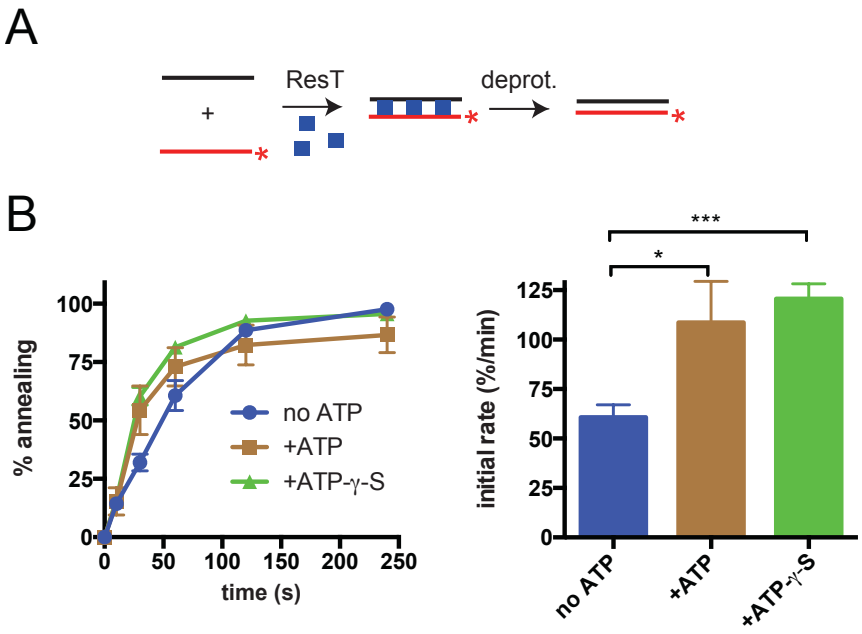
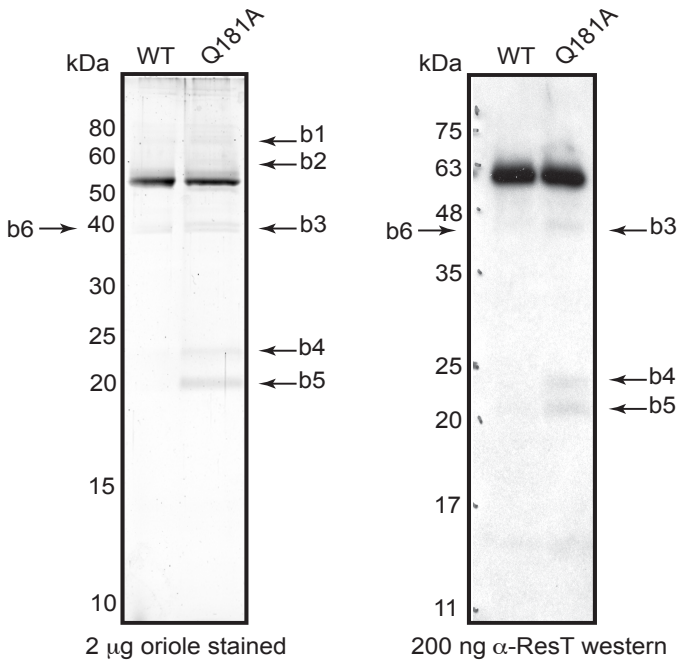


Figure S1. Single strand annealing assays performed in the presence of ATP.
A) Schematic representation of the single-strand annealing assay. Annealing reactions with 'naked DNA' involve a pair of complementary 35 nt oligonucleotides (OGCB664/665) with one oligo 5'-endlabeled with ^{32}P (asterisk, red line) partnered with an unlabeled oligo (black line). Addition of ResT (blue squares) to the complementary oligonucleotides induces annealing.
B) The results of annealing assays performed with 'naked DNA' substrates represented as % annealing vs. time plots and as bar graphs showing the initial rates of reactions without ATP and reactions supplemented with ATP or ATP-γ-S. Reactions were performed in triplicate and the mean and standard deviation is shown. Differences between the initial rates of annealing were examined using unpaired t-tests. * $P \leq 0.05$, and *** $P \leq 0.001$.

A



2 μg oriole stained 200 ng α-ResT western

B **Mass Spectrometry Summary Table**

Band	Spectra	Unique peptides	Unique peptide score	Percent coverage	Protein MW (Da)	Species	SwissProt Accession #	Protein name
b1	165	47	677.17	69.2	74915.1	*E. coli*	P77398	ArnA
b2	24	15	181.93	29.7	57499	*E. coli*	P0A6F5	GroL
$b3_{-1}$	61	20	277.98	46.7	40619.6	*E. coli*	P06987	HisB
$b3_{-2}$	164	45	573.41	62.6	54000	*Borrelia burgdorferi*	custom database	ResT
$b4_{-1}$	83	23	331.2	76.6	23811.2	*E. coli*	P0ACJ8	CRP
$b4_{-2}$	92	34	385.45	55.2	54000	*Borrelia burgdorferi*	custom database	ResT
$b5_{-1}$	23	11	105.36	23.2	54000	*Borrelia burgdorferi*	custom database	ResT
$b5_{-2}$	51	10	114.48	46	19149.6	*E. coli*	P0A7B8	HlsV
$b6_{-1}$	174	57	637.84	72.7	54000	*Borrelia burgdorferi*	custom database	ResT
$B6_{-2}$	47	21	235.36	57.4	40619.6	*E. coli*	P06987	HisB

