Regulation of Gene Expression through a Transcriptional Repressor that Senses Acyl-Chain Length in Membrane Phospholipids

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Figure S1. Identification of serine1157 as Snf1/AMPK phosphorylation site of Acc1, related to Figure 1

MS/MS spectrum of m/z 1031.2, which was identified as phosphopeptide AV(Sp)VSDLSYVANSQSSPLR from wild type Acc1. The complete y-ion series from \( y_3 \) to \( y_{15} \) is shown, confirming phosphorylation at serine1157.
Figure S2. Phenotypical characteristics of strains with modulated Acc1 activity, related to Figure 2

(A-C) Cells were grown overnight in YPD medium at 30°C and washed twice with sterile water. 5 µl aliquots of dilutions of OD_{600} = 1, 0.1, 0.01 and 0.001/ml were spotted onto the indicated plates and incubated at 30°C for 2 days (A-B) and 3 days (C). In A), the concentration of doxycycline (DC) was 2
µg/ml; in C) cells were grown on yeast extract peptone media containing non-fermentable carbon sources, glycerol (YPG), ethanol (YPE), or both (YPGE). (D) Visualization of lipid droplets with BODIPY™ 493/503 (green) and Nile Red (red) in cells grown to stationary phase (24h) at 30°C on +I medium or rich medium (YPD). Note the large number of damaged cells (transmission images) in the stationary culture of the snf1 mutant. Scale bar, 5 µm. (E) Total TAG levels in cells that were pre-cultured for 20h and shifted to fresh +I medium, for 3 hours. Lipid amounts were normalized to an internal standard (IS) as described in experimental procedures, and represented as mean ± SD; n=3.
Cells were pre-cultured logarithmically for 20h in +I medium and shifted to fresh media as indicated. (A) Growth of wild type (WT) and the acc1<sup>S/A</sup> mutant on media in the absence (−I) or presence (+I) of inositol. Standard deviations were below 5% and omitted from the diagram for better visibility. (B) Titration experiment to demonstrate the growth dependence of the acc1<sup>S/A</sup> mutant on inositol (1-5 µM). Doubling times were determined between 0-3 and from 3-6 hours after the shift to fresh medium. Results are presented as mean ± SD; n=3. (C) INO1 expression kinetics of acc1<sup>S/A</sup> opi1 strain in the presence (+I) or absence (−I) of inositol. ACT1 served as internal control; values were normalized to wild type grown for 1.5 hours on medium containing inositol (INO1 repressing conditions). Absence of the repressor Opi1 leads to full de-repression of INO1 in the acc1<sup>S/A</sup> background, independent of supplementation with inositol. Results are presented in mean ± SD; n=3. Data points labeled with “a” represent single measurements only.
Figure S4. Acc1 activity correlates with cellular inositol production, related to Figure 4

(A) Cells were grown overnight in YPD medium at 30°C and washed twice with sterile water. 5 µl of OD$_{600}$ = 1/ml aliquots were spotted on -I medium containing the indicated concentration of doxycycline (DC). After 2 days of growth at 30°C, the plates were sprayed with a suspension of an inositol auxotrophic tester strain (AID) and its growth around colonies was scored after further incubation for 2 days. $opi1$ and $cho2$ mutants served as positive controls, wild type (wt) as a negative control. Overproduction and excretion of inositol ($Opi^-$ phenotype) was determined by the appearance of halos around the spotted strains. (B) Model for the correlation between Acc1 activity and $INO1$ gene expression. A reduction of Acc1 activity in wild type (decreased $ACC1$ expression level in the tetO7-ACC1 strain in the presence of doxycycline or in wild type treated with SorA) leads to inositol overproduction (1), strains with Acc1 hyperactivity ($acc1^{S/A}$, $snf1$ mutants) are auxotrophic for inositol (3), wild type is inositol prototroph (2).
Figure S5. Synthesis of PA 32:1, related to Figure 7

(A) Strategy of 1-palmitoyl-2-palmitoleoyl-sn-glycerol-3-phosphate synthesis. Conditions: a) DMAP, DCC, palmitic acid, CCl₄, r.t., 3h; b) TsOH, MeOH, r.t., 8h; c) TBSCI, Et₃N, DMAP, DCM, 0°C-r.t., 3d; d) 4-pyrrolidinopyridine, DMAP, palmitoleic acid, CCl₄, r.t., 3h; e) Et₃N * 3HF, THF-MeCN (1:1), r.t., overnight; f) 1H-tetrazole, di-tert-butyl diisopropylphosphoramidite, m-CPBA, DCM-MeCN (1:1), 3h, r.t.-0°C; g) TFA, DCM, 3h, r.t.. (B) ¹H NMR spectra, (C) ¹³C NMR spectra and (D) ³¹P NMR spectra of 32:1 PA
**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

