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Supplementary Information for

Absolute yeast mitochondrial proteome quantification reveals trade-off between biosynthesis and energy generation during diauxic shift

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This PDF file includes:

Supplementary Materials and Methods
Figures S1 to S6
Tables S1 to S2
Legends for Datasets S1 to S7
References for SI

Other supplementary materials for this manuscript include the following:

Datasets S1 to S7

Materials and Methods

Experimental Model

All experiments were carried out using the prototrophic yeast *Saccharomyces cerevisiae* strain CEN.PK113-7D MitoLoc if not stated otherwise. For the integration of the fluorescent protein plasmid pMitoLoc (carrying preSu9-GFP and preCox4-mCherry genes) into *S. cerevisiae* CEN.PK 113-7D's genome, CRISPR-Cas9 integration was performed using the EasyClone-MarkerFree Vector Set (1).

Media and growth conditions

Batch cultivations were carried out in 2.5-litre bioreactors (DasGip GmbH, Jülich, Germany; working volume, 2 litres), equipped with off-gas O_2 and CO_2 sensors (BlueSens GmbH, Herten, Germany), pH, temperature and dissolved oxygen sensors. The minimal medium used contained per litre: 20 g glucose, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$. Vitamins and trace metals were added to the medium after autoclaving. The final concentration of trace metals in the medium was, per litre: 15.0 mg EDTA, 4.5 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 2H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 0.3 mg $CuSO_4 \cdot 5H_2O$, 0.4 mg $Na_2MoO_4 \cdot 2H_2O$, 4.5 mg $CaCl_2 \cdot 2H_2O$, 3.0 mg $FeSO_4 \cdot 7H_2O$, 1.0 mg H_3BO_3, and 0.10 mg KI. The final concentration of vitamins in the medium was, per litre: 0.05 mg biotin, 0.2 mg p-aminobenzoic acid, 1 mg nicotinic acid, 1 mg Ca-pantothenate, 1 mg pyridoxine-HCl, 1 mg thiamine-HCl, 25 mg myo-inositol.

Cells were grown at 30^{o}C at pH 5. Stirrer rate was 800 rpm, with an air-flow of 2 vvm, to give a dissolved oxygen tension (DOT) of at least 30%. Cultivations were monitored using off-gas analysis, pH, dO_2, temperature and external O_2 and CO_2 sensors (BlueSens GmbH, Herten, Germany). Batch cultivations started at the defined conditions using 1% of overnight culture inoculum, giving an OD of 0.1.

Dry weight and cell number determination

For the determination of cell dry weight (CDW) and quantification of extracellular metabolites, samples of culture medium were collected at the time of inoculation, after two hours from inoculation and then every hour for 20 h after inoculation. The

measurement of CDW was performed by filtering 10 ml of culture using pre-weighed 0.45 µm PESU membrane filters (Sartorius AG, Göttingen, Germany) that were dried by microwave heating (150W, 15 min) and then further dried in a desiccator for one week before weight determination.

Cell number and volume were determined based on electrical current exclusion using a CASY cell counter (Roche Innovatis AG, Bielefeld, Germany). 1 ml of cell culture was collected in pre-cooled Eppendorf tubes, then diluted $1/10^2$ to $1/10^4$ in an electrolyte/buffer (CASYton®, OLS OMNI Life Science, Bremen, Germany) and immediately analysed.

Sampling for proteomic analysis

For the whole-cell proteomic analysis, 5 ml (after 9 h) and 2 ml each (after 13 and 20 h) of cell culture was harvested into pre-cooled Falcon tubes and centrifuged (5 min, 4°C, 1,000 x g), and washed using ice-cold 1X PBS. Cells were then collected by centrifugation (21,000 x g, 30 s, 4°C), snap-frozen and stored at −80°C.

HPLC analysis of exometabolites

For the exometabolite analysis, 1 ml of culture was collected, filtered through a 0.45 µm nylon membrane filters (VWR, Radnor, PA, USA) and immediately stored at -20ºC. All samples were analysed on an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Bio-Rad (Hercules, CA, USA) HPX-87H column and a RI detector used for the detection and quantification of glucose and ethanol. Isocratic elution using 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min, and a column temperature of 45°C was used.

Cell confocal imaging

For the morphological analysis of yeast mitochondria, 500 µl of cell culture was harvested for each condition using ice-cold Eppendorf tubes. Cells were then washed with 1 ml ice-cold 1X PBA and chemically fixed using 500 µl of 4 g/l paraformaldehyde (PFA) in 3.6% sucrose solution and incubated at room temperature for 20 min.

After incubation with PFA, cells were washed twice with 1X PBA and resuspended in 20 µl of Vectashield mounting medium (Vector Labs Inc., Burlingame, CA, USA) and stored at 4°C. Prior to analysis, samples were diluted 1:20 in 20 µl Vectashield. Fixed and live cells were visualized using a Nikon A1 confocal fluorescent microscope (Nikon Instruments Inc, Melville, NY, USA), equipped with a CFI Apo λS 60x oil objective with nano-crystal coat and A1-F filter Cube (EX: 450/50; DM: 525/50; EM: 595/5 700/75), using 3.5 µl of sample applied to 10x poly-L-lysine coated slides. For each marker, the fluorescence was separated by excitation (eGFP: 488 nm; mCherry: 561 nm), with a minimum of 50 cells per condition imaged. Z-stacks were acquired with a step of 0.125 µm. For the measurement of the mitochondrial volume, images derived from Z-stack acquisition were first deconvolved using the automatic deconvolution tool of the NIS-Elements software (Version 4.11.0, Nikon Instruments Inc., Melville, NY, USA), the calculation of the volumes was performed by the NIS-Elements AR Measurement, part of the NIS-Elements software, which automatically calculated the volumes using measured area of the Z-stacks generated. The collected images were further analysed for the determination of the whole cell volume using ImageJ. For the creation of a 3D model of the Nikon A1 dedicated software, NIS-Elements Viewer was used for the conversion of the Z-stacks into a 3D model as shown in Figure 2c-e.

