Supplemental material

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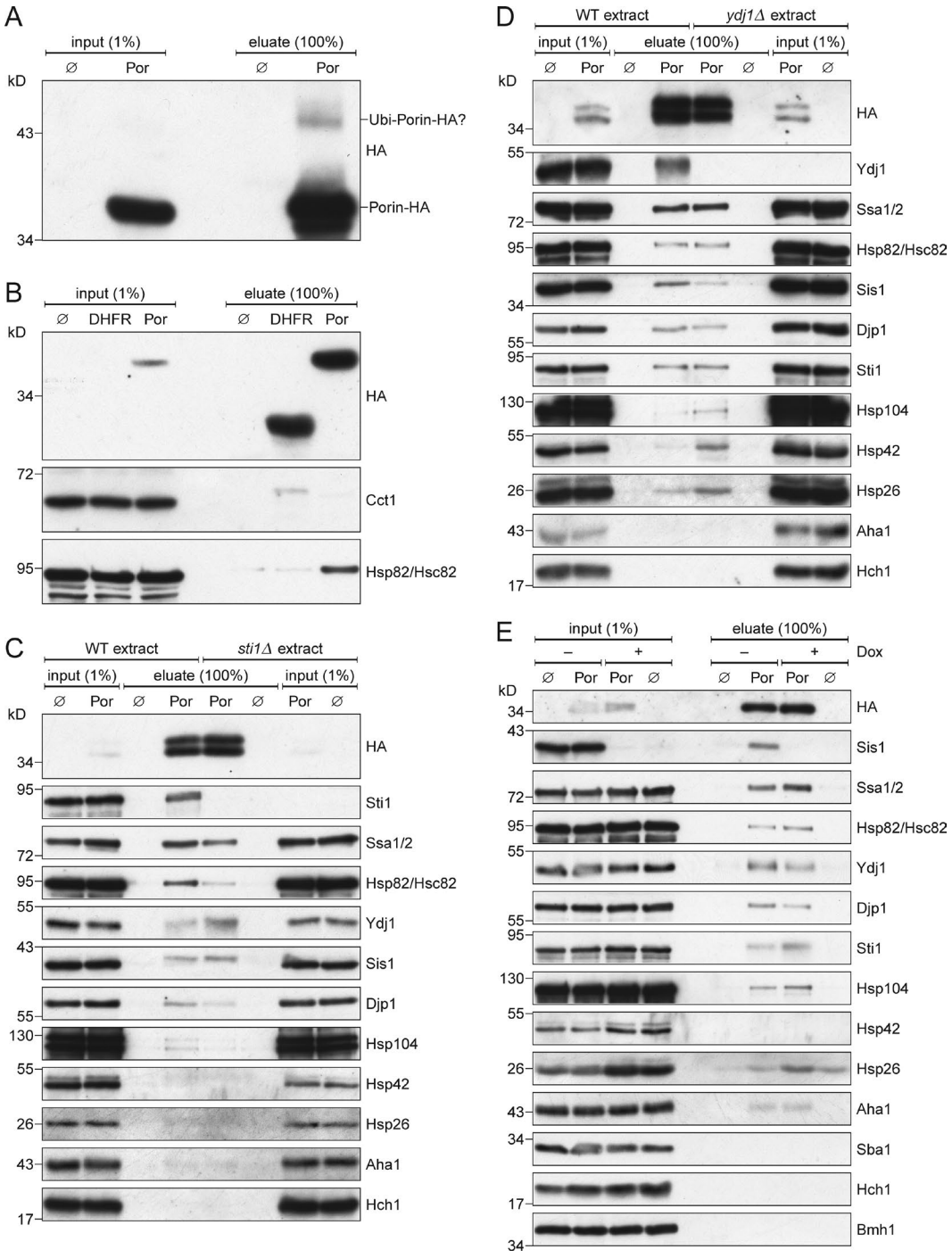

Figure S1. **The absence of a certain cochaperone affects the binding of β-barrel proteins to other (co)chaperones. (A and B)** In vitro translation reactions using WT yeast extracts without mRNA (Ø) or programmed with mRNA encoding DHFR-3HA (DHFR) or Porin-HA (Por) were subjected to a pull-down with anti-HA beads. Samples from the input and the eluate were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. A putative ubiquitinated species of Porin-HA is indicated. **(C and D)** In vitro translation reactions using yeast extracts prepared from WT, *sti1Δ* (C), or *ydj1Δ* (D) cells without mRNA (Ø) or programmed with mRNA encoding Porin-HA (Por) were subjected to a pull-down as in A. **(E)** Yeast extracts were prepared from cells that were left untreated (–Dox) or depleted for Sis1 by addition of doxycycline to the growth medium for 8 h (+Dox). The extracts were then used for in vitro translation followed by a pull-down assay as in A.