_Saccharomyces cerevisiae_ strains used in this study

| Strain     | Genotype                                      | Source          |
|------------|-----------------------------------------------|-----------------|
| wild type  | MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0      | Laboratory strain<sup>1</sup> |
| snf1       | MATα snf1::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf       |
| acc<sup>S/A</sup> | MATα ACC<sup>Ser<sup>157</sup>Ala</sup> his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study      |
| tetO7ACC1  | MATα leu2Δ1::tTA-LEU2 tetO7-ACC1 trp1-63 ura3-52 | Open Biosystems |
| snf1 tetO7ACC1 | MATα snf1Δ10 tetO7-ACC1 his3Δ1 leu2Δ1::tTA-LEU2 ura3Δ0 | A. Ulrich       |
| acc<sup>S/A</sup> opi1 | MATα ACC<sup>Ser<sup>157</sup>Ala</sup> opi1::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study      |
| Opi1-GFP   | MATα OPI1-GFP-HIS3MX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Open Biosystems |
| snf1 Opi1-GFP | MATα snf1::kanMX4 OPI1-GFP-HIS3MX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | This study      |
| acc<sup>S/A</sup> Opi1-GFP | MATα ACC<sup>Ser<sup>157</sup>Ala</sup> OPI1-GFP-HIS3MX6 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study      |
| ole1       | MATα ole1::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | This study<sup>2</sup> |
| opi1       | MATα opi1::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf       |
| cho2       | MATα cho2::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf       |
| AID        | MATα/α ino1Δ13/ino1Δ13 ade1/ade1             | S.A. Henry      |

<sup>1</sup>derived from sporulation of the diploid wild type strain BY4743

<sup>2</sup>derived from sporulation of a heterozygous diploid ole1/OLE1 parental strain on medium containing oleic acid (C18:1)

**Chemicals**

Palmitoleic acid (C16:1), tergitol (NP-40), 4-morpholineethanesulfonic acid (MES) and inositol were from Sigma-Aldrich, reduced glutathione from Merck, oleic acid from Larodan Fine Chemicals and phosphatidic acid (PA) species and phosphatidylcholine were from Avanti Polar Lipids; PA 32:1 was synthesized in this study.

**In vitro mutagenesis of ACC1**

The mutation generating the amino acid change from serine1157 to alanine (acc<sup>T</sup>S/A mutant) was introduced by site directed mutagenesis as described (Ho et al., 1989). Two mismatch PCR reactions were performed using genomic DNA from a wild type strain as template in order to generate overlapping
fragments at the region of the desired nucleotide exchange (forward primer 1: CAG AAT TTG AAG TGT CGG TGA C and reverse primer 1: G AAC AGG GCT GTT GCT GTT TCA GAT; forward primer 2: GA CAA ATC TGA AAC AGC AAC AGC CCT G and reverse primer 2: GAG AAA TCC AAA ACT GCC TTA CTC, changed nucleotides are in bold). Fragment sizes (686 bp and 987 bp, respectively) were checked on an agarose gel and both fragments were ligated in a subsequent PCR reaction without primers. The full length insert (1643 bp) was amplified (using forward primer 1 and reverse primer 2) and transformed into a wild type strain for genetic recombination. Cells were then plated on YPD-plates containing 1 μg/ml soraphen A for selection of mutants. The mutant generated in this fashion was back-crossed twice with a wild type strain. Correct nucleotide exchange was confirmed by sequencing.

Analysis of Acc1 by liquid chromatography/mass spectrometry

For qualitative analysis, Protein isolation of logarithmically growing cells was performed as previously described (Shirra et al., 2001). In brief, cells from wild type and the acc1S/A mutant strains were mechanically disrupted with glass beads in a Merckenschlager disintegrator (B. Braun Biotech Inc., Allentown, PA, USA) for 3 min under CO₂ cooling. Acc1 protein was enriched using Pierce® Monomeric Avidin Kit (ThermoScientific, Rockford, IL, USA). The 250 kDa Acc1 protein bands of wild type and acc1S/A mutant strains were excised from the gel and tryptically digested as described (Shevchenko et al., 1996). Peptide extracts were dissolved in 0.1% formic acid and separated on a nano-HPLC system (Ultimate 3000™, Dionex, Amsterdam, Netherlands). 70 μl samples were injected and concentrated on the loading column (LC Packings C18 PepMap™, 5 μm particle size, 100 Å pore size, 300 μm ID x 1mm) for 5 min using 0.1% formic acid as isocratic solvent at a flow rate of 20 μl/min. The column was then switched to the nanoflow circuit, and the sample was loaded on the nanocolumn (LC-Packings C18 PepMap™, 75 μm inner diameter x 150 mm) at a flow rate of 300 nl/min and separated using the following gradient: solvent A: water, 0.3% formic acid, solvent B: acetonitrile/water 80/20 (v/v), 0.3% formic acid; 0 to 5 min: 4% B, after 40 min 55% B, then for 5 min 90% B and 47 min re-equilibration at 4% B. The sample was ionized in a Finnigan nano-ESI source equipped with NanoSpray tips (PicoTip™ Emitter, New Objective, Woburn, MA, USA) and analyzed in a Thermo-Finnigan LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA, USA). The MS/MS data were analyzed by searching the NCBI non-redundant public database with SpectrumMill Rev. 03.03.084 SR4 (Agilent, Darmstadt, GER) software. Acceptance parameters were two or more identified distinct peptides according to Carr et al. (Carr et al., 2004). The MS/MS spectrum of the peptide confirming serine1157 as the Snf1 phosphorylation site is shown in Figure S1.

