SUPPLEMENTAL METHODS

Antibodies—Monoclonal mouse anti-FLAG (clone M2) and mouse anti-c-Myc (clone 9E10) as well as alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) antibodies were used for western blotting. All antibodies were obtained from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.).

Lipids—The free LCBs D-*erythro*-sphinganine (synthetic), D-*erythro*-sphing-4-enine (synthetic), and D-*ribo*-4-hydroxysphinganine (from yeast) as well as the acyl-CoAs C_{16} (palmitoyl) CoA, C_{18} (stearoyl) CoA, α-hydroxylated C_{18} (α-hydroxystearoyl) CoA, C_{20} (arachidoyl) CoA, C_{22} (behenoyl) CoA, C_{24} (lignoceroyl) CoA, and C_{26} (hexacosanoyl) CoA used in the *in vitro* Cer synthase assay were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). For the lipid analysis of *S. cerevisiae*, *N*-heptadecanoyl-D-*erythro*-sphing-4-enine (Cer containing a C_{17} fatty acid) from Avanti Polar Lipids was used as internal standard while for the lipid analysis of *P. pastoris*, *N*-pentadecanoyl-D-*erythro*-sphing-4-enine (Cer containing a C_{15} fatty acid) from Matreya LLC (Pleasant Gap, PA, U.S.A.; distributed by BIOTREND Chemikalien GmbH, Cologne, Germany) and *β*-D-glucosyl-(1→1)-*N*-lauroyl-D-*erythro*-sphing-4-enine (GlcCer containing a C_{12} fatty acid) from Avanti Polar Lipids were used.

Radiochemicals—[4,5-^{3}H]-D-*erythro*-sphinganine (ART 0460), [3-^{3}H]-D-erythro-sphing-4-enine (ART 0490), and [1-^{14}C]-stearoyl-CoA (ARC 0756) used in the in vitro Cer synthase assay were produced by American Radiolabeled Chemicals Inc. (Saint Louis, MO, U.S.A.; distributed by BIOTREND).

DNA Polymerases—The following proofreading DNA polymerases were used for the cloning steps: *PfuTurbo* (Agilent Technologies, Inc., Life Sciences and Chemical Analysis Group, Santa Clara, CA, U.S.A.), Phusion (Finnzymes Oy, Espoo, Finland; distributed by Biozym Scientific GmbH, Hessisch Oldendorf, Germany), and peqGOLD *Pwo* (PEQLAB Biotechnologie GmbH, Erlangen, Germany). *Taq* DNA polymerase (New England Biolabs Inc., Ipswich, MA, U.S.A.) and FastStart *Taq* DNA polymerase (Hoffmann-La Roche AG, Basel, Switzerland) were used for colony PCR. All other enzymes used for cloning were obtained from Fermentas Inc. (Burlington, ON, Canada) and New England Biolabs.

Integrative Expression Vector YIplac204GPD—An integrative expression vector for *S. cerevisiae* was constructed by combining elements from the vectors YIplac204 (1) and p424GPD (2). *GPD1* promoter, multiple cloning site, and *CYC1* terminator were cut out of p424GPD by first digesting with *Sac*I, blunting with T4 DNA polymerase, and finally digesting with *Kpn*I. This DNA fragment was gel-purified and ligated to YIplac204 cut with *Hinc*II and *Kpn*I. The resulting plasmid YIplac204GPD is an integrative vector suitable for expression of recombinant proteins under the control of the constitutive GPD promoter.

Cloning of P. pastoris BAR1—The open reading frame (ORF) of *P. pastoris BAR1* was amplified from genomic DNA (strain GS115) with Phusion DNA polymerase using primers ggatccatggattataaagatgatgatgataagtctGGTGTTGAAACATCTTCCTCT and gaattcctcgagttaTCAAGAACTCTCCTCATCATCA.[1] In its lowercase part, the forward primer contains a *Bam*HI restriction site followed by a start codon, a sequence encoding the FLAG epitope (DYKDDDD) and a serine codon as linker. The reverse primer contains the restriction sites *Eco*RI and *Xho*I as well as an extra stop codon. The PCR product was cloned in the vector pJET1.2/blunt (Fermentas) and checked by sequencing. The FLAG-tagged *BAR1* ORF was then subcloned in the integrative expression vector YIplac204GPD (see above) using the restriction sites *Bam*HI and *Xho*I, resulting in the plasmid Yiplac204GPD-BAR1.

An S. cerevisiae Strain Expressing Bar1p—Cells of the *S. cerevisiae* strain WBY616-LAG1 (also called RH6602; Ref. 3) were transformed with Yiplac204GPD-BAR1, which was linearized with *Eco*RV. Positive transformants were selected on CSM-Trp^{-} plates and checked by colony PCR with primer pairs AGTAAGTTGGCCGCAGTGTT/GCGCCTGTGAACATTCTCTT (integration at the *TRP1* locus) and TTGCAGTTATGACGCCAGAT/GCGCCTGTGAACATTCTCTT (original

TRP1 locus). The complementing plasmid pRS416-LAG1 (3) was counterselected by streaking out several times on CSM-Trp$^-$ plates (0.67% yeast nitrogen base; CSM-Trp$^-$ amino acid dropout mixture; 2% glucose; 2% agar; all w/v) containing 1 mg/ml 5-fluoroorotic acid. The resulting yeast strain WBY616-BAR1 expresses FLAG-tagged *P. pastoris* Bar1p as its only Cer synthase. Expression of FLAG-tagged Bar1p and absence of the complementing c-Myc-tagged *S. cerevisiae* Lag1p were confirmed by western blotting with anti-FLAG and anti-c-Myc antibodies (Supplemental Fig. S3*A*).