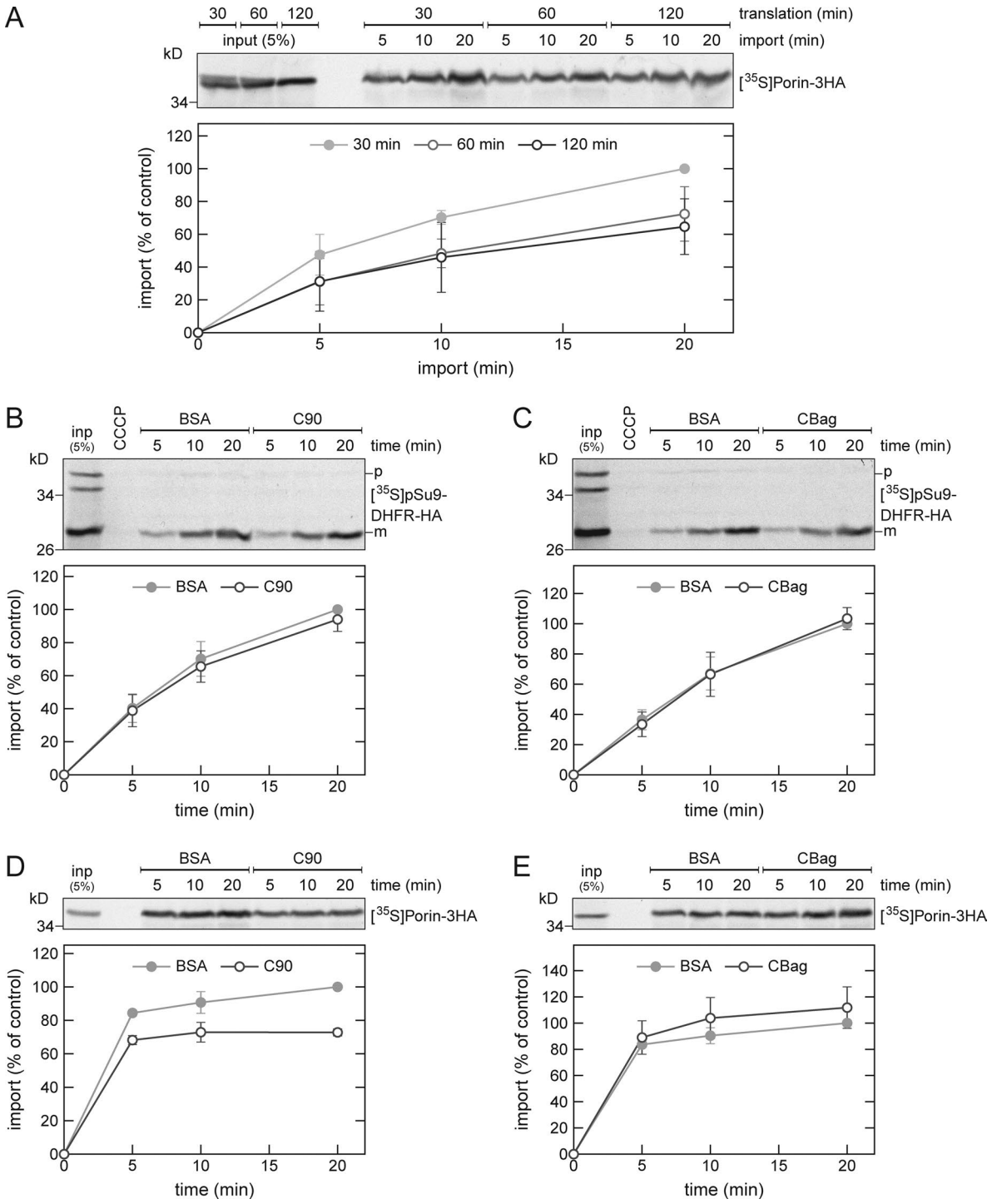

Figure S2. **The import of pSu9-DHFR-HA and urea-treated Porin-HA is not affected by chaperone inhibitors. (A)** Top, radiolabeled precursor molecules of Porin-HA were translated in yeast extract for the indicated times and were then used for in vitro import reactions using isolated mitochondria. After import for the indicated times, the mitochondria were subjected to carbonate extraction. The samples were analyzed by SDS-PAGE and autoradiography. Bottom, intensities of the bands corresponding to Porin-3HA from three independent experiments were quantified, and the mean intensity from the 20-min import of Porin-HA from the 30-min translation reaction was set to 100%. **(B–E)** Top, radiolabeled precursor molecules of pSu9-DHFR-HA (B and C) or urea-treated Porin-HA (D and E) were produced in yeast extract and were then subjected to in vitro import reactions using isolated mitochondria. Before the import reaction, the mitochondria were mixed with either 20 µM C90 or an equivalent amount of BSA (B and D) or the precursor protein–containing translation reactions were supplemented with either 20 µM CBag or an equivalent amount of BSA (C and E). After import for the indicated times, the mitochondria were treated with proteinase K (B and C) or were subjected to carbonate extraction (D and E). The samples were analyzed by SDS-PAGE and autoradiography. In a control reaction, the mitochondria were treated with the uncoupler CCCP before the import reaction (B and C). Bottom, intensities of the bands corresponding to Porin-3HA or the protease-protected, mature form (m) of pSu9-DHFR-HA from three independent experiments were quantified and the mean intensity from the 20 min import with BSA was set to 100%. Error bars represent ± SD. p and m, precursor and mature forms of pSu9-DHFR-HA, respectively.

