Supplemental material for

Characterization of yeast mutants lacking alkaline ceramidases *YPC1* and *YDC1*

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Supplemental Materials and Methods

Materials. FM4-64 was from Molecular Probes, 5'-Fluoroorotic Acid (FOA) from Toronto Research Chemicals, cycloheximide, phytosphingosine, 2,4-dinitrophenylhydrazine and Lucifer Yellow from Sigma. [^{14}C]serine was from ARC, St. Louis, MO; monomethylamine (33% in ethanol) was from Fluka AG, Buchs, Switzerland. Anti-DNP antibodies were from Dako. Protease inhibitors were from Roche Diagnostics GmbH, Mannheim, Germany. 3,3'-dihexyloxacarbocyanine iodide was from AnaSpec.

Synthetic Genetic Array. SGA analysis was performed as described previously (Collins et al., 2010). Briefly, the query strains (Y7092, FBY5162, FBY5173) were robotically crossed against an array of 4978 individual *MAT***a** knockouts of nonessential genes to generate double or triple mutant arrays. The resulting double and triple mutants were then screened for genetic interactions affecting cell growth. When selecting for triple mutants, the plates were further replicated in parallel on plates containing also Aureobasidin A at a concentration, which did not give visible growth inhibition of WT cells (0.03 µg/ml). They also were replicated onto plates containing 25 µM PHS, or 100 mM Ca^{2+}, or onto inositol free medium at 37°C and at 37°C in normal medium. The measurement of growth and following analysis and visualization of the high-throughput screen data were conducted with the help of the ScreenMill software (Dittmar et al., 2010). Additionally, the interactions pointed by the screen were verified by independent crosses, tetrad dissection or random sporulation and by serial dilution plating to assess colony sizes, cloning efficiency and growth rates.

Metabolic labeling of cells with [^{14}C]serine, lipid extraction, mild base treatment and thin-layer chromatography. Cells were grown in synthetic minimal medium. 3.0 OD_{600} units of exponentially growing cells (i.e. 3 ml of a culture having an OD_{600} of 1.0) were harvested, resuspended in 250 µl of the same medium supplemented with 10 µg/ml of cycloheximide (CHX). After 10 min of preincubation, 4 µCi of [^{14}C]serine were added and cells were incubated for 40 min at 30°C. Then the samples were diluted with 750 µl of fresh minimal medium supplemented with CHX and labeling was continued for a further 120 min. Labeling was terminated by adding NaN_3 and NaF (10 mM final concentrations) and chilling cells on ice. Cells were resuspended in chloroform:methanol (2:1) and broken with glass beads in the cold. The extract was kept apart and the pellet was re-extracted sequentially with chloroform:methanol (1:1) and EtOH:H_2O:Et_2O:Pyridin: 25% NH_4OH (15:15:5:1:0.018), which achieves quantitative extraction of all complex sphingolipids (Hanson and

Lester, 1980). Extracts were combined and solvent was evaporated under vacuum in a rotary evaporator. Incorporation into lipids usually amounted to 5 % of added radioactivity. Where indicated, lipids were subjected to mild base hydrolysis with mono-methylamine (MMA). Lipids were resuspended in 400 µl of MMA (33% in ethanol) or, as a negative control, in methanol, and incubated at 53°C for 1 hour. Then, solvents were evaporated under vacuum. All lipids were resolved by ascending TLC on silica gel plates after having been desalted by Folch partitioning as described (Folch et al., 1957). Extracts from metabolically labeled cells were resolved with chloroform/methanol/glacial AcOH (90:1:9) or $CHCl_3$:MeOH:KCl (55:45:5) solvent systems. When the untreated and deacylated lipid extract was run side by side, material from an equivalent number of cells was spotted. Radioactivity was detected and quantified by one- and two-dimensional radioscanning using a Berthold radioscanner and visualized by fluorography or radioimaging using the Bio-Rad Molecular Imager FX.

Isolation of detergent resistant membranes and Triton X-100 solubilization assay. For the isolation of detergent resistant membranes, published protocols were used (Bagnat et al., 2000; Malinska et al., 2004). Crude membranes corresponding to 200 µg protein were incubated in 300 µl cold TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and 1% Triton X-100 for 30 min on ice. Subsequently, the samples were overlaid with an Optiprep (Nycomed) step gradient and centrifuged for 3 h at 208,000 × g in a Beckman SW60 rotor at 4°C. After centrifugation, six equal fractions were collected, and the proteins were immunodetected on Western blots.

