HOKKAIDO UNIVERSITY

Title	Identification of the phytosphingosine metabolic pathway leading to odd-numbered fatty acids
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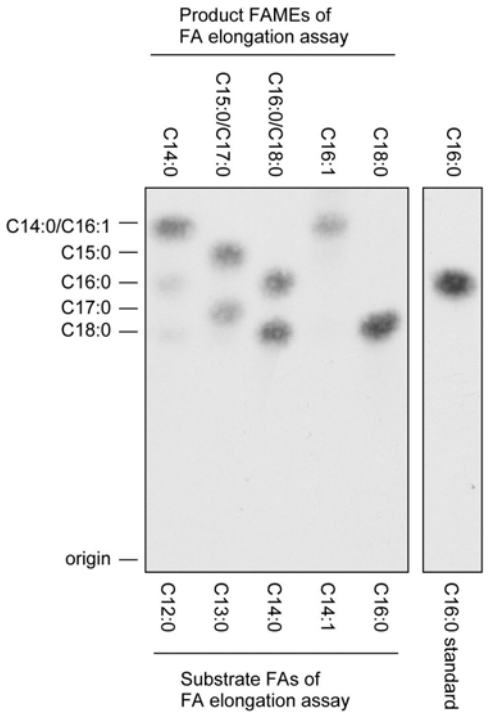
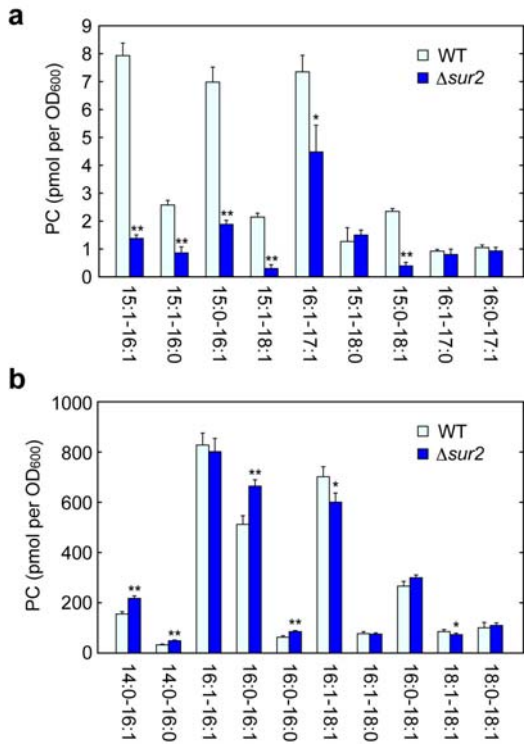
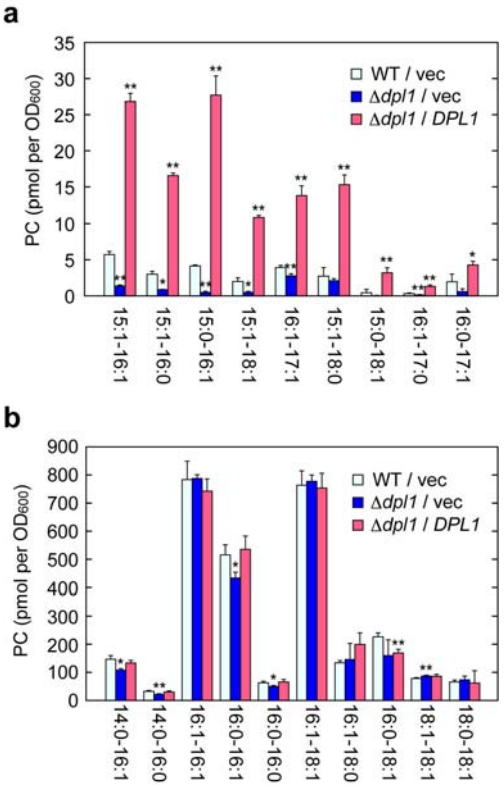