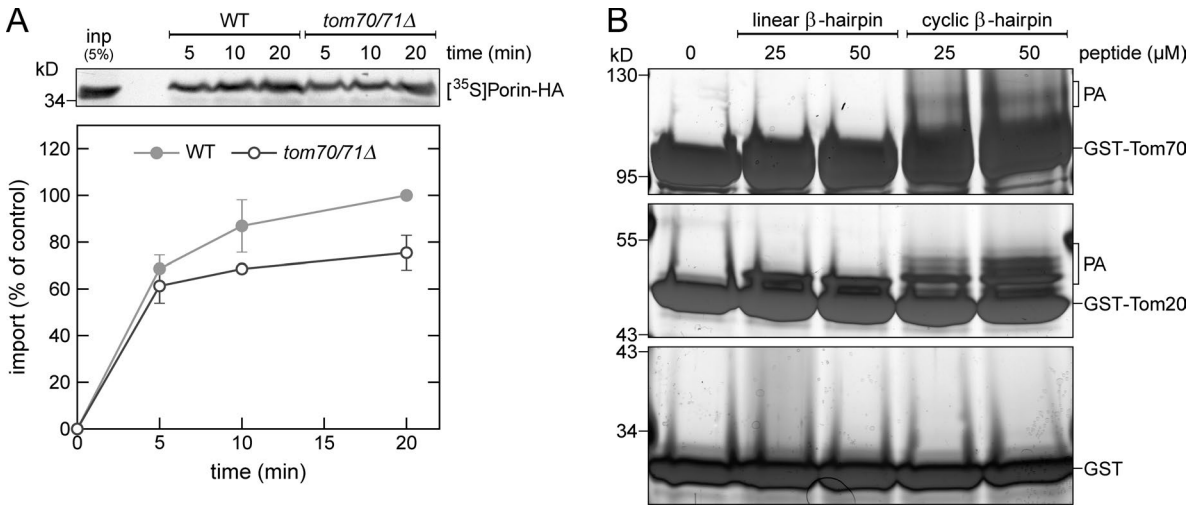

Figure S3. **The import receptor Tom70 is involved in the biogenesis of β-barrel proteins. (A)** Top, radiolabeled precursor molecules of Porin-HA were produced in yeast extract and subjected to in vitro import reactions using mitochondria isolated from a WT or a *tom70/71Δ* double deletion strain. After import for the indicated times, the mitochondria were subjected to carbonate extraction and analyzed by SDS-PAGE and autoradiography. Bottom, the intensities of the bands corresponding to Porin-3HA from three independent experiments were quantified, and the mean intensity from the 20-min import with WT mitochondria was set to 100%. Error bars represent ±SD. **(B)** The recombinant proteins GST-Tom70 (top), GST-Tom20 (middle), and GST (bottom) were incubated with a Bpa-containing linear or cyclic β-hairpin peptide at the indicated concentrations. The samples were illuminated with UV light before analysis by SDS-PAGE and silver staining. PAs are indicated.