ArnA is a bifunctional polymyxin resistance protein with oxidative decarboxylation and formylation activity.
GroL is an ATP-dependent chaperonin in the context of GroEL-GroES and unfolded substrate.
HisB is a bifunctional histidine biosynthesis protein with histidinol-phosphatase and imidazoleglycerol-phosphate dehydratase activity.
CRP is a camp-activated global transcriptional regulator.
HlsV is the protease subunit of the ATP-dependent HlsVU protease complex.

Figure S2. Assessing the purity of wild type ResT and ResT (181A).
A) The left panel shows 15% SDS-PAGE gel analysis of 2 μg of wild type (WT) and ResT (181A) preps visualized by oriole staining (image inverted). Possible contaminants are indicated by arrows and given a band designation (b1-6). The right panel shows 200 ng of each protein loaded to a 15% SDS-PAGE gel western blotted with a polyclonal α-ResT antibody to detect ResT cleavage fragments.
B) Summary of top hits in the b1-6 areas of the preparative gel against the *E. coli* database or the custom ResT databases (where appropriate). The function/activity of the low level contaminants is summarized below the Table. Material for analysis was derived from a 15% SDS-PAGE preparative gel run without molecular weight markers in which 4 μg of wild type (WT) and ResT (181A) preps were loaded and visualized by oriole staining. The b1-6 band regions were excised and subjected to in-gel tryptic digest followed by tandem MS-MS analyses. The results were run against an *E. coli* database and custom ResT and ResT (Q181A) databases constructed for the Spectrum Mill software.

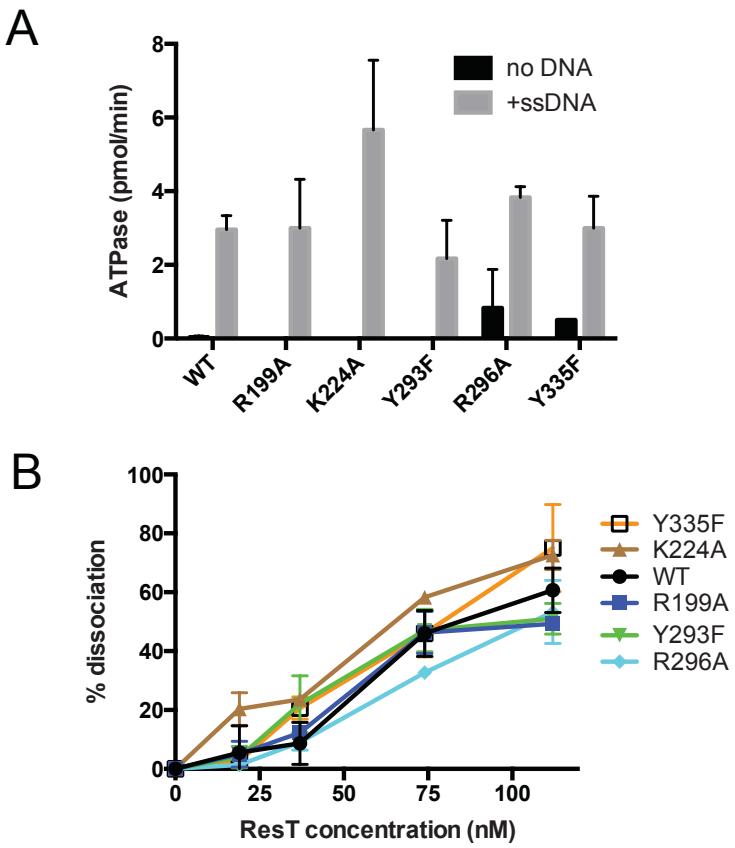


Figure S3. ATPase/helicase assays of ResT mutations in the telomere resolvase active site.
A) Summary of ATPase results of the indicated ResT variants, -/+ 10 μg/mL ϕX174 virion. ATPase assays containing 74 nM ResT were incubated at 37°C for 120 min. The mean and standard deviation of at least three independent experiments is shown.
B) % strand dissociation vs. ResT concentration curves of the indicated ResT variants. The helicase assays were performed by 60 min incubation at 37°C, with 15 nM substrate. The substrate was used was a partial duplex with a 26 bp duplex region with a 50 nt 3'-tail assembled with 5'-endlabeled OGCB666 and unlabeled OGCB692 (see Supplementary Table S1). Reactions were performed in triplicate and the mean and standard deviation are shown.

