Supplementary Material

Structural Basis for the Function of Tim50 in the Mitochondrial Presequence Translocase

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Generation and synthesis of Tim50 mutant forms

Site-directed mutants were created by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). $Tim50_{1\text{-}361}$ mutants were amplified and fused to a SP6 promotor by polymerase chain reactions, transcribed with the mMESSAGE mMACHINE Kit (Ambion, Austin, TX) and subsequently translated by using rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [^{35}S]methionine.[31]

Import of proteins into mitochondria

Mitochondria were isolated from a yeast strain expressing Tim23-protein A[15] and the corresponding wild-type strain grown in YPG (1% yeast extract, 2% bacto peptone, 3% glycerol) medium at 24°C. Mitochondria were preincubated for 2 min in import buffer (10 mM MOPS/KOH, pH 7.2, 3% [w/v] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine) at 25°C. Import reactions were started by the addition of radiolabeled precursor proteins and stopped by adding AVO-mix (8 μM antimycin A, 1 μM valinomycin, 20 μM oligomycin) on ice to dissipate the membrane potential.[31] Protease treatment was performed by adding 50 μg/ml proteinase K and stopped after 15 min on ice by the addition of 2 mM PMSF. Mitochondria were reisolated by centrifugation, washed with SEM buffer (10 mM MOPS/KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA) and analyzed by SDS-PAGE and digital autoradiography with a Storm 820 image analyzer (GE Healthcare).

Affinity chromatography

For protein complex isolation, mitochondria were solubilized in solubilization buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% glycerol) containing 1% digitonin.

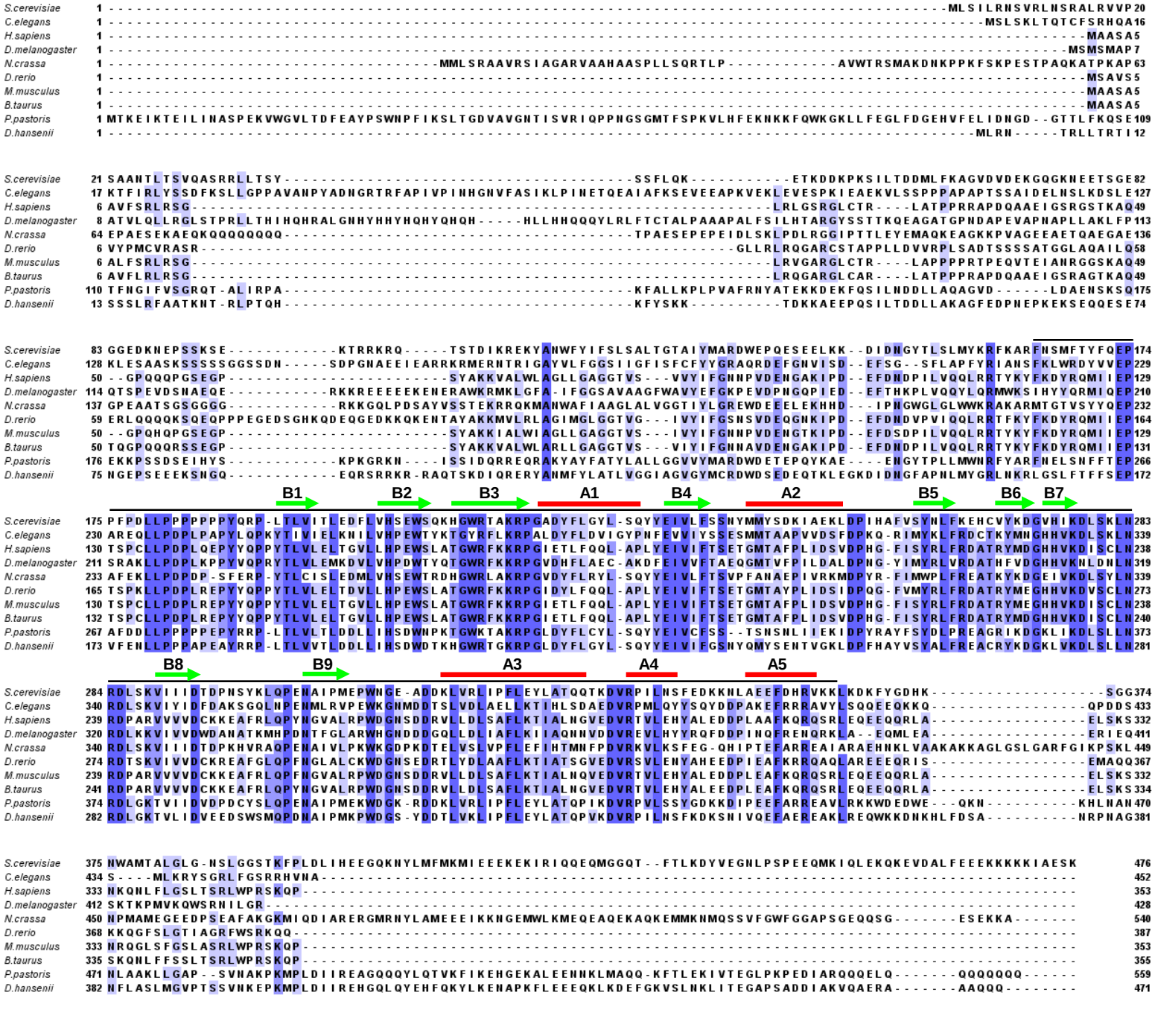
Protease inhibitors (2 mM PMSF, 2 mM Pefablock SC [Roche] and 4 μg/ml leupeptine) were added and the samples were incubated for 30 min at 4°C. 30 μl 50% IgG sepharose slurry per column was washed two times with 0.5 ml acetate buffer (0.5 M HAc/NH_4Ac, pH 3.5), two times with 2-fold solubilization buffer without digitonin and two times with solubilization buffer (see above). Sepharose beads were incubated with solubilized mitochondria and shaken for 90 min at 4°C. Columns were washed ten times with washing buffer (20 mM Tris/HCl, pH 7.4, 60 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.3% digitonin and 2 mM PMSF). Bound proteins were eluted with 50 μl SDS loading buffer without β-mercaptoethanol, incubated for 15 min at 37°C and subjected to SDS-PAGE. Gels were analyzed by immunoblotting or by digital autoradiography.