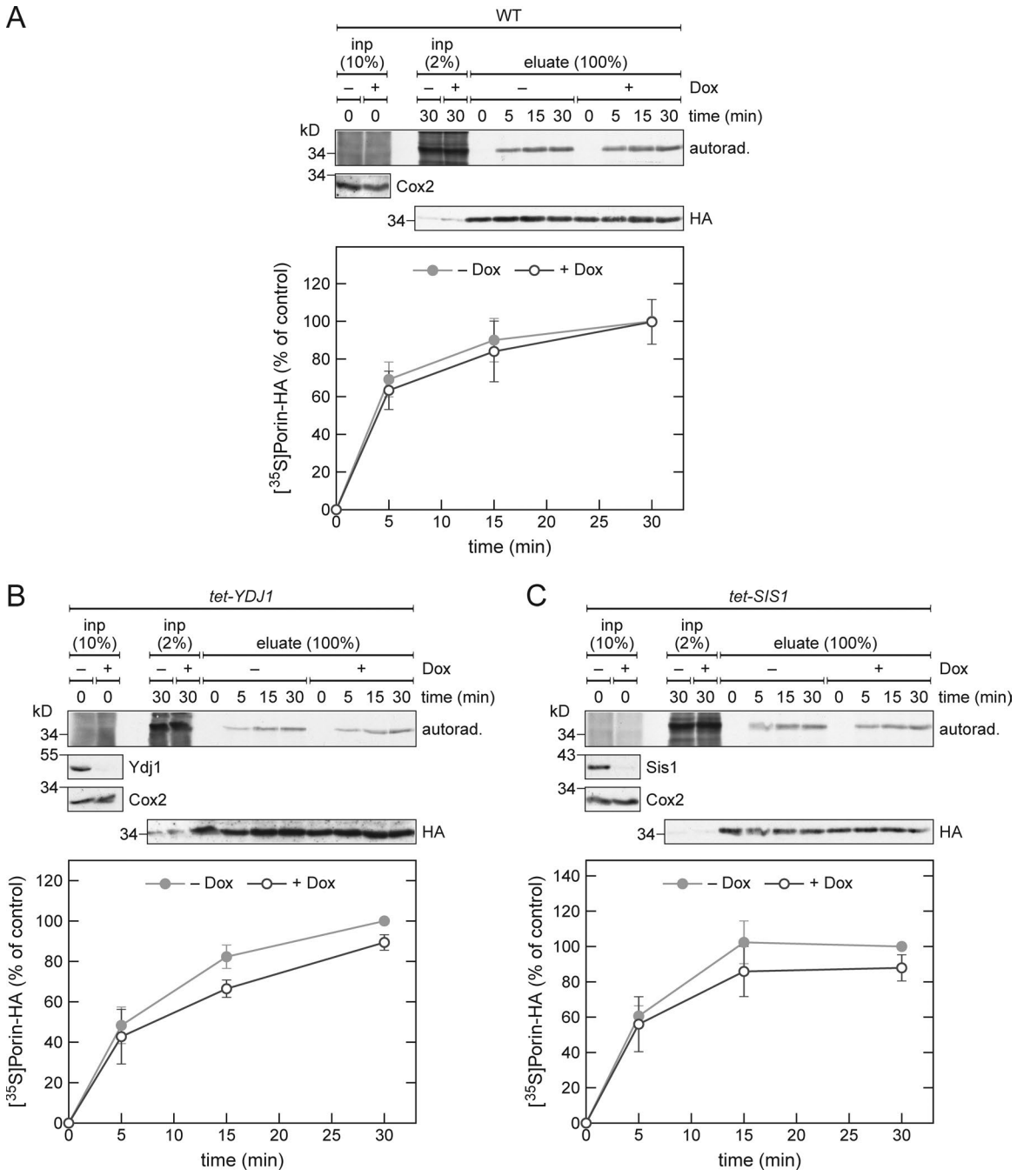

Figure S4. **The biogenesis of Porin-HA is not affected by doxycycline and only mildly reduced in the absence of Ydj1 or Sis1. (A–C)** Top, yeast cells harboring endogenously HA-tagged Porin from a WT strain (A) or from strains with a tetracycline-repressible promoter controlling the expression of *YDJ1* (B) or *SIS1* (C) were grown for 4 h in the absence (–Dox) or presence (+Dox) of doxycycline followed by 1 h of methionine starvation. Synthesis of radiolabeled proteins was initiated by addition of [^{35}S]Met to the medium, and cells were harvested after the indicated time periods. Then, a crude mitochondrial fraction was obtained, solubilized, and subjected to a pull-down with anti-HA beads. Input samples from the whole cell lysate (inp), and the eluates were analyzed by SDS-PAGE, autoradiography (autorad.), and immunodecoration with the indicated antibodies. Cox2 was used as a loading control. Bottom, intensities of