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Supplemental information

Impaired phosphatidylethanolamine metabolism activates a reversible stress response that detects and resolves mutant mitochondrial precursors

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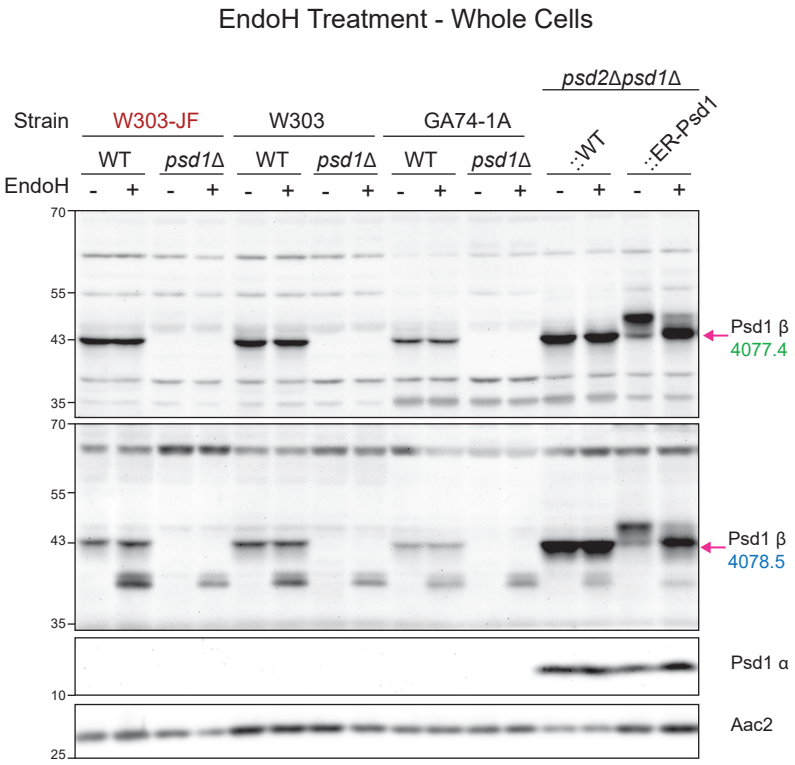

Figure S1. Absence of glycosylated Psd1 in strains used by Friedman et al., Related to Figure 1. Cell extracts derived from pairs of WT and *psd1*Δ yeast of the indicated backgrounds grown at 30°C in synthetic complete dextrose (SCD) were treated with EndoH as listed and analyzed by immunoblot using the designated anti-Psd1 antisera; Aac2 served as loading control. W303-JF is a kind gift from Jonathan Friedman (n=3).

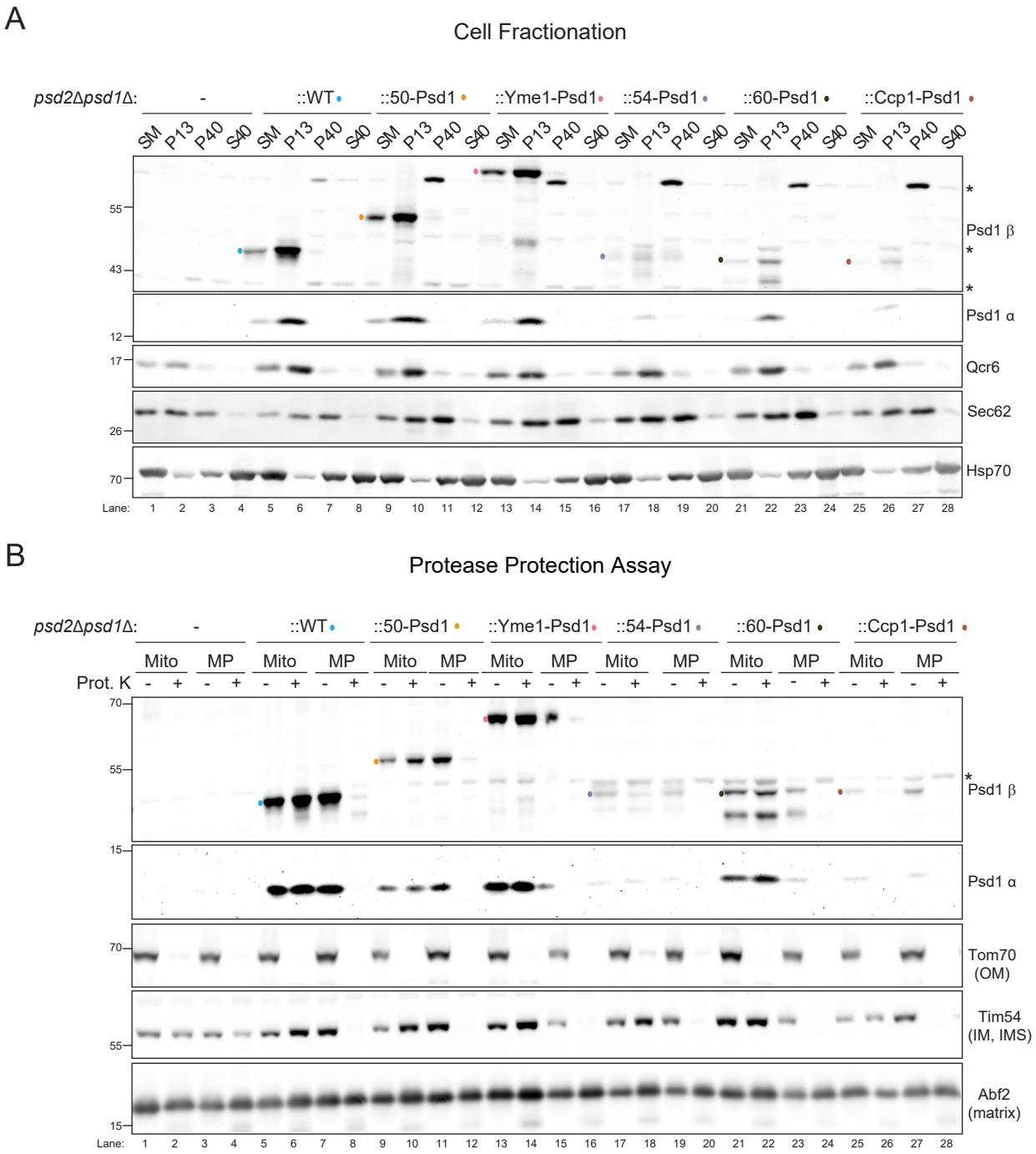

Figure S2. Assorted IM-Psd1 chimeras are properly localized, Related to Figure 2. (A) Subcellular fractions were collected from the indicated strains following growth in YPD medium. Equal amounts of each fraction were resolved by SDS-PAGE and immunoblotted for both subunits of Psd1 (β and α) mitochondrial (Qcr6), ER (Sec62), and cytosolic (Hsp70) markers. SM, starting material, P13, pellet of 13,000xg; P40, pellet of 40,000xg; and S40, supernatant of 40,000xg (n=3). (B) Intact mitochondria (Mito) and osmotically ruptured mitochondria (MP) from indicated yeast strains were treated as indicated with 100 μg proteinase K (Prot. K) and the resultant samples resolved by SDS-PAGE and immunoblotted for Psd1 (β and α subunits) and the compartment-specific markers Tom70 (OM), Tim54 (IMS), and Abf2 (matrix). For Psd1 immunoblots, different protein amounts, 10 μg (lanes 5-16) or 40 μg (lanes 1-4 and 17-28), were resolved due to differences in chimera abundance (n=3). *, nonspecific bands.

