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Supplemental Data

An RPA-Related Sequence-Specific DNA-Binding Subunit of Telomerase Holoenzyme Is Required for Elongation Processivity and Telomere Maintenance

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Strain Construction

Gene knockout constructs targeted the entire coding region for replacement by neo2. FZZ tagging constructs included the endogenous coding region sequence, the FZZ tag, a stop codon, 3' UTR sequence including the polyadenylation signal of *RPL29*, and neo2. Transformation and paromomycin selection for strains with a neo2 cassette were performed as previously described (Miller and Collins, 2000). Transgenes for integration at *BTU1* replaced the *BTU1* promoter and open reading frame with the gene of interest under expression control by ~1 kbp of the *MTT1* promoter (Shang et al., 2002). Transformation and paclitaxel selection for *BTU1* targeting in strain CU522 were performed as described (Witkin and Collins, 2004).

Extract Preparation

Cells were grown to mid-log phase ($3.5x10^5$ cells/mL) at 30°C in modified PPYS (1% proteose peptone, 0.2% yeast extract, 2% glucose, 50 μM $FeCl_3$) or modified Neff (0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose, 30 μM $FeCl_3$). Harvested cells were washed with 10 mM Tris-HCl pH 7.5 and lysed with ice-cold 0.2% Nonidet P-40 substitute (Igepal CA-630) and 0.1% Triton X-100 in T2EG50 buffer with 1/1000 volume of Sigma protease inhibitor cocktail (AEBSF, aprotinin, bestatin, E-64, leupeptin, pepstatin A) and 0.1 mM PMSF. Except for the

initial affinity purification used for mass spectrometry (Figure 1C), most purifications shown used 5 μM MG132 (Calbiochem) added to lysis buffer immediately prior to cell lysis. Lysis was allowed to proceed for 15 min at 4°C followed by centrifugation for 1 h at 4°C and 100,000-130,000 x *g* (S-100 extract) or 10,000 x *g* for 10 min (low-speed extract for rapid single-step purifications). Extract was used immediately or flash frozen in liquid nitrogen and stored at -80°C until use.

Nucleic Acid Purification and Hybridization

RNA was purified using TRIzol (Invitrogen) and resolved on 6% acrylamide/7 M urea gels before electrophoretic transfer to Hybond-N^+ (GE Heathcare). Blots were probed with end-labeled oligonucleotides. For genomic DNA purification, cells from 2 mL of culture were washed with 10 mM Tris-HCl pH 7.5 and lysed with 0.7 mL urea buffer (10 mM Tris-HCl pH 7.5, 0.35 M NaCl, 10 mM EDTA, 1% SDS, 42% urea). Lysate was extracted twice with PCI (phenol:chloroform:isoamyl alcohol, 25:24:1) and once with chloroform:isoamyl alcohol (24:1), 150 μl of 5 M NaCl was added, and DNA was precipitated with isopropanol. DNA spooled into a new tube was washed with 75% ethanol, dried, and resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA), treated with RNase A, and digested with restriction enzymes of choice for integration tests or with HindIII for rDNA telomere analysis. Digested DNA was recovered by PCI extraction and ethanol precipitation, resolved using non-denaturing 0.8% agarose/1X TBE for integration tests or denaturing 6% acrylamide/7 M urea/0.6X TBE for telomere blots, transferred to Hybond-N^+ membrane, and probed. Random hexamer labeling was used to make probes for integration tests; rDNA subtelomeric-sequence oligonucleotides were end-labeled to make probes for telomere blots. Data was collected with a Typhoon Trio Imager. Markers for the telomere blots should be considered approximate, because the DNA marker (Invitrogen) and *T.*

thermophila rDNA chromosome sequences are analyzed in denatured state and do not have balanced nucleotide content.