the bands corresponding to Porin-HA from three independent experiments were quantified and the mean intensity from the 30-min samples without doxycycline was set to 100%. Error bars represent ±SD.

Table S1. **Yeast strains used in this study.**

Strain	Genotype	Reference
sti1Δ	W303α, *sti1Δ::HIS3MX6*	Hoseini et al. (2016)
ydj1Δ	BY4741, *ydj1Δ::KanMX4*	EUROSCARF
tom70/71Δ	W303α, *tom70Δ::KanMX4, tom71Δ::NatNT2*	This study
tetO$_7$-Sis1	YMK120a, *sis1::tetO$_7$-SIS1 NatMX*	This study
tetO$_7$-Ydj1/Sis1	YMK120α, *sis1::tetO$_7$-SIS1 NatMX, ydj1::tetO$_7$-YDJ1 KanMX*	This study
Porin-3HA tetO$_7$-Ubi-L-Ydj1	YMK120α, *por1::POR1-3HA NatNT2, ydj1::tetO$_7$-Ubiquitin-Leu-YDJ1 HIS3MX*	This study
Porin-3HA tetO$_7$-Ubi-L-Sis1	YMK120α, *por1::POR1-3HA NatNT2, sis1::tetO$_7$-Ubiquitin-Leu-SIS1 HIS3MX*	This study
Porin-3HA tetO$_7$-Ubi-L-Ydj1/Sis1	YMK120α, *por1::POR1-3HA NatNT2, ydj1::tetO$_7$-Ubiquitin-Leu-YDJ1 HIS3MX, sis1::tetO$_7$-Ubiquitin-Leu-SIS1 KanMX*	This study

