Supporting Information

A stable tetramer is not the only oligomeric state that mitochondrial single-stranded DNA binding proteins can adopt.

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Protein over-expression and purification.

All the mtSSBs were over-expressed in *E. coli* BL21(DE3) cells by induction with 0.4 mM IPTG and grown overnight at 16 °C, in LB medium for the fungal mtSSBs and 2xYT for the *Xenopus* ones. The mtSSB from *Xenopus* species were purified using the same protocol as described for human mitochondrial SSB (1). All fungal mtSSBs and their mutant variants were purified with protocols similar to the one presented for *S. cerevisiae* Rim1 (1), with the following modifications. For *C. castellii* and *C. parapsilosis* mtSSBs cell pellets were resuspended in Buffer A (50 mM Tris–HCl pH 8.2, 1 mM EDTA, 15 mM spermidine, 10% (v/v) glycerol and 1 mM PMSF) + 600 mM NaCl, instead of Buffer A + 400 mM NaCl used for the other mtSSBs. After cells lysis, the supernatant after addition of 0.1-0.2 % polymin P was precipitated with 0.4 g/mL Am_2SO_4, followed by a strong anion exchange column (HighQ, Bio-Rad) and a single strand DNA cellulose column. Due to the inability to elute the *C. castellii* mtSSB from the ssDNA cellulose column, after HighQ this protein was purified with a POROS 50 HE heparin column (Applied Biosystems) using a salt gradient from 75 mM to 1 M NaCl. The fractions containing the purified mtSSBs were dialyzed in Storage Buffer (20 mM HEPES pH 7.4, 400 mM NaCl, 1 mM EDTA, 50% (v/v) glycerol) and stored at −20 °C. The quality of the purified proteins is shown in Figure S1A, together with *Sc*Rim1 and *Hsmt*SSB (1) as a reference.

2:1 binding model.

The data in Figure X were analyzed with a 2:1 DNA binding model, as recently described (1).

$$\mathbf{P_2} + \mathbf{S} \overset{\mathbf{K}}{\leftrightarrow} \mathbf{P_2S}$$

$$\mathbf{P_2S} + \mathbf{P_2} \overset{\mathbf{L}}{\leftrightarrow} \mathbf{P_4S}$$

$$v = \frac{\mathrm{K\,[P]+K\,L\,[P]^2}}{1+\mathrm{K\,[P]+K\,L\,[P]^2}} \tag{s1}$$

$$r_{obs} = \alpha_S\, r_S + \alpha_{P2S}\, r_{P2S} + \alpha_{P4S}\, r_{P4S} \tag{s2}$$

$$\alpha_S = \frac{1}{1+\mathrm{Q_1\,K\,[P]+Q_2\,K\,L\,[P]^2}} \tag{s3}$$

$$\alpha_{P2S} = \frac{\mathrm{Q_1\,K\,[P]}}{1+\mathrm{Q_1\,K\,[P]+Q_2\,K\,L\,[P]^2}} \tag{s4}$$

$$\alpha_{P4S} = \frac{\mathrm{Q_2\,K\,L\,[P]^2}}{1+\mathrm{Q_1\,K\,[P]+Q_2\,K\,L\,[P]^2}} \tag{s5}$$

$$Q_1 = \frac{F_{P2S}}{F_S}, Q_2 = \frac{F_{P4S}}{F_S} \quad \text{(s6)}$$

where r_S, r_{P2S} and r_{P4S} are the fluorescence anisotropies of the free DNA, and DNA bound to one and two dimers. The data we analyzed using Eqs. s1-6 with Scientist (Micromath, Saint Louis). While r_S is known, the variables r_{P2S} , r_{P4S} , Q_1, and Q_2 were fixed to values estimated by graphical inspection. Fitting for K and L was performed at the lowest DNA concentration to better estimate K and obtain an initial estimate of L; because of high affinities the estimated values have associated a large error. In the main text we report K_x and L_x, where the subscript x indicates the length of the ssDNA in nt. The solid line in the main figure are the analysis of the data with the following parameters:

Figure 3B (binding to FAM-dT_{20})
*Cn*Rim1:
$r_S = 0.04, r_{P2S} = 0.165, r_{P4S} = 0.27, Q_1 = 0.9, Q_2 = 0.98$
[DNA] = 45nM: K = (1.3 ± 0.5) x 10^9 M^{-1}, L = (1.4 ± 0.4) x 10^7 M^{-1}
*Cc*Rim1:
$r_S = 0.04, r_{P2S} = 0.19, r_{P4S} = 0.27, Q_1 = 0.75, Q_2 = 0.9$
[DNA] = 45nM: K = (2 ± 0.5) x 10^9 M^{-1}, L = (8 ± 2) x 10^7 M^{-1}

