

SUPPLEMENTARY INFORMATION

Mapping protein interactions in the active TOM-TIM23 supercomplex

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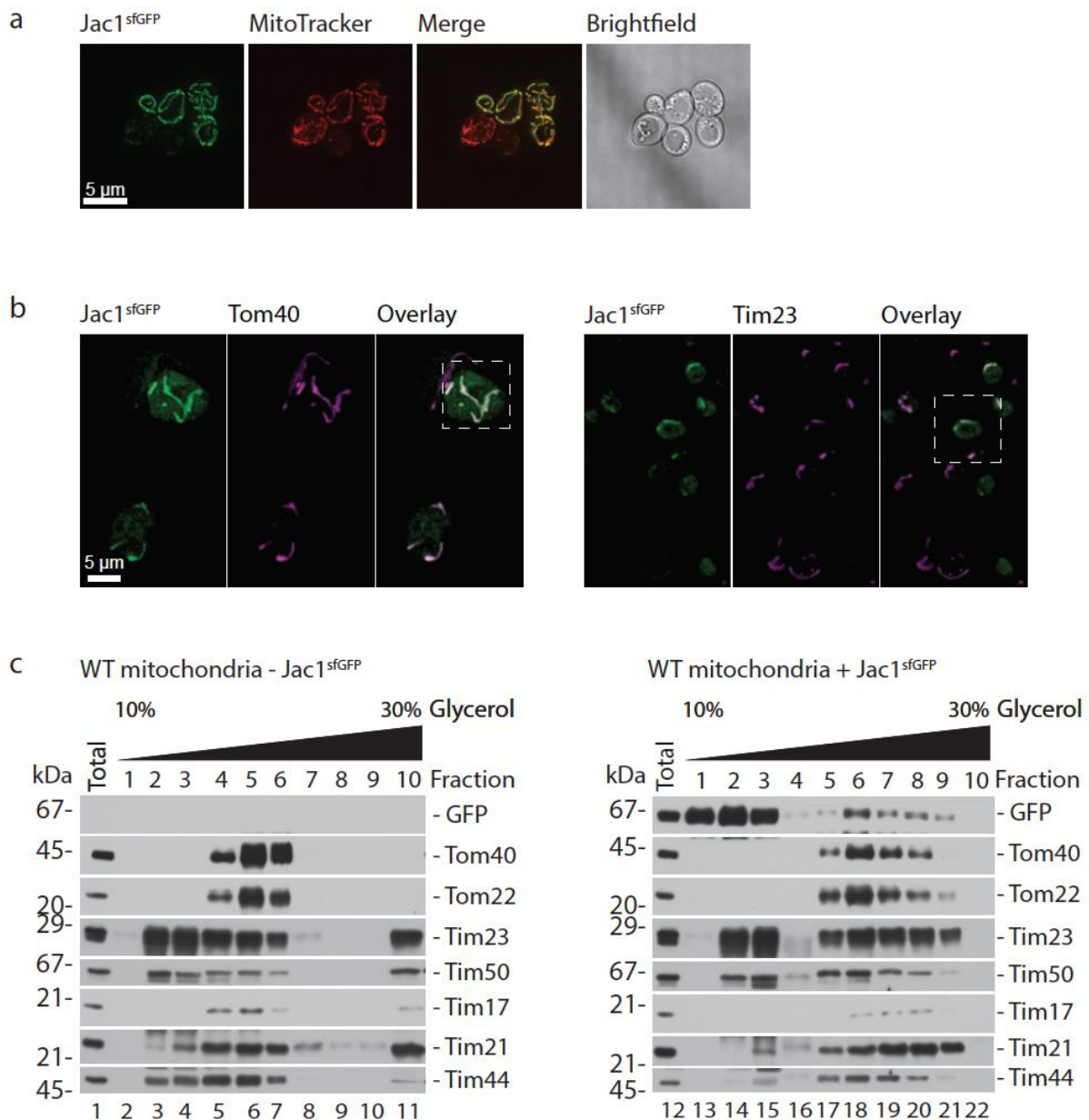
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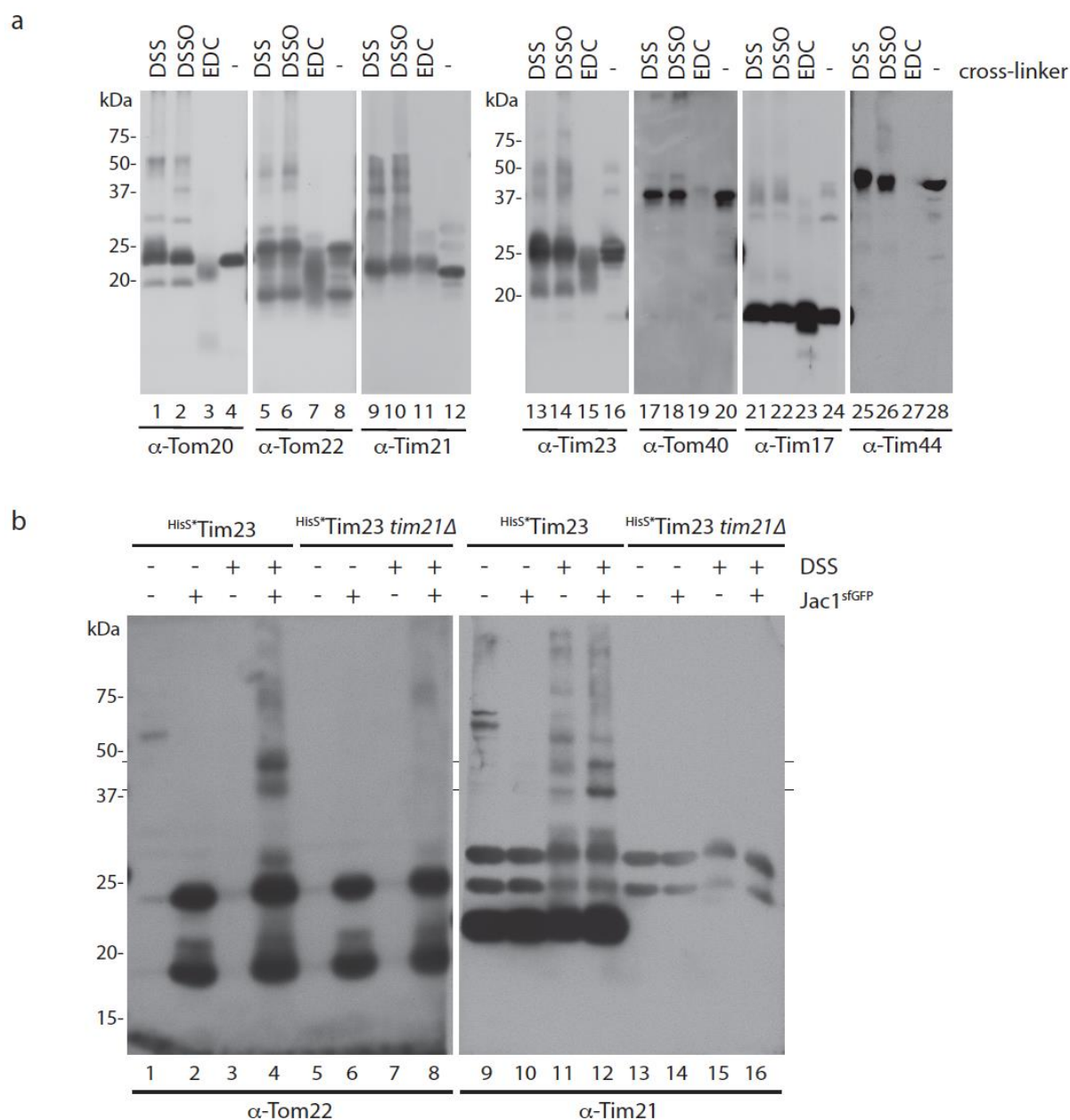
Content:

