

Reporting Summary

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Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study.

For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☒ ☐ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ 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

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

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).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

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

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

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

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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.