Figure 3C (*Cn*Rim1 binding to dT_{38}-FL):
$r_S = 0.05, r_{P2S} = 0.2, r_{P4S} = 0.27, Q_1 = 0.62, Q_2 = 0.88$
[DNA] = 50nM: K = (3 ± 1) x 10^9 M^{-1}, L = (3 ± 1) x 10^8 M^{-1}
[DNA] = 100nM: K = 3 x 10^9 M^{-1}, L = 4 x 10^8 M^{-1}
[DNA] = 200nM: K = 3 x 10^9 M^{-1}, L = 4 x 10^8 M^{-1}

Figure 3D (*Cc*Rim1 binding to dT_{38}-FL):
$r_S = 0.05, r_{P2S} = 0.22, r_{P4S} = 0.27, Q_1 = 0.5, Q_2 = 0.75$
[DNA] = 50nM: K = (6 ± 2) x 10^9 M^{-1}, L = (6 ± 2) x 10^8 M^{-1}
[DNA] = 100nM: K = 6 x 10^9 M^{-1}, L = 6 x 10^8 M^{-1}
[DNA] = 200nM: K = 6 x 10^9 M^{-1}, L = 6 x 10^8 M^{-1}

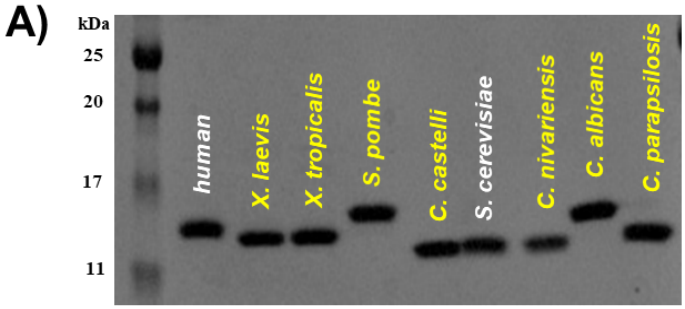
References

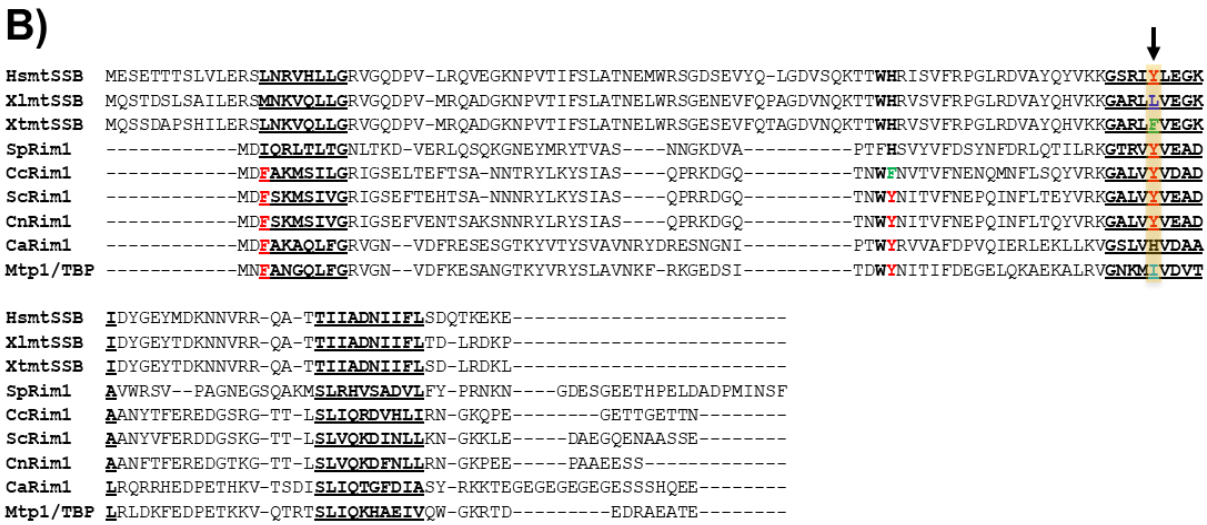
1. Singh, S. P., Kukshal, V., De Bona, P., Antony, E., and Galletto, R. (2018) The mitochondrial single-stranded DNA binding protein from S. cerevisiae, Rim1, does not form stable homo-tetramers and binds DNA as a dimer of dimers. *Nucleic Acids Res* **46**, 7193-7205

Table S1. Molecular weights and molar extinction coefficients of the studied mtSSBs.