Mitochondria isolation

Isolation of mitochondria was performed immediately after cell biomass sampling for whole-cell proteome analysis. Cells were harvested by centrifugation (5 min, 3,000 x g), then the pellet was resuspended in deionized water, followed by centrifugation again (5 min at 3,000 x g). Cells were incubated for 20 min at 30°C in 2 ml of Buffer A (100 mM Tris-H_2SO_4 pH 9.4, 10 mM DTT) per g of cell wet weight. Cells harvested after this step were then incubated with 7 ml/g cell wet weight zymolyase buffer (1.2 M sorbitol, 20 mMK_3PO_4 pH 7.4, 6 mg Zymolyase per cell wet weight 20-T, MP Biomedicals Life Sciences, Santa Ana, CA, USA), with the lytic activity of Zymolyase being used to digest the cell wall and create

spheroplasts, as confirmed by bright field microscopy. Cells were incubated with shaking for 1 hour at 30°C, and then all subsequent steps were carried out at 4°C. Spheroplasts were centrifuged for 5 min at 3000 x g, resuspended in 7ml/g sorbitol buffer (0.6 M sorbitol, 10 mM Tris-HCl pH 7.4, 1mM PMSF) and homogenised using a PYREX® Dounce Tissue Grinder (20 strokes for 3 times; Corning Inc., Corning, NY, USA). (The homogenisation step being used in order to open the cells and release intact organelles). The proceeding steps of differential centrifugation were then performed to determine the specific isolation of mitochondria based on their buoyant density. The cell lysate was centrifuged (5 min, 1,500 x g), followed by centrifugation of the supernatants (5 min, 4,000 x g). Crude mitochondrial fractions were pelleted by centrifugation (15 min, 12,000 x g), then mitochondrial pellets were resuspended in sucrose buffer (250 mM sucrose, 1mM EDTA, 10 mM MOPS-KOH, pH 7.2), and followed by a higher purity purification step using the Qproteome Mitochondria Isolation Kit (QIAGEN, Hilden, Germany). The mitochondrial fractions were finally shock-frozen in liquid nitrogen and stored at -80°C. The enrichment analysis of the mitochondrial fraction isolated at the different stages of the cell growth was performed via Western Blot analysis (2). Prior to protein immunoblotting, the protein concentration of the mitochondrial samples was determined using the Lowry-based DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

Mitochondrial Lipidomics

All solvents were purchased from Sigma – Aldrich (Merck, Kenilworth, NJ, USA). Mitochondrial lipid extraction was performed for all conditions and replicates by taking from each sample 1 mg mitochondrial protein previously measured using the DC™ Protein Assay (Bio-Rad). Before lipid extraction, the purified mitochondrial extracts were mixed with 20 µl of internal lipid standard (IS, Avanti polar lipids, Alabaster, AL, USA), providing a concentration of: 0.07 ppm of phosphatidylserine (PS) 17:0-14:1, 1.00 ppm of phosphatidylinositol (PI) 17:0-14:1,0.22 ppm of phosphatidylglycerol (PG) 17:0-14:1, 0.80 ppm of phosphatidylethanolamine (PE) 17:0-14:1, 0.85 ppm of phosphatidylcholine (PC)

17:0-14:1, 0.17 ppm of lysophosphatidic acid (LPA) 17:1, 0.43 ppm of phosphatidic acid (PA) 17:0, 1.20 ppm of diacylglycerol (DG) 10:0, 0.13 ppm of lysophosphatidylcholine (LPC) 17:1, 0.24 ppm of lysophosphatidylethanolamine (LPE) 17:1, 0.20 ppm of lysophosphatidylserine (LPS) 17:1, 0.16 ppm of lysophosphatidylinositol (LPI) 17:1, 0.17 ppm of ceramide (Cer) d18:1/17:0, 0.33 ppm of triacylglycerol (TG) 17:0-17:1-17:0, 0.58 ppm of Cardiolipin (CL) Mix I (14:1(3)-15:1 CL 15:0(3)-16:1 CL 22:1(3)-14:1 CL 24:1(3)-14:1 CL). The sample extraction was performed using the Methyl Tert-Butyl Ether (MTBE) method described as follows (3). Initially, 1.5 ml methanol was added to sample aliquots and vortexed for 15 min. Next, 5 ml of MTBE was added to the mixture that was then incubated for 1 h at room temperature. Following the addition of 1.25 ml MQ water, samples were incubated for 15 min then centrifuged (1500 x g, 10 min), inducing phase separation within the samples. First the upper organic phase was collected, then the entire extraction procedure was repeated on the lower phase. At the end of the subsequent extraction, the combined organic phases were dried, and lipids extracted and dissolved in 200 µl chloroform/methanol/water mixture (60/30/4.5 - v/v/v) and stored at -20°C until further analysis.

Before proceeding with lipid detection, a calibration curve was prepared with 8 concentrations points (from 0.1 to 7.5 µg/ml), using the internal lipid standard previously described. For sample analysis, an ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) was performed using a 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA, USA) and column UPLC BEH C18, 2.1 x100 1.7 µm (Column ID CMSI-HPLC-042; Waters Acquity, Milford, MA, USA). Mobile Phase A consisted of: MQ water + 1% 1M NH_4CO_2H + 0.1% HCO_2H, Mobile Phase B: acetonitrile:isopropanol (1:1, v/v) + 1M NH_4CO_2H + 0.1% HCO_2H.