Subunit Association Assays

Telomerase holoenzyme bound to FLAG antibody resin was treated with 10 μg RNase A or mock-treated in 100 μl T2MG (20 mM Tris pH 8.0, 1 mM $MgCl_2$, 10% glycerol) with 50 mM NaCl (T2MG50) supplemented with 0.1% Igepal and 5 mM β-mercaptoethanol for 15 min at room temperature (RT). For assaying r82 interaction with holoenzyme proteins, 5 μg r82 in 100 μl T2MG50 was incubated with holoenzyme for 30 min at RT prior to RNase treatment. For r82 reconstitution with intact holoenzyme, r82 and holoenzyme were incubated in blocking wash buffer (T2MG50, 0.1% Igepal, 5 mM DTT, 0.1 mg/ml heparin, 0.1 mg/ml casein) with or without 1 μg r82 in 100 μl volume for 30 min at RT. Activity was assayed in an aliquot of resin prior to elution, while proteins were examined by SDS-PAGE after elution with FLAG peptide.

Telomerase Activity Assays

Resin-bound samples were washed into T2MG supplemented with 5 mM β-mercaptoethanol. Activity assays reaction buffer had final concentrations of 50 mM Tris-acetate pH 8.0, 10 mM spermidine, 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.4 mM dTTP, 0.3 μM dGTP, 33 nM [α-^{32}P]dGTP (NEN/Perkin Elmer EasyTide 3000 Ci/mmol), and 50-100 nM $(GT_2G_3)_3$ primer. Reactions proceeded for 6-30 min at 30°C before quenching with TES (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.2% SDS). For the chase experiment, reactions were incubated for 10 min as noted above before addition of dGTP to 30 μM final (100-fold excess over the radiolabeled pulse) for a chase of another 10 min at 30°C. In assays including r82, r82, telomerase, and primer were incubated together at RT for 10 min before the addition of buffer and nucleotides. Products were extracted with PCI and precipitated with carriers (10 μg yeast tRNA, 10 μg glycogen, 25

μg linear polyacrylamide) and 0.3 M ammonium acetate in ethanol. Pellets were washed once with 75% ethanol, dried briefly, and resuspended in 5 μl of formamide with 10 mM EDTA and tracking dyes. Products were boiled, snap-cooled, and resolved on 9-10% acrylamide/7 M urea denaturing gels. Images were collected with a Typhoon Trio Imager and quantified with ImageQuant software.

Recombinant Protein Purification and Gel Mobility Shift

A synthetic gene encoding r82 was subcloned into pET28 and transformed into BL21 (DE3). Protein expression was induced with 0.1 mM IPTG at 16°C. Cells were resuspended in T2MG with 250 mM NaCl and sonicated to lyse, then 0.1% Igepal and an additional 50 mM NaCl were added before clearing the lysate. Tagged protein was bound to Ni-NTA agarose (Qiagen) with 20 mM imidazole at 4°C for 3 h before washing and eluting the same binding buffer supplemented to 300 mM imidazole. Purified protein was adjusted to 5 mM DTT and flash frozen.

All oligonucleotides were gel-purified before use (IDT DNA; see Supplemental Table 1 for probe and competitor sequences). Probe concentration was ~10 nM for 6-carboxyfluorescein-labeled oligonucleotides or ~1 pM for radiolabeled oligonucleotides; protein and competitor concentrations are given in Figure legends. Final binding buffer contained 10 mM Tris-HCl pH 8.0, 0.5 mM $MgCl_2$, 5% glycerol, 150 mM NaCl, 150 mM imidazole, 0.05% Igepal, 0.5 μg/μl BSA, and 0.1 μg/μl tRNA. Binding was conducted for 10 min at RT. Order-of-addition experiments verified that interaction equilibrium was reached within the incubation time. Samples were resolved on a 5% acrylamide gel (37.5:1 acrylamide:bisacrylamide) with 4% glycerol and 0.5X TBE run at 4°C. Data was collected using a Typhoon Trio Scanner, and binding affinity was calculated using ImageQuant software based on free probe signal.

Antibodies

Full-length recombinant p19 or p82 was used to generate rabbit polyclonal antibody. Rabbit polyclonal antibodies against p65, p75, and p45 were generated previously using full-length proteins (Witkin et al., 2007).