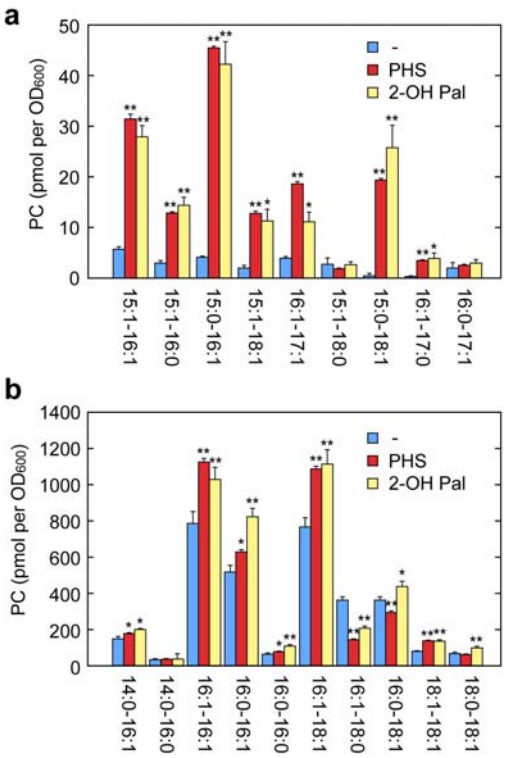
Supplementary Figure 1 | Preparation of FAME standard. Total membrane protein samples (20 μg) prepared from HEK 293T cells overexpressing the FA elongase ELOVL6 were incubated with 0.075 μCi [2-^{14}C]malonyl-CoA, 200 μM CoA, and 50 μM cold FA (C12:0, C13:0, C14:0, C14:1, or C16:0 FA) at 37 °C for 1 hr. Under these conditions, FAs were converted to acyl-CoAs by acyl-CoA synthetases and then elongated to acyl-CoAs with a longer chain-length by 2 or 4 carbons via 1 or 2 FA elongation cycles, respectively. The resulting acyl-CoAs were hydrolyzed to FAs by saponification. The produced ^{14}C-labeled FAs, as well as purchased [1-^{14}C]palmitic acid (C16:0), were methyl-esterified, extracted, and separated by reverse-phase TLC with chloroform/methanol/water (5:15:1, v/v).



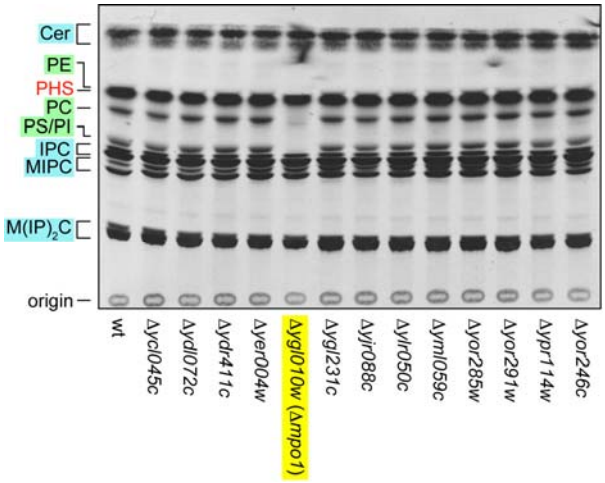
Supplementary Figure 2 | Odd-numbered PCs are decreased in Δ*sur2* cells. BY4741 (wild-type) and 3656 (Δ*sur2*) cells were grown in SC medium at 30 °C. Lipids were extracted from these cells and separated by normal-phase TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v). PC was collected from TLC plates, and its composition was analyzed by UPLC-MS with a Xevo G2 QTof LC/MS system. The data were analyzed with MassLynx software. Each bar represents the amount of each PC species (**a**, odd-numbered PC; **b**, even-numbered PC) and the mean ± S.D. from three independent experiments.



Supplementary Figure 3 | Overexpression of LCBP lyase Dpl1 causes an increase in odd-numbered PCs. BY4741 (wild-type) cells bearing the pAKNF316 (vector) plasmid (BY4741/pAKNF316), 3653 (Δ*dpl1*)/pAKNF316 cells, and 3653/pAN9 (*3xFLAG-DPL1*) cells were grown in SC-URA medium at 30 °C. Lipids were extracted from these cells and separated by normal-phase TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v). PC was collected from TLC plates and its composition was analyzed by UPLC-MS with a Xevo G2 QTof LC/MS system. The data were analyzed with MassLynx software. Each bar represents the amount of each PC species (**a**, odd-numbered PC; **b**, even-numbered PC) and the mean ± S.D. from three independent experiments. vec, vector.

