, 75 mM NaCl, 2 mM $MgCl_2$, 1 mM spermidine, 5 mM β-mercaptoethanol, 0.5 mM dATP, 0.5 mM dTTP, 0.33 μM [α-^{32}P]dCTP (300 Ci mmol^{-1}), 0.29 μM cold dCTP, and 0.2 mM each rNTP. When labeling with dATP, cold dATP was decreased to 10 μM and 0.33 μM [α-^{32}P]dATP (300 Ci mmol^{-1}) was added to the reaction instead of 0.33 μM [α-^{32}P]dCTP (300 Ci mmol^{-1}). When labeling with [γ-^{32}P]ATP, the cold ATP concentration was lowered to 25 μM. After incubation at 30°C, 100 μl of Stop Mix (3.6 M NH_4Acetate, 0.2 mg ml^{-1} glycogen, and a 16 nt ^{32}P-labeled loading control) and 500 μl ethanol were added to each 20 μl reaction. After incubating at -80°C for 30 min or more, the products were pelleted, washed with cold 70% ethanol, dried, then dissolved in 10 μl H_2O plus 10 μl 2x loading buffer (93% formamide, 30 mM EDTA, 0.1X TBE, and 0.05% each of bromophenol blue and xylene cyanol). Samples (9 μl) were loaded on a sequencing-style 10% acrylamide, 7 M urea, 1x TBE gel. The bromophenol blue dye was run to the bottom of the gel (about 1.75 h). The gel was removed from the glass plate using Whatman 3mm paper, then dried at 80°C under vacuum for 15- 30 min. Radiolabeled C-strand synthesis products were imaged on a Typhoon FLA9500 scanner (GE Lifesciences) and analyzed by ImageQuant TL v.8.1.0.0 (GE Lifesciences). Unless indicated otherwise, CST-Polα-primase activity was determined by total counts per lane corrected for any variation in the loading control. In some cases, individual reaction products were quantified.

Telomerase purification and reactions

Telomerase reactions were run to provide marker lanes for the C-strand synthesis reactions. Human telomerase expression and purification followed ref.[34]. To produce body-labeled products, telomerase was incubated at 30 °C for 1 h with 0.1 μM unlabeled 3xTEL DNA primer in 50 mM Tris-HCl pH 8.0, 50 mM KCl, 75 mM NaCl, 2 mM $MgCl_2$, 1 mM spermidine, 5 mM β-mercaptoethanol, 0.33 μM [α-^{32}P]dGTP (3000 Ci mmol^{-1}), 2.9 μM cold dGTP, 0.5 mM dATP, and 0.5 mM TTP. To produce end-labeled products, the reactions were identical except 5'-^{32}P-end-labeled 3xTEL DNA was substituted for the unlabeled primer, radiolabeled dGTP was omitted, and the cold dGTP concentration was 3.3 μM. For shorter products (< 30 nt), the body-labeled products have reduced gel mobility relative to the end-labeled products by one nt, because the latter products have an additional phosphate on their 5' ends. For long products (> 60 nt) the mobilities of the two sets of products converge. Note that neither set of telomerase products provides perfect markers for the C-strand products, because the latter initiate with a triphosphate; in addition, electrophoretic mobility is somewhat base-sequence dependent.

Thus, most of our size estimates are prefaced by the symbol ~ indicating an uncertainty of ± 1 nt.

Complete telomere replication reactions

G-strands were synthesized by incubating immunopurified human telomerase (2.0 nM) and 3xTEL DNA primer (20 nM) at 30 °C in 50 mM Tris-HCl pH 8.0, 50 mM KCl, 75 mM NaCl, 2 mM $MgCl_2$, 1 mM spermidine, 5 mM β-mercaptoethanol, 0.5 mM dATP, 0.5 mM dTTP, 3.3 μM dGTP, 2.9 μM dCTP, 0.33 μM ^{32}P dCTP and 0.2 mM each rNTP. At indicated times between 0 and 60 min, 19 μl was transferred to a tube containing 1 μl of CST (WT+pp, WT-pp, or g2.1; 50 nM final concentration) to initiate the synthesis of C-strands. After an additional 1 h incubation at 30 °C, 100 μl of Stop Mix (3.6 M NH_4AC, 0.2 mg ml^{-1} glycogen) and 500 μl ethanol were added to the 20 μl reaction. Ethanol precipitation, gel electrophoresis, and phosphorimager scanning then proceeded as described above for C-strand synthesis reactions.

Data availability statement

Primary data that are necessary to interpret, verify and extend the research in this article are freely available upon request to the authors.

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Author contributions

A.J.Z. and T.R.C. worked together to design and interpret the experiments, which were performed by A.J.Z.

Competing interests

T.R.C. is a scientific advisor for Storm Therapeutics and Eikon Therapeutics.

