The yeast acyltransferase Sct1p regulates fatty acid desaturation by competing with the desaturase Ole1p

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ABSTRACT The degree of fatty acid unsaturation, that is, the ratio of unsaturated versus saturated fatty acyl chains, determines membrane fluidity. Regulation of expression of the fatty acid desaturase Ole1p was hitherto the only known mechanism governing the degree of fatty acid unsaturation in Saccharomyces cerevisiae. We report a novel mechanism for the regulation of fatty acid desaturation that is based on competition between Ole1p and the glycerol-3-phosphate acyltransferase Sct1p/Gat2p for the common substrate C16:0-CoA. Deletion of SCT1 decreases the content of saturated fatty acids, whereas overexpression of SCT1 dramatically decreases the desaturation of fatty acids and affects phospholipid composition. Whereas overexpression of Ole1p increases desaturation, co-overexpression of Ole1p and Sct1p results in a fatty acid composition intermediate between those obtained upon overexpression of the enzymes separately. On the basis of these results, we propose that Sct1p sequesters C16:0-CoA into lipids, thereby shielding it from desaturation by Ole1p. Taking advantage of the growth defect conferred by overexpressing SCT1, we identified the acyltransferase Cst26p/Psi1p as a regulator of Sct1p activity by affecting the phosphorylation state and overexpression level of Sct1p. The level of Sct1p phosphorylation is increased when cells are supplemented with saturated fatty acids, demonstrating the physiological relevance of our findings.

INTRODUCTION The physical properties of a biological membrane are highly dependent on the lipid composition, which shows tremendous variation between different membranes and dependence on conditions (Dowhan, 1997). This variation is responsible for the plasticity of biomembranes, which plays a role in cellular adaptation to the environment and allows for cellular processes such as energy storage, signaling, transport, and membrane fusion and fission (Dowhan, 1997; Aguilar and de Mendoza, 2006; Hagen et al., 2010).

Membrane fluidity is an important physical property, which is to a large extent determined by the ratio of saturated versus unsaturated fatty acids (Los and Murata, 2004). Membrane fluidity is essential for maintaining the membrane barrier and for optimal functioning of membrane proteins (Singer and Nicolson, 1972; Martin et al., 2007). The presence of cis-double bonds in membrane lipid acyl chains causes disturbances in the acyl chain packing, which result in a much lower gel–to–liquid crystalline phase transition temperature of the membrane. A poikilothermic organism like the yeast Saccharomyces cerevisiae readily adapts the degree of desaturation of its acyl chains when shifted to another growth temperature to sustain membrane fluidity (Martin et al., 2007). The degree of

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Abbreviations used: G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; ESI-MS/MS, electrospray ionization tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; lyso-PA, phosphatidic acid; lyso-PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SGR, synthetic galactose-raffinose medium; SL, synthetic lactate medium; SSL, semi-synthetic lactate medium; TAG, triacylglycerol; TLC, thin-layer chromatography.

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desaturation of membrane lipids also depends on the function of the membrane they constitute. For example, the inner mitochondrial membrane harboring the enzyme complexes of the respiratory chain contains a higher amount of unsaturated phospholipid acyl chains than the plasma membrane that shields the cell from the extracellular milieu (Schneider et al., 1999). Accordingly, the increase in overall fatty acid desaturation observed following a shift of yeast cells from fermentable to nonfermentable carbon source reflects the full development of the mitochondrial network (Tuller et al., 1999).

The yeast fatty acid profile is considerably less complex than its mammalian or plant counterparts, which contain a wide variation of fatty acids differing in length and degree of unsaturation. It consists mainly of C16 and C18 fatty acids bearing either one or no double bond. Fatty acid synthesis in *S. cerevisiae* is initiated by the acetyl-CoA carboxylase Aac1p and continued by the cytosolic multienzyme fatty acid synthase complex consisting of Fas1p and Fas2p, yielding acyl-CoA's with an acyl chain length of 16 or 18 carbon atoms (Tehlivets et al., 2007). The endoplasmic reticulum (ER)-based fatty acid elongases Elo1p, Elo2p, and Elo3p can elongate the acyl-CoA's up to C26. Although essential, the very long acyl chains are very low in abundance (Rossler et al., 2003).

Monounsaturated fatty acids account for ~70–80% of total fatty acids and are synthesized from saturated fatty acyl-CoA substrates by the ER-resident A9-fatty acid desaturase Ole1p. Yeast can modulate the degree of acyl chain desaturation by varying the level of Ole1p expression. The expression of Ole1p is regulated at the level of transcription by temperature, carbon source, the presence of fatty acids in the growth medium, and oxygen levels (Martin et al., 2007). Transcriptional activation and mRNA stability of Ole1p requires ubiquitin-mediated cleavage of the homodimeric ER membrane proteins Spt23p and Mga2p by the 26S proteasome (Hoppe et al., 2000).

