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Supplemental Data

C. elegans Telomeres Contain G-Strand and C-Strand Overhangs that Are Bound by Distinct Proteins

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Supplemental Figures

Raices et al.,
supplemental Figure 1

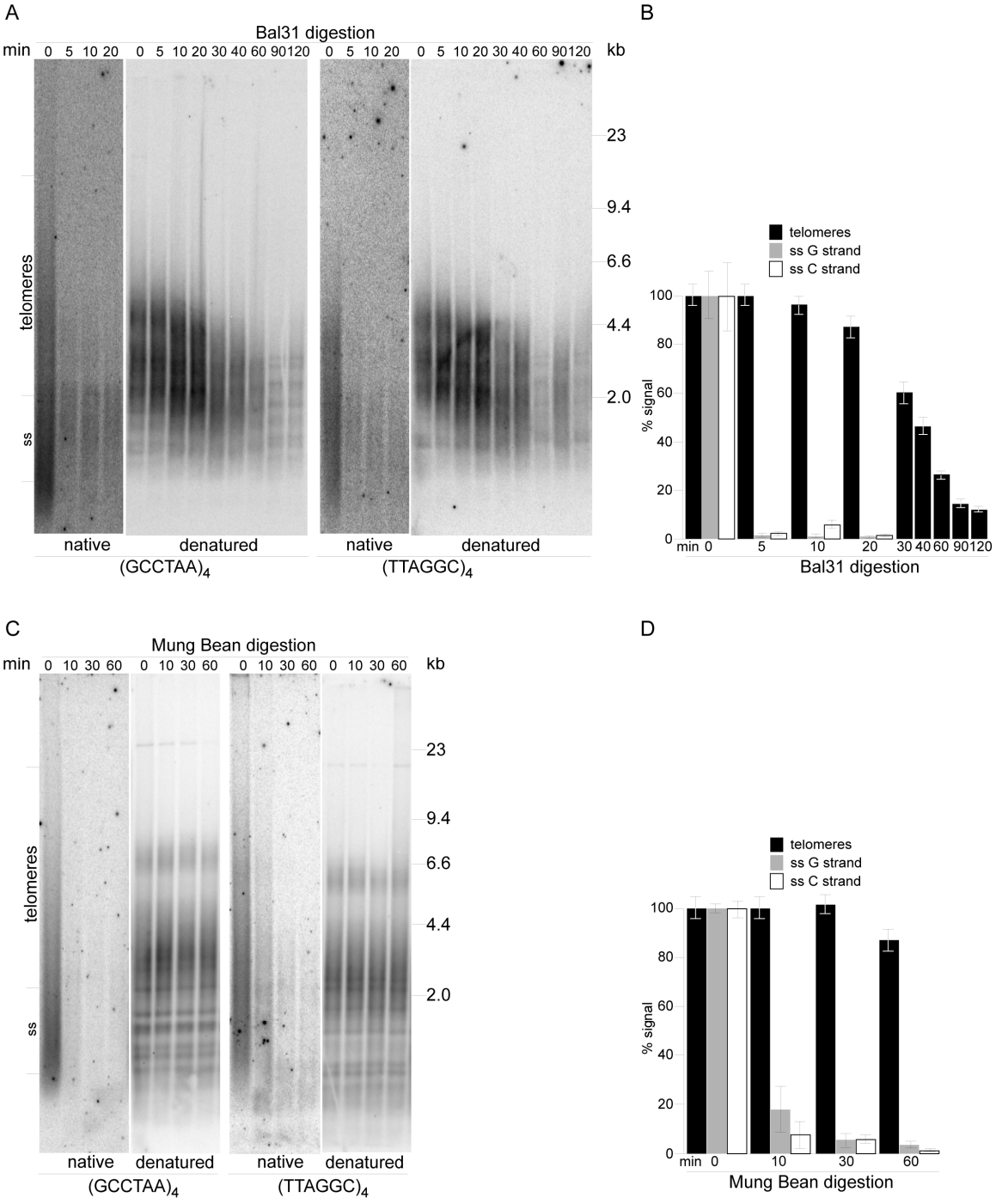

Figure S1. Bal-31 and Mung bean nuclease digests of *C. elegans* DNA

(A) *C. elegans* DNA was separated on agarose gels under native or denaturing conditions, after incubation with Bal-31 nuclease for the times indicated. The gels were hybridized with radioactively labeled $(GCCTAA)_4$ or $(TTAGGC)_4$ oligonucleotides as indicated. Single stranded telomeric DNA, telomeres and fragment size has been indicated. **(B)** Quantification of (A). Error bars indicate the standard deviation of three independent experiments. **(C)** As in A, except that the DNA was incubated with Mung bean nuclease prior to separation on the gels. **(D)** Quantification of (C). Error bars indicate the standard deviation of three independent experiments.