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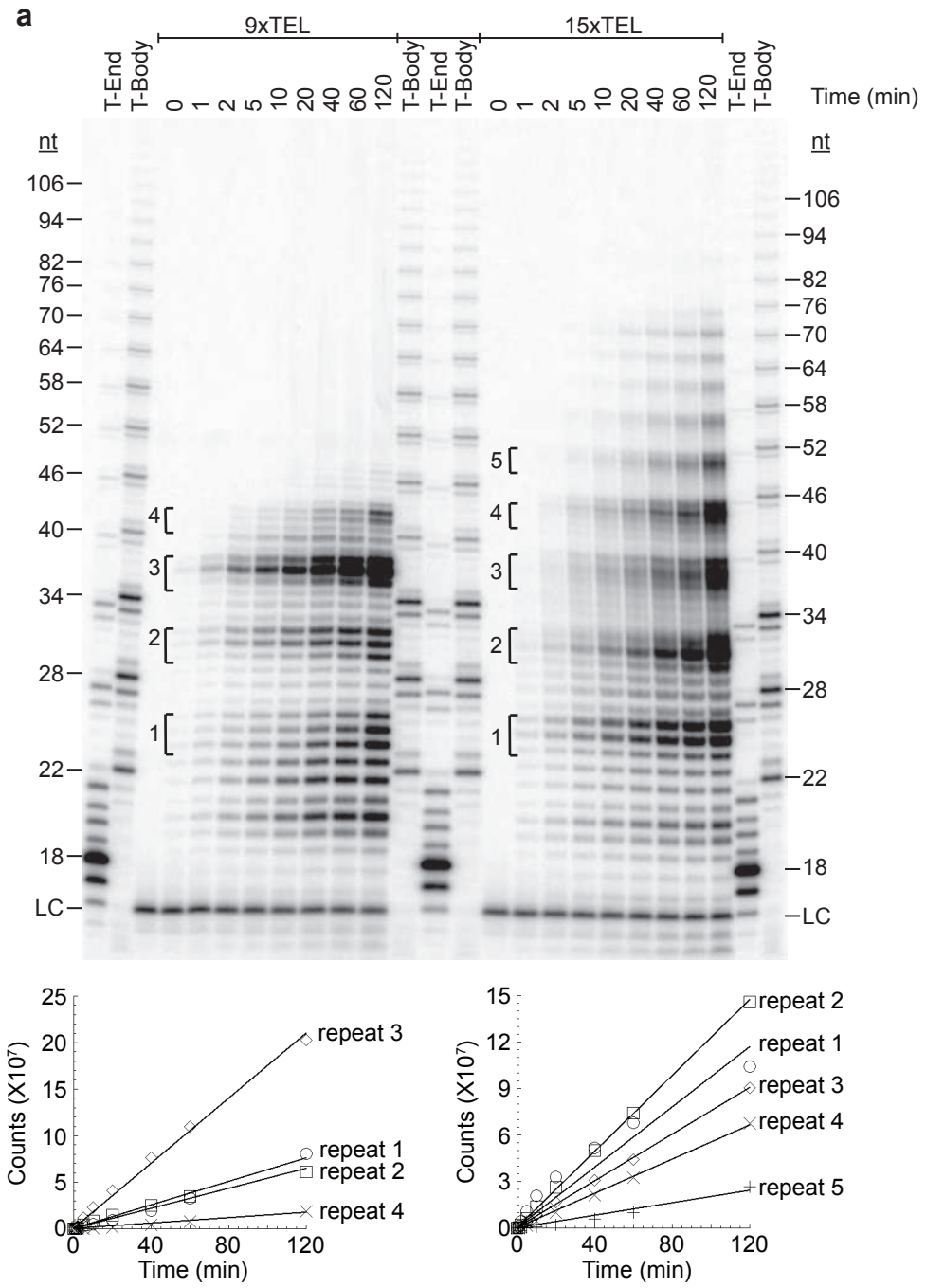
Fig.1


a
9xTEL
15xTEL
T-End
T-Body
0
1
2
5
10
20
40
60
120
Time (min)
nt
106
94
82
76
70
64
58
52
46
40
34
28
22
18
LC
1
2
3
4
5
Counts (X10⁷)
Time (min)
repeat 1
repeat 2
repeat 3
repeat 4
repeat 5

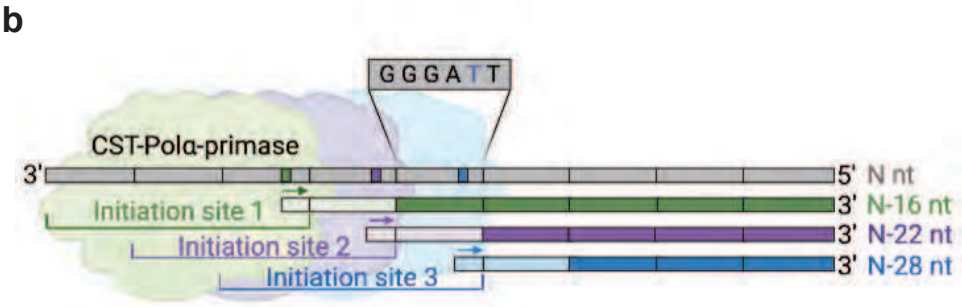

b
GGGATT
CST-Polα-primase
3'
5' N nt
Initiation site 1
3' N-16 nt
Initiation site 2
3' N-22 nt
Initiation site 3
3' N-28 nt

Fig. 1 | CST-Polα-primase uses telomeric repeats as origins of priming and replication.

a, Time courses of C-strand synthesis on telomeric DNA templates, products labeled with α-^{32}P-dCTP. Size markers are telomerase reaction products with end-labeled primer (T-End, 5'-phosphate) and body-labeled products (T-Body, 5'-hydroxyl) as described in Methods. LC, labeled oligonucleotide loading control. (Below) Quantification of C-strand synthesis products in the experiment directly above. **b**, Model explaining the observed ladders of C-strand reaction products. CST-Polα-primase binds the template (gray) at different sets of telomeric repeats (three alternatives shown as green, violet and blue), such that run-off synthesis gives products in six-nt increments. In each case, the RNA primer is indicated by a lighter shade, and telomeric repeats are indicated by vertical bars. Not shown: CST can also bind to some extent to the two terminal repeats, giving products six-nt longer than those produced by the binding modes illustrated here.