In a screen for gene products involved in acyl chain remodeling of the major membrane lipid phosphatidycholine (PC; Boumann et al., 2003), we identified the glycerol-3-phosphate (G3P) acyltransferase Sct1p/Gat2p. The *SCT1* gene was first characterized as a multicopy suppressor of a choline transport mutant also defective in phosphatidylethanolamine (PE) methylation (Matsushita and Nikawa, 1995), hence the name suppressor of choline transport 1. The 86-kDa enzyme Sct1p transfers acyl chains to the sn-1 position of G3P or dihydroxyacetonephosphate in the first committed step of glycerophospholipid synthesis, that is, the production of lysophosphatidic acid (lyso-PA; Zheng and Zou, 2001). The homologous protein Gpt2p/Gat1p catalyzes the same reactions, and deletion of *SCT1* and GPT2 is synthetically lethal. Both enzymes are integral membrane phosphoproteins localized to the ER (Bratschi et al., 2009).

In the present study, the analysis of *SCT1* deletion and overexpression strains demonstrates that Sct1p is a novel regulator of fatty acid desaturation by competing with the desaturase Ole1p for C16:0-CoA and sequestering C16:0 in lipids. The activity of Sct1p is regulated posttranslationally by phosphorylation. Moreover, the first component regulating Sct1p phosphorylation is reported, and the functional relevance of the phosphorylation of Sct1p is illustrated in wild-type yeast. To our knowledge, this is the first time that an acyltransferase is implicated in cellular fatty acid homeostasis.

**RESULTS**

**Deletion of *SCT1* changes the molecular species profile of PC by impairing acyl chain remodeling**

In a screen for gene products required for acyl chain remodeling of PC, we examined the effect of deleting genes encoding (potential) acyltransferases on the molecular species profile of PC in a *pct1* strain. Cells lacking Pct1p—the enzyme catalyzing the rate-limiting step of the CDP-choline pathway—rely on the methylation of PE for the synthesis of PC and require acyl chain exchange to obtain the steady-state molecular species profile of PC (Boumann et al., 2003). Among the candidate genes were the two G3P acyltransferases *SCT1* (GAT2) and GPT2 (GAT1). Figure 1A shows the PC species profiles of *pct1*, *sct1pct1*, and *gpt2pct1* cells grown to mid-log phase in SSL were analyzed by ESI-MS/MS in parent ion scans for *m/z* 184 in the positive ion mode. The relative abundance is shown for species that contribute at least 1% of total PC. Error bars represent the SD (pct1, n = 12; sct1pct1 and gpt2pct1, n = 3). (B) *pct1* and *sct1pct1* cells cultured in SL medium were pulse labeled with deuterium-labeled methionine for 10 min, followed by a 180-min chase with an excess of methionine. The species profile of PC synthesized during the pulse was determined at the indicated time points by ESI-MS/MS in parent ion scans for *m/z* 193 and is compared with the steady-state PC species profile (*m/z* 184). The relative abundance is shown for the four major PC species as mol% of total PC. Error bars represent the SD (n = 3).

![Figure 1](https://example.com/figure1.jpg)

**FIGURE 1:** Deletion of *SCT1* in a *pct1* strain modifies the PC species profile by impairing acyl chain exchange. (A) The PC species profiles of *pct1*, *sct1pct1*, and *gpt2pct1* cells grown to mid-log phase in SSL were analyzed by ESI-MS/MS in parent ion scans for *m/z* 184 in the positive ion mode. The relative abundance is shown for species that contribute at least 1% of total PC. Error bars represent the SD (pct1, n = 12; sct1pct1 and gpt2pct1, n = 3). (B) *pct1* and *sct1pct1* cells cultured in SL medium were pulse labeled with deuterium-labeled methionine for 10 min, followed by a 180-min chase with an excess of methionine. The species profile of PC synthesized during the pulse was determined at the indicated time points by ESI-MS/MS in parent ion scans for *m/z* 193 and is compared with the steady-state PC species profile (*m/z* 184). The relative abundance is shown for the four major PC species as mol% of total PC. Error bars represent the SD (n = 3).
To monitor postsynthetic modifications of PC, the cells were cultured on synthetic lactate medium (SL) and pulsed for 10 min with deuterium-labeled methionine, yielding PC with a (methyl-D2)2-labeled head group. The evolution of the molecular species profile of the newly synthesized PC during the chase with unlabeled methionine was recorded by ESI-MS/MS parent ion scans for m/z 193 and showed an almost twofold increase in 32:1 PC and a 20% decrease in 34:2 PC in the pct1 strain (Figure 1B, left), in agreement with Boumann et al. (2003). In contrast, in the sct1pct1 strain the molecular species profile of newly synthesized PC hardly changed during the chase (Figure 1B, right), indicating that the remodeling of PC by acyl chain exchange was strongly reduced.

The expression level of catalytically active Sct1p determines the cellular C16:0 content

To elucidate the role of Sct1p in PC remodeling, we first analyzed the fatty acid composition of the deletion strains sct1pct1 and gpt2pct1 (Figure 2A). Deletion of SCT1 decreased the cellular content of C16:0 by ~50%, which was compensated by an increase in C18:1. Deletion of GPT2 did not cause major changes in fatty acid composition. Overexpression of Sct1p from an episomal GAL1 promoter in sct1pct1 resulted in a fourfold increase in C16:0 content and at least twofold increases in C14:0 and C18:0 at the expense of the levels of C16:1 and C18:1 (Figure 2B), the overall content of saturated acyl chains increasing from ~12 to ~40%. The level of overexpression was estimated to be at least 30-fold using Sct1p with a C-terminal HIS6HA3 tag (Figure 2C). The tag did not significantly influence fatty acid composition (Figure 2B). The slower-migrating band observed upon overexpression of SCT1 (Figure 2C, apparent weight ~120 kDa) was previously assigned to the phosphorylated enzyme (Bratschi et al., 2009). Overexpression of a catalytically inactive form of Sct1p containing a mutation of a glycine residue in the active site (G253L; Lewin et al., 1999; Han et al., 2008) did not affect the fatty acid profile (Figure 2, B and C). From these results, we conclude that the enzyme activity of Sct1p determines fatty acid desaturation. The reduced PC remodeling in sct1pct1 is most likely a consequence of the reduced availability of C16:0.