Immunodepletion

Antibody resin was prepared with Protein A Sepharose 4 FastFlow (Amersham) and preimmune or p82-immununized serum: 20 µl of resin was incubated with 100 µl serum and 100 µl PBS for 1.5 h at RT, washed with PBS, washed with T2MG with IT (0.2% Igepal, and 0.1% Triton X-100) and 0.5 M NaCl, and washed into binding buffer (T2MG IT with 50 mM NaCl, 1/1000 volume of Sigma protease inhibitor cocktail, 0.5 mg/ml BSA). Holoenzyme purified on IgG resin and eluted with TEV protease was used for immunodepletion, with incubation at 4°C for 1 h before removing the unbound fraction and washing the resin twice in binding buffer at RT for 10 min.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Telomerase p82 I Similar to RPA1

(A) 3D-Jury structure prediction analysis of p82. The top five J-score predictions are shown.

(B) Primary sequence homology between predicted OB-fold domains in p82 and the OB-fold DNA-binding domains (DBD-A, DNA-B, DNA-C) of human RPA1 (hRPA1). Percent identity and additional similarity were calculated using ClustalW.

(C) Primary sequence identity comparison between p82, *T. thermophila* (Tt) RPA1, and hRPA1.

Figure S2. *TAP19*, *TAP50*, *TAP82,* and *TAP75* Can Be Targeted for Expression of C-Terminally Tagged Proteins

(A) Diagram of FZZ-tagging strategy.

(B) FZZ cassette integration status. Southern blots were performed on genomic DNA using a probe that recognizes DNA fragments of different sizes for endogenous (WT) and recombinant (neo2) chromosomes. Several independent clonal cell lines were examined for each targeting.

(C) Telomere length of rDNA chromosomes. Denaturing Southern blots were performed using a subtelomeric probe against the C-rich telomeric-repeat strand. For each targeting, a selected bulk population (P) and one clonal line derived from it (C) were examined.

Figure S3. Tagged Telomerase Holoenzyme Proteins Copurify Other Holoenzyme Proteins with an RNase-Resistant p75-p45-p19 Subcomplex

(A) Telomerase subunit association. Rapid single-step purification using FLAG antibody resin was carried out as in Figure 5A and analyzed by immunoblots (IB) for p65, p75, p45, and p19. TER was monitored to normalize for telomerase RNP recovery.

(B) Telomerase subcomplex association. Holoenzyme purification and RNase A treatment were performed as described for the experiment shown in Figure 4A, followed by immunoblot and Northern blot assays as in (A).

Figure S4. Purification of 82fzz Enriches the High-RAP Fraction of the TERTfzz Holoenzyme Population

Time course of activity assay reactions. TERTfzz holoenzyme was single-step purified on FLAG antibody resin for reactions performed using 25 nM primer.

Figure S5. High-RAP Activity Is Affected by Extract Production and r82

(A) Variable RAP of telomerase activity purified using extracts made under different conditions. Low-speed extracts were made with or without reducing agent (DTT), protease inhibitor cocktail (PIC), and addition of a proteasome inhibitor (MG132), followed by single-step purification of TERTfzz on FLAG antibody resin.

(B) Telomerase activity with r82 titration. Telomerase activity enriched by single-step purification of TERTfzz on IgG resin was assayed using 50 nM primer and the indicated concentration of r82. When r82 is added in molar excess to DNA primer in the activity assay reaction, r82 not bound to primer competes with telomerase product DNA binding. Activity and processivity both decrease due to this competition in vitro.

Figure S6. Recombinant p82 Preferentially Binds a Long Telomeric Sequence

A gel mobility shift assay was performed with 100- or 500-fold excess of competitor oligonucleotide.

Table S1. Sequence of Oligonucleotides

Notation	Oligonucleotide	Sequence (5'-3')
	$(GT_2G_3)_3$	GTTGGGGTTGGGGTTGGG
6FAM-NT	6FAM-$(AAT)_6$	6FAM-AATAATAATAATAATAAT
6FAM-TeloC3	6FAM-$(C_4A_2)_3$	6FAM-CCCCAACCCCAACCCCAA
6FAM-TeloG3	6FAM-$(T_2G_4)_3$	6FAM-TTGGGGTTGGGGTTGGGG
dsTelo3	6FAM-$(C_4A_2)_3$ and $(T_2G_4)_3$ annealed	
TeloG2	$(T_2G_4)_3$	TTGGGGTTGGGG
TeloG3	$(T_2G_4)_3$	TTGGGGTTGGGGTTGGGG
TeloG3-NT6	$(T_2G_4)_3$TATCGA	TTGGGGTTGGGGTTGGGGTATCGA
NT6-TeloG3	TATCGA$(T_2G_4)_3$	TATCGATTGGGGTTGGGGTTGGGG
hTeloG3	$(TAG_3T)_3$	TAGGGTTAGGGTTAGGGT
	$(TG_2)_6$	TGGTGGTGGTGGTGGTGG