Fig. 2

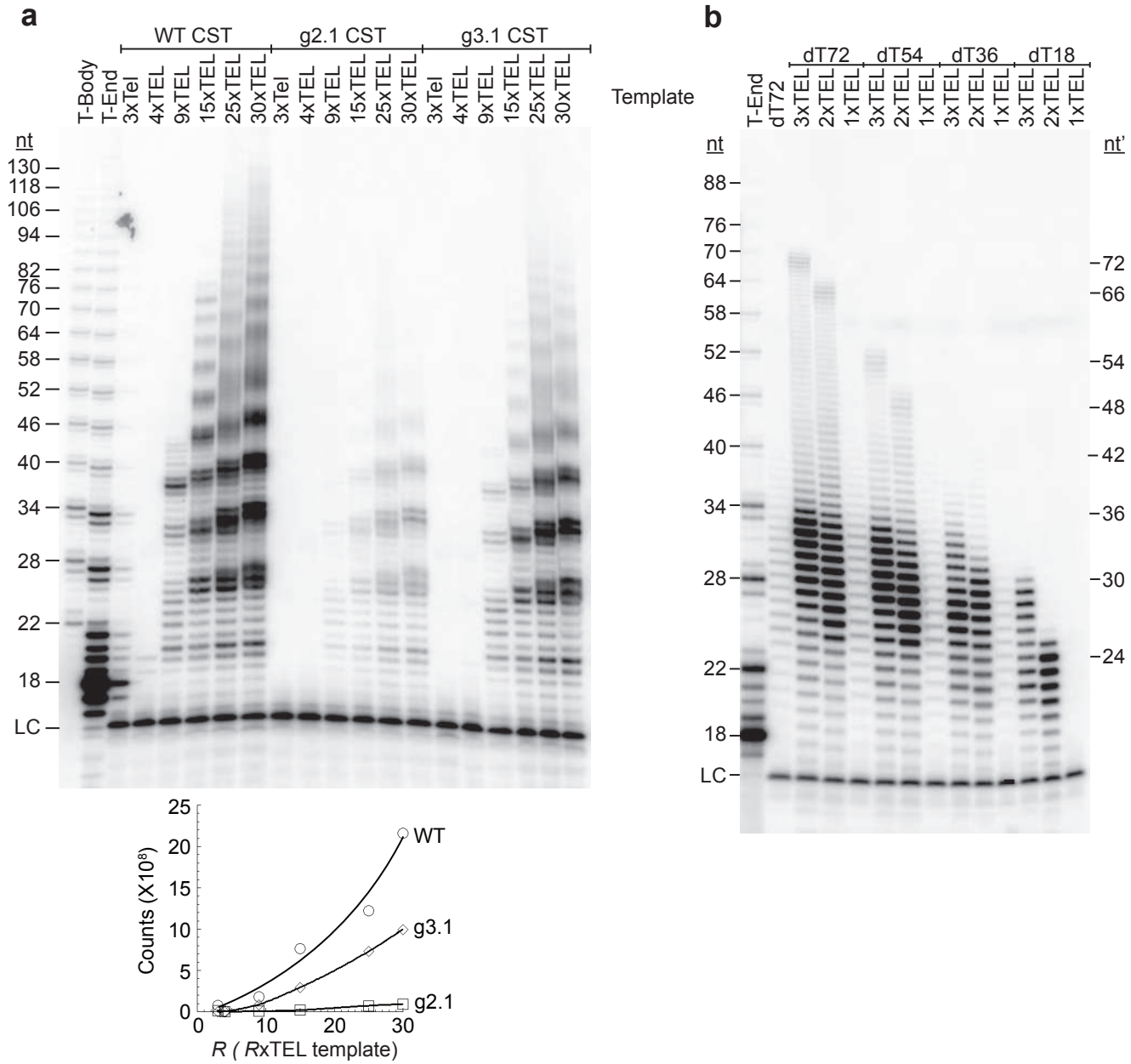
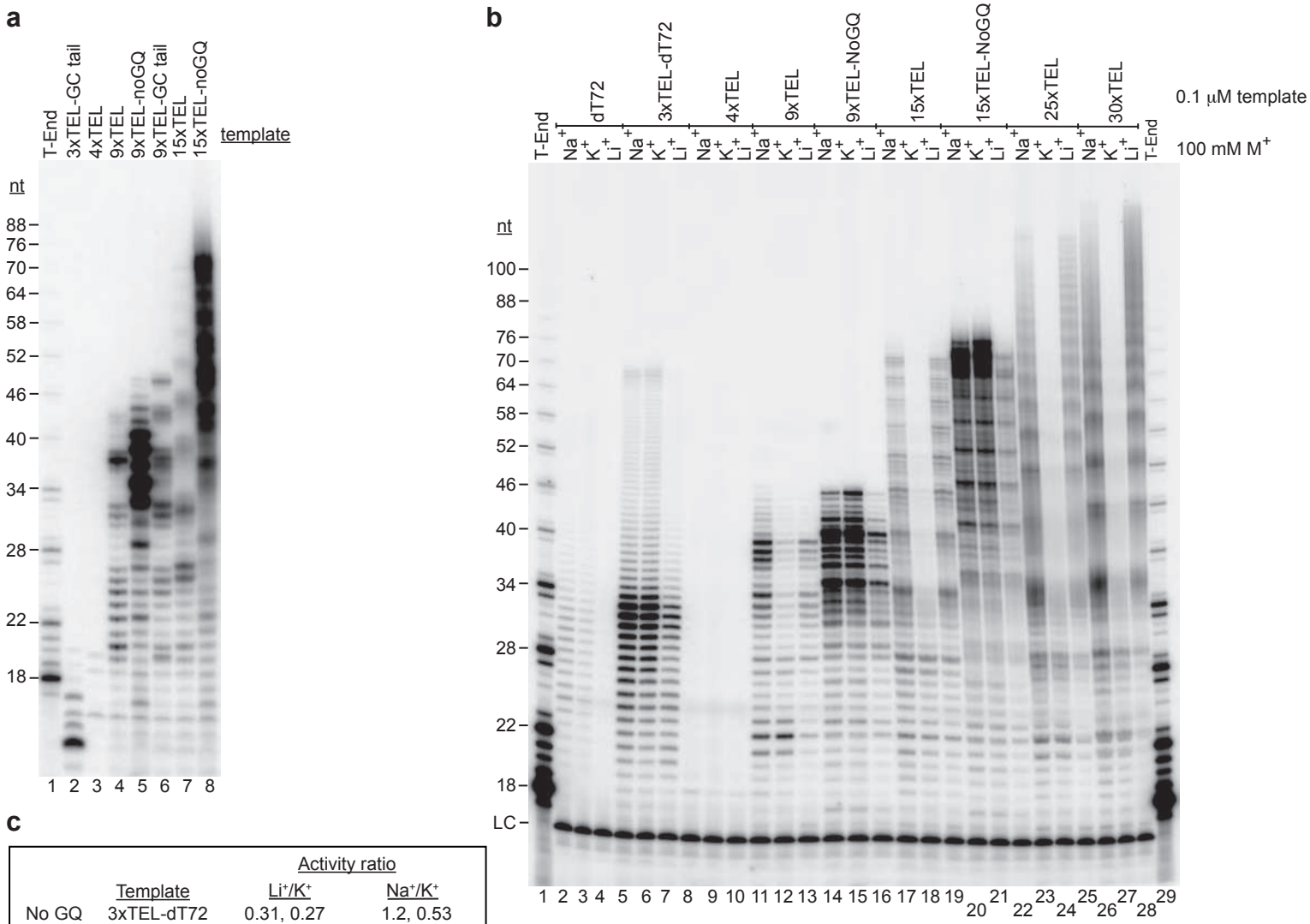


