Supplemental Materials

Molecular Biology of the Cell

Bruderek et al.

Table S1: *Plasmids used in this study*

Plasmid	Description	Reference
pBG8099	pYEDP1/8-2 harboring $cytb_2(84)_{\Delta}$-GFP under Gal promotor	Bernard Guiard
pMB028	pYEDP1/8-2 harboring $cytb_2(84)_{\Delta}$-GFP-$DHFR_{ds}$ under Gal promotor	This study
pMB029	pYEDP1/8-2 harboring GFP-$DHFR_{ds}$ under Gal promotor	This study
pMB031	removed insert in pYEDP1/8-2 (empty vector)	This study
pMB032	pYEDP1/8-2 harboring $cytb_2(84)_{\Delta}$-GFP-DHFR under Gal promotor	This study
pMB034	pUG35 harboring $cytb_2(167)_{\Delta}$-mCherry under Met_{25} promotor	This study
pTB10	pRS315 encoding *HSP78* under own promoter	This study
pRS314-*NUP120*-mCherry	pRS314 harboring Nup120-mCherry	(Skruzny *et al.*, 2009)
pWJ09	pRS314 encoding *FIS1* under own promoter	This study

Table S2: *Yeast strains used in this study*

Strain	Genotype	Source
WVY49 (WT)	MATα □□□□□□□□ lys2 ura3-52 leu2-3 112 trp1	Wolfgang Voos
BY4741	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0	Euroscarf
BY4741 rho-	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0	Euroscarf
ssc1-3	MATα □□□□□□□□ lys2 ura3-52 leu2-3 112 trp1 ssc1-3 (LEU2)	(Gambill *et al.*, 1993)
mdj1Δ	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0 mdj1Δ::KanMX	Euroscarf
hsp78Δ	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0 hsp78Δ::KanMX	Euroscarf
fzo1Δ	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0 fzo1Δ::KanMX	Euroscarf
fis1Δ	MATa his3Δ1 leu2Δ0, met15Δ0 ura3Δ0 fis1Δ::KanMX	Euroscarf

Michael Bruderek *et al.*,
"IMiQ: a novel protein quality control compartment protecting mitochondrial functional integrity"

Supplemental experimental procedures

Immunofluorescence microscopy

Cells were fixed in selective medium with 3% glycerol and 12 % formaldehyde. Cells were resuspended in paraformaldehyde buffer (100mM KH_2PO_4, 4 % paraformaldehyde, 45 mM NaOH) and incubated over night. Cell walls were digested by spheroplasts solution (1.2 M sorbitol, 50 mM KP_i, pH 7.4, 1 mM $MgCl_2$, and 3 mg/ml zymolyase 20T) and lysed by 5 % SDS. Fixed cells were settled on microscope slides and incubated with primary antibodies over night. Finally, cells were incubated with secondary antibodies (Cy3 a-rabbit IgG and Alexa 488 a-mouse IgG, Thermo Scientific, DE) and embedded by Roti-Mount FluoreCare DAPI (Roth, DE).

Import and degradation of radiolabeled preproteins in isolated mitochondria

Mitochondria were isolated from *S. cerevisiae* cells by standard procedures (Diekert *et al.*, 2001). Preprotein import into isolated mitochondria was performed as published (Ryan *et al.*, 2001). In brief, radiolabeled preprotein Su9(70)-DHFR was synthesized by *in vitro* transcription and translation in presence of [^{35}S]-methionine/cysteine using rabbit reticulocyte lysate (Promega, DE). For import reactions, isolated mitochondria were incubated in import buffer (250 mM sucrose, 10 mM MOPS/KOH pH 7.2, 80 mM KCl, 5 mM $MgCl_2$, 3% w/v BSA, 2 mM ATP, 2 mM NADH, 50 µM MnCl, 10 µM ZnCl) in presence or absence of a mitochondrial membrane potential ($\Delta\psi$). Import was triggered by addition of radiolabeled preprotein and incubation at 30 °C. After stopping the reaction on ice, non-imported preproteins were digested by proteinase K (PK) treatment. Subsequently, mitochondria were reisolated and analyzed by SDS-PAGE and digital autoradiography.