Raices et al.,
supplemental Figure 2

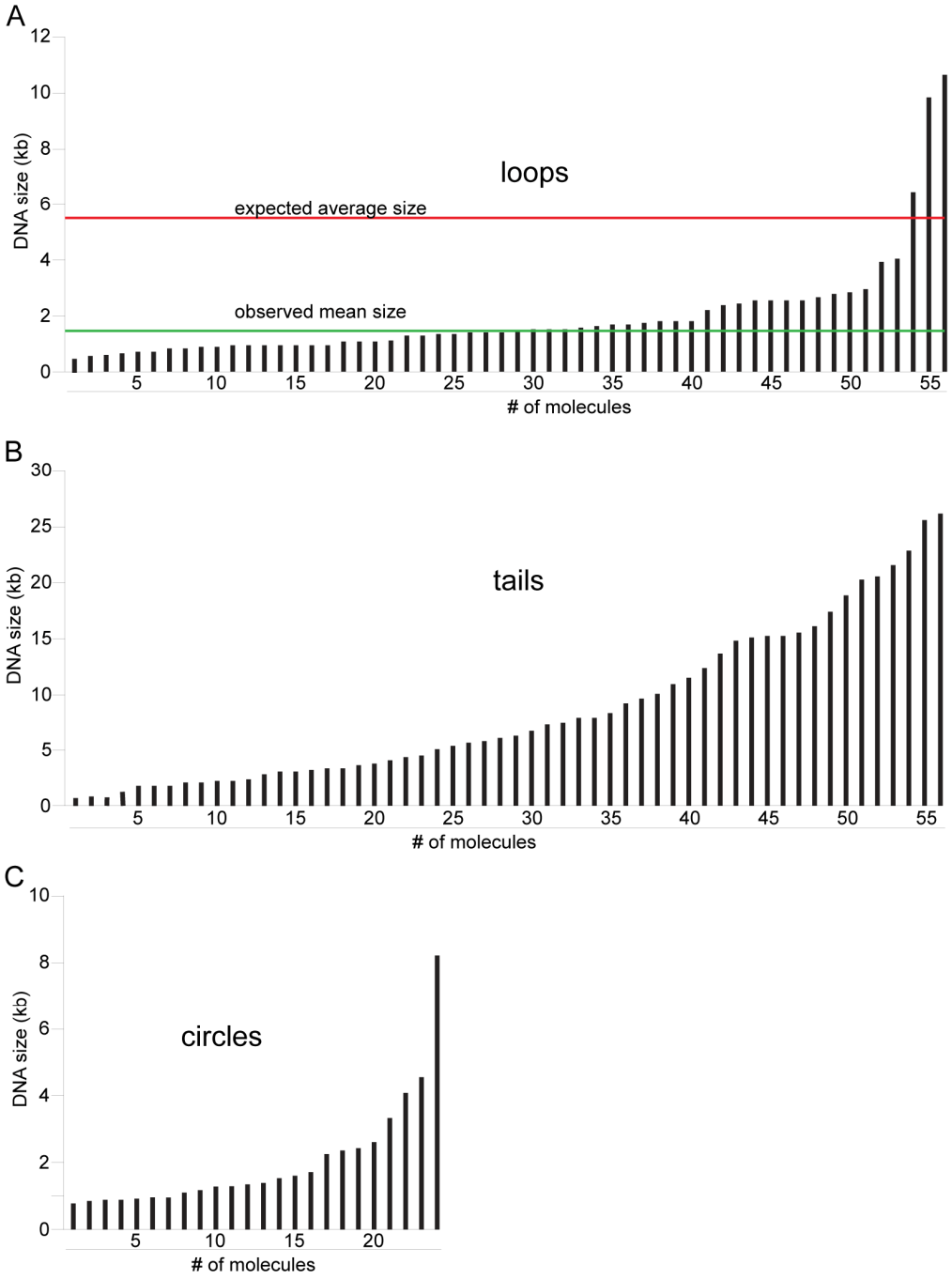

Figure S2. Size distribution of t loops and t circles

(A) Size distribution of 56 t loops. The x-axis represents molecule number, and the y-axis molecule size. The expected average loop size and the observed mean loop size are indicated. **(B)** Size distribution of the tails of the 56 molecules

in (A). The x-axis represents molecule number, and the y-axis molecule size. **(C)** Size distribution of 24 t circles. The x-axis represents molecule number, and the y-axis molecule size.