Quantitative analysis of Acc1 phosphorylation at position serine1157

For quantitative analysis, protein isolation of logarithmically growing cells was performed as described above but the avidin purification step was omitted due to possible degradation of Acc1 or loss of phosphorylation even in the presence of phosphatase inhibitors. The relative amount of Acc1 phosphorylation at position serine1157 from wild type, snf1 and acc1S/A mutant cells was determined as
The internal standard peptide mixture used for quantitative analysis of Acc1 phosphorylation was generated as described (Holzmann et al., 2009). In brief, the EtEP-peptides (GVTASVAGARAVSVSDLAYVANSQSSLR, GVTASVAGARAV(Sp)VSDLASVANSQSSLR), which were synthesized in house using solid-phase Fmoc chemistry, were purified and digested with trypsin. The resulting tryptic peptides were labeled with mTRAQ heavy (ABSCIEX, Foster City, CA) and quantified by nLC-MRM analysis on a 5500 QTRAP instrument (ABSCIEX, Foster City, CA) for the mTRAQ-heavy labeled equalizer peptide GVTASVAGAR. The individual EtEP-peptide digestions were then mixed in equimolar amounts, accordingly.

The tryptically digested Acc1 protein bands were labeled with mTRAQ light (ABSCIEX, Foster City, CA). The mTRAQ-light labeled Acc1 peptides were then spiked with an equimolar mixture of the mTRAQ-heavy labeled internal standard peptides (100 fmol each) and were immediately separated on a Dionex Ultimate 3000 RSLCnano HPLC, fitted with a C18 PepMap column (75 μm x 150 mm, 3 μ particle size, 100 Å pore size) (Dionex, Amsterdam, The Netherlands), developing a binary gradient from 98% solvent A (2% acetonitrile, 0.1% formic acid) and 2% solvent B (80% acetonitrile, 10% TFE, 0.08% formic acid) to 40% solvent B over 60 minutes at a flow rate of 275 nl/min, followed by a gradient from 40% solvent B to 90% solvent B over 5 minutes at a flow rate of 275 nl/min. The eluting peptides were analyzed on a 5500 QTRAP (ABSCIEX, Foster City, CA), essentially as described in (Holzmann et al., 2011). In brief, the mass-spectrometer was operated in MRM mode, monitoring four MRM transitions per peptide, under previously optimized collision energy (CE)- and collision cell exit potential (CXP)-settings. The integration of the respective XIC peaks was performed manually, using the MultiQuant 1.2 software (ABSCIEX, Foster City, CA). The abundance of the sample-derived peptides, relative to the internal standard peptides, was calculated from the ratios of the respective light to heavy XIC peak areas. The degree of phosphorylation was then calculated from the ratio of the sample-derived phosphorylated peptide to the sample-derived non-phosphorylated peptide.

Protein and phosphor-peptide identification, as well as tryptic digestion quality control, was performed by nLC-MS/MS analysis. Prior to their analysis by an LTQ-Orbitrap Velos, fitted with ETD (Thermo Fisher Scientific, Bremen, Germany), tryptic peptides prepared from Acc1 protein SDS-PAGE gel-bands were separated on a Dionex Ultimate 3000 RSLCnano, fitted with a C18 PepMap column (75 μm x 150 mm, 3 μ particle size, 100 Å pore size) (Dionex, Amsterdam, The Netherlands), developing a binary gradient from 98% solvent A (2% acetonitrile, 0.1% formic acid) and 2% solvent B (80% acetonitrile, 10% TFE, 0.08% formic acid), to 40% solvent B, over 115 minutes at a flow rate of 275 nl/min, followed by a gradient from 40% solvent B to 90% solvent B, over 5 minutes, at a flow rate of 275 nl/min. MS/MS data were generated in data-dependent mode (using CID and ETD for the 6 most intense signals recorded within MS1 full scans of the mass range 350-2000 m/z) and were interpreted by searching the NCBI non-redundant database with Mascot 2.2 (Matrix Science, London, UK).

