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**Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA binding protein dependent mechanism in *Saccharomyces cerevisiae***

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**Running Title:** Transcriptional changes in Acb1p depleted yeast cells

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**Key words:** Acyl-CoA binding protein (ACBP), phospholipids, fatty acids, inositol, transcriptional regulation.

**Abbreviations used:** OREs, oleate response elements; ER, endoplasmatic reticulum; LCACoA, long-chain acyl-CoA esters; L-FABP, liver fatty acid binding protein; ACBP, the acyl-CoA binding protein; PI, phosphatidylinositol; UAS<sub>INO1</sub>, inositol-sensitive upstream activating sequence; Opi<sup>-</sup>, overproduction of inositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; UPR, unfolded protein response.

## ABSTRACT

In the present study we have used DNA microarray and quantitative real-time analysis to examine the transcriptional changes occurring in response to cellular depletion of the yeast acyl-CoA binding protein, Acb1p. Depletion of Acb1p resulted in differential expression of genes encoding for proteins involved in fatty acid and phospholipid synthesis (e.g. *FAS1*, *FAS2*, *ACC1*, *OLE1*, *INO1* and *OPI3*), in glycolysis and glycerol metabolism (e.g. *GPD1* and *TDH1*), in ion transport/uptake (e.g. *ITR1* and *HNMI*), and in stress response (e.g. *HSP12*, *DDR2* and *CTT1*). Here, we show that transcription of the *INO1* gene, encoding the inositol-3-phosphate synthase, cannot be fully repressed by inositol and choline and that UAS<sub>INO1</sub>-driven transcription is enhanced in Acb1p depleted cells. In addition the reduced inositol-mediated repression of *INO1* transcription observed upon Acb1p depletion appears to be independent of the transcriptional repressor Opi1p. We also demonstrate that *INO1* and *OPI3* expression can be normalized in Acb1p depleted cells by supplementation of high concentrations of exogenous fatty acids or by overexpressing *FAS1* or *ACC1*. Together, these findings reveal an Acb1p-dependent connection between fatty acid metabolism and transcriptional regulation of phospholipid biosynthesis in yeast. Finally, expression of an Acb1p-mutant unable to bind acyl-CoA esters could not normalize the transcriptional changes caused by Acb1p depletion. This strongly implies that it is either the Acb1p/acyl-CoA ester complex directly or its ability to donate acyl-CoA esters to utilizing systems, which modulates gene expression.

## INTRODUCTION

In the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) fatty acids and fatty acid derivatives have been suggested to function as regulators of gene expression. This suggestion is supported by the fact that two oleate-activated transcription factors (Oaf1p and Pip2p), have been shown to be essential for fatty acid mediated induction of genes encoding peroxisomal enzymes and proteins [1]. Furthermore, transcriptional regulation of the yeast  $\Delta$ -9 desaturase, *OLE1*, by unsaturated fatty acids involves Spt23p and Mga2p [2].

It has been suggested that the regulatory properties of fatty acids are mediated through their activation to acyl-CoA esters, since fatty acids are unable to repress expression

of *ACC1* [3] and *OLE1* [4] and to induce expression of the peroxisomal enzymes acyl-CoA oxidase (*POX1*), and acyl-CoA synthetase (*FAA2*), [5] in *S. cerevisiae* depleted of the two acyl-CoA synthetases Faa1p and Faa4p. However, disruption of both *FAA1* and *FAA4* affects fatty acid import, and hence does not prove that fatty acid activation *per se* is required for fatty acid-mediated regulation of gene expression.

In *S. cerevisiae* long-chain acyl-CoA (LCACoA) esters are either synthesized endogenously by the fatty acid synthase (FAS) complex or produced from exogenously fatty acids imported by the fatty acid import machinery. Inside the cell acyl-CoA esters are bound to proteins in order to increase their solubility and to protect them from hydrolysis by thioesterases. A number of intracellular proteins have been reported to bind LCACoA esters including liver fatty acid binding protein (L-FABP), sterol carrier protein 2 (SCP-2) and the acyl-CoA binding protein (ACBP). A role of L-FABP in LCACoA ester metabolism is highlighted by the fact that L-FABP knockout mice display altered cytosolic LCACoA ester binding capacity and altered LCACoA ester acyl chain distribution [6]. Similarly, SCP-2/SCP-X knockout mice have impaired catabolism of methyl-branched fatty acyl-CoAs, inefficient import of phytanoyl-CoA into peroxisomes, and defective thiolytic cleavage of 3-ketopristanoyl-CoA [7]. However, *S. cerevisiae* expresses neither FABP [8] nor a SCP-2 homologue [9]. Thus, *S. cerevisiae* is well suited as a model for studying the function of ACBP since possible compensatory effects conducted by L-FABP and/or SCP-2 may be avoided.

Yeast ACBP, Acb1p, belongs to a large multigene family encoding ~10-kDa proteins, which binds LCACoA esters in a non-covalent and reversible manner with very high affinity ( $K_D$ , 1-10 nM) and specificity [10]. ACBP has been very highly conserved in evolution and it has been detected in all eukaryotic organism examined so far [11]. The high degree of sequence and structural conservation of ACBP among all eukaryotic species and the fact that human ACBP can complement ACBP function in yeast (our unpublished results), suggest that its function is associated with one or more basal cellular function(s) common to all cells [11].

*In vitro* experiments have shown that ACBP can attenuate the inhibitory effect of LCACoA esters on acetyl-CoA carboxylase and the mitochondrial adenine nucleotide translocase, stimulate the mitochondrial long-chain acyl-CoA synthetase, and is able to extract membrane-imbedded acyl-CoA esters and donate these to utilizing systems like

glycerolipid synthesis and  $\beta$ -oxidation [12,13]. Furthermore, it has been shown that overexpression of Acb1p and bovine ACBP in *S. cerevisiae* increases the acyl-CoA ester pool size [14,15]. Thus, ACBP may act as an acyl-CoA ester pool former and transporter *in vivo*. Despite this, the precise *in vivo* function of ACBP is currently unknown. Yeast cells lacking Acb1p activity display a variety of defects, including fragmented vacuoles, multi-layered plasma membrane, accumulation of vesicles of variable sizes, an 3- to 5.5-fold elevation of the *OLE1* mRNA level, and reduced levels of very long-chain fatty acids, ceramides and sphingolipids [4,16,17]. Due to the high affinity of ACBP for acyl-CoA esters and to the presence of cytosolic acyl-CoA hydrolases the intracellular free acyl-CoA ester concentration is expected to be very low [10]. It can therefore be expected that the Acb1p/acyl-CoA ester complex directly or indirectly through donation of acyl-CoA esters modulate acyl-CoA ester regulated and acyl-CoA ester consuming processes.

In the present study we have used whole genome cDNA microarrays and quantitative real-time PCR (RT-PCR) in order to identify transcriptional changes in response to cellular depletion of Acb1p. The present results suggest that depletion of Acb1p affects expression of genes encoding for proteins involved in fatty acid and phospholipid synthesis, glycolysis, glycerol metabolism, ion transport/uptake and in stress response. Moreover, this work uncovers an Acb1p-dependent connection between fatty acid biosynthesis and transcriptional regulation of phospholipid biosynthesis in yeast. Finally, we demonstrate for the first time that regulatory effects of Acb1p depend on its acyl-CoA ester binding property.

## EXPERIMENTAL

*Chemicals* - Bacto-peptone, yeast extract and yeast nitrogen base (YNB) were purchased from Difco (Diagnostic systems, Sparks, MD, U.S.A.). Secondary antibodies were obtained from Promega (Madison, WI, U.S.A.). Restriction enzymes and DNA/RNA modifying enzymes were either from New England Biolabs (Beverly, MA, U.S.A.) or Invitrogen (Carlsbad, CA, U.S.A.). All chemicals were of analytical grade.

*Yeast strains, media and growth* - The *S. cerevisiae* strains used in the present study are listed in Table 1. Y700 *pGAL-ACB1* is a conditional knockout strain constructed by insertion of the *GAL1* promoter in front of the *ACB1* gene, as described in [16]. In the wild type strain Y700 and the Y700 *pGAL-ACB1* strain single disruptions in *SCS2* and *PLD1*

(*SPO14*), in which the target ORF was disrupted by the *TRP1* marker, were constructed by PCR-mediated gene replacement using pFA6a-TRP1 template (kind gift of John R. Pringle, The University of North Carolina, Chapel Hill, North Carolina, U.S.A.) as described previously [18]. Disruption of the *HAC1* gene was achieved by homologous recombination of *Bam*HI-linearized pHAKO1 plasmid (kind gift of Peter Walter, University of California, San Francisco, CA, U.S.A.) in the wild type strain Y700 and the Y700 *pGAL-ACB1* strain. Correct integration of *TRP1* PCR products and pHAKO1 in Y700 and Y700 *pGAL-ACB1* cells was confirmed by PCR. Media used to grow yeast included yeast extract peptone dextrose (YPD), yeast extract peptone galactose (YPgal) and yeast nitrogen base dextrose (YNBD) made as previously described [17] and glucose-supplemented synthetic complete (SC) media made as described in [19]. *Myo*-inositol was omitted from the SC media or added to the concentration indicated. Choline was added to 1 mM and palmitic- or palmitoleic acid was added to 500  $\mu$ M where indicated. Yeast strains were kept on YPgal or yeast nitrogen base galactose (YNB-gal) plates and cultivated at 30 °C. Prior to any experiment yeast cells were inoculated in glucose-supplemented SC, YNBD or YPD media to an OD<sub>600</sub> of 0.005-0.01 and grown for 24 hours, diluted into fresh media to an OD<sub>600</sub> of 0.05-0.1 and grown to the OD<sub>600</sub> indicated. Depletion of Acb1p was confirmed by Western blotting after each experiment.

*Protein extraction, electrophoresis and Western blotting* - Protein extraction, electrophoresis and Western blotting was performed as described in [17] with the exception that PVDF membranes were used instead of nitrocellulose membranes. Antiserum towards anti-influenza hemagglutinin epitope (HA) was obtained from Roche Diagnostics (Hvidovre, Denmark).

*DNA microarray* - Yeast DNA microarrays were purchased from MWG (Ebersberg, Germany). 6250 gene specific oligonucleotide probes (40 mer) representing the complete *S. cerevisiae* genome were spotted on one single epoxy coated slide (1.8 x 3.6 cm). In addition each MWG Yeast Array contains 86 replicated spots and 32 *Arabidopsis* control spots.

*Yeast culture and total RNA isolation* - Wild type (Y700) and conditional *ACB1* knockout (Y700 *pGAL-ACB1*) cells were grown to an OD<sub>600</sub> of 0.9-1.3 in YPD, YNBD or glucose-supplemented SC media as described above. Following growth cells were collected by centrifugation (4000 rpm/10 min/4 °C) in 50 ml Falcon tubes half-filled with crushed ice. The supernatant was removed and cells were transferred to 1.5 ml microcentrifugation tubes

and pelleted by centrifugation (5000 rpm/3 min/4 °C). Harvested cells were frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted by the hot phenol method as previously described [20]. Deoxyribonuclease I digestion of the extracted RNA was performed as described by the manufacturer (MWG, Ebersberg, Germany).

*cDNA synthesis and post labelling* - RNA was isolated from Y700 and Y700 *pGAL-ACBI* cells grown in YPD media. For cDNA synthesis and post labelling the CyScribe cDNA Post labelling Kit was used. cDNA synthesis and post labelling was performed as described by the manufacturer (Amersham Biosciences, Piscataway, NJ, U.S.A.), except that amino allyl-modified cDNA was purified using the Qiagen QIAquick PCR Purification Kit in which the supplied wash and elution buffers were substituted with phosphate wash buffer [5 mM KPO<sub>4</sub> (pH 8.0) and 80 % ethanol] and phosphate elution buffer [4 mM KPO<sub>4</sub> (pH 8.5)], respectively, to avoid free amines.