Mitochondrial membrane potential measurement

After expression of aggregate reporter proteins for 10 h as described above, 20 OD_{600} units of cells were collected, resuspended in Teβ-buffer (200 mM Tris/HCl pH 8, 20 mM EDTA pH 8, β-mercaptoethanol) and incubated for 10 min at 30 °C. Cells were resuspended in spheroblast solution (1.2 M sorbitol, 50 mM KP_i pH 7.2, 1 mM $MgCl_2$) containing 3 mg/ml zymolyase and incubated for 30 min at 30 °C. Spheroblasts were washed twice with 1.2 M sorbitol and resuspended in storage buffer (250 mM sucrose, 10 mM MOPS/KOH pH 7.2, 80 mM KCl, 5 mM $MgCl_2$, 3% w/v BSA, 2 mM ATP, 2 mM NADH). For measurements, potential buffer (0.6 M sorbitol, 20 mM KP_i pH 7.2, 0.1% w/v BSA, 0.5 mM EDTA, pH 8) containing 5 mM L-malate and 5 mM L-glutamate was added. Potential-dependent fluorescence was measured by addition of 0.25 µM 3,3'-Dipropylthiadicarbocyanine iodide ($DiSC_3$). The mitochondrial potential was depleted by addition of 20 mM sodium azide causing a recovery of $DiSC_3$ fluorescence, used as experimental read out.

2-D PAGE

For 2D PAGE analysis of co-sedimentation upon mtGFP-$DHFR_{ds}$ expression, 1 mg mitochondria were resuspended in lysis buffer (0.5% Triton X-100, 50 mM KP_i pH 7.2, 10 mM DTT, 1 mM EDTA, 1x protease inhibitor (Roth, DE), 1 mM PMSF) and lysed by pipetting (20x) and shaking for 10 min at 4 °C. Lysed mitochondria were centrifuged at high speed (20000 xg) at 4°C and pellet was washed in wash buffer + triton X-100 (0.5% triton X-100, 20 mM KP_i pH 7.2, 1 mM PMSF) and subsequently washed again in wash buffer (20 mM KP_i pH 7.2, 1 mM PMSF). After reisolation, pellet was resuspended in rehydration buffer (7 M urea, 2 M thio-urea, 2% w/v CHAPS, 20 mM DTT, 0.8% v/v Pharmalyte (Biolytes) 3-10 NL, small amount of Bromophenol Blue) and was shaken vigorously at 20 °C for 10 min. For first-dimension IEF, sample was applied to dry 18 cm non-linear, pH 3 to 11 immobiline strips (GE Healthcare, UK), respectively, for overnight in-gel rehydration at 20°C in the dark in an IPGphor isoelectric focusing system (GE Healthcare, UK). Subsequently to re-swelling,

protein focusing was performed in stepwise increase of voltage (1 h 200 V, 1 h 500 V and 1 h 1000 V). Finally, the voltage was increased to 8000 V (slow increase over 2 h and 30 min) for 3 h and subsequently decreased to 500 V for 2 h and 30 min. Focused strip was equilibrated and reduced stepwise for 10 min, respectively in equilibration buffer (6 M urea, 1.5 mM Tris/HCl pH 8.8, 50 % v/v glycerol, 1 % w/v SDS) containing either 1 % w/v DTT or 2.5 % w/v iodoacetamide in the dark. Proteins were separated by SDS-PAGE on 11 % polyacrylamide gel at 0.5 W per gel for 1 hour and subsequently at 1.5 W per gel over night at 20 °C in an Ettan™ DALTsix system (GE Healthcare, UK). Protein detection was performed by gel fixation using fixation solution (40 % v/v methanol, 10 % v/v acetic acid) for 2 hours following staining using Coomassie Blue G250 (40 % v/v methanol, 10 % v/v acetic acid, 0.5 % w/v Coomassie Blue G250).