Fig. 2 | C-strand synthesis requires DNA binding by CST-Polα-primase, and a CST-Polα-primase binding site greatly enhances replication of a poly(dT) template. **a**, C-strand synthesis for 1 h with DNA templates (100 nM) comprising increasing numbers of telomeric repeats catalyzed by WT and two different DNA-binding mutants of CST-Pol α-primase (20 nM). The apparent products in lane 3 (WT CST and 3xTEL template) are spill-over from the adjacent marker lane; repeat experiments confirmed that there is no product formed. LC, Loading control. Below, quantification of the experiment of panel (**a**). Total incorporation, normalized to the loading control, as a function of the number of telomeric repeats in the template. **b**, Replication products of CST-Polα-primase on a poly(dT) template, dT72, and on templates with various numbers of telomeric repeats added to the poly(dT) sequence.

Fig. 3



c

	Template	Activity ratio Li^+/K^+	Activity ratio Na^+/K^+
No GQ	3xTEL-dT72	0.31, 0.27	1.2, 0.53
	9xTEL-NoGQ	0.28, 0.32	1.0, 1.1
	15xTEL-NoGQ	0.28, 0.21	1.0, 0.78
	Mean±SD	0.28±0.04	0.94±0.24
GQ	9xTEL	1.2, 3.1	3.4, 3.2
	15xTEL	3.3, 2.6	3.4, 2.6
	25xTEL	2.2, 2.7	4.2, 3.5
	30xTEL	4.4, 3.5	5.9, 3.9
	Mean±SD	2.9±0.09	3.8±1.0
GQ/No GQ		10.4*	4.0*

Fig. 3 | C-strand synthesis overcomes G-quadruplex structures in the template DNA.

a, Mutating the telomeric repeat sequence (no GQ) greatly increases C-strand synthesis. Compare lanes 4 and 5, 7 and 8. The GC-tail templates have added 10-nt GC-sequences on their 5' ends to test if the C-strand synthesis proceeds to the end of the template. Compare lanes 4 and 6. Although 3xTEL is inactive, adding the 10-nt tail to 3xTEL provides a template for C-strand synthesis (lane 2). **b,** C-strand synthesis when GQ structures are destabilized (LiCl) or stabilized (KCl). NaCl is an intermediate condition. CST-Polα-primase at 25 nM, DNA templates at 100 nM. **c,** Activity with 100 mM LiCl relative to that in 100 mM KCl, or 100 mM NaCl relative to 100 mM KCl, quantification of two experiments including that in panel (**b**). *P value <0.0001, two-tailed t-test.

