SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS:

Plasmids and Yeast Strains

The pGAL-OSH1, OSH2 and OSH3 multicopy plasmids (pOSH1, -2, -3) used in this study were a kind gift from C. Beh [1] and express the native untagged protein under control of the galactose promoter (GAL1-10). pGST-PAH1 was isolated from the GST-ORF yeast array [2] and confirmed by sequencing. It expresses Pah1 tagged at the N-terminus under control of the galactose promoter. HA-tagged Pah1 (pPAH1) and the catalytically dead D398E variant (pPAH1 D398E; containing the D398E mutation in the Pah1 coding sequence) were a kind gift from G. Carman [3]. The pOPI3 plasmid is based on pRS416 (CEN, URA3) [4] and expresses the Opi3 protein with an N-terminal myc tag (MEQKLISEEDL) under control of the constitutive portion of the *PHO5* promoter and was constructed by amplifying the *OPI3* coding sequence from yeast genomic DNA and cloning the PCR product into the Bgl2 (5') and SacI (3') restriction sites. pSCS2 was constructed as pOPI3 except that the *SCS2* gene was inserted. pCHO2 was obtained from the MoBY-ORF collection [5] and contains the *CHO2* gene with its endogenous promoter and terminator cloned into a centromere-based vector. RFP-ER contains the C-terminal transmembrane domain of Scs2 fused to monomeric DsRed in pRS416 (CEN, URA) under control of the PHO5 promoter [6].

All yeast strains are based on S288C. Deletion strains were obtained from freezer stocks of the haploid yeast deletion collection (BY4741, MAT a, KanMX), a gift from C. Boone. All deletion strains were confirmed by PCR. GFP-tagging of endogenous proteins was done by homologous recombination of PCR-generated fragments in haploids at the C-terminus of the endogenous protein using the pKT128 (SpHIS5) plasmid [7] in the BY7043 background [8]. Gene deletion strains were constructed in BY4741 using PCR-generated fragments from pKT127 (KanMX) [7] or in BY7092 using p4339 (NatR) [8]. Double and triple deletion strains were generated by standard yeast genetic techniques.

Yeast growth assays

10- fold serial dilutions of log phase cells were spotted using a pin-frogger (Sigma) onto agar plates containing synthetic defined (SD) media with the appropriate amino acid dropouts and either 2% glucose or 2% galactose as indicated. Ethanolamine, choline or monomethylethanolamine were added to SD media to a final concentration of 1 mM. All growth assays were performed at 30°C for at 24-48 hours. All growth assays were repeated independently at least two times.

Array-based genome-wide suppressor screen

The array-based genome-wide suppressor screen for the Δ*scs2*Δ*ice2* mutant was performed by modifying the synthetic genetic array (SGA) method [8] and using the GST-ORF collection (Open Biosystems) in place of the deletion mutant array [2]. GST-ORFs are expressed under control of the GAL1/10 promoter and tagged at the N-terminus with GST. The GST-ORF collection was arrayed using a RoToR HDA robot (Singer Instruments) at a density of 1536 spots per plate onto SD media containing glucose. This array was crossed with a Δ*scs2::NatMX* Δ*ice2::KanMX* mutant query strain (Y7092 background) grown on SD media containing choline and diploids were selected on medium also containing choline prior to sporulation for 5 days. Haploid Δ*scs2*Δ*ice2* double mutant progeny containing the GST-ORF plasmids were recovered on media lacking His, Arg, Lys, and containing canavanine, thialysine, G418, and Nat. The final pinning step was to media containing 2% galactose and lacking choline. Suppressors were identified by visual inspection of plates and GST-ORF plasmids were recovered and sequenced to confirm the identity of the suppressors.

Confocal microscopy

Yeast strains expressing GFP-tagged proteins were grown to log phase and imaged by squashing 1-5 microlitres of live yeast in media between a slide and cover slip. Yeast were examined at room temperature using a Zeiss LSM-5 Pascal confocal microscopy system equipped with a Zeiss 100X objective (Plan-neofluar, 1.3) and images were acquired with Zeiss Pascal software. All confocal microscopy images within a given experiment were taken with identical microscope settings to enable direct comparison between images and samples. Image brightness was adjusted uniformly for the entire image using Adobe Photoshop software. Quantification of Opi3-GFP (Fig. S2 B) from confocal images was done using Image J software (NIH) by drawing a line across the nuclear ER and determining the maximum pixel intensity from the resulting plot. Two separate measurements were taken per cell and averaged and a minimum of 20 cells per strain was analyzed. P values were calculated using an unpaired Student's two-tailed t-test. All experiments were repeated independently at least two times.