Peptide preparation and LC-MS

Gel slices were excised and subjected to tryptic in gel digestion (Rosenfeld *et al.*, 1992). In brief, slices were washed consecutively with water, 50 % acetonitrile (ACN), and 100 % ACN. Proteins were reduced with 20 mM DTT in 50 mM ammonium bicarbonate and alkylated with 40 mM iodoacetamide (in 50 mM bicarbonate). The slices were washed again and dehydrated with ACN. Dried slices were incubated with 400 ng sequencing grade trypsin at 37 °C overnight. The peptide extract was separated and remaining peptides extracted with 50 % ACN. Peptides were dried in a vacuum concentrator and stored at -20 °C.

Peptides were dissolved in 8 µl 0.1 % trifluoroacetic acid. 1 to 2 µl were injected onto a C18 trap column (20 mm length, 100 µm inner diameter) coupled to a C18 analytical column (200 mm length, 75 µm inner diameter), made in house with 3 µm ReproSil-Pur 120 C18-AQ particles (Dr. Maisch, Ammerbuch, DE). Solvent A was 0.1 % formic acid. Peptides were separated during a linear gradient from 0 % to 35% solvent B (90% ACN, 0.1% formic acid) within 30 or 45 min at a flow rate of 350 nl/min. The nanoHPLC was coupled online to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, DE) or an HCT Ultra ion trap mass spectrometer (Bruker Daltonik, DE). For Orbitrap analyses peptide ions between 330 and 1700 m/z were scanned in the Orbitrap detector with a resolution of 30,000 (maximum fill time 400 ms, AGC target 10^6). The 25 most intense precursor ions (threshold intensity 5000) were subjected to collision induced dissociation and fragments analyzed in the linear ion trap. Fragmented peptide ions were excluded from repeat analysis for 15 s. Raw data processing and analysis of database searches were performed with Proteome Discoverer software 1.4.1.12 (Thermo Fisher Scientific). Peptide identification was done with an in house Mascot server version 2.4.1 (Matrix Science Ltd, London, UK). MS2 data were searched against *S. cerevisiae* and human sequences from SwissProt (release 2013_03). Precursor Ion m/z tolerance was 10 ppm, fragment ion tolerance 0.6 Da. Tryptic peptides were searched with up to two missed cleavages. Low scoring spectrum matches were searched again with semitryptic specificity with up to one missed cleavage. Carbamidomethylation was set as a static modification (Cys). Oxidation (Met) and acetylation (protein N-terminus) were set as dynamic modifications. Mascot results from searches against SwissProt were sent to the percolator algorithm (Käll *et al.*, 2008) version 2.04 as implemented in Proteome Discoverer. Only proteins with two peptides (maximum posterior error probability 1 %) were considered and identified. HCT Ultra ion trap_ENREF_4 data were processed in ESI Compass 1.3 DataAnalysis Version 4.0 (Bruker). Mascot server searches were started from ProteinScape 3.0 against *S. cerevisiae* and human sequences from SwissProt with precursor and fragment ion tolerances of 0.6 Da and modification settings as above.

Supplemental references

Diekert, K., de Kroon, A.I., Kispal, G., and Lill, R. (2001). Isolation and subfractionation of mitochondria from the yeast Saccharomyces cerevisiae. Methods Cell Biol. *65*, 37-51.

Käll, L., Storey, J.D., MacCoss, M.J., and Noble, W.S. (2008). Assigning significance to peptides identified by tandem mass spectrometry using decoy databases. J. Proteome Res. *7*, 29-34.

Rosenfeld, J., Capdevielle, J., Guillemot, J.C., and Ferrara, P. (1992). In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem. *203*, 173-179.

Ryan, M.T., Voos, W., and Pfanner, N. (2001). Assaying protein import into mitochondria. Methods Cell Biol. *65*, 189-215.