Raices et al.,
supplemental Figure 3

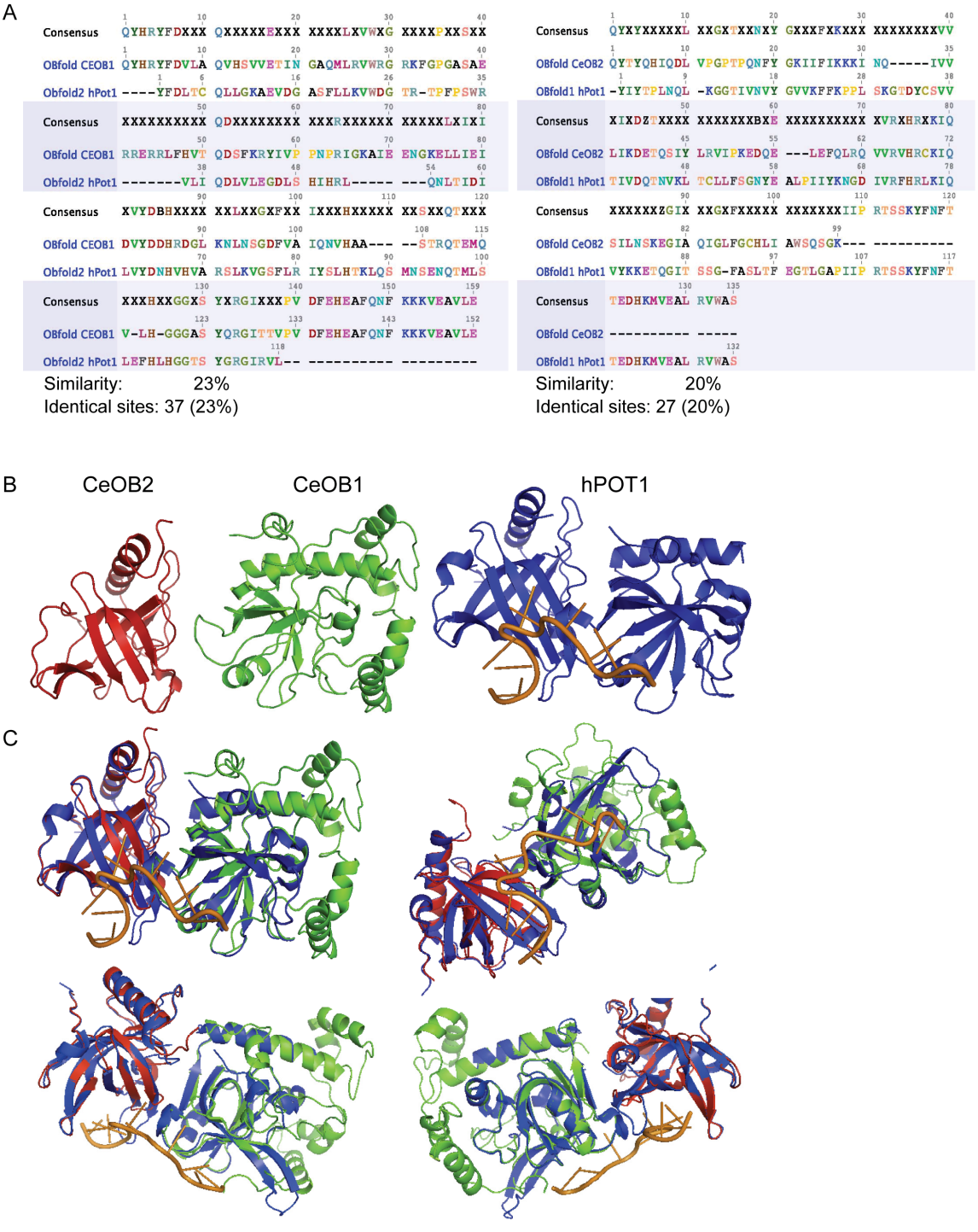

Figure S3. Amino acid and 3D alignment of CeOB1, CeOB2 and hPOT1

(A) Amino acid alignment of hPOT1 with the OB fold domains of CeOB1 (left panel) and CeOB2 (right panel). The consensus is shown above the alignments. Alignments were generated using Geneious software (Matrix: Blosum 62; gap open penalty: 12; gap extension penalty: 3; Algorithm: Global alignment with free ends and gaps) **(B)** 3D model prediction of CeOB1 (green), CeOB2 (red) and POT1 (blue) with DNA. Models were generated as described in the Experimental Procedures section. **(C)** Four different views of the merge of the predicted structures of CeOB1, CeOB2 and POT1.

Supplemental Tables

Table S1

Non cross-linked					
Fraction	% of molecules				n
	linear	looped	Circular	other	
35	80	1	18	1	72
36	82	4	14	0	50
37	75	5	20	0	44
Average	79	**3**	**17**	0	
Total					166
SD	3.6	2	3.5	0.6	

Cross-linked					
Fraction	% of molecules				n
	linear	looped	Circular	other	
35	58	23	12	0	26
36	79	10	11	0	71
37	70	12	18	0	57
Average	69	**15**	**14**	0	
Total					154
SD	11	7	4	0	

Table S1: Psoralen crosslinking increases the number of looped molecules in telomere-containing DNA fractions

