Supplemental Figures, Tables, and Methods

Shelterin is a Dimeric Complex with Extensive Structural Heterogeneity

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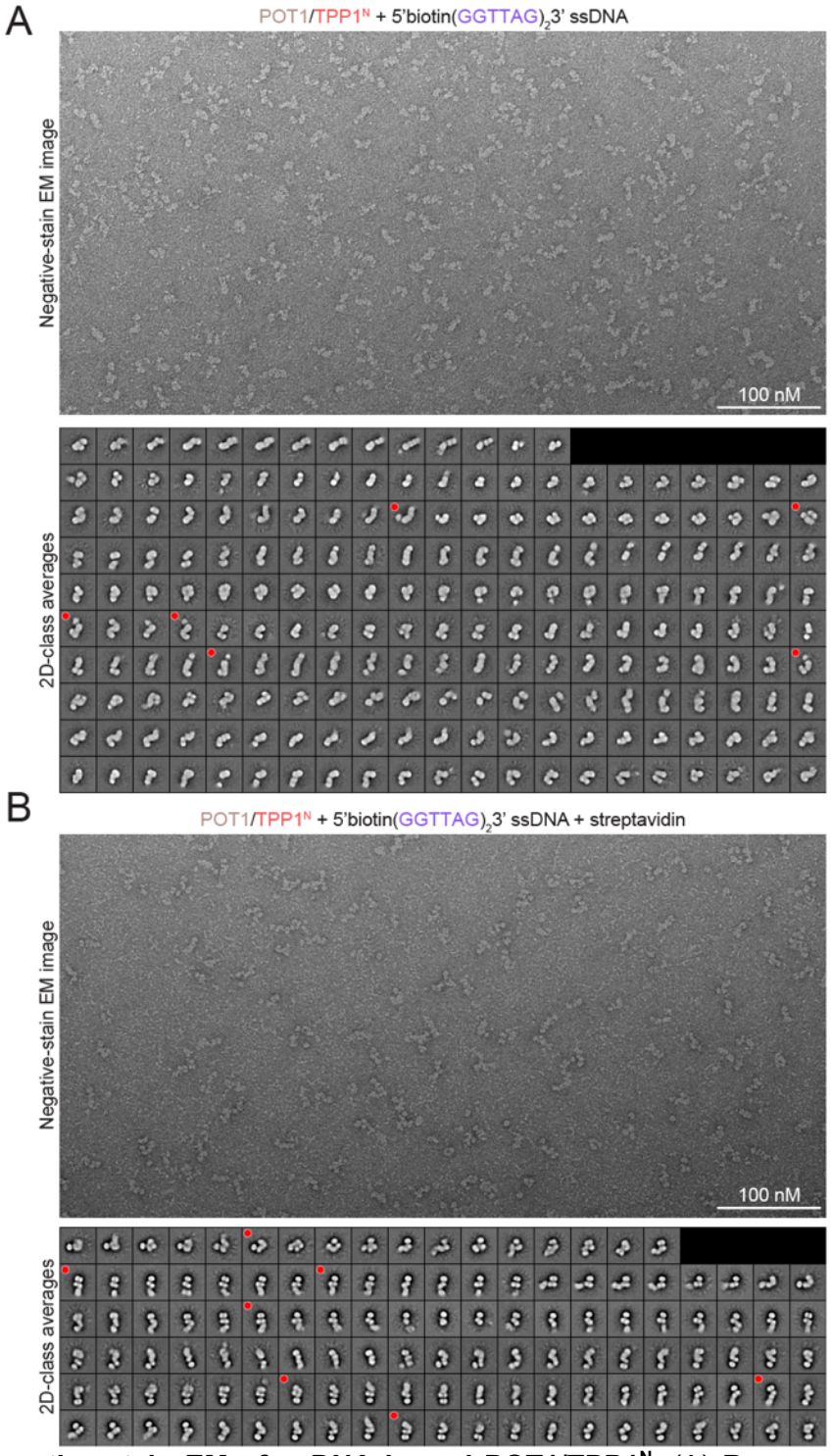
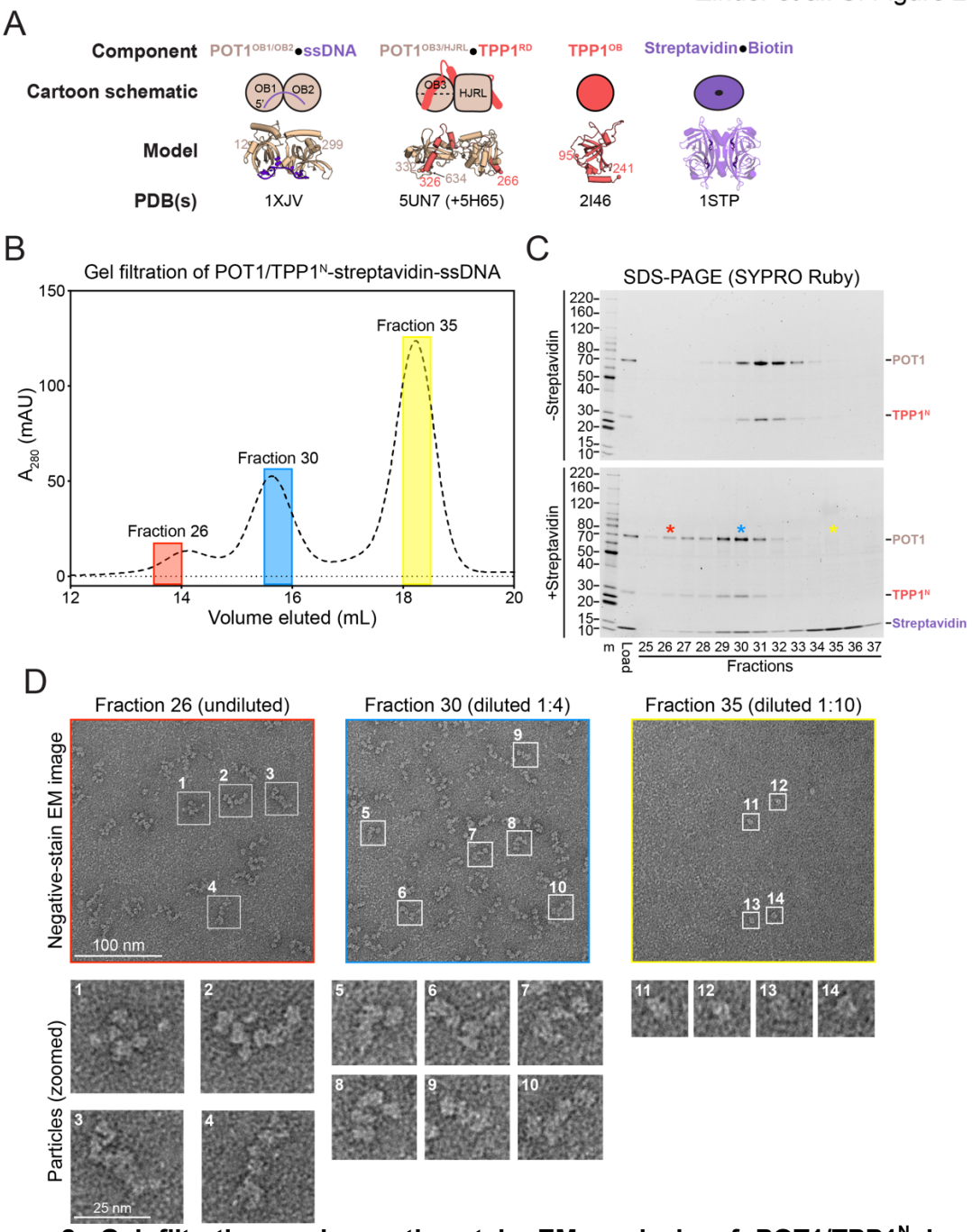
Running title: Structural Heterogeneity of Human Shelterin

Figures and Legends

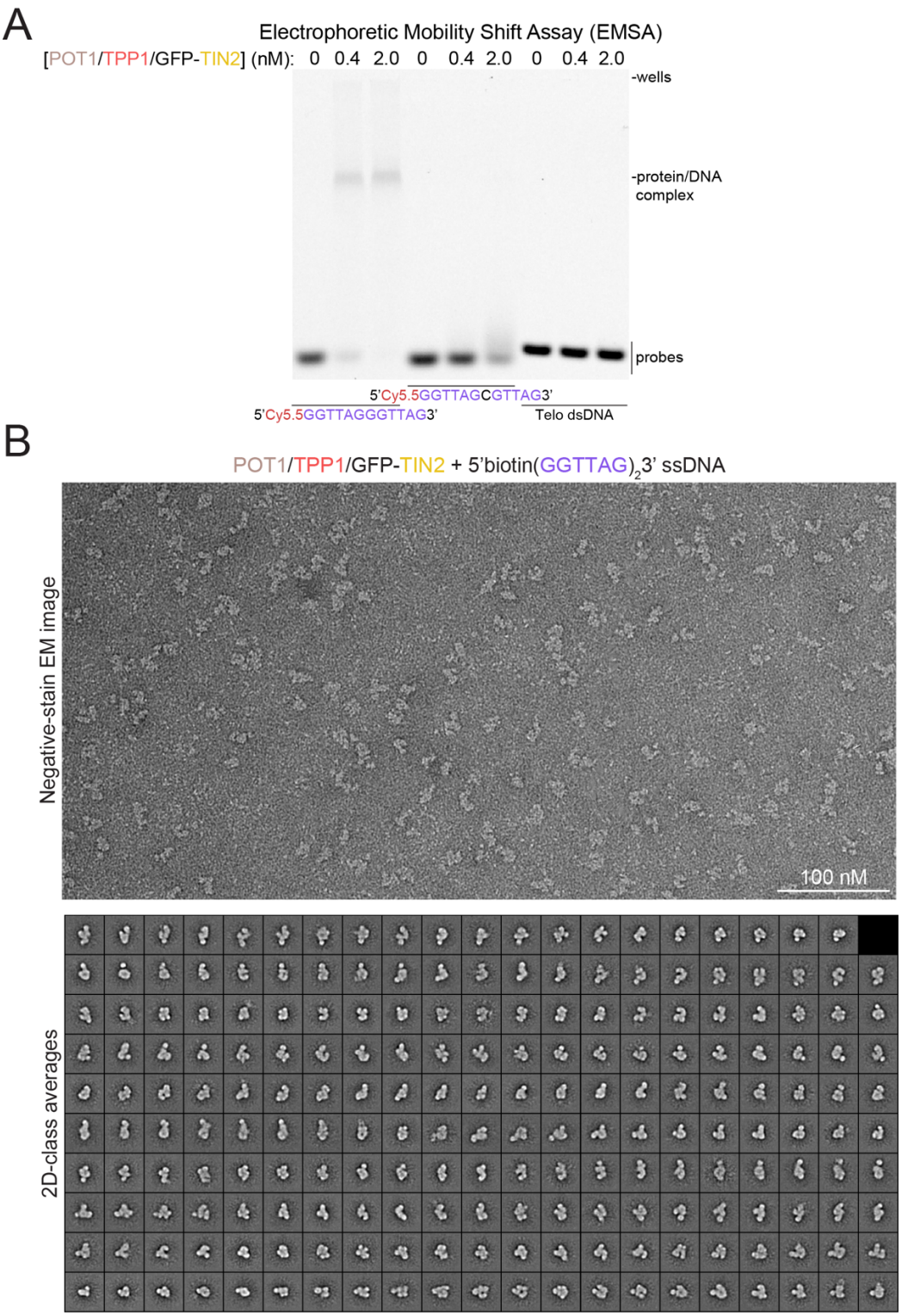


SI Figure 1. Negative-stain EM of ssDNA bound POT1/TPP1^{N}. (A) Raw negative-stain EM image and 2D-class averages of POT1/TPP1^{N} bound to 5'BiotinGGTTAGGGTTAG3' ssDNA. Classes selected for display in Figure 1D are indicated with red dots and may have been rotated and/or reflected. (B) Raw negative-stain EM image and 2D-class averages of POT1/TPP1^{N} bound to streptavidin-5'BiotinGGTTAGGGTTAG3' ssDNA. Classes selected for display in Figure 1E are indicated with red dots.