Supplemental figure legends

Figure S1. Immunofluorescence analysis of mitochondrial protein aggregation
A) Immunofluorescence of wt yeast cells expressing the aggregation reporter proteins *mt*GFP-DHFR and *mt*GFP-DHFR$_{ds}$ as indicated after 5 h of induction with galactose. Cells were fixed and incubated with antisera against GFP (green) and Ssc1 (red) as described in "Supplemental experimental procedures". Nuclei were visualized by staining with DAPI (blue). IMiQ structures are indicated by white arrows. Scale bar: 5 µm.
B) Immunofluorescence of wt yeast cells upon incubation at 42 °C (hs) for indicated time points. Mitochondrial protein Atp2 (green) was detected by immunostaining and nuclei were visualized by staining with DAPI (blue). Scale bar: 10 µm.
C) Aggregation behavior of Atp2 in heat stressed cells. Cells were incubated at 42 °C (hs) for the indicated times. Total cell extracts (T), supernatants (S) and aggregate pellet fractions after 20,000 xg centrifugation (P, 2 x amounts) were applied to SDS-PAGE and analyzed by Western blot using antibodies against Atp2. Mdh1 was used as soluble control protein of the mitochondrial matrix.

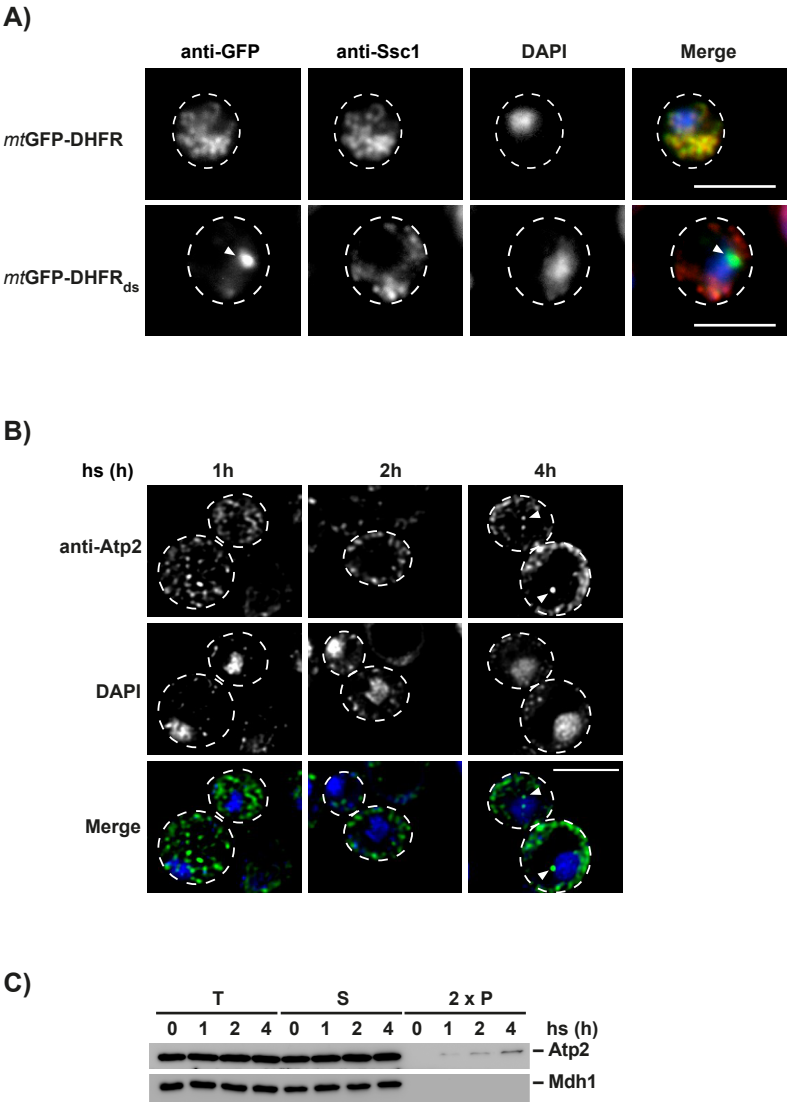
Figure S2. Aggregation behavior of destabilized reporter proteins
A) Expression of destabilized reporter and control proteins for the indicated times. Protein levels were analyzed by Western blot and immunodecoration with antibodies against GFP. The cytosolic protein Pgk1 was detected as loading control.
B) Differential centrifugation of wild type yeast cells expressing destabilized reporter protein or control proteins. Cells expressing the indicated reporter proteins for 10 h were lysed and pellet fractions (3x amounts) after centrifugation at 2,000 xg (P_2), 20,000 xg (P_{20}), and 125,000 xg (P_{125}) were analyzed by SDS-PAGE and Western blot as described in Fig. 2B. Total protein content is indicated by the respective Coomassie-stained blot membrane sections. (T, total cell extract; S, supernatant of 125,000 xg centrifugation).
C) Co-aggregation of endogenous proteins within the mitochondrial matrix. Cells expressing the indicated reporter proteins for 10 h were lysed and centrifuged at 20,000xg. Supernatant (S) and pellet fractions (P, 3x amounts) were analyzed by SDS-PAGE and Western blot using antibodies against the indicated proteins.
D) Co-aggregation of mitochondrial PQC system components. Wild type yeast cells expressing reporter proteins for 24 h were lysed and centrifuged at 20,000 xg. Total cell extracts (T), supernatants (S) and pellet fractions (P, 3x amounts) were analyzed by Western blot using antibodies against GFP and the indicated mitochondrial PQC components.