Additionally, the relative amounts of Acc1 serine1157 phosphorylation was estimated from the XIC peak areas of the 2+ and 3+ charged ions of the peptides AVSVSDLASVANSQSSLR and
AV(Sp)VSDLSYVANSQSSPLR, respectively. Manual data evaluation and integration of the XIC peaks was performed using the QualBrowser of the XCalibur 2.1 software (Thermo Fisher Scientific, Bremen, GER). The degree of phosphorylation was then calculated from the ratios of the peak areas of the phosphorylated to the non-phosphorylated peptide ions.

**Overexpression of INO1**

The INO1 open reading frame was amplified by PCR with the primers 5'-

GTGACGCGGCCGCATGACAGAAGATAATATTGCTCCATACCTCC-3' and 5'-

GTGACACTAGTTTACAAATCTCTCTCGAATCTTAGTTGTTTTG-3', digested with NotI and SpeI and subsequently ligated with the NotI/SpeI digested plasmid pSP-G1 (kindly provided by Dr. Jens Nielsen, Gothenburg, Sweden), resulting in the INO1 open reading frame under control of the TEF1 promoter. Wild type and acc1'S/A were transformed with the resulting plasmid pSP-G1/[INO1] and empty vector [EV].

**Fluorescence microscopy**

Microscopy was performed using a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) with spectral detection and a Leica 100x immersion objective (NA=1.4). BODIPY® 493/503 fluorescence was excited at 488 nm. Fluorescence emission was detected between 500-550 nm. Nile Red fluorescence was excited at 543 nm. Fluorescence emission was detected between 550-570 nm. Opi1-GFP fluorescence was excited at 488 nm, GFP fluorescence emission was detected in the range between 500-550 nm. Transmission images were recorded using differential interference contrast (DIC) optics.

**Lipid analysis**

Cell pellets were resuspended in 5 ml chloroform/methanol (Sigma-Aldrich/Roth) 2/1 (v/v) and a MasterMix, containing the non-natural occurring species 28:0 DAG, 36:0 TAG, 45:0 TAG, 51:0 TAG, 57:0 TAG, 24:0 PC, 34:0 PC, 38:0 PC, 24:0 PE, 34:0 PE, 34:0 PS, 24:0 PA, 28:0 PA and 34:0 PA was added as internal standard. Cells were broken with glass beads on a Multi Reax vibrating shaker (Heidolph Inc., Schwabach, Germany) for 30 min, and further 10 min after addition of 1 ml of 0.034% MgCl₂ solution. Lipids were extracted according to a slightly modified Folch method (Folch et al., 1957; Schneiter and Daum, 2006) and suspended in 1 ml chloroform/methanol (CM) 2/1 (v/v).

For analysis in positive ESI mode (PE, PC, DAG and TAG), a 100 µl aliquot of the lipid extract was diluted with 2-Propanol (1/5, v/v). For analysis in negative ESI mode (PA, PI and PS), a 100 µl aliquot of the lipid extract was dried and then dissolved in 100 µl CM/2-Propanol (1/10, v/v). Chromatographic separation was performed using an AQUITY-UPLC system (Waters, Manchester, UK) equipped with a BEH-C18-column, 2.1x150 mm, 1.7 µm (Waters). A binary gradient was applied. Solvent A consisted of water/methanol (1/1, v/v), solvent B was 2-propanol. Both solvents contained phosphoric acid (8 µM), ammonium acetate (10 mM) and formic acid (0.1 vol%). The linear gradient started at 45% solvent B and
increased to 100% solvent B within 32 minutes. The column compartment was kept at 50°C. A SYNAPT™G1 qTOF HD mass spectrometer (Waters) equipped with an ESI source was used for analysis with the following source parameters: capillary temperature 100°C, desolvatization temperature: 400°C, N₂ as nebulizer gas. The capillary voltage was 2.6 kV in positive and 2.1 kV in negative ionization mode. Leucine-enkephaline ([M+H⁺]: m/z 556.2771 and [M-H⁻]: m/z 554.2615) was used as reference substance in the lock-spray. Data acquisition was done by the MassLynx 4.1 software (Waters), and lipids were identified and analysed by “Lipid Data Analyser” software (Hartler et al., 2011).