FM4-64 staining to monitor endocytosis. FM4-64 was used to stain vacuoles and endosomes as described previously (Baggett et al., 2003). Yeast cells were cultured at 24˚C. Five separate aliquots of 1 ml each were centrifuged and cooled on ice. Each pellet was resuspended in 50 µl of ice cold FM4-64 (20 µg/ml). Tubes were incubated for 20 min in an ice-water bath to allow the dye to label the plasma membrane. For the time point zero, 1 ml ice-cold rich medium without any carbon source was added, cells were centrifuged 3 min at 300 × g, 4°C and washing was repeated. The pellet was resuspended in 50 µl of rich medium with no carbon source and kept on ice from this point onward. Remaining tubes were washed two times with cold rich medium containing a carbon source and finally resuspended in 1 ml of the same. These tubes were placed into a water bath at 26˚C with shaking. Tubes were removed after 5, 10, 20, and 45 min. When tubes were removed, the cells were washed twice with ice-cold rich medium without a carbon source and resuspended in 50 µl of rich medium with no carbon source and kept on ice. For fluorescent visualization, cells were mounted onto Concanavalin A (ConA)–coated cover slips and observed with a rhodamine/TRITC filter.

Lucifer yellow (LY) accumulation in the vacuole. Fluid-phase endocytosis was assayed using the dye LY as described previously (Baggett et al., 2003). Yeast cells were cultured in YPD medium overnight to an OD_{600} of ~ 0.1 at 30˚C. One ml aliquots of cell suspension were sedimented by centrifuging 2 min at 800 × g at room temperature. The cell pellet was resuspended in 90 µl of YPD

and then 10 µl of 40 mg/ml of LY was added. Holes were pierced through the top of the tubes to allow aeration of the cells during the LY uptake step. Tubes were incubated at 24°C for 1.5 hrs in the dark. Next, 1 ml of ice-cold phosphate buffer with 10 mM of NaN_3 and NaF was added and the tubes were centrifuged. Washing was repeated three times, resuspending the pellet between washes. Cells were mounted on ConA–coated cover slips and viewed by fluorescence microscopy using a FITC filter.

CPY secretion assay. Cells were grown overnight to OD_{600} 1-2. 10 OD_{600} units were collected, washed and resuspended in 1 ml of water. Tenfold dilutions of the various strains were deposited onto YPD plates, incubated for 3 days at 30°C and the next day overlaid with nitrocellulose. After 12 h of incubation at 30˚C, the nitrocellulose filter was washed with water and processed for Western blotting using anti-CPY antibodies.

Protein carbonylation assay. The level of protein carbonylation was assessed as described before (Dirmeier et al., 2002).

Supplemental Figure legends

Fig. S1. The localization of mtGFP, Vph1p, Sec63p, Sec7p and Sed5p is normal in yyΔΔ cells. WT and yyΔΔ cells expressing either mtGFP, *VPH1*-GFP, *SEC63*-GFP, *SEC7*-DsRed or GFP-*SED5* from single copy vectors were grown to exponential phase at 30°C, using galactose as a carbon source for the mtGFP and Vph1p expression. mtGFP contains GFP fused to the first 69 amino acids of the subunit 9 of the F_0 ATPase from *Neurospora crassa*, under control of the *GAL1* promoter (Westermann and Neupert, 2000).

Fig. S2. yyΔΔ cells show normal kinetics of endocytosis. a, WT and yyΔΔ cells were grown to early log phase on YPD medium at 24°C. Cells were incubated with FM4-64 (20 µg/ml final concentration) in an ice bath for 20 min, washed and then further incubated at 26°C. After 0, 5, 10, 20 and 45 min cells were visualized under the fluorescence microscope. **b**, exponentially growing cells were incubated for 1.5 h at 24°C in rich YPD medium containing lucifer yellow (LY, 4 mg/ml), washed and viewed under the fluorescent microscope.