Overexpression of Sct1p was found to severely reduce the growth of the sct1pct1 strain on agar plates (Figure 2D). In liquid synthetic galactose-raf fines (SGR) medium containing a minimal amount of glucose, growth of the overexpression strain was reduced to a lesser extent (Figure 2E). Previously, comparable growth rates in liquid culture were reported upon overexpression of Sct1p in a gpt2sct1 double-deletion background (Bratschi et al., 2009).

Overexpression of Sct1p increases lipid synthesis and affects glycerolipid composition

The consequences of overexpressing Sct1p and the induced rise in C16:0 for cellular lipid content and lipid synthesis of sct1pct1 cells were investigated by comparing the overexpression strain to the empty vector control. Overexpression of Sct1p resulted in a 1.6 ± 0.2-fold (±SD, n = 4) increase in phospholipid content based on a comparison of the amount of phosphorus recovered in cellular lipid extracts per OD600 unit. To investigate whether the rise in phospholipid content resulted from an increase in de novo lipid synthesis, we determined the incorporation of [14C]acetate into lipids. A 1.3 ± 0.2-fold (±SD, n = 4) increase was observed in the incorporation of [14C]acetate in the total lipid fraction per OD600 unit during 1 h of labeling. The distribution of the [14C] label between the phospholipid and the neutral lipid fractions was not dramatically changed, with 62 ± 7% of the [14C] label associated with phospholipids in the empty vector control versus 52 ± 11% in the overexpression strain (±variation, n = 2). ESI-MS/MS analysis revealed that overexpression of Sct1p increased the level of its product lyso-PA twofold, the increase being accounted for by C16:0 lyso-PA (Figure 3A). The level of

\[ \text{relative abundance} \ (%) \]

\[ \begin{array}{cccccc}
14:0 & 14:1 & 16:0 & 16:1 & 18:0 & 18:1 \\
0 & 20 & 40 & 60 & 0 & 20 & 40 & 60
\end{array} \]
A comparison of the incorporation of [14C]acetate in the lipids of pct1 and sct1pct1 cells cultured in SL medium did not reveal differences in total lipid synthesis, in the distribution of label between neutral lipids and phospholipids, and in the relative incorporation into different phospholipid classes. The relative incorporation of [14C]acetate into the neutral lipid classes was similar to that reported previously for an sct1 deletion strain and its parental wild type (Zaremba and McMaster, 2002), with an increase in the synthesis of TAG in pct1 versus sct1pct1 that was comparable to the increase induced by overexpression of Sct1p in sct1pct1 (Supplemental Figure S2C and Figure 3).

Similar to PA (Figure 3A), the molecular species profiles of the four major phospholipid classes revealed increased saturation upon Sct1p overexpression (Supplemental Figure S3), with rises in monounsaturated species predominating in PC and PE, and rises in disaturated species in phosphatidylserine (PS) and PI, all at the expense of the diunsaturated species. The increase in acyl chain saturation was strongest in PC, followed in decreasing order by PS, PI, and PE (Supplemental Figure S3C).

**Co-overexpression of Sct1p and Ole1p reveals competition for the common substrate C16:0-CoA**

The enzyme activity of Sct1p could enhance fatty acid saturation by sequestering C16:0 chains into lipids, thus depriving the desaturase Ole1p of its C16:0-CoA substrate. To address this possibility, we co-overexpressed Sct1p and Ole1p. Whereas overexpression of Sct1p caused a decrease in fatty acid desaturation as described earlier, overexpression of Ole1p increased fatty acid desaturation and slightly enhanced fatty acid length (Figure 4A). Co-overexpression gave rise to a fatty acid profile intermediate between those of the single-overexpression strains (Figure 4A). The expression level of Ole1p-Myc was similar in strains where pESC-OLE1 was cotransformed with pYES2 or pYES2-SCT1-HH (Figure 4B), and the expression level of Sct1p in sct1pct1 pYES2-SCT1-HH was not affected by cotransformation of pESC-OLE1 (unpublished data). Similar fatty acid profiles were obtained when an Ole1p-Myc-overexpressing strain was cotransformed with the vector encoding the His6HA3-tagged version of Sct1p (Supplemental Figure S4). Taken together, these results indicate that the two enzymes compete for the shared substrate C16:0-CoA.

Co-overexpression of Sct1p and Ole1p partially relieved the growth defect of cells overexpressing Sct1p (Figure 4C). Overexpression of Ole1p slightly impaired growth.

The growth phenotype and increased C16:0 content induced by overexpression of Sct1p are suppressed by deletion of the ACB1 and CST26 genes; Cst26p affects phosphorylation and expression of Sct1p

To get further insight into the mechanism by which Sct1p regulates fatty acid saturation and its biological significance, we screened for suppressors of the growth phenotype caused by overexpression of Sct1p. The screen comprised 10 double-knockout strains in which a gene involved in lipid metabolism was deleted in the pct1 background and yielded two suppressors.