P. pastoris Deletion Strains—For gene deletion by homolgous recombination, genomic regions flanking the open reading frame of the target gene were amplified by PCR. Knockout constructs containing a nourseothricin or a Zeocin™ resistance gene were generated either by cloning or by fusion PCR. After *P. pastoris* cells were transformed with these constructs, positive clones were picked from nourseothricin or Zeocin™ plates. Correct integration of the gene deletion cassettes into the genome of *P. pastoris* was checked by PCR. Details for strains generated in the course of this study are given below; references for previously published strains are listed in Table 1 (main text).

*Pichia pastoris BAR1 Deletion Strain bar1*Δ—The genomic regions flanking the *BAR1* ORF were amplified from genomic DNA (strain GS115) with *Pwo* DNA polymerase using the primers ccgcggTCCTAATGATGTGACGAAAC TG (upstream; *Sac*II), gcggccgcTTGTGTTTGTT AACTTGGCAATATTGG (upstream; *Not*I), gcggccgcATCTGGATGTACCTCATTCTAAA (downstream; *Not*I), and gaattcGTGGATCCATTG TTGCCACGTTC (downstream; *Eco*RI) containing the indicated restriction sites. The PCR products were cloned in the vector pGEM-T (Promega Corp., Fitchburg, WI, U.S.A.) and checked by sequencing. The flanking regions were then excised with *Sac*II and *Not*I (upstream region) or *Not*I and *Eco*RI (downstream region), gel-purified, and cloned simultaneously into the vector pBluescript II KS (−) (Stratagene) cut with *Sac*II and *Eco*RI. The resulting plasmid contains the upstream and downstream flanking regions of *BAR1* linked by a *Not*I site.

The nourseothricin resistance gene *NAT1* was cut out of the plasmid pSLNat (4) with *Not*I, gel-purified, and cloned in the *Not*I site of the plasmid described in the previous paragraph. The resulting plasmid pBSII-BAR1-KO contains the *NAT1* gene in an antiparallel orientation between the upstream and downstream flanking regions of *BAR1*.

The deletion cassette was excised from pBSII-BAR1-KO with *Sac*I and *Xho*I and used to transform cells of the strains GS115 (Invitrogen) and PPY12 (5). Positive transformants were selected on YPD plates (1% yeast extract; 2% peptone; 2% glucose; 2% agar; all w/v) containing 15 µg/ml nourseothricin and checked by colony PCR with primer pairs GCCAGGCTCTGATCTT TCC/CTTACATTCACGCCCTCCC (integration at the *BAR1* locus) and ggatccattATGGGTGTTG AAACATCTTCCTC/gcggccgcTCAAGAACTCT CCTCATCATC (wildtype *BAR1* locus).

*P. pastoris SCS7 Deletion Strain scs7*Δ—A deletion cassette containing the Zeocin™ resistance gene *Sh ble* was generated by triple fusion PCR analogous to Ref. 6. All reactions were performed with *PfuTurbo* DNA polymerase. The *SCS7* locus was amplified from genomic DNA (strain GS115) using primers GATTCATTCCCCACAGGTTG and CCAAAGC TCCGAAACAGAAG. From this PCR product, ≈ 500 bp directly flanking each side of the *SCS7* ORF were amplified with primers TACCAAGAAGCCCATGACCAA and cattttgaag ctatggtgtgtgAGTTTCGGTGGATTTCTAATTGA (upstream) and gaaggctttaatttgcaagctggAGTATTT ATAAAGTTATGTAAGCAA and CTTTCTCCAA CGATGCTTCCA (downstream) with their lowercase part being complementary to the *Sh ble* gene. The *Sh ble* was amplified from the plasmid pGAPZ B (Invitrogen) using primers tcaaatagaaatccaccgaaactCACACACCATAGCTTC AAAATG and ttgcttacataactttataaatactCCAGCTT GCAAATTAAAGCCTTC with their lowercase part being complementary to the genomic regions flanking the *SCS7* ORF. The upstream and downstream sequences and the *Sh ble* gene were fused in a single PCR for 12 rounds without primers. From this, the full-length fusion product

was amplified in a separate reaction with primers TACCAAGAAGCCCATGACCAA and CTTTCT CCAACGATGCTTCCA. The knock-out cassette was ligated into the *Sma*I site of the pBluescript II KS (−)-vector (Stratagene) and checked by sequencing.

The knock-out cassette was excised from the plasmid and used to transform cells of the strain GS115. Transformants were selected on YPD plates containing 100 µg/ml Zeocin™. Correct integration of the knock-out cassette was confirmed by colony PCR with the primer pairs GATTCATTCCCCACAGGTTG/ATGCCGATAT ACTATGCCG, CCAAAGCTCCGAAACAGA AG/CGGCATAGTATATCGGCAT (integration at the *SCS7* locus), and GATTCATTCCCCACAGG TTG/CCAAAGCTCCGAAACAGAAG (wildtype *SCS7* locus).

P. pastoris Δ*8-Desaturase Deletion Strain delta8*Δ—The ORF of the Δ8-desaturase including ≈ 500 bp of upstream and downstream sequences was amplified by PCR from genomic DNA (strain GS115) with *PfuTurbo* DNA polymerase using primers cgggatccAAGTGGGTTAGCAACGAAA AGC and ggggtaccATTGTAATTTGGTGTTGGG AGAC containing the restriction sites *Bam*HI and *Kpn*I. The PCR product was cloned in the vector pGEM-T (Promega), checked by sequencing and then subcloned in the vector pLitmus28i (New England Biolabs). A portion of the Δ8-desaturase ORF including the three catalytically essential histidine boxes (7) was cut out with *Hind*III and *Xba*I.

The Zeocin™ resistance gene *Sh ble* was amplified by PCR from the plasmid pGAPZ B (Invitrogen) with *PfuTurbo* DNA polymerase using primers ccatgggaagcttCACACACCATAGC TTCAAAATG and gctctagaCCAGCTTGCAAAT TAAAGCCTTC including the restriction sites *Hind*III and *Xba*I. The PCR product was cloned in the vector pGEM-T (Promega) and checked by sequencing. The *Sh ble* gene was cut out with *Hind*III and *Xba*I and used to replace the *Hind*III/*Xba*I fragment from the plasmid constructed in the previous step. The resulting plasmid pL28iPp8desZeo contains a genomic fragment comprising the Δ8-desaturase gene where an essential part of the ORF has been replaced by the *Sh ble* gene.