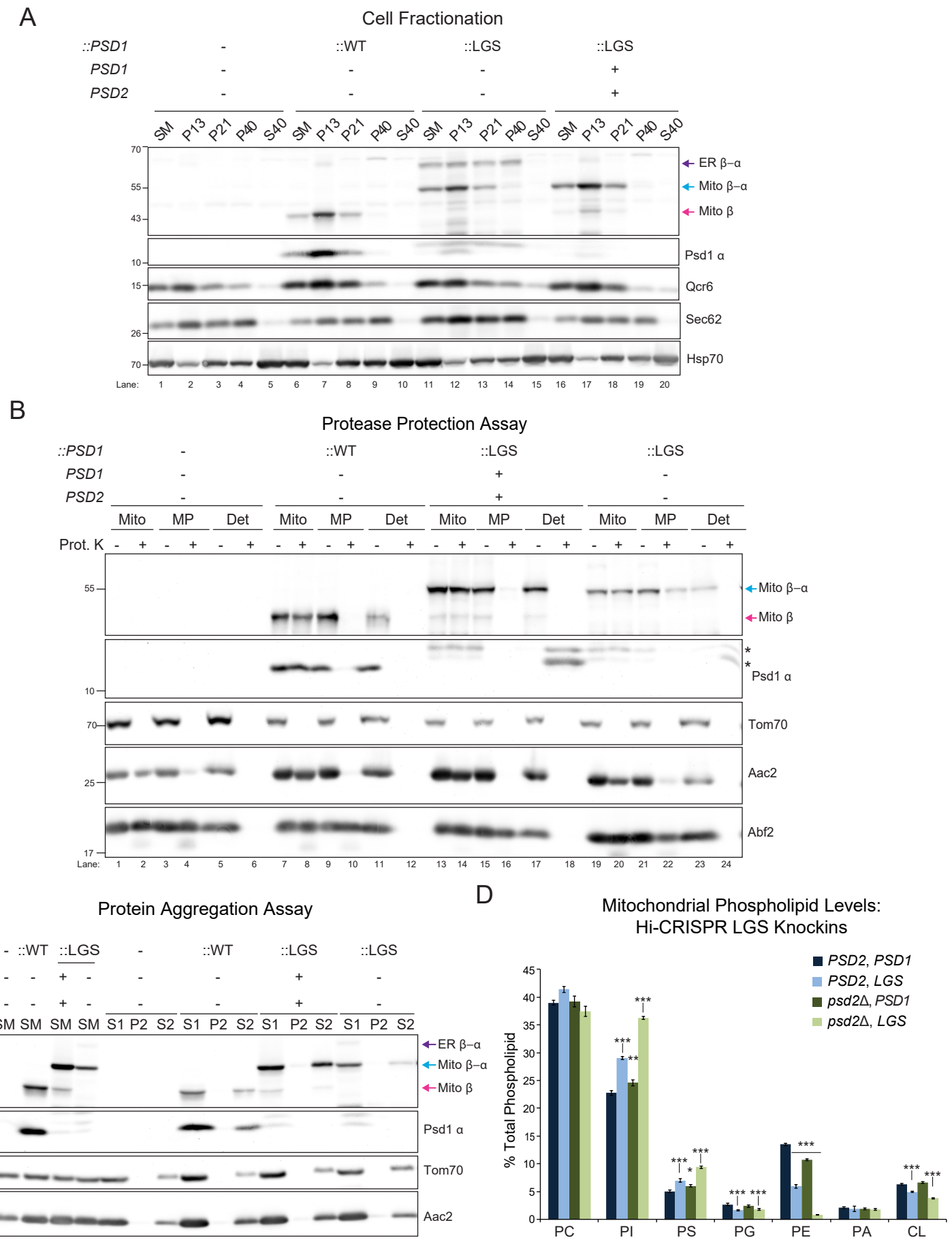

Figure S3. Glycosylated mutant Psd1 co-fractionates with the ER and is not aggregation prone, Related to Figure 3. (A) Subcellular fractions were collected from the indicated strains following growth in YPD medium. Equal amounts of each fraction were resolved by SDS-PAGE and immunoblotted for both subunits of Psd1 (β and α), and mitochondrial (Qcr6), ER (Sec62), and cytosolic (Hsp70) markers. SM, starting material, P13, pellet of 13,000x*g*; P40, pellet of 40,000x*g*; and S40, supernatant of 40,000x*g* (n=3). (B) Protease protection assay in intact mitochondria (Mito), osmotically ruptured mitochondria (MP), or deoxycholate-solubilized mitochondria (Det.) treated as indicated with 100 µg proteinase K (Prot. K). The resultant samples were resolved by SDS-PAGE and immunoblotted for Psd1 (β and α subunits), and the compartment-specific markers Tom70 (OM), Aac2 (IMS), and Abf2 (matrix). Aac2 was detected using a monoclonal antibody, 6H8, which recognizes an N-terminal epitope present in the IMS (n=3). *, nonspecific bands. (C) Mitochondria isolated from the indicated strains grown in YPD at 30°C were solubilized with digitonin and separated into a supernatant (S1) and pellet (P1) by centrifugation. The resulting pellet (P1) was re-extracted with TX-100 and again fractionated into a supernatant (S2) and pellet (P2) by centrifugation. Collected fractions were resolved by SDS-PAGE and immunoblotted as indicated. SM, starting material (n=3). (D) Mitochondrial phospholipid levels from control and CRISPR-LGS knockin strains analyzed in Fig. 3G (mean ± SEM for n=6). Significant differences (1 symbol $P \leq 0.05$; 2 symbols $P \leq 0.01$; 3 symbols $P \leq 0.001$) compared to WT (*PSD2*, *PSD1*) were calculated by one-way ANOVA with Holm-Sidak pairwise comparison.

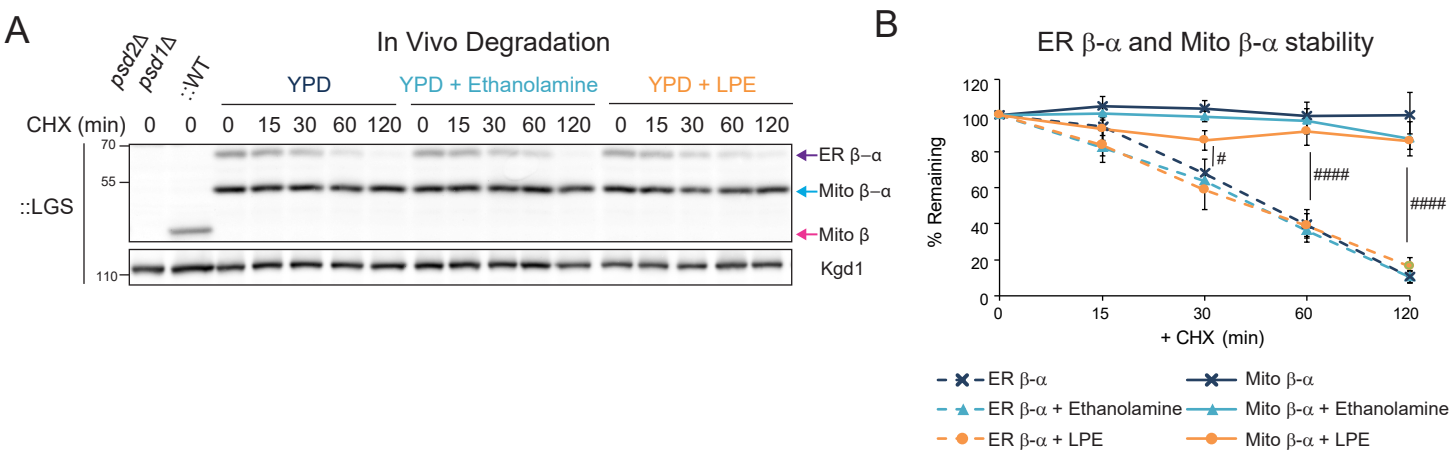

Figure S4. LPE and ethanolamine do not alter the stability of glycosylated non-functional mutant Psd1, related to Figure 6. (A) In vivo degradation assay. Cell extracts from the indicated strain were isolated at the designated times following growth in YPD containing cycloheximide (CHX) only or CHX containing LPE or ethanolamine. Samples were resolved by SDS-PAGE and immunoblotted as indicated (n=5). (B) The percentages of nonfunctional glycosylated ER β-α and mitochondrial β-α remaining at each time point were quantified (mean ± SEM for n=5). Statistical differences (1 symbol $P \leq 0.05$; 4 symbols $P \leq 0.0001$) between ER β-α and Mito β-α was determined at each timepoint by one-way ANOVA with Tukey's multiple comparisons test.