Linear, looped, circular and other molecules were counted in the three fractions with the highest content of telomeres. The number of molecules counted (n), the average and standard deviation (SD) are indicated.

Table S2

Sequence Name	Structural similarity to hPOT1	Domains or motifs	Mutant Strain	Telomeric Phenotype
F57C2.3 (CeOB1)	2nd OB fold	none	TM1400	Very long telomeres/ increased G- OH signal and t-circles
3R5.1	2nd OB fold	none	RB1636	Not detected
F39H2.5	2nd OB fold	β-lactamase	TM1354	Progressive telomere shortening
B0280.10 (CeOB2)	1st OB fold	none	TM1620	Long heterogeneous telomeres / increased t-circles

Table S2: POT1 like sequences in the *C. elegans* genome

Sequence names, homology region, recognizable domains and telomeric phenotype of the deletion are listed.

Table S3

	HH PRED				
	Probability	E-value	P-value	Score	aa
CeOB1					
hPOT1	100	2.3 E-43	0	264.8	152-294
TEBPα	99	1.7 E-11	1.1 E-15	83.8	211-312
CeOB2					
TEBPα	97.7	1.8 E-5	1.2 E-09	53.2	34-212
hPOT1	97.3	0.00018	1.2 E-8	46.7	2-144
	I-TASSER (C-score of the best model)				
CeOB1	-2.83				
Ce OB2	-3.45				

Table S3: CeOB1 and CeOB2 align with the OB folds of hPOT1 and TEBPα.