A linear gradient from 35% to 80% was applied for solvent B over 2 min followed by an increase to 100% B over 5 min. The temperature of the column was kept at 50°C and the injection volume was of 1 µl per sample, with each sample being analysed in triplicate. For the Q-TOF-MS we used QTOF 6520 Agilent instrument with electrospray ionization (ESI), MS scan m/z 100-1700, collecting

1.67 spectra/sec in positive mode. Pooled extract of mitochondria isolated during the glucose phase samples was injected six times as quality control (QC). Using the Agilent Profinder, the mass, retention time (RT), and area of the IS were extracted from each injection with a % relative standard deviation (RSD) below 10. Finally, 106 lipids were identified from the mitochondrial extract using LipidMatch (4). To perform relative quantitation, each identified lipid was quantified using the calibration standard from the same class or group and also based on the RT. The concentration ratio, calculated from the calibration curve, was multiplied by the IS concentration to acquire the concentration (µg/ml).

Protein extraction for proteomic analysis

1 mg of yeast cell pellets and mitochondria pellets in 500 µL of lysis buffer (50 mM triethylammoinium bicarbonate (TEAB), 2 % sodium dodecyl sulfate (SDS)) were lysed, respectively. Samples were homogenized using a FastPrep®-24 instrument (Matrix-C (red) FAST tubes MP Biomedicals, OH, USA) for 5 repeated 40 second cycles at 6.5 m/s, with 30-60 second pauses in between. Samples were centrifuged (400 x g, 2 min) then supernatants were transferred to new Eppendorf tubes. The supernatants were centrifuged again (21,100 x g, 10 min) to remove cell debris then supernatants were transferred to new Eppendorf tubes again. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the Benchmark™ Plus microplate reader (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) solutions as standards. The reference pool of mitochondrial samples for quantification was prepared by combining equal amounts of all mitochondrial samples. The whole-cell-proteome reference pool was prepared from the aliquots of lysates from samples of all conditions analysed.

Tryptic Digestion and TMT (tandem mass tags) labelling of proteins

Aliquots containing 25 µg of total protein from each sample and 25 µg from the reference pool were used for the TMT sample preparation; 50 µg of the pooled mitochondrial sample was spiked with 10.6 µg of the UPS2 Proteomics Dynamic Range Standard Set (Sigma-Aldrich, Saint-Louis, MO) for label-free quantification.

Samples were reduced by the addition of 2 M DL-dithiothreitol (DTT) to a final concentration of 100 mM and incubated at 56°C for 30 min. The samples were then trypsin digested using the filter-aided sample preparation (FASP) method modified from Wiśniewski *et al.* (2009) (5). In brief, reduced samples, which had been diluted to 500 µl by addition of 8M urea, were applied on Nanosep® 30k Omega filters (Pall Life Sciences, Port Washington, NY, USA) then 200 µl 8M urea was used to repeatedly wash away the SDS. Alkylation was performed with 10 mM methyl methanethiosulfonate (MMTS) diluted in digestion buffer (1% sodium deoxycholate (SDC), 50 mM TEAB) for 30 min at room temperature and filters were then repeatedly washed with digestion buffer. Trypsin (Pierce Trypsin Protease, MS Grade, Thermo Fisher Scientific, Waltham, MA, USA) in digestion buffer was added in a ratio of 1:100 relative to protein amount and then samples were incubated at 37°C overnight. Another portion of trypsin was then added and incubated for 3 h. Peptides were collected by centrifugation (21,100 x g, 10 min) and subjected to isobaric mass tagging reagent TMT® according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The labelled samples were combined with the TMT sets, and then samples were concentrated using vacuum centrifugation with SDC being removed by acidification with 10% TFA.

The TMT-sets were fractionated into 44 primary fractions by basic reversed-phase chromatography (bRP-LC) using a Dionex Ultimate 3000 UPLC system (Thermo Fischer Scientific, Waltham, MA, USA). Peptide separations were performed using a reversed-phase XBridge BEH C18 column (3.5 µm, 3.0x150 mm, Waters Corporation) and a linear gradient from 3% to 40% solvent B over 17 min followed by an increase to 100% B over 5 min. Solvent A was 10 mM ammonium formate buffer at pH 10.00 and solvent B was 90% acetonitrile, 10% 10 mM ammonium formate at pH 10.00. The primary fractions were concatenated into final 20 fractions,evaporated and reconstituted in 15 µl of 3% acetonitrile, 0.2% formic acid for nLC MS analysis.

The pooled sample for the label-free quantification was fractionated into 44 primary fractions as described above, but the primary fractions were concatenated

into 10 final fractions. The combined fraction (16+17+36+37) was treated using the HiPPR Pierce detergent removal kit (Thermo Fisher Scientific, Waltham, MA, USA) and 25mM TEAB as an exchange buffer. The final fractions were evaporated and reconstituted in 15 µl of 3% acetonitrile, 0.2% formic acid for nLC MS analysis.

LC-MS/MS Analysis

Fractions were analysed on an Orbitrap Fusion Tribrid mass spectrometer interfaced with Easy-nLC1200 liquid chromatography system (both Thermo Fisher Scientific, Waltham, MA, USA). Peptides were trapped on an Acclaim Pepmap 100 C18 trap column (100 µm x 2 cm, particle size 5 µm, Thermo Fischer Scientific) and separated on an in-house packed analytical column (75 µm x 30 cm, particle size 3 µm, Reprosil-Pur C18, Dr. Maisch) using a linear gradient from 5% to 35% B over 75 min followed by an increase to 100% B for 5 min at a flow of 300 nL/min. Solvent A was 0.2% formic acid in water and solvent B was 80% acetonitrile, 0.2% formic acid. For the TMT-labelled peptides, MS scans were performed at 120 000 resolution, m/z range 380-1380, MS/MS analysis was performed in a data-dependent, with top speed cycle of 3 s for the most intense doubly or multiply charged precursor ions. Most intense precursors were fragmented in MS2 by collision induced dissociation (CID) at 35 collision energy with a maximum injection time of 50 ms, and detected in the ion trap followed by multinotch (simultaneous) isolation of the top 10 MS2 fragment ions, with m/z 400-1400, selected for fragmentation (MS3) by higher-energy collision dissociation (HCD) at 60% and detection in the Orbitrap at 50,000 resolution, m/z range 100-500. Precursors were isolated in the quadrupole with a 0.7 m/z isolation window and dynamic exclusion within 10 ppm, for 45 seconds being used for m/z-values already selected for fragmentation. For label-free quantification, MS3 fragmentation was not performed, and the same MS1 and MS2 scan settings were used, aside from the precursor isolation window of 1.2 m/z and the top speed duty cycle of 1 s.