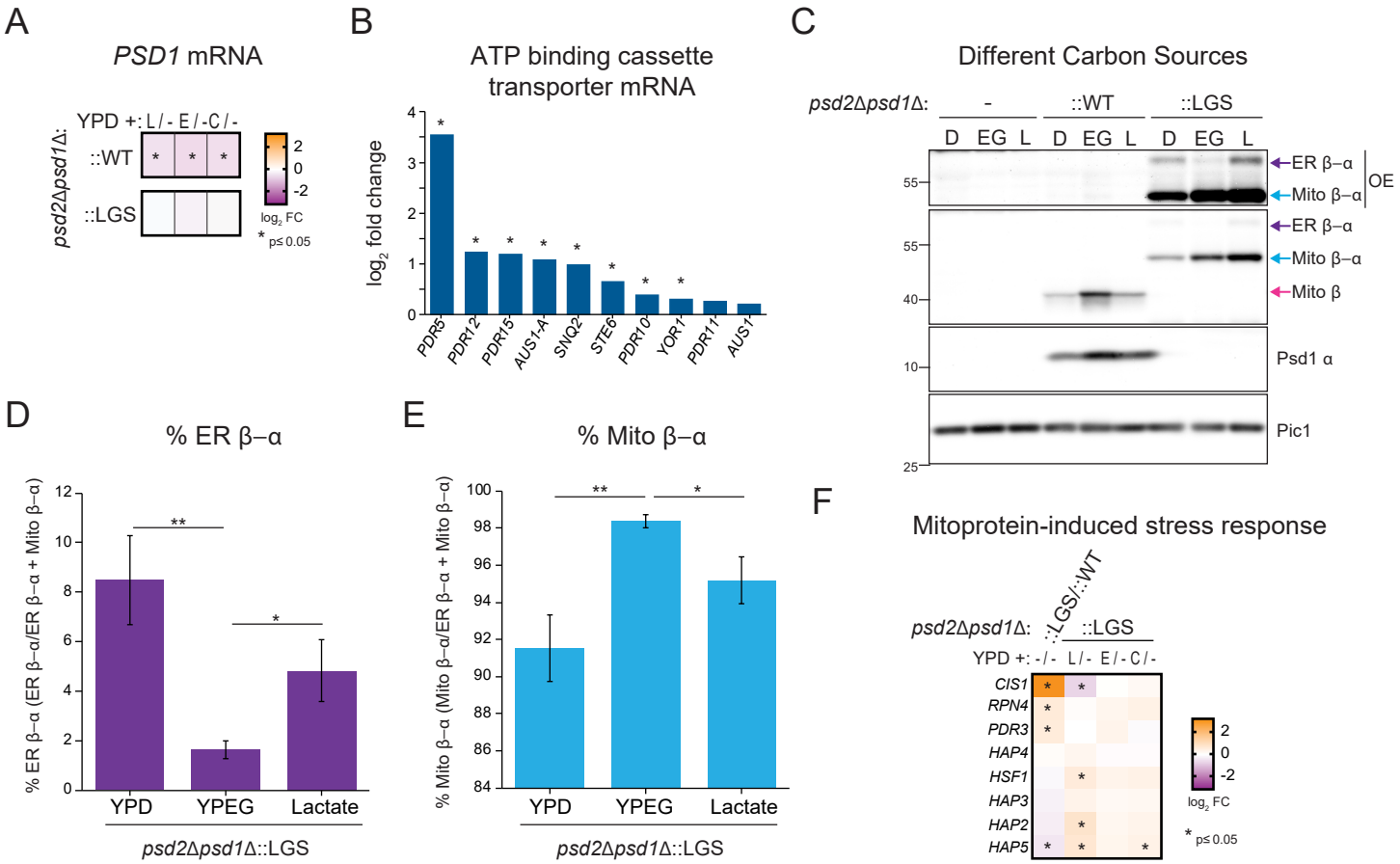

Figure S5. Additional transcriptomic and metabolic comparisons, Related to Figures 6 and 7. (A) *PSD1* mRNA levels in ::WT and ::LGS yeast cultured in dextrose alone compared to dextrose supplemented with LPE (L), ethanolamine (E), or choline (C). padj values that were significant (P ≤ 0.05) are designated (*). (B) Relative abundance of ATP binding cassette transporter genes in ::LGS vs ::WT (*symbols padj values P ≤ 0.05). (C) The indicated strains were pre-cultured at 30°C in YPD (D), YPEG (EG) and Rich Lactate (L) and after isolation of cell extracts, the α and β subunits of Psd1 were analyzed by immunoblotting. Pic1 served as a loading control (n=4). OE, over-exposed. (D and E) The relative abundance of (D) ER β-α, and (E), Mito β-α in Psd1-LGS expressing yeast was determined (means ± SEM for n=4). Statistical differences (1 symbols P ≤ 0.05; 2 symbols P ≤ 0.01) were determined by unpaired Student's t-test. (F) Heatmaps of Mitoprotein-induced stress genes in ::LGS vs ::WT and how each supplement does or does not affect their expression relative to ::LGS grown in YPD alone. padj values that were significant (P ≤ 0.05) are designated (*).

TABLE S1, Related to Transparent Methods – Yeast Strains

Strain	Genotype	Source
GA74-1A	*MAT*a, *his3-11,15, leu2, ura3, trp1, ade8 [rho+, mit+]*	Carla Koehler
W303	*MAT*a, *his3-11, ura3-1,15, trp-1-1, ade2-1, can1-100 [rho+, mit+]*	Cathy Clarke
D273-10B	*MATa [rho + , mit +]*	Carla Koehler
BY4741	*MAT*a, *his3-1*, *leu2-0*, *met15-0*, *ura3-0 [rho + , mit +]*	Euroscarf
*psd1*ΔGA74-1A	*MAT*a, *psd1Δ::HIS3MX6, leu2, ura3, trp1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
*psd2*ΔGA74-1A	*MAT*a, *psd2Δ::HIS3MX6, leu2, ura3, trp1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
*psd2Δpsd1*Δ	*MAT*a, *psd2Δ::HIS3MX6, leu2, ura3, psd1□::TRP1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
*psd1*Δ W303	*MAT*a, *psd1Δ::HIS3MX6, ura3-1,15, trp1-1, ade2-1, can1-100 [rho+, mit+]*	(Calzada et al., 2019)
*psd1*Δ D273-10B	*MATa, psd1Δ::KANMX6, [rho + , mit +]*	(Calzada et al., 2019)
*psd1*Δ BY4741	*MATa, psd1Δ::KANMX4,his3-1, leu2-0, met15-0, ura3-0 [rho + , mit +]*	Euroscarf
ρ^0	*MATa, his3-11,15, leu2, ura3, trp1, ade8 [rho-, mit +]*	(Calzada et al., 2019)
*cho1*Δ	*MATa, cho1Δ::HIS3MX6, leu2, ura3, trp1, ade8 [rho + , mit +]*	(Onguka et al., 2015)
*cho1Δpsd2Δpsd1*Δ	*MATa, psd2Δ::HIS3MX6, leu2, ura3, psd1Δ::TRP1, *cho1 HI-CRISPR ade8 [rho + , mit +]*	(Calzada et al., 2019)
*tam41*Δ	*MATa, tam41Δ::HIS3MX6, leu2, ura3, trp1, ade8 [rho + , mit +]*	This study
psd2Δpsd1Δ::WT Psd1	*MATa, psd2Δ::HIS3MX6, Psd1-3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
psd2Δpsd1Δ::ER-Psd1	*MATa, psd2Δ::HIS3MX6, CPY*mPsd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
psd2Δpsd1Δ::Psd1^{S463A}	*MATa, psd2Δ::HIS3MX6, Psd1*^{S463A}*3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
psd2Δpsd1Δ::Psd1^{H345A}	*MATa, psd2Δ::HIS3MX6, Psd1*^{H345A}*3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{D210A}	*MATa, psd2Δ::HIS3MX6, Psd1*^{D210A}*3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
1A::Psd1$^{LGS/AAA}$	*MAT*a, *his3-11,15, Psd1*$^{LGS/AAAA}$*3XFLAG::LEU2, ura3, trp1, ade8 [rho+, mit+]*	This study
*psd1*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *psd1Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, trp1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
*psd2*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *psd2Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, trp1, ade8 [rho+, mit+]*	This study
*psd2Δpsd1*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *psd2Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Onguka et al., 2015)
*cho1*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *cho1Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, trp1, ade8 [rho+, mit+]*	This study
*psd2Δpsd1Δcho1*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *psd2Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, psd1Δ::TRP1, ade8 [rho+, mit+] CRISPR*cho1*	This study
*psd2Δpsd1Δpdr5*Δ::Psd1$^{LGS/AAA}$	*MAT*a, *psd2Δ::HIS3MX6, pdr5Δ::CloNat/nourseothricin, Psd1*$^{LGS/AAAA}$*3XFLAG, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
ρ^{0}*::* Psd1$^{LGS/AAA}$	*MATa, Psd1*$^{LGS/AAAA}$*3XFLAG::LEU2, ura3, trp1, ade8 [rho-, mit+]*	This study
tam41□:: Psd1$^{LGS/AAA}$	*MATa, tam41Δ::HIS3MX6, Psd1*$^{LGS/AAAA}$*3XFLAG::LEU2, ura3, trp1, ade8 [rho+, mit+]*	This study
Psd1$^{LGS/AAA}$	*MAT*a, *his3-11,15, *Psd1*$^{LGS/AAA}$ *HI-CRISPR, ura3, trp1, ade8 [rho+, mit+]*	This study
*psd2*Δ, Psd1$^{LGS/AAA}$	*MAT*a, *psd2Δ::HIS3MX6, *Psd1*$^{LGS/AAA}$ *HI-CRISPR, ura3, trp1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{R139A}	*MATa, psd2Δ::HIS3MX6, Psd1*^{R139A}*3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study