CeOB1 and CeOB2 protein sequences were used as a seed for the structure prediction program HHpred. HHpred creates a Hidden Markov Model (HMM) profile from such an alignment and compares this profile to a database of HMM profiles for known structures. In a search of a database of HMM profiles for structures found in the Protein Data Bank, the second OB-fold of hPot1 (1xjv_A) and TEBPα (1jb7_A) were the top-ranked hits for CeOB1 with *E*-values and probability scores shown in the table. For CeOB2, the top-ranked hits were the first OB-fold TEBPα and hPot1 with the *E*-values and probability scores shown in the table. Similar results were obtained in the structure-prediction metaserver 3D-Jury.

The 3D models were created using I-TASSER (http://zhang.bioinformatics.ku.edu). When analyzing CeOB1 the C-score of the first (best) model in the prediction was -2.83. For CeOB2 the C-score of the best model was -3.45. The 3D models created by I–TASSER were plotted using the MacPymol software.

These models were aligned to the crystal structure of hPOT1 using the DaliLite program (http://www.ebi.ac.uk/DaliLite) and plotted using the MacPymol.

Table S4

	Soluble protein (%)	Minimal repeat number	Active protein (%)	K_D value (nM)
CeOB1	50	2	10	15
CeOB2	10	1	7	90

Table S4: Binding properties of CeOB1 and CeOB2

Percentage of soluble protein, minimal number of telomeric repeats required for binding, active fraction and K_D values are listed.

Supplemental Experimental Procedures

Strains

The strains used in this study include the following: N2, N2 ancestral, CC2, CF512, CB3191 and CB3192 (*Caenorhabditis* Genetics Center, Minneapolis, MN), and the TM1400 and T1620 mutant strains (Shohei Mitani National Bioresource Project for the Experimental Animal Nematode *C. elegans*, Japan).

Two-Dimensional Gel Electrophoresis

2D telomere blots were performed as described previously (Cesare and Griffith, 2004). The first dimension telomeric *Alu*I/*Mbo*I restriction fragments were separated overnight at RT in a 0.5% agarose gel in 0.5X TBE (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA) at 1V/cm. The gels were stained with 300 ng ethidium bromide/ml ethidium bromide in 0.5X TBE, destained in 0.5X TBE, and visualized by UV light. The appropriate lanes were then excised. A 1.1% agarose gel in 0.5X TBE containing 300 ng of ethidium bromide/ml was poured around the excised slab, and the second-dimension electrophoresis was carried out at 4°C in 0.5X TBE containing 300 ng of ethidium bromide/ml with the following conditions: 15 V/cm, 4 h. The gels were visualized using UV light. In-gel hybridization was done as described above. The signal was visualized using a Typhoon 9400 PhosphoImager (Amersham/GE Healthcare). Images were analyzed using ImageQuant software (Molecular Dynamics). A large scale preparation of *C. elegans* wild type CC2 embryos and isolation of nuclei was performed by bleaching adult hermaphrodites as previously described (Hope,

1999). Embryos (a total volume of 10 ml) were quickly frozen in liquid Nitrogen in an equal volume of 1X PBS and stored at -80°C until used.

Nuclei Preparation and DNA purification for EM analysis

A large scale preparation of *C. elegans* wild type CC2 embryos was performed by bleaching adult hermaphrodites as previously described (Hope, 1999). Embryos (a total volume of 10 ml) were quickly frozen in liquid Nitrogen in an equal volume of 1X PBS and stored at -80°C until used.

Nuclei purification was performed as described (Hope, 1999). Embryos were thawed in ice and transferred to a pre-chilled Wheaton stainless-steel tissue grinder (clearance 0.0005 inches/12.5µm). An equal volume of 2X Nuclear Preparation Buffer containing 20mM Hepes pH7.6, 20mM KCl, 3mM $MgCl_2$, 2mM EGTA, 0.5M sucrose, 1mM PMSF and 1x Complete Protease Inhibitor Cocktail (Roche) was added. Homogenization was performed with three sets of 5 strokes. To ensure complete homogenization and that nuclei were intact, a small aliquot of the preparation was visualized under a dark field microscope and under a fluorescent microscope by adding DAPI to 1µg/ml.