Deletion of ACB1, coding for the yeast acyl-CoA-binding protein that has been implicated in the termination of fatty acid synthesis and in fatty acid elongation (Schjerling et al., 1996; Gaigg et al., 2001), partially restored the growth defect (Figure 5A, left) and partially reduced the accompanying rise in C16:0 content (Figure 5B) conferred by overexpression of Sct1p. Immunoblot analysis verified that the level of overexpression of Sct1p was not affected by deletion of ACB1 (Figure 5C). Closer inspection of the fatty acid profiles of phosphatidic acid (PA) increased hardly if at all, suggesting that the acylation of lyso-PA is rate limiting in PA synthesis. The molecular species profile of PA showed the appearance of disaturated PA and an increase in 32:1 and 34:1 at the expense of the diunsaturated and C18:1-containing PA species (Figure 3A), consistent with the change in cellular acyl chain composition. Consistent with the overall increase in cellular lipid synthesis and content, [14C]acetate labeling showed that the synthesis of PA was increased (Figure 3B). In addition, the incorporation of [14C]acetate in phosphatidylinositol (PI) and triacylglycerol (TAG) increased at the expense of PE and steryl ester in the phospholipid and neutral lipid classes, respectively, when Sct1p was overexpressed (Figure 3B). The changes in phospholipid and neutral lipid synthesis were mirrored in the steady-state phospholipid and neutral lipid composition (Supplemental Figure S2, A and B).
The upper band was shown to correspond to the phosphorylated enzyme (Bratschi et al., 2009), these findings strongly suggest that phosphorylation inactivates Sct1p and that Cst26p regulates (de)phosphorylation of Sct1p.

We conclude that deletion of \( CST26 \) fully suppresses the growth phenotype and increase in C16:0 content conferred by overexpression of Sct1p by a mechanism that most likely involves inactivation of Sct1p by increased phosphorylation and decreased expression.

Phosphorylation of Sct1p is enhanced by the presence of C16:0 in the culture medium

The foregoing results indicate that the level of expression and the phosphorylation state of Sct1p are potentially critical parameters in regulating fatty acid desaturation. To examine whether yeast uses these regulatory mechanisms to control the activity of chromosomally expressed Sct1p, we monitored the expression of chromosomally HIS6HA3-tagged Sct1p under conditions known to influence
fatty acid composition. Varying the growth temperature from 30 to 37°C or the carbon source (glucose vs. lactate; Martin et al., 2007) did not affect the expression level or phosphorylation state of Sct1p (Supplemental Figure S5). A more straightforward way to alter the fatty acid composition is by supplementing fatty acids to the culture medium. A slow-migrating band appeared upon addition of C16:0 to wild-type (WT) cells in yeast extract/peptone/dextrose (YPD), and the intensity of the slow-migrating band increased twofold upon addition of C16:0 to pct1 cells in synthetic glucose medium (Figure 6). In contrast, addition of C18:1 did not affect the migration pattern of Sct1p-HA. Densitometric analysis of the blots revealed that 10 and 30% of Sct1p-HA were phosphorylated in the WT and pct1 cells grown in the presence of C16:0, respectively, versus 0 and 15% with no added fatty acid or C18:1 present.

**DISCUSSION**

On the basis of the results presented, we propose a new mechanism for the control of fatty acid desaturation at the level of acyl chain incorporation into PA that is juxtaposed to the regulation of Ole1p activity (Martin et al., 2007). We propose that Sct1p shields C16:0-CoA and other saturated acyl-CoAs from the introduction of a double bond by Ole1p by preferentially sequestering them into lipids (Figure 7). We furthermore propose that the competition for substrate with the desaturase Ole1p is determined by the activity of Sct1p, which in turn depends on the expression level and the phosphorylation state of the enzyme. In the following we discuss the new competition model and the regulation of Sct1p activity by phosphorylation, including the role of Cst26p, the growth phenotype of Sct1p overexpression strains, the effects of SCT1 deletion and overexpression on lipid metabolism, and the implications of our findings for yeast and higher eukaryotes.

**A new paradigm for regulation of fatty acid desaturation involving competition between a desaturase and an acyltransferase**

Although the competition for substrate between Sct1p and Ole1p primarily revolves around C16:0-CoA, the preferred substrate of Sct1p in vivo (Zheng and Zou, 2001), our results indicate that also C18:0-CoA and C14:0-CoA are subject to competition between Sct1p and Ole1p (Figures 2B and 4A). Overexpression of Ole1p caused an increase in average acyl chain length, as was observed previously (Kajiwara et al., 2000), suggesting that Ole1p promotes elongation to C18:0-CoA by efficient desaturation to C18:1-CoA. Consistently, a shortening of fatty acids was observed upon ablation of unsaturated fatty acid synthesis (Pineau et al., 2008). Overexpression of Sct1p was found to decrease the average cellular fatty acid length, suggesting that Sct1p also competes for substrate with the fatty acid elongases that use C16:0-CoA as substrate, although it must be noted that the role of the fatty acid elongases in the synthesis of C18:0-CoA is not fully established (Rossler et al., 2003). According to the proposed mechanism, the substrate preferences and the activities of Sct1p, Ole1p, and the fatty acid elongases determine the cellular fatty acid composition (Figure 7). The influence of additional enzymes that consume large amounts of acyl-CoA, including the other enzymes involved in PA synthesis, Gpt2p, Ale1p, and Slc1p, remains to be investigated. The fatty acid composition of the gpt2 deletion strain (Figure 2A) and its reported lack of substrate preference (Zheng and Zou, 2001) suggest that Gpt2p is not a major player in regulating the degree of fatty acid desaturation.