psd2Δpsd1Δ::Psd1^{R189A}	*MATa, psd2Δ::HIS3MX6, Psd1^{R189A}3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{K192A}	*MATa, psd2Δ::HIS3MX6, Psd1^{K192A}3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{R196A}	*MATa, psd2Δ::HIS3MX6, Psd1^{R196A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{Q228A}	*MATa, psd2Δ::HIS3MX6, Psd1^{Q228A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{K230A}	*MATa, psd2Δ::HIS3MX6, Psd1^{K230A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{H243A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H243A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{H374A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H374A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{R385A}	*MATa, psd2Δ::HIS3MX6, Psd1^{R385A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{H431A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H431A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{K452A}	*MATa, psd2Δ::HIS3MX6, Psd1^{K452A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{VGAT}	*MATa, psd2Δ::HIS3MX6, Psd1^{VGAT} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{VGSI}	*MATa, psd2Δ::HIS3MX6, Psd1^{VGSI} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1$^{VG(x2)}$	*MATa, psd2Δ::HIS3MX6, Psd1$^{VG(x2)}$ 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Psd1^{ts}	*MATa, psd2Δ::HIS3MX6, Psd1$^{ts\ (K356R,\ F397L,\ E429G,\ and\ M448T)}$ 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1$^{ts\text{-}S463A}$	*MATa, psd2Δ::HIS3MX6, Psd1$^{ts\text{-}S463A}$3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{N168A}	*MATa, psd2Δ::HIS3MX6, Psd1^{N168A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{E171A}	*MATa, psd2Δ::HIS3MX6, Psd1^{E171A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{D176A}	*MATa, psd2Δ::HIS3MX6, Psd1^{D176A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{E185A}	*MATa, psd2Δ::HIS3MX6, Psd1^{E185A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{D343A}	*MATa, psd2Δ::HIS3MX6, Psd1^{D343A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{H346A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H346A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{H348A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H348A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{D352A}	*MATa, psd2Δ::HIS3MX6, Psd1^{D352A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{R358Q}	*MATa, psd2Δ::HIS3MX6, Psd1^{R358Q} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Zhao et al., 2019)
psd2Δpsd1Δ::Psd1^{H360A}	*MATa, psd2Δ::HIS3MX6, Psd1^{H360A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{N379A}	*MATa, psd2Δ::HIS3MX6, Psd1^{N379A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{N383A}	*MATa, psd2Δ::HIS3MX6, Psd1^{N383A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{E384A}	*MATa, psd2Δ::HIS3MX6, Psd1^{E384A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{N440A}	*MATa, psd2Δ::HIS3MX6, Psd1^{N440A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)
psd2Δpsd1Δ::Psd1^{E454A}	*MATa, psd2Δ::HIS3MX6, Psd1 ^{E454A} 3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	(Ogunbona et al., 2017)

psd2Δpsd1Δ::Tim50-Psd1	*MATa, psd2Δ::HIS3MX6, Tim50-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Yme1-Psd1	*MATa, psd2Δ::HIS3MX6, Yme1-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This Study
psd2Δpsd1Δ::Tim54-Psd1	*MATa, psd2Δ::HIS3MX6, Tim54-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study
psd2Δpsd1Δ::Mic60-Psd1	*MATa, psd2Δ::HIS3MX6, Mic60-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This Study
psd2Δpsd1Δ::Ccp1-Psd1	*MATa, psd2Δ::HIS3MX6, Ccp1-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho+, mit+]*	This study

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-yeast Psd1	(Tamura et al., 2012)	4077; 4078
Mouse monoclonal anti-FLAG	Sigma-Aldrich	Cat#F3165
Mouse monoclonal anti-yeast Aac2	(Panneels et al., 2003)	6H8
Mouse monoclonal anti-yeast Sec62	David Meyer	Purple Top
Rabbit polyclonal anti-yeast Tom70	(Baile et al., 2013)	7306.2
Rabbit polyclonal anti-yeast Qcr6	(Baile et al., 2013)	MGB73.2
Rabbit polyclonal anti-Hsp70	(Claypool et al., 2006)	SH-1T
Rabbit polyclonal anti-yeast Tim54	(Hwang et al., 2007)	7303
Mouse monoclonal anti-yeast Cox2	Abcam	Cat#ab110271
Rabbit polyclonal anti-yeast Kgd1	(Glick et al., 1992)	453.3
Mouse monoclonal anti-Ubiquitin	Santa Cruz Biotechnology	Cat #sc-8017
Rabbit polyclonal anti-yeast Pic1	(Whited et al., 2013)	3676.1
Rabbit polyclonal anti-yeast Cho1	(Choi et al., 2010)	N/A
Rabbit polyclonal anti-TOMM20	Sigma-Aldrich	Cat#HPA011-562
Mouse monoclonal anti-Calnexin	Millipore	Cat#MAB3126
Goat polyclonal anti-Flag epitope tag	Novus Biological	NB600-344
Rabbit polyclonal anti-yeast Tam41	This Paper	7301
Rabbit polyclonal anti-yeast Abf2	(Calzada et al., 2019)	5477
Rabbit polyclonal AP anti-Kar2	(Huyer et al., 2004)	
ImmunoPure Antibody Donkey Anti-Rabbit IgG (H+L) Peroxidase Conjugated	Thermo Scientific	Cat#31458
ImmunoPure Antibody Donkey Anti-Mouse IgG (H+L) Peroxidase Conjugated	Thermo Scientific	Cat#31432
Goat Anti-Mouse IgG (H+L) Secondary antibody DyLight® 550	Thermo Scientific	Cat#84540
Goat Anti-Rabbit IgG (H+L) Secondary antibody DyLight® 550	Thermo Scientific	Cat#84546
Bacterial and Virus Strains		
Escherichia coli DH5 alpha Competent Cells	This study	N/A
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Ethanolamine hydrochloride	Sigma-Aldrich	Cat#236381
Endoglycosidase H	NEB	Cat#P0702L
Choline chloride	Sigma-Aldrich	Cat#C7527-500
Tris(2-carboxyethyl)phosphine hydrochloride	Sigma-Aldrich	Cat#C4706
Iodoacetamide	Sigma-Aldrich	Cat# I6125
Dithiothreitol	Fisher	Cat#BP172-25
Cycloheximide	Acros Organic	Cat#66-81-9
MG132 (Z-Leu-Leu-Leu-al)	Sigma-Aldrich	Cat#M7449
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma-Aldrich	Cat#C2759
USP2Core	LifeSensors Inc	Cat# DB501
Instant Non-Fat Dry Milk	Giant	N/A

Digitonin D-3200	BIOSYNTH international, Inc	Cat#11024-24-1
Yeast Nitrogen Base (YNB) without amino acids	Research Products International	Cat#Y20040
Complete Supplement Mixture (CSM) Drop-out: complete	Formedium	Cat#DCS0019
D(+) Glucose Anhydrous	Formedium	Cat#GLU02
Phosphorus-32 Radionuclide	PerkinElmer	NEX053001MC
Acetic Acid, Sodium Salt, [1-14C]	PerkinElmer	NEC084H001MC
Anti-DYKDDDDK G1 Affinity Resin	GenScript	Cat#L00432
Critical Commercial Assays		
Pierce BCA Protein Assay Kit	ThermoFisher Scientific	Cat#23225
RNeasy MinElute Cleanup kit	Qiagen	Cat#74204
Deposited Data		
Tandem Mass Tag and Mass Spectrometry data	MassIVE repository massive.ucsd.edu	ftp://MSV000086558@massive.ucsd.edu
RNA sequence data	Gene Expression Omnibus	GSE162987
Experimental Models: Cell Lines		
HEK293	ATCC	Cat#CRL-1573
Experimental Models: Organisms/Strains		
Refer to Table 1		
Oligonucleotides		
CIS1 forward, 5'-AATGCAGGAGTTGCTTACA-3'	This study	N/A
CIS1 reverse, 5'-TTCTTCCGCTATAGTCTCCAA-3'	This study	N/A
ACT1 forward, 5′-GTATGTGTAAAGCCGGTTTTG-3′	This study	N/A
ACT1 reverse, 5′-CATGATACCTTGGTGTCTTGG-3′	This study	N/A
PSD1 forward, 5′-CCAGTAGCACAAGGCGAAGA-3′	This study	N/A
PSD1 reverse, 5′-GACATCAAGGGGTGGGAGTG-3'	This study	N/A
LGS/AAA mutation CRISPR-Cas9 gene block: 5'CTTTGGTCTCACCAAAAC*TATTGGGAGGGATGCCTTTGGTTAAGGGTGAAGAAATGGGTGGCTTTGAA*GCGGCAGCC*ACTGTTGTACTTTGTTTTGAAGCTCCCACTGAATTTAAGTTCGATGTTAGGAAATGGGTGGCTTTGAATT*GTTTTAGAGAGAGACCTTTC-3'	This study	N/A
Recombinant DNA		
pRS305	(Sikorski and Hieter, 1989)	N/A
pCRCT	(Bao et al., 2015)	Addgene plasmid 60621
Software and Algorithms		
Quantity One Software	Bio-Rad Laboratories	https://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software?ID=1de9eb3a-1eb5-4edb-82d2-68b91bf360fb

SigmaPlot 11 software	Systat Software, San Jose, CA	http://www.sigmaplot.co.uk/products/sigmaplot/
Prism 8	GraphPad	https://www.graphpad.com/scientific-software/prism/
SWISS-MODEL	SWISS MODEL	https://swissmodel.expasy.org
RStudio Version 1.3.959	RStudio	https://rstudio.com/products/rstudio/download/

TRANSPARENT METHODS

Molecular Biology

All of the Psd1 constructs used in this study were subcloned into the pRS305 plasmid. The following constructs have been previously described: WT Psd1 containing a COOH-terminal 3XFLAG tag, the autocatalytic LGS/AAA mutant, and ER-Psd1 (Onguka et al., 2015); the Psd1 point mutants analyzed in Figures 3A and 4B (Ogunbona et al., 2017; Zhao et al., 2019); and human PISD with 3XFLAG tag (Zhao et al., 2019). Additional point mutations analyzed in Fig. 4A (R139A, R189A, K192A, R196A, Q228A, K230A, H243A, H374A, R385A, H431A, K452A, $V_{402}GAT_{405}$, $V_{407}GSI_{410}$, and VG(x2)) were generated by overlap extension using pRS305Psd3XFLAG as template. To direct Psd1 to the IM using information from other IM proteins with a similar topology, the first 101 amino acids of Psd1, which includes its mitochondrial targeting sequence and single transmembrane domain, were replaced by the equivalent regions of the following single pass IM proteins: Tim50 (1-132), Mic60 (1-57), Yme1 (1-251), Tim54 (1-54), and Ccp1 (1-36). All constructs were verified by DNA sequencing.