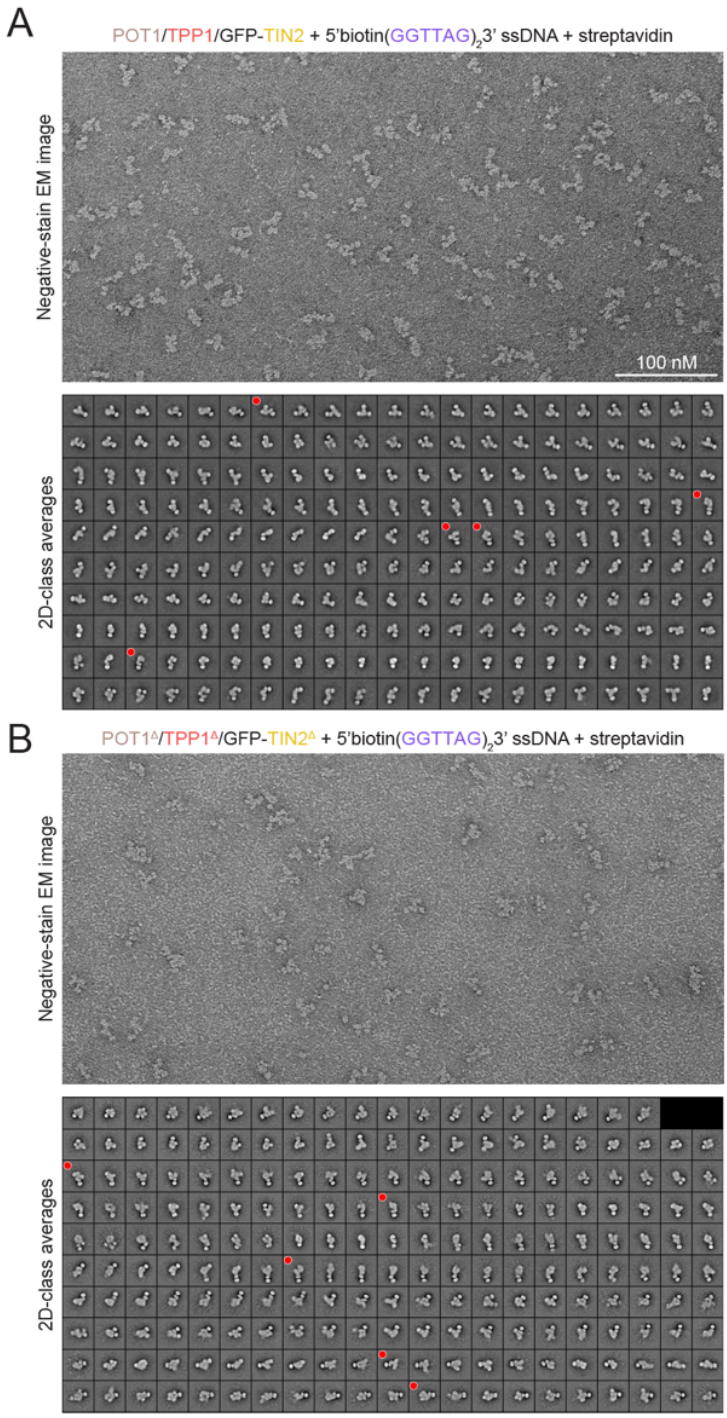
Zinder et al. SI Figure 2



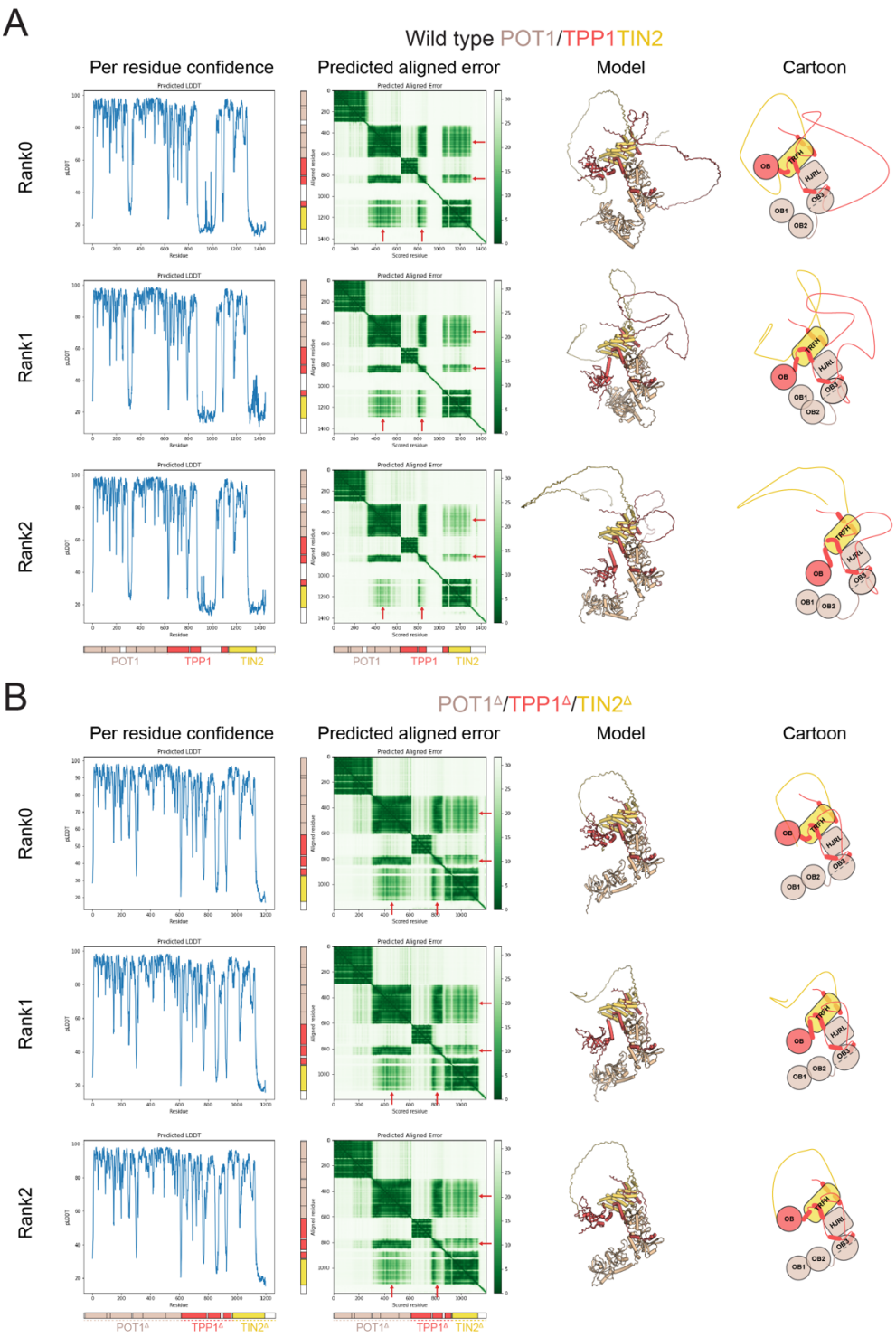
SI Figure 2. Gel filtration and negative-stain EM analysis of POT1/TPP1^{N} bound to streptavidin-ssDNA as a tool to assign domains. (A) Schematic representations, crystal structures, and PDB IDs of streptavidin and structured domains within POT1/TPP1^{N}. Cα atoms of the last modelled residues are shown as spheres with amino acid numbers indicated. Models rendered using Pymol. (B) Superose 6 increase 10/300 GL gel-filtration trace for absorbance at 280 nm of streptavidin-5'BiotinGGTTAGGGTTAG3' ssDNA from Figure 1D with selected fractions highlighted. (C) SDS-PAGE of fractions from gel filtration. Gel is 8-16% polyacrylamide Tris-glycine and stained with SYPRO Ruby. Asterisks correspond to the selected fractions in panel A. Molecular-weight markers are shown in kDa. (D) Raw negative-stain EM images of the indicated fractions with selected particles highlighted and zoomed below.



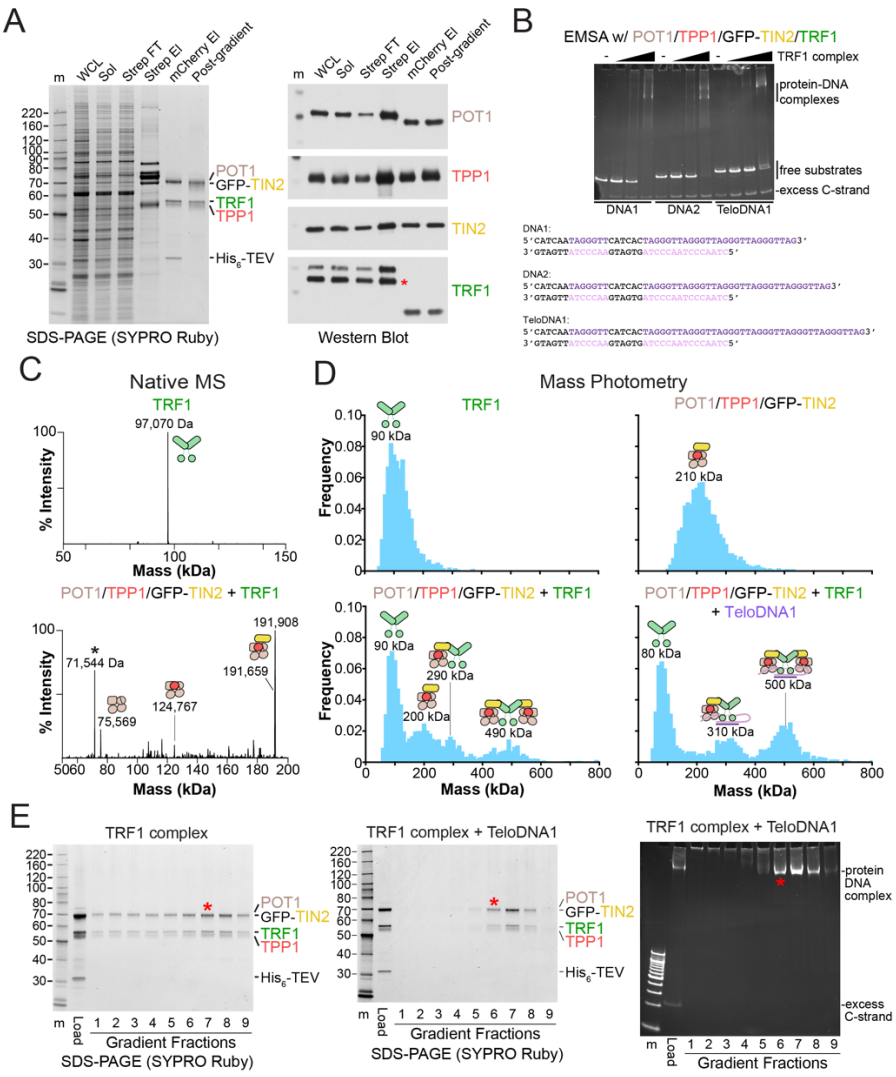
SI Figure 3. DNA binding and negative-stain EM analysis of POT1/TPP1/GFP-TIN2. (A) DNA-binding activity of POT1/TPP1/GFP-TIN2 on telomeric and mutant DNAs. Protein concentrations and ssDNA sequences are indicated. 'Telo dsDNA' with sequence 5'CATCAATAGGGTTCATCCTAGGGTTGTACTG3' was labeled with Cy5.5 dUTP by Klenow polymerase. Probe concentration is 0.25 nM. (B) Raw negative-stain EM image and 2D-class averages of POT1/TPP1/GFP-TIN2 bound to 5'BiotinGGTTAGGGTTAG3' ssDNA.