*Hybridization, washing and scanning* - For hybridization a formamide-based hybridization buffer from MWG was used. Cy3 and Cy5 labeled cDNA was pooled, dried, resuspended in 30 µl preheated (42 °C/10 min) formamide-based buffer (MWG, Ebersberg, Germany) and incubated at 95 °C for 3 min. This hybridization mixture was spun down briefly and applied to the yeast DNA microarray under a clean cover slip. Slides were then placed into a wet hybridization chamber (GeneMachines<sup>®</sup>, Ann Arbor, MI, U.S.A.) and incubated for 18-22 hours in a 42 °C water bath. After incubation microarrays were washed as described by the manufacturer (MWG, Ebersberg, Germany). Arrays were then dried by centrifugation at 500 g for 2 min at room temperature and scanned at 532 nm (Cy3) and 635 nm (Cy5) in a GMS-418 scanner (Genetic MicroSystems<sup>™</sup>, Woburn, MA, U.S.A.). Overlay images were constructed in ImaGene version 4.2 (BioDiscovery, El Segundo, CA, U.S.A.).

*Experimental setup and data analysis* - RNA isolated from five different cultures of either Y700 or Y700 *pGAL-ACBI* cells grown in YPD media was pooled in two different tubes. cDNA synthesised from Y700 and Y700 *pGAL-ACBI* RNA was labeled with Cy3 and Cy5, respectively. The experiment was repeated on independent cultures with opposite Cy3/Cy5 labelling. Overlay images were processed using the microarray spot finding and quantification software ImaGene version 4.2 (BioDiscovery, El Segundo, CA, U.S.A.). Fluorescent spots were located, and extracted intensity values were corrected for local background by subtracting the intensity level around each spot from the spot's signal intensity. Intensities corrected for background were further processed and analysed using

Acuity version 4.0 (Axon Instruments, Foster City, CA, U.S.A.) software. Data from each microarray was normalized using print-tip locally weighted linear regression (lowess) normalization and average  $\log_2 (R/G)$  values (M values) were calculated. In dual colour experiments *R* and *G* represent processed and normalized intensity values measured at red and green channel, respectively. Subsequently, data were filtered in order to remove unreliable data from the dataset. Spots flagged bad and data with intensity levels at 532 nm (Cy3) and 635 nm (Cy5) below the highest intensity level of the 32 *Arabidopsis* control spots were removed. Furthermore, data that were not present in both microarrays were also excluded. M values were then transferred to EXCEL (Microsoft®) in which standard deviation (SD) were calculated. Data with a SD of more than 0.6 were removed, however, to avoid removing data which are e.g. highly upregulated in both arrays but have a SD of more than 0.6 the list of removed data was checked for such important data. Finally, genes considered to be differentially expressed were identified using a fold-change filter in which the cut-off was set to an absolute M value of 0.70 (corresponds to a ratio of 1.6 between wild type and *Acb1p* depleted cells).

*Quantitative real-time PCR, RNA isolation and cDNA synthesis* - Total RNA was isolated from cells cultured and harvested as described above. RNA was extracted by the hot phenol method as previously described [20] and treated with deoxyribonuclease I as described by MWG (Ebersberg, Germany). cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and random hexamers (deoxy-NTP<sub>6</sub>) (Amersham Biosciences, Piscataway, NJ, U.S.A.). Quantitative real-time PCR (Q-RT-PCR) was performed on an ABI PRISM 7700 RealTime PCR-machine (Applied Biosystems, Foster City, CA, U.S.A.) using 2x SYBR Green JumpStart™ Taq ReadyMix™ and Sigma Reference Dye (Sigma-Aldrich, St. Louis, MO, U.S.A.) as described by manufacturer. Reactions were incubated at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s and 72 °C for 45 s. Fluorescence signals were measured during elongation and all reactions were performed in duplicate. The levels of the target mRNAs were examined and normalized to the level of the *ACT1* gene (actin) using the  $2^{-\Delta\Delta C_T}$  method [21]. Primers for Q-RT-PCR were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, U.S.A.), and efficacy and specificity were tested by dilution experiments and melting curves, respectively. Used primer sets are listed in Supplementary



Table S1. Primer pairs were designed to amplify a fragment of ~50 bp, except of the *HAC1* primer pairs which amplify a fragment of 91 bp.

*β-galactosidase assay in yeast* - Y700 and Y700 *pGAL-ACB1* cells transformed with plasmid pJH359 bearing an *INO1-CYC1-lacZ* fusion (kind gift of Susan Henry, Cornell University of Ithaca, New York, U.S.A., [22]) were grown overnight in appropriate medium, diluted to an OD<sub>600</sub> of 0.05-0.1 and grown to an OD<sub>600</sub> of 0.6-0.8. β-galactosidase activity in yeast was determined as described in [23]. Activity was calculated as β-galactosidase units:  $[A_{420}/(\text{OD}_{600} \text{ of assayed culture} \cdot \text{volume assayed} \cdot \text{time})]$ ,  $A_{420}$  is the absorbance of the product *o*-nitrophenol (ONP), OD<sub>600</sub> is the optical density of the culture at the time of assay, volume is the amount of the culture used in the assay in ml and time is measured in min.

*Complementation of INO1 expression by increased fatty acid synthesis* - Increased fatty acid synthesis was achieved by transforming the wild type strain Y700 and the Y700 *pGAL-ACB1* strain with high-copy plasmids expressing either *ACC1* (pG1M-ACC1, kind gift of Keiji Furukawa, Kikumasa sake brewing Co.Ltd. Japan, [24]) or *FAS1* (pJS229, kind gift of Hans-Joachim Schüller, Universität Greifswald, Greifswald, Germany, [25]). The corresponding empty vectors served as controls. Total RNA was extracted from exponentially growing cells grown in selective SC-media supplemented with 0.2 mM inositol, and used for Q-RT-PCR analysis.

*Assay for the overproduction of inositol phenotype* - To test for the overproduction of inositol (Opi<sup>-</sup>) phenotype *Acb1p* depleted cells and wild type cells were dotted on synthetic inositol-free plates, and grown at 30 °C. Plates were then sprayed with a suspension of a diploid tester strain (AID, provided by Susan Henry) homozygous for *ino1* and *adel* and incubated at 30 °C as previously described [26].

*LC-ESI mass spectrometry of lipids* - The number of *Acb1p* depleted cells, at a given OD<sub>600</sub>, is reduced compared to wild type cells, implying that *Acb1p* depleted cells are bigger than wild type cells (our unpublished data). Thus, whenever quantifying the relative phospholipid content of *Acb1p* depleted cells compared to wild type cells we therefore correct for differences in cell numbers. Lipids were extracted from 25 OD<sub>600</sub> units of exponentially growing cells (OD<sub>600</sub> ~1) as described by Hanson and Lester using procedure IIIA [27]. Aliquots corresponding to 10 OD<sub>600</sub> units of cells dissolved in 65 °C CHCl<sub>3</sub>-CH<sub>3</sub>OH-H<sub>2</sub>O (16:16:5, solvent A) were mixed with internal standard lipids extracted from 10 OD<sub>600</sub> units of cells grown on <sup>13</sup>C-glucose as the only carbon source. The solvent was

evaporated under a stream of N<sub>2</sub> and the lipids were redissolved in 10 µl 65 °C solvent A by stirring with a rotating pestle (30000 rpm) for 5 min. Immediately after mixing 1 µl was injected on to a PVA SIL HPLC column (YMC Europe GmbH, D-46514 Scherbeck, Germany, 1 x 150 mm) and eluted at 45 µl/min. Solvent B [hexane-isopropanol (98:2)], C [CHCl<sub>3</sub>-isopropanol (65:35)] and D [CH<sub>3</sub>OH] were changed linearly over time to give ratios B:C:D as follows: 0 min 100:0:0; 22 min 12:88:0; 30 min 10:74:16; 45 min 8:61:31; 50 through 65 min 0:0.100; 70 through 72.5 min 0:100:0; 75 through 82.5 100:0:0. Ions in the effluent were ionized by electrospray ionization with an electrode potential of 3500 volt and the masses detected by a Bruker Esquire-LC ion trap mass spectrometer. The <sup>12</sup>C/<sup>13</sup>C ratio between ion counts for each individual <sup>12</sup>C-species and one selected <sup>13</sup>C-specie in each lipid class were calculated for both strains. Alteration in the relative amount of individual lipid species between wild type and *Acb1p* depleted cells were calculated by dividing the wild type <sup>12</sup>C/<sup>13</sup>C ratio with the corresponding <sup>12</sup>C/<sup>13</sup>C ratio of *Acb1p* depleted cells. Specie % composition based on the sum of ion counts for all specie within a certain lipid class was calculated for all lipid classes. The content of a given phospholipid class in *Acb1p* depleted cells was normalized to wild type levels (wild type 100 %) and calculated using the following formula:  $\Sigma [\% \text{ individual phospholipid specie in Wt} / ((\text{Wt } ^{12}\text{C}/^{13}\text{C}) / (\Delta ^{12}\text{C}/^{13}\text{C}))]$ , where  $\Delta$  is *Y700 pGAL-ACB1* and Wt is *Y700*.

## RESULTS

*Genome wide changes in gene expression of S. cerevisiae in response to Acb1p depletion* - DNA microarray profiling revealed that the effect of *Acb1p* depletion on gene expression is not restricted to *OLE1* [4,16]. A total of 134 genes were identified as being differentially expressed by more than 1.6-fold of which 44 were downregulated and 90 were upregulated in *Acb1p* depleted cells compared to wild type cells.

*Confirmation of microarray data by quantitative real-time PCR* - Expression of 22 selected genes, of which 10 have been shown to be differently expressed by microarray analysis, were examined by Q-RT-PCR (Table 2). The 10 genes identified by microarray analysis were all shown to be differential expressed to the same degree by Q-RT-PCR analysis (Table 2). We propose that the identified changes in gene expression measured by microarray analysis may reflect actual changes in mRNA levels in response to *Acb1p*

depletion. However, in the present work we primarily focus on microarray data, which have been confirmed by Q-RT-PCR.

*Genes associated with lipid metabolism* - The present microarray and Q-RT-PCR analysis show that genes encoding for proteins involved in fatty acid biosynthesis (*ACCI*, *FASI*, *FAS2*, and *OLE1*) all are upregulated in *Acb1p* depleted cells compared to wild type cells (Table 2 and Supplementary Table S2). Consistent with previous findings the gene encoding the  $\Delta$ -9 desaturase in yeast, *OLE1*, was found to be upregulated approximately 4-fold in YNBD (results not shown) and approximately 2-fold in YPD media in *Acb1p* depleted cells (Table 2) [4,16].

The microarray and Q-RT-PCR data also revealed that genes encoding for enzymes involved in phospholipid biosynthesis (*INO1*, *CHO1*, *CHO2* and *OPI3*) are upregulated in *Acb1p* depleted cells (Table 2). Moreover, by Q-RT-PCR we observed that the expression of both *PSD1* and *PSD2*, which also encode for phospholipid biosynthesis enzymes, are increased by 1.3-fold in *Acb1p* depleted cells compared to wild type cells (Table 2). This suggests that synthesis of glycerophospholipids may be affected in cells exhausted of *Acb1p*. In particular, the *INO1* gene encoding the inositol-3-phosphate synthase (Ino1p) that catalyzes synthesis of inositol-3-phosphate, the rate-limiting and committed step in the synthesis of phosphatidylinositol (PI), was strongly affected by *Acb1p* depletion. Although below the detection level of the microarrays *INO1* mRNA levels were found by Q-RT-PCR to be upregulated ~150-fold upon *Acb1p* depletion (Table 2). *INO1* mRNA and Ino1p levels were therefore further investigated under different growth conditions.