**Phosphorylation of Sct1p inhibits its activity in vivo and is regulated by Cst26p**

The G3P acyltransferases Sct1p and Gpt2p were recently demonstrated to be phosphoproteins, and phosphorylation was proposed to regulate their activity (Bratschi et al., 2009). The enhanced phosphorylation of Sct1p and the accompanying complete reversal of the increased acyl chain saturation observed in the cst26 strain overexpressing SCT1 indicate that Sct1p is inactivated by...
phosphorylation. Preliminary experiments addressing the mechanism by which phosphorylation affects Sct1p activity showed that treatment with phosphatase of cell homogenates from an Sct1p-overexpressing strain does not affect the enzyme activity in vitro, suggesting that phosphorylation exerts an indirect effect on Sct1p activity in vivo, for example, by affecting access to substrate. The decrease in expression level of overexpressed Sct1p in the cst26 background may be a consequence of degradation of the phosphorylated enzyme. The functional significance of Sct1p phosphorylation in determining yeast fatty acid composition is evidenced by the increase in phosphorylation of chromosomally expressed Sct1p following exogenous addition of C16:0. Yeast has been shown to readily incorporate exogenous C16:0 in glycerolipids (Wagner and Paltauf, 1994). The modulation of Sct1p phosphorylation could provide a feedback mechanism by which yeast controls the incorporation of saturated acyl chains into its lipids.

A number of key enzymes in yeast lipid biosynthesis, including CTP synthase, PS synthase, and choline kinase, are regulated by phosphorylation involving protein kinases A and C (Carman and Kersting, 2004). Recently the Mg2+-dependent PA phosphatase Pah1p was found to be inactivated by phosphorylation of an amphipathic helix that controls membrane translocation (O’Hara et al., 2006; Karamanos et al., 2010). The major lipase Tg1p that mobilizes free fatty acids from TAG for the synthesis of PA is activated by the cyclin-dependent kinase Cdk1p/Cdc28p, linking lipid synthesis to the cell cycle (Kurat et al., 2009). A genome-wide screen identified 16 kinases that use Sct1p as a substrate in vitro, including the cyclin-dependent kinase Pho85p and the p21-activated kinase Ste20p (Pтеcek et al., 2005). Ste20p was linked to lipid metabolism, as it downregulates sterol uptake (Lin et al., 2009) and binds to and is activated by PA (Harkins et al., 2008). Therefore, it is tempting to speculate that Ste20p phosphorylates and deactivates Sct1p as PA levels rise, which would implicate Ste20p in the regulation of fatty acid desaturation in addition to its role in sterol homeostasis. Future research should identify the kinase(s) and phosphatase(s) acting on Sct1p and elucidate how its regulation by phosphorylation interfaces with other regulatory cascades.

It is remarkable that Cst26p was found to be required for maintaining the dephosphorylated state of Sct1p under conditions of Sct1p overexpression. Cst26p/Psi1p was only recently identified as an acyl-CoA-dependent acyltransferase involved in the acyl chain remodeling of PI, incorporating C18:0 at the sn-1 position (Le Guedard et al., 2009). The decreased cellular C18 content in cst26pc1 (Figure 5B) is consistent with the loss of C18:0-CoA consumption by Cst26p reducing the activity of the elongases by a decrease in the substrate-to-product ratio. The inactivation of overexpressed Sct1p upon deletion of CST26 could reflect a feedback mechanism to decrease the cellular content of saturated fatty acids when their incorporation into PI is impaired. Cst26p may inhibit the kinase or activate the phosphatase acting on Sct1p via its impact on the molecular species profile of PI or downstream phosphoinositides. Alternatively, Cst26p may be a dual-function protein with independent roles in PI metabolism and in the regulation of Sct1p activity.

Growth defect and adaptation of membrane lipid composition

The extent of the decrease in fatty acid desaturation correlates with the severity of the reduction of growth in the co-overexpression experiment (Figure 4), indicating that the increased acyl chain saturation plays a pivotal role in the growth defect conferred by Sct1p overexpression. The dramatic increase in fatty acyl chain saturation probably reduces membrane fluidity, impairing membrane processes. The growth phenotype and the increase in fatty acid saturation observed in liquid culture (Figure 2) resemble the effects of switching off fatty acid desaturation by inactivating heme synthesis (Pineau et al., 2008), a condition inducing ER stress (Pineau et al., 2009).