Min Supplemental Figure 1

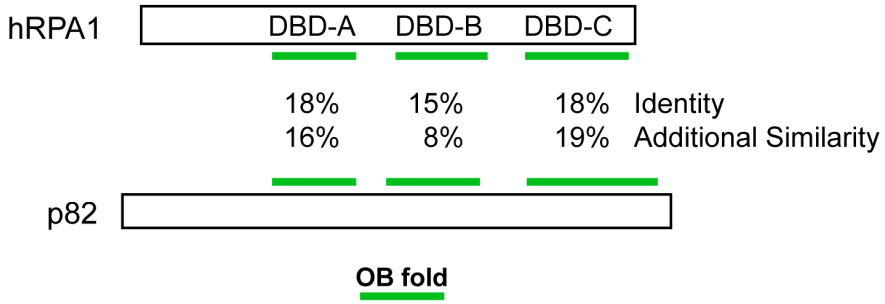
A

3D-Jury Analysis of p82

Model	J-score	PDB Hit
PSIB_01	136.50	1fgu - ssDNA-binding domain of the large subunit of RPA
PSIB_02	112.25	1l1o - Structure of the human RPA trimerization core
FFA3_02	107.75	1fgu - ssDNA-binding domain of the large subunit of RPA
FFA3_01	104.75	1jmc - ssDNA-binding domain of human RPA (183-420) bound to ssDNA
BASI_01	101.75	1fgu - ssDNA-binding domain of the large subunit of RPA

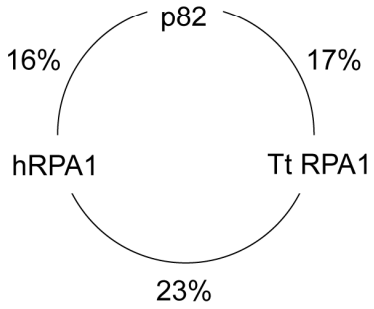
B

Comparison of OB folds in p82 and hRPA1



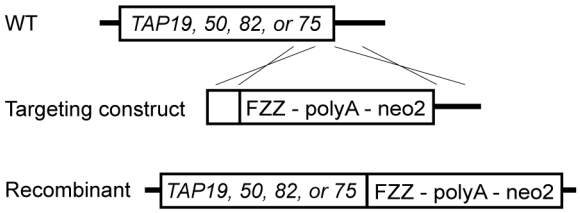
C

Primary Sequence Identity



Min Supplemental Figure 2

A



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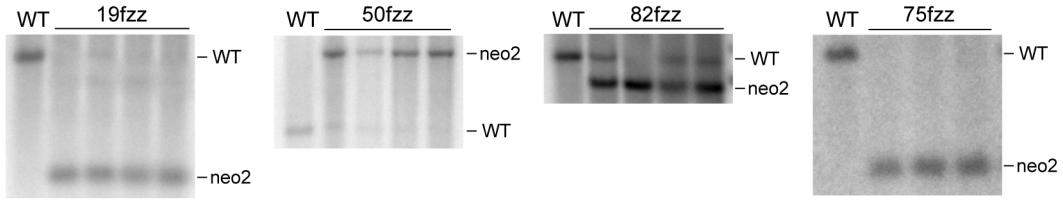