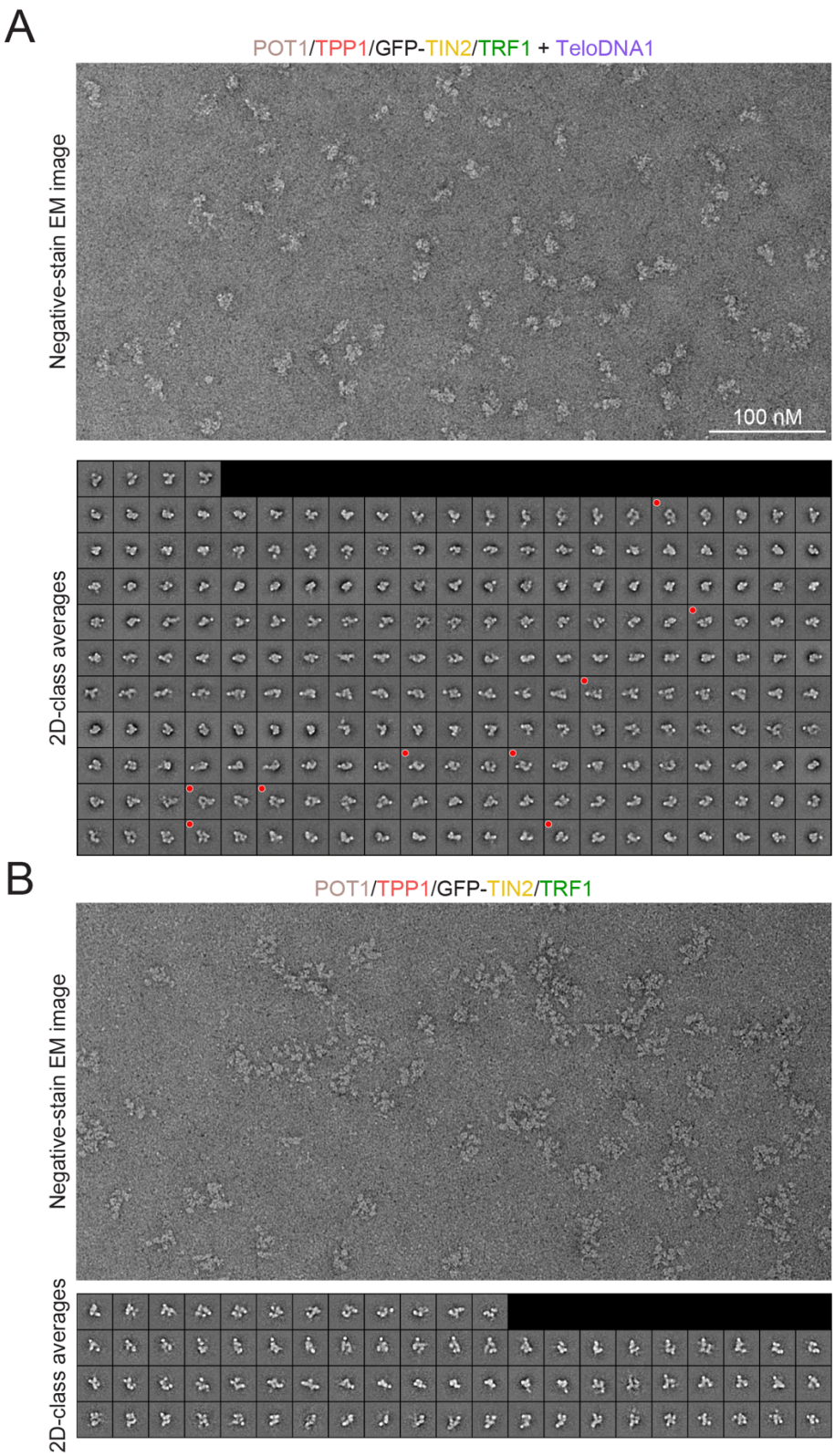
SI Figure 4. Negative-stain EM of wild-type and 3xΔ POT1/TPP1/GFP-TIN2 bound to streptavidin-ssDNA. (A) Raw negative-stain EM image and 2D-class averages of POT1/TPP1/GFP-TIN2 bound to streptavidin-5'BiotinGGTTAGGGTTAG3' ssDNA. Classes selected for display in Figure 2D are indicated with red dots and may have been rotated and/or reflected. (B) Raw negative-stain EM image and 2D-class averages of $POT1^{\Delta}$/$TPP1^{\Delta}$/GFP-$TIN2^{\Delta}$ bound to streptavidin-5'biotinGGTTAGGGTTAG3' ssDNA. Classes selected for display in Figure 2D are indicated with red dots and may have been rotated and/or reflected.



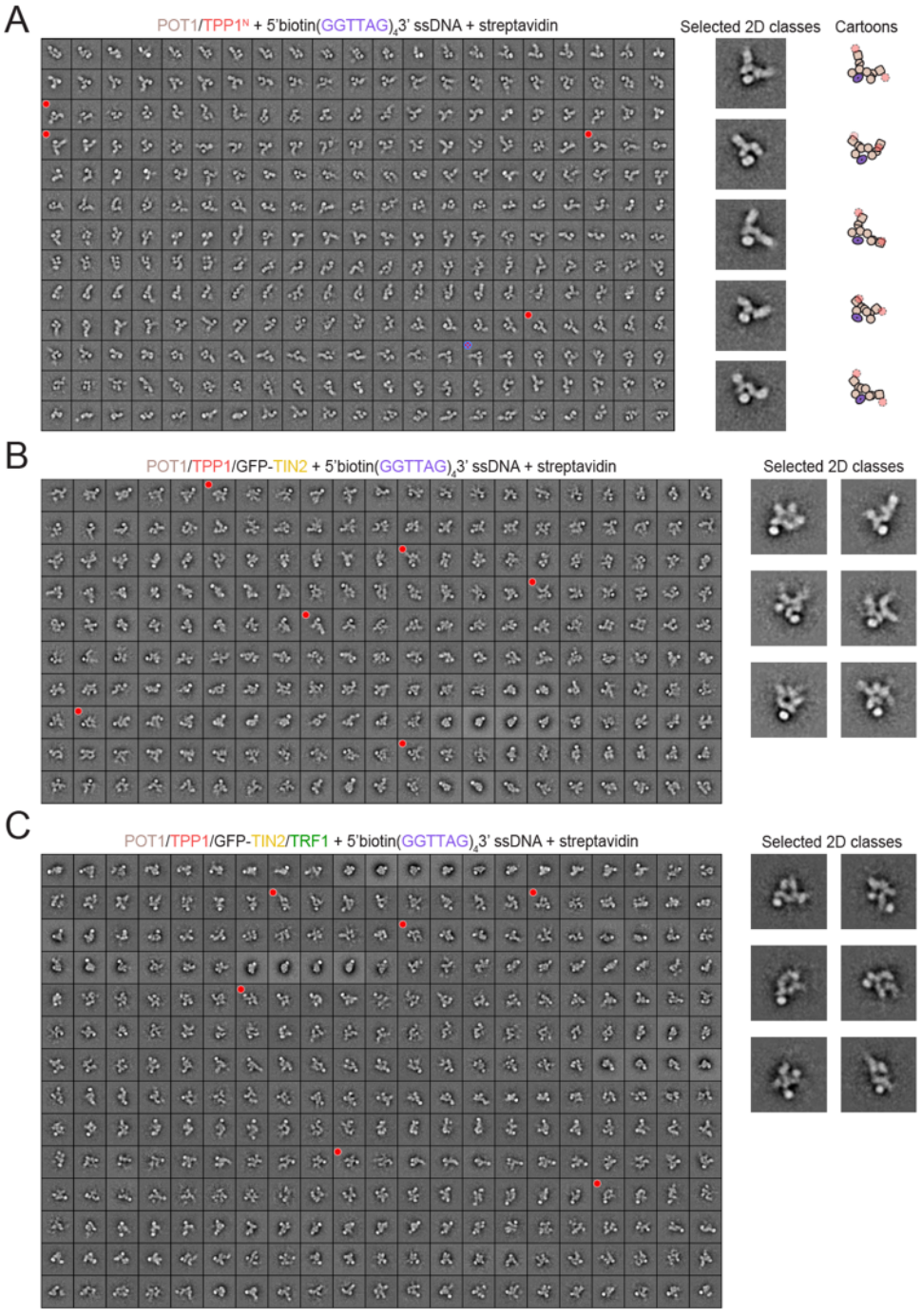
SI Figure 5. AlphaFold-multimer modelling of POT1/TPP1/TIN2. Per-residue confidence, predicted aligned error, structural models (rendered in Pymol) and cartoon schematics for the top 3 of 10 ranked (based on average pLDDT) models for (A) full-length or (B) 3xΔ POT1/TPP1/TIN2. Domain schematics for each protein are shown alongside their corresponding plots. Structural models were aligned based on the POT1 OB3/HJRL. Red arrows in the predicted aligned error chart highlight regions of intermediate error between TIN2's TRFH domain and POT1's C-terminal domains.