Data matching for protein identification and relative quantification

MS raw data files for each TMT set were merged for identification and relative quantification using Proteome Discoverer version 2.2 (Thermo Fisher Scientific,

Waltham, MA, USA). The *Saccharomyces cerevisiae* ATCC 204508 / S288c reference proteome database was downloaded from Uniprot (6) (February 2018, 6049 sequences) and used for the database search on the TMT files. The concatenated database containing the above-mentioned sequences and the 48 UPS protein sequences was used for the processing of the UPS2-spiked reference files. A database search for each set was performed using the Mascot search engine (Matrix Science, London, UK) with MS peptide tolerance of 5 ppm and MS/MS tolerance for identification of 600 millimass units (mmu). Tryptic peptides were accepted with 1 missed cleavage. Variable modifications of methionine oxidation, and fixed modifications of cysteine methylthiolation were used for all searches. TMT-6 on lysine and peptide N-termini was set as a fixed modification for the TMT-labelled peptides. Percolator was used for the peptide-spectrum match (PSM) validation with the strict false discovery rate (FDR) threshold of 1%.

Quantification was performed in Proteome Discoverer 2.2, Minora feature detection node was used for precursor ion quantification in the label-free experiments, with the maximum intensity values used for quantification. Abundance values for all unique and shared peptides were used for the IBAQ calculation. Abundances from the 3 technical replicates were averaged and divided by the number of theoretically observable peptides for a protein to yield the IBAQ intensity (the number of observable peptides being calculated using an in-house Python script). The known absolute amount values of the UPS2 standard proteins were used to scale the Log-transformed IBAQ intensity values versus Log-transformed protein concentration. Only UPS2 proteins with at least 2 identified unique peptides were used for scaling. For the relative quantification of the TMT-labelled samples, the TMT reporter ions were identified in the MS3 higher energy collisional dissociation (HCD) spectra with a mass tolerance of 3 milli mass units (mmu), the signal-to-noise (S/N) abundances of the reporter ions for the unique peptides were used for relative quantification. The resulting TMT reporter abundance values for each sample were normalized within Proteome Discoverer 2.2 to the total peptide amount.

Statistical test

Unless indicated otherwise, all statistical tests were carried out using the Welch's t-test. Graphics and statistical computing were performed using the software environment R (7).