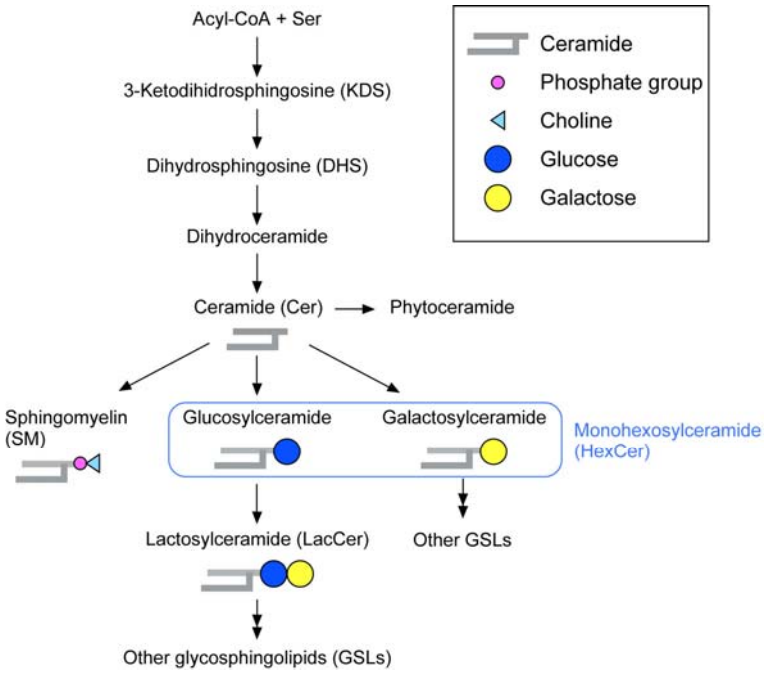


Supplementary Figure 4 | Addition of PHS or 2-hydroxypalmitic acid to the medium causes an increase in odd-numbered PCs. BY4741 (wild-type) cells were grown in SC medium at 30 °C. Cells were untreated or treated with 10 μM PHS (Enzo Life Sciences) or 10 μM 2-hydroxypalmitic acid (Wako Pure Chemical Industries, Osaka, Japan) for 4 hr. Lipids were extracted from these cells and separated by normal-phase TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v). PC was collected from TLC plates, and its composition was analyzed by UPLC-MS with a Xevo G2 QTof LC/MS system. The data were analyzed with MassLynx software. Each bar represents the amount of each PC species (**a**, odd-numbered PC; **b**, even-numbered PC) and the mean ± S.D. from three independent experiments.

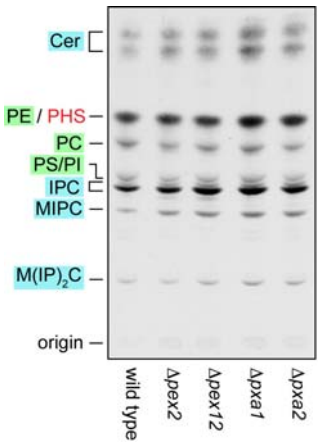


Supplementary Figure 5 | Impaired metabolism of PHS to glycerophospholipids in Δ*mpo1* cells.