The homogenate was centrifuged at 4000g for 10 min at 4°C to pellet the nuclei. Nuclei were then washed and re-suspended in homogenization buffer containing 10 mM Tris-HCl pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.2% NP-40 (IGEPAL) and 5% sucrose, supplemented with 200 µg of AMT (4′-aminomethyl trioxsalen; Sigma)/ml for cross-linking. One third of the sample was kept aside to use as non-crosslinked control. The rest was transferred to a glass petri dish and UV

crosslinked for 30 min using a 365nM UV light. The sample was kept on ice with constant and gentle stirring throughout the procedure. Crosslinked and control nuclei were lysed in TENS-PK overnight at 50°C. Samples were extracted 3 times with Phenol-Chloroform-Isoamyl alcohol, ethanol precipitated, washed with 70% ethanol and resuspended in 10 mM Tris HCl (pH 7.5), 0.1 mM EDTA. Telomeric restriction fragments were obtained in the presence of 2 µg/ml purified Single Strand Binding protein (SSB) from *E. coli*, using *Alu*I and *Mbo*I restriction enzymes. Efficiency of digestion was verified on agarose gels and DNA was quantified using Hoechst 33423 fluorimetry. After digestion DNA was purified with TENS-PK, phenol extracted and ethanol precipitated overnight at -20°C. 2 mg of DNA were obtained for each sample. The DNA was fractionated using a Pharmacia (Piscataway) Gradifrac system with a C16/70 column (Amersham, Piscataway) packed with Bio-Gel A15m matrix (Bio-Rad) as described previously (Cesare and Griffith, 2004; Cesare et al., 2003). DNA (50 ng) from purification samples was applied to a nylon membrane using a slot blot manifold and was visualized using ^{32}P end-labeled oligonucleotide $[TTAGGC]_4$ as a probe. The membrane was visualized using a PhosphoImager and quantified using ImageQuant software (Molecular Dynamics).

Electron microscopy

T-loops and t-circles were visualized by surface spreading of telomeric fractions from size exclusion chromatography on a denatured protein film as described previously (Griffith et al., 1999). To confirm that the DNA was telomeric, DNA was digested with DdeI (New England Biolabs) for 1h and passed over a 2 ml column of 2% agarose beads (Agarose Bead Technology)

equilibrated with 10 mM Tris-HCL (pH 7.6), and 0.1 mM EDTA followed by EM analysis.

Telomeric single stranded DNA was visualized by incubating telomeric fractions from size exclusion chromatography with 500 ng of *E. coli* Single Strand Binding protein (SBB) prepared in the JDG laboratory for 30 min on ice. Free protein was removed using 2% agarose beads as above and the purified protein-DNA fractions mixed with a buffer containing 2.5 mM spermidine and incubated on glow charged carbon foil grids for 3 min. Carbon grids were washed in water and dehydrated in a series of ethanol washes, air-dried and rotary shadowcast with tungsten. Samples were analyzed using an FEI Tecnai 12 transmission electron microscope (FEI inc) at 40 KV and images were captured on a Gatan slow-scan CCD camera and supporting software (Gatan Inc). Image size and contrast was adjusted using Adobe Photoshop.

For EM of CeOB1 and CeOB2 at t-loops the proteins CeOB1, CeOB2, SSB, or GST (~50ng) were incubated with telomeric DNA in buffer containing 20mM HEPES (pH 8.0), 100mM KCL, 1mM DTT for 5 min at RT. Protein-DNA complexes were fixed with 0.6% glutaraldehyde and mounted on carbon coated copper grids and rotary shadowcast with tungsten.