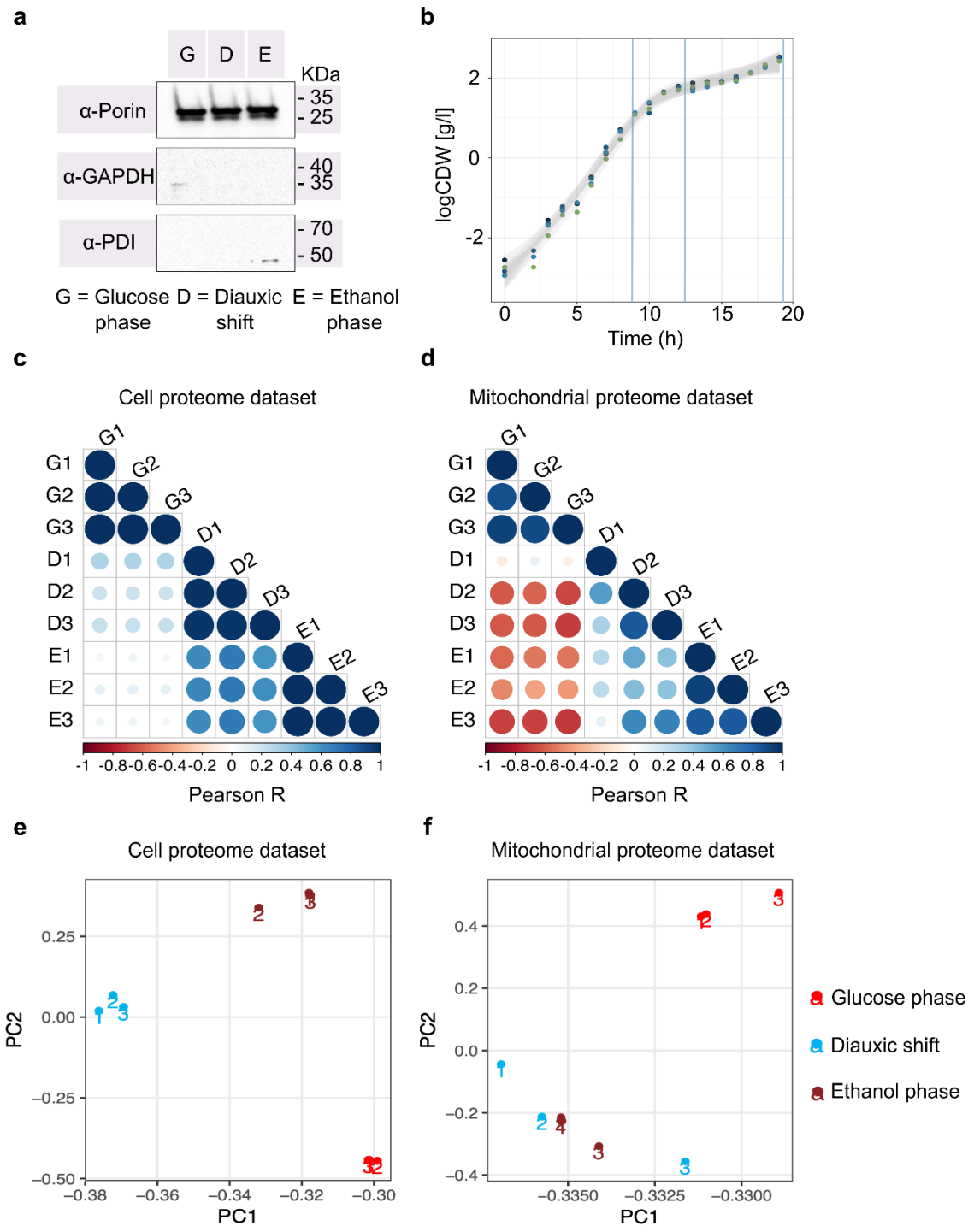

Fig. S1

Validation of and comparisons between samples and replicates for mitochondrial proteome analysis

a, Verification of mitochondrial enrichment by Western blot analysis. Following mitochondria isolation, aliquots of crude mitochondria extracts from the three growth stages were subjected to protein extraction via TCA precipitation. 20 µg of mitochondrial

proteins were separated using Gradient SDS Polyacrylamide Gel and then probed with antibodies specific for markers for mitochondria enrichment (porin) and cytosol contamination and ER contamination (respectively glyceraldehyde 3-phosphate dehydrogenase, GAPDH, and protein disulfide isomerase, PDI).
b, Yeast growth and biomass increase expressed as the logarithm of the cell dry weight (CDW) in grams per litre of culture. The blue vertical lines correspond to the time chosen for the yeast cell harvesting and mitochondrial isolation (number of biological replicates=4).
c-d, Correlogram representing Pearson's correlation coefficient (R) between the three biological replicates of the cellular and mitochondrial absolute proteomic datasets. G1; G2 and G3 = samples collected during glucose phase (9 hours from the inoculation). D1; D2 and D3 = samples collected during the diauxic shift (13 hours from the inoculation). E1; E2 and E3 = samples collected during the ethanol phase (20 hours from the inoculation). The intensity of the colour in the right bar indicates the degree of correlation.
e-f, Principal component analysis (PCA) of cellular and mitochondrial proteomic data according to biological replicates and the metabolic growth phase. **e**, The first principal component explains 73.25 % of the variance, while the second principal component explains 21.21%. **f,** The first principal component is accounting for 95.07% and the second principal component is accounting for 3.15% of the total variation

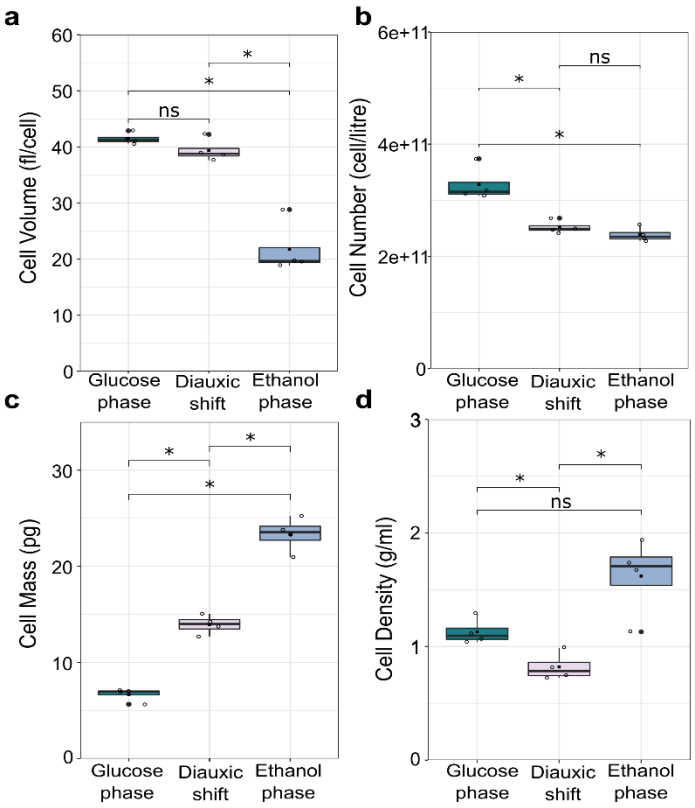

Fig. S2

Biophysical properties of cells during changes in metabolism

a-b, Cell volume and cell number were measured by electric current exclusion using the CASY capillary particle cell counter, using four biological replicates of samples collected during the glucose phase, diauxic shift and ethanol phase. Due to the high-throughput nature of the CASY instrument, each replicate included measurement of ~ 10,000 cells/run.

c, Mass per cell unit, measured by dividing cell dry weight (CDW), which was measured every two hours during cell growth, by the cell number.

d, Cell density as determined by dividing cell mass by cell volume for each condition. Here, individual cell mass was determined by dividing cell wet weight (CWW) over total cell number.

