Supplementary methods

Protein expression and purification. His_{10}-Tim21 (residues 103-239) (Chacinska *et al*, 2005) was treated with subtilisin for 1 h at 4°C and stable fragments were analyzed by SDS-PAGE and ESI-MS (SCIEX API 165 single quadrupole mass spectrometer (Perkin Elmer) equipped with a Nucleosil C8-HD reverse phase column (Macherey & Nagel)). $Tim21_{IMS}$ (103-225) was amplified by PCR and ligated into pPROEXHTa (Invitrogen) to allow expression of a fusion protein with cleavable N-terminal His-tag. BL21(DE3) RIL cells (Stratagene) were grown in TB medium containing ampicillin and chloramphenicol. After addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at OD_{600} of 1.5, His-tagged $Tim21_{IMS}$ was expressed at 37°C. Cells were harvested after 5 h, suspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and lysed using a French press. The soluble fraction was loaded on Ni-NTA-agarose and $Tim21_{IMS}$ eluted with buffer B (buffer A containing 250 mM imidazole). After dialysis against 20 mM Tris, 100 mM NaCl, pH 8, the solution was treated with TEV protease and passed over Ni-NTA-agarose. The flow-through was collected and further purified by gel filtration using a Superdex 75 column. Fractions containing $Tim21_{IMS}$ were desalted and concentrated to 90 mg/ml. Due to the cloning vector used residues GAMG remained at the N-terminus uncut. The SeMet derivative was expressed essentially the same way as the native protein according to standard protocols. The purified SeMet protein was concentrated to 26 mg/ml. ESI-MS exhibited a single peak at 14923 Da (calculated mass 14922 Da), confirming a full SeMet incorporation.

GST-$Tom22_{IMS}$ (120-152) was expressed and purified as described (Chacinska *et al*, 2005). For cleavage of the GST tag, GST-$Tom22_{IMS}$ was dialyzed against PBS and treated with thrombin (4 units per mg GST-$Tom22_{IMS}$) at room temperature overnight. $Tom22_{IMS}$ was isolated by gel filtration using a Superdex 75 column.

Peptide scan analyis. Cellulose-bound peptide scans were generated by automated spot synthesis (Landgraf *et al*, 2004). Analysis of the peptide scan was performed essentially as described (Brix *et al*, 1999, 2000). To analyze binding of Tim21 to

Tom22, the prepared peptide membrane was incubated with 250 nM intermembrane space domain of Tim21 (Chacinska *et al*, 2005) in binding buffer (100 mM KCl, 5% (w/v) sucrose, 0.05% Tween 20, 0.05% (w/v) bovine serum albumin, 30 mM Tris-HCl, pH 7.6) for 60 min at 25°C with gentle shaking. After washing the peptide-bound protein was electro-transferred onto polyvinylidene difluoride membrane using a semidry blotter as described (Brix *et al*, 1999, 2000). The membrane was analyzed by Western-blotting with anti-Tim21 antibodies, a fluorescence blotting substrate (ECF, GE Healthcare), and a fluorescence scanning system (STORM, GE healthcare). For quantification and subtraction of the local background for each peptide spot, the program Imagequant (GE Healthcare) was used. For correlation of the peptide binding values with the primary structure, the values for peptides containing a given amino acid were added and divided by the number of peptides (typically 6 – 7 different peptides/amino acid), yielding normalized units (Brix *et al*, 1999).

Peptide competition analysis. The intermembrane space domain of Tim21 (aa 103-239) was bound to Ni-NTA-Sepharose as described (Chacinska *et al*, 2005). After washing the domain was preincubated with P3 and $P3_{AA}$ (P3 Asp131Ala, Asp135Ala) peptides in the presence of 50 mM NaCl for 30 min. Mitochondria were solubilized in buffer containing 20 mM HEPES, pH 7.5, 140 mM NaCl, 10% glycerol, 20 mM imidazole and 0.5 % (w/v) Triton-X100. Mitochondrial extracts were incubated with immobilized Tim21 in the presence or absence of P3 and $P3_{AA}$. After washing elution fractions were analyzed by SDS-PAGE and immunodecoration with the antibodies against Tom40 and Tim23.