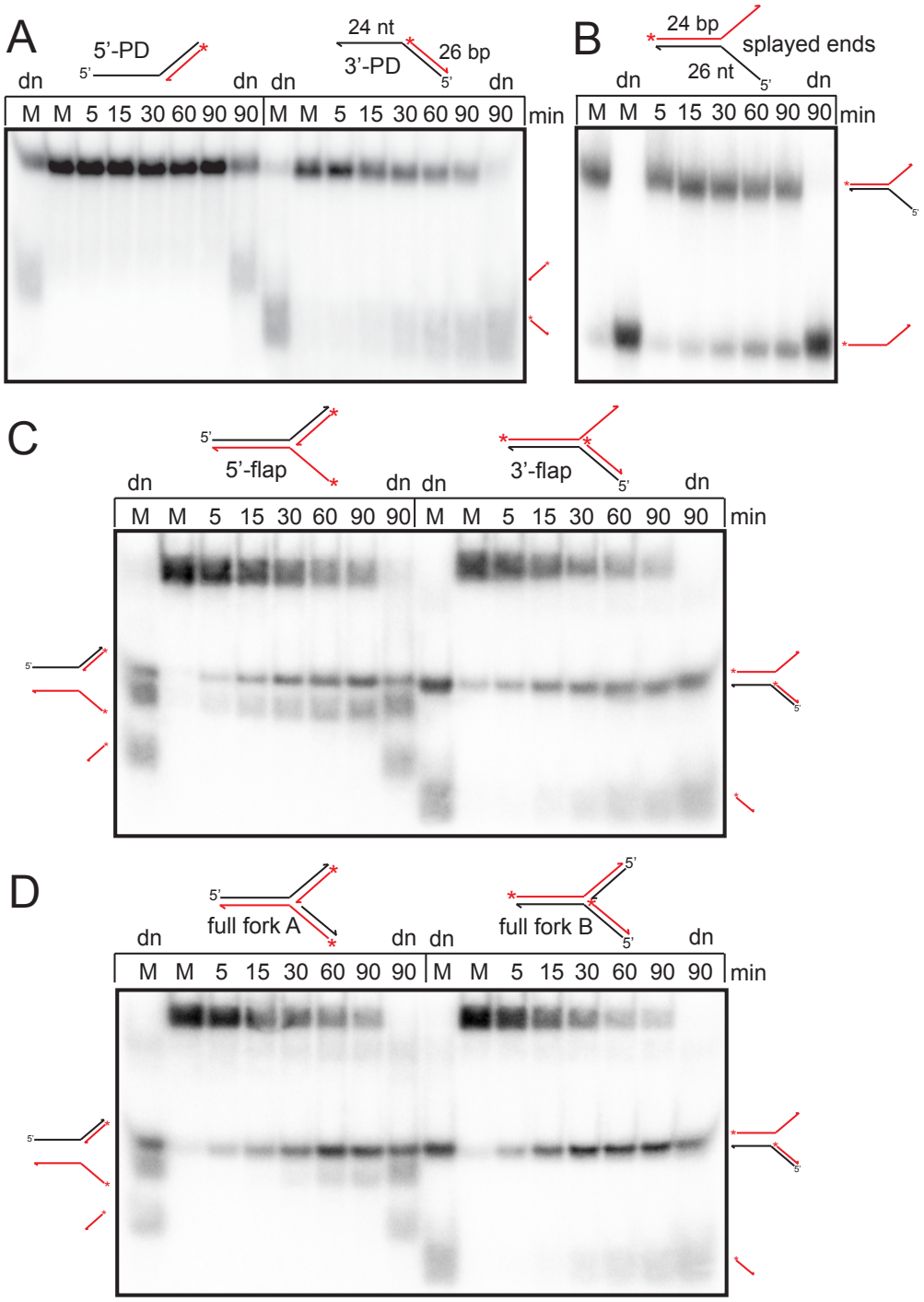


Figure S4. DNA unwinding assays with ResT (Q181A) and synthetic replication fork mimics. 8% PAGE 1X TAE/0.1%SDS gel analysis of timecourse reactions of ResT (Q181A) with various assemblies of a replication fork mimic with heterologous arms. The partial duplex, splayed end, 3'- and 5'-flaps and replication fork mimics assayed are diagrammed above the gel panels. The red asterisk indicates a 5'-endlabel and the red line indicates DNA strands that are radiolabeled, unlabeled strands are shown in black. M, denotes mock incubated substrate; dn, indicates heat denaturation prior to gel loading. The schematics to the side of the gels indicate the gel migration position of the various possible products of substrate unwinding. Master reactions were incubated at 37°C and aliquots were withdrawn at the times indicated above the gel panels, substrate was present at 15 nM and ResT (Q181A) at 37 nM.