Quantification of PM-ER contact sites by transmission electron microscopy

For thin-section electron microscopy, cells were prepared essentially as described previously [9]. Briefly, log-phase cells growing in SD media with galactose and choline were harvested. Cells were washed twice with water and incubated in freshly prepared potassium permanganate solution (1.5%) twice for 30 min at 4°C. The cells were washed several times in water, and dehydrated with increasing

concentrations of acetone and embedded in Spurr's resin. Sections approximately 50 nm thick were cut with a Leica UCT microtome and stained with 4% uranyl acetate for 20 minutes, followed by Reynold's lead citrate at room temperature for 6 min. Thin sections were viewed on a Hitachi H7600 transmission electron microscope (located at the UBC Bioimaging Facility). To quantify PM-ER contacts in each cell we identified sections of ER, which are electron-dense linear structures, within 60 nm of and parallel to the PM in TEM images at 80,000 x magnification (approx. 5-10 images per cell). PM-ER contact length was measured by tracing each segment using ImageJ software (National Institutes of Health). The whole-cell perimeter was measured from a single 20,000 x magnification image for each cell by tracing the plasma membrane. Contact length was calculated as the average of the lengths of the contacts for a given cell. The ER:PM ratio was calculated as the sum of the length of the contacts divided by the length of the plasma membrane perimeter for each cell. The contact frequency (per µm) was calculated by dividing the number of contacts in a given cell by the perimeter of that cell. A minimum of 15 budded cells were analyzed per condition. Measurements were assumed to fit a gaussian distribution and P values were calculated using an unpaired Student's one-tailed t-test.

In vivo methylation assay

To monitor conversion of PE to PC, 100 ml of cells were grown in SD media containing either 2% raffinose and 2% galactose or 2% dextrose to an OD600 of 0.5. For the experiments shown in Fig. 1 G and Fig. S1 D, SD media was supplemented with 1 mM choline to support growth of the Δ*cho2* and Δ*scs2*Δ*ice2* mutants, otherwise yeast were grown in the absence of choline. Cells were collected by centrifugation, washed once in 0.67% Yeast Nitrogen Base (YNB) then resuspended in 12 ml YNB + 2% raffinose, 2% galactose (YNB-RG). After addition of 24 µCi of [^{3}H]-ethanolamine, cells were shaken at 37 °C for 0.5 - 1 h. Cells were then collected by centrifugation, washed once in 1.6 ml YNB-RG, resuspended in 120 mL SD media and shaken at 30 °C. Aliquots of 20 ml were removed every hour, collected by centrifugation, washed once in 1 ml water, and then frozen for subsequent analysis.

After thawing cell pellets and washing once in 1 ml water, cells were resuspended in 200 µl water and glass beads added to the top of the meniscus. Cells were lysed by three 30 s cycles of agitation in a Precellys 24 tissue homogenizer. The lysate was collected by washing the beads twice with 800 µl water and transferred to a glass tube. Lipids were extracted by the addition of 6 ml 2:1 methanol:chloroform, and vortexed once, followed by addition of 2 ml of 0.9% w/v NaCl and vortexing once more [10]. Phase separation was achieved by centrifugation at 1500 g for 1 min. The upper aqueous phase was discarded and the lower organic phase transferred to a clean tube. The solvent was evaporated under a stream of nitrogen and the resultant lipid film resuspended in 60 µl chloroform

containing 2 mg/ml PS/PE/PC standards. Following addition of 140 µl of HPLC buffer (90:3:1 acetonitrile:methanol:phosphoric acid) [10], the entire sample was injected into a GE Healthcare AKTA FPLC system and lipids separated on a LiChrospher 60 Si column. Fractions corresponding to the PE and PC peaks were collected and activity determined by scintillation counting. A background reading corresponding to column flow-through between the PE and PC fractions was subtracted from the counts in the PE and PC fractions. PE to PC conversion rates were calculated from the initial linear portion of the assays (minimum of three time points, beginning at t=0 h) and regression analysis was performed using GraphPad Prism software to determine the slopes and the associated error. Experiments were replicated independently at least two times. PC synthesis rates are presented as [PC/(PC+PE)]/h. P values were calculated using an unpaired Student's two-tailed t-test.