Positive transformants were selected on YPD plates containing 100 µg/ml Zeocin™ and checked by colony PCR with primer pairs GAGTTTCGAGGGATCCGATG/ATGCCGATAT ACTATGCCG, CGGCATAGTATATCGGCAT/CA CCGGTCAACAGCTATGTC (integration at the Δ8-desaturase locus) and GAGTTTCGAGGGAT CCGATG/CACCGGTCAACAGCTATGTC (wildtype Δ8-desaturase locus).

P. pastoris Strains Overexpressing GCS—A plasmid containing the ORF of the *P. pastoris* GCS in the expression vector pPIC3.5 (Invitrogen) (8) was linearized with *Bgl*II and used to transform the strains *gcs*Δ, *delta4*Δ, and *scs7*Δ (Table 1). Positive transformants were selected on MD plates (1.34% yeast nitrogen base; 0.4 µg/ml biotin; 2% glucose; 2% agar; all w/v) followed by Mut^+/Mut^S screening on MM plates (1.34% yeast nitrogen base; 0.4 µg/ml biotin; 0.5% methanol; 2% agar; all w/v). The resulting strains *gcs*ΔGCS, *delta4*ΔGCS, and *scs7*ΔGCS overexpress the GCS under the control of the methanol-inducible alcohol oxidase *AOX1* promoter.

LCB Analysis—Strong alkaline hydrolysis of sphingolipids with $Ba(OH)_2$ and derivatisation of the LCBs with 1-fluoro-2,4-dinitrobenzene (Sigma-Aldrich) was performed as described previously (9). The resulting 2,4-dinitrophenyl-derivatized LCBs were separated by reverse-phase HPLC with methanol/acetonitrile/2-propanol, 10:3:1 (v/v/v) against water. Elution was monitored with a UV detector at 350 nm.

In the case of *S. cerevisiae*, the analysis was performed on a 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with an ET 250/4 Nucleosil 120-5 C18, 250 × 4.6 mm column (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Separation was achieved by isocratic elution at 84% organic solvent for 10 min, followed by a linear gradient to 100% organic solvent (see above) in 45 min, and a final isocratic step for 15 min.

In the case of *P. pastoris*, the analysis was performed on a Spectra SYSTEM HPLC (Thermo Fisher Scientific, Waltham, MA, U.S.A.) equipped with a Multospher RP18-5 250 × 4 mm column

(CS-Chromatographie Service GmbH, Langerwehe, Germany). Separation was achieved using a concave gradient from 84 to 100% organic solvent in 50 min followed by isocratic elution for 5 min.

Drop Dilution Test—The *P. pastoris* strains GS115 and GS115-*bar1*Δ were grown in liquid YPD medium until stationary phase. The cultures were diluted with water to an optical density of 1.40 ± 0.02. From this, a dilution series was prepared by diluting the culture 10-fold with water in each step. 5 µl of each dilution were applied to a YPD plate and incubated at 30°C for 3 days. The image was acquired using a CanoScan flatbed scanner (Canon Inc., Tokyo, Japan).

P. pastoris Strains Expressing Marker Proteins for ER, ER Exit Sites, and Golgi—The plamids pIB2-DsRed-HDEL (DsRed containing an ER retention signal; Ref. 10) and pLAS202 (GFP-tagged *cis*-Golgi marker Vrg4p; L. Satkamp and B. S. Glick, unpublished) were linearized by digestion with *Dra*III and *Sal*I, respectively. Cells of the *P. pastoris* strains PPY12 and PPY12-*bar1*Δ were transformed with the linearized plasmids and selected on MD plates supplemented with arginine. Expression of the recombinant proteins was confirmed by fluorescence microscopy.

The plasmid p53.Glu (GluGlu-tagged ER exit site marker Sec12p; Ref. 11) was linearized with *Xho*I. Cells of the *P. pastoris* strains PPY12 and PPY12-*bar1*Δ expressing either DsRed-HDEL or Vrg4p-GFP were transformed with the linearized plasmid and selected on MD plates without amino acids. Integration was confirmed by colony PCR with primers TGACACCCTCTTTCCAGCTT and CAGCCAATTCTGAGTGGTCA. All expression constructs were kindly provided by Benjamin Glick, University of Chicago, Chicago, IL, U.S.A.

Yeast Culture and Staining Procedures—P. pastoris strains PPY12 and PPY12-*bar1*Δ expressing either DsRed-HDEL or Vrg4p-GFP in combination with GluGlu-tagged Sec12p were grown in liquid YPD medium until an optical density of 0.5 to 0.7. The cells were fixed in a 100 mM potassium phosphate buffer, pH 6.5, containing 3.7% formaldehyde for 1 hour, washed twice with potassium phosphate buffer, and stored at 4°C until fluorescent staining.

For calcofluor white staining, fixed cells were applied to a poly-L-lysine-coated cover glass, incubated with ready-made calcofluor white solution in 100 mM phosphate buffer, pH 7.0 (Sigma-Aldrich), and observed after 15–30 min.

For phalloidin-rhodamine staining, a 100 µM stock solution of phalloidin-tetramethylrhodamine B (Sigma-Aldrich) in methanol was diluted to 4 µM in PBS containing 0.1% triton X-100. Fixed cells were incubated with the diluted phalloidin-rhodamine solution at 4°C for 40 min with shaking. The cells were washed with PBS and applied to a poly-L-lysine-coated cover glass.