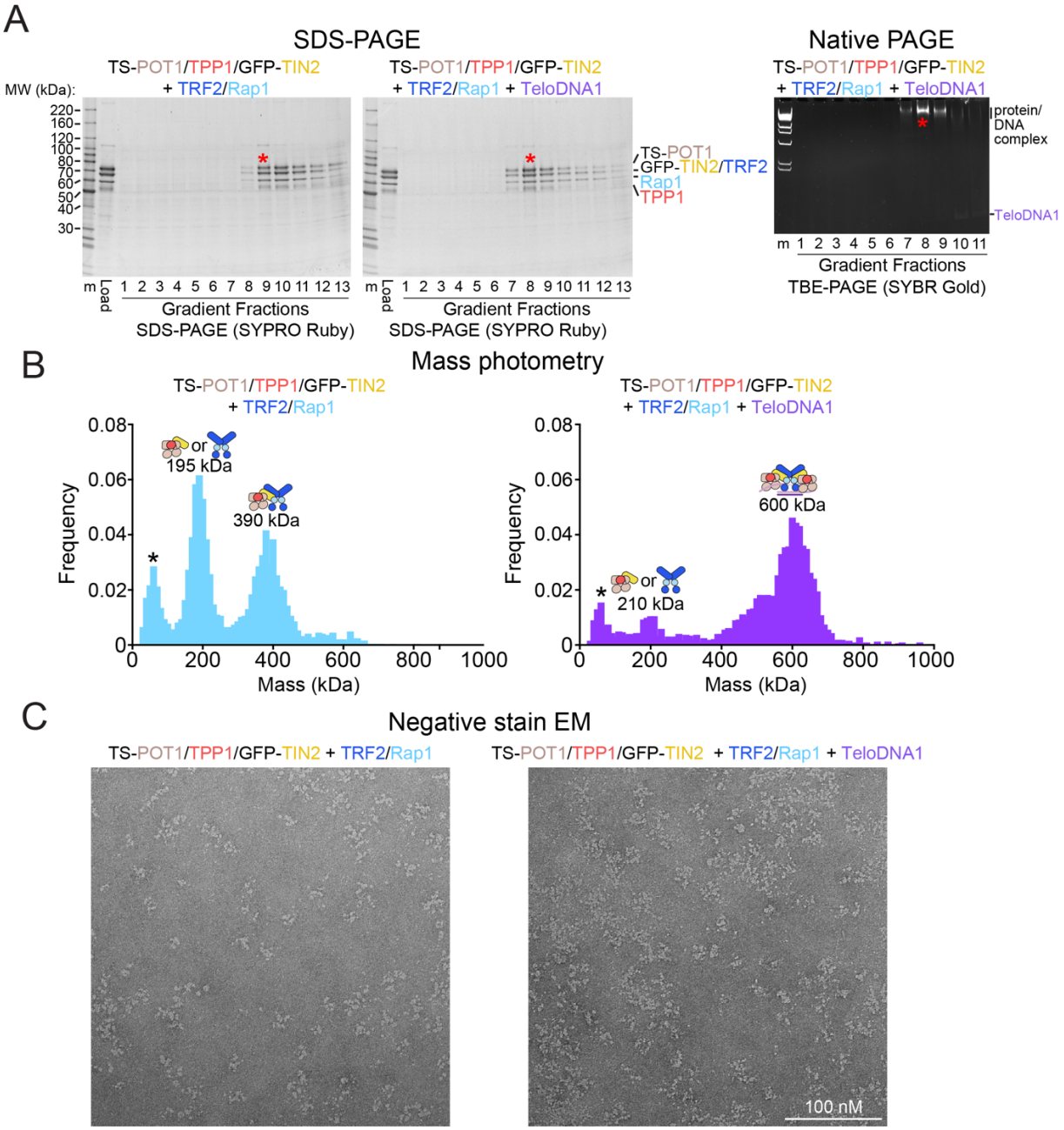
SI Figure 6. Biochemical analyses and reconstitution of the TRF1 complex. (A) SDS-PAGE and Western blot summary of TRF1 complex purification. Gel is 8-16% polyacrylamide Tris-glycine and stained with SYPRO Ruby. Molecular-weight markers are shown in kDa. WCL: whole-cell lysate; Sol: soluble extract; FT: flow-through; El: eluate. Asterisk corresponds to an unknown species derived from the mCherry tag. (B) Qualitative EMSA of the TRF1 complex post mCherry elution binding to the indicated DNAs. DNAs were present at 50 nM and the approximate concentration of the TRF1 complex was determined by GFP absorbance at 488 nm. Gel is 4-20% polyacrylamide TBE and stained with SYBR Gold. T1comp: TRF1 complex. (C) Native MS of purified and phosphatase-treated TRF1 and POT1/TPP1/GFP-TIN2 samples. The measured masses (in Da) are indicated along with cartoons that correspond to complexes. Asterisk indicates a species that likely corresponds to HSP70 contamination. (D) Mass photometry of TRF1 complex components and reconstituted TRF1 complex. Final protein concentrations were 50 nM $(TRF1)_2$, 25 nM POT1/TPP1/GFP-TIN2, 6 nM $(TRF1)_2$ + 12 nM POT1/TPP1/GFP-TIN2, and 6 nM $(TRF1)_2$ + 12 nM POT1/TPP1/GFP-TIN2 + 6 nM TeloDNA1. Peak maxima are indicated along with cartoons that correspond to complexes of that molecular weight to within 10%. (E) SDS-PAGE of fractions from glycerol-gradient purification. Lower number fractions have higher glycerol concentrations. SDS-PAGE gel is 8-16% polyacrylamide Tris-glycine and stained with SYPRO Ruby and native TBE gel is 4-20% polyacrylamide TBE and stained with SYBR Gold. Asterisks indicate fractions that were used for negative-stain EM analysis.



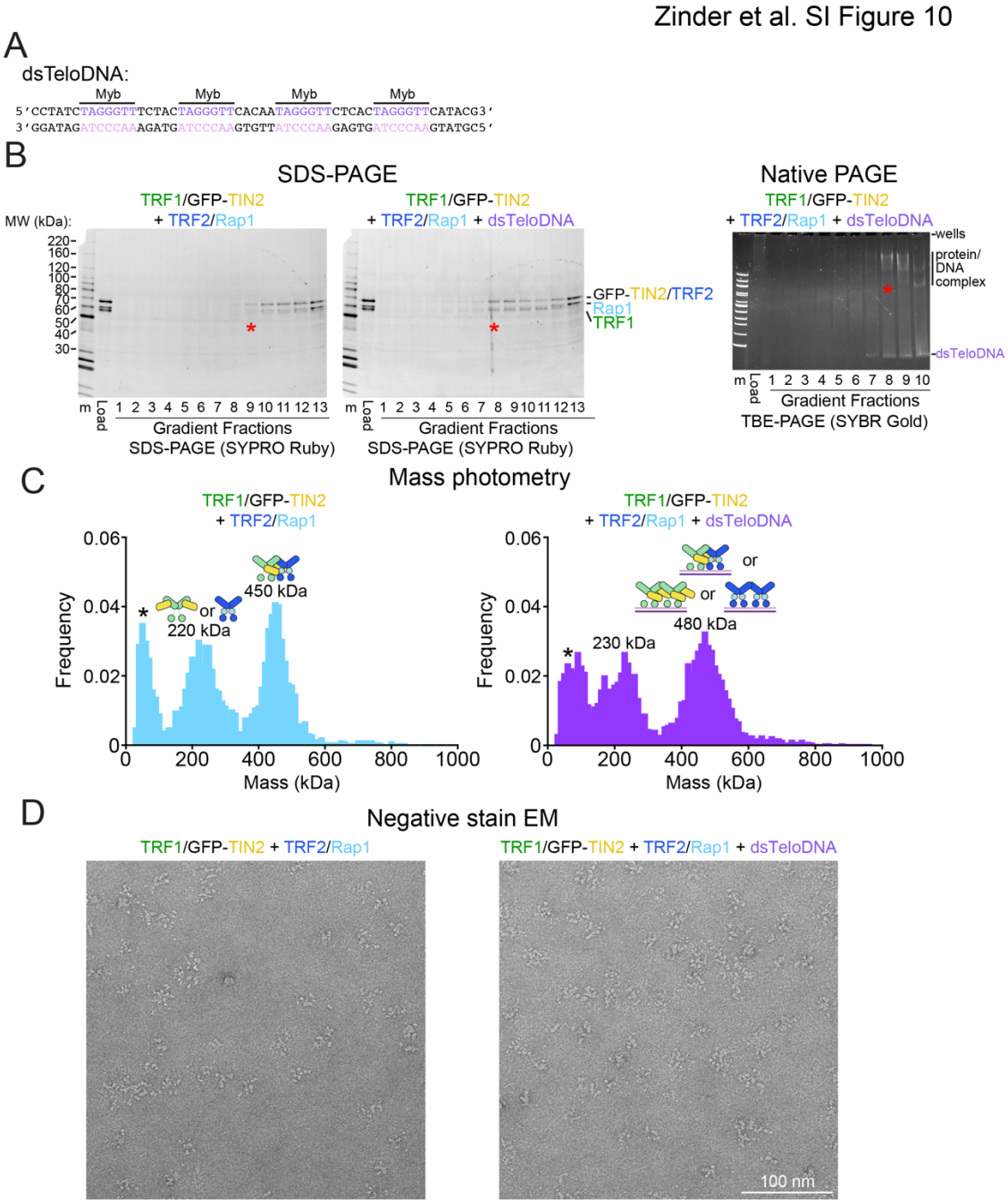
SI Figure 7. Negative-stain EM of the TRF1 complex. (A) Raw negative-stain EM image and 2D-class averages of the TRF1 complex bound to TeloDNA1 ssDNA. Classes selected for display in Figure 3F are indicated with red dots. (B) Raw negative-stain EM image and 2D-class averages of the TRF1 complex.



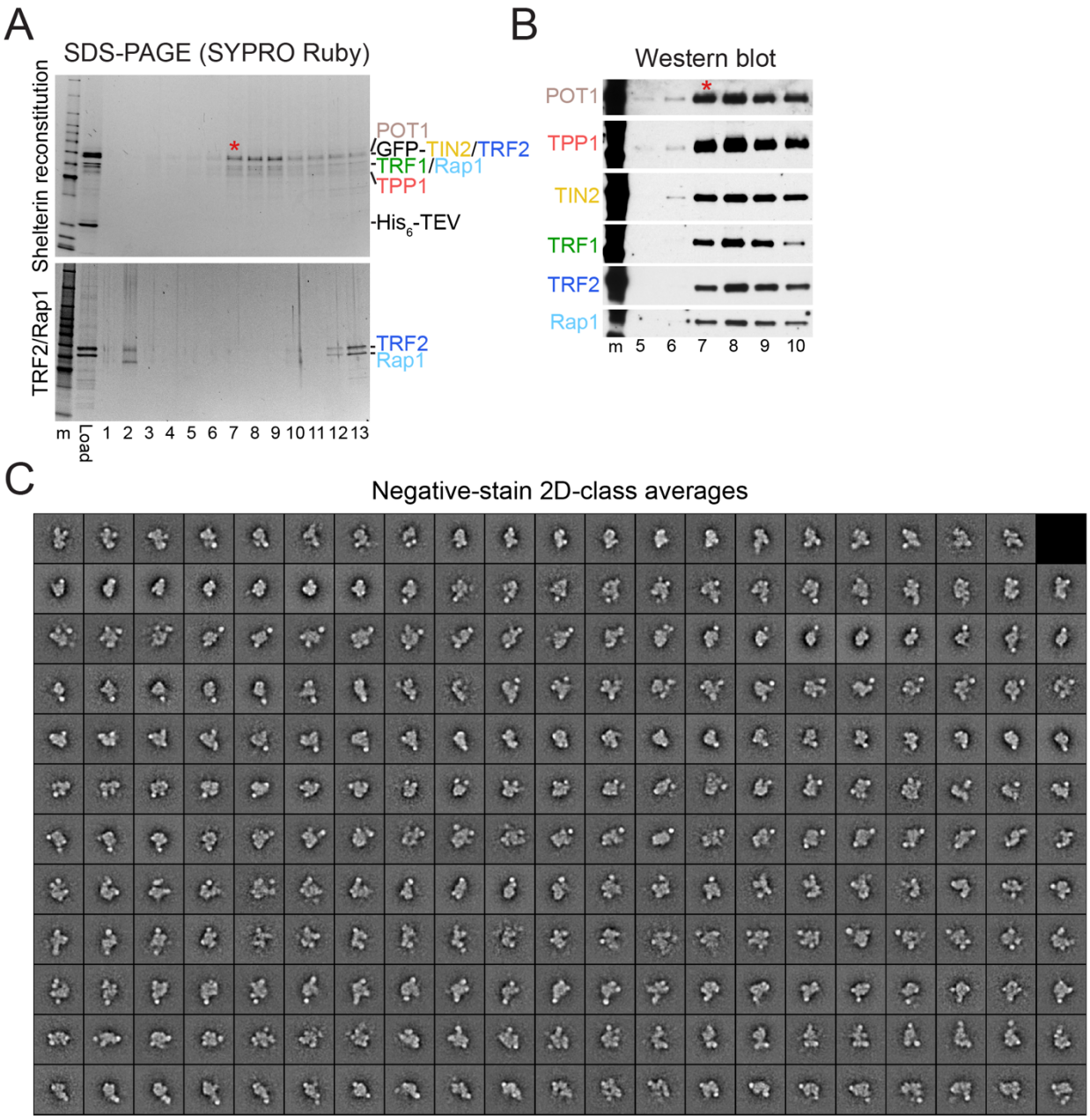
SI Figure 8. Negative-stain EM of shelterin subcomplexes bound to DNA containing two POT1-binding sites. (A) 2D-class averages of POT1/TPP1^{N} bound to streptavidin-5'Biotin(GGTTAG)$_4$3' ssDNA. Selected classes showing streptavidin associated with a curved U-shape with cartoon interpretations are indicated with red dots and expanded with cartoon interpretations (right side). (B) 2D-class averages of POT1/TPP1/GFP-TIN2 bound to streptavidin-5'Biotin(GGTTAG)$_4$3' ssDNA. Selected classes showing streptavidin associated with a curved U-shape are indicated with red dots and expanded (right side). (C) 2D-class averages of the TRF1 complex bound to streptavidin-5'Biotin(GGTTAG)$_4$3' ssDNA. Selected classes showing streptavidin associated with a curved U-shape are indicated with red dots and expanded (right side).