For total fatty acid analysis, a 200 µl aliquot of lipid extract was subjected to transmethylation and GC-MS measurements. Routinely, 100 µg of butylhydroxytoluene (BHT, Sigma-Aldrich) were added to the samples to prevent lipid oxidation. After drying under a stream of nitrogen, 0.5 ml toluene (Merck) and 2 ml of 14% methanolic boron trifluoride complex (Sigma-Aldrich) were added and incubated for 1 hour at 100°C. Samples were cooled on ice, and 1 ml of ice-cold water and 2 ml of hexane/chloroform 4:1 (v/v) were added and mixed on an overhead shaker for 15 min. Phase separation was achieved by centrifugation at 3,000 rpm for 3 min and the upper phase was collected. Extraction was repeated by adding 1 ml ice-cold water and 2 ml of hexane/chloroform 4/1 (v/v); upper phases were combined and dried under a stream of nitrogen. Fatty acid methyl esters (FAMEs) were dissolved in 100 µl hexane (Roth) and GC-MS was performed using a Trace GC/DSQ–MS (Thermo Scientific, Vienna, AUT). 1 µl sample solution was injected (injector temperature 250°C) and separated on a 60 m DB5-MS column with a temperature gradient from 80 to 300°C (Agilent, Waldbronn, GER). MS data were acquired in positive EI ionization mode (70 eV, source temperature: 280°C, mass range: 50-700 m/z). Data analysis was performed using the XCalibur 1.4 software (Thermo). The peaks in the extracted ion chromatograms of m/z 74 and m/z 87 (=MacLafferty-fragments of FAME) were integrated. C17:0-FAME (derived from C17-containing lipid species in the MasterMix) was used as internal standard.

**Relative quantification of INO1 gene expression**

Total RNA was isolated using RNeasy® Mini Kit including a DNA digestion with RNase-free DNase Set (Qiagen). 1 µg of RNA was transcribed into cDNA using oligo(dT)12-18 primers (0.5 µg), PCR grade dNTP mix (0.5 mM), First Strand Buffer (1x), DTT (10 mM) and 100 Units SuperScript® III Reverse Transcriptase (all Invitrogen). Real-Time PCR was conducted on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) and TaqMan® probes and primers (**INO1**: TaqMan® probe 5'-Fam-CTG TTG CCC ATG GTT AGC CCA AAAC G-Tamra-3', forward primer 5'-GGA ATG ACG TTT ATG CTC TTT A-3', reverse primer 5'-GTC CCA ACC AGA GAC GAC AAA-3'; **ACT1**: TaqMan® probe 5'-Fam-TGC AAA CCG CTG CTC AAT CTT CAA T-Tamra-3'; forward primer 5'-CGC CTT GGA CTT CTA ACA AGA AG-3', reverse primer 5'-GAC CAT CTG GAA GTT CGT AGG ATT-3'). In brief, the Real-Time PCR reaction of 25 µl contained 0.5 µM primers, 0.2 µM TaqMan® probe, 1x Master Mix and 5µl aliquots of 1:50 dilutions of transcribed RNA. All
reactions were performed in technical duplicate. Non-template control (RNA) and non-reaction control (H$_2$O) were routinely performed. The thermal program for the PCR run included stage 1: 95°C, 10 min and stage 2: 95°C, 0.5 min and 60°C, 1 min for a total of 40 cycles. Relative quantification was done using the comparative ΔΔCT method (see StepOnePlus$^\text{TM}$ user manual from Applied Biosystems).

**Opi1 overexpression and purification**

The *OPI1* open reading frame was amplified by PCR with the primers 5'-gactaGGATCCTCTGAAAATCAACGTGGATTATCAGAGG-3' and 5'-gactaCCCGGGTTAGTCTTGCTATCCACGTTGCCTG-3', digested with BamHI and Smal and subsequently ligated with the BamHI/Smal digested plasmid pYEX4-T1 (CLONTECH), resulting in *OPI1* fused to the 3' end of the GST open reading frame. Wild type yeast cells were transformed with the resulting plasmid pYEX4-T1 GST-Opi1.

For GST-Opi1 overexpression, cells were cultivated overnight in 5 ml minimal SD medium lacking uracil to stationary phase, rediluted in 1 liter of the same media containing 50 µM copper sulfate (inducing conditions), and grown for another 16 hours. 5 g cell wet weight were harvested, washed once with water and resuspended in 25 ml lysis buffer (50 mM Tris/Cl, 1 M NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM EDTA, pH 7.5). Cell breakage was performed using a Merckenschlager disintegrator (Braun Melsungen, Germany) for 3 min with CO$_2$ cooling. Cell debris and glass beads were sedimented at 4000 rpm for 5 min at 4°C. Membrane fractions were sedimented at 20,000 rpm for 20 min at 4°C and the clear supernatant was loaded on a column manually packed with 500 µl Glutathione Sepharose 4 Fast Flow (GE Healthcare). After washing with 15 ml lysis buffer, Opi1-GST was eluted ten times with 1 ml elution buffer each (50 mM Tris/Cl, 1 M NaCl, 1 mM DTT, 15 mM reduced glutathione, pH 8.0). Fractions were checked for fusion protein content and purity by Coomassie staining, and fractions with the highest fusion protein content were pooled and used in the Opi1 binding assay.