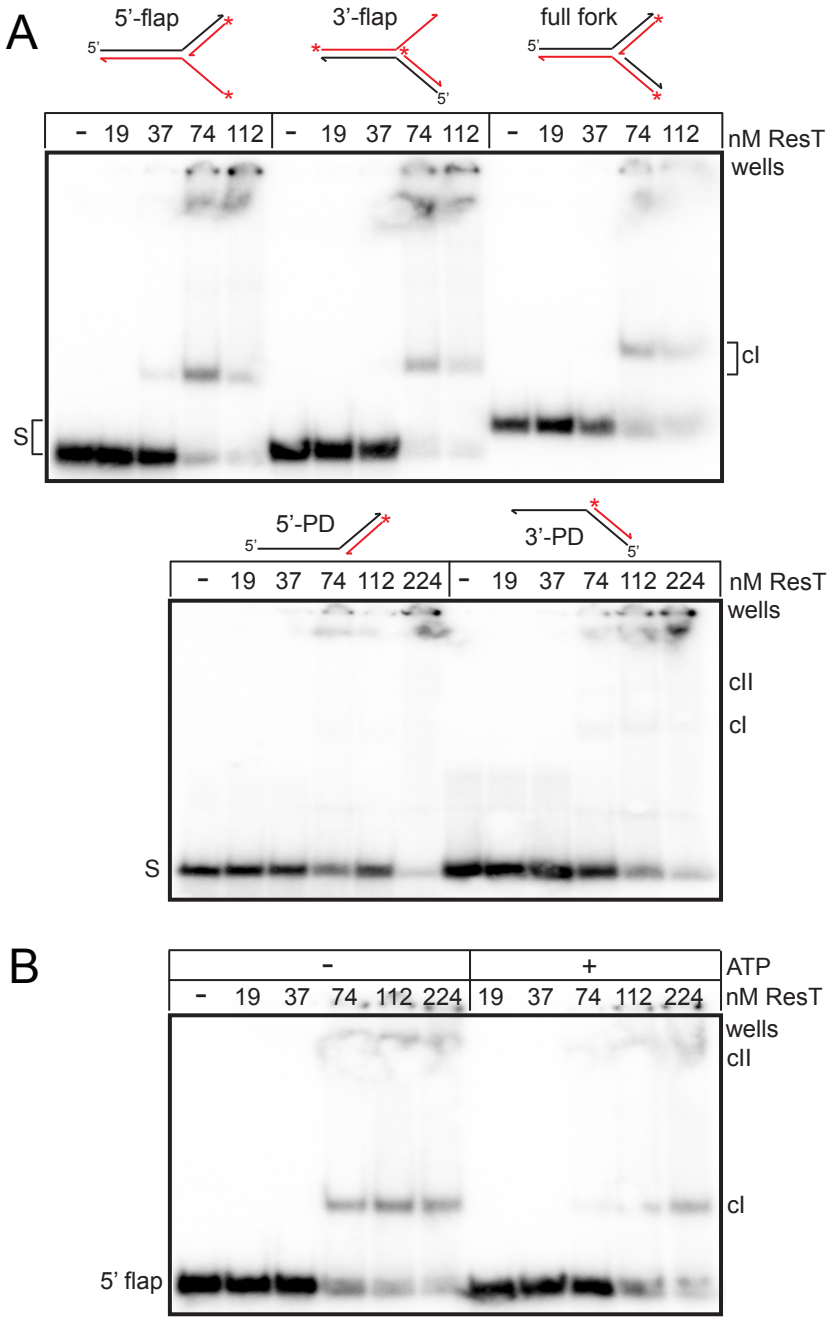


Figure S5. Electrophoretic mobility shift assays (EMSA) with wild type ResT and replication fork mimics. A) 6% PAGE 0.5X TBE EMSA analysis of wild type ResT with partial duplex (PD), 5'-, 3'-flap and full fork assemblies. The 5'-endlabeled substrates (S) are diagrammed above the gels and were present at 5.25 nM. ResT was added to the concentrations indicated in the loading key above the gels and incubated with substrate at 0°C for 20 min prior to gel loading. See the Supplemental Material and Methods section for additional details. Discrete ResT-DNA complexes are indicated by the designation cI and cII to the right of the gels. ResT has a tendency to produce wellshifts at higher concentrations, the position of the wells is indicated.
B) 6% PAGE 0.5X TBE EMSA analysis comparing binding of ResT to the 5'-flap substrate in conditions -/+ 2 mM ATP.