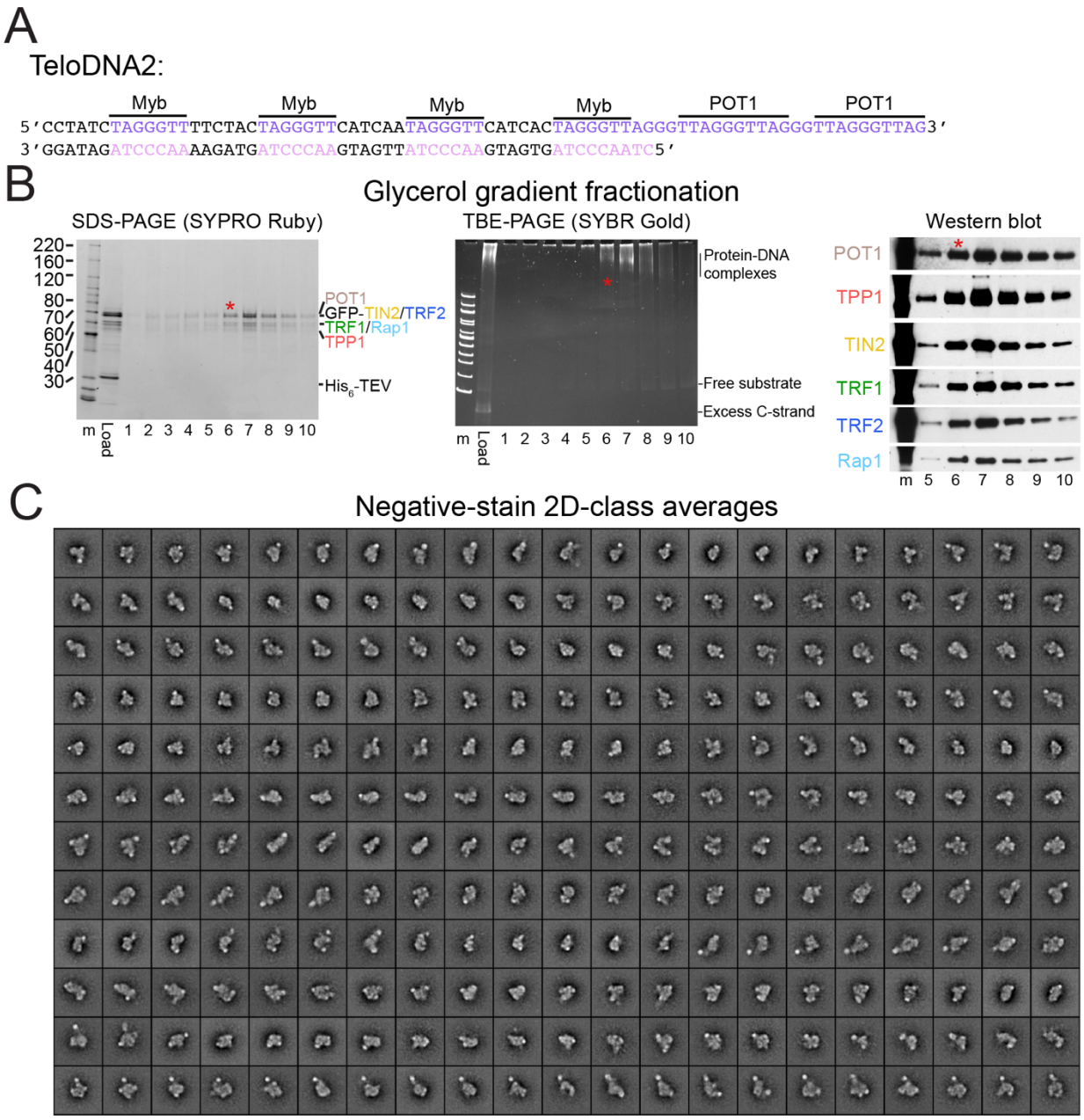
SI Figure 9. Reconstitution and analysis of POT1/TPP1/TIN2/TRF2/Rap1. (A) SDS- and native PAGE of glycerol-gradient fractions of the TRF2/Rap1 + POT1/TPP1/GFP-TIN2 reconstitution in the absence or presence of TeloDNA1. Lower number fractions have higher glycerol concentrations. Gel for SDS-PAGE is 8-16% polyacrylamide Tris-glycine run in MOPS-SDS buffer and stained with SYPRO Ruby and molecular-weight markers are shown in kDa. Gel for native PAGE is 4-20% polyacrylamide TBE and stained with SYBR Gold. Red asterisks indicate the fractions used for negative-stain EM and mass photometry analysis. (B) Mass photometry of reconstituted complexes. Final protein concentrations are unknown. Peak maxima are indicated along with cartoons that correspond to complexes of that molecular weight to within ~10%. Asterisk indicates a peak derived from the buffer. (C) Raw negative-stain EM images of reconstituted complexes.



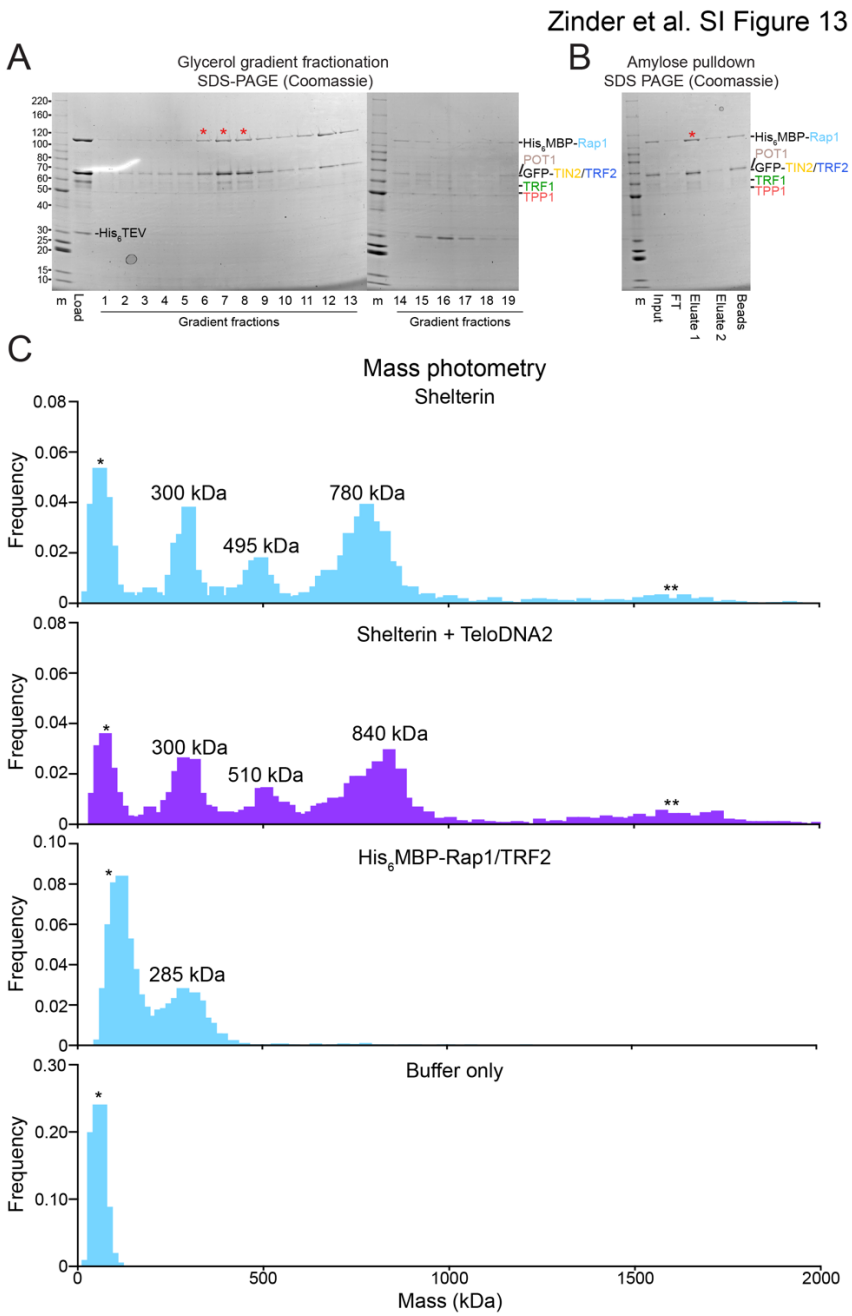
SI Figure 10. Reconstitution and analysis of TRF1/TIN2/TRF2/Rap1. (A) DNA sequence of dsTeloDNA with binding sites for TRF1/2 Myb domains indicated. (B) SDS- and native PAGE of glycerol-gradient fractions of the TRF2/Rap1 + POT1/TPP1/GFP-TIN2 reconstitution in the absence or presence of dsTeloDNA. Lower number fractions have higher glycerol concentrations. Gel for SDS-PAGE is 8-16% polyacrylamide Tris-glycine run in MOPS-SDS buffer and stained with SYPRO Ruby and molecular-weight markers are shown in kDa. Gel for native PAGE is 4-20% polyacrylamide TBE and stained with SYBR Gold. Red asterisks indicate the fractions used for negative-stain EM and mass photometry analysis. (C) Mass photometry of reconstituted complexes. Final protein concentrations are unknown. Peak maxima are indicated along with cartoons that correspond to complexes of that molecular weight to within ~10%. Asterisk indicates a peak derived from the buffer. (D) Raw negative-stain EM images of reconstituted complexes.