*Regulation of INO1 expression in Acb1p depleted cells* - Most of the genes involved in phospholipid synthesis e.g. *INO1*, *OPI3*, *PIS1*, *CHO1* and *CHO2* are characterized by the presence of a cis-acting inositol-sensitive upstream activating sequence (UAS<sub>INO1</sub>), also called the inositol/choline-responsive element (ICRE), in their promoters (for a review see [28]). Transcription of UAS<sub>INO1</sub> containing genes is positively regulated by Ino2p and Ino4p, which binds to UAS<sub>INO1</sub> elements and activate transcription when cells are grown in the absence of inositol and choline. Repression occurs in the presence of high concentrations of inositol and choline, and is mediated by the negative regulator Opi1p (for a review see [29]). In the absence of inositol and choline, Opi1p is sequestered in the ER-membrane by binding to phosphatidic acid (PA) and Scs2p [30]. Upon addition of inositol and choline, PA is quickly consumed for the synthesis of PI and PC, followed by translocation of Opi1p to the nucleus [30]

where it represses UAS<sub>INO1</sub> dependent expression indirectly presumably by interacting with the Ino2p transcriptional activator.

*INO1* mRNA and the corresponding Ino1p level were both found to be significantly upregulated in *Acb1p* depleted cells compared to wild type cells when grown in both rich (YPD) and in minimal media (SC) supplemented with 0.2 mM inositol (Figure 1). This suggests that full repression of *INO1* mRNA and Ino1p by inositol requires the presence of *Acb1p*. Consistent with this, we did not observe a significant difference in UAS<sub>INO1</sub>-driven  $\beta$ -galactosidase activity between wild type and *Acb1p* depleted cells when cultured in inositol-free medium (results not shown). In contrast, when grown in minimal media (YNBD) supplemented with 0.02 mM or 0.2 mM inositol, UAS<sub>INO1</sub>-driven  $\beta$ -galactosidase expression was approximately 3-fold higher in *Acb1p* depleted cells compared to wild type cells (Figure 2). Upregulation of *INO1* transcription in *Acb1p* depleted cells may therefore be due to increased UAS<sub>INO1</sub>-driven transcription as a result of reduced inositol-mediated repression. This notion is supported by the fact that other genes containing UAS<sub>INO1</sub> elements in their promoter incl. *OPI3*, *CHO1* and *CHO2* also are upregulated in *Acb1p* depleted cells (Table 2). In a recent microarray study a total of 36 Ino2p-Ino4p target genes were identified [31]. Upon *Acb1p* depletion 20 of these genes were found to be upregulated (by at least 1.5-fold), 3 were downregulated (by at least 1.5-fold), and 13 genes could not be shown to be differently expressed when compared to wild type cells. This indicates that inositol-mediated repression of Ino2p-Ino4p target genes at steady-state depend on *Acb1p*. Since Ino2p, Ino4p and *Opi1p* regulate transcription of UAS<sub>INO1</sub> containing genes, mRNA levels of these transcription factors were measured by Q-RT-PCR. We found that the mRNA level of *INO2* and *INO4* was increased ~2-fold in *Acb1p* depleted cells compared to wild type cells when grown in rich medium whereas no significant changes was observed for cells grown in minimal medium (results not shown). The mRNA level of *OPI1* was increased ~1.3-fold in *Acb1p* depleted cells compared to wild type cells when grown in rich medium whereas the level was decreased ~1.3-fold when cells were grown in minimal medium (results not shown).

*Acb1p* depletion reduces inositol-mediated repression of *INO1* by an *Opi1p* independent regulatory mechanism - Since *INO1*, *CHO1*, *CHO2* and *OPI3* were identified to be upregulated in *Acb1p* depleted cells, we speculate that the transcriptional repressor *Opi1p* is sequestered in the ER membrane of these cells due to higher levels of PA and/or *Scs2p*.

Disruption of *SCS2* reduced *INO1* expression in both wild type and *Acb1p* depleted cells, however, *INO1* mRNA levels were still increased by ~60-fold (Figure 3A) and ~5.5-fold (Figure 3B) in *Acb1p* depleted cells grown in YPD and SC media containing 0.2 mM inositol, respectively. A similar expression pattern was observed for *OPI3* upon *SCS2* disruption (results not shown). Moreover, *SCS2* mRNA levels were unaffected by *Acb1p* depletion (results not shown) and relative PA levels were unchanged or lower in *Acb1p* depleted cells compared to wild type cells (Table 3 and below). Finally, overexpression of *OPI1* did not reduce *INO1* mRNA levels of *Acb1p* depleted cells to that of wild type cells (results not shown). Taken together these observations suggest that *Acb1p* depletion reduces inositol-mediated repression of *INO1* and other Ino2p-Ino4p target genes by an *Opi1p* independent regulatory mechanism.

*Acb1p* depleted *S. cerevisiae* exhibit an overproduction of inositol phenotype but do not accumulate phosphatidic acid - The overproduction of inositol (*Opi<sup>-</sup>*) phenotype has previously been associated with deregulation of the *INO1* gene [32]. Therefore we tested *Acb1p* depleted cells for an *Opi<sup>-</sup>* phenotype [32]. The appearance of growth of an inositol dependent strain around *Acb1p* depleted cells grown on synthetic inositol-free plates, shows that *Acb1p* depletion leads to inositol secretion (Figure 4A). Moreover, it has been shown that strains carrying deletions in either *CHO1*, *CHO2* and *OPI3* have defects in phosphatidylcholine (PC) synthesis, are unable to repress *INO1* in the presence of inositol and exhibit a conditional *Opi<sup>-</sup>* phenotype due to accumulation of PA [32,33]. Expression of *INO1* and the *Opi<sup>-</sup>* phenotype can be normalized in these strains by adding exogenous choline, which alleviate PA accumulation by increasing PC synthesis via the CDP-choline pathway [28,34]. Thus, if accumulation of PA causes increased *INO1* expression in *Acb1p* depleted cells supplementation of both choline and inositol would be expected to repress *INO1* mRNA and *Ino1p* levels further in both wild type and *Acb1p* depleted cells compared to supplementation with inositol alone. However, to our surprise addition of choline to inositol containing media lead only to a minor decrease in the *INO1* mRNA and *Ino1p* levels of *Acb1p* depleted cells compared to wild type cells (Figures 1A and 1B). Hence, increased *INO1* expression in *Acb1p* depleted cells may not be caused by accumulation of PA as a result of reduced PC synthesis via the phosphatidylethanolamine (PE) methylation pathway. Increased phospholipase D1 (*Pld1p*)-mediated turnover of PC has also been reported to result in derepression of the *INO1* gene [26] probably by causing accumulation of PA. However, in a *pld1*-null background *INO1* levels were still increased by ~90-fold (Figure 3A) and ~4.5-fold

(Figure 3B) upon *Acb1p* depletion when cells were grown in YPD and SC media containing 0.2 mM inositol, respectively. Hence, increased PC turnover does not seem to cause derepression of *INO1* in *Acb1p* depleted cells. This is in good agreement with our previously data showing that PC turnover is only slightly reduced in *Acb1p* depleted cells compared to wild type cells [16]. In agreement with above results no increase was found in the relative PC and PA level of *Acb1p* depleted cells when compared to wild type cells (Table 3 and below). Taken together these results indicate that neither changes in PC metabolism nor accumulation of PA is responsible for increasing *INO1* mRNA and Ino1p levels in cells depleted of *Acb1p*.

*The mechanism causing induction of INO1 in Acb1p depleted cells is independent on HAC1 expression* - Eukaryotic cells respond to accumulation of unfolded proteins in the ER by activating a stress response pathway known as the unfolded protein response (UPR) pathway (for a review see [35]). In *S. cerevisiae* the UPR pathway may also be activated in response to inositol starvation and it is coupled to inositol biosynthesis as cells defective in UPR activation, require exogenous inositol for growth and express low levels of *INO1* [36]. In yeast, it has been proposed that Hac1p negatively regulates the activity of Opi1p [37]. Interestingly, the genes *HAC1*, *PDH1*, *KAR2* and *ORM2*, were identified in the present study as being upregulated in cells depleted of *Acb1p* (Table 2), indicative of induction of the UPR pathway. Disruption of *HAC1* diminished *INO1* mRNA levels in wild type cells and cells depleted of *Acb1p* (Figures 3A and 3B). However, *INO1* mRNA levels were still increased by ~40-fold (Figure 3A) and ~5-fold (Figure 3B) in *Acb1p* depleted cells compared to wild type cells when cells were grown in YPD and SC media containing 0.2 mM inositol, respectively. These results clearly demonstrate that the observed reduction in inositol-mediated repression of *INO1* in cells lacking *Acb1p* is due to other reasons than induction of the UPR pathway. This suggestion is supported by a recent microarray study showing that Opi1p-mediated repression of *INO1* in response to inositol cannot be prevented by constitutively activation of the UPR pathway [31].

*Phospholipid analysis by LC-ESI tandem mass spectrometry* - To examine whether the observed induction of genes encoding enzymes involved in phospholipid biosynthesis in *Acb1p* depleted cells are caused by changes in the overall phospholipid content, we measured relative levels of PC, PI, PE, phosphatidylglycerol (PG), phosphatidylserine (PS) and PA using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with <sup>13</sup>C-labelled yeast lipids as internal standards. *Acb1p* depleted cells grown in

YPD media exhibited a ~30 % increase in the relative level of the major glycerolphospholipids PI and PE and unchanged levels of PC, PG and PA compared to wild type cells (Table 3). However, the PS level was decreased by ~20 % in the *Acb1p* depleted cells. For *Acb1p* depleted cells grown in SC media supplemented with 0.2 mM inositol it was found that the relative level of PA, PI, PS and PE was reduced by ~20 % whereas the PC level was unchanged compared to wild type cells. In contrast, the relative PG level was decreased by ~50 % (Table 3). These data indicate that a reduction in the relative phospholipid content is not solely responsible for induction of phospholipid biosynthesis genes in *Acb1p* depleted cells, since only the relative PS level was consistently reduced under all conditions. *Acb1p* may therefore act downstream of or independently of the signal produced through phospholipid metabolism to affect *INO1* expression. However, it should be noticed that the relative phospholipid specie composition is altered in *Acb1p* depleted cells compared to wild type cells (Supplementary Table S3-S8). *Acb1p* depletion causes a shift from C<sub>34:1</sub> species mainly towards C<sub>34:2</sub> and C<sub>32:1</sub> or C<sub>32:2</sub> species in the PI, PG, PS and PA classes independent on whether cells are grown in YPD or SC media supplemented with 0.2 mM inositol (Supplementary Table S3-S8). These results confirm that *Acb1p* depletion leads to changes in the relative specie composition of phospholipids as previously reported [17]. It can therefore not be excluded that changes in the relative specie composition of phospholipids in *Acb1p* depleted cells causes the observed alteration in expression of genes encoding for enzymes involved in phospholipid biosynthesis.