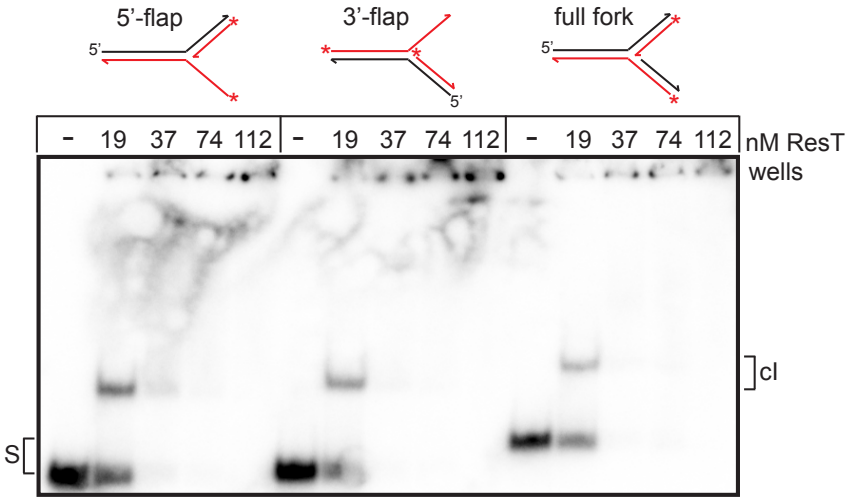
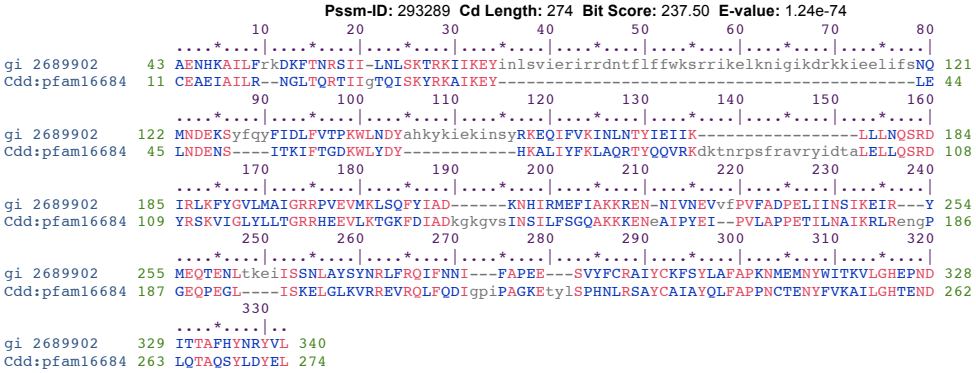


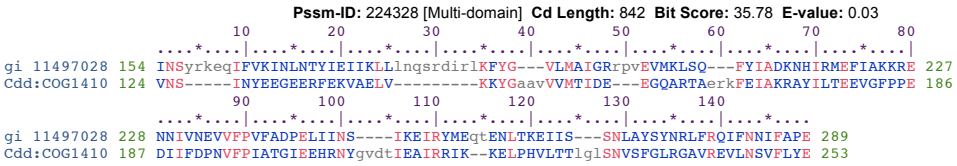
Figure S6. EMSA analysis of ResT (F92AF93AW94A) with replication fork mimics.
6% PAGE 0.5X TBE EMSA analysis of ResT (F92AF93AW94A) with 5'-, 3'-flap and full fork assemblies. The 5'-endlabeled substrates (S) are diagrammed above the gels and were present at 5.25 nM. ResT was added to the concentrations indicated in the loading key above the gels and incubated with substrate at 0^{o}C for 20 min prior to gel loading. Discrete ResT-DNA complexes are indicated by the designation cI to the right of the gels. ResT has a tendency to produce wellshifts at higher concentrations, the position of the wells is indicated.

A

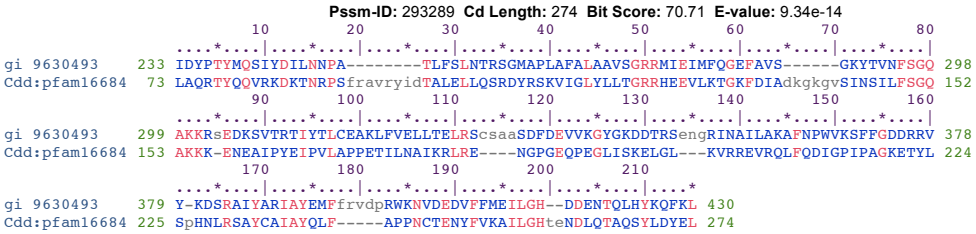
ResT telomere resolvase domain alignment



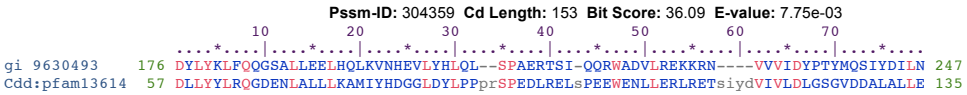
ResT MetH (cobalamine/pterin) domain alignment from DELTA BLAST



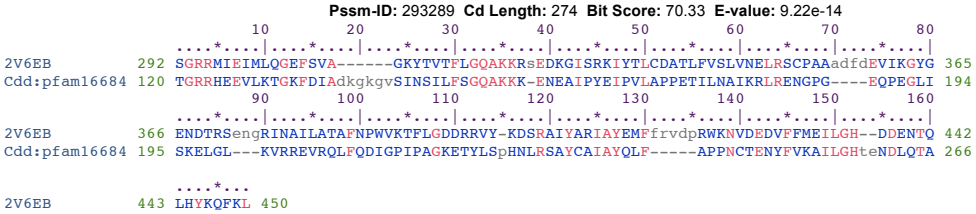
TelN ‘protelomerase’ telomere resolvase domain alignment