Samples were measured for each condition (n=3) and biological replicate (n=4). Statistical analyses were performed using paired *t*-test. Statistically significant differences are indicated as follows: ns (not significant) $p<1$; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

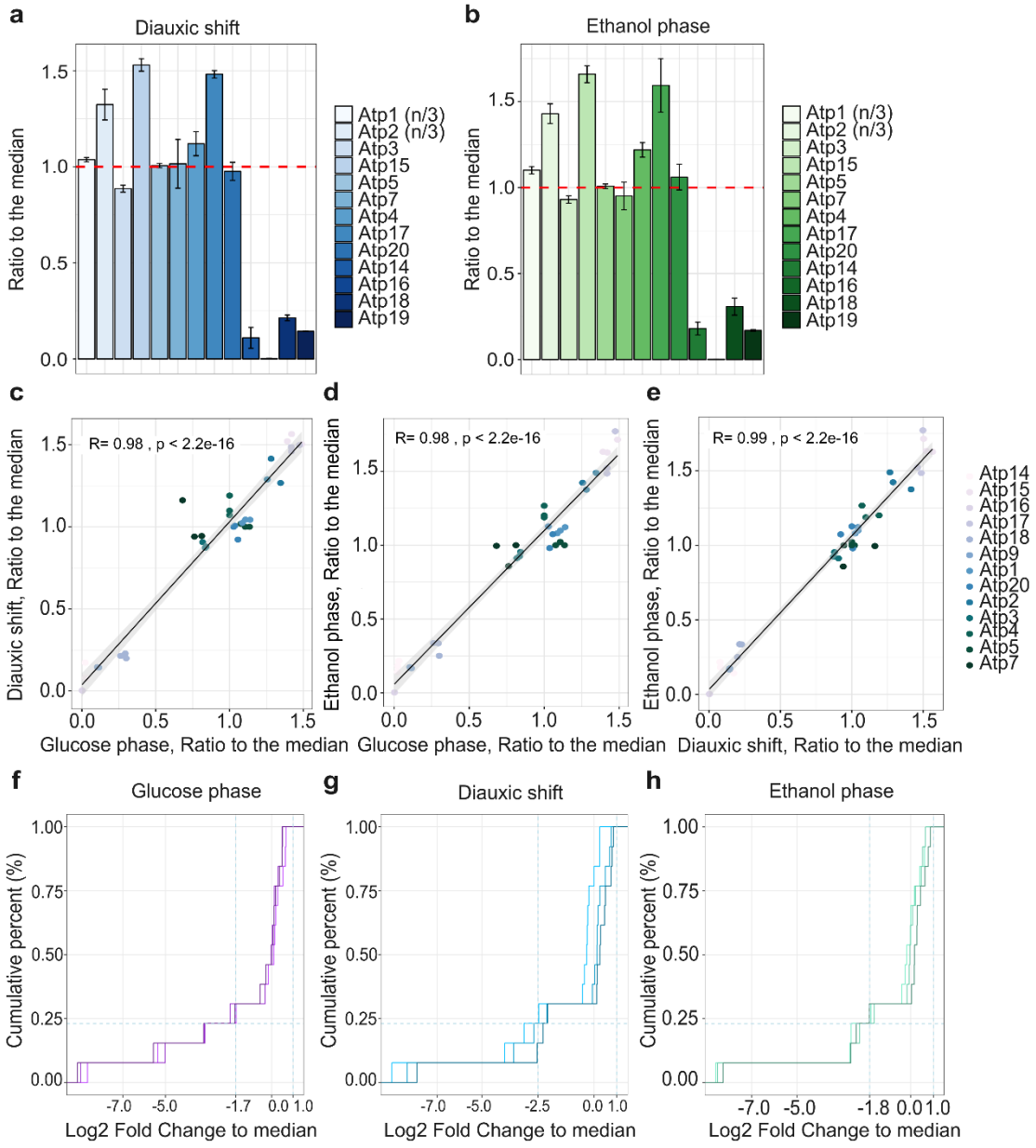

Fig. S3

Stoichiometry of the ATP synthase complexes inferred by absolute proteome data

a-b, Stoichiometry of the subunits of ATP synthase in the diauxic shift (**a**) and ethanol phase (**b**), expressed as the ratio to the median, taking the theoretical stoichiometry of *ATP1* and *ATP2* (3 subunits per complex) into account. Data were calculated as the mean of three biological replicates. Subunits are represented by different bar fill colour.

c-e, Comparison of the stoichiometry of the ATP synthase subunits between the three stages of growth. Different point colours represent the subunits.

f-h, Cumulative percentage distribution of the stoichiometric correlation among the ATP synthase complex's subunits versus the Log2 fold change from the median during the glucose phase, diauxic shift and ethanol phase. Each graph shows three biological replicates for each condition.

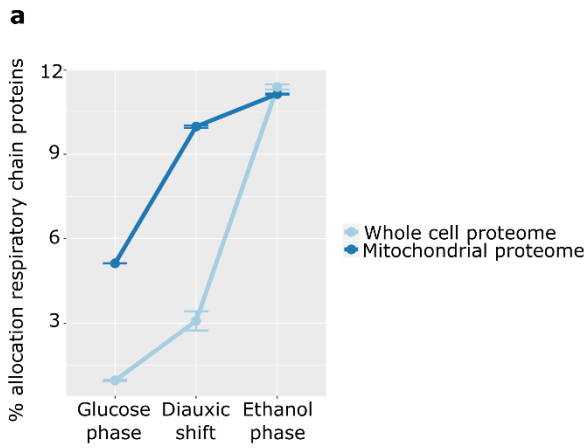

Fig. S4

Comparison between the percentage allocation of the respiratory chain protein complexes in the mitochondrial and cellular proteome during glucose phase, diauxic shift and ethanol phase of yeast growth

a, Protein complexes of the respiratory chain in mitochondrial protein and whole-cell proteome in the three stages of growth, calculated as the mean percentage allocation. Data are mean ± SD of three biological replicates.