Supplementary Reference

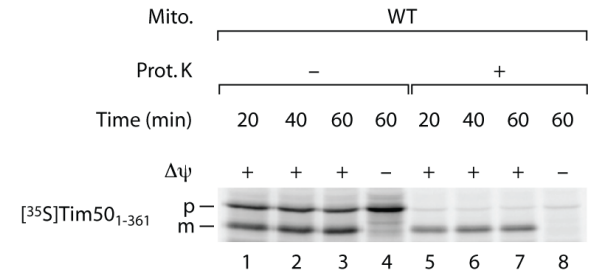
31. Stojanovski, D., Pfanner, N. & Wiedemann, N. (2007). Import of proteins into mitochondria. *Methods Cell Biol.* **80**, 783-806.

Supplementary Figures

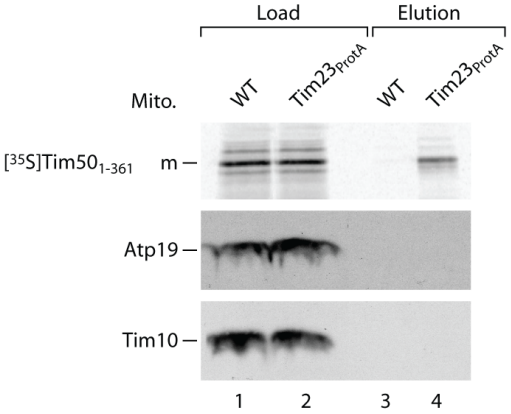
Supplementary Fig. S1. Sequence alignment of the Tim50 family members. The program ClustalW was utilized to align the Tim50 sequences from ten species. The conserved regions are highlighted in blue color. The fragment used for crystallization of $Tim50_{IMS}$ is marked by a black bar. The α-helices A1 to A5 and the β-strands B1 to B9 are labeled by red bars and green arrows, respectively. The protruding β-hairpin is formed by B2, B3 and the connecting loop.