Yeast strain generation and growth conditions

Yeast strains used in this study are listed in Table S1 and were derived from GA74-1A, BY-4741, W303 or D273-10B. To integrate assorted pRS305-based constructs into the yeast genome, AflII-linearized plasmids were transformed into GA74-1A yeast strains of the indicated genotype (in general, *psd1*Δ*psd2*Δ; however, the autocatalytic mutant LGS/AAA Psd1 mutant was additionally transformed into WT yeast containing or lacking mtDNA (*rho*-), and *psd1*Δ, *psd2*Δ, *cho1*Δ, and *tam41*Δ single deletion strains), stable integrants selected on synthetic dropout medium lacking Leu (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mixture synthetic-leu, 2% dextrose), and expression of each Psd1 construct verified by immunoblot.

Two approaches were used to generate deletion strains: 1) PCR-mediated gene replacement of the entire reading frame (Wach et al., 1994) and 2) homology-integrated (HI)-Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) gene editing in yeast (Bao et al., 2015; Ogunbona et al., 2017). The *tam41*Δ strain and *pdr5*Δ strains were generated by replacing the entire *TAM41* or *PDR5* open reading frames with HISMX6 or CloNat/nourseothricin, respectively. All gene deletions were confirmed by either immunoblot (*tam41*Δ; Figure 7C) or genomic PCR (*pdr5*Δ). The *psd1*Δ*psd2*Δ*cho1*Δ strains was generated using a CRISPR-Cas9 gene block designed to target *CHO1* (Calzada et al., 2019).

LGS/AAA mutations were inserted into the *PSD1* open reading frame of WT and *psd2*Δ yeast using a HI-CRISPR system (Bao et al., 2015; Calzada et al., 2019; Ogunbona et al., 2017). Briefly, the CRISPR-Cas9 gene block (gBlock) was designed using Benchling online CRISPR guide design tools to target *PSD1* and assembled into the plasmid pCRCT, a gift from Huimin Zhao (Addgene plasmid 60621), using the Golden Gate assembly method (Engler et al., 2009). The gBlock was composed of the CRISPR-Cas9 target (20bp), a 100 base pair (bp) homology-directed repair template with 50bp homology arms on either side flanking the Cas9 cutting site, and the codons encoding the LGS motif mutated to AAA by designing homology repair templates encoding these changes *psd1*$^{LGS/AAA}$ (BsaI restriction site; *homology arms*; **LGS/AAA mutations**; *3' CRISPR target* ; 5'-CTTTGGTCTCACCAAAAC*TATTGGGAGGGATGCCTTTGGTTAAGGGTGAAGAAATGGGT GGCTTTGAA***GCGGCAGCC***ACTGTTGTACTTTGTTTTGAAGCTCCCACTGAATTTAAGTTC GATGTTAG**GAAATGGGTGGCTTTGAATT**GTTTTAGAGAGAGACCTTTC-3'). The gblock was assembled into the pCRCT plasmid using the Golden Gate assembly method (Engler et al., 2009), and successfully confirmed by DNA sequencing. Following transformation of targeting plasmid into WT or *psd2*Δ yeast, positive clones were identified by immunoblot.

To determine if the various IM-directed Psd1 chimeras were functional, yeast strains were grown overnight in YPD and equal amounts (serial 1:4 dilutions starting at 0.00012 OD_{600}'s in 3µl of sterile water) spotted onto SCD plates in the absence or presence of 2 mM ethanolamine hydrochloride. To assess respiratory capacity, serially diluted strains were spotted on synthetic complete ethanol glycerol (SCEG; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% (w/v) complete amino acid mixture, 1% (v/v) ethanol, 3% (v/v) glycerol), and synthetic complete lactate (SC-LAC; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% (w/v) complete amino acid mixture, 0.05% (w/v) dextrose, 2% (v/v) lactic acid, 3.4 mM $CaCl_2$-$2H_2O$, 8.5 mM NaCl, 2.95 mM $MgCl_2$-$6H_2O$, 7.35 mM KH_2PO_4, and 18.7 mM NH_4Cl, pH 5.5) plates. Plates, which contained 2% (w/v) agar (Himedia, cat RM301), were incubated at 30°C or 37°C for the indicated days.

For routine growth, yeast were grown in synthetic complete dextrose medium (SCD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% (w/v) complete amino acid mixture, 2% (w/v) dextrose) with or without 2mM ethanolamine, or YPD (1% (w/v) yeast extract, 2% (w/v) tryptone, 2% (w/v) dextrose) unless otherwise noted. For growth assays involving various supplements, overnight pre-cultures of the indicated yeast strains grown in YPD were inoculated to an OD_{600} of 0.2 in YPD media alone, or YPD supplemented with 2mM ethanolamine, 2mM choline, 1% (v/v) Tergitol (Type NP-40), 1% (v/v) Tergitol + 0.5mM lyso-PE, or 10 µM CCCP, and grown at 30ºC overnight with vigorous shaking.

Quantitation of mitochondrial Psd1

Quantification of Psd1 levels in three different batches of mitochondria isolated from WT or *psd1*Δ yeast grown in rich lactate medium (1% (w/v) yeast extract, 2% (w/v) tryptone, 0.05% (w/v) dextrose, 2% (v/v) lactic acid, 3.4mM $CaCl_2$-$2H_2O$, 8.5mM NaCl, 2.95mM $MgCl_2$-$6H_2O$, 7.35mM KH_2PO_4, 18.7mM NH_4Cl, pH 5.5) was determined by immunoblot using a recHis_6Psd1, generated and purified previously (Tamura et al., 2012), standard curve.

Preparation of cell lysates for immunoblot analysis

Unless otherwise indicated, the equivalent of 2.0 OD_{600} of yeast cells were harvested by centrifugation at 1690 *x g* for 10 min. Cell pellets were resuspended in 1mL of MilliQ grade

water and treated with a final concentration of 120mM sodium hydroxide (NaOH) and 1% (v/v) β-mercaptoethanol (β-ME) ice with periodic inversion for 10 min on. 100% (w/v) trichloroacetic acid (TCA) was added for a final concentration of 6% and proteins precipitated on ice for 10 min with periodic inversion. Pellets were collected by centrifugation at 21,000 *x g* for 2 min, washed with 1 mL 100% acetone, and re-centrifuged at 21,000 *x g* for 2 min. Pellets were dissolved with 60µL 0.1M NaOH at room temperature prior to addition of 60µL 2X reducing sample buffer (RSB) and denaturation at 95ºC for 5 min. Equal sample volumes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblot.

EndoH treatment in whole cells

Yeast cultures maintained on YPD plates were grown overnight in SCD or YPD liquid medium prior to harvesting 10 OD_{600} of each culture by centrifugation at 1690 *x g* for 10 min. Cell pellets were resuspended in 250µL of lysis buffer (20mM HEPES-KOH pH 7.4, 150mM potassium acetate, 2mM magnesium acetate, 1mM phenylmethylsulfonyl fluoride (PMSF)) and transferred to a 1.5mL microfuge tube containing ~50µL of glass beads. Samples were vortexed at level 10 for 30 min at 4ºC. Unbroken cells were separated by centrifugation at 425 *x g* for 5 min and the supernatant transferred to a microfuge tube containing 10µL of 10% (w/v) sodium dodecyl sulfate (SDS) and 2µL of β-ME (final concentration 0.5% (w/v) SDS and 1% (v/v) β-ME). Samples were incubated at 95ºC for 10 min and cooled at room temperature. Each sample was divided into two 100µL aliquots and diluted with an equal volume of 0.5M sodium citrate pH 5.5. One aliquot served as an untreated control; 1µL of Endoglycosidase H (EndoH; New England BioLabs) was added to the other sample. Samples were then incubated for 4 hrs at 37ºC. Proteins were precipitated from the entire sample by addition of 100% (w/v) TCA to a final concentration of 20% and incubated on ice for 10 min. Pellets were collected by centrifugation at 21,000 *x g* for 10 min at 4ºC and resuspended in 40µL of 0.1M NaOH and 40µL of 2X RSB as described above. Equal volumes of untreated and EndoH-treated samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblot.