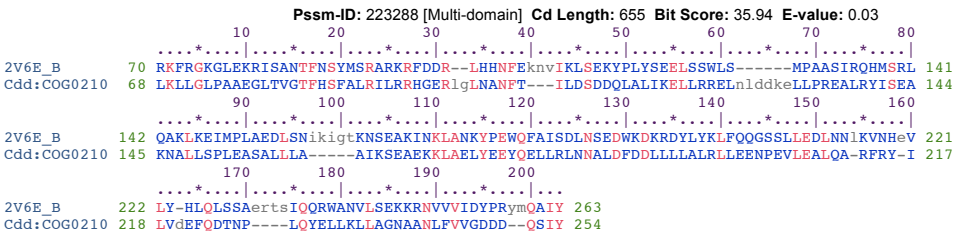
TelN ‘protelomerase’ P-loop NTPase domain alignment



TelK ‘protelomerase’ telomere resolvase domain alignment



TelK ‘protelomerase’ DELTA-BLAST UvrD domain alignment



B

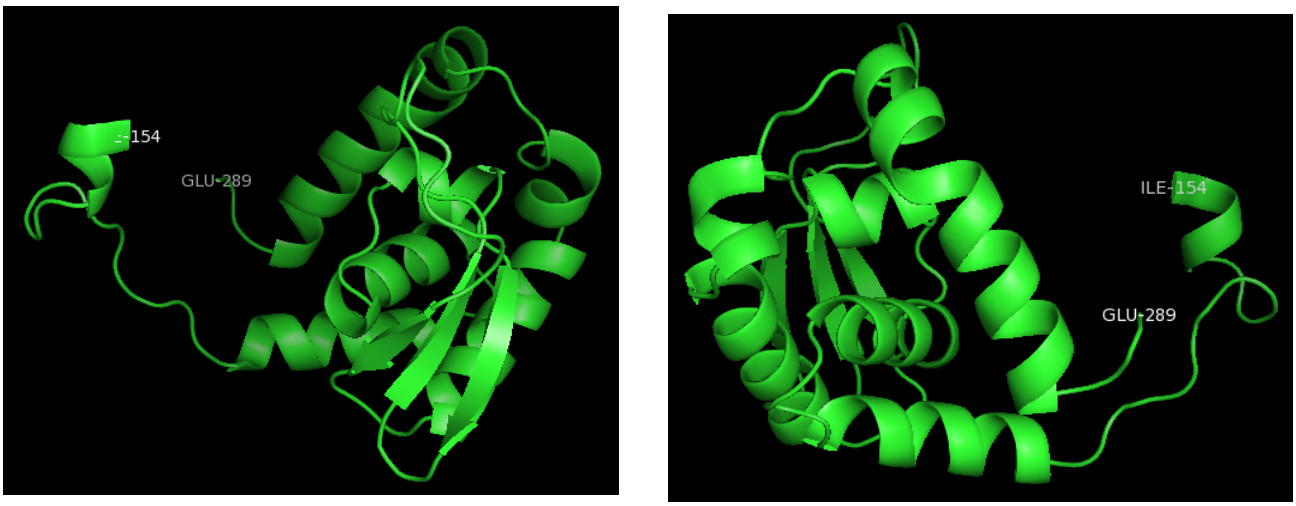


Figure S7. Putative conserved domains identified in the telomere resolvases, ResT, TelN and TelK.
A) Show are alignments of ResT, TelN and TelK against putative conserved domains identified by PSI-BLAST and DELTA-BLAST searches (46, 47). In each telomere resolvase shown there is the expected strong homology to the telomere resolvase domain (pfam 16684) and a weak putative conserved domain homology to the pterin-binding domain of MetH (ResT), the AAA_31 sub-type of P-loop NTPase's (TelN) and to the DNA helicase UvrD (TelK).
B) One 2 One threading results for ResT (154-289) region against *Agrobacterium tumefaciens* telomere resolvase, TelA, the closest telomere resolvase relative to ResT with structural data available (pdb accession 4e0g). The ResT (154-289) model is shown in two orientations related by a 90^{o} counter clockwise rotation. The 154 and 289 residues are labeled. The Phyre2 bioinformatics One 2 One threading software from Imperial College was employed (49).

Table S1. Oligonucleotides used in this study.