In vitro Opi3 methylation assay

Microsomes containing Opi3 were isolated from wild type strain D273-10B as described [11], and pelleted through 20% (w/v) sucrose in buffer (0.6 M sorbitol, 50 mM Tris-HCl pH 7.5) at 130,000 g for 1 h (SW41 rotor, Beckman) before use. [3H]-PME-loaded mitochondria from a Δ*opi3* strain SH414 prepared as described [11] were subjected to lipid extraction. The dried mitochondrial lipid extract was hydrated in buffer, and large unilamellar vesicles (liposomes) were obtained by extrusion through 200 nm pore size filters after 10 freeze-thaw cycles [12]. Microsomes at 0.5 mg protein/ml were incubated with [3H]-PME-loaded liposomes at 0.4 mM phospholipid-phosphorous in buffer supplemented with 5 mM S-adenosyl-L-methionine at 30°C for 2 h. Lipids were extracted before or after separating liposomes from microsomes on a 20% (w/v) sucrose cushion in buffer also containing 1 mM EDTA by centrifugation at 130,000 g for 1 h. Recovery of [3H]-labeled lipid was determined by liquid scintillation counting, and the conversion of [3H]-PME was quantitated after thin layer chromatography as described [13]. Lipid extraction was performed (according to [14]) and phospholipids were quantitated (according to [15]). Protein concentrations were determined using the BCA method (Pierce) with 0.1% (w/v) SDS added and BSA as a standard. Experiments were replicated independently at least three times. P values were calculated using an unpaired Student's two-tailed t-test.

NBD-PC, NBD-PE and FM4-64 cellular uptake assays

The assays were performed essentially as described in (Pomorski et al., 2003). Briefly, stock solutions of NBD-PE and NBD-PC (10 mM; Avanti Polar Lipids) were prepared in DMSO. Log phase cells were pre-incubated at 4°C and NBD-lipids were added to a final concentration of 100 µM, vortexed thoroughly, and incubated at either 4°C or room temperature for the indicated length of time. After

incubation, cells were washed in ice-cold media without glucose, containing 2% sorbitol and 20 mM NaN_3. Cells were then mounted on a microscope slide for confocal microscopy. For FM4-64 labeling, log phase cells were incubated either at 4°C or room temperature with 40 μM FM 4-64 for 15 and 60 minutes. Cells were then washed twice in ice-cold media and mounted on a microscope slide for confocal microscopy. Experiments were replicated independently at least two times.

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SUPPLEMENTARY FIGURES:

Figure S1. (A) Yeast growth assays of mutants containing either a plasmid expressing Cho2 (+pCHO2) or empty vector (-) on SD media lacking choline. (B) Yeast growth assays of mutants on SD media in the absence (-) or presence of 1 mM monomethylethanolamine (+MME). (C) Yeast growth assays of indicated mutants on SD media without choline or ethanolamine (-), with choline (+Cho) or with ethanolamine (+Etn). (D) Incorporation of [^{3}H]-ethanolamine into PC over time in wild type, Δ*cho2* and Δ*scs2*Δ*ice2* yeast grown in the presence of choline. Plotted is the fraction of counts in PC out of total PE+PC counts at each time point. (E) Incorporation of [^{3}H]-ethanolamine into PC over time in wild type and Δ*scs2*Δ*ice2* cells expressing Opi3 from a plasmid grown in the absence of choline. Plotted is the fraction of counts in PC out of total PE+PC counts at each time point. Error bars = SD.

Figure S2. (A) Yeast growth assays of WT and Opi3-GFP yeast on SD media lacking choline. (B) Quantification of Opi3-GFP in the nuclear ER of WT and Δ*scs2*Δ*ice2* mutant yeast (n = number of cells; error bars = SD). (C) Images of WT and Δ*scs2*Δ*ice2* yeast cells (transformed with the indicated plasmids) expressing endogenous Tcb3-GFP grown on SD media containing galactose. Asterisks indicate regions with disorganized pmaER in the mutants. Scale bars = 2 μm. (D) Incorporation of [^{3}H]-ethanolamine into PC over time in wild type and Δ*scs2*Δ*ice2* cells expressing Osh3 from a plasmid grown in the absence of choline. Plotted is the fraction of counts in PC out of total PE+PC counts at each time point. Error bars = SD.