Overexpression of Sct1p results in increased synthesis of PA and a consequent overall increase in cellular lipid content. In contrast to deletion of SCT1, overexpression does not affect the relative rates of synthesis of TAG versus sterolesters in the neutral lipid fraction (Zaremberg and McMaster, 2002). Among the glycerophospholipids, the synthesis of PI increases at the expense of PE. The PI synthetic Pis1p may have a preference for C16:0-containing CDP-DAG and hence convert more CDP-DAG into PI upon Sct1p overexpression. Alternatively, the increase in PA synthesis due to Sct1p overexpression may deplete the UASINO genes, enhancing the production of inositol, which in turn promotes the synthesis of PI at the expense of PS, the biosynthetic precursor of PE (Kelley et al., 1988; Loewen et al., 2004). Irrespective of the mechanism involved, lowering the PE content contributes to maintaining membrane fluidity when yeast faces a rise in saturated acyl chains, as the smaller dimensions of the PE head group allow tighter packing of acyl chains than the head groups of other phospholipids (Silvius et al., 1986). Cells overexpressing Sct1p not only reduce their PE content, but also seem to protect PE from the excess of C16:0, as the change in saturation is smaller in PE than in the other major phospholipids. The rise in PI/PE ratio in response to increased saturation is fully consistent with the finding that overexpression of PI synthesize suppresses the growth defect conferred by a mutation in the RSP5 gene impairing fatty acid desaturation (Kaliszewski et al., 2006).

It is worth noting that the increase of C16:0 in PE reduces its nonbilayer propensity (Koynova and Caffrey, 1994; Boumann et al., 2006). As a consequence, overexpression of Sct1p renders PE more suitable as a bilayer-forming lipid, which may account for the discovery of the SCT1 gene as a multicopy suppressor of a mutant defective in PC synthesis (Matsushita and Nikawa, 1995). The increase in fatty acid saturation probably allowed PE to substitute for PC and rescue cell growth. This is to our knowledge the first time that these results receive an adequate explanation.

PC is the preferred phospholipid for accommodating C16:0 upon overexpression of Sct1p in the pct1-deletion background. In the absence of an active CDP-choline route, PC acquires C16:0 acyl chains from its precursor PE and by acyl chain remodeling (Figure 1B). Compared to the methylation of PE, the CDP-choline route more readily incorporates C16:0 into PC (Boumann et al., 2003). The latter property may account for the connection between turnover of PC through the CDP-choline route and Sct1p activity reported previously. Deletion of SCT1 was shown to result in a 10-fold decrease in the turnover of PC through the CDP-choline route, whereas deletion of GPT2 caused a fivefold increase in turnover of PC through the CDP-choline route (Zaremberg and McMaster, 2002). Our observation that the extent of phosphorylation of chromosomally expressed Sct1p is higher in pct1 than in wild-type cells (Figure 6) may reflect this connection. Taken together, these findings substantiate the incorporation of C16:0 into PC as an adaptation to changes in fatty acid composition and assign PC a function as reservoir of C16:0. Of interest, mutants disturbed in the synthesis of TAG become sensitive to exogenous C16:0 when the PE methylation pathway is inactivated (Garbarino et al., 2009).

Regulation of fatty acid desaturation in mammalian cells may involve a glycerol-3-phosphate acyltransferase

Mammalian cells harbor 4 glycerol-3-phosphate acyltransferase (GPAT) isozymes, mitochondrial GPAT1 and GPAT2 and microsomal
GPAT3 and GPAT4, which contain the same active-site motifs as their yeast functional homologues (Lewin et al., 1999; Wendel et al., 2009). GPAT1 seems to have a specificity for C16:0-CoA similar to that of Sct1p (Hammond et al., 2002), whereas the other GPATs do not show pronounced substrate preferences. Of interest, GPAT1-deficient mice display a reduced stearoyl-CoA desaturase 1 (SCD1) expression (Hammond et al., 2005), and overexpression of GPAT1 in mice specifically induces expression of SCD1 by an unknown mechanism not secondary to increased de novo lipogenesis (Linden et al., 2009). GPAT1 seems to have a specificity for C16:0-CoA similar to their yeast functional homologues (Lewin et al., 2002), whereas the other GPATs do not show pronounced substrate preferences.

**TABLE 1:** Yeast strains and plasmids used in this study.

| Strain/plasmid | Genotype | Source |
|----------------|----------|--------|
| BY4741         | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | EUROSCARF |
| sc1            | Isogenic to BY4741 except sc1::kanMX4 | EUROSCARF |
| gpt2           | Isogenic to BY4741 except gpt2::kanMX4 | EUROSCARF |
| acb1           | Isogenic to BY4741 except acb1::kanMX4 | EUROSCARF |
| cst26          | Isogenic to BY4741 except cst26::kanMX4 | EUROSCARF |
| pct1           | Isogenic to BY4741 except pct1::LEU2 | This study |
| sc1pct1        | Isogenic to BY4741 except pct1::LEU2 sc1::kanMX4 | This study |
| gpt2pct1       | Isogenic to BY4741 except gpt2::kanMX4 pct1::LEU2 | This study |
| acbpct1        | Isogenic to BY4741 except acb1::kanMX4 pct1::LEU2 | This study |
| cst26pct1      | Isogenic to BY4741 except cst26::kanMX4 pct1::LEU2 | This study |
| BY4741 SCT1-HH | Isogenic to BY4741 except SCT1-6His-3HA-loxP-KanMX-loxP | This study |
| pct1 SCT1-HH   | Isogenic to BY4741 SCT1-HH except pct1::LEU2 | This study |
| pYES2          | High copy vector containing the GAL1 promoter and the URA3 gene | Invitrogen |
| pYES2-SCT1     | pYES2 containing the SCT1 gene | Zheng and Zou (2001) |
| pYES2-SCT1-HH  | pYES2 containing the SCT1 gene with a C-terminal His6-HA3 tag | This study |
| pYES2-SCT1[G253L]HH | pYES2 containing the SCT1 gene with a C-terminal His6-HA3 tag and a Gly to Leu mutation at amino acid 253 | This study |
| pESC-HIS       | High copy vector containing GAL1 and GAL10 promoters and the HIS3 gene | Stratagene |
| pESC-OLE1      | pESC-HIS containing the OLE1 gene with a C-terminal Myc tag behind the GAL1 promoter | This study |