Fig. 4

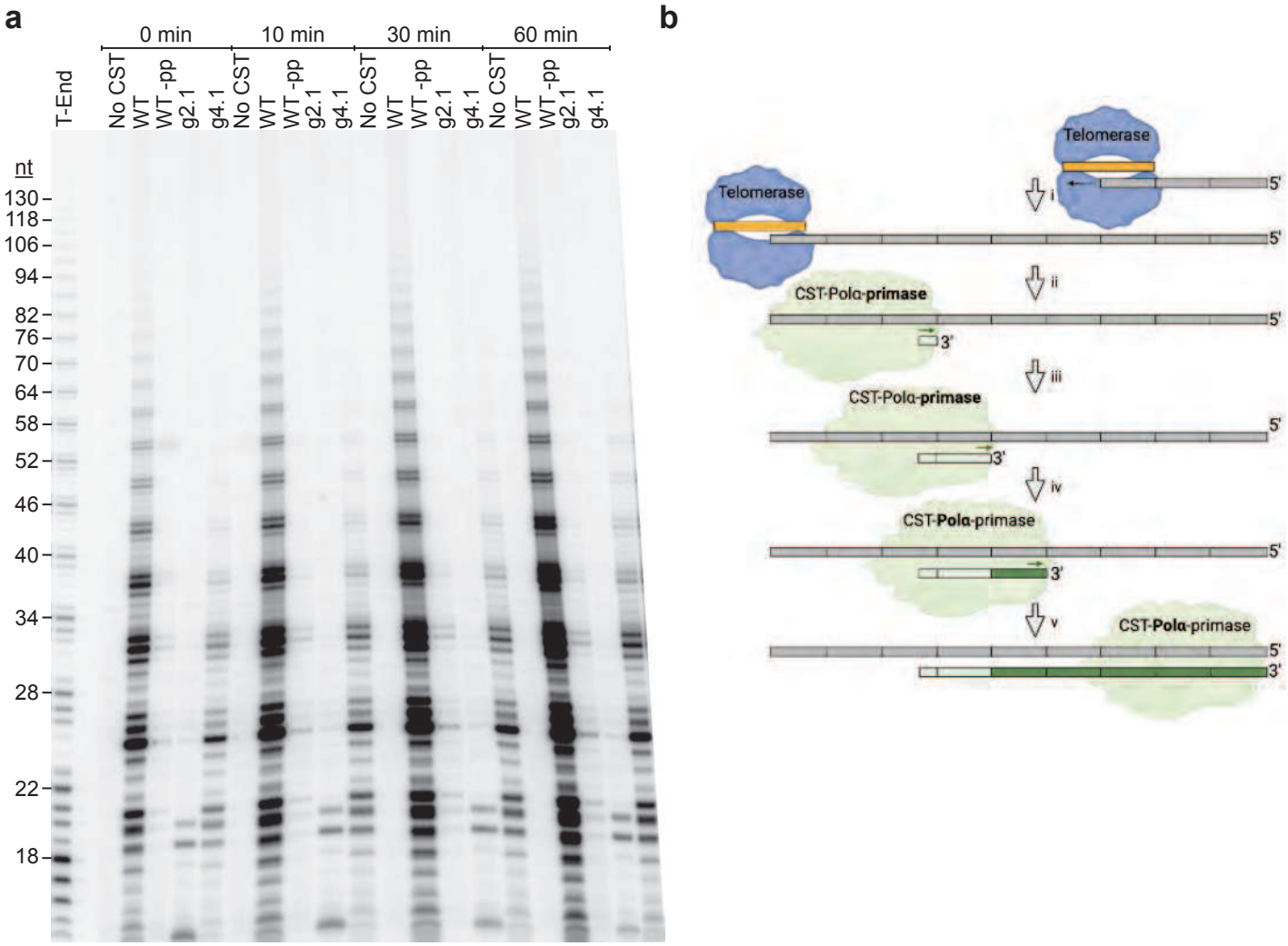