*Supplementation with fatty acids or overexpression of FAS1 or ACC1 can normalize inositol-mediated repression of INO1 in Acb1p depleted cells* – A link between fatty acid metabolism and *INO1* expression has previously been reported by Shirra *et al.* [38] who found that inhibition of acetyl coenzyme A carboxylase (*Acc1p*) activity restored expression of *INO1* in *SNF1* disrupted cells. Moreover, it was shown that supplementation with palmitoleic acid (C<sub>16:1</sub>) relieve inositol auxotrophy and permit *INO1* expression in cells lacking *SNF1* [38]. In addition Gaspar *et al.* [39] suggested that the observed burst in PI synthesis following addition of inositol depend on *de novo* fatty acid synthesis, further indicating a link between fatty acid biosynthesis and regulation of phospholipid biosynthesis. Addition of palmitic acid (C<sub>16:0</sub>) to inositol containing media normalizes *INO1* and *OPI3* expression in *Acb1p* depleted cells to that of wild type cells (Figure 5A and 5B). In contrast palmitoleic acid only partly normalizes *INO1* and *OPI3* expression in *Acb1p* depleted cells to

that of wild type cells (Figure 5A and B). Notably palmitoleic acid increases *INO1* and *OPI3* expression in wild type cells (Figure 5A and B). This shows that palmitic acid and palmitoleic acid affect inositol-mediated repression of Ino2p-Ino4p target genes differently in both wild type and *Acb1p* depleted cells. However, a signal generated from fatty acid metabolism may be involved in regulation of inositol-mediated repression of Ino2p-Ino4p target genes. Wenz *et al.* have previously shown that expression of *FAS1*, but not *FAS2*, from a high-copy plasmid increases FAS activity by 2.8-fold [25]. We therefore hypothesized that ectopic expression of *FAS1* and *ACC1*, the rate-limiting enzyme in fatty acid biosynthesis, from a high-copy plasmid would increase fatty acid/acyl-CoA ester availability and thereby reduce *INO1* expression in *Acb1p* depleted cells. Overexpression of *FAS1* reduces the ratio in *INO1* mRNA levels between *Acb1p* depleted cells and wild type cells from ~6.5 to ~2.6 (Figure 6A). No further effect on *INO1* expression was observed when *FAS1* and *ACC1* were overexpressed at the same time (Figure 6A). Notably, when *ACC1* was overexpressed in cells transformed with the empty *FAS1*-vector the ratio in *INO1* mRNA levels between *Acb1p* depleted cells and wild type cells was only reduced from ~6.5 to ~4.7 (Figure 6A). However, ectopic expression of *ACC1* from a high-copy plasmid in cells only transformed with this plasmid reduced the ratio in *INO1* mRNA levels from ~7.4 to ~2.2 (Figure 6B). A possible explanation for this observation is that transformation with two plasmids might affect the copy number of each one, and therefore the expression of individual proteins. *INO1* mRNA levels could not be reduced in *Acb1p* depleted cells when *FAS2* was overexpressed, further supporting that observed effects are caused by increased availability of fatty acids/acyl-CoA esters (results not shown). Together, these results show that increased availability of fatty acids/acyl-CoA esters in *Acb1p* depleted cells normalizes expression of genes involved in phospholipid biosynthesis. Thus, *Acb1p* might facilitate removal of *de novo* synthesized acyl-CoA esters, from the yeast FAS under normal physiological conditions, thereby making them available for further metabolism and regulatory processes.

Interestingly, addition of 500  $\mu$ M palmitic acid to inositol-free plates repress the *Opi*<sup>-</sup> phenotype (Figure 4A) in accordance with the observed effect of palmitic acid on inositol uptake [16] and membrane abnormalities (our unpublished results) in *Acb1p* depleted cells. However, palmitic acid is unable to reduce the high *INO1* mRNA and Ino1p levels of both wild type and *Acb1p* depleted cells cultured in inositol-free media (Figures 4B and 4C). This implies that high *INO1* expression not necessarily is associated with the *Opi*<sup>-</sup> phenotype.



*The in vivo function of Acb1p depends on ligand binding* - Depletion of Acb1p in yeast results in slow growth and aberrant organelle morphology incl. fragmented vacuoles, multi-layered plasma membranes and accumulation of vesicles of variable sizes [16,17]. Ectopic expression of Acb1p in Acb1p depleted cells normalizes vacuole morphology (our unpublished data) and *INO1* mRNA levels (Figure 7), whereas expression of Acb1p<sup>Y28F,K32A</sup>, which exhibit a 1000-fold reduced affinity for acyl-CoA esters [40], is unable to restore *INO1* mRNA levels (Figure 7) and vacuole morphology (our unpublished data). The minor effect of Acb1p<sup>Y28F,K32A</sup> on *INO1* expression in Acb1p depleted cells is likely due to the fact that it is overexpressed and binds acyl-CoA esters with very low affinity. Ectopic expression of rat L-FABP, which binds acyl-CoA esters with a  $K_D$  of 14-1000 nM [41,42], in Acb1p depleted cells was also unable to normalize the *INO1* mRNA level (results not shown). This shows that the function of Acb1p is closely linked to its ability to bind acyl-CoA esters with high affinity and supports a role of Acb1p in acyl-CoA trafficking.

## DISCUSSION

In the present study we identified transcriptional changes of genes encoding for proteins involved in fatty acid and phospholipid synthesis, glycolysis, stress response, glycerol metabolism, and in ion transport/uptake in response to Acb1p depletion (Table 2 and Supplementary Table S2). Depletion of Acb1p was shown to increase transcription of *INO1*, *CHO1*, *CHO2* and *OPI3* all encoding proteins required for phospholipid biosynthesis. Common for all of these genes are the presence of UAS<sub>INO1</sub> elements in their promoter regions (for a review see [28]). Regulation via UAS<sub>INO1</sub> elements involves the transcriptional activators Ino2p and Ino4p and the transcriptional repressor Opi1p [30]. Nuclear localization of Opi1p is regulated by PA and Scs2p, which bind and detain Opi1p to the ER-membrane. We show that the observed reduction in inositol-mediated repression of UAS<sub>INO1</sub>-driven transcription in Acb1p depleted cells is not caused by altered *OPI1* levels or changes in PA and *SCS2* levels. Moreover, *INO2* and *INO4* mRNA levels were not significantly altered in Acb1p depleted cells grown in minimal media compared to wild type cells (results not shown). The ~3-fold increase in *INO1* mRNA levels of Acb1p depleted cells grown in minimal media (Figure 3B) may therefore not be caused by increased levels of these transcriptional activators. Furthermore, Ino2p and Ino4p binds constitutively to the *INO1*

promoter, and it has been suggested that occupancy of the *INO1* promoter by Ino2p/Ino4p is insufficient for its activation [37]. Taken together these data strongly suggest that the reduction in inositol-mediated repression of Ino2p-Ino4p target genes in *Acb1p* exhausted cells occur by an Ino2p, Ino4p, and Opi1p independent mechanism. However, it cannot be excluded that the *Acb1p*/acyl-CoA ester complex directly or indirectly regulates the activity of the Ino2p, Ino4p, and Opi1p transcription factors. A possible mechanism could be that *Acb1p* depletion alter Opi1p phosphorylation, which has been shown to affect its regulator activity [43].

Activation of the UPR pathway has previously been linked to increase phospholipid biosynthesis in yeast [37]. However, we did not find any connection between induction of the UPR pathway and increased expression of Ino2p-Ino4p target genes in *Acb1p* depleted cells. Moreover, *HAC1* disruption reduced the expression of *OPI3* by ~2-fold in *Acb1p* depleted cells grown in YPD media whereas *OPI3* expression in wild type cells were unaffected by *HAC1* disruption (results not shown). The absence of an effect of *HAC1* disruption on *OPI3* expression in wild type cells confirms, as recently suggested [31], that the UPR pathway does not seem to be directly involved in transcriptional regulation of Ino2p-Ino4p target genes.

A large body of evidence suggests that *Acb1p* functions as an acyl-CoA ester transporter and pool former. This lead us to investigate the possibility that the altered expression of phospholipid synthesizing genes, incl. *INO1*, in *Acb1p* depleted yeast is due to absence of fatty acid metabolites. Supplementation of exogenous palmitic acid to *Acb1p* depleted cells normalizes *INO1* and *OPI3* mRNA levels to that of wild type cells (Figure 5A and 5B), in contrast to palmitoleic acid, which was found to partly normalize *INO1* and *OPI3* levels in *Acb1p* depleted cells relatively to wild type cells (Figure 5A and B). The difference in the effect of palmitic acid and palmitoleic acid may be due to that palmitic acid has additional effects on *Acb1p* depleted cells compared to palmitoleic acid.

Supplementation of palmitic acid to *Acb1p* depleted cells partly restores inositol uptake [16] and reduces inositol secretion (Figure 4A). This may suggest that reduced inositol-mediated repression of Ino2p-Ino4p target genes incl. *INO1* in *Acb1p* depleted cells, simply is a cellular response to low intracellular inositol levels. However, this is not likely to be the case since *Acb1p* depleted cells, although unable to completely normalize *INO1* levels upon inositol addition, respond to exogenous inositol by repressing *INO1* transcription (Figure 1A). Disruption of *SCS2*, which previously has been shown to reduce *INO1*

expression in response to inositol depletion [44], did not normalize *INO1* expression in *Acb1p* depleted cells (Figures 3A and 3B). Thus, further supporting the notion that *INO1* deregulation in *Acb1p* depleted cells is caused by other means than lack of inositol. The steady-state expression of *GPD1* and *TDH1*, which is only slightly affected by inositol [45], is dramatically increased in *Acb1p* depleted cells (Table 2) suggesting that at least part of the transcriptional response accompanying *Acb1p* depletion is inositol independent.

Overall above observations raise the possibility that reduced inositol-mediated repression of Ino2p-Ino4p target genes in *Acb1p* depleted cells is due to decreased availability of fatty acids and/or acyl-CoA esters. This is strongly supported by the observation that ectopic overexpression of *FAS1* and *ACCI* reduces ratios in *INO1* mRNA levels between *Acb1p* depleted cells and wild type cells from ~6.5 and ~7.4 to ~2.6 and ~2.2, respectively (Figures 6A and 6B). Notably, overexpression of *FAS2*, which is unable to enhance FAS activity [25], in *Acb1p* depleted cells did not reduce *INO1* mRNA levels (results not shown). This suggests that the *Acb1p* mediated removal of *de novo* synthesized acyl-CoA esters from the yeast FAS [16] can be overcome by increasing the availability of fatty acids and/or acyl-CoA esters through enhancement of *de novo* fatty acid biosynthesis or supplementation with fatty acids (Figures 5 and 6). These results strongly support the hypothesis that altered availability of fatty acids and/or fatty acid derivatives may be involved in the mechanism by which *Acb1p* depletion alter inositol-mediated repression of Ino2p-Ino4p target genes.

In addition to affecting the expression of phospholipid biosynthesis genes, *Acb1p* depletion also increases the expression of fatty acid synthetic genes incl. *ACCI*, *FAS1*, *FAS2*, and *OLE1* (Table 2 and Supplementary Table S2). This indicates that the expression of these genes also is regulated by the availability of specific acyl-CoA esters, made available by *Acb1p*/acyl-CoA ester complexes. We have previously shown that depletion of *Acb1p* in the temperature-sensitive mutant of *ACCI*, *mtr7-1*, already at permissive temperature results in lethality [16], supporting the notion that *Acb1p* effects the availability of acyl-CoA esters.

The acyl-specie composition of phospholipids is altered upon *Acb1p* depletion (Supplementary Table S3-S8). Thus, it cannot be ruled out that this can affect the expression of Ino2p-Ino4p target genes. One possible model consistent with our results is that *Acb1p* recruits LCACoA esters to phospholipid remodelling enzymes thereby affecting the acyl-

specie composition of phospholipids, which then affect the expression of Ino2p-Ino4p target genes.

Several studies have shown that fatty acids and fatty acid derivatives affect gene expression directly in both prokaryotes and eukaryotes [46,47]. A number of these studies suggest that the active specie is acyl-CoA ester. In *Escherichia coli* fatty acid synthesis and degradation is transcriptionally coordinated by the acyl-CoA ester regulated transcription factor FadR (for a review see [46]). Eukaryotic peroxisome proliferator activated receptors (PPARs) are activated by fatty acids and induce transcription of multiple genes involved in lipid metabolism [47], while LCACoA esters have been reported to antagonize this effect [48]. Immunogold electron microscopy (EM) has shown that ACBP/Acb1p is found in the nucleus of a number of mammalian cells [49,50] and in yeast cells (our unpublished results). The Acb1p/acyl-CoA complex may therefore modulate gene expression by donating acyl-CoA esters to gene regulatory components in the nucleus. Such a transport function is compatible with the recent suggestion implying that ACBP and HNF-4 $\alpha$  interact and ACBP stimulate HNF-4 $\alpha$ -mediated transactivation of HNF-4 $\alpha$  target genes in hepatocytes [50].