Figure S3. (A) Incorporation of [^{3}H]-ethanolamine into PC over time in wild type and Δ*scs2*Δ*ice2* cells expressing GST-Pah1 from a plasmid grown in the absence of choline. Plotted is the fraction of counts in PC out of total PE+PC counts at each time point. Error bars = SD. (B) Growth assays of wild type and Δ*cho2* yeast expressing GST-Pah1 from a plasmid grown on SD media containing galactose and lacking choline. (C) Yeast grown on SD media with Cho (-) and with Cho and 1 mM diothiothreitol (+DTT). (D) Images of Δ*scs2*Δ*ice2* yeast expressing endogenous Opi3-GFP and transformed with a plasmid expressing GST-Pah1 grown on SD medium containing galactose and lacking choline. Scale bar = 2 μm.

Figure S4. Representative transmission electron microscopy images of wild type and Δ*scs2*Δ*ice2* yeast expressing GST-Pah1 from a plasmid (+PAH1) grown on SD media containing galactose and choline. pmaER has been indicated on the images by tracing in red and nuclear ER has been traced in blue. n, nucleus; v, vacuole. Scale bars = 500 nm.

Figure S5. (A) Lipid radiolabel recovered after separation of liposomes and microsomes in the *in vitro* Opi3 assay. Radiolabel recovered from the top and bottom of the sucrose cushion after sedimentation is plotted as a percentage of total label before sedimentation. Note that ~30% of total label was not recovered after sedimentation. (B) Endocytosis assay. Uptake of FM 4-64 dye at 4°C (cold) or room temperature (RT) for 15 min in the indicated strains. Note that at 4°C the FM 4-64 dye remained in the plasma membrane whereas at RT it was endocytosed and labelled internal membranes. (C) Lipid transport assay. The indicated strains were labelled with either NBD-PC or NBD-PE for the indicated times at 4°C and imaged. Cells were additionally labeled with FM 4-64 under the same conditions to ensure endocytosis was blocked for the duration of the assay. Δ*lem3* cells were used as a control to show specificity of uptake of NBD-labelled lipids by the non-endocytic lysolipid transport pathway [16]. Asterisks mark unlabeled cells in the Δ*lem3* control. Note that during the time course of the assay, FM 4-64 remained in the plasma membrane whereas NBD lipids were transported in a Lem3-dependent manner to internal membranes that included pmaER (arrows) and the nuclear ER (arrowheads). No differences in transport of NBD-PC or NBD-PE to the ER in the Δ*scs2*Δ*ice2* mutant were observed. Scale bars = 2 μm.