For immunostaining of GluGlu-tagged Sec12p, fixed cells were washed with a 100 mM phosphate/citrate buffer, pH 5.9, containing 1.2 M sorbitol. Spheroplasting was performed in the same buffer supplemented with 0.75 mg/ml Zymolyase® 20T (Seikagaku Corp., Tokyo, Japan) and 25 mM β-mercaptoethanol for 75 min at 30°C with gentle shaking. The cells were washed twice with phosphate/citrate buffer containing sorbitol and applied to a poly-L-lysine-coated cover glass.

The cover glass was submerged in methanol at −20°C for 6 min followed by acetone at −20°C for 30 s. After the solvent had evaporated, PBS containing 2% (w/v) milk powder was applied as blocking buffer and incubated for 2 hours. Incubation with monoclonal mouse anti-GluGlu primary antibody (Covance Inc., Princeton, NJ, U.S.A.) diluted 1 : 150 in blocking buffer was at room temperature for 2 hours, incubation with MFP555-conjugated goat anti-mouse secondary antibody (MoBiTec, Göttingen, Germany) diluted 1 : 100 in blocking buffer was at 4°C over night. The cells were washed 5 times with blocking buffer after each incubation and rinsed with PBS after the secondary antibody incubation. The samples were mounted in 90% (v/v) glycerol containing 1 mg/ml *p*-phenylenediamine.

Microscopy was performed on a BX51 fluorescence microscope equipped with a UPlanFl 100×/1.30 objective and a ColorView II digital camara (Olympus Corp., Tokyo, Japan). Images were recorded using analySIS Docu software (Soft-Imaging-Systems GmbH, Münster, Germany) and edited with ImageJ (http://rsb.info.nih.gov/ij).

FOOTNOTE

[1] In all primer sequences, the part complementary to the template DNA is printed in uppercase letters while an (optional) 5' extension is shown in lowercase. Any restriction sites incorporated into the 5' extension are underlined

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SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1 (Excel file)

Cer and GlcCer species detected by UPLC/MS in the lipid extracts from *P. pastoris* and *S. cerevisiae* as well as in the *in vitro* Cer synthase assay with unlabeled substrates.

SUPPLEMENTAL TABLE 2 (Excel File)

IPC, MIPC, and $M(IP)_2C$ species detected by LC/MS-MS in the lipid extracts from *P. pastoris*.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. S1. **No phenotypes could be found in the *P. pastoris* strain *bar1*Δ. *A,*** In a drop dilution test, growth of the *P. pastoris* strains GS115 and GS115-*bar1*Δ on YPD plates is indistinguishable. ***B,*** No differences between the *P. pastoris* strains PPY12 and PPY12-*bar1*Δ were observed by calcofluor white staining (cell wall), phalloidin-rhodamine staining (actin cytoskeleton), and the localization of the marker proteins DsRed-HDEL (ER), GluGlu-tagged Sec12p (ER exit sites), and Vrg4p fused with GFP (Golgi).

Supplemental Fig. S2. **In contrast to *P. pastoris*, sphingolipids from *S. cerevisiae* contain both C_{18} and C_{20} LCBs.** HPLC chromatograms of LCBs liberated by strong alkaline hydrolysis of whole cells of the *S. cerevisiae* strain WBY616-LAG1 (top) and of a lipid extract from the *P. pastoris* strain GS115 (bottom). The proportion of the only detectable C_{20} LCB, t20:0, is 57% of total LCBs for *S. cerevisiae*, but only 4.3% for *P. pastoris*. The retention times are not comparable between the two chromatograms because different HPLC columns and different elution gradients were used. The identity of the LCBs was confirmed by comparison with commercially available standards and previously published data (*S. cerevisiae*: Ref. 12; *P. pastoris:* Ref. 6) as well as by HPLC/MS (*P. pastoris* only: Ref. 13).

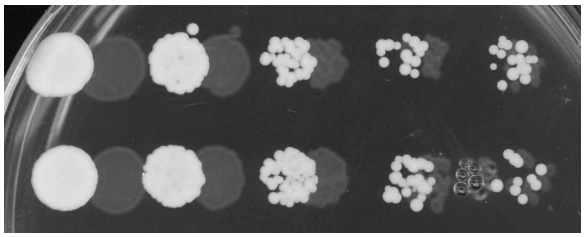
Supplemental Fig. S3. **Bar1p shows Cer synthase activity *in vitro*.** The data shown here differ from Fig. 4A in the main text by the use of ^{3}H- or ^{14}C-labeled substrates and longer incubation times (with ^{3}H-labelled LCBs). All experiments were performed once. ***A,*** Western blot of microsomal preparations confirming the expression of FLAG-tagged Bar1p and the absence of c-Myc-tagged Lag1p in the *S. cerevisiae* strain WBY616-BAR1. The strain WBY616-LAG1, in contrast, expresses only c-Myc-tagged Lag1p. Only the microsomal preparation from WBY616-BAR1 is able to produce ^{3}H-labeled Cer in an *in vitro* assay with [^{3}H]sphinganine or [^{3}H]sphing-4-enine and C_{18}-(stearoyl)-CoA as precursors. ***B,*** *left* Bar1p accepts acyl-CoAs with chain lengths ranging from 16 to 22 with a maximum at 18. A microsomal preparation of the *S. cerevisiae* strain WBY616-BAR1 was incubated for 30 min with ^{3}H-labelled sphinganine or sphing-4-enine and unlabeled acyl-CoAs of different chain lengths. ***B,*** *right* Bar1p prefers the dihydroxy LCBs sphinganine and sphing-4-enine over the trihydroxy LCB 4-hydroxysphinganine. A microsomal preparation of the *S. cerevisiae* strain WBY616-BAR1 was incubated for 5 min with unlabeled sphinganine, sphing-4-enine, or 4-hydroxysphinganine and ^{14}C-labeled C_{18}-(stearoyl)-CoA.