Figure S3. 2D-PAGE analysis of co-sedimenting mitochondrial proteins in presence of *mt*GFP-DHFR$_{ds}$.
Mitochondria isolated from wild type yeast cells expressing the destabilized reporter protein *mt*GFP-DHFR$_{ds}$ were lysed and centrifuged at 20,000 xg. The pellet fraction was analyzed by 2D-PAGE as described in Experimental procedures. Proteins in a range between 20-140 kDa and pI 3-11 are shown. Protein spots identified by LC-MS are listed. Indicated are protein name, subcellular or submitochondrial localization (according to SGD; Saccharomyces genome database), molecular weight and pI of the mature protein, number

of peptides found in LC-MS, and functional description. * Molecular weight and pI of full-length protein (transit peptide not known).

Figure S4. Mitochondrial quality and functionality are maintained in cells containing IMiQ aggregate deposits.
A) Determination of mitochondrial membrane potential ($\Delta\psi$). Spheroblasts were prepared from cells expressing destabilized or control proteins for 10 h and analyzed using $DiSC_3$ fluorescence as described.
B) *In vitro* import of cytosolic precursor proteins. Radiolabeled Su9(70)-DHFR was imported for indicated time points into isolated and energized mitochondria containing *mt*GFP-$DHFR_{ds}$ or control proteins (10 h expression) as described. Where indicated, the mitochondrial membrane potential was abolished by addition of 8 µM antimycin A, 0.5 µM valinomycin and 2 µM oligomycin ($-\Delta\psi$). Import reactions were analyzed by SDS-PAGE and digital autoradiography.
C) Analysis of mitochondrial protein complex integrity in isolated mitochondria containing destabilized reporter proteins. Mitochondria isolated from yeast cells were lysed by 0.05% digitonin and analyzed either by 10 % or 12% BN-PAGE and Western blot. Protein components of mitochondrial complexes located in the outer membrane (Tom40; TOM translocase complex), inner membrane (Cox4; respiratory complex IV) or mitochondrial matrix (Atp2; F-ATPase complex and Kgd1; ketoglutarate dehydrogenase complex) were detected by the respective antibodies. As loading controls, samples of lysed isolated mitochondria were applied to SDS-Page and proteins were detected by Western blot.