Single Telomere Length Analysis (STELA)

Genomic DNA was prepared as described above and diluted to a 20ng/µl concentration. A mix of 20 ng of DNA and 1 µl of 10 µM oligo (telorette) in a 2 µl volume was incubated at 60°C for 10 min. Ligation was carried out as described (Cheung et al., 2004) using T4 ligase (Invitrogen). The ligase was then

inactivated by incubation at 70°C for 15 min. Ligated DNA was used as template in subsequent PCR reactions. A 15 µl PCR reaction contained the 250pg of ligated DNA, 1X PCR buffer IV (ABgene), 2 mM MgCl2, 0.5 µM each of the teltail primer and the subtelomeric-region-specific primer, 0.3 mM of each dNTP (Amersham) and 1.5U Extensor Hi-Fidelity PCR Enzyme Mix (ABgene). Thermal cycling conditions were the following: initial denaturation at 94°C for 3 min, 25 cycles of 94°C for 20s, 64°C for 30s, and 70°C for 8 min, followed by final elongation at 70°C for 10 min. PCR products were separated on 1% agarose gel. In gel hybridization was performed as described above using as probe a ^{32}P end-labeled $[TTAGGC]_4$ oligonucleotide. Signals were detected using a Typhoon 9400 PhosphorImager (Amersham/GE Healthcare).

The oligonucleotide sequences used in the STELAs were modified from (Cheung et al., 2004) as follows:

Teltail 5′-TGCTCCGTGCATCTGGCATC-3′, Telorette 503 5′-GACAGCTATGACTGCTCCGTGCATCTGGCATCTAAGCCT-3′, Telorette 503b 5′-GACAGCTATGACTGCTCCGTGCATCTGGCATCTAAGCCTAAGCCT-3′, Unrelated Linker 5’-GACAGCTATGACTGCTCCGTGCATCTGGCATC-3’, Subtel VL 5′-GATGC-GCAGCTAACTATAGGAC-3′, Subtel VR 5′-ACCAACAAGGGAACACGATAACTA-3′, Telorette INV-1 5′-AGGCTTAGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3′, Telorette INV-2 5′-TAGGCTTGATGCCAGATGCACGGAGCAGTCATAGCTGTC3′, Telorette INV-3 5′-TTAGGCTGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3′, Telorette INV-4 5′-GGCTTAGGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3′,

Telorette INV-5 5′-CTTAGGCGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3′, Telorette INV-6 5′-GCTTAGGGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3′, Unrelated Linker INV 5’-TCCGATGATGCCAGATGCACGGAGCAGTCATAGCTGTC-3’

Sequence alignments and 3D models

Alignments for CeOB1 (Wormbase accession number F57C2.3) and CeOB2 (Wormbase accession number B0280.10) were generated independently, and the alignments were based on a consensus between the alignments provided by the structure-prediction programs HHPred, 3D-Jury and I-TASSER. For pair wise structure comparison between CeOB1 or CeOB2 and hPOT1 we used the DaliLite program (http://www.ebi.ac.uk/DaliLite) using the crystal stucture of hPOT as template (http://www.rcsb.org/pdb/explore.do?structureId=1XJV) (Lei et al., 2004). The 3D models created by I–TASSER and DaliLite were plotted using the MacPymol software.

Protein expression and purification

CeOB1 and CeOB2 were cloned in a GATEWAY-modified pET28 vector carrying an N-terminal 9-His tag. The constructs were expressed in *E. coli* BL21 (DE3). Freshly transformed bacterial cultures with relevant plasmids, were grown in 2L liquid LB medium containing 100µg/ml carbenicillin at 37 °C to A_{600} = 0.6. After that, cultures were set on ice for 30 min, IPTG was added to a final concentration of 250µM and cultures were incubated 16 h at 18 °C in the presence of 2% ethanol. Cells were pelleted at 5,000g for 20 min, resuspended in 20ml of lysis buffer TBS (50 mM Tris-HCl pH 8, 500 mM NaCl, 1X Complete Protease Inhibitor