Figure S1

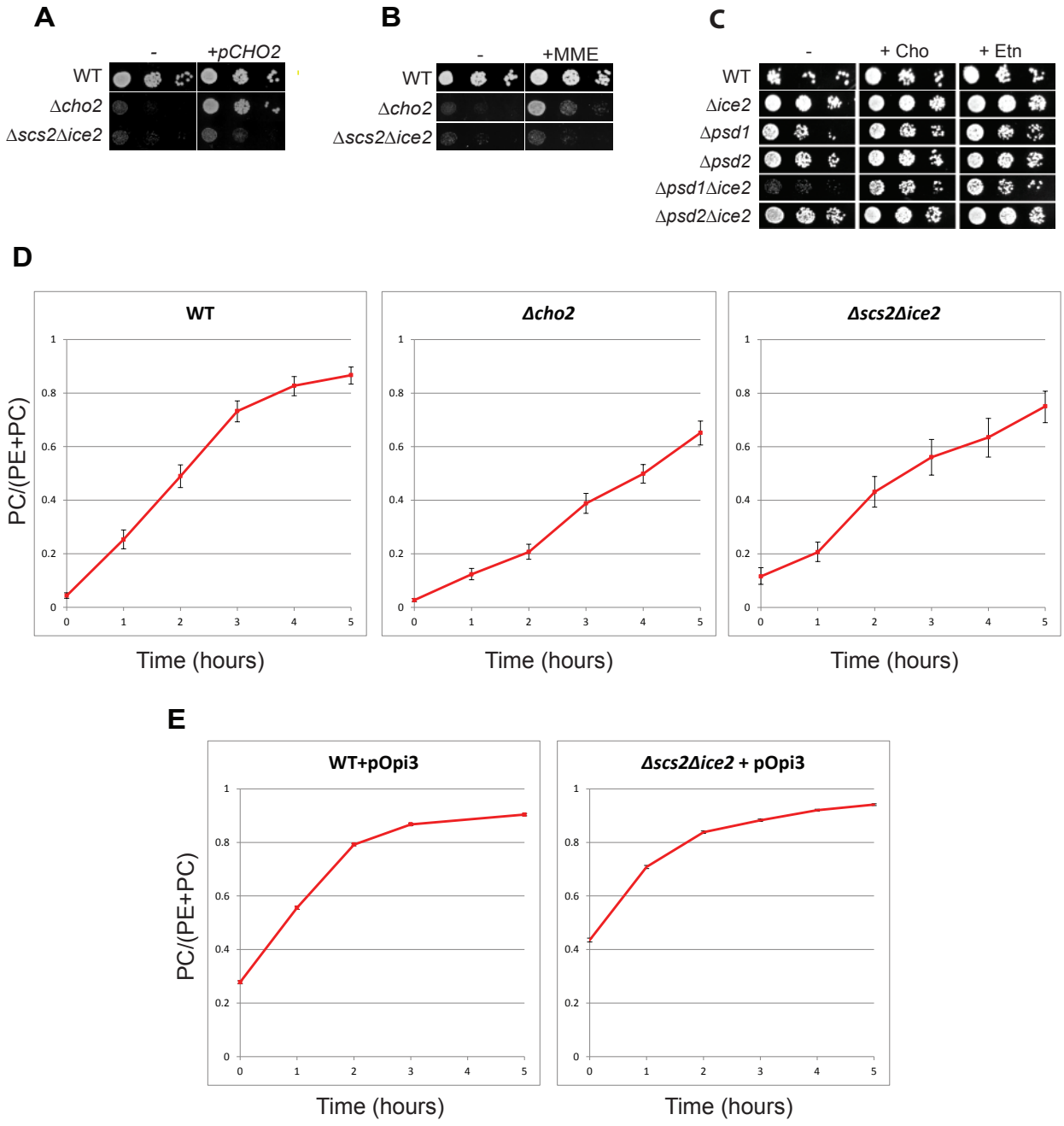

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Figure S2