In conclusion we suggest that fatty acid derivates are involved in regulation of Ino2p-Ino4p target genes and that increased expression of Ino2p-Ino4p target genes in Acb1p depleted cells is caused by decreased availability of acyl-CoA esters due to the absence of Acb1p.

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## REFERENCES

- 1 Baumgartner, U., Hamilton, B., Piskacek, M., Ruis, H. and Rottensteiner, H. (1999) Functional analysis of the Zn(2)Cys(6) transcription factors Oaf1p and Pip2p. Different roles in fatty acid induction of beta-oxidation in *Saccharomyces cerevisiae*. *J.Biol.Chem.* **274**, 22208-22216
- 2 Zhang, S., Skalsky, Y. and Garfinkel, D. J. (1999) MGA2 or SPT23 is required for transcription of the delta9 fatty acid desaturase gene, OLE1, and nuclear membrane integrity in *Saccharomyces cerevisiae*. *Genetics* **151**, 473-483
- 3 Kamiryo, T., Parthasarathy, S. and Numa, S. (1976) Evidence that acyl coenzyme A synthetase activity is required for repression of yeast acetyl coenzyme A carboxylase by exogenous fatty acids. *Proc.Natl.Acad.Sci.U.S.A* **73**, 386-390
- 4 Choi, J. Y. and Martin, C. E. (1999) The *Saccharomyces cerevisiae* FAT1 gene encodes an acyl-CoA synthetase that is required for maintenance of very long chain fatty acid levels. *J.Biol.Chem.* **274**, 4671-4683
- 5 Faergeman, N. J., Black, P. N., Zhao, X. D., Knudsen, J. and DiRusso, C. C. (2001) The Acyl-CoA synthetases encoded within FAA1 and FAA4 in *Saccharomyces cerevisiae* function as components of the fatty acid transport system linking import, activation, and intracellular Utilization. *J.Biol.Chem.* **276**, 37051-37059
- 6 Martin, G. G., Huang, H., Atshaves, B. P., Binas, B. and Schroeder, F. (2003) Ablation of the liver fatty acid binding protein gene decreases fatty acyl CoA binding capacity and alters fatty acyl CoA pool distribution in mouse liver. *Biochemistry* **42**, 11520-11532
- 7 Seedorf, U., Raabe, M., Ellinghaus, P., Kannenberg, F., Fobker, M., Engel, T., Denis, S., Wouters, F., Wirtz, K. W., Wanders, R. J., Maeda, N. and Assmann, G. (1998) Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function. *Genes Dev.* **12**, 1189-1201
- 8 Kurlandzka, A., Rytka, J., Gromadka, R. and Murawski, M. (1995) A new essential gene located on *Saccharomyces cerevisiae* chromosome IX. *Yeast* **11**, 885-890
- 9 Tahotna, D., Hapala, I., Zinser, E., Flekl, W., Paltauf, F. and Daum, G. (1993) Two yeast peroxisomal proteins crossreact with an antiserum against human sterol carrier protein 2 (SCP-2). *Biochim.Biophys.Acta* **1148**, 173-176
- 10 Faergeman, N. J. and Knudsen, J. (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem.J.* **323** ( Pt 1), 1-12
- 11 Burton, M., Rose, T. M., Faergeman, N. J. and Knudsen, J. (2005) Evolution of the acyl-CoA binding protein (ACBP). *Biochem.J.* **392**, 299-307

- 12 Rasmussen, J. T., Rosendal, J. and Knudsen, J. (1993) Interaction of acyl-CoA binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor. *Biochem.J.* **292** ( Pt 3), 907-913
- 13 Rasmussen, J. T., Faergeman, N. J., Kristiansen, K. and Knudsen, J. (1994) Acyl-CoA-binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for beta-oxidation and glycerolipid synthesis. *Biochem.J.* **299** ( Pt 1), 165-170
- 14 Knudsen, J., Faergeman, N. J., Skott, H., Hummel, R., Borsting, C., Rose, T. M., Andersen, J. S., Hojrup, P., Roepstorff, P. and Kristiansen, K. (1994) Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. *Biochem.J.* **302** ( Pt 2), 479-485
- 15 Mandrup, S., Jepsen, R., Skott, H., Rosendal, J., Hojrup, P., Kristiansen, K. and Knudsen, J. (1993) Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. *Biochem.J.* **290** ( Pt 2), 369-374
- 16 Gaigg, B., Neergaard, T. B., Schneider, R., Hansen, J. K., Faergeman, N. J., Jensen, N. A., Andersen, J. R., Friis, J., Sandhoff, R., Schroder, H. D. and Knudsen, J. (2001) Depletion of acyl-coenzyme A-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *Saccharomyces cerevisiae*. *Mol.Biol.Cell* **12**, 1147-1160
- 17 Faergeman, N. J., Feddersen, S., Christiansen, J. K., Larsen, M. K., Schneider, R., Ungermann, C., Mutenda, K., Roepstorff, P. and Knudsen, J. (2004) Acyl-CoA-binding protein, *Acb1p*, is required for normal vacuole function and ceramide synthesis in *Saccharomyces cerevisiae*. *Biochem.J.* **380**, 907-918
- 18 Longtine, M. S., McKenzie, A., III, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**, 953-961
- 19 Sherman, F. (1991) Getting started with yeast. *Methods Enzymol.* **194**, 3-21
- 20 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) In *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 21 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408
- 22 Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L. and Henry, S. A. (1991) Analysis of sequences in the *INO1* promoter that are involved in its regulation by phospholipid precursors. *Nucleic Acids Res.* **19**, 1687-1693
- 23 Guarente, L. (1983) Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* **101**, 181-191

- 24 Furukawa, K., Yamada, T., Mizoguchi, H. and Hara, S. (2003) Increased ethyl caproate production by inositol limitation in *Saccharomyces cerevisiae*. *J.Biosci.Bioeng.* **95**, 448-454
- 25 Wenz, P., Schwank, S., Hoja, U. and Schuller, H. J. (2001) A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene *FAS2* by the *FAS1* gene product. *Nucleic Acids Res.* **29**, 4625-4632
- 26 Patton-Vogt, J. L., Griac, P., Sreenivas, A., Bruno, V., Dowd, S., Swede, M. J. and Henry, S. A. (1997) Role of the yeast phosphatidylinositol/phosphatidylcholine transfer protein (*Sec14p*) in phosphatidylcholine turnover and *INO1* regulation. *J.Biol.Chem.* **272**, 20873-20883
- 27 Hanson, B. A. and Lester, R. L. (1980) The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*. *J.Lipid Res.* **21**, 309-315
- 28 Henry, S. A. and Patton-Vogt, J. L. (1998) Genetic regulation of phospholipid metabolism: yeast as a model eukaryote. *Prog.Nucleic Acid Res.Mol.Biol.* **61**, 133-179
- 29 Carman, G. M. and Kersting, M. C. (2004) Phospholipid synthesis in yeast: regulation by phosphorylation. *Biochem.Cell Biol.* **82**, 62-70
- 30 Loewen, C. J., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A. and Levine, T. P. (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. *Science* **304**, 1644-1647
- 31 Jesch, S. A., Liu, P., Zhao, X., Wells, M. T. and Henry, S. A. (2006) Multiple endoplasmic reticulum-to-nucleus signaling pathways coordinate phospholipid metabolism with gene expression by distinct mechanisms. *J.Biol.Chem.* **281**, 24070-24083
- 32 Greenberg, M. L., Reiner, B. and Henry, S. A. (1982) Regulatory mutations of inositol biosynthesis in yeast: isolation of inositol-excreting mutants. *Genetics* **100**, 19-33
- 33 Hirsch, J. P. and Henry, S. A. (1986) Expression of the *Saccharomyces cerevisiae* inositol-1-phosphate synthase (*INO1*) gene is regulated by factors that affect phospholipid synthesis. *Mol.Cell Biol.* **6**, 3320-3328
- 34 Griac, P., Swede, M. J. and Henry, S. A. (1996) The role of phosphatidylcholine biosynthesis in the regulation of the *INO1* gene of yeast. *J.Biol.Chem.* **271**, 25692-25698
- 35 Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**, 451-454
- 36 Cox, J. S., Shamu, C. E. and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* **73**, 1197-1206

- 37 Brickner, J. H. and Walter, P. (2004) Gene recruitment of the activated INO1 locus to the nuclear membrane. *PLoS.Biol.* **2**, e342
- 38 Shirra, M. K., Patton-Vogt, J., Ulrich, A., Liuta-Tehlivets, O., Kohlwein, S. D., Henry, S. A. and Arndt, K. M. (2001) Inhibition of acetyl coenzyme A carboxylase activity restores expression of the INO1 gene in a *snf1* mutant strain of *Saccharomyces cerevisiae*. *Mol.Cell Biol.* **21**, 5710-5722
- 39 Gaspar, M. L., Aregullin, M. A., Jesch, S. A. and Henry, S. A. (2006) Inositol induces a profound alteration in the pattern and rate of synthesis and turnover of membrane lipids in *Saccharomyces cerevisiae*. *J.Biol.Chem.* **281**, 22773-22785
- 40 Kragelund, B. B., Poulsen, K., Andersen, K. V., Baldursson, T., Kroll, J. B., Neergard, T. B., Jepsen, J., Roepstorff, P., Kristiansen, K., Poulsen, F. M. and Knudsen, J. (1999) Conserved residues and their role in the structure, function, and stability of acyl-coenzyme A binding protein. *Biochemistry* **38**, 2386-2394
- 41 Frolov, A., Cho, T. H., Murphy, E. J. and Schroeder, F. (1997) Isoforms of rat liver fatty acid binding protein differ in structure and affinity for fatty acids and fatty acyl CoAs. *Biochemistry* **36**, 6545-6555
- 42 Rolf, B., Oudenampsen-Kruger, E., Borchers, T., Faergeman, N. J., Knudsen, J., Lezius, A. and Spener, F. (1995) Analysis of the ligand binding properties of recombinant bovine liver-type fatty acid binding protein. *Biochim.Biophys.Acta* **1259**, 245-253
- 43 Sreenivas, A., Villa-Garcia, M. J., Henry, S. A. and Carman, G. M. (2001) Phosphorylation of the yeast phospholipid synthesis regulatory protein *Opi1p* by protein kinase C. *J.Biol.Chem.* **276**, 29915-29923
- 44 Kagiwada, S. and Zen, R. (2003) Role of the yeast VAP homolog, *Scs2p*, in INO1 expression and phospholipid metabolism. *J.Biochem.(Tokyo)* **133**, 515-522
- 45 Jesch, S. A., Zhao, X., Wells, M. T. and Henry, S. A. (2005) Genome-wide analysis reveals inositol, not choline, as the major effector of *Ino2p*-*Ino4p* and unfolded protein response target gene expression in yeast. *J.Biol.Chem.* **280**, 9106-9118
- 46 Black, P. N., Faergeman, N. J. and DiRusso, C. C. (2000) Long-chain acyl-CoA-dependent regulation of gene expression in bacteria, yeast and mammals. *J.Nutr.* **130**, 305S-309S
- 47 Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M. and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc.Natl.Acad.Sci.U.S.A* **94**, 4318-4323
- 48 Murakami, K., Ide, T., Nakazawa, T., Okazaki, T., Mochizuki, T. and Kadowaki, T. (2001) Fatty-acyl-CoA thioesters inhibit recruitment of steroid receptor co-activator 1 to alpha and gamma isoforms of peroxisome-proliferator-activated receptors by competing with agonists. *Biochem.J.* **353**, 231-238



- 49 Elholm, M., Garras, A., Neve, S., Tornehave, D., Lund, T. B., Skorve, J., Flatmark, T., Kristiansen, K. and Berge, R. K. (2000) Long-chain acyl-CoA esters and acyl-CoA binding protein are present in the nucleus of rat liver cells. *J.Lipid Res.* **41**, 538-545
- 50 Petrescu, A. D., Payne, H. R., Boedecker, A., Chao, H., Hertz, R., Bar-Tana, J., Schroeder, F. and Kier, A. B. (2003) Physical and functional interaction of Acyl-CoA-binding protein with hepatocyte nuclear factor-4 alpha. *J.Biol.Chem.* **278**, 51813-51824

## FIGURE LEGENDS

Figure 1. *INO1* mRNA and protein levels during different growth conditions in Acb1p depleted cells. Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700, black columns) and *pGAL-ACB1* (Acb1p depleted Y700, open columns) cells grown in minimal (SC) media supplemented with choline and/or inositol as indicated (A) or in rich (YPD) media (C). Immunoblot analysis of protein extracts from *S. cerevisiae* containing Ino1p-HA. Wild type (Y700 Ino1p-HA) and *pGAL-ACB1* (Acb1p depleted Y700 Ino1p-HA) cells were grown in SC media supplemented with choline and/or inositol as indicated (B) or in YPD media (D). The blots in (B) and (D) were re-probed with an antibody against Vac8p to serve as loading control. Data in (A) and (B) are shown as means  $\pm$  S.D. of three independent experiments and Western blots are representative of three independent experiments.