Cocktail (Roche) and 1mM PMSF) and lysed using an EmusliFlex-C3 (Avestin) using two cycles of 15,000 psi. Lysates were clarified by centrifugation at 25,000 g for 40 min at 4 °C. Pellets were discarded and protein were purified from the soluble fraction. Packed Talon Beads (Clontech-BD Bioscience) were equilibrated in TBS, added to the cleared lysate in the presence of 8mM Imidazol and rotated for 2 h at 4 °C. Beads were washed three times for 30 min at 4 °C in 15 ml TBS, and transferred to a column. Bound protein was eluted in the presence of 200 mM Imidazol. Protein concentration was measured using the Bradford assay (BioRad) using a BSA standard dilution curve. Protein purifications were checked on a Coomassie-stained 12% (w/v) SDS-PAGE gel and confirmed using using the Monoclonal Anti-polyHis Antibody H1029 (Sigma).

Oligonucleotide sequences for mobility shift assays

3R CE-G 5'-TCGAACGATGGGTTAGGCTTAGGCTTAGGC-3', 3R CE-C 5'-GCCTAAGCCTAAGCCTAAGCTTCGTACCC-3', 3R human 5'-TCGAACGATG-GGTTAGGGTTAGGGTTAGGG-3', S. cerevisiae 5'-TGGGTGTGTGGGTGTGT-GGGTGTGGGTGTG-3', NR oligo 5'-TCGAAGCATGGGTCGAAGCATGGGTCGAAG-3', 3R CE-G INV 5'-TTAGGCTTAGGCTTAGGCTCGAACGATGGG-3', 3R CE-C INV 5'-TCGAACGATGGGGCCTAAGCCTAAGCCTAA-3'.

K_D measurement

The equilibrium dissociation constants (K_D) for CeOB1 and CeOB2 were determined by quantitative analysis of mobility shift assays as described previously (Travers and Buckle). As recombinant proteins are often not fully

active, the active portion of each protein preparation was determined in order to get an accurate [P] value for the K_D equation. This was accomplished by using an oligo saturation assay. Bound and free DNA were separated by EMSA and quantified by PhosphorImager analysis. In these assays the minimum number of repeats that can bind CeOB1 and CeOB2 were used as oligos (5' CGTCAATCGAACGATGAGTTAGGCTTAGGC 3' and 5' CGTCAAGGTAGATCGAACGATGAGGCCTAA 3', respectively).

Sequences for generation of HA-tagged CeOB1 and CeOB2 constructs

Primers used for CeOB1 were:

CeOB1-HA

5'GAATGCTATTAGGCATAGTTGGGACGTCATATGGATATTGTGGTGGATCTC TCGGTCTTCG3' and

CeOB1-N 5'CCCGGGATGTCCTCGTTCGACCGTCGTATT 3'

Primers used for CeOB2 were:

CeOB2-HA

CCCGGGATGTATCCATATGACGTCCCAGACTATGCCATGCAATACACTTACC AGCACATT 3' and

CeOB2-C 5'GAATGCTATTAAATATTAATATTGTAGAAAACAAC 3'

Chromatin immunoprecipitations

Mixed-stage worms were grown on HG plates seeded with OP50 bacteria and harvested. The compact worm pellets were washed 3 times with 1X PBS and cross-linked in 1X PBS containing 1% formaldehyde, followed by incubation for 30 min at 22–25 °C. After centrifugation, the worm pellet was washed three more

times with 1X PBS, was frozen in liquid nitrogen and stored at -80°C. ChIPs were performed as described previously (Verdun et al., 2005) with minor variations. Briefly, 1 µg of antibody (anti-HA, anti-Flag or IgG control) was used for the immunoprecipitations, and the lysates were normalized by protein concentration. For every precipitation, 550 µg of total protein was used. For the quantifications, the signal resulting from hybridizations with the CeRep55 repeat probe 5'CTGGCTTCCCACTATATTTTACTCT3' (http://www.sanger.ac.uk/Projects/C_elegans/REPEATS/gifs/CeRep55.gif) was subtracted from the telomeric signal to account for nonspecific background.

Supplemental References

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