Table S2. **Primers used in this study**

Construct	Primer	Sequence (5′–3′)
Porin-3HA	TJ207	AGTTGTCTGAACCTGTTCACAAGCTAGGTTGGTCTTTGTCCTTCGACGCTCTGAAGCTTTACCCATACGATGTTCCTG
	TJ208	CGAGCACATATATGGTATATAGTGAACATATATATATTAGATATATACGTGAGCTCGATTACAACAGGTGTTGTCC
tetO$_7$-Sis1	TJ196	GGATAAGTTGTTTGCATTTTAAGATTTTTTTTTTAATACATTCACATCAACAGTATAGCGACCAGCATTCACATACG
	TJ198	GCACTTGGAGATACTCCAAGTAAATCATAAAGTTTTGTCTCCTTGACCATAAGCTTATCGATACCGTCGATCCCC
tetO$_7$-Ydj1	TJ192	CATATCTTTTGATAGAACATAATTAAAAATTATCCAAACTGAATTCTACACAGTATAGCGACCAGCATTCACATACG
	TJ194	GCAGTTACTGGAACACCTAGAATATCGTAAAACTTAGTTTCTTTAACCATAAGCTTATCGATACCGTCGATCCCC
tetO$_7$-Ubi-L-Ydj1	TJ192	CATATCTTTTGATAGAACATAATTAAAAATTATCCAAACTGAATTCTACACAGTATAGCGACCAGCATTCACATACG
	TJ193	GTGGCAGTTACTGGAACACCTAGAATATCGTAAAACTTAGTTTCTTTAACCAAACCACCTCTCAATCTCAAGACCAAG
tetO$_7$-Ubi-L-Sis1	TJ196	GGATAAGTTGTTTGCATTTTAAGATTTTTTTTTTAATACATTCACATCAACAGTATAGCGACCAGCATTCACATACG
	TJ197	TTAGCACTTGGAGATACTCCAAGTAAATCATAAAGTTTTGTCTCCTTGACCAAACCACCTCTCAATCTCAAGACCAAG
tom70Δ	TJ326	GCAAGATTCGGAAGTGAAATTACAGCTCACATCTAGGTTCTCAATTGCCAGACATGGAGGCCCAGAATACCCTCC
	TJ327	TTACTTAGTTTTTGTCTTCTCCTAAAAGTTTTTAAGTTTATGTTTACTGTCAGTATAGCGACCAGCATTCACATAC
tom71Δ	TJ292	TTTTGTATATATCTCTACATACTTGTATATACCGAACATAAGAAGCTCTTGCCAGATCTGTTTAGCTTGCCTCG
	TJ293	TATCCAGTATTAACTAAAAGTATATATTTGACCAATACCTGACATATCTTGAGCTCGATTACAACAGGTGTTGTCC
pGEM4polyA	TJ154	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCGGCCGCCCGGTCTCCCTATAGTGAGTCGTATTAATTTC
	TJ155	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAGCTTGCATGCCTGCAGGTCGACTC
3HA	TJ024	CCC-GGATCCATACCCATACGATGTTCC
	TJ025	CCC-GTCGACTTAACCAGCGTAATCTGG
DHFR-3HA	TJ009	CCC-GGTACCATGGTTCGACCATTGAACTGC
	TJ010	CCC-GGATCCCCGTCTTTCTTCTCGTAGAC
yk DHFR-3HA	TJ144	CACAC-GGTACCAAAAAAATGTCTGTTCGACCATTGAACTGCATC
	TJ010	CCC-GGATCCCCGTCTTTCTTCTCGTAGAC
yk Porin-3HA	TJ149	CACAC-GGTACCAAAAAAATGTCTCCTCCAGTTTACAGC
	TJ150	CACACGGATCCCCAGCGTCGAAGGACAAAGAC
yk Tom40-3HA	TJ180	CACACGAGCTCAAAAAAATGTCTGCACCAACTCCATTAGC
	TJ181	CACACGGATCCCCCAATTGAGGAAGAGCTTGCAATG
yk Tob55(bbd)-3HA	TJ184	CACACGAGCTCAAAAAAATGTCTAAAACGTTTACAGCGAAGACAG
	TJ183	CACACGGATCCCCTAAAAATGCCAGACCAAGACCAAAC
yk NcTom40	TJ187	CACACGAGCTCAAAAAAATGGCTTCGTTTTCCACCG
	TJ188	CACACGGATCCTTAAAAGGGGATGTTGAGGGAC
yk Tom40	TJ180	CACACGAGCTCAAAAAAATGTCTGCACCAACTCCATTAGC
	TJ186	CACACGGATCCTTACAATTGAGGAAGAGCTTGCAATG
yk Om14	TJ300	CACACGAGCTCAAAAAAATGTCTGCAACTGCTAAACAC
	TJ301	CACACGGATCCCCTTTCTTGTCGTATCTGGAGTAG
yk Cyc3	TJ312	CACACGAGCTCAAAAAAATGGGTTGGTTTTGGGCAG
	TJ313	CACACGGATCCCCAGGGGCGGAGGACGAAG
yk Tim23	TJ302	CACACGAGCTCAAAAAAATGTCGTGGCTTTTTGGAG
	TJ303	CACACGGATCCCCTTTTTCAAGTAGTCTTTTCTTGACAC
yk Yah1	TJ314	CACACGAGCTCAAAAAAATGCTGAAAATTGTTACTCGGG
	TJ315	CACACGGATCCCCACTAAAATCGTTGTTATTAACGTTTC
yk pSu9-DHFR-3HA	TJ157	CACACGAATTCAAAAAAATGTCTGCCTCCACTCGTGTCCTC
	TJ158	CACACGGTACCACCAGCACCAGCACCGGAAGAGTAGGCGCGC
hp18(VDAC, Bpa258)	TJ090	GGTATTAAACTGTAGCTGTCAGCTCTTC
	TJ091	GAAGAGCTGACAGCTACAGTTTAATACC
hp18(VDAC)-DHFR-3HA	TJ014	CCCGAATTCATGAAGCCAGGTATTAAACTGAC