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**MATERIALS AND METHODS**

**Strains and culture conditions**

The yeast strains listed in Table 1 were maintained on YPD agar plates (1% yeast extract, 2% bactopeptone, and 2% glucose). Strains harboring the pYES2 and/or pESC-HIS plasmids were obtained using the high-efficiency transformation protocol (Gietz and Schiestl, 2007) and maintained on plates containing synthetic glucose medium lacking uracil and/or histidine, respectively. Synthetic medium contained the following per liter: 6.7 g of yeast nitrogen base without amino acids (Difco, Franklin Lakes, NJ), 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, 20 mg of tryptophan, 40 mg of uracil, and either 20 g of glucose (synthetic glucose medium) or 22 ml of 90% (vol/vol) lactic acid and 1 g of glucose, adjusted to pH 5.5 using KOH (SL). Strains were cultured aerobically at 30°C in semisynthetic lactate medium (SSL; Daum et al., 1982) or in SL. Strains containing the pYES2 and/or pESC-HIS plasmids were cultured in synthetic glucose medium without uracil and/or histidine, respectively, for 24 h, and then shifted to SGR medium containing 1% raffinose and 2% galactose at OD600 0.02 to induce the GAL1 promoter and cultured for another 24 h. Growth was monitored by measuring the OD at 600 nm on a single-beam spectrophotometer (Novaspec II; Pharmacia, GE Healthcare, Piscataway, NJ). Cells were harvested at mid-logarithmic growth phase (OD600 between 0.3 and 0.7).

**Growth phenotypes** were analyzed by culturing cells to mid-log phase in synthetic glucose medium, washing them twice with water, and spotting 15 μl of cell suspension serially diluted to OD600 0.002. Cells

**To compare** Sct1p expression patterns in the presence and absence of exogenous fatty acids, cells were cultured for 16 h in YPD or synthetic glucose medium and then transferred to YPD containing 1% (wt/vol) Brij35 with or without 0.5 mM C16:0 or 0.5 mM C18:1, or to synthetic glucose medium containing 0.2% Brij35 with or without 0.1 mM C16:0 or 0.1 mM C18:1 at OD600 0.002. Cells
were grown for another 24 h, harvested at mid-logarithmic growth phase, and subjected to Western blotting as described later.

**Molecular biology**

The PCT1 gene was deleted in the strains BY4741, sct1, gpt2, acb1, and cts26 (Table 1) to yield the corresponding knockout strains by LEU2 insertion in PCR-mediated gene disruption using primers PCT1-LEU2-1 and -2 (Supplemental Table S1) as previously described (Boumann et al., 2003).

Chromosomal SCT1 was tagged with a His6HA3 epitope by amplification of the 6His-3HA-loxP-KanMX-loxP cassette from the pU6H3HA plasmid (De Antoni and Gallwitz, 2000) with primers SCT1-HH-1 and -2 (Supplemental Table S1) and subsequent insertion in the BY4741 and pct1 strains (Table 1).

The pYES2 (Invitrogen, Carlsbad, CA) and pESC-HIS (Stratagene, Santa Clara, CA) vectors were used to construct galactose-inducible overexpression vectors in which the expression of His6HA3-tagged Sct1p and Myc-tagged Ole1p was under control of the GAL1 promoter (Table 1). The pYES2-SCT1-HH overexpression vector was constructed by amplification of SCT1-6His-3HA from pct1 SCT1-HH chromosomal DNA using primers pYES-SCT1-1 and -2 (Supplemental Table S1) and insertion of the amplified fragment into the BamHI and XhoI restriction sites of pYES2 (Table 1). The pESC-OLE1 vector was constructed by amplification of OLE1 from BY4741 chromosomal DNA with primers pESC-OLE1-1 and -2 and insertion of the amplified fragment into the Apal restriction site of pESC-HIS plasmid (Stratagene).

An inactive version of His6HA3-tagged Sct1p was obtained by introducing a mutation of Gly to Leu in the active site (Supplemental Table S1; Lewin et al., 1999; Han et al., 2008). Site-directed mutagenesis was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the pYES2-SCT1-HISHA plasmid and primers SCT1-G253L-1 and -2 to construct the pYES2-SCT1(G253L)- HISHA vector (Han et al., 2008).

Correct integration of PCR-amplified fragments in the genome was verified by PCR. Correct amplification of the genes inserted in the vectors was verified by DNA sequencing.