Oligo name	Oligo sequence	Use
OGCB455	5'-GATCATATCCTTTCTTTAAACTTCTATCATTGATTCTTACTAG TCTTTACCTTACTATACTTCTATCAGTTTATCGATTCTTCTTTA-3'	87 nt ssDNA; used in Fig. S1B.
OGCB456	5'-GATCTAAAGAAGAATCGATAAACTGATAGAAGTATAGTAAG GTAAAGACTAGTAAGAATCAATGATAGAAGTTTAAAGAAAGGA TAT-3'	Complement of 455 to make a duplex DNA; used in Fig. S1B.
OGCB409	5'-TCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTCG CGGATT TCGACACAATTTATCAGGCGATGATACAAATCTCCGT TGTACTTTGTTTCGCGCTTGGTATAATCGCTGGGGGTCAAAGA T-3'	126 nt bottom strand of polarity test substrate; used in Figs. 3 & 4.
OGCB666	5'-GTTACTTAGCCGGAACGAGGCGCAGA-3'	26 nt strand to make polarity test substrate; used in Figs. 3 & 4.
OGCB667	5'-ATCTTTGACCCCCAGCGATTATACCA-3'	26 nt strand to make polarity test substrate; used in Figs. 3 & 4.
OGCB669	5'-GTCGGATCCTCTAGACAGCTCCATGATCACTGGCACTGGT AGAATTCGGC-3'	50 nt leading template strand for fork mimic; used in Figs. 5, S4-S6.
OGCB670	5'-CAACGTCATAGACGATTACATTGCTACATGGAGCTGTCTAG AGGATCCGA-3'	50 nt lagging template strand for fork mimic; used in Figs. 5, S4-S6.
OGCB671	5'-TGCCGAATTCTACCAGTGCCAGTGAT-3'	26 nt nascent leading strand for fork mimic; used in Figs. 5, S4-S6.
OGCB672	5'-TAGCAATGTAATCGTCTATGACGTT-3'	26 nt nascent lagging strand for fork mimic; used in Figs. 5, S4-S6.
OGCB681	5'-TGTGGAATGCTACAGGCGTTCTAGTTTGTACTGGTGACGAA ACTCAGTGTTACGGTACATGGGTTCCTATTGGGCTTGCTATCC CTGAAAATGAGGGTGG-3'	100 nt top strand for D-loop substrates; used in Fig. 6.
OGCB686	5'-CCACCCTCATTTTCAGGGATAGCAAGCCCAATAGGGGTAC CGAGCTCGAATTCACTGGCCGTCGTTCCAGTACAAACTACAA CGCCTGTAGCATTCCACA-3'	100 nt bottom strand for D-loop substrates; used in Fig. 6.
OGCB687	5'-AACGCCGGCCAGTGAATTCGAGCTCGGTACC-3'	31 nt strand with complementarity to the centre of 686; used to make D-loop in Fig. 6.
OGCB688	5'-AGTCTTAAGCCTTGACTAGTCAGCTTGACTAAGCGATTGAC TAACGACGGCCAGTGAATTCGAGCTCGGTACCC-3'	74 nt strand to make 5'-D-loop; used in Fig. 6.
OGCB689	5'-AACGACGGCCAGTGAATTCGAGCTCGGTACCCAGTCTTAA GCCTTGACTAGTCAGCTTGACTAAGCGATTGACT-3'	74 nt strand to make 3'-D-loop; used in Fig. 6.
OGCB692	5'-TCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTC GCGGATTTCGACACAATTTATCAGGCGATGATACAAAT-3'	76 nt strand to make 3'-partial duplex with 50 nt 3'-tail, use with 666; used in Fig. S3.

OGCB562	5’-GAAATAATTAAGCTTCTACTGAATGCGAGTCGAGATATTA GATTAAAATTT-3’	Mutagenic oligo used to make the Q181A mutation; use with 563.
OGCB563	5’-AAATTTTAATCTAATATCTCGACTCGCATTCAGTAGAAGCT TAATTATTTC-3’	Mutagenic oligo used to make the Q181A mutation; use with 562.
OGCB524	5’-AGGATTAGAAGAGATAATACTTTTTTATTTGCGGCGAAAT CAAGAAGAATAAAAGAATTAAAA-3’	Mutagenic oligo used to make the F93AW94A mutation; use with 525.
OGCB525	5'-TTTTAATTCTTTTATTCTTCTTGATTTCGCCGCAAATAAAAA AGTATTATCTCTTCTAATCCT-3'	Mutagenic oligo used to make the F93AW94A mutation; use with 524.
OGCB540	5’-AGAGATAATACTTTTTTAGCGGCGGCGAAATCAAGAAGA-3’	Mutagenic oligo used to make the F92A mutation in the F93AW94A background; use with 541.
OGCB541	5’-TCTTCTTGATTTCGCCGCCGCTAAAAAAGTATTATCTCT-3’	Mutagenic oligo used to make the F92A mutation in the F93AW94A background; use with 540.
OGCB127	5’-GATCtcctctaaccattgcTCATTATACTAAAAGATAATAAATTAAT TTATTATTAATTAGTATAAATA-3'	Used to construct Type 2 *rTel* used in Figs. 2 and 4. Use with 128.
OGCB128	5’-GATCTATTTATACTAATTAATAATAAATTAATTTATTATCTTT TAGTATAATGAgcaatggttagagga-3’	Used to construct Type 2 *rTel* used in Figs. 2 and 4. Use with 127.
OGCB674 -5’P	5’-ATTCTTCTTCATTCTTCTATTATTA-3’	5’-phosphorylated nascent lagging strand for mobile fork in Fig. 7; use with 676, 697 and 698.
OGCB676	5’-TAATAATAGAAGAATGAAGAAGAAT-3’	Nascent leading strand for partially mobile fork used in Fig. 7; use with 674-5’P, 697 and 698.
OGCB697	5’-AAAATAACACAATCAAAATATTGTGGATAATCAATCCATTC TGAATATTCTTCTTCATTCTTCTATTATTA-3’	Leading arm template strand for partially mobile fork; in Fig. 7; use with 674-5’P, 676 and 698.
OGCB698	5’-TAATAATAGAAGAATGAAGAAGAATTGAATGAATGGATTGA TTATCCACAATATTTTGATTGTGTTATTTT-3’	Lagging arm template strand for partially mobile fork; in Fig. 7; use with 674-5’P, 676, and 697.