Supplementary Figures 1 to 4

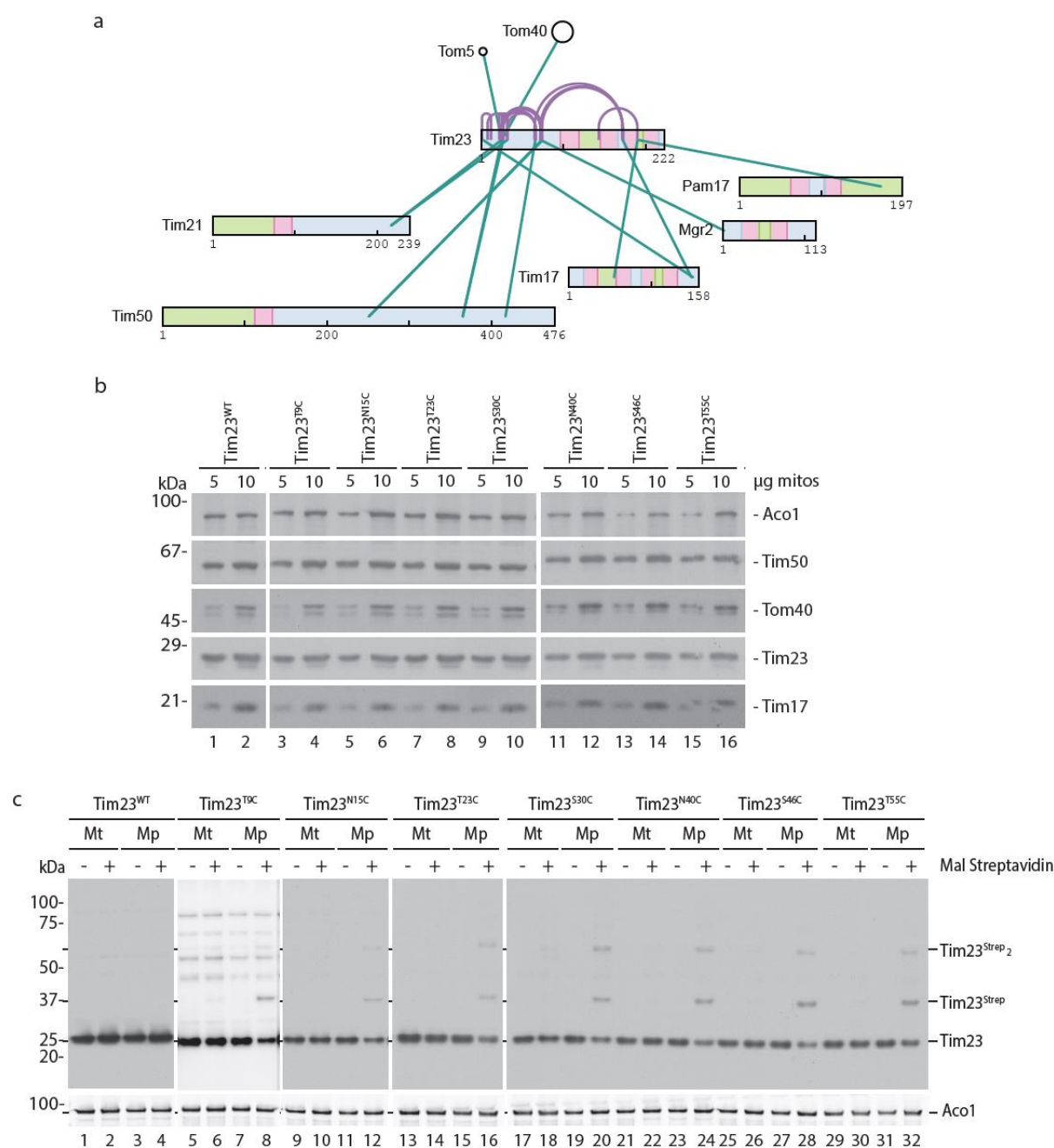
Supplementary Tables 1 to 4



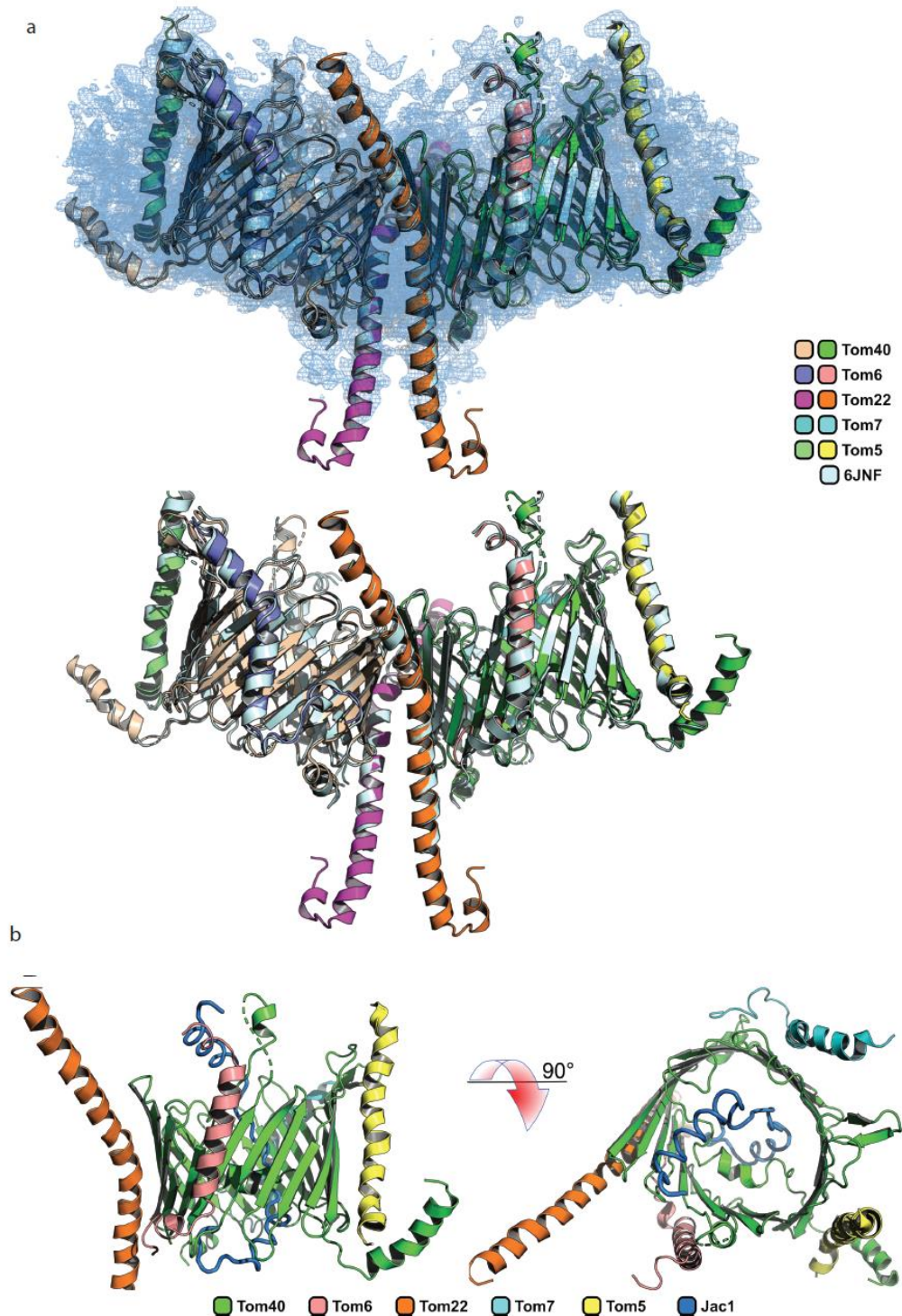
Supplementary Fig. 1. Supercomplex-forming Jac1^{sfGFP} localizes to mitochondria. **a** Yeast cells were transformed with Jac1^{sfGFP} (green)-encoding plasmid. After induction of expression, cells were co-stained with MitoTrackerTM Orange CMTMRos (red). Merged green and red fluorescence images are shown, indicating co-localization (yellow). (Scale bar = 5 μm). Representative images from three independent experiments are depicted. **b** Two-colour confocal microscopy of yeast cells expressing Jac1^{sfGFP}. Cells were labelled with antibodies against GFP (green) and Tom40 (magenta) or Tim23 (magenta). (Scale bar, 5 μm). Representative images from three biological replicates are depicted. **c** Solubilized wild type mitochondria were fractionated on 10-30% glycerol gradients following import of Jac1^{sfGFP}. Fractions were collected from top, TCA precipitated, and analyzed by SDS-PAGE followed by western blotting. Total: 2% of mitochondria loaded on the gradient. Representative experiment from a biologic triplicate is shown.