Figure 2. UAS<sub>INO1</sub>-driven gene expression is increased in Acb1p depleted cells. Wild type (Y700, black columns) and *pGAL-ACB1* (Acb1p depleted Y700, open columns) cells were grown in YNBD (-inositol) medium supplemented with inositol as indicated. Cells were transformed with the *INO1-CYCI-lacZ* plasmid and  $\beta$ -galactosidase activity was measured to assess the ability of UAS<sub>INO1</sub> elements to drive *lacZ* expression in wild type and Acb1p depleted cells.  $\beta$ -galactosidase activity unit was defined as OD<sub>420</sub>/min/ml. Data are means  $\pm$  S.D. of three independent experiments.

Figure 3. *INO1* mRNA levels in wild type and Acb1p depleted cells disrupted in *SCS2*, *PLD1* or *HAC1*. Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700) and *pGAL-ACB1* (Acb1p depleted Y700) cells disrupted in *SCS2*, *PLD1* or *HAC1* following

growth in rich (YPD) media (A) or minimal (SC) media supplemented with 0.2 mM inositol (B). Data are means  $\pm$  S.D. of three independent experiments.

Figure 4. *Acb1p* depleted cells exhibits an overproduction of inositol ( $Opi^-$ ) phenotype. (A) Wild type (Y700) and *pGAL-ACB1* (*Acb1p* depleted Y700) cells were tested for their ability to secrete inositol to the surrounding media during conditions of supplementation with or without 0.5 mM palmitic acid. (B) Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700, black columns) and *pGAL-ACB1* (*Acb1p* depleted Y700, open columns) cells grown in YNBD (-inositol) media supplemented with or without 0.05 % Brij 58 and 0.5 mM palmitic acid as indicated. (C) Immunoblot analysis of protein extracts from *S. cerevisiae* containing Ino1p-HA. Wild type (Y700 Ino1p-HA) and *pGAL-ACB1* (*Acb1p* depleted Y700 Ino1p-HA) cells were grown in YNBD (-inositol) media supplemented with or without 0.05 % Brij 58 and 0.5 mM palmitic acid as indicated. The blot in (C) was re-probed with an antibody against Vac8p to serve as loading control. Data in (B) are shown as means  $\pm$  S.D. of three independent experiments and the Western blot is representative of three independent experiments.

Figure 5. Fatty acid supplementation normalizes *INO1* and *OPI3* expression upon *Acb1p* depletion. Quantitative real-time PCR analysis of *INO1* (A) and *OPI3* (B) mRNA levels in wild type (Y700, black columns) and *pGAL-ACB1* (*Acb1p* depleted Y700, open columns) cells is shown. Cells were grown in rich (YPD) media containing 0.05 % Brij58 detergent in the presence or absence of 0.5 mM palmitic acid ( $C_{16:0}$ ) or 0.5 mM palmitoleic acid ( $C_{16:1}$ ) as indicated. Data are means  $\pm$  S.D. of three independent experiments.

Figure 6. Ectopic overexpression of *ACC1* and *FAS1* decreases *INO1* expression in *Acb1p* depleted cells. (A) Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700, black columns) and *pGAL-ACB1* (*Acb1p* depleted Y700, open columns) cells transformed in pairs with combinations of empty vectors or expression vectors as indicated. Transformants were grown in selective medium (SC – Ura – Trp + 0.2 mM inositol). (B) Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700, black columns) and *pGAL-ACB1* (*Acb1p* depleted Y700, open columns) cells transformed with either empty vector or expression vector as indicated. Transformants were grown in selective

medium (SC – Trp + 0.2 mM inositol). Data are means  $\pm$  S.D. of three independent experiments.

Figure 7. *Acb1p* affect gene expression in an acyl-CoA ester dependent manner. Quantitative real-time PCR analysis of *INO1* mRNA levels in wild type (Y700), *pGAL-ACB1* (*Acb1p* depleted Y700), *pGAL-ACB1 + ACB1* (*Acb1p* depleted Y700 complemented with *ACB1*) and *pGAL-ACB1 + ACB1<sup>Y28F,K32A</sup>* (*Acb1p* depleted Y700 complemented with mutant *ACB1*) cells cultured in SC media supplemented with 0.2 mM inositol. Data are means  $\pm$  S.D. of three independent experiments.

**Table 1. Strains used in the present work**

Strain	Genotype	Source or reference
Y700	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3</i>	Gaigg <i>et al.</i> , 2001
Y700 <i>pGAL-ACB1</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, pGAL-ACB1::KanMX</i>	Gaigg <i>et al.</i> , 2001
Y700 <i>INO1-HA<sub>3</sub></i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, INO1-HA<sub>3</sub>::His3MX</i>	the present study
Y700 <i>pGAL-ACB1 INO1-HA<sub>3</sub></i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, pGAL-ACB1::KanMX, INO1-HA<sub>3</sub>::His3MX</i>	the present study
Y700 <i>scs2Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, SCS2::TRP1</i>	the present study
Y700 <i>pGAL-ACB1 scs2Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, pGAL-ACB1::KanMX, SCS2::TRP1</i>	the present study
AID	<i>MATa/MATα, ade1/ade1 ino1/ino1</i>	S. Henry
Y700 <i>pld1Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, PLD1::TRP1</i>	the present study
Y700 <i>pGAL-ACB1 pld1Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, pGAL-ACB1::KanMX, PLD1::TRP1</i>	the present study
Y700 <i>hac1Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, HAC1::URA3</i>	the present study
Y700 <i>pGAL-ACB1 hac1Δ</i>	<i>MATa, ade2-1 trp1 can1-100 leu2-112 his3-11 his3-15 ura3, pGAL-ACB1::KanMX, HAC1::URA3</i>	the present study

**Table 2. Comparison of fold change (Acb1p depleted cells versus wild type cells) obtained by microarray and quantitative real-time PCR (Q-RT-PCR) analysis**

GeneID	Gene	Function	Process	Fold change <sup>a</sup>	
				Microarray analysis	Q-RT-PCR <sup>b</sup>
<b>Stress response</b>					
ygr088w	CTT1	catalase T, cytosolic	response to stress	no data <sup>c</sup>	3.1 ± 0.14
ybr072w	HSP26	heat shock protein	response to stress	no data <sup>c</sup>	5.9 ± 1.4
yo1052c-a	DDR2	heat shock protein DDRA2	response to stress	6.9 ± 0.71	2.7 ± 0.66
yll026w	HSP104	heat shock protein	response to stress	no data <sup>c</sup>	3.1 ± 0.41
yfl014w	HSP12	heat shock protein	response to stress	no data <sup>c</sup>	6.8 ± 1.6
<b>C-compound and carbohydrate metabolism</b>					
ydl022w	GPD1	glycerol-3-phosphate dehydrogenase (NAD <sup>+</sup> ), cytoplasmic	intracellular accumulation of glycerol	6.4 ± 1.8	5.5 ± 0.89
yjl052w	TDH1	glyceraldehyde-3-phosphate dehydrogenase 1	glycolysis; gluconeogenesis	4.8 ± 0.16	3.3 ± 0.91
<b>Lipid metabolism</b>					
ygl055w	OLE1	stearoyl-CoA desaturase	fatty acid desaturation	1.9 ± 0.75	1.9 ± 0.45
yjl196c	ELO1	fatty acid elongation protein	fatty acid elongation	no data <sup>c</sup>	1.2 ± 0.11
yk1182w	FAS1	fatty-acyl-CoA synthase, β chain	fatty acid biosynthesis	1.6 ± 0.25	1.6 ± 0.41
yp1231w	FAS2	fatty-acyl-CoA synthase, α chain	fatty acid biosynthesis	1.8 ± 0.17	1.6 ± 0.26
yhr007c	ERG11	cytochrome P450 lanosterol 14α-demethylase	ergosterol biosynthesis	no data <sup>c</sup>	-1.9 ± 0.10
yjl153c	INO1	myo-inositol-3-phosphate synthase	phosphatidylinositol biosynthesis	no data <sup>c</sup>	151 ± 10.7
ygr170w	PSD2	phosphatidylserine decarboxylase 2	phosphatidylserine biosynthesis	no data <sup>c</sup>	1.3 ± 0.11
ygr157w	CHO2	phosphatidylethanolamine N-methyltransferase	phosphatidylcholine biosynthesis	1.8 ± 0.51	1.6 ± 0.02
yjr073c	OPI3	methylene-fatty-acyl-phospholipid synthase	phosphatidylcholine biosynthesis	2.9 ± 0.68	6.5 ± 0.55
yn1169c	PSD1	phosphatidylserine decarboxylase 1	phosphatidylserine biosynthesis	no data <sup>c</sup>	1.3 ± 0.17
ypr113w	PIS1	CDP diacylglycerol--inositol 3-phosphatidyltransferase	phosphatidylinositol biosynthesis	no data <sup>c</sup>	1.1 ± 0.07
yer026c	CHO1	CDP-diacylglycerol serine O-phosphatidyltransferase	phosphatidylserine biosynthesis	1.8 ± 0.24	1.7 ± 0.14
<b>Others</b>					
yp1154c	PEP4	aspartyl protease	vacuolar protein, catabolism	1.8 ± 0.14	1.7 ± 0.32
yor061w	CKA2	casein kinase II alpha' chain	G2/M and G1/S transition etc.	no data <sup>c</sup>	-1.2 ± 0.12
yfl031w	HAC1	transcription factor	unfolded protein response	no data <sup>c</sup>	5.6 ± 0.39
ylr350w	ORM2	strong similarity to ORM1	response to unfolded protein	2.8 ± 0.43	Not determined
ycl043c	PDI1	protein disulfide-isomerase precursor	protein folding	2.2 ± 0.17	Not determined
yjl034w	KAR2	nuclear fusion protein	response to unfolded protein	1.9 ± 0.98	Not determined

<sup>a</sup> Positive values represent fold upregulation in Acb1p depleted cells compared to wild type cells whereas negative values represent fold downregulation in Acb1p depleted cells compared to wild type cells. Data are means ± S.D. of either three or more independent experiments (Q-RT-PCR) or of two independent experiments (Microarray analysis).

<sup>b</sup> For each Q-RT-PCR analysis RNA from three or four different cell cultures were used for cDNA synthesis and mean values were calculated. *ACT1* was used as endogenous reference.

<sup>c</sup> Data has been removed because intensities values were below that of *Arabidopsis* controls or because data was not present in both (dye-reversal) arrays.