**Preparation of unilamellar liposomes**

1 ml solutions of 0.5 mM dioleoyl-PC (36:2 PC) or mixtures of 0.4 mM 36:2 PC and 0.1 mM of either dipalmitoyl-PA (32:0 PA), 1-palmitoyl-2-palmitoleoyl-PA (32:1), distearoyl-PA (36:0 PA) or dioleoyl-PA (36:2 PA) in CHCl$_3$/MeOH 95:5 (v/v) were prepared. The solvent was evaporated at 30°C under a stream of nitrogen and the lipids were dried overnight in vacuum. Lipid films were resuspended in 1 ml hydration buffer (50 mM Tris, 1 M NaCl, 1 mM DTT, pH 8.0) and hydrated at room temperature for 2 hours under vortexing every 15 min. Samples were 15 times extruded according to the Extrusion Technique protocol from Avanti Polar Lipids using a Lipids Mini Extruder (Avanti Polar Lipids) and a polycarbonate filter (Whatman, pore size 0.2 µm) and stored at 4°C until usage.

**Synthesis of 1-palmitoyl-2-palmitoleoyl-sn-glycero-3-phosphate (32:1 PA)**
All chemicals were purchased from Sigma-Aldrich or Alfa Aesar and were used without further purification. $^1$H-NMR and $^{13}$C spectra were recorded on a Bruker 300 MHz instrument using CDCl$_3$, MeOD and CF$_3$COOD as solvents. Chemical shifts (δ) are expressed in ppm downfield from TMS as internal standard. The letters s, d, t, q, and m are used to indicate a singlet, doublet, triplet, quadruplet, and multiplet. Silica gel flash chromatography separations were performed on a Biotage SP1 instrument using petroleum ether/ethyl acetate or chloroform/methanol mixtures as eluent. TLC analysis was carried out on Merck pre-coated silica gel 60-F$_{254}$ plates. Mass spectra were recorded on a Synapt HDMS Q-TOF MS by direct infusion with a flow rate of 20 µl/min. An ESI ion source was used with the following settings: Capillary voltage: 2.6 kV for positive ESI or 2.1 kV for negative ESI; Sampling cone: 40 V; Extraction cone: 4.5 V; Scan time: 1 sec. The strategy for the synthesis of PA 32:1 was modified from Tallman et al. (Tallman et al., 2007) and is schematically shown in Figure S7A.

**Synthesis of 2.** A solution of DCC (1.85 g, 8.97 mmol) in CCl$_4$ (12.5 mL) was added dropwise to a solution of 1 (1.00 mL, 8.13 mmol), palmitic acid (2.30 g, 8.97 mmol) and DMAP (1.15 g, 9.41 mmol) in CCl$_4$ (12.5 mL). A white precipitate was formed which was removed by filtration after quantitative esterification (3h, TLC). The solvent was evaporated under reduced pressure and the crude product was purified by preparative column chromatography using petroleum ether/ethyl acetate as eluent yielding in 96 % (2.81 g, 7.58 mmol) of 2 as a white solid. $^1$H NMR (300 MHz, CDCl$_3$) δ 4.33 (td, $J = 11.0$, 6.2 Hz, 1H), 4.24 – 4.05 (m, 3H), 3.75 (dd, $J = 8.4$, 6.2 Hz, 1H), 2.35 (t, $J = 7.5$ Hz, 2H), 1.63 (dd, $J = 13.4$, 6.4 Hz, 2H), 1.45 (s, 3H), 1.39 (s, 3H), 1.27 (bs, 24H), 0.89 (t, $J = 6.6$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 173.63, 109.80, 73.65, 66.34, 64.50, 34.11, 31.92, 29.68, 29.67, 29.65, 29.59, 29.45, 29.36, 29.25, 29.12, 26.68, 25.39, 24.89, 22.69, 14.12. HDMS ESI C$_{22}$H$_{42}$O$_4$Na [M+Na]$^+$ m/z calcd = 393.2981, found = 393.2974.

**Synthesis of 3.** p-Toluenesulfonic acid (47.0 mg, 0.273 mmol) was added to a solution of 2 (2.00 g, 5.40 mmol) in MeOH (80 mL). After 8 h stirring at room temperature the solution was concentrated and the product was isolated in 57 % (1.02 g, 3.10 mmol) as a white solid by preparative column chromatography using petroleum ether/ethyl acetate as eluent. $^1$H NMR (300 MHz, CDCl$_3$) δ 4.29 – 4.10 (m, 2H), 3.95 (d, $J = 3.3$ Hz, 1H), 3.65 (dt, $J = 11.1$, 8.7 Hz, 2H), 2.58 (s, 1H), 2.37 (t, $J = 7.6$ Hz, 2H), 2.15 (s, 1H), 1.63 (dd, $J = 13.9$, 6.9 Hz, 2H), 1.27 (s, 24H), 0.89 (t, $J = 6.6$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 174.37, 70.26, 65.16, 63.32, 34.16, 31.92, 29.69, 29.65, 29.60, 29.45, 29.36, 29.25, 29.13, 24.91, 22.70, 14.13. HDMS ESI C$_{19}$H$_{38}$O$_4$Na [M+Na]$^+$ m/z calcd = 353.2668, found = 353.2654.