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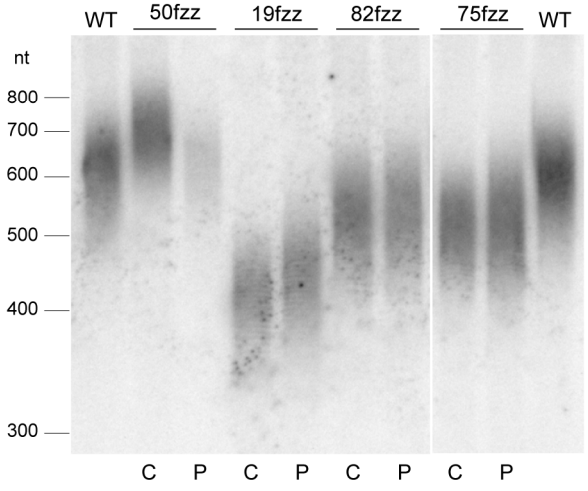
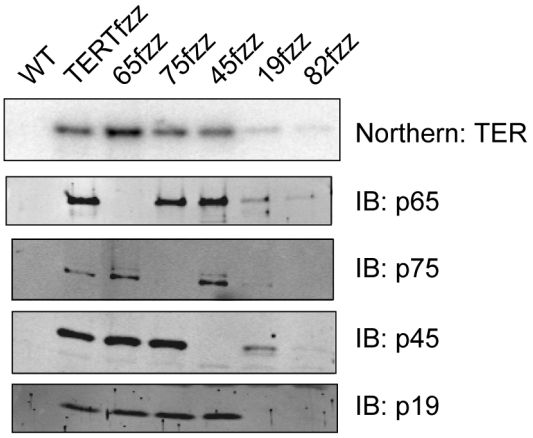


Min Supplemental Figure 3

A



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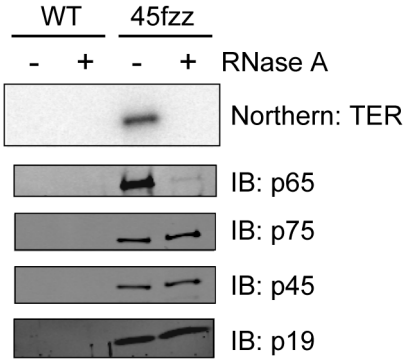

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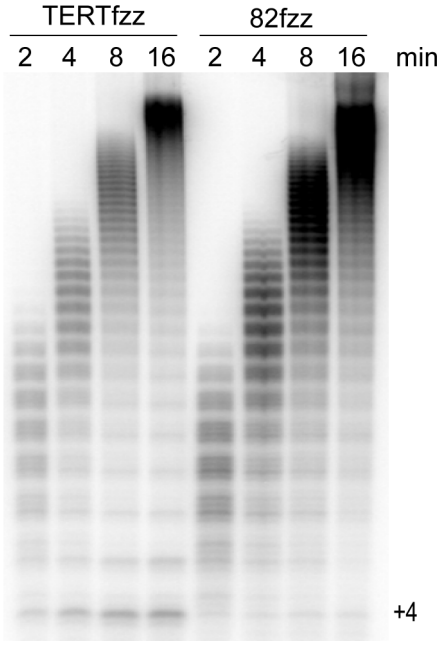

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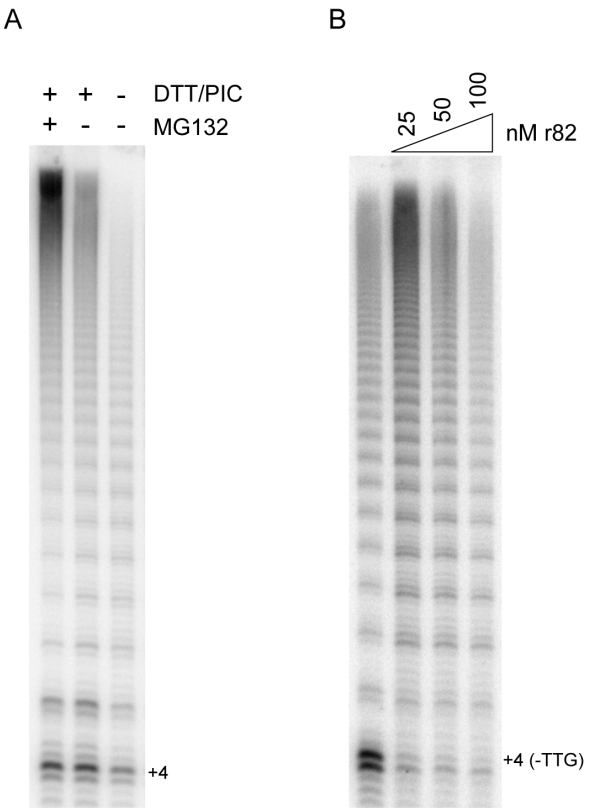

Min Supplemental Figure 6