**Table 3. The relative phospholipid content of Y700 *pGAL-ACB1* cells compared to Y700 cells**

	Phospholipid class					
	PA (%)	PI (%)	PG (%)	PS (%)	PE (%)	PC (%)
<i>YPD media</i>						
Y700 <i>pGAL-ACB1</i> versus Y700 <sup>1</sup>	95 ± 7.8	128 ± 15.2	89 ± 18.9	78 ± 2.5	129 ± 7.8	98 ± 15.5
<i>SC media (+ ino.)</i> <sup>2</sup>						
Y700 <i>pGAL-ACB1</i> versus Y700 <sup>1</sup>	81 ± 6.8	80 ± 4.4	46 ± 7.6	72 ± 20.8	80 ± 19.1	96 ± 18.1
<i>SC media (- ino.)</i> <sup>3</sup>						
Y700 <i>pGAL-ACB1</i> versus Y700 <sup>1</sup>	120 ± 22.5	223 ± 0.9	66 ± 8.4	125 ± 18.9	139 ± 23.5	100 ± 15.9

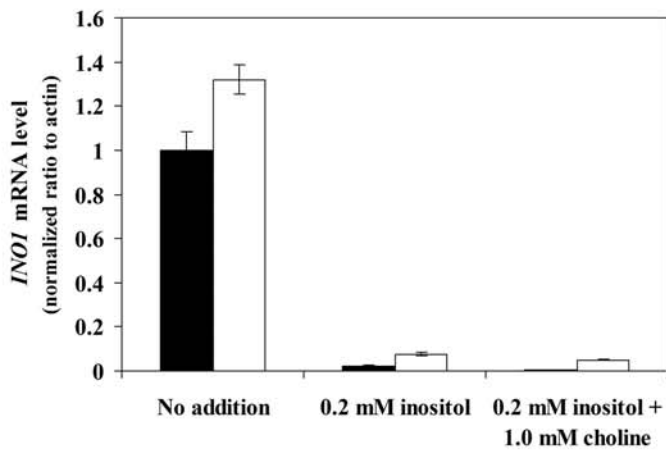
<sup>1</sup>The content of a given phospholipid class in *Acb1p* depleted cells was normalized to wild type levels (wild type 100 %) and calculated using the following formula:  $\Sigma [\% \text{ individual phospholipid specie in Wt} / ((\text{Wt } ^{12}\text{C}/^{13}\text{C}) / (\Delta ^{12}\text{C}/^{13}\text{C}))]$ , where  $\Delta$  is Y700 *pGAL-ACB1* and Wt is Y700. Data are means ± S.D. of two independent experiments.

<sup>2</sup>SC media supplemented with 0.2 mM inositol.

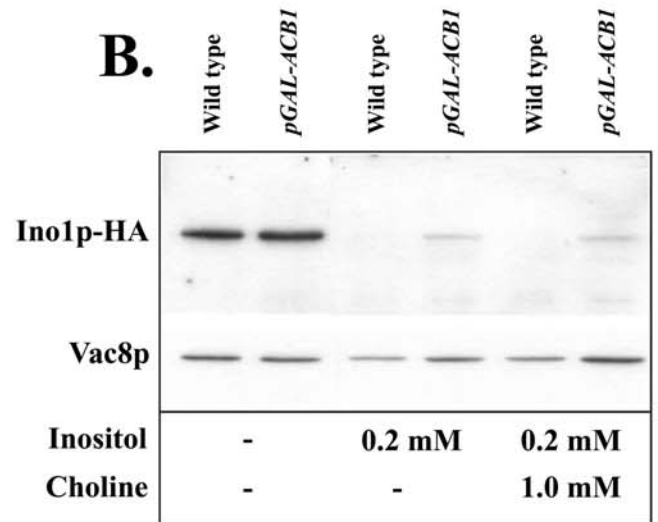
<sup>3</sup>SC media without inositol.

# Figure 1

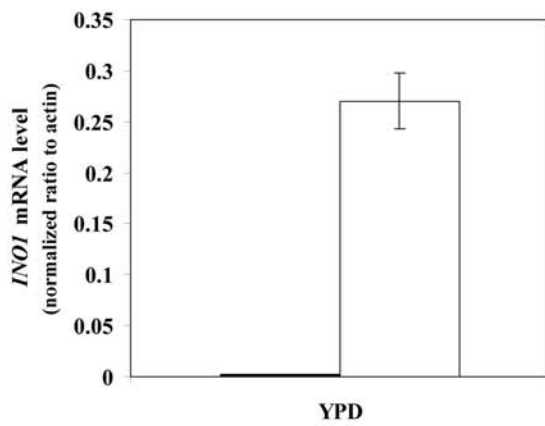
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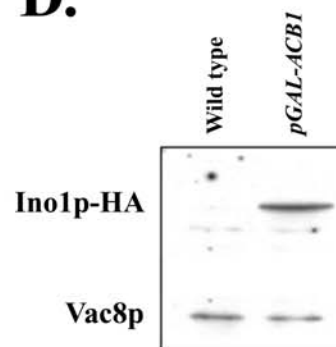
**B.**