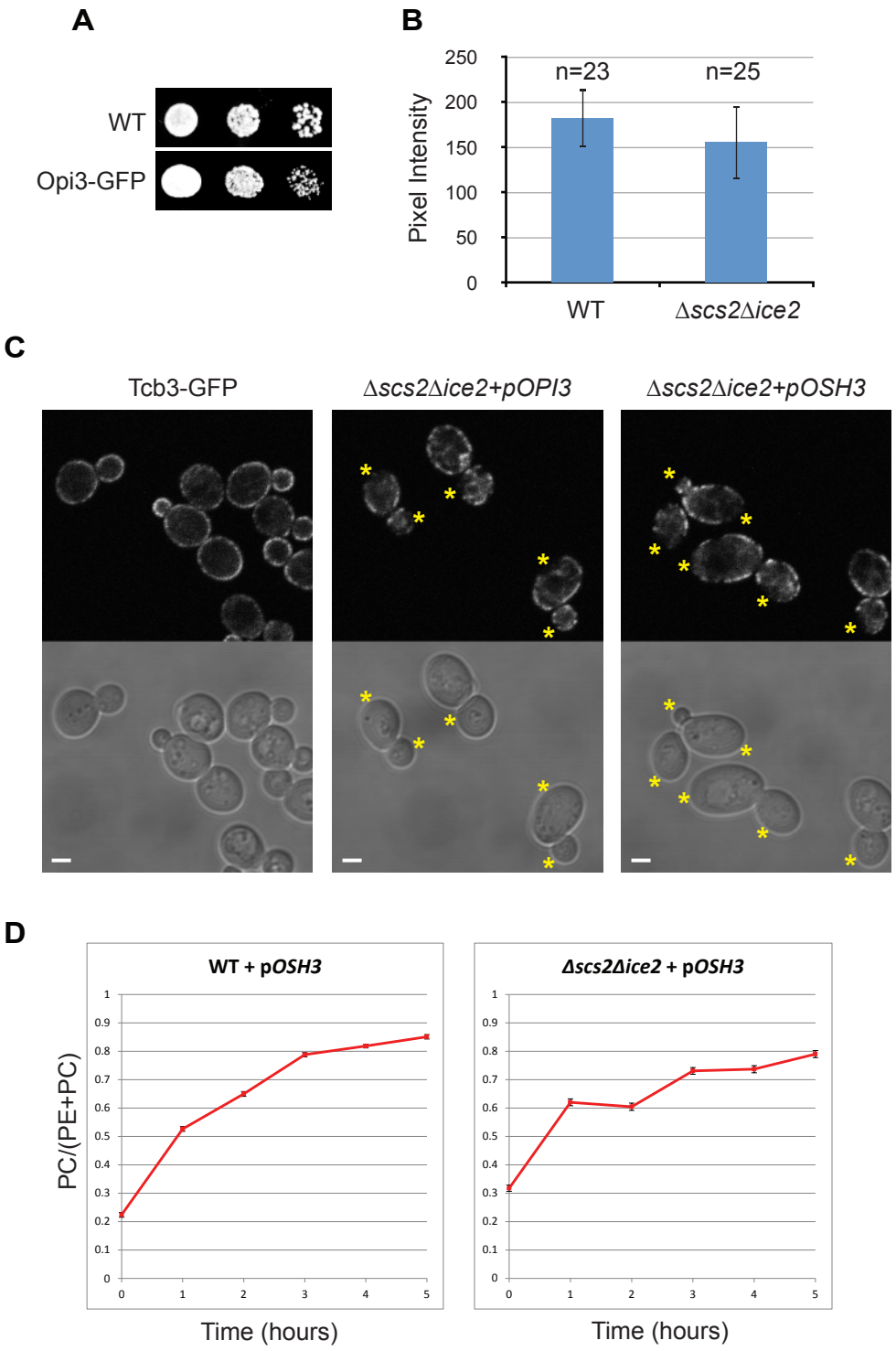

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Figure S3

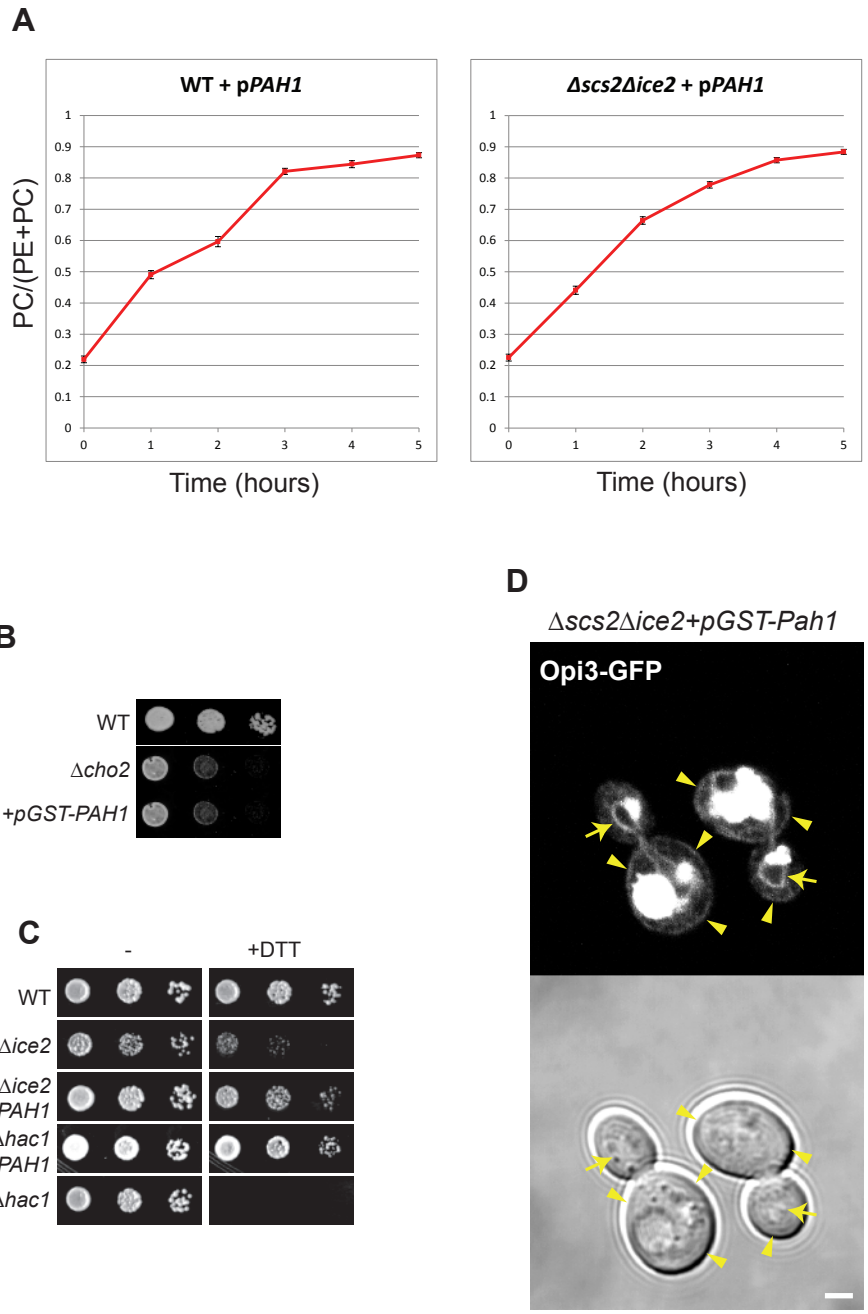