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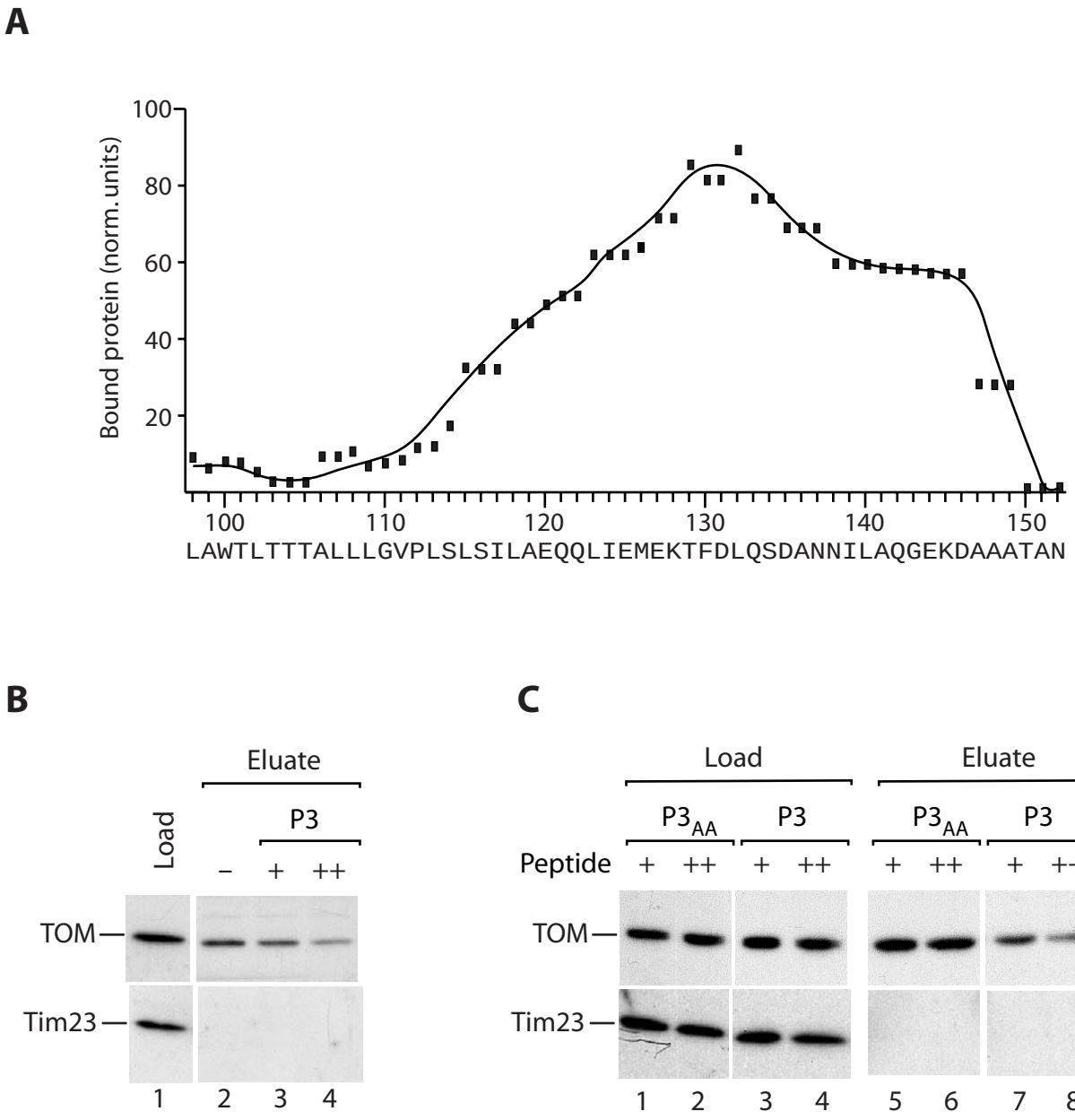
Supplementary Table 1. Interactions between α-helices and other parts of $Tim21_{IMS}$ up to 3.5 Å distance using the program "O"

H-bonds:

Helix residue		Interacting residue		Distance (Å)
Asp105	OD1	Ser158	OG	2.64
	OD2		N	3.09
Arg111	NH1	Pro198	O	2.84
	NH2		O	2.99
Asp121	OD2	Tyr213	OH	2.68
Arg123	NH2	Asp130	OD1	2.85
	NE		OD2	2.78
	NH2	Glu136	O	2.84

Hydrophobic interactions:

Helix residue		Interacting residue		Distance (Å)
Phe109	CE2	Ile156	CD1	3.48
	CZ		CD1	3.48
Ala112	CB	Met203	CE	3.35
Met115	SD	Phe200	CE2	3.49
AsN119	ND2	Leu215	CD2	3.16
Ile122	CD1	Tyr213	CD2	3.49



Supplemental Fig 1 Analysis of the Tom22-Tim21 interaction. (**A**) The purified intermembrane space domain of Tim21 was applied to a Tom22 peptide scan consisting of overlapping 20-mer peptides covalently bound to a cellulose matrix. After washing peptide-bound protein was analyzed by Western -blotting with anti-Tim21 antibodies. Signals were quantified and converted to sequence-specific normalized units as described at *EMBO reports* online (http://www.emboreports.org). (**B**) The intermembrane space domain of Tim21 was immobilized on Ni-NTA agarose and preincubated in the presence or absence of P3 peptide followed by incubation with solubilized mitochondria in the presence or absence of P3 peptide as described at *EMBO reports* online (http://www.emboreports.org). +, 75 µM; ++, 100 µM peptide. Bound proteins were analyzed by Western-blot analysis using antibodies against Tom40 (TOM) and Tim23. Load, 5%; Eluate, 100% (**C**) Competition of Tom22 binding to the intermembrane space domain of Tim21 by wild-type P3 peptide and the mutant form $P3_{AA}$ (P3 Asp131Ala, Asp135Ala) was performed as described under (B). Load, 5%; Eluate, 100%.