SI Figure 11. Reconstitution and negative-stain EM of shelterin. (A) SDS-PAGE of glycerol-gradient fractions of TRF2/Rap1 in the presence or absence of the TRF1 complex. Lower number fractions have higher glycerol concentrations. Gel is 4-12% Bis-Tris run in MOPS-SDS buffer and stained with SYPRO Ruby. Molecular-weight markers are shown in kDa. Asterisk corresponds to the fraction used for negative-stain EM analysis. (B) Western blot analysis of peak fractions from the glycerol gradient in panel A. Asterisk corresponds to the fraction used for negative-stain EM analysis. (C) Negative-stain EM 2D-class averages of reconstituted shelterin.



SI Figure 12. Reconstitution and negative-stain EM of shelterin bound to DNA. (A) DNA sequence of TeloDNA2 with binding sites for TRF1/2 Myb domains and POT1 OB1/2 indicated. (B) SDS-PAGE, native PAGE, and Western blot of the reconstituted shelterin-TeloDNA2 complex. Lower number fractions have higher glycerol concentrations. Gel is 4-12% Bis-Tris run in MOPS-SDS buffer and stained with SYPRO Ruby for SDS-PAGE and 4-20% polyacrylamide TBE and stained with SYBR Gold for TBE-PAGE. Molecular-weight markers are shown in kDa. Asterisk corresponds to the fraction used for negative-stain EM analysis. (C) Negative-stain 2D-class averages of reconstituted shelterin bound to DNA.



SI Figure 13. Amylose pulldown and mass photometry of His6MBP-Rap1 containing shelterin. (A) SDS-PAGE of glycerol-gradient fractions from the shelterin reconstitution using His_6MBP-Rap1. Lower number fractions have higher glycerol concentrations. Gel is 8-16% Tris-Glycine run in Tris-Glycine-SDS buffer and stained with Coomassie Blue. Molecular-weight markers (m) are shown in kDa. Red asterisks correspond to the fractions used for the amylose pulldown. (B) Amylose pulldown of reconstituted His_6MBP-Rap1-containing shelterin. Gel is 8-16% Tris-Glycine run in Tris-Glycine-SDS buffer and stained with Coomassie Blue. Red asterisk corresponds to the fraction used for mass photometry. FT, flowthrough. (C) Mass photometry data from Figure 4E with data at higher molecular weights included. Additionally, mass photometry measurements are shown for TRF2/His_6MBPRap1 and buffer alone. A single asterisk indicates a peak arising from buffer contaminants and double asterisk indicates species far outside of the instrument's calibrated range.

Tables

Supplementary Table 1. Negative-stain EM Summary

Proteins	DNA	Figure(s)	Micrographs	Particles	Particles Per Class
POT1/TPP1^{N}	5'Biotin$(GGTTAG)_2$3'	1D, SI1A	100	40,955	80-200
POT1/TPP1^{N} + streptavidin	5'Biotin$(GGTTAG)_2$3'	1E, SI1B	75	24,820	80-200
POT1/TPP1/GFP-TIN2	5'Biotin$(GGTTAG)_2$3'	SI3B	100	32,813	60-150
POT1/TPP1/GFP-TIN2 + streptavidin	5'Biotin$(GGTTAG)_2$3'	2C, SI4A	140	33,117	60-150
POT1$^{\Delta}$/TPP1$^{\Delta}$/GFP-TIN2$^{\Delta}$ + streptavidin	5'Biotin$(GGTTAG)_2$3'	2C, SI4B	110	30,154	60-150
POT1/TPP1/GFP-TIN2/TRF1	TeloDNA1	3F, SI7A	100	22,851	40-100
POT1/TPP1/GFP-TIN2/TRF1	None	SI7B	100	15,058	40-100
POT1/TPP1^{N} + streptavidin	5'Biotin$(GGTTAG)_4$3'	SI8A	110	31,399	40-100
POT1/TPP1/GFP-TIN2 + streptavidin	5'Biotin$(GGTTAG)_4$3'	SI8B	150	29,112	40-100
POT1/TPP1/GFP-TIN2 + TRF1 + streptavidin	5'Biotin$(GGTTAG)_4$3'	SI8C	200	36,202	40-100
Shelterin	None	4D, SI11C	300	31,939	60-150
Shelterin	TeloDNA2	4D, SI12C	300	31,285	60-150

Supplementary Table 2. Intra-protein crosslinks within POT1/TPP1/GFP-TIN2

Protein	Residue 1	Residue 2	Lowest score	# of precursors	Interacting domains
POT1	427	433	2.0E-18	126	HJRL
TIN2	81	119	9.5E-18	36	TRFH
POT1	355	433	4.9E-30	12	OB3-HJRL
TIN2	101	106	7.0E-17	55	TRFH
POT1	422	433	6.0E-25	62	HJRL
POT1	121	433	1.6E-21	6	OB1-HJRL
TIN2	121	353	9.5E-18	5	TRFH-CTD
POT1	171	234	2.7E-16	145	OB2

POT1	412	433	1.1E-15	14	HJRL
POT1	121	355	4.0E-15	15	OB1-OB3
POT1	121	469	4.3E-15	10	OB1-HJRL
POT1	433	469	5.1E-15	15	HJRL
POT1	121	234	1.6E-14	9	OB1-OB2
POT1	353	469	7.6E-14	3	OB3-HJRL
POT1	85	355	4.1E-13	5	OB1-OB3
POT1	234	469	7.0E-11	6	OB2-HJRL
POT1	85	433	8.3E-11	1	OB1-HJRL
POT1	234	355	5.1E-10	4	OB2-OB3
POT1	33	355	7.7E-10	7	OB1-OB3
POT1	234	433	1.1E-09	3	OB2-HJRL
POT1	33	353	1.3E-09	8	OB1-OB3
POT1	234	289	1.5E-09	48	OB2
POT1	85	469	2.8E-09	8	OB1-HJRL
POT1	121	121	3.0E-08	9	OB2
POT1	353	433	3.0E-08	1	OB3-HJRL
POT1	234	353	1.4E-07	3	OB2-OB3
POT1	182	469	3.0E-07	5	OB2-HJRL
POT1	355	379	3.6E-06	6	OB3
POT1	131	353	3.7E-06	4	OB1-OB3
POT1	407	433	7.0E-06	6	HJRL
POT1	379	433	3.1E-05	3	OB3-HJRL
TIN2	233	235	3.4E-05	3	CTD
TIN2	62	81	1.5E-04	10	TRFH
POT1	469	504	2.1E-04	5	HJRL
POT1	131	469	2.5E-04	4	OB1-HJRL
POT1	85	131	4.1E-04	1	OB2
POT1	131	234	5.6E-04	7	OB1-OB2
TIN2	101	119	6.1E-04	3	TRFH
POT1	353	355	6.6E-04	1	OB3
POT1	379	469	8.0E-04	4	OB3-HJRL
POT1	469	469	8.8E-04	2	HJRL
POT1	355	469	1.4E-03	1	OB3-HJRL
POT1	422	469	1.9E-03	8	HJRL
POT1	131	355	2.8E-03	6	OB1-OB3

Supplementary Table 3. Inter-protein crosslinks within POT1/TPP1/GFP-TIN2

Protein1	Residue	Protein 2	Residue	Lowest score	# of precursors	Interacting domains
POT1	433	TPP1	232	1.2E-20	19	HJRL-OB
POT1	433	TPP1	170	5.2E-21	19	HJRL-OB
POT1	469	TPP1	232	1.7E-07	11	HJRL-OB
POT1	121	TIN2	81	2.0E-14	10	OB1-TRFH
POT1	469	TPP1	170	2.2E-12	9	HJRL-OB
POT1	234	TIN2	81	3.3E-08	8	OB2-TRFH
TIN2	81	TPP1	170	2.1E-06	7	TRFH-OB
POT1	433	TIN2	101	9.3E-15	6	HJRL-TRFH
POT1	121	TIN2	98	1.3E-06	6	OB1-TRFH
POT1	469	TPP1	233	2.8E-11	5	HJRL-OB
POT1	85	TPP1	492	3.0E-08	5	OB1-TIN2BD
POT1	355	TPP1	492	3.9E-05	5	OB3-TIN2BD
POT1	234	TIN2	131	4.6E-04	5	OB2-TRFH
POT1	234	TPP1	170	2.3E-13	4	HJRL-OB
POT1	353	TPP1	170	6.2E-12	4	HJRL-OB
POT1	355	TIN2	101	7.9E-07	4	OB3-TRFH
POT1	234	TIN2	106	1.9E-06	4	OB2-TRFH
POT1	234	TIN2	101	3.0E-05	4	OB2-TRFH
POT1	469	TIN2	106	2.3E-15	3	HJRL-TRFH
POT1	85	TPP1	170	9.6E-14	3	OB1-OB
POT1	469	TPP1	492	2.6E-06	3	HJRL-TIN2BD
POT1	430	TIN2	101	6.4E-11	2	HJRL-TRFH
POT1	433	TPP1	492	3.7E-10	1	HJRL-TIN2BD
POT1	234	TIN2	119	2.4E-07	1	OB2-TRFH
POT1	85	TIN2	101	2.0E-06	1	OB1-TRFH
POT1	433	TIN2	98	9.9E-06	1	HJRL-TRFH
POT1	234	TIN2	233	4.4E-05	1	OB2-CTD
POT1	469	TIN2	235	4.4E-04	1	HJRL-CTD
POT1	427	TPP1	170	9.4E-04	1	HJRL-OB
POT1	353	TPP1	492	1.3E-03	1	OB3-TIN2BD

POT1	433	TIN2	81	2.8E-03	1	HJRL-TRFH

Supplementary Table 4. Mass measurements of shelterin and shelterin subcomplexes with and without incubation with telomerase DNA