**Synthesis of 4.** TBDMSCl (309 mg, 2.05 mmol) in DCM (2 mL) was added dropwise to a solution of 3 (600 mg, 1.82 mmol), DMAP (22.4 g, 0.183 mmol) and triethylamine (279 mg, 2.00 mmol) in DCM (4 mL) at 0°C. Afterwards, the ice bath was removed and the reaction mixture was stirred at room temperature for two days. As the starting material was not consumed (TLC), further TBDMSCl (100 mg, 0.663 mmol) was added. After stirring for additional 24 h TLC analysis still indicated remaining substrate. Anyway, the mixture was diluted with 25 mL DCM and extracted with distilled water (25 mL). The organic phase was dried with MgSO$_4$, and concentrated under reduced pressure. Chromatographic purification using petroleum ether/ethyl acetate as eluent resulted 43 % (344 mg, 0.773 mmol) of 4 as a yellowish oil. $^1$H
NMR (300 MHz, CDCl₃) δ 4.23 – 4.09 (m, 2H), 3.89 (dd, J = 10.5, 5.3 Hz, 1H), 3.66 (dd, J = 15.7, 10.1, 5.1 Hz, 2H), 2.53 (d, J = 5.3 Hz, 1H), 2.35 (t, J = 7.5 Hz, 2H), 1.63 (dd, J = 14.1, 7.3 Hz, 2H), 1.27 (s, 24H), 0.95 – 0.88 (m, 12H), 0.09 (s, 6H).

¹³C NMR (75 MHz, CDCl₃) δ 173.97, 69.99, 64.97, 63.68, 34.20, 31.92, 29.69, 29.65, 29.60, 29.46, 29.36, 29.27, 29.14, 25.83, 25.64, 24.94, 22.69, 18.26, 14.12, -5.46.

HDMESI C₃₃H₅₁O₃Si [M-(H₂O)+H]^+ m/z calcd = 427.3607, found = 467.3605.

**Synthesis of 5.** DCC (134 mg, 0.651 mmol) in CCl₄ (3 mL) was added dropwise to a solution of 4 (250 mg, 0.562 mmol), palmitoleic acid (159 mg, 0.624 mmol), and 4- pyrrolidinopyridine (99.1 mg, 0.669 mmol) in CCl₄ (2 mL). After 3 h stirring at room temperature, the reaction mixture was filtered and concentrated. Purification by column chromatography (petroleum ether/ethylacetate) afforded the product as colorless oil in 94 % (338 mg, 0.496 mmol) yield.

¹H NMR (300 MHz, CDCl₃) δ 5.36 (t, J = 5.6 Hz, 2H), 5.14 – 5.04 (m, 1H), 4.36 (dd, J = 11.8, 3.6 Hz, 1H), 4.17 (dd, J = 11.8, 6.2 Hz, 1H), 3.73 (d, J = 5.3 Hz, 2H), 2.32 (t, J = 7.2 Hz, 4H), 2.03 (d, J = 5.6 Hz, 4H), 1.67 – 1.59 (m, 2H), 1.35 – 1.24 (m, 2H), 0.97 – 0.84 (m, 8H), 0.07 (s, 3H).

¹³C NMR (75 MHz, CDCl₃) δ 173.46, 173.08, 129.99, 129.71, 71.67, 62.44, 61.44, 60.25, 34.34, 34.17, 31.93, 31.78, 29.70, 29.66, 29.64, 29.48, 29.36, 29.30, 29.19, 29.13, 29.12, 29.08, 28.99, 27.17, 25.75, 24.93, 22.69, 18.20, 14.12, -5.47. HDMESI (M+H)^+ m/z calcd for C₄₁H₈₁O₅Si [M+H]^+ = 681.5853, found = 681.5868.

**Synthesis of 6.** To a solution of 5 (310 mg, 0.455 mmol) in 6 mL THF:MeCN (1:1) was added HF * Et₃N (860 µL) at room temperature. The reaction mixture was stirred overnight. Afterwards the reaction was quenched with saturated NaHCO₃ (10 mL). The product was extracted with DCM (3 * 15mL) and the combined organic phases were washed with brine (30 mL) and dried over Na₂SO₄. Removal of the solvent resulted in 96 % of compound 6 (246 mg; 0.435 mmol) as yellow oil.

¹H NMR (300 MHz, CDCl₃) δ 5.53 – 5.25 (m, 2H), 5.20 – 5.00 (m, 1H), 4.45 – 4.20 (m, 2H), 3.74 (d, J = 5.0 Hz, 2H), 3.05 (dd, J = 14.4, 7.2 Hz, 1H), 2.42 – 2.26 (m, 4H), 2.08 – 1.96 (m, 4H), 1.61 (dd, J = 16.4, 9.5 Hz, 4H), 1.40 – 1.21 (m, 40H), 0.89 (t, J = 6.3 Hz, 6H).