**Pulse-chase labeling with deuterium-labeled methionine**

Cells grown to mid-log phase in SL medium were collected by filtration, washed with amino acid-free SL, and resuspended at OD900 0.2–0.4 in 300 ml of SL medium supplemented with 120 mg/l (0.78 mM) (methyl-o3)2-l-methionine (Cambridge Isotope Laboratories, Andover, MA) instead of 20 mg/l l-methionine. After 10 min, cells were collected by filtration, washed with amino acid-free SL, and transferred within 3 min to 250 ml SL medium supplemented with 300 mg/l l-methionine, and incubation at 30°C was continued. At various time points, 50-ml aliquots of cell culture were collected, inactivated by adding KCN, NaF, and NaN3 at 15 mM each, and stored on ice until further processing. All media used for washing and resuspension were at 30°C.

**Lipid analysis by mass spectrometry**

Cell homogenates were obtained by vortexing yeast cells corresponding to ~25 OD900 units in the presence of glass beads (Boumann et al., 2003) and subjected to lipid extraction (Bligh and Dyer, 1959). Total lipid extracts corresponding to 300 nmol of phospholipid-phosphorus (Rouser et al., 1970) were dissolved in 200 μl of chloroform:methanol 1:1 (vol/vol) containing 10 mM ammonium acetate and analyzed by ESI-MS/MS on an API3000 triple quadrupole instrument (Applied Biosystems, Foster City, CA). Samples were injected into the instrument with a Hamilton Gastight 1710 syringe and a Harvard Apparatus Pump 11 at a constant flow rate of 3 μl/min. PC and (methyl-D3)3-PC were detected in parent ion scans for m/z 184 and 193, respectively, in the positive ion mode, PE in neutral loss scans for 141 atomic mass units in the positive ion mode, PI in parent ion scans for m/z 241 in the negative ion mode, and PS in neutral loss scans for 87 atomic mass units in the negative ion mode. Other settings were as previously described (Brügger et al., 1997). Signal intensities were quantified using Analyst software (Applied Biosystems/MD Sciex, Toronto, Canada), taking into account the three most abundant [M + H]+ isotopic signals. An equimolar mixture of biologically relevant PC species (Avanti Polar Lipids, Alabaster, AL) indicated that the signal intensities are representative of the relative abundance of PC species under the conditions used. Yeast samples destined for analysis of lyso-PA and PA were lyophilized and subjected to butanol extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described (Scherer et al., 2009, 2010).

**Fatty acid analysis**

Yeast total lipid extracts prepared as earlier and corresponding to 1 μmol of phospholipid phosphorus were transesterified by heating at 70°C for 2 h in 2.5% (vol/vol) H2SO4 in methanol. The fatty acid methyl esters were extracted in hexane and separated on a Trace GC ultra gas chromatograph (Thermo Electron, Interscience, Louvain-la-Neuve, Belgium) using a Restek Stabilwax column and a temperature gradient from 170 to 210°C. Fatty acid methyl esters were identified and signal intensities were calibrated using a fatty acid methylster standard (Nu-Chek-Prep, Elysian, MN).

**[14C]Acetate labeling and analysis of lipid composition by thin-layer chromatography**

Cells grown to mid-log phase at 30°C in 5 ml of SGR medium without uracil were labeled with 75 μCi [1-14C]acetate (56 mCi/mmol, GE Healthcare) for 1 h at 30°C, harvested by centrifugation, and resuspended in 1 ml 5% trichloroacetic acid. Subsequently cells were pelleted, washed twice with water, homogenized, and subjected to lipid extraction as described. Lipid extracts were subjected to liquid scintillation counting, determination of total phospholipid-phosphorus content (Rouser et al., 1970), and analysis of neutral lipid and phospholipid composition by thin-layer chromatography (TLC; de Kroon et al., 1997; Schneiter and Daum, 2006). The radioactive spots on the TLC plates were visualized and quantified using a Personal Molecular Imager FX PhosphorImager (Bio-Rad, Hercules, CA). Steady-state phospholipid and neutral lipid composition was analyzed by TLC (de Kroon et al., 1997; Schneiter and Daum, 2006) using lipid extracts corresponding to 300 nmol of phospholipid-phosphorus. Spots were visualized with I2 vapor, and phospholipid-containing spots were scraped off. The amount of inorganic phosphorus in TLC spots was measured according to Rouser et al. (1970) after destruction in 70% perchloric acid for 3 h at 180°C.

**Western blotting**

Logarithmically growing cells were harvested, washed, resuspended in 0.1 M NaOH, and incubated at room temperature for 5 min, pelleted, resuspended in SDS–PAGE sample buffer containing 4% β-mercaptoethanol, and incubated for 4 min at 95°C (Kushnirov, 2000). Samples corresponding to 10 μg of protein (as determined by the Pierce BCA protein assay kit [Thermo Fisher Scientific, Rockford, IL], using Pierce NanoBlotting buffer as detergent and bovine serum albumin as a standard) were analyzed by 10% SDS–PAGE followed by Western blotting with antibodies against HA (mouse anti-HA 12CA5; Roche Diagnostics, Indianapolis, IN), Myc (mouse anti-Myc 9E10; Roche Diagnostics, Indianapolis, IN), and actin (mouse actin 1B4; Sigma, St. Louis, MO) as described.
anti-c-Myc immunoglobulin 9E10, sc-40; Santa Cruz Biotechnology, Santa Cruz, CA), and Sec61p (a kind gift from R. Schekman, University of California, Berkeley, CA), using horseradish peroxidase–conjugated secondary antibodies, followed by detection with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

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