Fig. S3. CPY and Gas1p are targeted normally in yyΔΔ. a, tenfold dilutions of the various strains were deposited onto YPD plates, incubated for 3 days at 30°C and overlaid with nitrocellulose. The nitrocellulose filter was processed for Western blotting using anti-CPY antibodies. *vps4*Δ cells are deficient in vacuolar targeting and serve as positive control. **b**, cell membranes of WT and yyΔΔ cells containing either *CAN1*-GFP, *GAS1*-GFP, or *SEC63*-GFP were incubated with 1% of Triton X-100 on ice for 30 min and then loaded at the bottom of a step-density Optiprep gradient (Bagnat et al., 2000). After centrifugation, six fractions were collected and analyzed in a Western blot for the presence of the GFP-marked proteins, the *SEC63*-GFP serving as a detergent sensitive control. Fractions 1-2 contain the detergent resistant membranes floating on top of the gradient, fractions 4 – 6 the soluble proteins

not associated with detergent resistant membrane domains.

Fig. S4. Serine incorporation into lipids in yyΔΔ cells is qualitatively normal. a, WT and yyΔΔ cells were cultured with or without 3 μg/ml of AbA for 1 h. Then the cells were labeled with [^{14}C]serine for 160 min at 30°C in the same medium as used for preincubation. The extracted lipids were deacylated or not with MMA, therewith leaving sphingolipids intact but hydrolyzing labeled glycerophospholipids. Lipids were resolved by TLC in chloroform:methanol:glacial AcOH (90:1:9). **b**, the same as in A but the lipids were resolved in $CHCl_3$:MeOH:0.25% KCl in H_2O (55:45:5), with IPC-C, IPC-D and MIPC highlighted with a red asterisk. Cers = ceramides.

Fig. S5. a, **hydroxylation or desaturation of fatty acids decreases their affinity for Ypc1p**. Reverse ceramidase activity of microsomal detergent extracts of 1Δ.YPC1 cells were assayed in presence of various concentrations of unlabeled fatty acids (0 – 300 nmol) as described in Fig. 5C. **b**, **long chain base specificity of Ypc1p-dependent microsomal reverse ceramidase activity**. The Ypc1p-dependent ceramide synthase activity was assayed under standard conditions but replacing PHS by LCBs that are not normally present in yeast cells. The amounts of ceramide-[^{3}H]C16 are indicated as a percentage of the amounts obtained in the standard assay (5 nmol PHS). Result of a test done in duplicate is indicated.

Fig. S6. Growth of *ypc1*Δ, *ydc1*Δ and yyΔΔ cells on non-fermentable carbon sources. *Ypc1*Δ, *ydc1*Δ, yyΔΔ cells and their isogenic WT were grown to exponential phase, collected and resuspended at OD_{600} of 1.0 in media with different carbon sources, such as dextrose (2%, YPD), ethanol (3%, YPEthanol), glycerol (2%, YPGlycerol) and lactate (2%, YPLactate). Cell density was measured at indicated times by measuring OD_{600}.

Fig. S7. Protein carbonylation in the presence of H_2O_2. yyΔΔ and WT strains were grown to exponential phase in YPD medium, cultures were supplemented with 1 mM H_2O_2 and further grown for 24 hours at 30˚C. After culturing, cells had reached densities of $OD_{600} \approx 10$ and were harvested, spheroplasts were prepared and lysed in hypotonic medium. ER-derived microsomes and mitochondrial membranes were isolated by sedimentation at 12,100 × g, remaining cellular membranes by subsequent centrifugation at 100’000 × g yielding also the cytosolic supernatant fraction. 5 μg of the proteins from each fraction were derivatized with 2,4-dinitrophenylhydrazine. The derivatized proteins were separated by SDS-PAGE and probed with anti-DNP antibodies on a Western blot.

Fig. S8. Chronological life span of *ypc1*Δ and *ydc1*Δ cells. *Ypc1*Δ*::kanMX* and *ydc1*Δ*::kanMX* deletions in the BY background (EUROSCARF collection) were grown to stationary phase ($OD_{600} \approx$ 15) in YPD and then transferred to sterile water (day 0). Cells were kept at 25°C without shaking and CFUs were determined by plating cells at the indicated days onto YPD onto 4 plates at different dilutions. Viability is given as percent of colonies counted at day 0 (=100%), which was > 400 CFUs for all strains. (Due to caloric restriction and to the possibility to feed on dying cells, the CFUs drop

relatively slowly.)

Fig. S9. Localization of Ypc1p-GFP in exponentially growing and stationary cells.
FY1679.YPC1-GFP cells were viewed after having been grown at 30°C in complete synthetic medium to late log phase viewed (OD_{600} = 0.9) and stationary phase (OD_{600} of 10). Cells were analyzed using a Delta vision Deconvolution microscope (Applied Precision, Issaquah, WA) with 100x oil objective and individual Z stacks are shown. (This microscope is different from the one used for Fig. 6B). Diffuse cytosolic fluorescence comes from other Z stacks and is due to amplification. Comparison with Nomarski pictures shows that vacuoles are spared. The white bar represents 6.4 µm. Two consecutive Z stacks of the same cells are shown in the red box.