Mitochondrial isolation and fractionation

Isolation of crude mitochondria and cell fractionation by gravity centrifugation was adapted and modified from (Claypool et al., 2006). Briefly, strains were cultured in 150 mL of designated media shaking at 30°C for ~36 hours, then 100 OD_{600} was transferred to two Fernbach flasks containing 950 mL of the same medium used for pre-cultures which were then grown at 30°C overnight for an OD_{600} of ~2.5–3.5. Cell pellets, which were collected by centrifugation for 5 min at 6,000 *x g*, were resuspended in water and re-collected at 2000 *x g* for 5 min. The washed yeast pellet was resuspended in 50 mL of 0.1 M Tris-SO_4, pH 9.4 containing 15 mM dithiothreitol, and incubated for 20 min at 30°C shaking at 220 rpm. Following a 5 min spin at 2000 *x g*, yeast pellets were washed with 40 mL of 1.2 M sorbitol, 20 mM KPi, pH 7.4, collected again at 2000 × *g* for 5 min, and resuspended with 1.2 M sorbitol, 20 mM KPi, pH 7.4 (2 mL/gram of yeast) spiked with Zymolyase 20 T (3 mg/gram of yeast; Nacalai Tesque, Inc.) and shaken at 220 rpm for 1 h at 30°C. Following centrifugation at 3500 *x g* for 5 min at 4ºC, pellets were washed with 1.2 M sorbitol, 20 mM KPi, pH 7.4 and collected at 3500 × *g* for 5 min at 4°C; this washing step was performed twice. The final washed pellet was resuspended in 50 mL of 0.6 M sorbitol, 20 mM KOH-MES, pH 6.0 (BB6.0 buffer) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and subjected to 15 strokes in a tight-fitting (type A) glass dounce kept on ice. 200 µL was set aside and analyzed as the starting material (SM) for cell

fractionation studies. Cell homogenates were centrifuged for 5 min at 1700 *x g* and the supernatant set aside. The pellet was again resuspended with ~50 mL BB6.0 containing PMSF, subjected to 15 strokes in a tight-fitting glass dounce kept on ice, and centrifuged for 5 min at 1700 *x g*. The resulting supernatant was combined with that generated from the first round of dounces. The combined supernatants were centrifuged for 10 min at 13,500 *x g* to sediment mitochondria-enriched pellets (P13). The P13 fraction was resuspended in 35 mL of BB6.0, homogenized with two strokes of a telfon pestle, spun at 1700 *x g* 5 min to remove debris from the supernatant which was again centrifuged at 13,500 *x g* for 10 min. The same procedure was performed a second time with the resulting pellet, except that 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4 (BB7.4) was used in place of BB6.0 and PMSF was omitted. When additional subcellular fractions were collected, 35 mL of the supernatants from the original 13,500 *x g* centrifugation step was centrifuged at 21,500 *x g* for 15 min to remove remaining mitochondria (P21), and the resulting supernatant centrifuged at 40,000 *x g* for 30 min to separate ER-enriched membranes (P40) from light membranes and cytosol (S40). Protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Catalog No. 23225) and aliquots of SM, P13, P21, P40 and S40 were frozen in liquid nitrogen and stored at -80 °C.

EndoH Treatment in crude mitochondria and microsomal fractions

100µg of each P13 and P40 sample was resuspended with 20µL of 1X glycoprotein denaturing buffer (New England BioLabs) and incubated at 100°C for 10 min. For removal of N-glycans, samples were incubated in a final volume of 40µL containing 1X G5 reaction buffer (New England BioLabs) and incubated at 37°C for 4 hrs in the absence (mock) or presence of 1µL EndoH. 30µg of each sample was resolved by SDS-PAGE and analyzed by immunoblot.

TMT Proteomics

Protein processing

P40 samples, collected through subcellular fractionation and quantified by BCA assay as described above, were resuspended and denatured in 8 M urea with 100 mM ammonium bicarbonate, pH 7.8. Disulfide bonds were reduced by incubation for 45 min at 57°C with a final concentration of 10 mM Tris (2-carboxyethyl) phosphine hydrochloride (Catalog no C4706, Sigma Aldrich). A final concentration of 20 mM iodoacetamide (Catalog no I6125, Sigma Aldrich) was then added to alkylate these side chains and the reaction was allowed to proceed for one hour in the dark at 21°C. Aliquots of 100 µg protein were taken and diluted to 1 M urea using 100 mM ammonium bicarbonate, pH 7.8. Trypsin (V5113, Promega) was added at a 1:100 ratio and the samples were digested for 14 hours at 37 °C.

Tandem Mass Tag (TMT) labeling and cation exchange-based fractionation of peptides

The digested lysates were desalted using C18 Omix tips (Cat number A57003100, Agilent) and dried down in 50 µg portions. For each of the conditions, 50 µg of digested peptides were labeled with the TMT 10plex reagent (Cat number 90113, ThermoFisher). Dried pellets were resuspended in 50 µL of 1X phosphate buffered saline and to each tube, one aliquot of the respective TMT reagent, which was resuspended in 41 µL of acetonitrile, was added. The reaction was allowed to proceed at 21°C for one hour and then quenched via the addition of 8 µL of 1 M ammonium bicarbonate. The samples were dried down and then desalted using Omix tips. The individual TMT labeled samples were pooled and then fractionated using strong cation exchange chromatography on an AKTA Pure 10 (GE Healthcare) equipped with a Luna 5 µm

100 angstrom 150 x 2.1 mm strong cation exchange (SCX) column (Catalog no 00F-4398-B0, Phenomenex). Buffer A was 5 mM KH_2PO_4 in 30% acetonitrile (Catalog no 34998, Sigma Aldrich), pH 2.7. Buffer B was 350 mM KCl (Catalog no PX1405 EM Science) in buffer A. A 200 µL/min gradient was run from 0% B to 50% B over 10 mL, then up to 100% B over 1 mL. A total of 12 fractions were collected during the peptide elution portion of the gradient.

Mass spectrometry (MS)

Individual fractions from the strong cation exchange (SCX) chromatography were desalted using ZipTips (ZTC18S096, EMD Millipore), dried down and resuspended in 0.1% formic acid (Catalog no 94138, Honeywell). Fractions were analyzed by LC-MS on an Orbitrap Fusion Lumos (ThermoFisher) equipped with an Easy NanoLC1200 HPLC (ThermoFisher). Peptides were separated on a 75 µm × 15 cm Acclaim PepMap100 separating column (Thermo Scientific) downstream of a 2 cm guard column (Thermo Scientific). Buffer A was 0.1% formic acid in water. Buffer B was 0.1% formic acid in 80% acetonitrile. Peptides were separated on a two-hour gradient from 0% B to 35% B. Peptides were collisionally fragmented using HCD mode. Precursor ions were measured in the Orbitrap with a resolution of 120,000. Fragment ions were measured in the Orbitrap with a resolution of 50,000. The spray voltage was set at 2.2 kV. Orbitrap MS1 spectra (AGC 1×106) were acquired from 400-1800 m/z followed by data-dependent HCD MS/MS (collision energy 42%, isolation window of 0.7 Da) for a three second cycle time. Charge state screening was enabled to reject unassigned and singly charged ions. A dynamic exclusion time of 60 seconds was used to discriminate against previously selected ions.

Database search

The LC-MS/MS data was searched against a *Saccharomyes cerevisiae* (strain ATCC 204508 / S288c) database downloaded from Uniprot on 4/30/2018. Proteome Discoverer version 2.1.1.21 (ThermoScientific) was used to interpret and quantify the relative amounts from the diagnostic fragment ions of the TMT reagent. The database search parameters were set as follows: two missed protease cleavage sites were allowed for trypsin digested with 5 ppm precursor mass tolerance and 0.02 Da for-fragment ion quantification tolerance. Trypsin was set as the protease with up to two missed cleavages allowed. Oxidation of methionine, pyroglutamine on peptide amino termini and protein N-terminal acetylation were set as variable modifications. Carbamidomethylation (C; +57Da) was set as a static modification. TMT 10plex was set as a constant modification on peptide amino termini and lysine residue side chains. Data was searched using the Sequest HT algorithm and the results were filtered via Percolator with a decoy database false discovery rate (FDR) set to < 1% as a filter for peptide identification (Spivak et al., 2009). Volcano plots were generated by RStudio Version 1.3.959.

Overexpression of PISD in HEK cells

HEK cells were cultured in DMEM (Gibco) with 10% heat-inactivated FBS (Gibco). Cells were seeded at 2×10^6 cells per plate on 10-cm plates 24 hours prior to transfection. Cells were transfected using 30 µL of Lipofectamine 3000 (Thermo Fisher Scientific) reagent and 30 µL of P3000™ Reagent with 15 µg of plasmid DNA per plate. Media was changed 48 hours after transfection. Cells were collected for immunofluorescence 72 hours after transfection.