Figure S5. Phenotype complementation of yeast mutant cells.
A) Growth rates of *fis1*Δ yeast cells expressing the destabilized *mt*GFP-$DHFR_{ds}$ fusion protein. Control strains were transfected with single copy plasmid pWJ09 that encoded the full length *FIS1* gene under its own promoter. After galactose induction for 10 h, cells from the indicated mutant strains were spotted on selective plates containing fermentable (2 % glucose) or non-fermentable carbon source (3 % glycerol) and incubated at 30 or 37 °C to assess mitochondrial function.
B) Growth rates of *hsp78*Δ yeast cells expressing the destabilized *mt*GFP-$DHFR_{ds}$ fusion protein. Control strains were transfected with single copy plasmid pTB10 that encoded the full length *HSP78* gene under its own promoter. After galactose induction for 10 h, cells from the indicated mutant strains were subjected to a short heat shock and recovery as indicated in Fig. 6C. Cells were then incubated at a lethal temperature of 50 °C for 1 h and spotted on selective plates containing 2 % glucose.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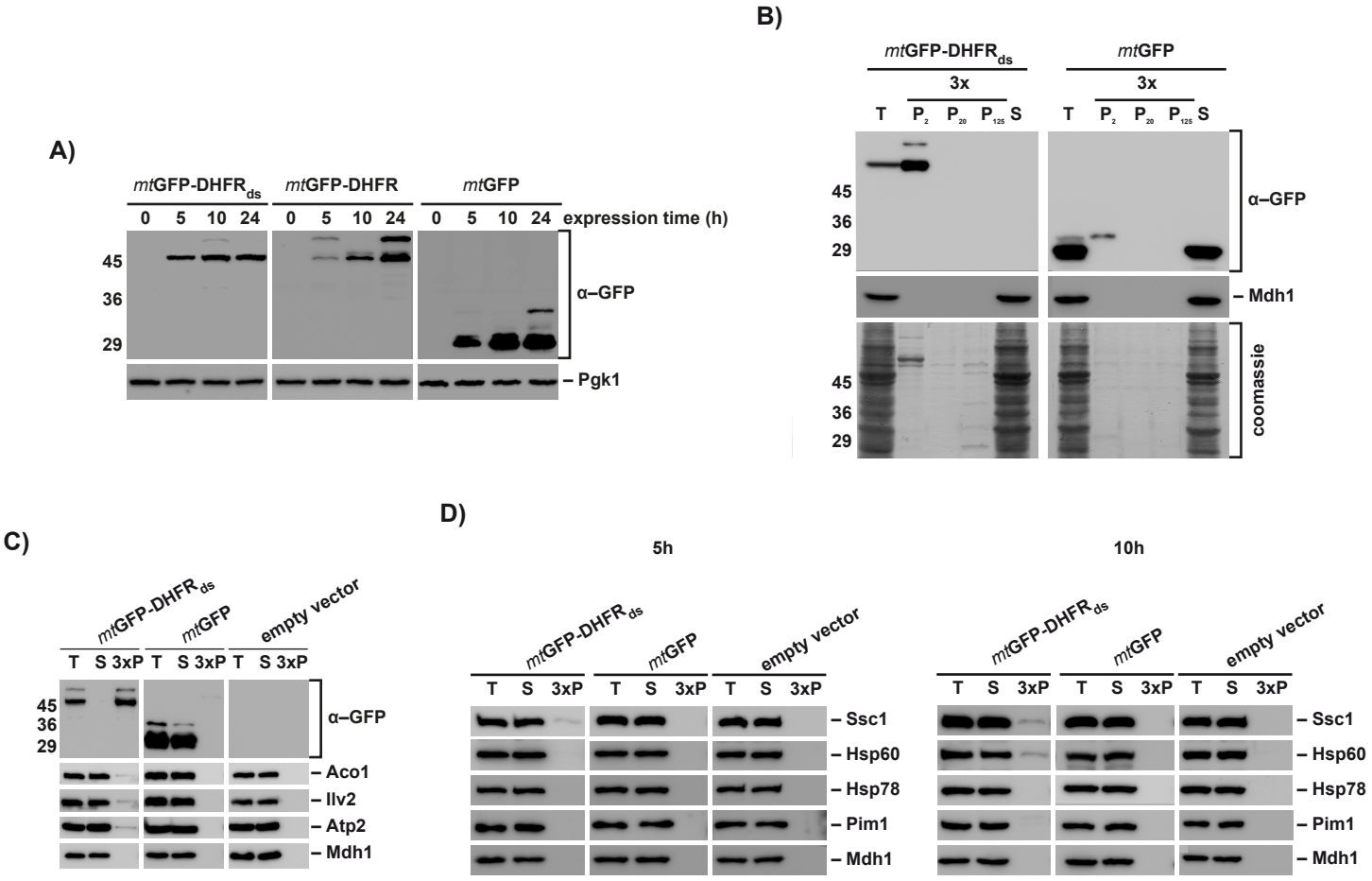
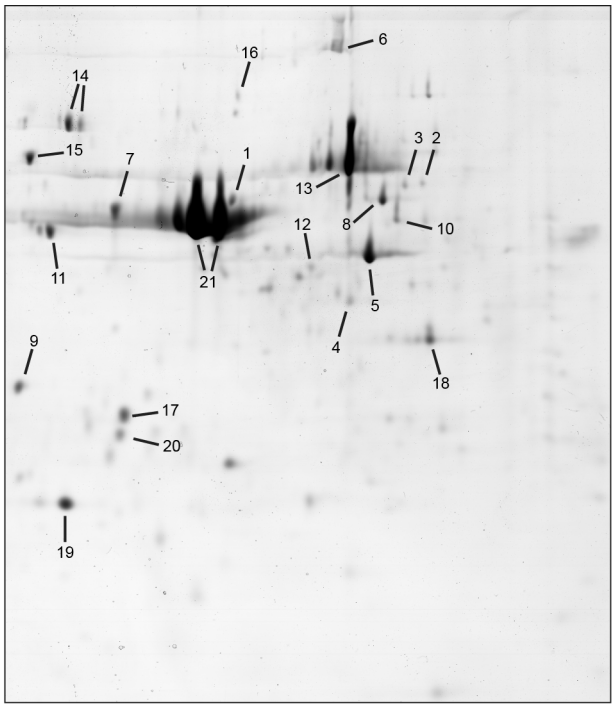
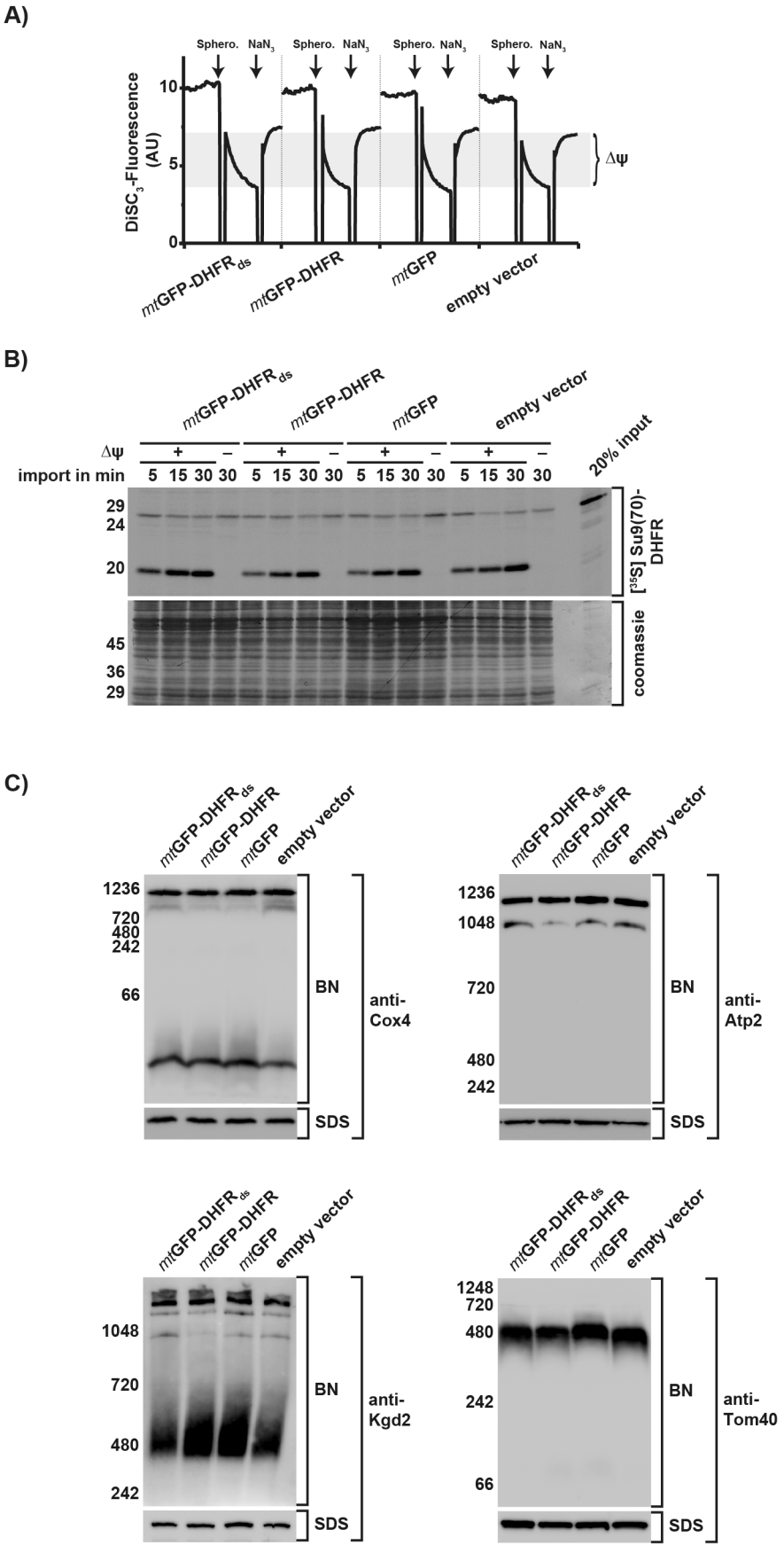