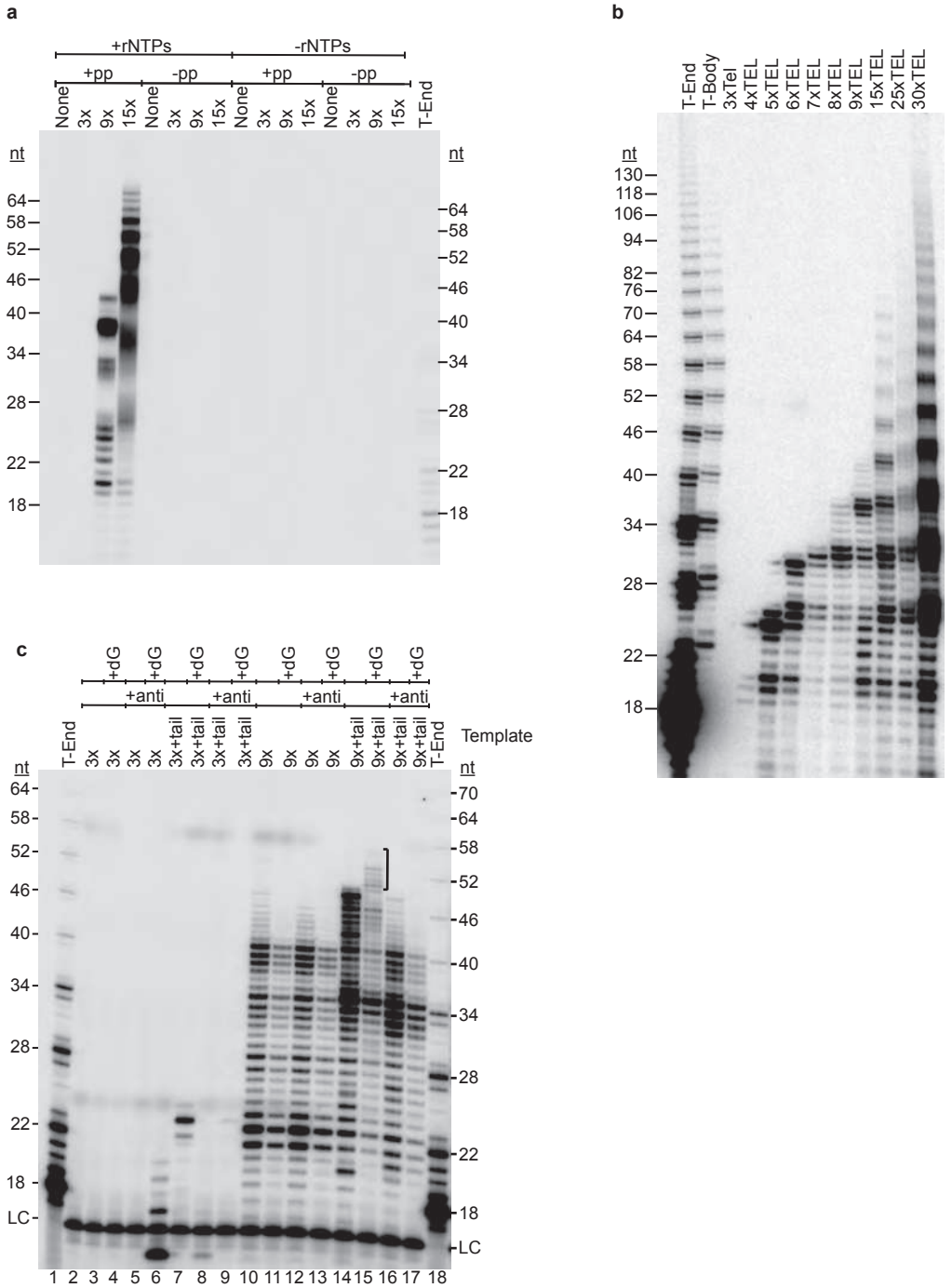
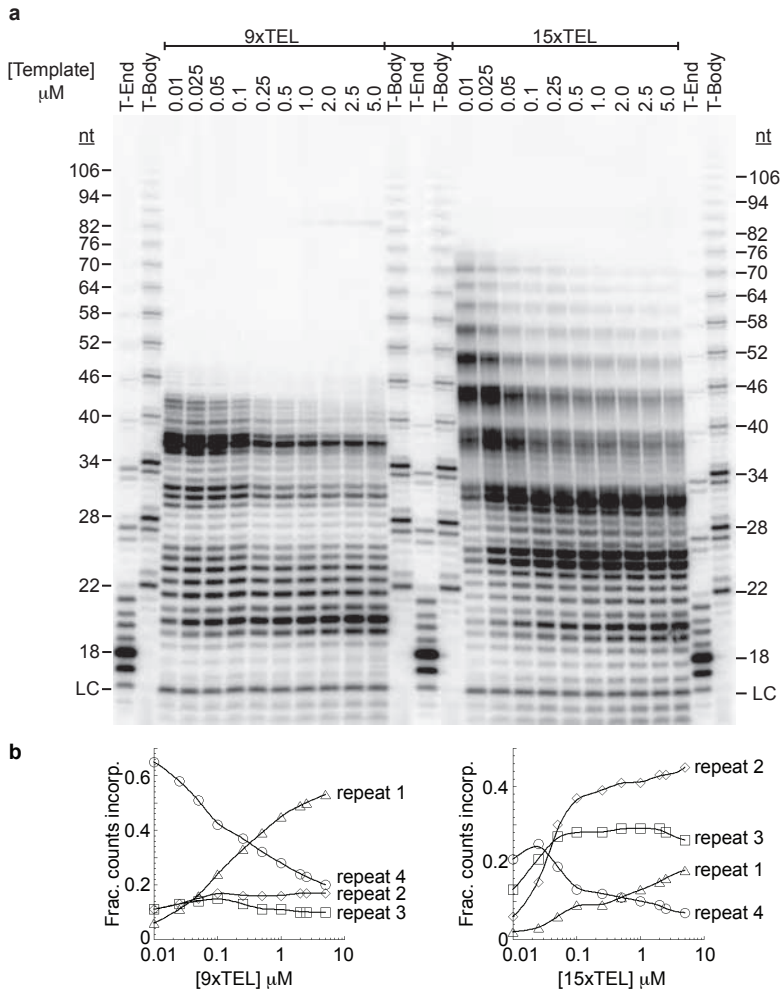