Supplementary Fig. 2. Chemical cross-linking of supercomplex subunits. a Supercomplex isolation was carried out from HisS*Tim23 mitochondria subjected to Jac1^{sfGFP} import. Isolation was performed using SUMO* protease. Isolated complex was treated with DMSO, 2 mM DSS, 2 mM DSSO and 10 mM EDC. Samples were analyzed by SDS-PAGE and western blotting. DSS: disuccinimidyl suberate, DSSO: disuccinimidyl sulfoxide, EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. Three independent experiments were carried out. **b** TIM23 complex was isolated from HisS*Tim23 or HisS*Tim23 *tim21* Δ mitochondria in the absence or presence of Jac1^{sfGFP}. Eluted samples were treated with DMSO or 2 mM DSS. Samples were analyzed by SDS-PAGE and western blotting. Three replicates were carried out.



Supplementary Fig. 3. Tim23 N-terminus localizes to the IMS. **a** Overview of intra- and inter- protein cross-links obtained for Tim23. Colour code indicates domain location. blue: IMS, pink: IMM and green: matrix. **b** Purified mitochondria from wild type cells and cells expressing Tim23 cysteine mutants were subjected to SDS-PAGE and immunoblotting. Representative image from two independent experiments. **c** Cysteine modification assay for Tim23 was carried out in mitochondria and mitoplasts from wild type and Tim23 cysteine mutants in the presence or absence of maleimide streptavidin. Samples were subjected to SDS-PAGE and western blotting. Representative image from three replicates.



Supplementary Fig. 4. Extended structure of the TOM complex. **a** *de novo* extension of intermembrane space (IMS) domain of Tom22 (magenta and orange) based on available TOM complex structures (PDB id: 6JNF, EMD ID: 9851). Rosetta tools were utilized for extending the structure. Superposition of the published TOM complex atomic model (PDB id: 6JNF) supports the extended (modelled) TOM complex. **b** Single TOM complex subunit with front and top view. Colour code indicates individual subunits.

Supplementary Table 1: List of identical inter-protein cross-links obtained by three methods: 1) TIM23 complex isolation followed by cross-linking, 2) whole mitochondria *in organello* cross-linking, and 3) *in organello* cross-linking followed by TIM23 complex isolation, in resting (- Jac1^{sfGFP}) or active (+ Jac1^{sfGFP}) state.

Cross-linker	Inter-protein cross-links	Approach 1 Isolation followed by cross-linking		Approach 2 <i>in organello</i> cross-linking		Approach 3 <i>in organello</i> cross-linking followed by isolation	
		- Jac1 ^{sfGFP}	+ Jac1 ^{sfGFP}	- Jac1 ^{sfGFP}	+ Jac1 ^{sfGFP}	- Jac1 ^{sfGFP}	+ Jac1 ^{sfGFP}
EDC	Tom70 ¹⁰³ - Tom20 ¹⁸³	-	+	-	+	NA	NA
EDC	Tom5 ¹ - Tom20 ⁹⁰	-	+	-	+	NA	NA
EDC	Tom5 ¹ - Tom20 ¹⁸²	-	+	+	-	NA	NA
EDC	Tom5 ¹ - Tom20 ¹⁸³	-	+	+	-	NA	NA
DSS	Tim23 ¹⁹⁰ - Tim17 ⁵⁵	+	-	-	-	+	-
DSS	Hsp70 ⁴⁴⁵ - Mge1 ¹⁰⁴	+	+	+	+	+	+
DSS	Hsp70 ⁸³ - Mge1 ¹¹⁹	-	+	-	+	+	+
DSS	Hsp70 ⁴⁴⁴ - Tim44 ²²⁸	-	+	-	-	-	+
DSS	Hsp70 ⁵¹⁴ - Tim44 ²¹⁵	+	-	-	-	+	-
DSS	Hsp70 ⁵⁷⁴ - Tim44 ¹⁹⁸	+	+	-	-	+	+
DSS	Pam16 ³⁴ - Pam18 ¹⁰¹	+	+	+	-	+	+
DSS	Pam16 ⁸⁹ - Pam18 ¹³⁵	-	+	-	-	-	+

-: cross-link not detected

+: cross-link detected

NA: not analyzed

Supplementary Table 2: List of yeast strains generated in this study.