Supplemental Fig. S4. **Minor species of Cer and GlcCer in the *P. pastoris* strains GS115, *bar1*Δ, *gcs*Δ, and *scs7*Δ.** This figure is the same as Fig. 4 in the main text, but with a different axis scaling to show minor species of Cer and GlcCer. Columns which are open at the top are off scale.

Supplemental Fig. S5. **Minor species of Cer and GlcCer in the *P. pastoris* strains GS115, *gcs*Δ, *c9*Δ, *delta8*Δ, and *delta4*Δ.** This figure is the same as Fig. 6 in the main text, but with a different axis scaling to show minor species of Cer and GlcCer. Columns which are open at the top are off scale.

Supplemental Fig. S6. **Minor species of Cer and GlcCer in the *P. pastoris* strains GS115, *gcs*ΔGCS, *delta4*ΔGCS, and *scs7*ΔGCS.** This figure is the same as Fig. 7 in the main text, but with a different axis scaling to show minor species of Cer and GlcCer. Columns which are open at the top are off scale.

A)

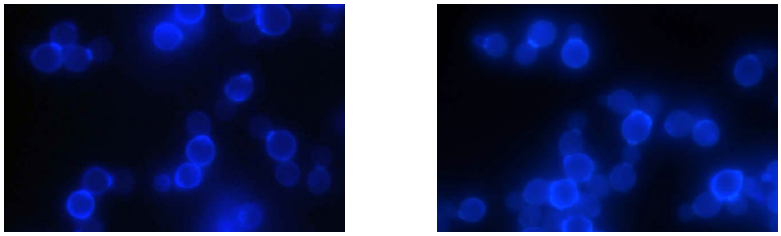


WT

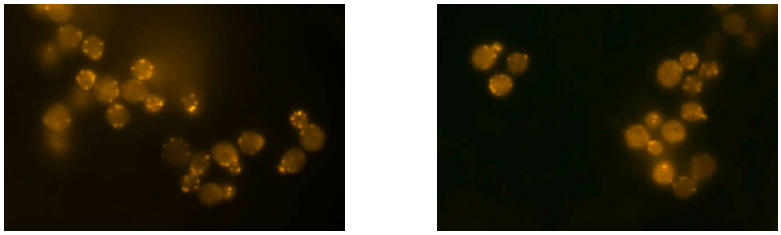
*bar1*Δ

B)

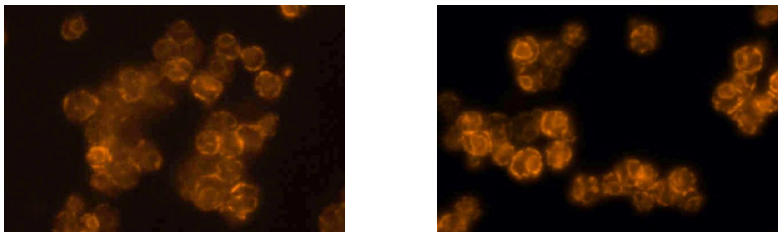
WT *bar1*Δ



calcofluor white



phalloidin-rhodamine



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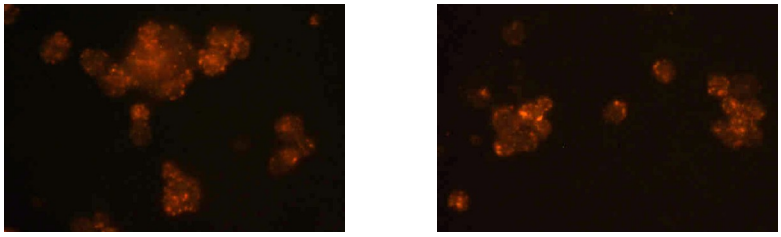