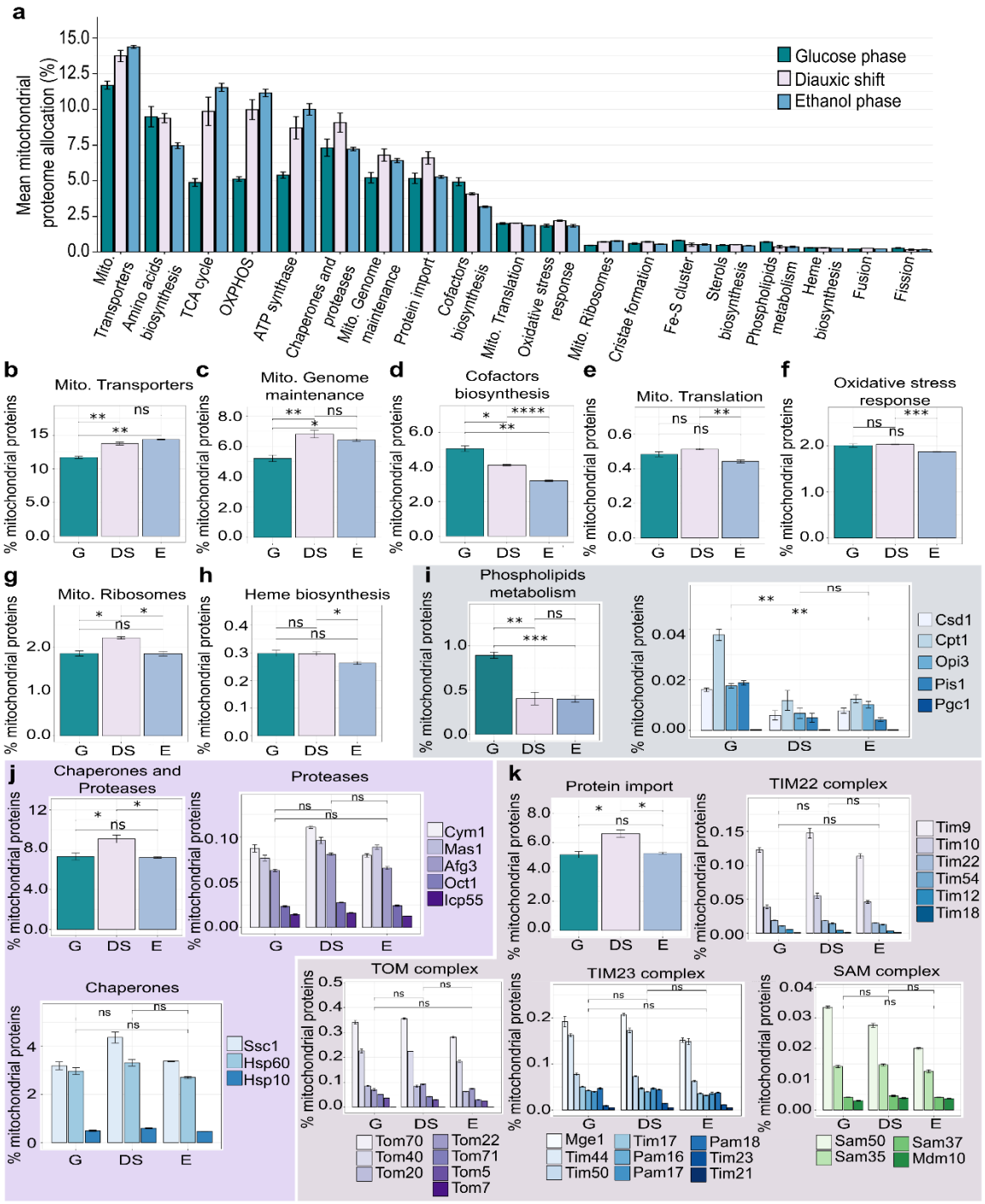

Fig. S5

Overall mitochondria proteome allocation and focus on the nine functional groups and details on the phospholipid metabolism and protein import machinery

a, Allocation of the whole mitochondrial proteome in the three stages of growth, calculated as the mean percentage allocation. Data are mean ± SD of three biological replicates. The specific identifications are reported in Dataset S7.

b-h, Allocation of selected mitochondrial processes. Data are mean ± SD of three biological replicates. Statistical analyses were performed using paired *t*-test.

i, Allocation of mitochondrial proteins involved in the phospholipid's metabolism with a focus on the enzymes Cds1, Cpt1, Opi3, Pis1 and Pgc1 involved in the biosynthesis of the bilayer-forming phospholipids PC, PI and PG. Data are mean ± SD of three biological replicates. Statistical analyses were performed using paired *t*-test.
j, Allocation of mitochondrial chaperones and proteases involved in the mechanisms of protein import with focus on the proteases Cym1, Mas1, Afg3, Oct1 and Icp55 and the chaperones Ssc1, Hsp60 and Hsp10. Data are mean ± SD of three biological replicates. Statistical analyses were performed using paired *t*-test.
k, Allocation of mitochondrial proteins involved in the mitochondrial import machinery with a focus on the protein forming the SAM, TIM23, TIM22 and TOM complexes. Data are mean ± SD of three biological replicates. Statistical analyses were performed using paired *t*-test.
Statistically significant differences are indicated as follows: ns $p<1$; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.
G, glucose phase; DS, diauxic shift; E, ethanol phase.
PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