Supplemental Tables

Table S1. Yeast *Saccharomyces cerevisiae* strains.

Strains	Genotype	Reference
WT (BY4742)	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF, (Frankfurt, GE)
yyΔΔ (FBY2182)	BY4742, but *ypc1Δ::kanMX ydc1Δ::kanMXΔ::natMX4*	This study
ypc1Δ	BY4742, but *ypc1Δ::kanMX*	EUROSCARF
ydc1Δ	BY4742, but *ydc1Δ::kanMX*	EUROSCARF
Y7092	*MAT*α *can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ ura3Δ0 met15Δ0*	C. Boone
ypc1Δ (FBY5162)	Y7092, but *ypc1Δ::LEU2*	This study
yyΔΔ (FBY5173)	Y7092, but *ypc1Δ::LEU2 ydc1Δ::natMX4*	This study
WT.URA3 (FBY5319)	*MAT*α *can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0::loxP-LEU2-loxP ura3Δ0 met15Δ0* containing pNP302	This study
WT.YPC1 (FBY5320)	*MAT*α *can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0::loxP-LEU2-loxP ura3Δ0 met15Δ0*	This study
yyΔΔ.GAL-YPC1 (FBY5179)	As FBY2182, but containing pBF842	This study
yyΔΔ.GAL-HIS3 (FBY5181)	As FBY2182, but containing pBF841	This study
yyΔΔ.YPC1 (FBY5182)	As FBY2182, but containing pYPC1-URA3	This study
yyΔΔ.URA3 (FBY5180)	As FBY2182, but containing pRS316	This study
WT.Fus-Mid (FBY5280)	As BY4742, but containing pTPQ55	This study
yyΔΔ.Fus-Mid (FBY5290)	As FBY2182, but containing pTPQ55	This study
FY1679.YPC1-GFP	*MAT***a**/α *ura3-52/ura3-52 trp1Δ63/TRP1 leu2Δ1/LEU2 his3Δ200/HIS3* containing pYPC1-GFP	(Natter et al., 2005)
yyΔΔ.CAN1-GFP (FBY5171)	As FBY2182, but containing pCAN1-GFP	This study
WT.CAN1-GFP (FBY5172)	As BY4742, but containing pCAN1-GFP	This study
yyΔΔ.GAS1-GFP (FBY5187)	As FBY2182, but containing Yep24-GAS1.GFP	This study
WT.GAS1-GFP (FBY5186)	As BY4742, but containing Yep24-GAS1.GFP	This study
yyΔΔ.FUR4-GFP (FBY5197)	As FBY2182, but containing YCplac33-FUR4.GFP	This study
WT.FUR4-GFP (FBY5204)	As BY4742, but containing YCplac33-FUR4.GFP	This study
yyΔΔ.PMA1-GFP (FBY5274)	As FBY2182, but containing pPMA1-GFP	This study
WT.PMA1-GFP (FBY5273)	As BY4742, but containing pPMA1-GFP	This study
yyΔΔ.SEC63-GFP (FBY5165)	As FBY2182, but containing pSEC63-GFP	This study
WT.SEC63-GFP (FBY5166)	As BY4742, but containing pSEC63-GFP	This study
ypc1Δvps4Δ (FBY5293)	*MAT***a** *can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ypc1Δ::LEU2 vps4Δ::kanMX*	This study
vps4Δ	*MAT***a** *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 vps4Δ::kanMX*	EUROSCARF
yyΔΔ.PHM5-GFP	As FBY2182, but containing pPHM5416	This study