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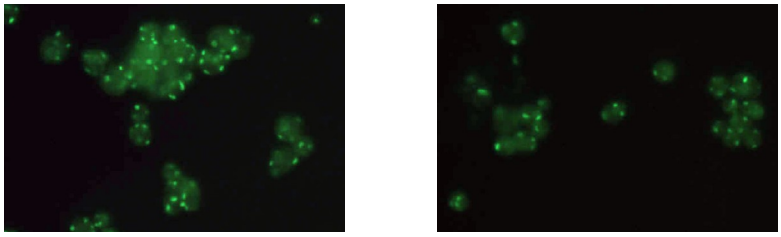
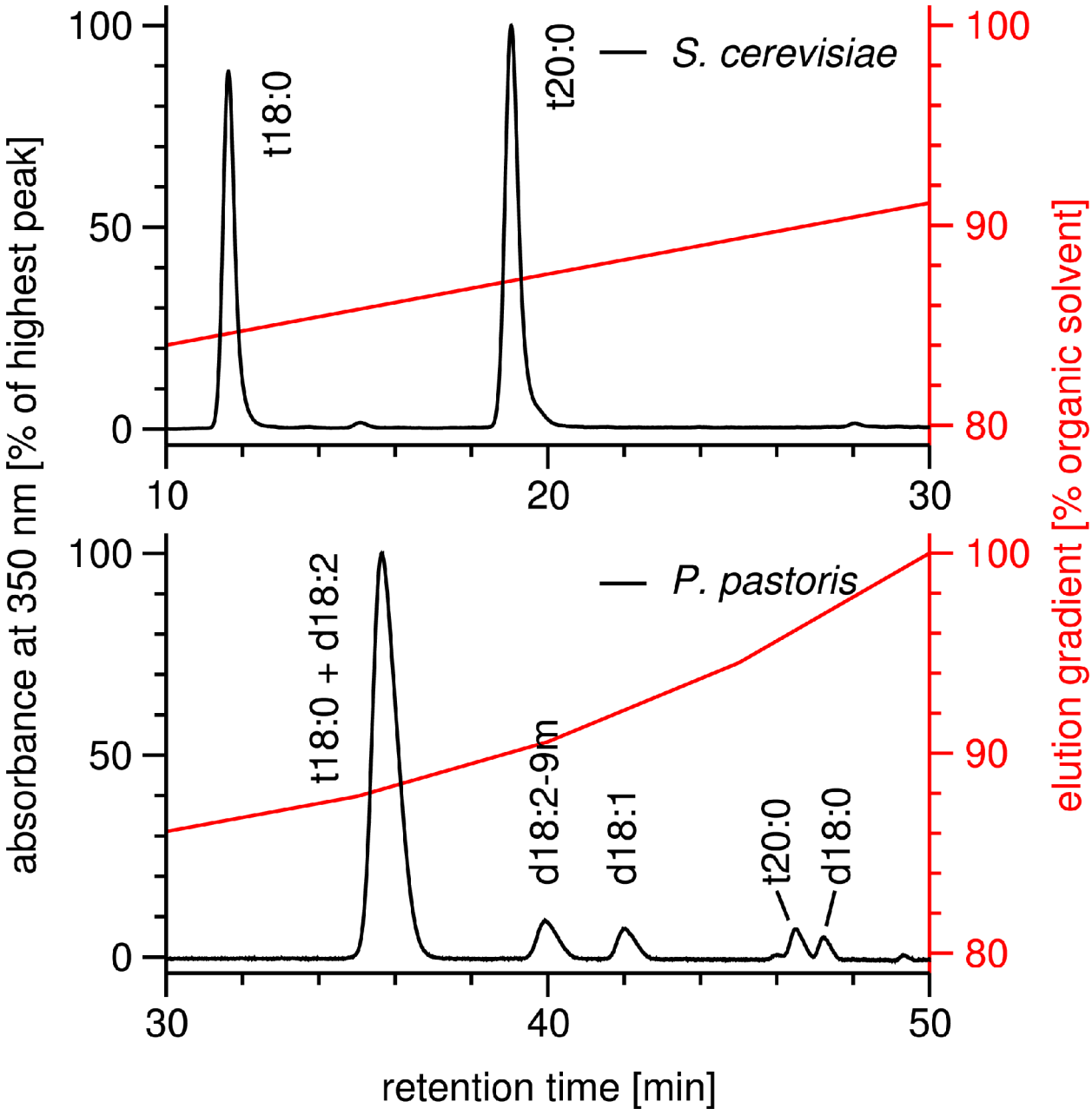
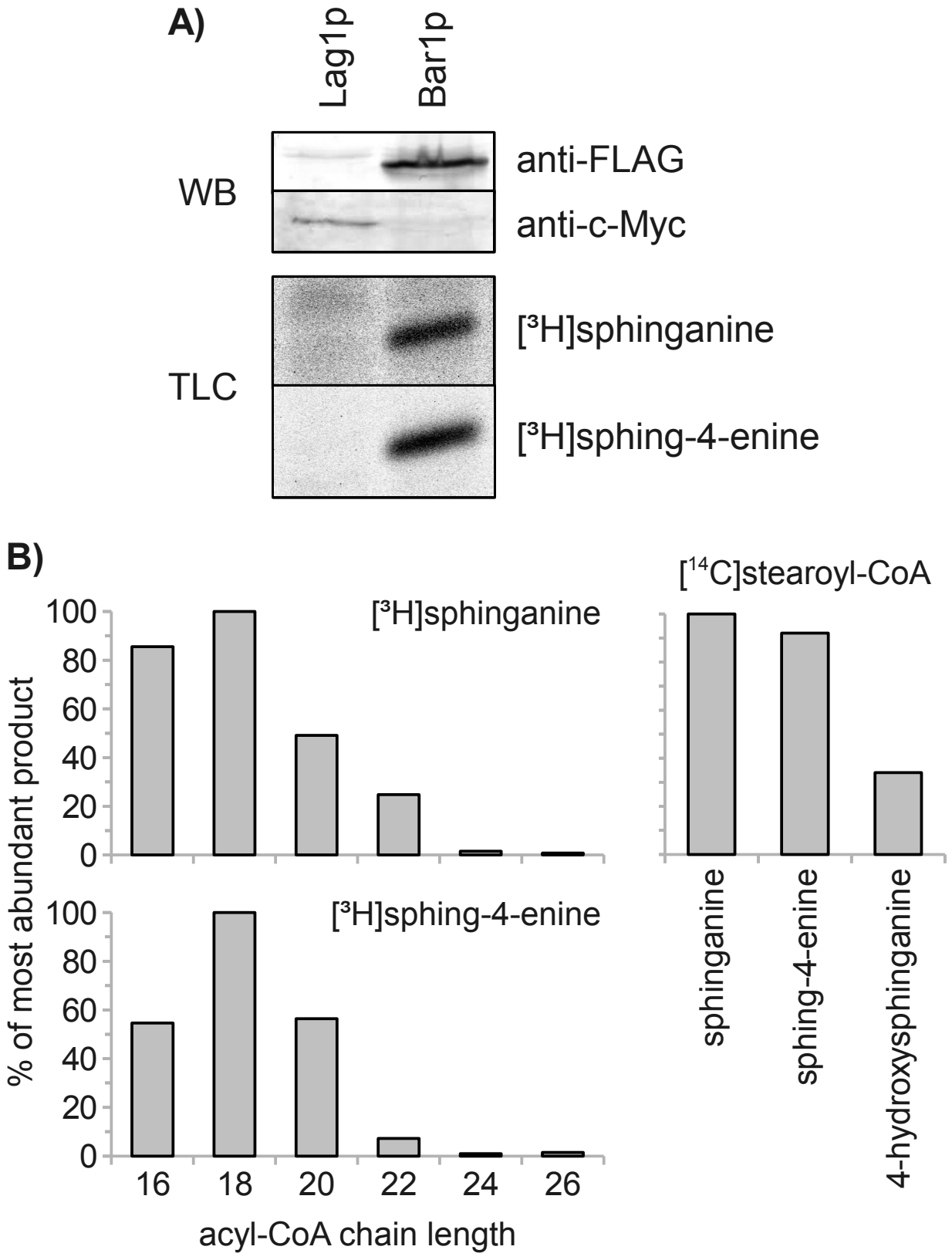