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Figure S4

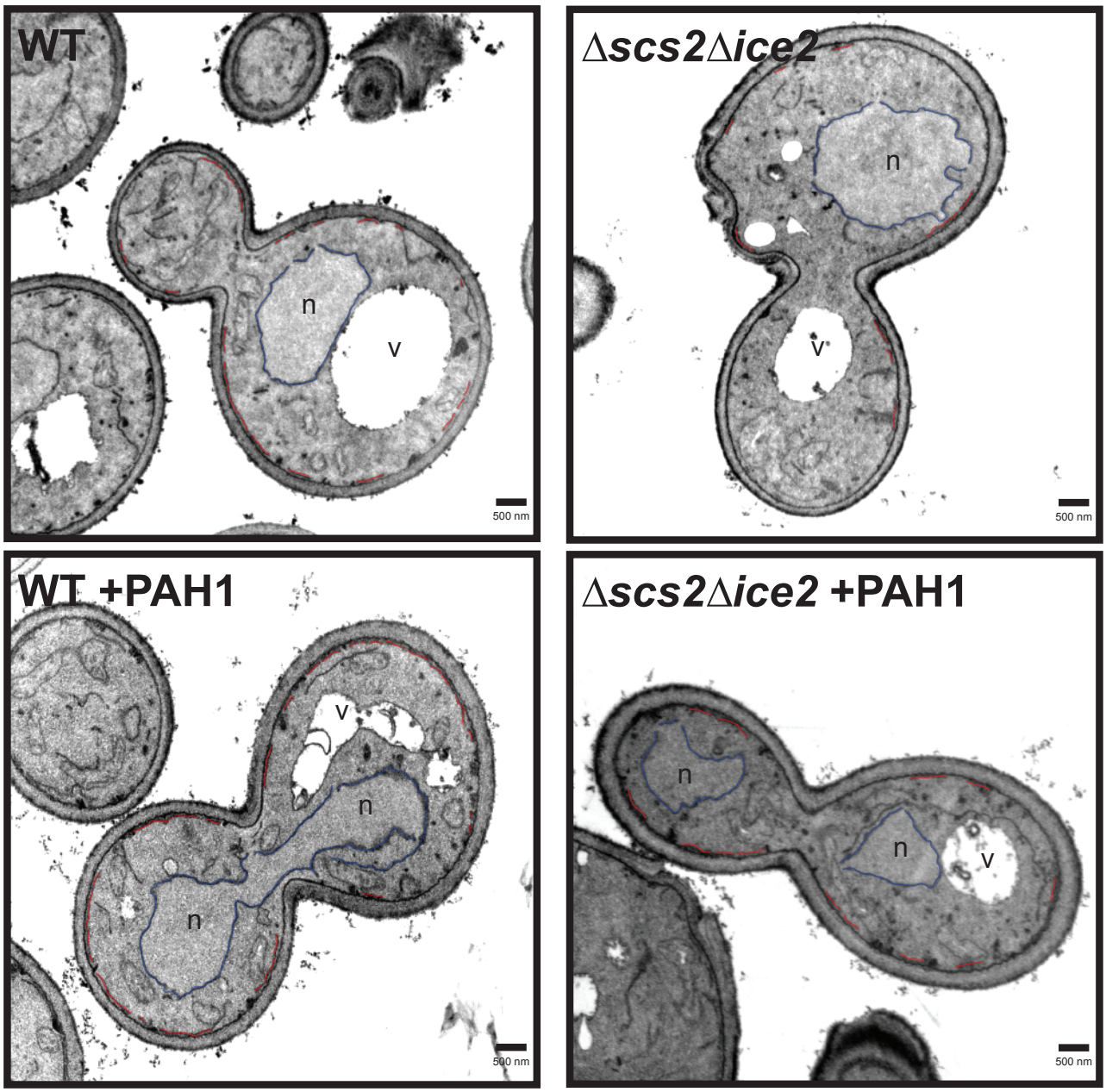

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