Complex/Subcomplex	Expected Mass (Da)	Measured Mass	
		From Native MS (Da)	From Mass Photometry (kDa)
$(TRF1)_2$	97,057	97,070	80 - 90
Twinstrep-POT1/TPP1/GFP-TIN2	191,614	191,623	170 - 210
Twinstrep-POT1/TPP1/GFP-TIN2/ $(TRF1)_2$	288,690	288,806	270 - 290
(Twinstrep-POT1/TPP1/GFP-TIN2/ $TRF1)_2$	480,304	480,700	470 - 490
$(TRF1)_2$/GFP-TIN2	164,312	164,325	-
Twinstrep-POT1/TPP1	124,377	124,472	-
Twinstrep-POT1	75,438	75,504	-
(Twinstrep-POT1/TPP1/GFP-TIN2/$TRF1)_2$ + TeloDNA1	505,116	-	500
$(TRF2/Rap1)_2$	200,023	-	195 - 230
$(TRF1/GFP\text{-}TIN2)_2$	231,187	-	220 - 230
$(TRF2/His_6MBP\text{-}Rap1)_2$	286,420	-	280 - 300
POT1/TPP1/GFP-TIN2/$(TRF2/Rap1)_2$	391,601	-	390
$(TRF1/GFP\text{-}TIN2/TRF2/Rap1)_2$	431,191	-	450
$(TRF2/Rap1)_4$ + dsTeloDNA	433,904	-	480
$(TRF1/GFP\text{-}TIN2)_4$ + dsTeloDNA	496,232	-	480
$(TRF1/GFP\text{-}TIN2/TRF2/Rap1)_2$ + dsTeloDNA	465,049	-	480
POT1/TPP1/GFP-TIN2/ $(TRF2/His_6MBP\text{-}Rap1)_2$	474,130	-	495
$(POT1/TPP1/GFP\text{-}TIN2/\ TRF1)_2$	472,473	-	495
POT1/TPP1/GFP-TIN2/ $(TRF2/His_6MBP\text{-}Rap1)_2$ + TeloDNA2	515,005	-	510
$(POT1/TPP1/GFP\text{-}TIN2/\ TRF1)_2$ + TeloDNA2	521,176	-	510
(POT1/TPP1/GFP-TIN2/TRF2/Rap1)$_2$ + TeloDNA1	583,179	-	600
(POT1/TPP1/GFP-TIN2/ TRF1/TRF2/$His_6MBP\text{-}Rap1)_2$	758,568	-	780
(POT1/TPP1/GFP-TIN2/ TRF1/TRF2/$His_6MBP\text{-}Rap1)_2$ +TeloDNA2	799,443	-	840

Supplemental Methods

Protein Expression Constructs

BiGBac cloning resulted in the following vectors (in order of appearance in the paper): pBIG1b-POT1/Twinstrep-eGFP-ENLYFQ/GGS-TPP1(87-337), pBIG1b-Twinstrep-ENLYFQ/GGS-POT1/TPP1(87-544)/eGFP-GGLEVPFQGPGS-TIN2(1-352), pBIG1b-Twinstrep-GFP-ENLYFQ/GGS-(1-300-GGSGGS-331-634)/TPP1(87-337-GGSGGS-483-544)/eGFP-GGLEVPFQGPGS-TIN2(1-270), pBIG2ab-(Twinstrep-ENLYFQ/GGS-POT1/TPP1(87-544)/eGFP-GGLEVPFQGPGS-TIN2(1-352))A(mCherry-ENLYFQ/GGS-TRF1)B, pLIB-MHHHHHH-MBP-LEVLFQ/GPGS-TRF1, pBIG1a-MHHHHHH-MBP-LEVLFQ/GPGS-Rap1/TRF2(42-542). The '/' indicates where TEV or 3C proteases are expected to cleave. In all cases, GFP is enhanced GFP (Uniprot C5MKY7), the TIN2 gene was derived from a synthetic gene that was codon optimized for expression in insect cells, and TRF1 lacks residues 296-315 (corresponding to the dominant splice variant in human cells)

Detailed Protein Purification Protocols

For POT1/TPP1^{N}, cryo-ground powder was resuspended in 50 mL lysis buffer (20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl, 0.1% v/v Triton TX-100, 8% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF, made fresh in isopropanol), 1x cOmplete EDTA-free protease inhibitor (Millipore-Sigma), 0.05 mg/mL casein (Millipore-Sigma)). The resuspension was centrifuged at 40,000 x g and the supernatant (soluble lysate) added to 2 mL of NHS-activated sepharose resin (Thermo Fisher) onto which a purified GFP-nanobody had been immobilized. The slurry was incubated with end-over-end rotation at 8^{o}C for 2 hrs, centrifuged at 200 x g for 1 min, the supernatant decanted, the resin resuspended in 10 mL wash buffer (20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl, 0.05% v/v Tween-20), and transferred to a disposable column. The flow-through was discarded and the column washed twice more with 10 mL wash buffer before resuspension in 5.5 mL wash buffer and transferred to a conical flask. To this slurry, 200 µg of purified His$_6$-TEV protease (made in-house) was added, and the mixture was incubated at 8^{o}C with end-over-end rotation overnight. The next morning the slurry was transferred to a disposable column and the flow-through collected. This flow-through was filtered through 0.22 µm and injected onto a HiLoad 16/600 Superdex200 pg column equilibrated in degassed wash buffer + 5% glycerol using an Akta FPLC (Cytiva). Fractions were collected and analyzed by SDS-PAGE with Coomassie blue staining, and those containing co-eluting POT1 and TPP1^{N} were pooled, concentrated to 8 mg/mL using an Amicon YM-30 centrifugal filtration device (Millipore-Sigma), aliquoted, flash frozen in liquid nitrogen,

and stored at -80°C until needed. POT1/TPP1^{N} used in EMSAs was further purified by diluting in 10 volumes of 20 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.1 mM TCEP-HCl, 0.5% Tween-20, 5% v/v glycerol, binding to a 1 mL ReSource Q anion exchange column equilibrated in the same buffer, and eluting with a gradient to 40% 20 mM HEPES-KOH pH 7.5, 1 M NaCl, 0.1 mM TCEP-HCl, 0.05% Tween-20, 5% glycerol. Two peaks were observed and fractions from the first peak (centered at approximately 200 mM NaCl) were pooled, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed.

POT1/TPP1/GFP-TIN2 was purified in a similar manner to POT1/TPP1^{N}, but the soluble lysate was instead added to 2 mL streptactin-XT high-capacity resin (IBA biosciences). The slurry was incubated with end-over-end rotation at 8°C for 30 min and the beads were washed as with POT1/TPP1^{N}. The complex was eluted with five 2-mL additions of 50 mM Biotin-Tris pH 7.5, 300 mM NaCl, 0.5 mM TCEP-HCl, 10% v/v dimethylsulfoxide (DMSO) and adjusted to 150 mM NaCl with the addition of 10 mL of 20 mM HEPES-KOH pH 7.5, 0.1 mM TCEP-HCl, 0.05% Tween-20. This solution was then filtered through 0.22 μm and injected onto a 1 mL HiTrap-Heparin HP column (Cytiva) that had been equilibrated in Buffer A (20 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.1 mM TCEP-HCl, 0.05% Tween-20). After injection, a gradient to 0-50% Buffer B (Buffer A containing 1 M NaCl) was run and fractions collected. Visibly green fractions were pooled, adjusted to 5.5 mL, filtered through 0.22 μm, and injected onto a HiLoad 16/600 Superdex200 pg column equilibrated in degassed wash buffer + 5% glycerol. Fractions were collected and analyzed by SDS-PAGE, and those containing co-eluting POT1, TPP1, and GFP-TIN2 were pooled, concentrated to 1.8-5 mg/mL using centrifugal filtration, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed.

POT1/TPP1/GFP-TIN2 3xΔ was purified in a similar manner to POT1/TPP1/GFP-TIN2, but a 1-mL ReSourceQ anion-exchange column (Cytiva) was substituted for Heparin and the tag on POT1 was cleaved overnight with 100 μg TEV protease prior to gel filtration. Fractions from gel filtration that contained all three components were pooled, concentrated to 1.5 mg/mL using centrifugal filtration, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed.

POT1/TPP1/GFP-TIN2/TRF1 was purified in a similar manner to POT1/TPP1/GFP-TIN2, but instead of adjusting the streptactin eluate to low salt and separating over heparin, the eluate was directly added to 0.5 mL of sepharose resin onto which a purified mCherry-nanobody had been immobilized. The slurry was incubated with end-over-end rotation at 8°C for 1 hr, washed as previously (using 5-mL wash volumes instead of 10 mL), resuspended in 1 mL wash buffer and eluted with overnight incubation at 8°C using 100 μg of purified His_6-TEV protease. The

next morning the slurry was transferred to a disposable column, the flow-through and a 1 mL wash were collected, pooled and concentrated to 500 µL using a Microcon YM-100 device (Millipore-Sigma) and gently added to the top of two 11-mL 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl. These gradients were then centrifuged at 41,000 RPM using an SW41-Ti rotor (Beckman) followed by fractionated from the bottom (~600 µL/fraction, ~19 fractions total) using a peristaltic pump (GE) and a Model 2110 fraction collector (Bio-Rad). Fractions were analyzed by SDS-PAGE and those that contained all four components were pooled, concentrated to 2-4 mg/mL, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed.

For TRF1, the soluble lysate was added to 2 mL amylose resin (NEB) equilibrated in wash buffer and incubated for 3 hrs at 8°C with end-over-end rotation. The beads were washed as previously and eluted with five 2-mL additions of wash buffer containing 50 mM maltose. The amylose eluate was filtered and purified over heparin as previously, except the gradient was run from 20-100% Buffer B. Pooled fractions from the heparin elution were adjusted to 5.5 mL and the MBP tag cleaved with the addition of 200 µg purified 3C protease (made in-house) and overnight incubation at 8°C. The cleaved protein was purified by gel filtration as previously. Fractions containing TRF1 were pooled, concentrated to 1.5-4 mg/mL using centrifugal filtration, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed.

TRF2/Rap1 was purified using the same protocol as TRF1, except the amylose elution step was performed in 20 mM HEPES-KOH pH 7.5, 200 mM NaCl, 0.5 mM TCEP-HCl, 50 mM maltose and the heparin gradient was run from 10-60% Buffer B. After gel filtration, fractions containing TRF2/Rap1 were pooled, concentrated to 2-8 mg/mL using centrifugal filtration, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until needed. For complexes that retained the His_6MBP tag on Rap1, the lysis and wash buffers included an additional 15 mM imidazole-HCl pH 7.5. Furthermore, nickel-NTA resin (Qiagen) was used instead of amylose, the protein was eluted from the nickel-NTA column using 20 mM HEPES-KOH pH 7.5, 200 mM NaCl, 0.5 mM TCEP-HCl, 250 mM imidazole-HCl pH 7.5, and the 3C protease cleavage step was omitted.

For TRF1/GFP-TIN2, the soluble lysate was added to 1.5 mL mCherry-nanobody resin and incubated for 3 hrs at 8°C with end-over-end rotation. The beads were washed as previously, resuspended in 1.5 mL wash buffer and eluted with overnight incubation at 8°C using 300 µg of purified His_6-TEV protease. The next morning the slurry was transferred to a disposable column, the flow-through and a 1.5 mL wash were collected, and the beads were further washed with 7.5 mL 20 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.1 mM TCEP-HCl

which was pooled with the eluted protein. This solution was then injected onto a MonoS 5/50 GL column (Cytiva) that had been equilibrated in Buffer A and eluted with a gradient to 50% Buffer B. Visibly green fractions (in a peak centered around ~300 mM NaCl) were pooled, concentrated to 1.5 mg/mL and adjusted to 5% v/v glycerol using centrifugal filtration, aliquoted, flash frozen in liquid nitrogen, and stored at -80^{o}C until needed.