Vrg4p-GFP

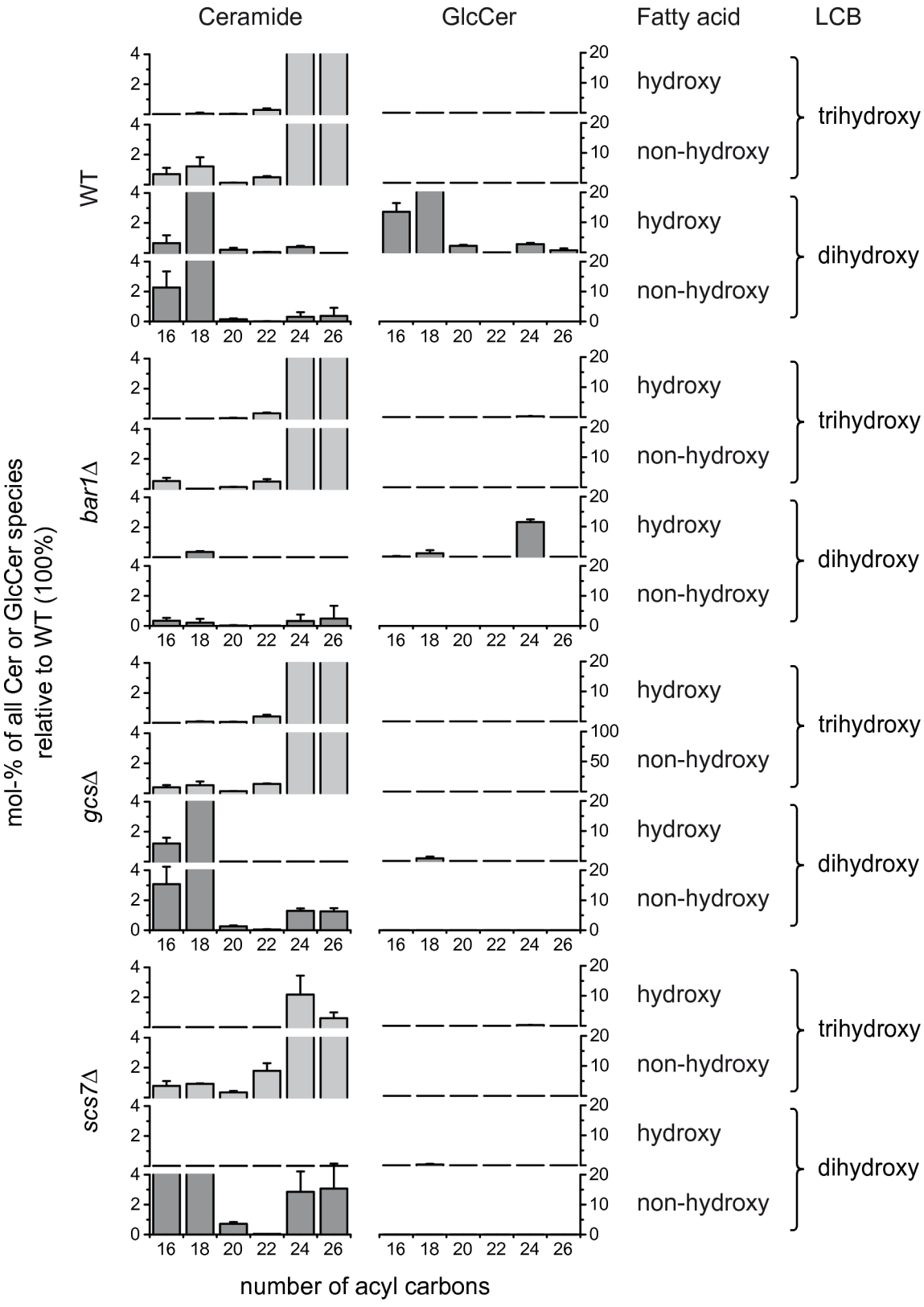
Supplemental Fig. S1



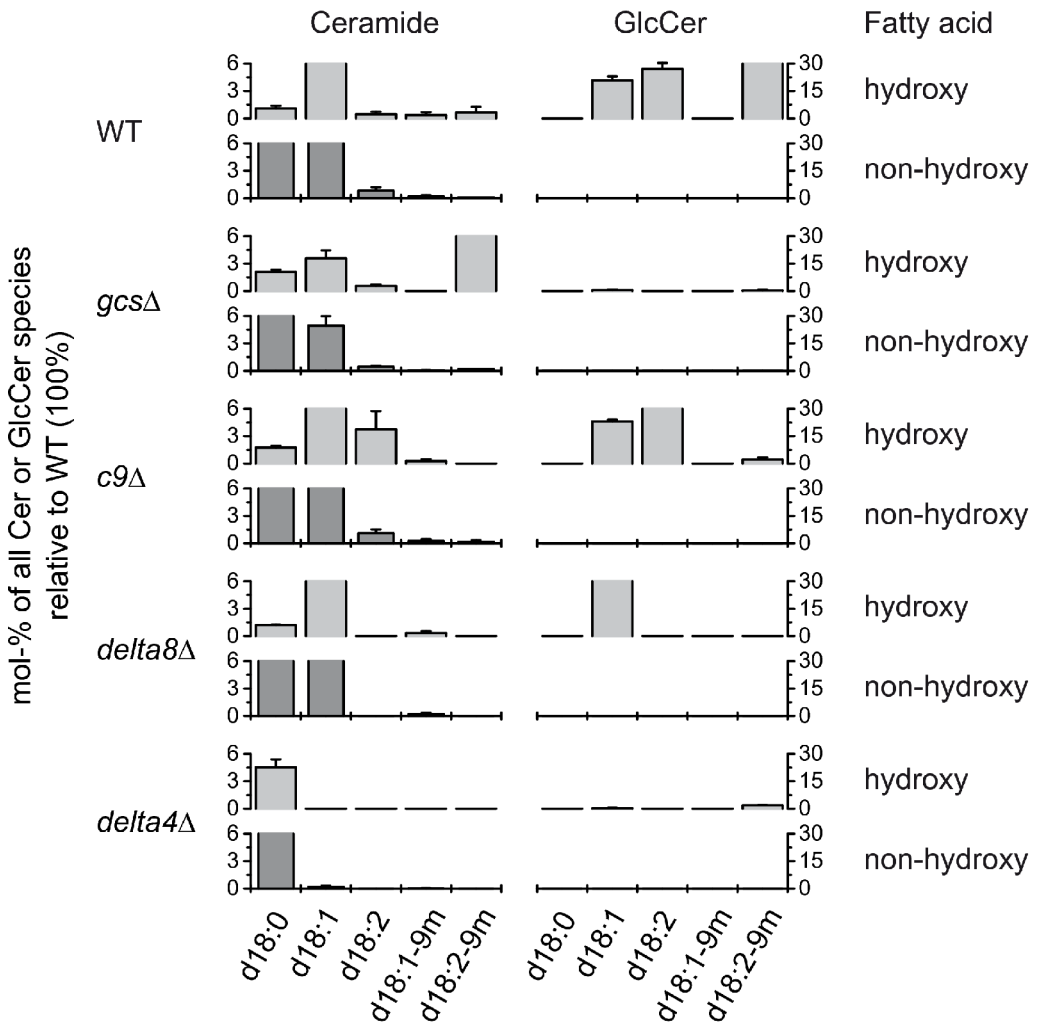
Supplemental Fig. S2