Immunofluorescence

Cover slips were fixed in phosphate-buffered saline buffer containing 4% (v/v) paraformaldehyde at 37°C for 15 min followed by quenching with ammonium chloride (50 µM). Fixed cover slips were permeabilized in 0.25% (v/v) Triton X-100. Samples were incubated with

the following primary antibodies: anti-FLAG (NB600-344; Novus Biological) for PISD, anti-TOMM20 (Sigma; HPA011562) for detection of mitochondria, and anti-calnexin (Millipore; MAB3126) for detection of ER. Appropriate Alexa Fluor–labelled secondary antibodies (Invitrogen) were applied next. Finally, cover slips were mounted for imaging with Dako fluorescent mounting medium (S3023; Agilent Technology).

Imaging and image analysis

Images were captured using 488 nm, 568 nm and 640 lasers on Olympus spinning disc confocal system (Olympus SD-OSR) using 100x lens (UAPON 100XOTIRF) running MetaMorph software in super-resolution mode. Images were originally captured as 11 z-stacks spanning 0.2 μm between each stack. Image stacks were compressed to a single image using max intensity projection and combined into a composite image using ImageJ. To obtain the intensity plot, a line selection was made in the composite image, pixel intensity profile was obtained along the line selection using "get profile" function for each channel and plotted in a line graph.

Phospholipid Analyses

To determine the steady phospholipid profiles in *psd2*Δ*psd1*Δ transformed with the assorted IM-directed Psd1 chimeras, starter cultures were diluted to an OD_{600} = 0.4 in 2 mL of rich lactate medium supplemented with 10 μCi/mL $^{32}P_i$ and grown shaking at 240 rpm for ∼24 hr in a 30°C water bath. For the remaining phospholipid analyses, starter cultures were diluted to an OD_{600} = 0.1 in 2 mL of YPD supplemented with 0.5 μCi/mL ^{14}C-Acetate and grown shaking at 240 rpm for ∼24 hr in a 30°C water bath. Where indicated, cultures additionally contained 2 mM choline, 2mM ethanolamine or 1% (v/v) Tergitol + 0.5mM lyso-PE. Yeast were centrifuged at 1690 *x g* for 5 min, washed with 2 mL MilliQ grade water and re-centrifuged at 1690 *x g* for 5 min. The yeast pellets were resuspended in 0.3 mL MTE buffer (0.65M mannitol, 20mM Tris, pH 8.0, and 1 mM EDTA) containing 1mM PMSF, 10 μM leupeptin, and 2 μM pepstatin A, transferred to a 1.5 mL microcentrifuge tube containing 0.1 mL glass beads, and each tube sealed with parafilm. Yeast were disrupted by vortexing on high for ∼30 min at 4 °C. Glass beads and unbroken yeast were removed after a 2 min 250 × *g* centrifugation at 4°C. The resulting extract was used to assess cellular phospholipid levels. To sediment a crude mitochondrial pellet, the cellular extract was centrifuged for 5 min at 13,000 × *g* at 4°C. Following liquid scintillation, equal amounts of labeled cell extract or crude mitochondria were transferred to 5 mL borosilicate tubes containing 1 mL chloroform and 0.5 mL methanol and vortexed for 30 min on medium-high at room temperature. Phase separation was initiated by adding 0.3 mL 0.9% (w/v) NaCl to each sample which was then vortexed on high for 1 min. Samples were centrifuged at 1000 rpm in a clinical centrifuge for 5 min at room temperature, and the upper aqueous phase aspirated into radioactive waste. The organic phase was washed with 0.25 mL 1:1 Methanol:H_2O, vortexed on high for 30 seconds at room temperature, and centrifuged as before. The lower organic phase was transferred to a new tube and dried down under a stream of nitrogen. Just prior to resolving the phospholipids by thin layer chromatography, dried lipid extracts were resuspended in 13 μL of chloroform and loaded on SILGUR-25 (Machery-Nagel) TLC plates that had been pretreated with 1.8% (w/v) boric acid in 100% ethanol and activated at 95 °C for at least 30 min. Phospholipids were resolved using chloroform/ethanol/H_2O/triethylamine (30:35:7:35) after which the plates were air-dried for 30 min and developed using a K-screen and FX-Imager (Bio-Rad Laboratories).

Reverse transcription-quantitative real-time PCR

Yeast were grown in YPD at 30°C to an OD_{600} of 0.5-0.7, and RNA was extracted using hot phenol extraction (Amin-ul Mannan et al., 2009). 1uL of Turbo DNase (TURBO DNA-free Kit; Invitrogen) was used to remove DNA from 10 µg of RNA, RNeasy MiniElute Cleanup Kit (Qiagen) to purify RNA, and SuperScript VILO Master Mix (Invitrogen) to reverse-transcribe 1 µg of RNA into cDNA. The qPCR was done exactly as described in (Ogunbona et al., 2018) using the FastStart Universal SYBR Green Master Rox (Roche), a 1:10 dilution of synthesized cDNA as template, and 100nM of primers for a total reaction volume of 20 µL. The primers used are *CIS1* forward, 5'-AATGCAGGAGTTGCTTACA-3'; *CIS1* reverse, 5'-TTCTTCCGCTATAGTCTCCAA-3'; *PSD1* forward, 5′-CCAGTAGCACAAGGCGAAGA-3′; *PSD1* reverse, 5′-GACATCAAGGGGTGGGAGTG-3′; and the housekeeping genes *ACT1* forward, 5′-GTATGTGTAAAGCCGGTTTTG-3′; and *ACT1* reverse, 5′-CATGATACCTTGGTGTCTTGG-3′. For controls, we included no-template and minus-RT controls. Samples were run using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). The Ct value differences between *ACT1* and target genes (*PSD1* or *CIS1*) were computed to compare mRNA levels in the tested strains.

Submitochondrial localization

The submitochondrial localization assay, adapted and modified from (Claypool et al., 2006), determines the accessibility of proteins to protease (100 µg/mL Proteinase K) in intact mitochondria, mitoplasts with osmotically ruptured OMs, and detergent solubilized mitochondria. For intact mitochondria, 150 µg of mitochondria were placed in two tubes and resuspended in 1mL of BB7.4 lacking or containing Proteinase K. In parallel, 600 µg of mitochondria was centrifuged at 8,000 *x g* for 5 min at 4°C and the resulting pellet resuspended in 200 µL of BB7.4 and then aliquoted into four microfuge tubes, each containing 50 µL. To rupture the OM only, 950 µL of 20 mM K^+HEPES pH 7.4 lacking or containing Proteinase K was added to two tubes which were vortexed on medium for 15 sec. To provide access to all mitochondrial compartments, 950 µL of 0.5% (w/v) deoxycholate in 20 mM K^+HEPES pH 7.4 lacking or containing Proteinase K was added to the final two tubes which were vortexed on medium for 15 sec. All samples were incubated on ice for 30 minutes after which 20 mM PMSF was added prior to centrifuging each sample at 21,000 × *g* for 10 min at 4 °C. For intact mitochondria and OM-ruptured mitoplasts, after aspirating the supernatant, the pellet was resuspended in 180 µL of BB7.4 + 1 mM PMSF and transferred to a new tube that contained 20 µL of 100% (w/v) TCA. For detergent solubilized mitochondria, the supernatants were transferred to new tubes that contained 0.2 mL of 100% (w/v) TCA. All tubes were incubated on ice for 1 h, and centrifuged at 21,000 *x g* for 10 min at 4 °C. After the resulting supernatants were discarded, the pellets were washed with 0.5 mL of cold acetone, spun at 21,000 × *g* at 4°C for 2 minutes, and the supernatants aspirated. 30 µL of 0.1 M NaOH was added to tubes to resuspend the pellets, after which 30 µL of 2X RSB was added. Samples were incubated at 95°C for 5 minutes and then resolved by SDS-PAGE and analyzed by immunoblot.

Aggregation Assay

For the detergent based aggregation assay, 200 µg of mitochondria was centrifuged at 21,000 *x g*, 4°C for 5 min to pellet mitochondria. Post-aspirating the supernatant, the pellets were resuspended in 40 µL 1.5% (w/v) digitonin lysis buffer (20 mM HEPE-KOH, pH 7.4, 100 mM NaCl, 20 mM Imidazole, 1mM $CaCl_2$, 10% glycerol) containing 1mM PMSF, 10 µM leupeptin,

and 2 μM pepstatin A. The samples were incubated on ice and flicked to mix every 10 min for 30 min. Next, samples were centrifuged at 21,000 *x g*, 4°C for 10 minutes and the supernatants (S1) transferred to new tubes containing 50μL 2X RSB. Pellets were resuspended with 40 μL 1% (v/v) TX-100 lysis buffer (20 mM HEPE-KOH, pH 7.4, 100 mM NaCl, 20 mM Imidazole, 1mM $CaCl_2$, 10% glycerol) supplemented with 1mM PMSF, 10 μM leupeptin, and 2 μM pepstatin A, kept on ice and flicked to mix every 10 min. After 30 min, samples were centrifuged at 21,000 *x g*, 4°C for 10 minutes and the supernatants (S2) transferred to new tubes containing 50μL 2X RSB; the pellet (P2) was resuspended in 90 μL of 1X RSB. All samples were incubated for 5 min at 95°C, resolved by SDS-PAGE and analyzed by immunoblot.