Strain	Genotype
HisS ⁺ Tim23 <i>tim21</i> Δ	HisS ⁺ Tim23 <i>tim21</i> ::TRP
BY4741-pRG13	BY4741 + [pRG13-Jac1 ^{sfGFP} (LEU2)]
Tim21 ^{FLAG}	YPH499 <i>tim21</i> ::TIM21 ^{FLAG} (HIS3)
Tim21 ^{FLAG} +Tim21 ^{WT}	YPH499 <i>tim21</i> ::TIM21 ^{FLAG} (HIS3) + [pFL39-TIM21 (TRP)]
YPH499-pRG26	YPH499 + [pRG26 (TRP)]
YPH499-pRG27 (Tim23 ^{ALFA})	YPH499 + [pRG27-pTIM23 ^{ALFA} (TRP)]
YPH499-pRG28 (Tim17 ^{ALFA})	YPH499 + [pRG28-pTIM17 ^{ALFA} (TRP)]
YPH499-pRG30 (Tim50 ^{ALFA})	YPH499 + [pRG30-pTIM50 ^{ALFA} (TRP)]
YPH499-pRG33 (Tim44 ^{ALFA})	YPH499 + [pRG33-pTIM44 ^{ALFA} (TRP)]
YPH499-pRG35 (Pam18 ^{ALFA})	YPH499 + [pRG35-pPAM18 ^{ALFA} (TRP)]
YPH499-pRG37 (Pam16 ^{ALFA})	YPH499 + [pRG37-pPAM16 ^{ALFA} (TRP)]
YPH499-R673 (Tim23 ^{WT})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 (HIS3)]
YPH499-pRG39 (Tim23 ^{T9C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 T9C (HIS3)]
YPH499-pRG40 (Tim23 ^{N15C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 N15C (HIS3)]
YPH499-pRG41 (Tim23 ^{T23C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 T23C (HIS3)]
YPH499-pRG42 (Tim23 ^{S30C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 S30C (HIS3)]
YPH499-pRG44 (Tim23 ^{N40C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 N40C (HIS3)]
YPH499-pRG45 (Tim23 ^{S46C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 S46C (HIS3)]
YPH499-pRG46 (Tim23 ^{T55C})	MB29 <i>tim23</i> Δ + [pRS413-pORFt TIM23 T55C (HIS3)]

Supplementary Table 3: List of plasmids used in this study.

Plasmids	Description
GST-nGFP-Strep (R286)	GST-Thrombin-GFP Nanobody-Strep in pGEX-4T3 (Amp)
pRG9	14HIS-SUMO-Jac1 ^{sfGFP Q7R} in pSUMO (Kan)
Tim44 ^{C-term} (R662)	Tim44 residues 244-431 in pPROEX HTc (Amp)
Tim44 (1014)	Tim44 full length (without presequence) in pQE60 (Amp)
pRG13	Jac1 ^{sfGFP Q7R} in p425Gal1 (Amp, LEU2)
pRG26	ALFA-ALDH terminator in pRS414 (Amp, TRP1)
pRG27	Tim23 in pRG26 (Amp, TRP1)
pRG28	Tim17 in pRG26 (Amp, TRP1)
pRG30	Tim50 in pRG26 (Amp, TRP1)
pRG33	Tim44 in pRG26 (Amp, TRP1)
pRG35	Pam18 in pRG26 (Amp, TRP1)
pRG37	Pam16 in pRG26 (Amp, TRP1)
pFL39 (R84)	empty vector for yeast expression (Amp, TRP1)
pGB 9607-7 (R85)	Tim21 in pFL39 for expression in yeast (Amp, TRP1)
R673	pORFt Tim23 in pRS413 (Amp, HIS3)
pRG39	pORFt Tim23 T9C in pRS413 (Amp, HIS3)
pRG40	pORFt Tim23 N15C in pRS413 (Amp, HIS3)
pRG41	pORFt Tim23 T23C in pRS413 (Amp, HIS3)
pRG42	pORFt Tim23 S30C in pRS413 (Amp, HIS3)
pRG44	pORFt Tim23 N40C in pRS413 (Amp, HIS3)
pRG45	pORFt Tim23 S46C in pRS413 (Amp, HIS3)
pRG46	pORFt Tim23 T55C in pRS413 (Amp, HIS3)

Supplementary Table 4: Antibodies used in this study.