Fig. 4 | Reconstitution of complete telomere replication.
a, C-strands were labeled with α-^{32}P-dCTP. Time between initiation of telomerase reaction and addition of CST-Polα-primase is indicated at top. CST-Polα-primase is present at 75 nM. WT – pp is CST largely depleted of Polα-primase by conducting IP purification at high (300 mM) salt concentration. g2.1 is a DNA-binding defective mutant of CST, and g4.1 is a control mutant of CST that retains DNA binding but is present at only one-third the concentration as WT CST due to lower yield during purification.
b, Model for reconstituted telomere replication. (i) Telomerase (RNA template in orange) binds to the 3xTEL DNA primer (gray) and extends it with telomeric repeats (gray rectangles). (ii) Telomerase dissociates, and CST-Polα-primase (green) binds to telomeric repeats and begins RNA primer synthesis with ATP. (iii) Primase synthesizes RNA primer, ~ 8 nt. (iv) Template-primer pair is handed off to Polα, which catalyzes C-strand DNA synthesis (green bar). (v) Polα continues DNA synthesis, with its association to the template stabilized by CST binding to telomeric repeats at each step.

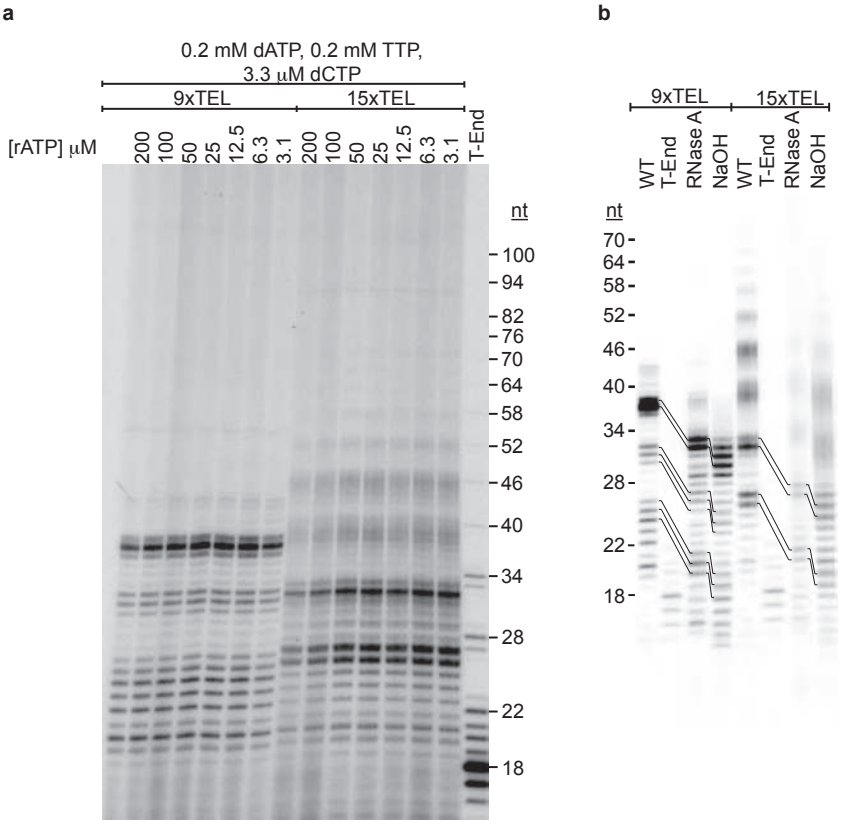
Extended data figures



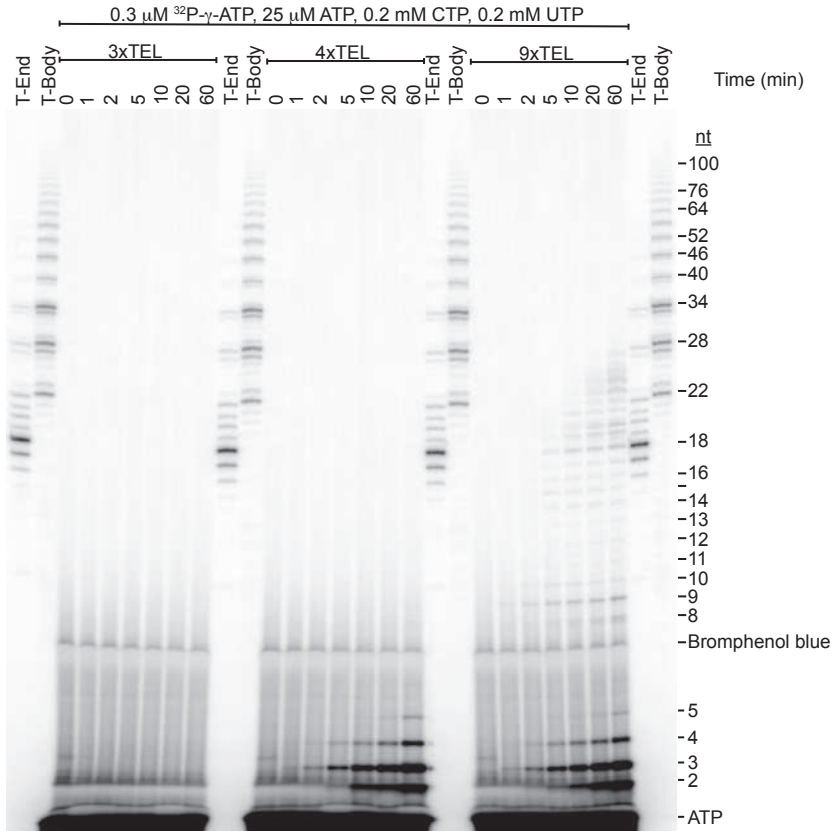
Extended Data Fig. 1 | Validation of the C-strand synthesis reaction on telomeric DNA templates.
a, Product formation requires DNA template, CST-Polα-primase, and ribonucleotides (rNTPs). +pp: CST-Polα-primase. -pp: CST with Pol α-primase removed by 300 mM NaCl elution. DNA templates are None, or 100 nM 3xTEL, 9xTEL and 15xTEL. Nucleotide (nt) sizes are based on telomerase reaction with a 5'-end-labeled primer (T-End). **b**, Longer DNA templates give larger ladders of C-strand products. Templates consist of the number of telomeric repeats indicated. 3xTEL and 4xTEL do not support DNA synthesis. T-Body is a marker lane of body-labeled telomerase products, which run about one nt slower than the T-End products; the T-End products have a 5'-phosphate which increases their electrophoretic mobility. **c**, Adding a 10-nt tail to the 5' ends of templates confirms that at least some products run off the end of the template, and adding an antisense oligonucleotide to pair with the 10-nt tail shows that C-strand synthesis stops at double-stranded DNA. (Lanes 1 – 4) 3xTEL, which binds CST-Polα-primase, is too short to support C-strand synthesis. (Lanes 6 – 9) 3xTEL with a 10-nt tail now synthesizes a C-strand product, which is fully extended when dGTP is included in the reaction mix, and which is eliminated by adding the antisense oligonucleotide that base-pairs with the tail. (Lanes 10 – 13), 9xTEL C-strand products are the same length independent of dGTP or antisense oligonucleotide (the bands are fainter in lanes 11 and 13, but the product size distribution is the same). (Lanes 14 – 17), 9xTEL with a 10-nt tail now show longer extension upon inclusion of dGTP, but that additional extension is eliminated when the tail becomes base-paired with the antisense oligonucleotide. LC, loading control.