Homology model

Homology modeling of Psd1 based on the *E. coli* PSD structure (PDB code: 6L06) was performed using SWISS-MODEL (https://swissmodel.expasy.org) (Waterhouse et al., 2018). Psd1 sequence (residues 120-500), except the signal sequence and the transmembrane region at the N-terminus, was used for modeling.

Cycloheximide and proteasome inhibition experiments

Cycloheximide (CHX) degradation experiments were adapted and modified from (Claypool et al., 2011). Starter cultures of yeast grown in YPD at 30ºC overnight were diluted to an OD_{600} = 1 in a total volume of 10 mL YPD or YPD + 2 mM choline and incubated for 5 min at 30ºC shaking at 220 rpm. To inhibit cytosolic protein synthesis, CHX was added at 200 μg/mL. For the MG132 drug treatment, starter cultures of yeast grown in YPD at 30ºC overnight were collected and then resuspended to an OD_{600} = 1 in a total volume of 10 mL SCD + 2mM choline. Following the addition of 80 μM of MG132 or an equivalent volume of DMSO, samples were incubated for 1 hour at 30ºC shaking at 220 rpm prior to the addition of 200 μg/ml of CHX. At the indicated time points, 1 OD_{600} units of cells were transferred to a tube containing an equal volume of ice cold 2X azide mixture (20 mM NaN_3 and 0.5 mg/ml bovine serum albumin) and yeast pellets collected following 1690 *x g* centrifugation for 10 min at 4ºC. The pellets were frozen at -80ºC until all time points were collected at which point cell lysates were harvested as already described.

RNA-Sequence

Yeast strains were grown in YPD (D), YPD containing 2 mM ethanolamine (E), YPD containing 2 mM choline (C), or YPD containing 1% (v/v) tergitol and 0.5 mM lyso-phosphatidylethanolamine (L), with shaking at 220 rpm, to an OD_{600} between 0.5-0.7. RNA was extracted using the hot phenol extraction (Amin-ul Mannan et al., 2009). At least 50 ng/μl (for a total of 2 μg) of RNA per sample were sent to Novogene for quality tests, library construction, sequencing, and data analysis, detailed below.

Data analysis

Saccharomyces cerevisiae release 98 reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5). HTSeq v0.6.1 was used to count the read numbers mapped of each gene. FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis between two conditions/groups

(three or four biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). The resulting p-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR).

Heatmaps

Genes presented in Figures 7E and 7F exhibited the largest degree of increase or decrease in fold change (FC) in LGS mutant (::LGS) YPD versus WT (::WT) YPD, all having adjusted p-values of less than 0.05. Genes encoding retrotransposons, putative proteins of unknown function, or those corresponding to dubious open reading frames were excluded. Heatmaps were created with Prism 8.

Data access

The high-throughput sequencing data from this study have been submitted to the Gene Expression Omnibus under accession number GSE162987.

In Vivo Retro-translocation assay

The *in vivo* retro-translocation assay was adapted and modified from (Neal et al., 2018). Cells were grown to log phase (OD_{600} 0.2-0.3) at 30ºC and 15 OD_{600}'s of cells were pelleted. Cells were resuspended in H_20, centrifuged and lysed with the addition of 0.5 mm diameter silica beads (Biospec) and 400µL of XL buffer (1.2 M sorbitol, 5 mM EDTA, 0.1 M KH_2PO_4, final pH 7.5) with protease inhibitors, followed by vortex in 1 min intervals for 6-8 min at 4ºC. Lysates were combined and clarified by centrifugation at 2,500 *x* g for 5 min. Clarified lysate was ultracentrifuged at 100,000 *x* g for 15 min to separate pellet (P100) and supernatant fraction (S100). P100 pellet was resuspended in 200µL of 1.5% (w/v) digitonin lysis buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 20 mM Imidazole, 1mM $CaCl_2$, 10% glycerol) with protease inhibitors and 5 mM N-ethyl maleimide (NEM, Sigma) and incubated for 2 hours at 4ºC followed by centrifugation of extract for 30 min at 4ºC. Clarified extract was transferred to 600 µL of basic lysis buffer (20mM HEPES-KOH, pH7.4, 100mM NaCl, 20mM Imidazole, 1mM $CaCl_2$, 10% glycerol) with protease inhibitors and NEM containing 30 µL 1:1 anti FLAG Affinity Gel (Genscript). S100 supernatant was added directly to 600 µL of basic lysis buffer with protease inhibitors and NEM containing 30 µL 1:1 anti FLAG Affinity Gel (Genscript). All samples were incubated overnight at 4ºC. Samples were washed once with 0.1% (w/v) Digitonin wash buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 20 mM Imidazole, 1mM $CaCl_2$), washed once with 0.1% (w/v) High Salt Digitonin wash buffer (20 mM Tris-Cl, pH 7.4, 250 mM NaCl, 20 mM Imidazole, 1mM $CaCl_2$) and washed once more with 0.1% (w/v) Digitonin Low Salt wash buffer (20 mM Tris-Cl, pH 7.4, 20 mM Imidazole, 1mM $CaCl_2$), aspirated to dryness, and resuspended in 2X Urea sample buffer (8 M urea, 4% SDS, 1mM DTT, 125 mM Tris, pH 6.8), and incubated at 55ºC for 10 min. Samples were resolved by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal anti-ubiquitin (Fred Hutchinson Cancer Center, Seattle), anti-FLAG for Psd1-α, anti-Psd1(Tamura et al., 2012) for Psd1-β , Mito β-α, and ER β-α, and goat anti-rabbit or anti-mouse (Bio-Rad) conjugated with horseradish peroxidase (HRP) to recognize the primary antibodies. Western Lightning® Plus (Perkin Elmer, Watham, MA) chemiluminescence reagents were used for immunodetection.

Proteolytic removal of ubiquitin from retrotranslocated Psd1 LGS mutant

Ubiquitin removal was accomplished with the broadly active Usp2 ubiquitin protease as described (Garza et al., 2009), except that human recombinant Usp2Core (LifeSensors Inc., Malvern, PA) was used, and leupeptin and NEM were excluded from all buffers. Briefly, 100 µL

of S100 supernatant containing the *in vivo* retrotranslocated Psd1-LGS mutant was incubated with 20 µL of Usp2Core (5 µg) for 1 hr at 37ºC. The reaction was quenched with 200 µL of SUME (1% SDS, 8 M Urea, 10 mM MOPS, pH 6.8, 10 mM EDTA) with protease inhibitors and retrotranslocated Psd1-LGS mutant was immunoprecipitated as described above. 20 µL of the bound material was used for detection of Psd1-LGS mutant with anti-Psd1 antibody.

Antibodies

Antibodies employed in this study include rabbit antibodies specific to yeast Psd1 (Tamura et al., 2012), Qcr6 (Baile et al., 2013), Hsp70 (Claypool et al., 2006), Pic1 (Whited et al., 2013), Tom70 (Baile et al., 2013), Tim54 (Hwang et al., 2007), Abf2 (Calzada et al., 2019), Kgd1 (Glick et al., 1992), Cho1 (Choi et al., 2010), Kar2 (Huyer et al., 2004); and mouse monoclonal antibodies against Sec62 (gift of David Meyers, University of California, Los Angeles, Los Angeles, CA), Aac2 (Panneels et al., 2003), FLAG (clone M2, catalog number F3165, Sigma), Ubiquitin (clone P4D1, catalog number sc-8017, Santa Cruz Biotechnology), and Cox2 (Anti-MTCO2 antibody [4B12A5] ab110271, Abcam). Mature Tam41 (Arg35-stop codon) was cloned into pET28a (Novagen) downstream of the encoded His_6 tag, induced in BL21-CodonPlus(DE3)-RIL *Escherichia coli* and affinity purified with Ni^{2+} agarose (Qiagen). The purified His_6Tam41 protein was used as an antigen in rabbits (Pacific Immunology). Other antibodies used were horseradish peroxidase-conjugated (Thermo Fisher Scientific), and fluorescence-conjugated secondary antibodies (Pierce).

Miscellaneous

Quantity One Software (Bio-Rad Laboratories) was used to quantify immunoblots and TLC plates. Statistical comparisons were performed using SigmaPlot 11 software (Systat Software, San Jose, CA) or Prism 8 (GraphPad); *P* values of ≤ 0.05 were deemed significant. All the graphs show means, and error bars are standard errors of the mean (SEM). All figure panels are representative of at least three biological replicates, unless otherwise notes.

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