Antibody	Source	Identifier	Dilution
GFP (western blot)	Roche	11814460001	1:1000
GFP (microscopy)	Thermo Fisher Scientific	A-11120	
Anti-ALFA HRP-conjugated	NanoTag Biotechnologies	N1502-HRP	1:500
FLAG	Sigma	F3165	1:500
Peroxidase Streptavidin (SA-HRP)	Jackson ImmunoResearch Lab	016-030-084	1:500
Goat anti Rabbit IgG (H+L) HRPO	Jackson ImmunoResearch Lab	111-035-144	1:10,000
Tim23	Rabbit polyclonal, self-made	3846	1:500
Tim23 ^{IMS}	Rabbit polyclonal, self-made	133	1:750
Tim17	Rabbit polyclonal, self-made	4968	1:200
Tim50	Rabbit polyclonal, self-made	3314	1:400
Tim21	Rabbit polyclonal, self-made	3111	1:400
Tim44	Rabbit polyclonal, self-made	3869	1:250
Hsp70	Rabbit polyclonal, self-made	4945	1:50,000
Pam16	Rabbit polyclonal, self-made	3872	1:200
Pam18	Rabbit polyclonal, self-made	3955	1:200
Pam17	Rabbit polyclonal, self-made	5017	1:400
Tom70	Rabbit polyclonal, self-made	3530	1:400
Tom40	Rabbit polyclonal, self-made	4901	1:200
Tom22	Rabbit polyclonal, self-made	3533	1:400
Tom20	Rabbit polyclonal, self-made	3534	1:400
Tom5	Rabbit polyclonal, self-made	162	1:500
Aco1	Rabbit polyclonal, self-made	5004	1:500

Description of additional supplementary files

Title: Supplementary Movie 1

Description: Movie depicting components of the modelled TOM-TIM23 supercomplex and the integrated intra- and inter-protein cross-links obtained from DSS, EDC and SDA cross-linking.

Title: Supplementary Data 1

Description: Mass spectrometric analyses using three approaches. 1) TIM23 complex isolation in absence or presence of Jac1sfGFP followed by DSS, SDA and EDC cross-linking, 2) DSS and EDC cross-linking on purified mitochondria in absence or presence of Jac1sfGFP, and 3) DSS cross-linking of mitochondria in absence or presence of Jac1sfGFP followed by TIM23 complex isolation. For approach 3, Tim23 WT residues are mentioned in brackets next to the HisS*-tagged residue number.

Title: Supplementary Data 2

Description: Overview of critical inter-protein cross-links obtained from the three cross-linking approaches.

Reporting Summary

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- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☒ ☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

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Software and code

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Data collection	Inspector software version 0.14.13919 was used for the Abberior STED microscope, as distributed by the microscope manufacturer. DeltaVision softWoRx software (GE Healthcare, Applied Precision) was used for standard fluorescence images.
Data analysis	General import and western blot analysis: ImageQuantTL v8.1 (GE Healthcare); ImageJ v1.47 (NIH); Prism8 (GraphPad Software). Mass spectrometry analysis: Proteome Discoverer (v. 1.4, Thermo Fisher Scientific); pLink 1 (v. 1.23); MaxQuant (v. 1.6.0.1); pLink 2 (v. 2.3.9), xiNET (http://crosslinkviewer.org/). Structural analysis: ROSETTA 2020.46.61480 (DiMaio et al. 2015); Coot 0.9.5 (Emsley et al. 2010); 2.2 Open-source (www.pymoll.org).