S.cerevisiae
C.elegans
H.sapiens
D.melanogaster
N.crassa
D.rerio
M.musculus
B.taurus
P.pastoris
D.hansenii
B1
B2
B3
A1
B4
A2
B5
B6
B7
B8
B9
A3
A4
A5

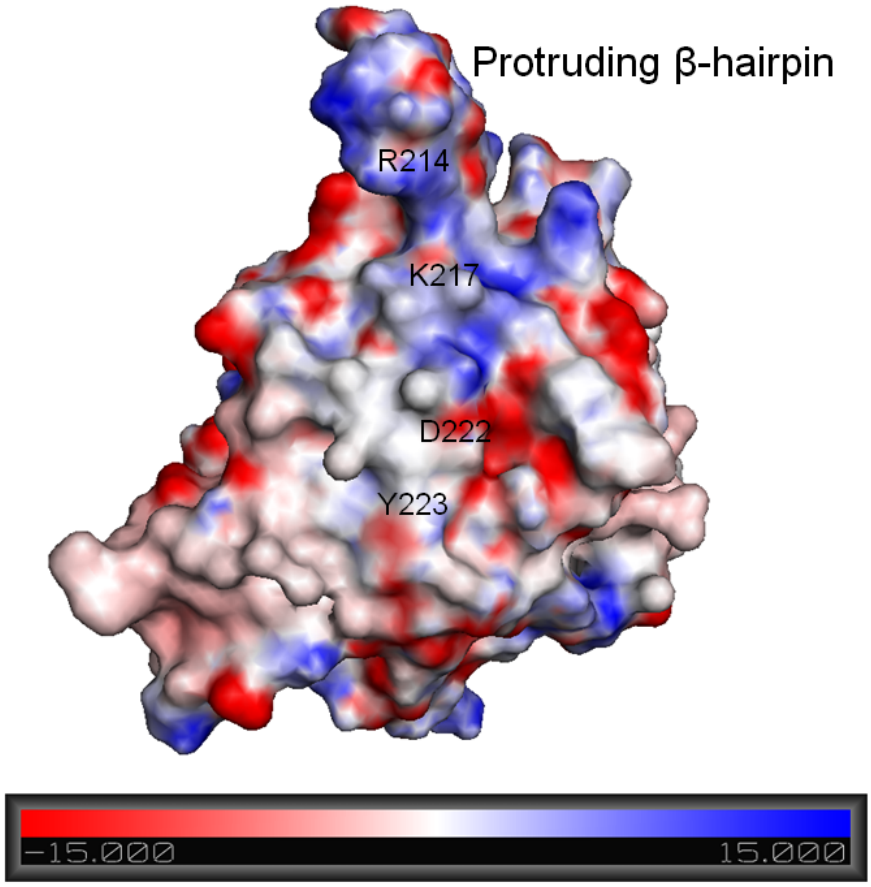
(a)



(b)



Supplementary Fig. S2. Import of truncated Tim50 into isolated yeast mitochondria and interaction with Tim23. (a) $Tim50_{1\text{-}361}$ was synthesized in reticulocyte lysate and labeled with [^{35}S]methionine. Mitochondria were isolated from yeast wild-type (WT) cells. $Tim50_{1\text{-}361}$ was imported into the isolated mitochondria and processed to the mature form in a membrane potential (Δψ)-dependent manner (lanes 1-3). Mature $Tim50_{1\text{-}361}$ was protected against externally added proteinase K (lanes 5-7). The mitochondria were separated by SDS-PAGE and radiolabeled $Tim50_{1\text{-}361}$ was detected by digital autoradiography. p, precursor; m, mature. (b) $Tim50_{1\text{-}361}$ was imported into mitochondria isolated from a yeast strain containing protein A-tagged Tim23 and into control mitochondria (WT). After the import reaction, the mitochondria were lysed with digitonin and subjected to affinity purification with IgG-Sepharose. The eluate containing Tim23 and associated proteins is shown in lane 4. The samples were separated by SDS-PAGE and analyzed by digital autoradiography, showing that $Tim50_{1\text{-}361}$ interacts with mitochondrial Tim23. Control proteins, Atp19 and Tim10, were detected by Western blotting. Load, 10%; elution, 100%.



Supplementary Fig. S3. Electrostatic surface potential drawing of $Tim50_{IMS}$. The Tim50 molecule is rotated along the vertical axis by ~90° from the orientation in Fig. 1a. The negatively charged region is shown in red and the positively charged region is shown in blue. The protruding β-hairpin is labeled. The residues R214 and K217 that are involved in binding Tim23 are labeled. Residues D222 andY223, which were replaced for mutagenesis studies, are also labeled.