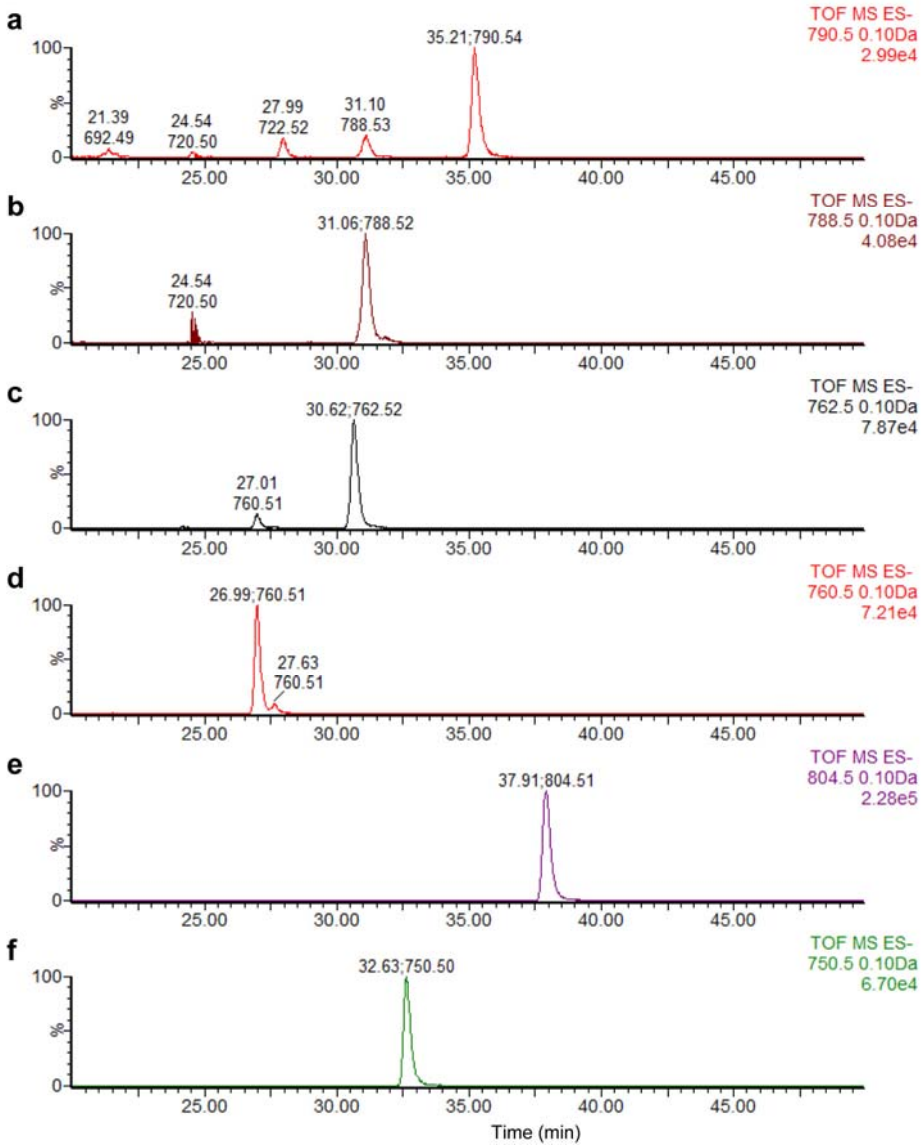
BY4741 (wild-type), 3452 (Δ*ycl045c*), 3769 (Δ*ydl072c*), 4247 (Δ*ydr411c*), 325 (Δ*yer004w*), 4378 (Δ*ygl010w*/Δ*mpo1*), 4598 (Δ*ygl231c*), 2565 (Δ*yjr088c*), 6440 (Δ*ylr050c*), 511 (Δ*yml059c*), 2541 (Δ*yor285w*), 1587 (Δ*yor291w*), 5530 (Δ*ypr114w*), and 2502 (Δ*yor246c*) cells were incubated with 0.1 μCi [^{3}H]PHS at 30 °C for 3 hr. Lipids were extracted, separated by normal-phase TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v), and detected by autoradiography.