	MW (Da) (w/o MTS)	**ε_{280} (M^{-1} cm^{-1})**
***Hsmt*SSB**	15326	19940
***Hsmt*SSB^{Y109R}**	15319	18450
***Xlmt*SSB**	14,885	15470
***Xtmt*SSB**	15072	15470
***Sp*Rim1**	14520	14440
***Sp*Rim1^{Y91H}**	14494	12950
***Sp*Rim1^{Y91R}**	14513	12950
***Cc*Rim1**	13139	12950
***Cn*Rim1**	13039	12950
***Ca*Rim1**	14479	12950
***Ca*Rim1^{H87Y}**	14505	14440
***Ca*Rim1$^{H87Y/F18I}$**	14471	14440
***Ca*Rim1$^{\Delta 25}$**	11799	11460
***Ca*Rim1$^{H87Y/\Delta 25}$**	11825	12950
***Ca*Rim1Δ18**	12634	12950
***Ca*Rim1$^{H87Y/\Delta 18}$**	12660	14440

Figure S1. A) SDS-PAGE of some of the purified mtSSBs used in this study. **B)** Sequence alignment of the mature forms of mtSSBs; highlighted are few of the amino acids and regions discussed in the manuscript. **C)** Distribution of amino acids (~200 yeast mtSSBs) at the conserved position indicated by the arrow in B).





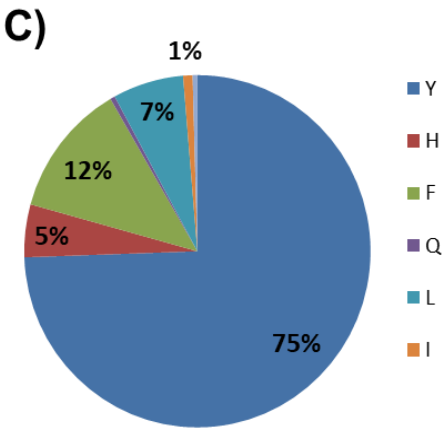
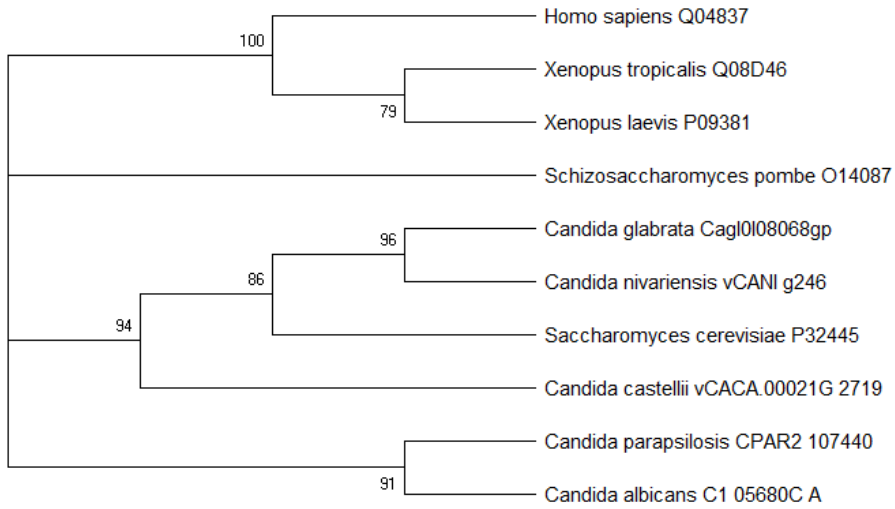


Figure S2. A) Phylogenetic tree of mtSSBs was constructed with MEGA (https://www.megasoftware.net/) using Maximum-likelihood statistical method. The multiple sequence alignment was done by inbuilt MUSCLE program. Phylogenetic-tree was created bootstrap with 2000 replicates with 50% cutoff value. **B)** Amino acid identity of selected yeast mtSSBs. In parenthesis is the sum of identical and strongly similar amino acids.

A)



B)

	C. glabrata	*C. nivariensis*	*S. cerevisiae*	*C. castellii*	*C. parapsilosis*	*C. albicans*
C. glabrata	---	95.1 (98)	89.1 (99)	79.2 (91)	30.5 (56)	29 (50)
C. nivariensis	95.1 (98)	---	87.2 (97)	77.5 (94)	28.3 (54)	28 (50)
S. cerevisiae	89.1 (99)	87.2 (97)	---	78.2 (96)	30.5 (56)	29.9 (50)
C. castellii	79.2 (91)	77.5 (94)	78.2 (96)	---	30.5 (56)	27.4 (51)
C. parapsilosis	30.5 (56)	28.3 (54)	78.2 (96)	30.5 (56)	---	47 (67)
C. albicans	29 (50)	28 (50)	29.9 (50)	27.4 (51)	47 (67)	---

