ONLINE METHODS

RNA construct design and preparation. Templates for RNA mutants were cloned using QuikChange (Agilent) with ptetTELO as the template[32]. The cpRNA constructs were generated using two rounds of PCR with ptetTELO as the template and then inserted into pUC19; the sequence of the linker joining the natural 5' and 3' ends of TER is TTTTGGATCC[15]. RNA was synthesized by T7 RNA polymerase in run-off transcription of EarI-digested plasmids containing the template for TER, TER mutants or cpRNAs. Transcriptions ran overnight at 37 °C prior to hammerhead cleavage with 25 mM $MgCl_2$ at room temperature for 1 h. Samples were then ethanol precipitated prior to purification on a denaturing gel (6% v/v polyacrylamide, 7 M urea and 1X TBE). Purified RNAs were eluted and concentrated in 5 mM sodium cacodylate, pH 6.5.

Construction of dye-labeled telomerase RNA. Cy5-labeled telomerase RNA was constructed as described[33]. Briefly, synthesized RNA fragments harboring a site-specific reactive amine group (Dharmacon) were conjugated to amine-reactive Cy5 (GE Lifesciences) and purified by reverse phase HPLC on a C8 column. Full-length telomerase RNAs were generated by DNA-splinted RNA ligation with T4 DNA ligase (NEB). Oligonucleotides used had the following sequences: TER 1-31 fragment 5'-AUACCCGCUUAAUUCAUUCGAUCUGUAAUA, U36 TER 32-95 fragment 5'-P-GAAC[5-N-U]GUCAUUCAACCCCAAAAAUCUAGUGCUGAUAUAACCUUCACCAAUUAGGUUCAA-AUAAG, U63 TER 32-95 fragment 5'-P-GAACUGUCAUUCAACCCCAAAAAUCUAG-UGC[5-N-U]GAUAUAACCUUCACCAAUUAGGUUCAAAUAAG, TER 96-159 fragment 5'-P-UGGUAAUGCGGGACAAAAGAC UAUCGACAUUUGAUACACUAUUUAUCAAUGGA-UGUCUUAUUUU, Stem III DNA Splint 5' TGTCCCGCATTACCACTTATTTGAACCTAA 3', Stem II DNA Splint 5' GTTGAATGACAGTTCTATTACAGATCTGAA 3'

Telomerase reconstitution and immunoprecipitation. Telomerase was reconstituted using the TnT Quick Coupled Transcription-Translation System (Promega) as previously described, with ^{35}S-methionine (Perkin Elmer) included for quantitation[16,34]. p65 was purified as previously described[16]. Final RNA and p65 concentrations in the translation reactions were 400 nM and 2 μM, respectively. Assembled telomerase was immunoprecipitated with T7-tag antibody-conjugated agarose (Novagen). ^{35}S-methionine was quantitated using a scintillation counter, and all samples were diluted with 1x tTB (50 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, 5 mM DTT)+ 30% v/v glycerol to normalize for the amount of ^{35}S-TERT.

PAGE purified full-length Cy5-labeled RNAs were pre-bound with recombinantly expressed and purified p65, and reconstituted in the TnT system with FLAG-labeled TERT. Telomerase reconstituted with dye-labeled RNA showed comparable activity to wild-type RNA as previously described[19]. Telomerase was purified by immunoprecipitation with anti-FLAG resin (Sigma) and eluted with FLAG peptide (Sigma) as described[34].

Direct telomerase activity assays. Telomerase activity assays were performed as previously described[16,34]. After incubating for 1 h at 30 °C, reactions were stopped

with 3.6 M sodium acetate, pH 5.2, 1 mg glycogen and 2,000-6,000 counts of a recovery control (a 5'- end labeled and gel-purified 63-mer DNA oligonucleotide of random sequence), with the exception of the assays shown in Supplementary Fig. 2b, which were incubated at 30 °C for 1.5 h to maximize signal. Reactions conducted at the same time received equal counts of recovery control.

Real time detection of telomerase binding to DNA primers by smFRET. smFRET-based telomerase binding assays were conducted as previously described[22]. Biotinylated primers (IDT) labeled at the n-2 alignment residue with Cy3 (GE Lifesciences) were immobilized on a quartz slide through a biotin-streptavidin linkage (biotinylated polyethylene glycol from Laysan Bio, streptavidin from Invitrogen). Telomeric primers used: D, Biotin – TGTGTGTGTGTGTGTG [5-Amino-C6-dT] TGGG; E, Biotin – TGTGTGTGTGTGTGTG [5-Amino-C6-dT] TGGGG; F, Biotin – TGTGTGTGTGTGTGTG [5-Amino-C6-dT] TGGGGT; A, Biotin – TGTGTGTGTGTGTGTG [5-Amino-C6-dT] TGGGGTT; B, Biotin – TGTGTGTGTGTGTGTG [5-Amino-C6-dT] TGGGGTTG.

Immunopurified telomerase complexes were flowed over immobilized primers in telomerase binding and imaging buffer (20 mM Tris pH 8.0, 5 mM $MgCl_2$, 10% w/v glucose, 10% v/v glycerol, 2 mM trolox, 1μg ml^{-1} catalase, and 1.5 mg ml^{-1} glucose oxidase). Binding events were identified by anti-correlated changes in donor and acceptor dye fluorescence detected using a prism-type TIRF microscope and an Andor Ixon CCD camera using 100 ms integration time. FRET was measured over the course of the binding event using the formula $E = 1/[1+\gamma(I_D/I_A)]$, where E is FRET efficiency, I_D is donor intensity and I_A is acceptor intensity. γ is a factor that adjusts for differences in dye quantum yields, and can be useful in correcting FRET efficiency when there is a protein-induced Cy3 enhancement as was observed in a subset of our traces. Because we cannot distinguish between acceptor bleaching events and enzyme dissociation from the primer, we could not determine γ by the previously established method[35]. Instead, we approximated γ as $(I_{D1} + I_{A1})/(I_{D2} + I_{A2})$ where $I_{D1} + I_{A1}$ represents the sum of the donor and acceptor intensity before protein binding and $I_{D2} + I_{A2}$ represents the sum of the donor and acceptor intensities after binding. γ was determined individually for each trace and was consistent with previously reported values of protein-induced Cy3 enhancement[35]. There was no correlation between the mean dyesum intensity and the width of the FRET histogram in our experiments, demonstrating that differences in the widths of the FRET distributions are not due to variations in the signal intensity.

We do not know the extent to which the environment of the dye might hinder its rotational freedom, affecting the observed FRET efficiencies. We cannot measure dye anisotropy accurately, as any measurement would include signal from inactive or unbound complexes. For other systems, deviations in anisotropy values have not been observed to change the direction of the FRET efficiency trend[36], and in our case it seems unlikely that such a photo-physical artifact would coincidentally give rise to the opposite changes in FRET that we observe for the two TER labeling sites.

Each experiment consisted of traces obtained from 40 200-second movies, which generated between 23-106 separate binding events. Binding event traces

were analyzed in Matlab (Mathworks) and each experiment was used to generate histograms consisting of all the FRET values observed at each time point over the course of all of binding events. Gaussian distributions were fit to the histograms to determine the center of the distribution. Plotting and fitting of the smFRET data was performed in Origin (Originlab). The experiment was repeated in triplicate for each primer permutation with both RNA labeling sites.

Figure preparation. Figures were generated in Adobe Illustrator.

Methods only references

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