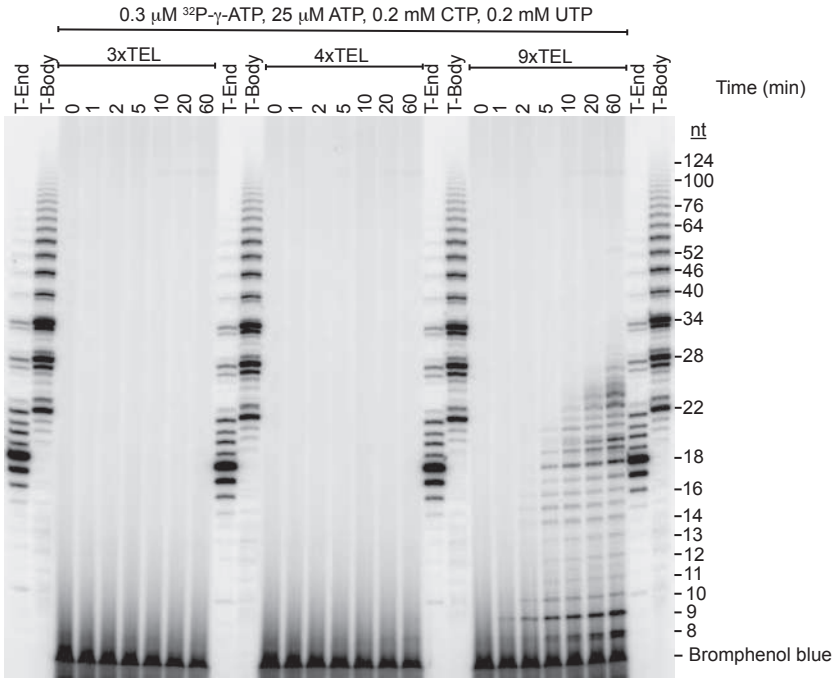
Extended Data Fig. 2. | Increasing DNA template concentration to test for processive C-strand synthesis versus distributive re-priming.
a, Reaction of 25 nM CST-Polα-primase with increasing concentrations of 9xTEL or 15xTEL DNA template. **b**, Quantification of groups of extension products ("repeats") from (**a**). Numbering of repeats is the same as in Fig. 1a. Increase of counts of repeat 1 products with increasing template concentration is interpreted as distributive re-priming, whereas the fraction of repeat 4 or 5 products that persists at high template concentration (1 μM template is 40-fold excess over concentration of CST-Polα-primase) is interpreted as processive extension.



Extended Data Fig. 3 | RNA primers initiate with ATP and are approximately 8 nt long.
a, Reactions with γ-^{32}P-ATP (trace concentration) as the only labeled nucleotide, with increasing concentrations of unlabeled ATP as indicated. Label incorporation decreases at 100 and 200 μM rATP, because above K_m, dilution of the radiolabeled rATP is no longer compensated by increased reaction velocity. **b,** Hydrolysis of the RNA primer with NaOH or RNase A shortens the dC-labeled C-strand. WT, wild-type CST-Polα-primase, T-End, 5'-end labeled telomerase reaction products as size markers. RNase A, reaction products from lane WT were boiled and then treated with RNase A. NaOH, reaction products from lane WT were treated with NaOH. The 8-nt reduction in dC-labeled product size upon NaOH treatment indicates that the C-strands initiate with pppAACCCUAA/dCdC-dC…, where the RNA primer is underlined and the slash indicates the 3'-most site of hydrolysis. RNase A, which cleaves after pyrimidines, would then cleave as follows: pppAACCU/AAdCdC-dC…. This would result in the RNase A products being two nt longer than the NaOH products, as observed. The lines connecting the bands on the gel indicate the reduction in product size upon NaOH or RNase A treatment.



4 hr exposure



4 day exposure

Extended Data Fig. 4 | RNA primers synthesized by CST-Polα-primase on three different DNA templates, directly visualized by omitting deoxynucleotides from the reaction.
Top half, production of short RNA oligonucleotides initiating with pppA on the 4xTEL and 9xTEL DNA templates. Their mobilities are consistent with 2, 3 and 4 nt, as indicated, although these sizes have not been independently confirmed. These products may represent abortive initiation by primase. Bottom half, the bottom of the gel containing the unincorporated ^{32}P-ATP was cut off to allow a longer exposure of the larger products. The 9xTEL template allowed synthesis of RNA primers of length 8 and 9 nt; size estimates have +/- 1 nt uncertainty because they are based on the telomerase size markers, which have a different 5' end and different base composition. There are also some longer products formed (14 – 27 nt), presumably indicating some ability of Polα to incorporate ribonucleotides when there are no dNTPs in the reaction. The 4xTEL template, which supported synthesis of the putative abortive initiation products, was too short to allow synthesis of the 8 – 9 nt primers.