Figure S3. A) Tryptophan quenching of *Sp*Rim1 (black) or *Ca*Rim1 (blue) upon binding to poly(dT). Note that *Sp*Rim1 harbors a tyrosine at the conserved position corresponding to W62 of *Ca*Rim1 (W54 in *Ec*SSB). **B)** Occluded site-size calculated from the data in A), at different salt concentrations. **C)** Stoichiometry of *Sp*Rim1 determined from the change in Cy3 fluorescence of oligo-dT substrates of different lengths. **D)** Stoichiometry of *Sp*Rim1 and *Sc*Rim1 bound to a 68-mer, monitored via the change in FRET between the ends of the ssDNA.

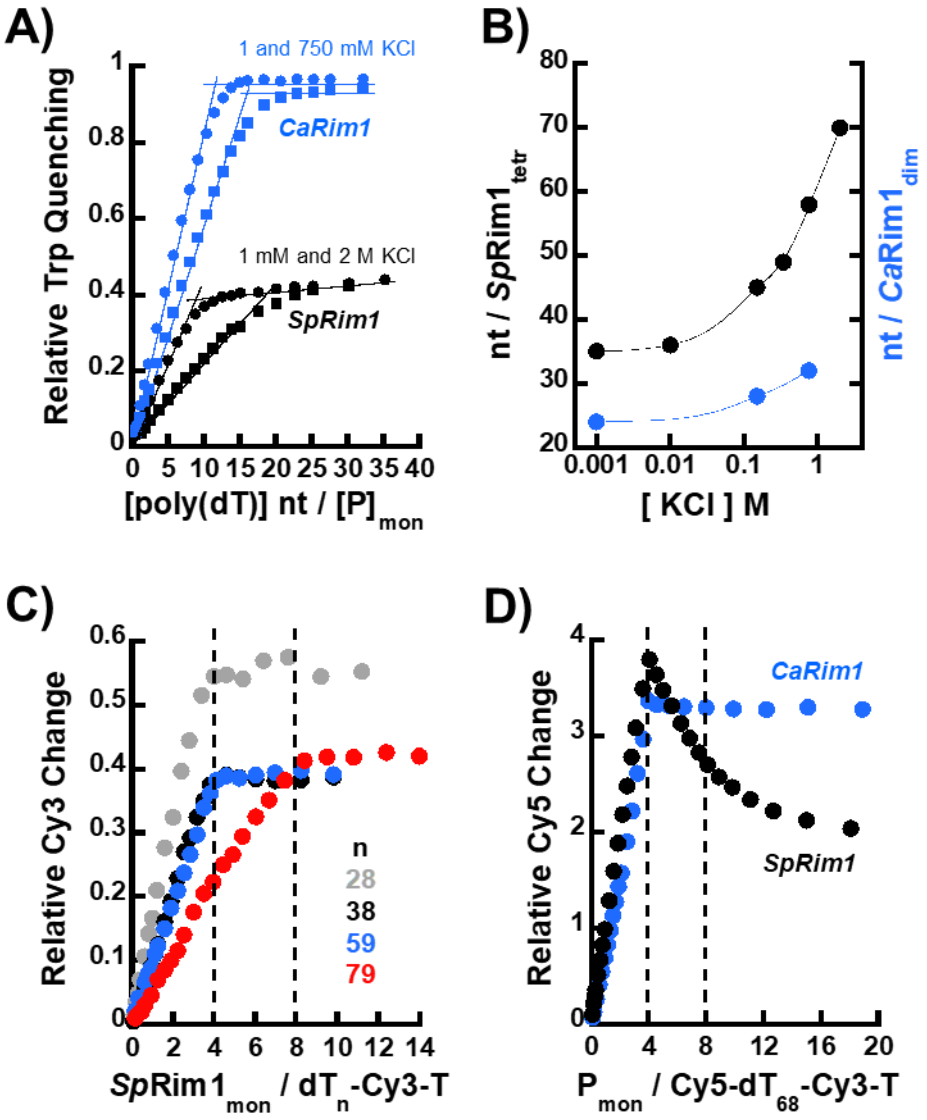


Figure S4. A) Sedimentation equilibrium absorbance profiles at three rotor speeds of a Cy3-labeled 28-mer in the presence of a large excess of CaRim1. The red line are the fits to a single exponential. **B)** Stoichiometry of *Ca*Rim1-ssDNA complexes calculated from experiments in A) and different lengths of ssDNA. **C)** Sedimentation equilibrium absorbance profile of *Ca*Rim1$^{H87Y/\Delta25}$ at three rotor speeds. Red lines are the fits with a single exponential.