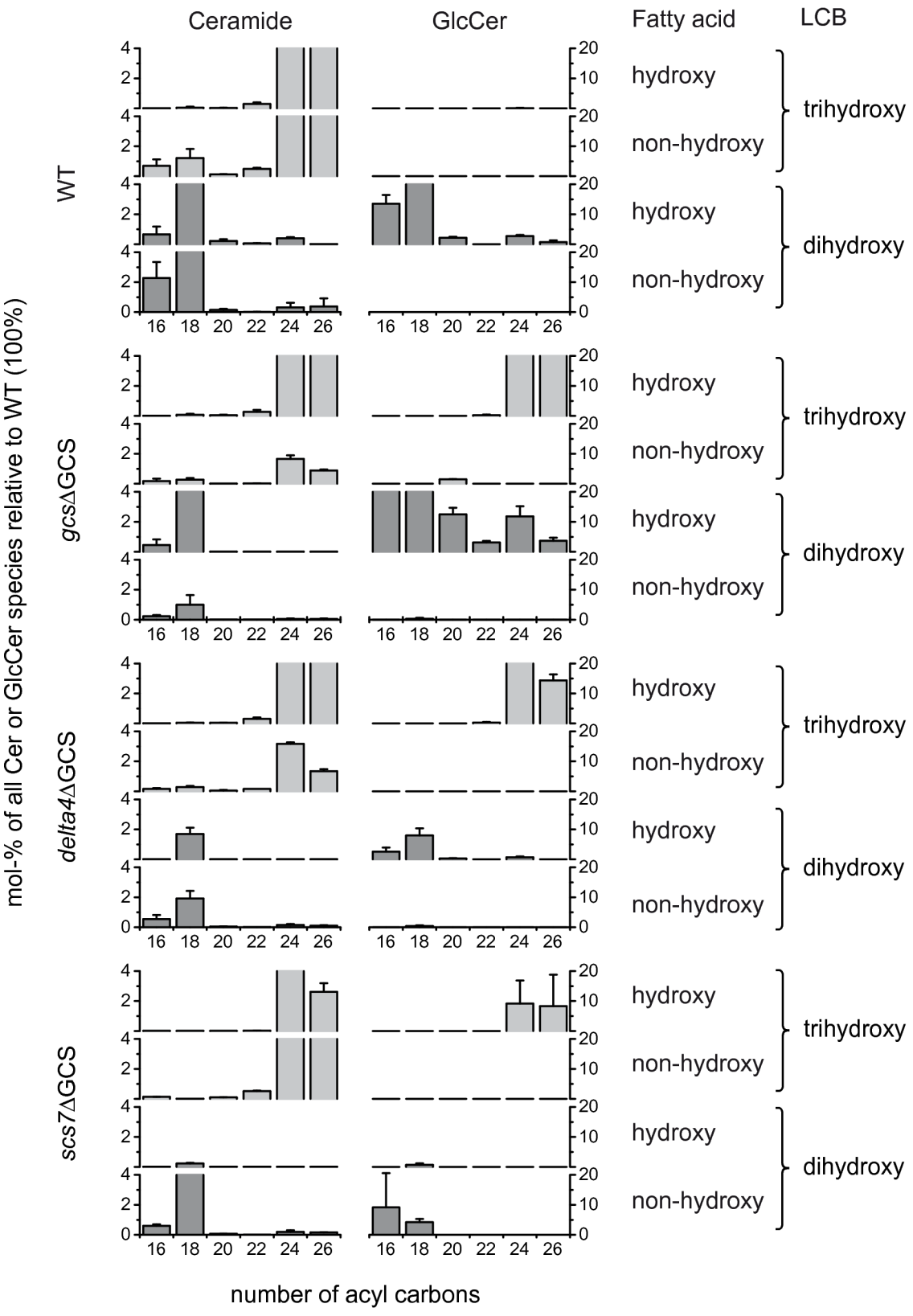
Supplemental Fig. S3



Supplemental Fig. S4



Supplemental Fig. S5



Supplemental Fig. S6