Table S2. **Primers used in this study *(Continued)***

Construct	Primer	Sequence (5′–3′)
	TJ010	CCCGGATCCCCGTCTTTCTTCTCGTAGAC
mk DHFR-3HA	TJ141	CACACGGTACCGCCACCATGGTTCGACCATTGAACTGC
	TJ010	CCCGGATCCCCGTCTTTCTTCTCGTAGAC
mk Porin-3HA	TJ148	CACACGGTACCGCCACCATGGCTTCTCCTCCAGTTTACAGCG
	TJ150	CACACGGATCCCCAGCGTCGAAGGACAAAGAC
mk VDAC1-3HA	TJ151	CACACGGTACCGCCACCATGGCTGTGCCACCCACG
	TJ153	CACACGGATCCCCTGCTTGAAATTCCAGTCCTAGAC
3HA NatNT2	TJ205	CACACAAGCTTTACCCATACGATGTTCCTGACTATG
	TJ206	CACACGGATCCCTTCGAGCGTCCCAAAACCTTC
pMK632	TJ203	GGAGGGTATTCTGGGCCTCCATGTC
	TJ204	GTATGTGAATGCTGGTCGCTATACTG
KanMX/HIS3MX	TJ201	GACATGGAGGCCCAGAATACCCTCC
	TJ202	CAGTATAGCGACCAGCATTCACATAC