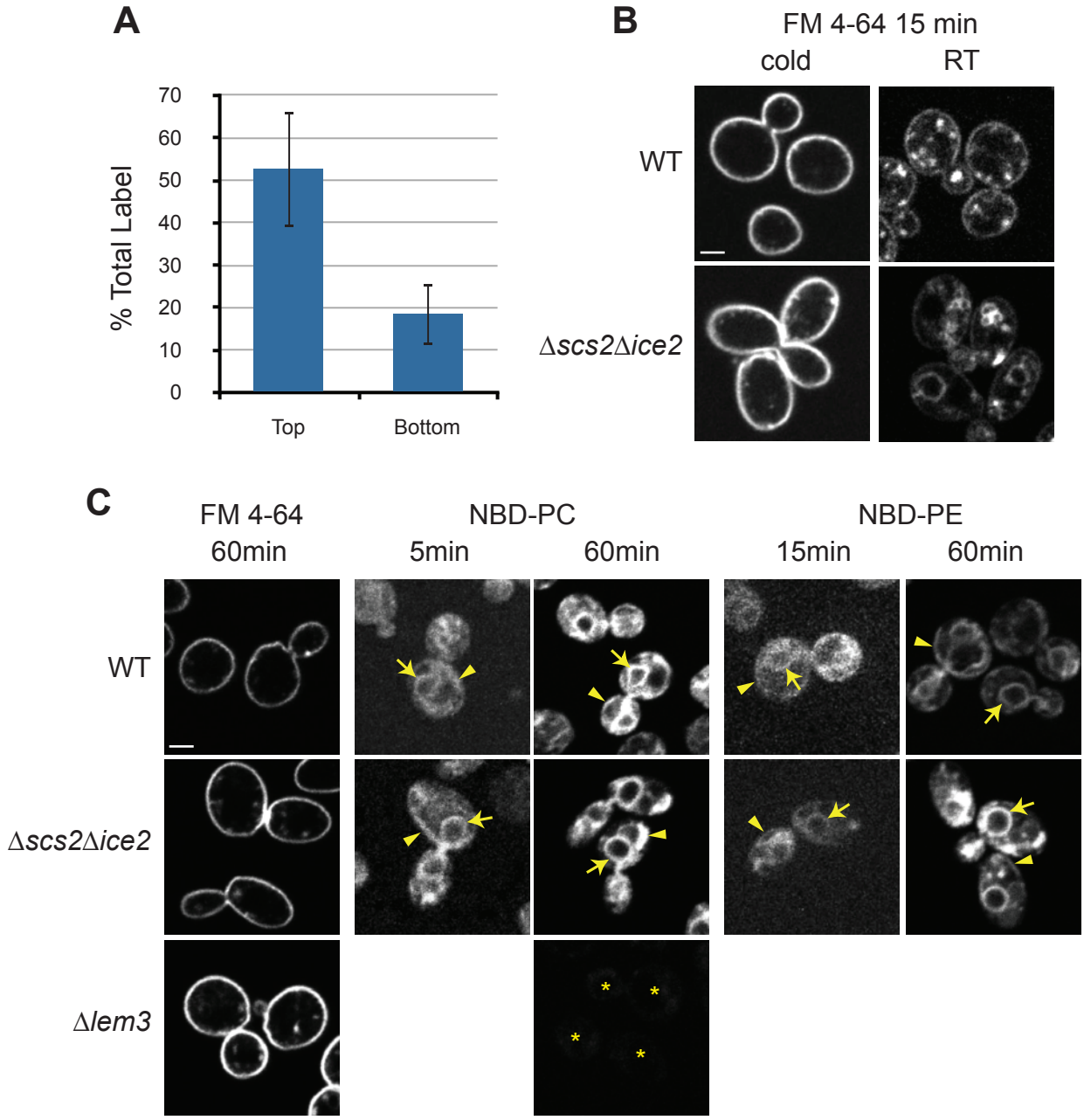
Figure S5



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