WT.PHM5-GFP	As BY4742, but containing pPHM5416	This study
yyΔΔ.SNC1-GFP	As FBY2182, but containing pGS416-SNC1	This study
WT.SNC1-GFP	As BY4742, but containing pGS416-SNC1-GFP	This study
yyΔΔ.SSO1-GFP	As FBY2182, but containing pSSO1416-GFP	This study
WT.SSO1-GFP	As BY4742, but containing pSSO1416-GFP	This study
yyΔΔ.STE2-GFP	As FBY2182, but containing pSTE2416	This study
WT.STE2-GFP	As BY4742, but containing pSTE2416	This study
yyΔΔ.mtGFP (FBY5184)	As FBY2182, but containing pYES-mtGFP	This study
WT.mtGFP (FBY5183)	As BY4742, but containing pYES-mtGFP	This study
yyΔΔ.VPH1-GFP (FBY5169)	As FBY2182, but containing pVPH1-GFP	This study
WT.VPH1-GFP (FBY5170)	As BY4742, but containing pVPH1-GFP	This study
yyΔΔ.SEC7-RFP (FBY5193)	As FBY2182, but containing pTQ128	This study
WT.SEC7-RFP (FBY5195)	As BY4742, but containing pTQ128	This study
yyΔΔ.SED5-GFP (FBY5201)	As FBY2182, but containing pSED5-GFP	This study
WT.SED5-GFP (FBY5202)	As BY4742, but containing pSED5-GFP	This study
YPK9	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52*	(Jiang et al., 1998)
1Δ.YPC1	YPK9 *lag1Δ::TRP1* containing pPK183	This study
2Δ.YPC1	YPK9 *lag1Δ::TRP1 lac1Δ::URA3* containing pPK183	(Jiang et al., 2004)
FBY7478	*MAT* **a/α** *his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, ura3Δ0/ura3Δ0, met15Δ0/met15Δ0, LYP1/lyp1Δ, CAN1/can1Δ::STE2pr-Sp_his5*	
FBY7479	*MAT* **a/α***his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, ura3Δ0/ura3Δ0, met15Δ0/met15Δ0, LYP1/lyp1Δ, CAN1/can1Δ::STE2pr-Sp_his5 ypc1::kanMX4/YPC1 ydc1::kanMX4::natMX/YDC1*	This study
FBY7480	*MAT* **a/α** *his3Δ1/his3Δ1, leu2Δ0/LEU2, lys2Δ0/LYS2, ura3Δ0/ura3Δ0, met15Δ0/met15Δ0, LYP1/lyp1Δ, CAN1/can1Δ::STE2pr-Sp_his5, ypc1::kanMX4/ypc1::kanMX::ura3::LEU2, ydc1::kanMX4::natMX/ydc1::natMX*	This study

Table S2. Plasmids.

pNP302	CEN ARS *URA3, ADH1* promoter	C. De Virgilio
pYPC1-URA3	*YPC1* in pNP302	This study
pBF841	2µ *HIS3, GAL* promoter	N. Ramachandra
pBF842	*YPC1* in pBF841	N. Ramachandra
pSTE2416	*STE2*-GFP in pRS416 CEN *URA3, TPI1* promoter	F. Reggiori
pPHM5416	GFP-*PHM5* in pRS416 CEN *URA3, TPI1* promoter	(Reggiori and Pelham 2001)
pSSO1416	GFP-*SSO1* in pRS416 CEN *URA3, TPI1* promoter	F. Reggiori
pGS416-SNC1	GFP-*SNC1* in pRS416 CEN *URA3, TPI1* promoter	(Lewis et al., 2000) H. Pelham
pSED5-GFP	GFP-*SED5* CEN *URA3* http://www2.brc.riken.jp/cache/dna/8658	A. Nakano
pTQ128	*SEC7*-DsRed in CEN *LEU2, ADH1* promoter	K. Simons
pYES-mtGFP	mtGFP in 2µ *URA3, GAL* promoter	(Westermann and Neupert, 2000)
pVPH1-GFP	*VPH1*-GFP CEN *URA3*	R. Schneiter
pSEC63-GFP	*SEC63*-GFP in 2µ *URA3*	R. Schneiter
pCAN1-GFP	*CAN1*-GFP in 2µ *URA3, ADH1* promoter	W. Tanner
pFUR4-GFP	YCplac33-*FUR4*-GFP CEN *URA3*, endogenous promoter	W. Tanner
pPMA1-GFP	*PMA1*-GFP CEN *URA3*, endogenous promoter	R.Schneiter
YEp24-GAS1.GFP	*GAS1*-GFP in 2µ *URA3*, endogenous promoter	L. Popolo
pTPQ55	Fus-Mid-GFP in CEN *URA3, GAL* promoter	K. Simons
pYPC1-GFP	*YPC1*-GFP in pRS416 *URA3 TEF1* promoter	(Natter et al., 2005)
pPK183	*YPC1* in 2µ with endogenous promoter *LEU2*	(Jiang et al., 2004)

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