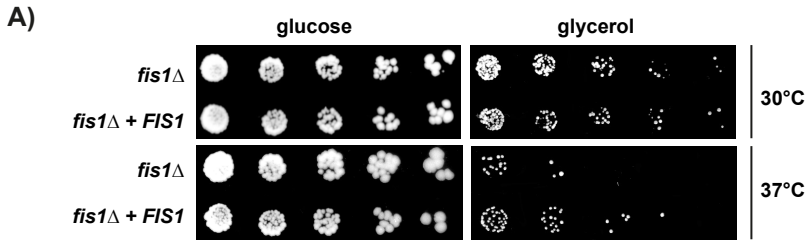
Bruderek *et al.*, Supplm. Fig. 2

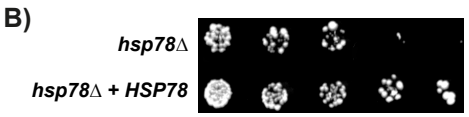


Number	Name	Description [a]	Localisation [b]	MW [kDa] [c]	pI [d]
1	Ald4	Amino acid biosynthesis	Matrix	53,9	5.9
2	Ilv1	Amino acid biosynthesis	Matrix	63,8 *	9,3 *
3	Ilv3	Amino acid biosynthesis	Matrix	62,8 *	8,8 *
4	Ilv5	Amino acid biosynthesis	Matrix	39,1	6,7
5	Cit1	TCA cycle	Matrix	49,1	7,0
6	Kgd1	TCA cycle	Matrix	110,9	6,8
7	Kgd2	TCA cycle	Matrix	50,4 *	9,4 *
8	Lpd1	TCA cycle	Matrix	51,5	7,0
9	Pdb1	TCA cycle	Matrix	36,4	4,5
10	Atp1	electron transport chain	Inner membrane	51,6	7,7
11	Atp2	electron transport chain	Inner membrane	51,0	4,8
12	Cor1	electron transport chain	Inner membrane	47,3	6,3
13	Cyb2	electron transport chain	Inner membrane space	56,5	6,5
14	SSC1	Mitochondrial chaperone	Matrix	68,0	5,0
15	Hsp60	Mitochondrial chaperone	Matrix	57,7	4,6
16	Mef1	Mitochondrial translation	Matrix	79,6	5,9
17	Pil1		Lipid droplet	38,3	4,4
18	Gal7		Cytosol	42,7	7,9
19	Hsp26		Cytosol	23,9	5,2
20	Lsp1		Cytosol	38,0	4,5
21	GFP			26,9	5,6



Bruderek *et al.*, Supplm. Fig. 4





Bruderek *et al.*, Supplm. Fig. 5

Sequence plasmid pMB028

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Sequence plasmid pMB029

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