Table S3. **Plasmids used in this study**

Plasmid	Insert	Reference
pGEM4polyA	Poly-A stretch (72 × A)	This study
pRS316-Atg32-3HAn	Atg32 with internal 3 × HA-tag	Okamoto et al. (2009)
pGEM4polyA-3HA	C-terminal 3 × HA-tag	This study
pRS426-TPI-3HA	C-terminal 3 × HA-tag	This study
pGEM4-pSu9-DHFR	Presequence of *N. crassa* ATP synthase subunit 9 (aa M1-S69)-*M. musculus* DHFR	Pfanner et al. (1987)
pGEM4-Tom40	Tom40	Paschen et al. (2003)
pGEM4-Tob55	Tob55	Paschen et al. (2003)
pGEM4-NcTom40	*N. crassa* Tom40	Rapaport and Neupert (1999)
pGEM4-VDAC1	*H. sapiens* VDAC1	Engl et al. (2012)
pGEM4-hTom40	*H. sapiens* Tom40	Engl et al. (2012)
pGEM4polyA-DHFR-3HA	*M. musculus* DHFR-3 × HA-tag	This study
pGEM4polyA-yk-DHFR-3HA	Yeast kozak sequence (AAAAAAATGTCT) *M. musculus* DHFR-3 × HA-tag	This study
pGEM4polyA-yk-Porin-3HA	Yeast kozak sequence (AAAAAAATGTCT) Porin-3 × HA-tag	This study
pGEM4polyA-yk-Tom40-3HA	Yeast kozak sequence (AAAAAAATGTCT) Tom40-3 × HA-tag	This study
pGEM4polyA-yk-Tob55(Δ1-120)-3HA	Yeast kozak sequence (AAAAAAATGTCT) Tob55 β-barrel domain (aa K121-end)-3 × HA-tag	This study
pGEM4polyA-yk-NcTom40	Yeast kozak sequence (AAAAAAATGGCT) *N. crassa* Tom40	This study
pGEM4polyA-yk-Tom40	Yeast kozak sequence (AAAAAAATGTCT) Tom40	This study
pGEM4polyA-yk-pSu9-DHFR-3HA	Yeast kozak sequence (AAAAAAATGTCT) presequence of *N. crassa* ATP synthase subunit 9 (aa M1-S69)-*M. musculus* DHFR-3 × HA-tag	This study
pGEM4polyA-yk-Om14-3HA	Yeast kozak sequence (AAAAAAATG) Om14-3HA	This study
pGEM4polyA-yk-Cyc3-3HA	Yeast kozak sequence (AAAAAAATG) Cyc3-3HA	This study
pGEM4polyA-yk-Tim23-3HA	Yeast kozak sequence (AAAAAAATG) Tim23-3HA	This study
pGEM4polyA-yk-Yah1-3HA	Yeast kozak sequence (AAAAAAATG) Yah1-3HA	This study
pBpa2-PGK1+3SUP4-tRNA$_{CUA}$	tRNA aaRS + tRNA$_{CUA}$	Chen et al. (2007)
pRS426-TPI-hp18(VDAC)-DHFR-3HA	Hairpin 18 of *H. sapiens* VDAC1 (aa K252-A283)-*M. musculus* DHFR-3 × HA-tag	This study
pRS426-TPI-hp18(VDAC, Bpa258)-DHFR-3HA	Hairpin 18 of *H. sapiens* VDAC1 (aa K252-A283; Thr258Bpa)-*M. musculus* DHFR-3 × HA-tag	This study
pGEM4polyA-mk-DHFR-3HA	Mammalian kozak sequence (GCCACCATGG) *M. musculus* DHFR-3 × HA-tag	This study
pGEM4polyA-mk-Porin-3HA	Mammalian kozak sequence (GCCACCATGG) Porin-3 × HA-tag	This study
pGEM4polyA-mk-VDAC1-3HA	Mammalian kozak sequence (GCCACCATGG) *H. sapiens* VDAC1-3 × HA-tag	This study
pGEM4-Porin	Porin	Mayer et al. (1993)
pFA6a-NatNT2	NatNT2 cassette	Janke et al. (2004)
pFA6a-KanMX4	KanMX4 cassette	Wach et al. (1994)
pFA6a-HIS3MX6	HIS3MX6 cassette	Wach et al. (1997)
pFA6a-3HA-NatNT2	3 × HA-tag CYC1 terminator NatNT2 cassette	This study
pMK632	NatMX cassette tetO$_7$-CYC1 promoter-Ubiquitin-Leucin-HA-tag	Gnanasundram and Koš (2015)
pMK632Kan	KanMX cassette tetO$_7$-CYC1 promoter-Ubiquitin-Leucin-HA-tag	This study
pMK632His	HIS3MX cassette tetO$_7$-CYC1 promoter-Ubiquitin-Leucin-HA-tag	This study

If not indicated otherwise, gene sequences are from *S. cerevisiae*

Table S4. **Antibodies used in this study**

Antibody directed against	Species	Dilution	Identifier
Aha1	Rabbit	1:2,000	N/A
Bmh1	Rabbit	1:1,000	N/A
Cct1	Rabbit	1:500	N/A
Cox2	Rabbit	1:1,000	N/A
Djp1	Rabbit	1:2,000	N/A
***H. sapiens* GAPDH**	Mouse	1:1,000	CSA-335 (Stressgen)
HA	Rat	1:1,000	11867423001 (Roche)
Hch1	Rabbit	1:4,000	N/A
***H. sapiens* Hsc70**	Rat	1:1,000	ADI-SPA-815 (Enzo)
Hsp104	Rabbit	1:25,000	N/A
Hsp26	Rabbit	1:4,000	N/A
Hsp42	Rabbit	1:4,000	N/A
Hsp82/Hspc82	Rabbit	1:20,000	N/A
Porin	Rabbit	1:50,000	N/A
Rps3	Rabbit	1:50,000	N/A
Sba1	Rabbit	1:2,500	N/A
Sis1	Rabbit	1:20,000	N/A
Ssa1/2	Rabbit	1:20,000	N/A
Sti1	Rabbit	1:10,000	N/A
Tob55	Rabbit	1:2,000	N/A
Tom40	Rabbit	1:10,000	N/A
Tom70	Rabbit	1:2,000	N/A
Ydj1	Rabbit	1:10,000	N/A

If not indicated otherwise, target proteins are from *S. cerevisiae*.

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