Supplemental Materials and Methods

Substrate labeling, assembly and purification

All substrates were assembled by 5'- ^{32}P endlabeling the oligos with T4 polynucleotide kinase (PNK) and [γ^{32}P]ATP (37^{o}C, 1 hour, using, 66 nM [γ^{32}P]ATP and 4 units of T4 PNK) followed by annealing reactions conducted in a waterbath brought to a boil and allowed to slowly cool to room temperature overnight. The annealing buffer contained 25 mM HEPES (pH 7.6), 0.1 mM EDTA and 50 mM NaCl. The substrate assemblies used to make the replication fork mimics for Figures 5 & S4 were excised from 8% PAGE 1X TAE gels. The DNA was recovered from the gels by crush and soak (4^{o}C, overnight) followed by application of the soak over 0.45 μm acetate spin filters (costar columns) followed by ethanol precipitation to concentrate the recovered substrates.

Single-strand annealing assays

Annealing reactions with 'naked DNA' were performed by mixing fifteen-nanomolar unlabeled oligonucleotide OGCB665 into buffer containing 25 mM HEPES (pH 8.2), 2 mM $MgCl_2$, 1 mM DTT, 100 μg/ml bovine serum, 50 mM NaCl and 2 mM ATP or ATP-γ-S. This mixture was chilled on ice for 2 min, to inhibit the rate of spontaneous annealing. 15-nM 5**'** -^{32}P endlabeled oligonucleotide OGCB664 and ResT were added followed by incubation at 30^{o}C. Timepoints were taken by removing 18 μL aliquots, from the 120 μL master annealing reactions, to tubes with pre-aliquoted sodium dodecyl sulphate (SDS) stop dye containing an excess unlabeled OGCB664 to prevent further annealing after reaction termination. The 5X stop dye contains 100 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 3% SDS, 30% glycerol, 0.024% bromophenol blue and 600nM unlabeled reporter oligonucleotide.

Electrophoretic analysis was performed on 20 X 20 cm vertical gels with 8% PAGE 1XTAE/0.1% SDS at 13 V/cm for 105 min followed by gel drying, exposure of the gels to phosphor screens and analysis on a BioRad FX phosphorimaging machine. Reactions were performed in triplicate and the data were quantitated with BioRad's Quantity One software according to the manufacturer's instructions. Reaction curves and statistics were generated with Prism's GraphPad 6.0.

Electrophoretic mobility shift assays

Binding reactions with 5.25 nM 5'-^{32}P-endlabeled substrates were performed in binding buffer (25 mM HEPES [pH 8.2], 2 mM mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, 78 ng/mL heparin sulphate, 50 mM NaCl) by incubation at 0^{o}C for 20 min with the concentration of ResT indicated in the figure. Where indicated, ATP was added to a final concentration of 2 mM. The samples were prepared for gel loading by addition of 5X load dye to a 1X concentration. 5X load dye contains 200 mM EDTA (pH 8), 32% glycerol, and 0.024% bromophenol blue. 10 cm x 10 cm gels 6% PAGE 0.5X TBE gels were run at 15V/cm in the cold room until the bromophenol blue was 3 cm from the bottom of the gel. The gels were dried and exposed to a phosphorimaging screen for visualization.

Mass Spectroscopy Analysis

Material for analysis was derived from a 15% SDS-PAGE preparative gel run without molecular weight markers in which 4 μg of wild type (WT) and ResT (181A) preps were loaded and visualized by oriole staining. The b1-6 band regions were excised and subjected to in-gel tryptic digest followed by tandem MS-MS analyses. The results were run against an *E. coli* database and custom ResT and ResT (Q181A) databases constructed for the Spectrum Mill software. The MS analysis was performed by the Core Mass Spectroscopy Facility of the College of Medicine, University of Saskatchewan, Saskatoon, Canada.

Supplementary References

46. Boratyn, G.M., Schaffer, A.A., Agarwala, R., Altschul, S.F., Lipman, D.J. and Madden, T.L. (2012) Domain enhanced lookup time accelerated BLAST. *Biol Direct*, **7**, 12.

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49. Kelley, L.M., S., Yates, CM., Wass, MN. and Sternberg, MJE. (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc*, **10**, 845–858.