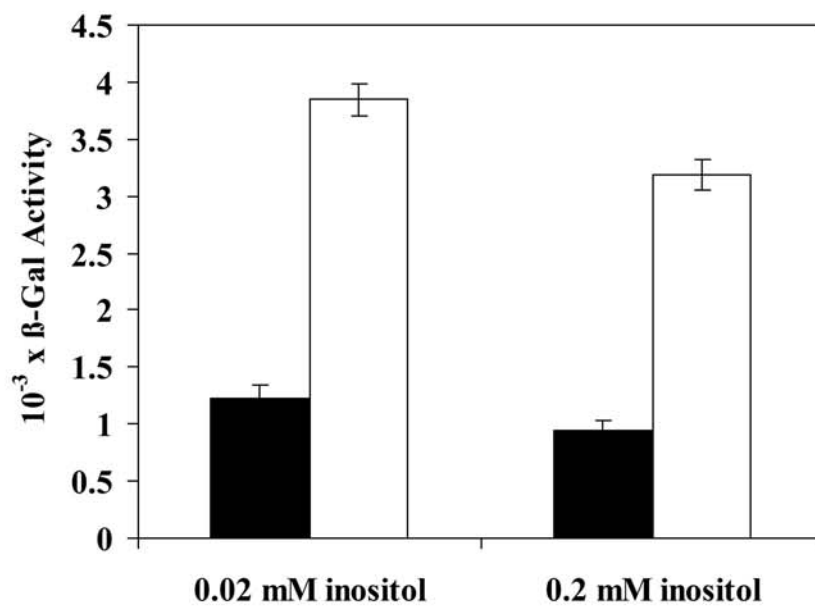
**C.**



**D.**



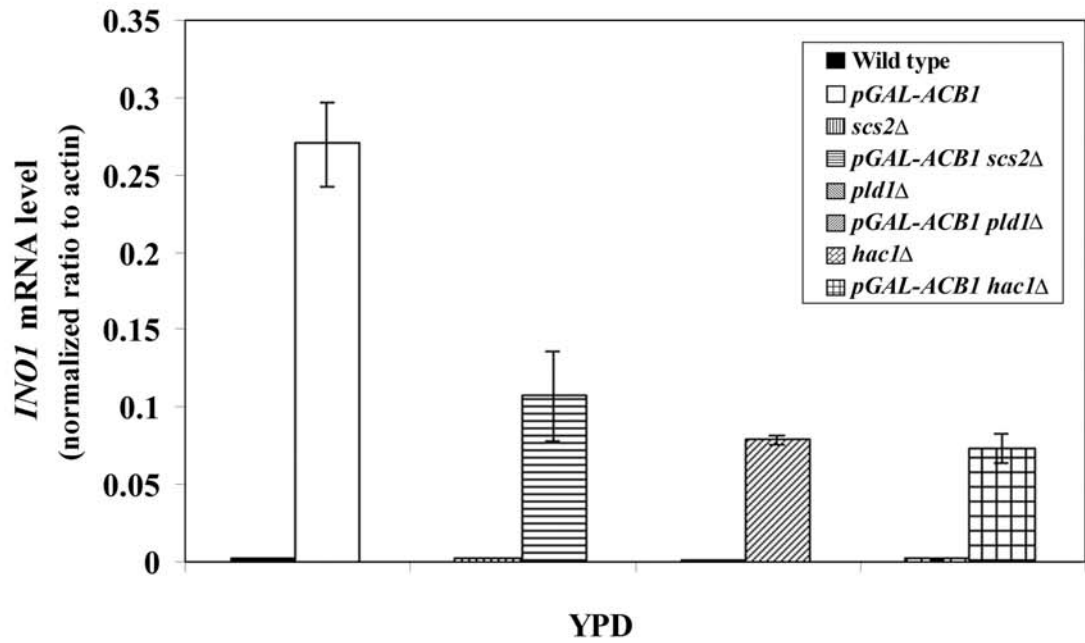
## Figure 2



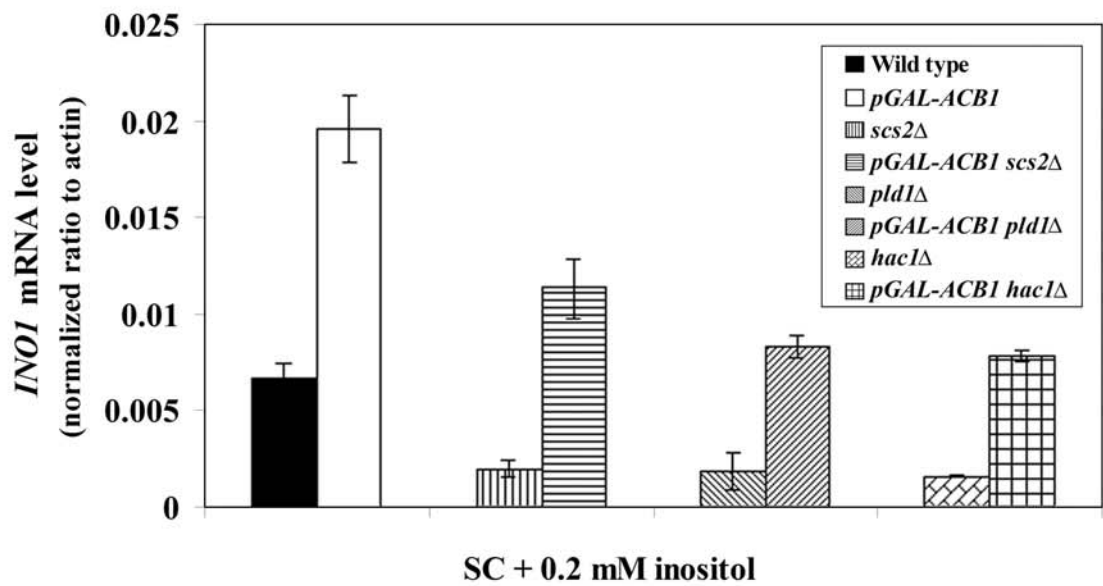


# Figure 3

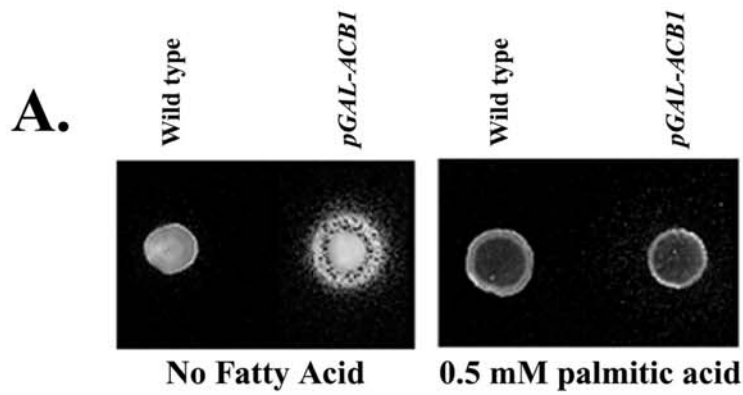
**A.**



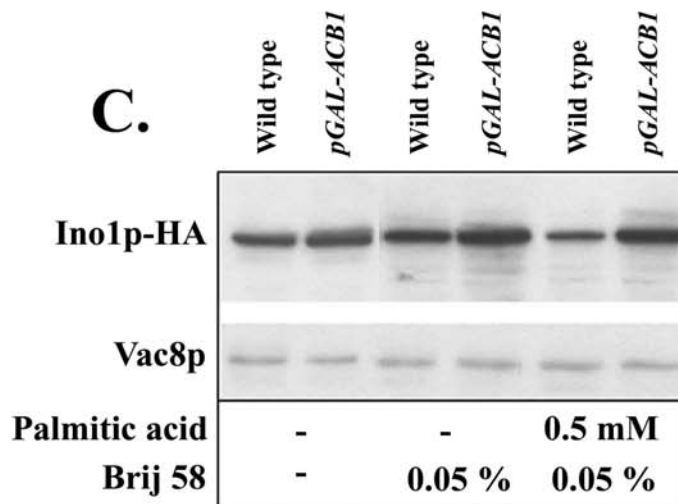
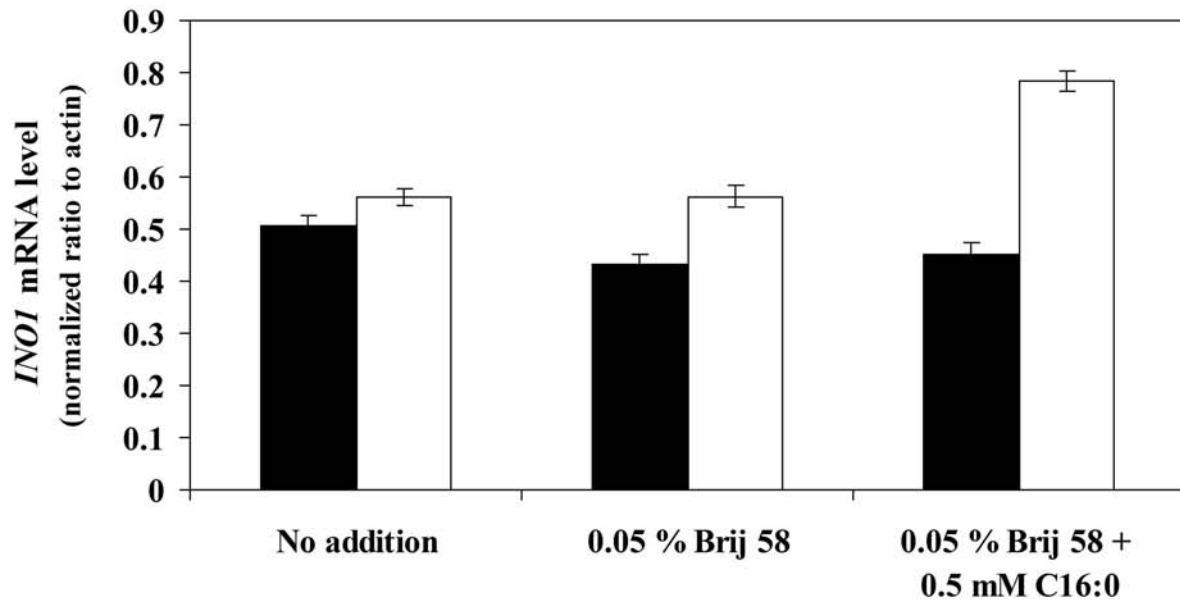
**B.**



## Figure 4

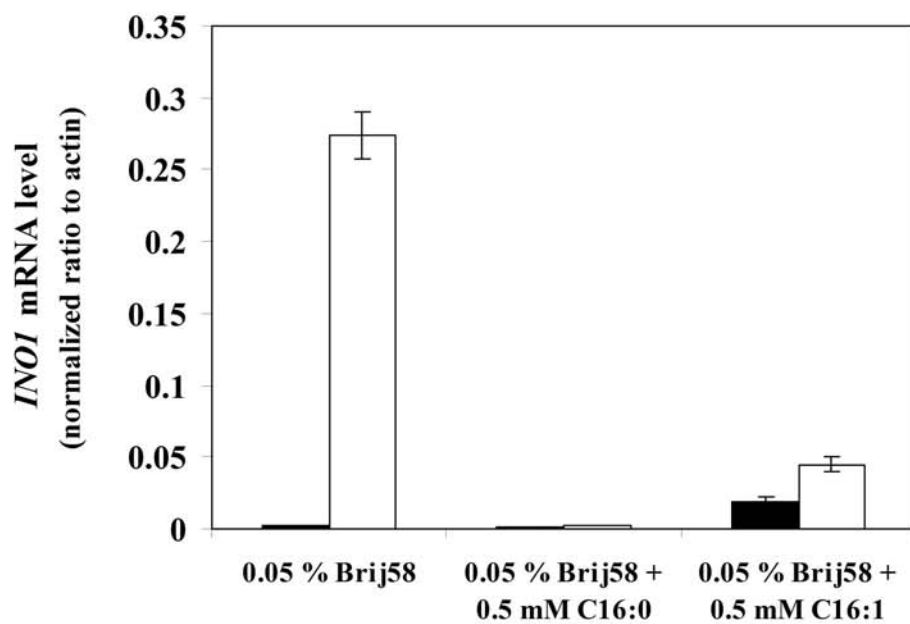


**B.**

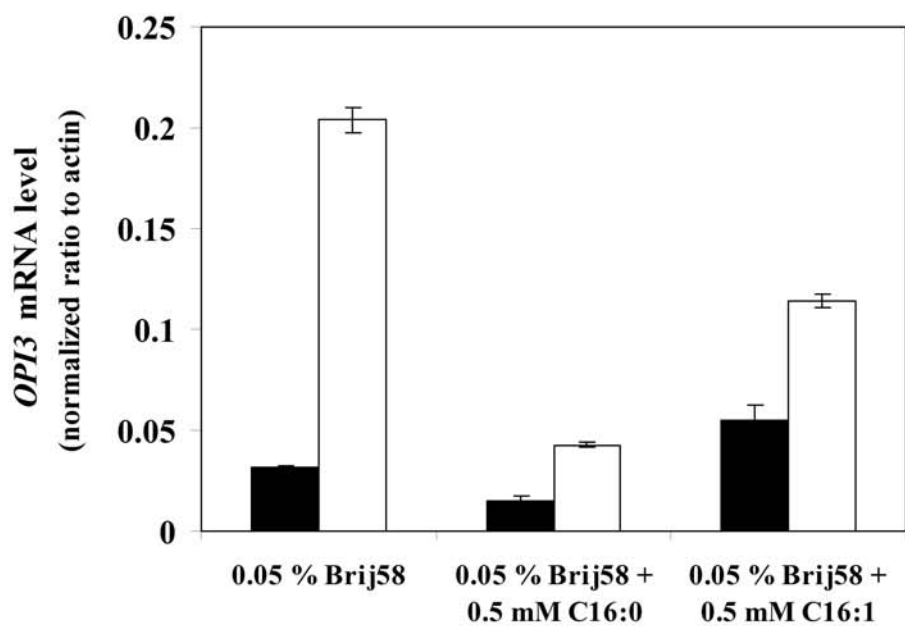


## Figure 5

**A.**

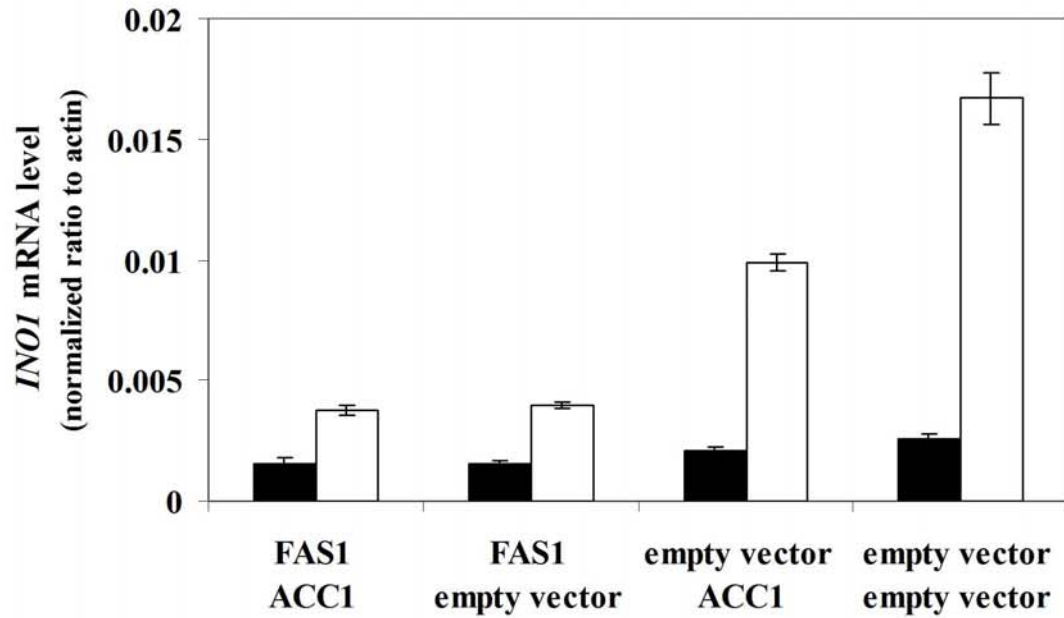


**B.**

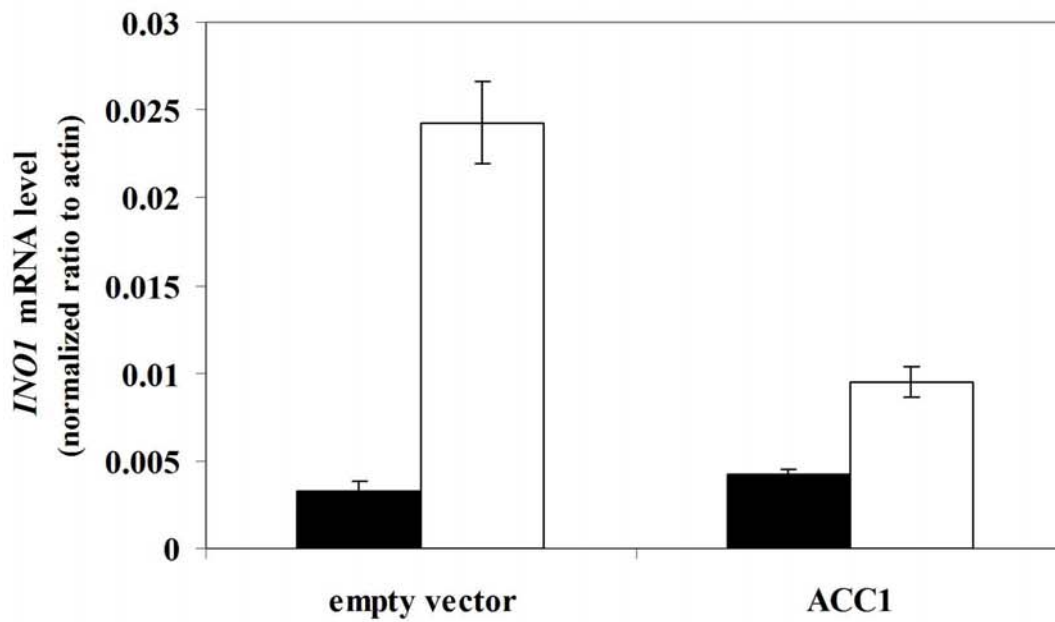


## Figure 6

**A.**



**B.**



# Figure 7

