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# Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

## 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<br>*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<br>*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 | Fluorescence microscopy: cellSens 1.17 software (Olympus)<br>Image Reader LAS-3000 for Western blot signals<br>Image Quant TL for autoradiography<br>AB2 Luminescence Spectrometer Version 5.50 for membrane potential measurements<br>TargetP software was used to determine the iMTS of Ubx2 |
| Data analysis | ImageJ 2.0.0-rc-41/1.50d open source image software and Adobe Photoshop CS5 to process data<br>Adobe Illustrator CS5 to assemble figures<br>Multi-Gauge software for quantifications |

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/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research 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 list of figures that have associated raw data
- A description of any restrictions on data availability

All data of this study are available in the article and the extended data. Uncropped versions of all blots and gels are presented in Supplemental Figure 1.

# 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 | Samples size was chosen without the use of statistical measures. The amount of mitochondrial and cellular proteins used for the biochemical assays was chosen based on previous experiences with this specific type of experiments and commonly used sample sizes in the field of research. For key experiments, several test runs were performed to determine the optimal sample size used. |
| Data exclusions | All relevant data shown. No data were excluded from this study. |
| Replication | All experiments of this study have been reproduced using the same experimental set-up (amount of cell extracts, mitochondrial proteins, buffers, detergents, affinity matrices etc.) with similar results. The number of the independent replications is stated in the Figure Legends. For Western blots and autoradiography, representative results are shown. For all shown data, successful replications are available. |
| Randomization | The clones of the yeast strains were selected randomly. |
| Blinding | No blinding was performed. The yeast strains have to be verified before isolation of materials for cell biological and biochemical analysis. |

# 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 |
|---|---|
| ☐ | ☒ Antibodies |
| ☐ | ☒ Eukaryotic cell lines |
| ☒ | ☐ Palaeontology |
| ☒ | ☐ Animals and other organisms |
| ☒ | ☐ Human research participants |
| ☒ | ☐ Clinical data |

## Methods

| n/a | Involved in the study |
|---|---|
| ☒ | ☐ ChIP-seq |
| ☒ | ☐ Flow cytometry |
| ☒ | ☐ MRI-based neuroimaging |

## Antibodies

| | |
|---|---|
| Antibodies used | Antibodies against proteins from baker´s yeast Saccharomyces cerevisiae were generated in rabbits using peptides (Ubx2, Cdc48, Ufd1, Hrd1, Doa10, Msp1, Tom40, Tom5, Tom70, Sam50, Mim1, Om14, Om45, Porin, Pam17, Tim23, Tim44, Tim50, Rip1, Cox4, Mdj1, Sod2 precursor, Sec61, Sss1, Ssa1, Pgk1, Cox1, Cyt1, Cox2) or recombinant proteins (Tom20, Tom22, Tom40). The antisera were used in 1:250-1:1000 dilution. Antisera against Ufd1 and Sam50 were affinity purified and used in 1:40 dilution. The anti-HA (12CA5) antibody was obtained from Roche (Cat. No. 11583816001). The anti-ubiquitin antibody (P4D1) (Cat. sc-8017) and the anti-DHFR (A9) (Cat. sc-377091) were obtained from Santa Cruz Biotechnology. The following dilutions were used: 1:2000 (anti HA), 1:100 (anti-ubiquitin) and 1:1000 (anti-DHFR). |
| Validation | The specificity of the antibody raised against a protein from baker´s yeast (Saccharomyces cerevisiae) was controlled by comparing total cell extracts or mitochondrial lysates from wild-type yeast cells and the corresponding deletion strain or strains expressing a tagged version of the protein of interest via SDS-PAGE and Western blotting. Absence or size shift of the signal in cellular fractions of the mutant strain confirmed the specificity of the antibody signal. |

## Eukaryotic cell lines

Policy information about cell lines

| | |
|---|---|
| Cell line source(s) | All yeast strains used in this study are described in Supplementary Information Table 1. The following yeast strains were obtained from Euroscarf: BY4741, ubx2Δ, vms1Δ, doa1Δ, dfm1Δ, msp1Δ, cdc48-2, cdc48-3, ufd1-2, pre9Δ, shp1Δ.<br><br>The yeast strains YPH499, 334, YWO2, npl4-1, Tom22His, Por1HA, HATom5, HATom6, HATom7, tom20Δ, tom70Δ, mim1Δ, |

sam37Δ, 333+pCYB2-ΔDHFR, 334-pCYB2-DHFR-HB have been described:

Becker, T. et al. The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins. J. Cell Biol. 194, 387-395 (2011).

Bömer, U. et al. The sorting route of cytochrome b2 branches from the general mitochondrial import pathway at the preprotein translocase of the inner membrane. J. Biol. Chem. 272, 30439-30446 (1997).
Ellenrieder, L. et al. Separating mitochondrial protein assembly and endoplasmic reticulum tethering by selective coupling of Mdm10. Nat. Commun. 7, 13021 (2016).

Jarosch, E. et al. Protein translocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat. Cell Biol. 4, 134-139 (2002).

Müller, C. S. et al. Cryo-slicing BN-MS – a novel technology for high-resolution complexome profiling. Mol. Cell. Proteomics 15, 669-681 (2016).

Opaliński, Ł. et al. Recruitment of cytosolic J-proteins by TOM receptors promotes mitochondrial protein biogenesis. Cell Rep. 25, 2036-2043.

Sikorski, R. S. & Hieter, P. A. system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27 (1989).

Wenz, L. S. et al. Sam37 is crucial for formation of the mitochondrial TOM-SAM supercomplex, thereby promoting β-barrel biogenesis. J. Cell. Biol. 210, 1047-1054 (2015).

The following yeast strains were newly generated for this study: pam17Δ BY4741, pam17Δ YWO2, ubx2Δ vms1Δ, ubx2Δ msp1Δ, ubx2Δ pam17Δ, cdc48-2 pam17Δ, pre9Δ pam17Δ, doa1Δ pam17Δ, vms1Δ pam17Δ, ufd1-2 pam17Δ, npl4-1 pam17Δ, ubx2Δ+UBX2, ubx2Δ pam17Δ + UBX2, ubx2Δ+UBX2ΔiMTS-L, Tom40HA, Tom40HA ubx2Δ, Tom40HA ubx2Δ+UBX2ΔUBA, Tom40HA ubx2Δ+UBX2ΔUBX, Tom40HA ubx2Δ+UBX2ΔiMTS-L, Tom40HA vms1Δ, Tom40HA doa1Δ, Tom40HA ufd1-2, Ubx2HA, Ubx2His, Hrd1HA, Doa10ProtA, ubx2Δ 334, ubx2Δ+ pCYB2DHFR-HB, ubx2Δ+pCYB2ΔDHFR-HB, 334 + pYEp352, ubx2Δ + pYEp352, ubx2Δ+UBX2-GFP

Authentication

The yeast strains were selected by several rounds of growth on selection medium. Total cell extracts were prepared and the deletion or tagging of a yeast genes was confirmed by the absence of size shift of the corresponding gene product after Western blotting and immunodetection with the corresponding antisera.

Mycoplasma contamination

Mycoplasma contamination is not a problem for yeast cultures. Therefore, the mycoplasma contamination was not analyzed.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified line were used.