Sample Preparation for Negative-stain EM

For POT1/TPP1^{N}, an aliquot of purified protein was thawed, centrifuged at 20,000 x g, and the concentration of the supernatant was measured. 550 pmol of protein was then added to a tube containing 1.1 nmol of 5'Biotin(GGTTAG)$_2$ DNA (Thermo Fisher) in a volume of 100 µL dilution buffer (20 mM HEPES-KOH pH 7.5, 200 mM KCl, 0.1 mM TCEP-HCl, 10% glycerol). This mixture was then incubated on ice for 10 min, after which point the volume was adjusted to 550 µL with dilution buffer (final concentrations: 1 µM protein complex, 2 µM DNA). After centrifugation at 20,000 x g for 1 min, the supernatant was injected onto a Superose 6 increase 10/300 GL column (Cytiva) that had been equilibrated in degassed dilution buffer. Fractions were collected and analyzed by SDS-PAGE and those of interest were analyzed by negative-stain EM. If necessary, samples were diluted with dilution buffer to a concentration that achieved a high density of well-separated particles on the grid. For streptavidin labeling, 1.1 nmol of DNA was mixed with 3.3 nmol of D-biotin-Tris pH 7.5, and then 2.2 nmol purified streptavidin (New England BioLabs) was added. This mixture was incubated on ice for 10 min, then another 10 nmol of biotin added and the volume adjusted to 100 µL. Subsequent steps were performed as before. When 5'Biotin(GGTTAG)$_4$ DNA was used, the concentrations of DNA, D-biotin, and streptavidin were reduced by a factor of four. For POT1/TPP1/GFP-TIN2 wt and 3xΔ, a similar protocol was used, except that final concentrations were 0.4 µM complex, 0.8 µM 5'Biotin(GGTTAG)$_2$ DNA, 1.6 µM streptavidin (if present), and 2.4 µM D-biotin (if present).

For POT1/TPP1/GFP-TIN2/TRF1, the eluate from the mCherry column after concentration was used as an input. 220 pmol of the eluate (by GFP absorbance at 488 nm) was added to 110 pmol TeloDNA1 or buffer and the volume adjusted to 220 µL with dilution buffer lacking glycerol (final concentrations are 1 µM GFP-TIN2 in the TRF1 complex by A_{488}, 0 or 0.5 µM TeloDNA1). This mixture was incubated on ice for 2 hrs and then 200 µL was added to the top of 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 200 mM KCl, 0.5 mM TCEP-HCl. The gradients were centrifuged and fractionated as before. Fractions were analyzed by SDS-PAGE with SYPRO Ruby staining, and the DNA-containing sample was additionally

analyzed by native TBE-PAGE with SYBR Gold staining. Fractions of interest were further analyzed by negative-stain EM.

For POT1/TPP1/GFP-TIN2 and POT1/TPP1/GFP-TIN2/TRF1 bound to streptavidin-5'Biotin$(GGTTAG)_4$ DNA, 165 pmol of DNA was mixed with 500 pmol D-biotin, and then 330 pmol of streptavidin was added. The reaction was incubated for 10 min on ice and then another 1 nmol of D-biotin was added and the volume adjusted to 100 µL with dilution buffer. To this sample, 330 pmol of POT1/TPP1/GFP-TIN2 was added and the mixture incubated for 10 min on ice. Then, either buffer or buffer plus 165 pmol of $(TRF1)_2$ was added to a final volume of 220 µL and the mixture was incubated on ice for another 1 hr before separation by ultracentrifugation, fractionation, and analysis as above.

For the POT1/TPP1/TIN2/TRF2/Rap1 reconstitution, 150 pmol of $(TRF2/Rap1)_2$ was mixed with 300 pmol POT1/TPP1/GFP-TIN2 and incubated on ice for 10 min. Then, either buffer or buffer plus 160 pmol TeloDNA1 was added to a final volume of 220 µL. This sample was incubated on ice for 2 hrs, then added to the top of 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.5 mM TCEP-HCl, and separation by ultracentrifugation, fractionation, and analysis was performed as above. Peak fractions used for mass photometry were diluted 1:10 into the same buffer lacking glycerol.

For the shelterin core reconstitution, 220 pmol of $(TRF2/Rap1)_2$ was mixed with 220 pmol $(TRF1/GFP\text{-}TIN2)_2$ and incubated on ice for 10 min. Then, either buffer or buffer plus 220 pmol dsTeloDNA (made fresh by annealing equimolar HPLC purified 5'CCTATCTAGGGTTTCTACTAGGGTTCACAATAGGGTTCTCACTAGGGTTCATACG3' to 5'CGTATGAACCCTAGTGAGAACCCTATTGTGAACCCTAGTAGAAACCCTAGATAGG3' oligos) was added to a final volume of 220 µL. These samples were dialyzed against 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.1 mM TCEP-HCl, 5% v/v glycerol at 8^{o}C for 5 hours then added to the top of 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.5 mM TCEP-HCl, and separation by ultracentrifugation, fractionation, and analysis was performed as above. Peak fractions used for mass photometry were diluted 1:10 into the same buffer lacking glycerol.

For the shelterin reconstitution, 260 pmol the TRF1 complex (eluate from the mCherry step) was mixed with 130 pmol of $(TRF2/Rap1)_2$ and incubated on ice for 10 min. Then, either buffer or buffer plus 200 pmol TeloDNA2 was added to a final volume of 220 µL (final concentrations were 1.2 µM GFP-TIN2 in the TRF1 complex by A488, 0.6 µM $(TRF2/Rap1)_2$, 0 or 0.9 µM TeloDNA2). This sample was incubated on ice for 2 hrs then added to the top of 10-

30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.5 mM TCEP-HCl and separation by ultracentrifugation, fractionation, and analysis as above.

DNA Substrates

All unlabeled oligonucleotides were purchased from Thermo Fisher and purified using standard desalting unless otherwise specified. All 5'Biotin oligonucleotides were ordered from Thermo Fisher with HPLC purification. All single-stranded 5' Cy5.5 DNAs were ordered from Integrated DNA Technologies with HPLC purification. The Cy5.5-labeled telomeric dsDNA was generated by mixing 540 pmol of HPLC purified 5'CATCAATAGGGTTCATCCTAGGGTTGTACTG3' DNA with 650 pmol of HPLC purified 5'CAGTACAACCCTAGGATGAACCCTATT3' in 25 µL annealing buffer (10 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.2 µm filtered), heating to 98^{o}C for 2 min, and cooling quickly to 12^{o}C in a thermocycler. The annealed product was labeled by fill-in synthesis using 5 U Klenow exo- (New England Biolabs) and a dNTP mix containing 40 µM each of dATP, dCTP, dGTP and SulfoCy5.5-dUTP (Lumiprobe). These reactions were then exchanged into annealing buffer using a BioSpin P6 column (Bio-Rad) and the concentration was attained by measuring A_{673} and using an extinction coefficient of 211,000 $M^{-1}cm^{-1}$. To make TeloDNA1, 7 nmol of 5'CATCAATAGGGTTCATCACTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG3' was annealed to 10.5 nmol of 5'CTAACCCTAGTGATGAACCCTATTGATG3' by heating to 98^{o}C and cooling quickly to 12^{o}C in 100 µL annealing buffer. The volume was then adjusted to 550 µL with annealing buffer and injected onto a Superdex200 10/300 GL column equilibrated in phosphate-buffered saline (PBS, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4). Fractions were analyzed by TBE-PAGE with SYBR Gold (Invitrogen) staining and those containing primarily the annealed product were pooled, concentrated to 100 µL in a Microcon YM-10, and stored at -20^{o}C. dsTeloDNA was made by mixing 500 pmol HPLC purified 5'CCTATCTAGGGTTTCTACTAGGGTTCACAATAGGGTTCTCACTAGGGTTCATACG3' and 500 pmol HPLC purified 5'CGTATGAACCCTAGTGAGAACCCTATTGTGAACCCTAGTAGAAACCCTAGATAGG3' in 50 µL annealing buffer and heat/cooling as above. TeloDNA2 was generated in a similar manner to TeloDNA1, except the sequences used were 5'CCTATCTAGGGTTTTCTACTAGGGTTCATCAATAGGGTTCATCACTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG3' and 5'CTAACCCTAGTGATGAACCCTATTGATGAACCCTAGTAGAAAACCCTAGATAGG3'.

Sample Preparation for Mass Photometry

For the TRF1 complex, 1 nmol of $(TRF1)_2$, 2 nmol POT1/TPP1/GFP-TIN2, and 0 or 1.5 nmol of TeloDNA1 were mixed together and diluted to 250 µL in 20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl. After a 2-hr incubation at 4°C, 250 µL of the reconstitution was loaded onto each of two 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl and centrifuged, fractionated, and analyzed as before. Fractions containing the complex were pooled, concentrated in a Microcon YM-30, buffer exchanged 1:10 into 20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl, flash frozen in liquid nitrogen and stored at -80°C. For shelterin, 400 pmol of the TRF1 complex (by absorbance at 488 nm) was mixed with 300 pmol of $(TRF2/His_6MBP\text{-}Rap1)_2$ and adjusted to 200 µL with 20 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 mM TCEP-HCl. This sample was dialyzed against 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.1 mM TCEP-HCl, 5% v/v glycerol overnight using a 3500 Da cutoff 100-500 µL capacity slide-a-lyzer MINI device (Thermo Fisher) followed by 4 additional hours of dialysis against same buffer lacking glycerol the next morning. The sample was then loaded onto each of two 10-30% glycerol gradients in 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.1 mM TCEP-HCl and centrifuged, fractionated, and analyzed by SDS PAGE. Fractions containing the complex were pooled, incubated with 30 µL amylose beads with end-over-end rotation for 3 hrs at 8°C. The beads were then washed three times with 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.1 mM TCEP-HCl, and finally eluted with 50 mM maltose in the the same buffer. The eluate was isolated from beads by centrifugation through a 0.22 µm filter using a Spin-X centrifugal filtration device (Corning) and immediately used at a 1:8 final dilution in mass photometry measurements. For the DNA-containing sample, the eluate was first mixed with an equal volume of 60 nM TeloDNA2 (final concentration of 7.5 nM) and incubated on ice for 30 min prior to measurement.

Prior to analysis, samples were thawed, centrifuged at 20,000 x g, and the concentration of the supernatant was measured. Samples were then diluted to 1 µM in 20 mM HEPES-KOH pH 7.5, 300 mM NaCl, 0.1 mM TCEP-HCl, and mixed together if indicated. Immediately prior to measurement, samples were diluted to 4x final concentration in MP buffer (20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.1 mM TCEP-HCl, filtered three times through 0.22 µm), then 2.5 µL was added to 7.5 µL MP buffer in a sealed well on a glass slide and light scattering was recorded for 1 min.

Antibodies and Western Blotting

Polyclonal antibodies were previously generated by inoculating rabbits with the following purified proteins as antigens (produced in insect cells unless otherwise specified): full-length TRF1, full-length TRF2, full-length TIN2, full-length Rap1, and GST-TPP1(87-337) produced in *E. coli*. Affinity purification of the resulting sera generated primary antibody stocks that were used at 1:2000 dilutions in PBS + 0.05% Tween and 1% v/v milk. A rabbit polyclonal antibody against POT1 was purchased from Proteintech and used at a 1:1000 dilution.

Protein samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature with PBS + 0.05% Tween containing 5% milk prior to incubation with the primary antibody for 60 min at room temperature. Membranes were then washed three times with PBS + 0.05% Tween, incubated with a 1:10,000 dilution of anti-rabbit IgG-HRP (Millipore-Sigma) for 45 min at room temperature in PBS + 0.05% Tween and 1% milk, washed three times, blotted dry, and finally treated with West Pico PLUS luminescence reagent (Thermo Fisher). Membranes were exposed to film for 0.5-5 min, then the film was developed and scanned using the transparency setting of a conventional scanner.