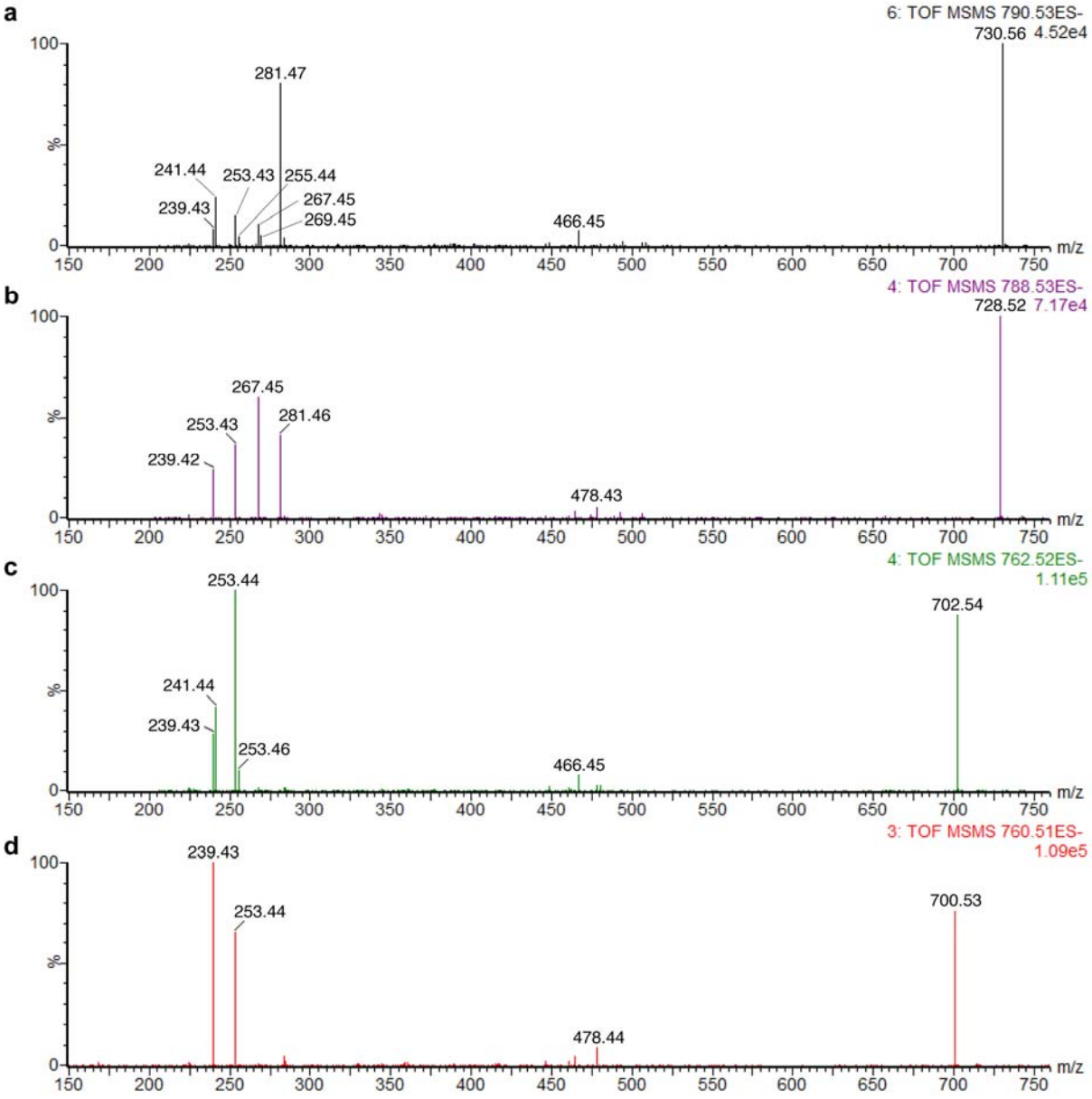
Supplementary Figure 6 | Sphingolipid biosynthetic pathway in mammals.



Supplementary Figure 7 | PHS is metabolized normally in peroxisome mutants. BY4741 (wild-type), 1214 (Δ*pex2*), 601 (Δ*pex12*), 2105 (Δ*pxa1*), and 5038 (Δ*pxa2*) cells were incubated with 0.1 μCi [^{3}H]PHS at 30 °C for 3 hr. Lipids were extracted, separated by normal-phase TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v), and detected by autoradiography.



Supplementary Figure 8 | Ion chromatograms of PCs containing odd-numbered FAs. (**a**–**d**) Ion chromatograms corresponding to PC species containing odd-numbered FAs were selected from total ion chromatograms. Selected *m/z* were (**a**) 790.5, which corresponds to PC with chain-lengths of 15:0–18:1, 15:1–18:1, 16:0–17:1, or 16:1–17:0; (**b**) 788.5 (15:1–18:1 or 16:1–17:1); (**c**) 762.5 (15:0–16:1 or 15:1–16:0); and (**d**) 760.5 (15:1–16:1). (**e**,**f**) Ion chromatograms of PC standards (15:0–15:0, *m/z* = 750.5 (**e**) and 18:1–16:0, *m/z* = 804.5 (**f**)) are presented. The numbers above the peaks represent retention times and *m/z* values. The numbers on the right of the spectra represent the ion intensities of the selected peaks.



Supplementary Figure 9 | MS-MS spectra of PCs containing odd-numbered FAs. MS-MS spectra of the selected ions are represented (**a**, *m/z* = 790.5; **b**, *m/z* = 788.5; **c**, *m/z* = 762.5; and **d**, *m/z* = 760.5). The *m/z* values of the FA fragments are listed in the Supplementary Table 3. The numbers above the peaks represent *m/z* values. The numbers on the right of the spectra represent the ion intensities of the highest peaks.