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Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The following structures available on the Protein Data Bank (PDB) were utilized for modelling: the TOM complex, PDB id: 6JNF (<http://doi.org/10.2210/pdb6JNF/pdb>); Jac1, PDB id: 3UO3 (<http://doi.org/10.2210/pdb3UO3/pdb>); Tim21, PDB id: 2CIU (<http://doi.org/10.2210/pdb2CIU/pdb>); Tim50, PDB id: 3QLE (<http://doi.org/10.2210/pdb3QLE/pdb>) and Tim23, PDB id: 7CLV (<http://doi.org/10.2210/pdb7CLV/pdb>). Data and mass spectrometry datasets supporting the findings of

this manuscript are available within the paper, its extended data and the source data file. The cross-linking mass spectrometry data generated in this study have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository under accession code XYZ (hyperlink and identifier awaited). Additional information is available from the corresponding author upon request. Source data is provided with this paper.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical method was used to predetermine sample size. Sample size for each experiment was determined based on prior experience and established protocol within the field for that type of experiment (Schendzielorz et al, Nature Communications, 2018). For fluorescence microscopy of yeast cells to check for expression of GFP fusion protein, at least 10 images were taken with multiple cells in each image. For radioactive import experiment, sample size of at least 3 was utilized (Schendzielorz et al, Nature Communications, 2018).
Data exclusions	All cross-links obtained are reported in Supplementary Data 1. However, those cross-links which were topologically not possible were not considered for further analysis and structural modeling.
Replication	We carried out at least three biological replicates of all experiments, unless indicated differently, for both import and western blot analysis. All attempts at replication were successful. Cross-linking mass spectrometry analysis with DSS for Approach 1 was done as a triplicate, and once for SDA and EDC samples.
Randomization	The two samples for all rounds of cross-linking followed by mass spectrometry were constant. One was without fusion protein Jac1sfGFP, other was with. Single colony clones for bacteria or yeast following transformation were picked randomly and confirmed by sequencing and immunoblotting. For all other experiments, randomization does not apply since there were no other separate group allocation for samples.
Blinding	Blinding during data acquisition and analysis does not apply since there were no different treatment groups.

Reporting for specific materials, systems and methods

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Materials & experimental systems

n/a	Involved in the study
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Human research participants
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used

All antibodies against mitochondrial proteins in this study were self-made by injecting recombinant protein or peptide into rabbits and have been used in previous publications (Schendzielorz et al, Nature Communications, 2018). Specifically, the dilutions used for each are: anti-Tim23 (1:500), anti-Tim23IMS (1:750), anti-Tim17 (1:200), anti-Tim50 (1:400), anti-Tim21 (1:400), anti-Tim44 (1:250), anti-Hsp70 (1:50,000), anti-Pam18 (1:200), anti-Pam16 (1:200), anti-Pam17 (1:400), anti-Tom70 (1:400), anti-Tom40 (1:200), anti-Tom22 (1:400), anti-Tom20 (1:400), anti-Tom5 (1:500), anti-Aco1 (1:500). Additionally, the following commercial primary antibodies with their catalogue numbers and dilutions were used: GFP for western blotting (Roche, 11814460001, 1:1000); GFP for microscopy (Thermo Fisher Scientific, A-11120); ALFA (NanoTag Biotechnologies, N1502-HRP, 1:500); FLAG (Sigma, F3165, 1:500), Peroxidase Streptavidin (Jackson ImmunoResearch, 016-030-084, 1:500). Secondary Goat anti Rabbit IgG (H+L) HRPO (Jackson ImmunoResearch, 111-035-144, 1:10,000) was used. A list of antibodies, their source and dilution is provided as Supplementary Table 4.

Validation

Antibody specificity for self-made antibodies was confirmed by western blot with deletion or tagged yeast strains, and have also been utilized in previous publications (Schendzielorz et al, Nature Communications, 2018). Commercial antibodies have been validated

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	All yeast strains used in this study are described and reported in the Methods section and in Supplementary Table 2.
Authentication	Mutant yeast strains underwent several rounds of growth on selective media before being used for experiments and were confirmed by PCR and sequencing of genomic DNA, western blotting with whole cell extracts or with 5-FOA shuffling.
Mycoplasma contamination	Yeast strains and cultures were not tested for mycoplasma contamination since this is not an issue for them.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified line were used.