¹³C NMR (75 MHz, CDCl₃) δ 173.79, 173.39, 130.01, 129.68, 72.10, 61.99, 61.51, 34.27, 34.10, 31.92, 31.78, 29.72, 29.69, 29.65, 29.47, 29.36, 29.27, 29.17, 29.12, 29.10, 28.98, 27.22, 27.15, 24.92, 24.88, 22.69, 22.65, 14.11. HDMESI (M+H)^+ m/z calcd for C₃₅H₆₇O₅ [M+H]^+ = 567.4989, found = 567.4996.

**Synthesis of 7.** To a solution of 6 (100 mg, 0.176 mmol) and di-tert-butyl diisopropylphosphoramidite (60.0 mg, 0.216 mmol) in DCM (1 mL) was added 1H-tetrazole (16.0 mg, 0.228 mmol) in 1 mL MeCN. The reaction mixture was stirred for 2 h at room temperature. Afterwards m-CPBA (41.0 mg; 0.238 mmol) was added at 0°C and the resulting mixture was stirred for 1 h. The reaction was quenched with 10% NaS₂O₃ and partitioned. The organic layer was washed with 10% K₂CO₃ and dried over MgSO₄. Preparative column chromatography (petroleum ether/ ethyl acetate) yielded in 47 % (62.5 mg, 82.3 µmol) of compound 7 as colorless oil.

¹H NMR (300 MHz, CDCl₃) δ 5.42 – 5.29 (m, 2H), 5.28 – 5.20 (m, 1H), 4.37 (dd, J = 11.9, 4.1 Hz, 1H), 4.22 – 4.05 (m, 3H), 2.32 (td, J = 7.5, 4.0 Hz, 4H), 2.07 – 1.97 (m, 4H), 1.62 (d, J = 3.0 Hz, 4H), 1.50 (s, J = 1.0 Hz, 18H), 1.35 – 1.23 (m, 40H), 0.89 (t, J = 6.5 Hz, 6H).

¹³C NMR (75 MHz, CDCl₃) δ 173.28, 172.82, 129.99, 129.69, 82.79, 82.73, 82.69, 82.63, 69.64, 69.52, 64.37, 64.29,
61.98, 34.20, 34.06, 31.92, 31.78, 29.84, 29.78, 29.69, 29.66, 29.63, 29.48, 29.36, 29.28, 29.19, 29.12, 29.11, 29.07, 28.98, 27.22, 27.17, 24.85, 24.83, 22.69, 22.65, 14.11, 14.10. $^{31}$P NMR (121 MHz, CDCl$_3$) $\delta$ -9.93. HRMS ESI C$_43$H$_{84}$O$_6$P $[\text{M+H}]^+$ m/z calcd = 759.5904, found = 759.5914. 

Synthesis of 8. TFA (230 µL) was added to a solution of 7 (48.0 mg, 63.2 µmol) in DCM (2 mL). The reaction was quenched with MeOH after stirring 3 h at room temperature. The mixture was diluted with benzene and concentrated. A final column chromatography purification using methanol/chloroform as eluent resulted in PA 32:1 (22.6 mg, 34.9 µmol) as white solid in 55 % yield. $^1$H NMR (300 MHz, CDCl$_3$:CD$_3$OD 6:1 + 1 drop CF$_3$COOD) $\delta$ 5.32 – 5.18 (m, 2H), 5.17 – 5.06 (m, 1H), 4.26 (dd, $J$ = 11.9, 3.4 Hz, 1H), 4.15 – 3.90 (m, 3H), 2.23 (dd, $J$ = 13.7, 7.3 Hz, 4H), 1.91 (d, $J$ = 5.5 Hz, 4H), 1.51 (s, 4H), 1.24 – 1.12 (m, 40H), 0.78 (t, $J$ = 6.4 Hz, 6H). $^{13}$C NMR (75 MHz, CDCl$_3$:CD$_3$OD 6:1 + 1 drop CF$_3$COOD) 173.78, 173.34, 129.76, 129.46, 69.69, 69.58, 61.93, 54.28, 53.98, 53.68, 53.37, 53.07, 52.77, 52.44, 49.42, 49.14, 48.85, 48.57, 48.28, 48.00, 47.71, 33.90, 33.81, 31.67, 31.53, 29.46, 29.44, 29.40, 29.24, 29.10, 29.03, 28.95, 28.87, 28.81, 28.72, 26.94, 24.58, 22.41, 22.38, 13.70. $^{31}$P NMR (121 MHz, CDCl$_3$:CD$_3$OD 6:1 + 1 drop CF$_3$COOD) $\delta$ 3.53. HDMS ESI C$_{35}$H$_{66}$O$_9$P $[\text{M-H}]^-$ m/z calcd = 645.4495, found = 645.4500. NMR spectra of 32:1 PA are shown in Figure S7B.
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