Supplementary Table 1 | Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	1
325	BY4741, Δ*yer004w::KanMX4*	2
511	BY4741, Δ*yml059c::KanMX4*	2
601	BY4741, Δ*pex12::KanMX4*	2
1214	BY4741, Δ*pex2::KanMX4*	2
1587	BY4741, Δ*yor291w::KanMX4*	2
2105	BY4741, Δ*pxa1::KanMX4*	2
2427	BY4741, Δ*lcb4::KanMX4*	2
2502	BY4741, Δ*yor246c::KanMX4*	2
2541	BY4741, Δ*yor285w::KanMX4*	2
2565	BY4741, Δ*yjr088c::KanMX4*	2
3452	BY4741, Δ*ycl045c::KanMX4*	2
3653	BY4741, Δ*dpl1::KanMX4*	2
3656	BY4741, Δ*sur2::KanMX4*	2
3769	BY4741, Δ*ydl072c::KanMX4*	2
4247	BY4741, Δ*ydr411c::KanMX4*	2
4378	BY4741, Δ*mpo1::KanMX4*	2
4598	BY4741, Δ*ygl231c::KanMX4*	2
5038	BY4741, Δ*pxa2::KanMX4*	2
5530	BY4741, Δ*ypr114w::KanMX4*	2
6440	BY4741, Δ*ylr050c::KanMX4*	2
6550	BY4741, Δ*hfd1::KanMX4*	2
AOY13	BY4741, Δ*faa1::KanMX4* Δ*faa4::NatNT2*	3
DEY195	BY4741, Δ*pep4::MET15*	4
YOY158	BY4741, *SEC61-mCherry::KanMX4*	This study
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	5
KHY13	SEY6210, Δ*dpl1::TRP1*	6
KHY20	SEY6210, Δ*lcb3::LEU2*	7
KHY21	SEY6210, Δ*dpl1::TRP1* Δ*lcb3::LEU2*	6
KHY49	SEY6210, Δ*lcb3::LEU2* Δ*ysr3::URA3* Δ*lpp1::HIS3* Δ*dpp1::TRP1*	This study
KHY80	*MAT**a** leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ade2-101* Δ*lcb4::LEU2* Δ*dpl1::LYS2* Δ*lcb3::LEU2* Δ*lpp1::HIS3* Δ*dpp1::TRP1*	This study
KHY543	SEY6210, Δ*pep4::LEU2* Δ*akr1::URA3* Δ*akr2::KanMX4*	8

Supplementary Table 2 | Selected *m/z* values for PC ([M+HCOO]⁻) species in Q1 of MS analysis

m/z	PC species*
748.4	14:0-16:1
750.4	14:0-16:0
760.4	15:1-16:1
762.4	15:0-16:1, 15:1-16:0
764.4	15:0-16:0
774.4	16:1-16:1
776.4	16:0-16:1
778.4	16:0-16:0
788.4	15:1-18:1, 16:1-17:1
790.4	15:0-18:1, 15:1-18:0, 16:0-17:1, 16:1-17:0
792.4	15:0-18:0, 16:0-17:0
802.5	16:1-18:1
804.5	16:0-18:1, 16:1-18:0
830.5	18:1-18:1
832.5	18:0-18:1

*PC species determined by MS/MS analysis were listed.

Supplementary Table 3 | The *m/z* values of FA fragment ions in MS analysis

FA fragment	*m/z*
C14:1	225.3
C14:0	227.3
C15:1	239.3
C15:0	241.3
C16:1	253.3
C16:0	255.3
C17:1	267.3
C17:0	269.3
C18:1	281.3
C18:0	283.3

Supplementary Methods

Preparation of FAME standard

To prepare ^{14}C-labeled FAs ranging from C14-C18, C12-C16 acyl-CoAs were subjected to FA elongation reactions using the FA elongase ELOVL6, which exhibits high activity toward C12-C16 acyl-CoAs[9], and [2-^{14}C]malonyl-CoA as a carbon donor. HEK 293T cells were transfected with the pCE-puro 3xFLAG-ELOVL6 plasmid[10]. Twenty-four hours after transfection, total membrane fractions were prepared and subjected to FA elongation reactions, which was performed as described previously[10] using 0.075 μCi [2-^{14}C]malonyl-CoA (55 mCi/mmol; Moravek Biochemicals, Brea, CA), 200 μM CoA, and 50 μM cold FA (C12:0, C13:0, C14:0, C14:1, or C16:0 FA; Sigma). The resulting ^{14}C-labeled FAs and purchased [1-^{14}C]palmitic acid (C16:0) were methyl-esterified as described previously[3].

Supplementary References

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