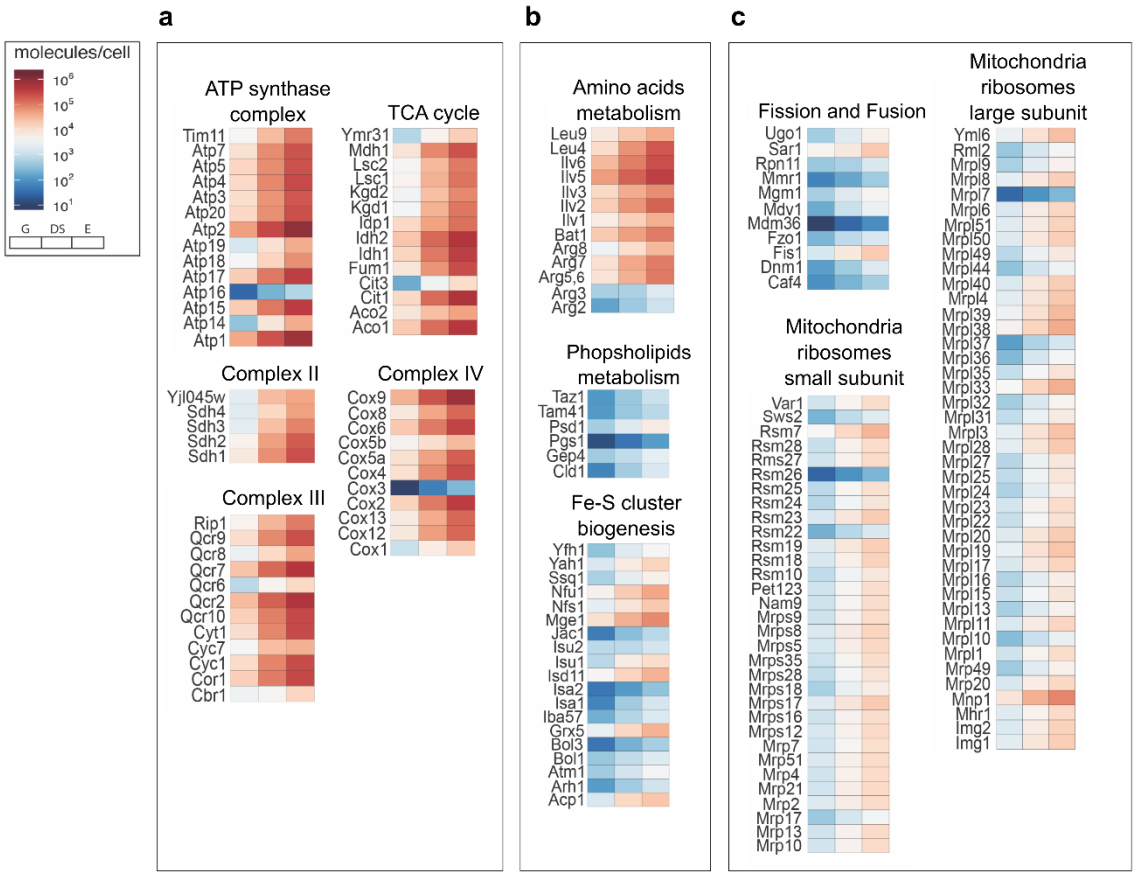

Fig. S6

Absolute quantification of the growth stage-dependent mitochondrial proteome

Absolute copy numbers of proteins in selected mitochondrial processes in the three stages of growth. Colours indicate the mean absolute copy number per cell and growth stage (n = 3).

a, Proteins involved in the respiratory chain, oxidative phosphorylation complexes and the TCA cycle.

b, Biosynthetic processes. **c**, Processes involved in mitochondrial biogenesis.

G, glucose phase; DS, diauxic shift; E, ethanol phase; TCA, tricarboxylic acid cycle; Fe-S, iron-sulfur

Table S1

Yeast strains used in this study

Organism	Description	Source
Saccharomyces cerevisiae CEN.PK113-7D	MATa, *URA3, HIS3, LEU2, TRP1*, MAL2-8c, *SUC2*	Euroscarf
Saccharomyces cerevisiae CEN.PK113-7D MitoLoc	MATa, *URA3, HIS3, LEU2, TRP1*, MAL2-8c, *SUC2* preSu9-GFP, preCox4-mCherry	This study

Table S2

Plasmids used in this study for the generation of the strain *Saccharomyces cerevisiae* CEN.PK113-7D MitoLoc

Organism	Description	Source
pMitoLoc	2µ vector with preSu9-GFP and preCox4-mCherry fluorescent markers for colocalization analysis of yeast mitochondria, with nourseothricin (NAT) resistance marker	Vowinckel *et al.,* (2015) (8)
pCfB3035	2µ X-4 EasyClone-Marker Free backbone vector for genomic insertion of 1-2 genes and promoters into chromosome X-site 4 (236336..237310)	Jessop-Fabre *et al.,* (2016) (1)
pCfB3035 MitoLoc	pCfB3035 preSu9-GFP, preCox4-mCherry	This study
pCfB2312	2µ vector with TEF1-promoter-driven Cas9 protein and CYC terminator with kanMX resistance marker	Jessop-Fabre *et al.,* (2016) (1)
pCfB3042	2µ EasyClone-MarkerFree guiding RNA (gRNA) vector for directing Cas9 to cut at site X-4	Jessop-Fabre *et al.,* (2016) (1)

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Dataset S1 (data provided in a separate file)
Absolute mitochondrial proteome for the three biological replicate samples during the glucose phase, diauxic shift and ethanol phase.

Dataset S2 (data provided in a separate file)
Absolute cellular proteome for the three biological replicate samples during the glucose phase, diauxic shift and ethanol phase.

Dataset S3 (data provided in a separate file)
List of p-values and log2 fold-changes (log2FC) calculated for the absolute mitochondrial proteome used in the generation of the Volcano plots (Figure3 a-c) and to identify significant changes in protein abundance.

Dataset S4 (data provided in a separate file)
Differentially expressed proteins in the three metabolic phases of yeast growth graphically showed in the Venn diagram (Figure 3 d-e).

Dataset S5 (data provided in a separate file)
GO term enrichment for significantly regulated mitochondrial proteins.

Dataset S6 (data provided in a separate file)
GO term groups associated with each cellular protein.

Dataset S7 (data provided in a separate file)
GO term groups associated with each mitochondrial protein.