Yeast PAH1-encoded phosphatidate phosphatase controls the expression of CHO1-encoded phosphatidylserine synthase for membrane phospholipid synthesis

Received for publication, June 12, 2017, and in revised form, June 30, 2017 Published, Papers in Press, July 3, 2017, DOI 10.1074/jbc.M117.801720

Gil-Soo Han and George M. Carman

From the Department of Food Science and the Rutgers Center for Lipid Research, New Jersey Institute for Food, Nutrition, and Health, Rutgers University, New Brunswick, New Jersey 08901

Edited by Dennis R. Voelker

The PAH1-encoded phosphatidate phosphatase (PAP), which catalyzes the committed step for the synthesis of triacylglycerol in Saccharomyces cerevisiae, exerts a negative regulatory effect on the level of phosphatidate used for the de novo synthesis of membrane phospholipids. This raises the question whether PAP thereby affects the expression and activity of enzymes involved in phospholipid synthesis. Here, we examined the PAP-mediated regulation of CHO1-encoded phosphatidylserine synthase (PSS), which catalyzes the committed step for the synthesis of major phospholipids via the CDP–diacylglycerol pathway. The lack of PAP in the pah1Δ mutant highly elevated PSS activity, exhibiting a growth-dependent up-regulation from the exponential to the stationary phase of growth. Immunoblot analysis showed that the elevation of PSS activity results from an increase in the level of the enzyme encoded by CHO1. Truncation analysis and site-directed mutagenesis of the CHO1 promoter indicated that Cho1 expression in the pah1Δ mutant is induced through the inositol-sensitive upstream activation sequence (UASINO), a cis-acting element for the phosphatidate-controlled Henry (Ino2–Ino4/Opi1) regulatory circuit. The abrogation of Cho1 induction and PSS activity by a CHO1 UASINO mutation suppressed pah1Δ effects on lipid synthesis, nuclear/endoplasmic reticulum membrane morphology, and lipid droplet formation, but not on growth at elevated temperatures. Loss of the DGK1-encoded diacylglycerol kinase, which converts diacylglycerol to phosphatidate, partially suppressed the pah1Δ-mediated induction of Cho1 and PSS activity. Collectively, these data showed that PAP activity controls the expression of PSS for membrane phospholipid synthesis.

The Saccharomyces cerevisiae PAH1-encoded PAP1,2 (EC 3.1.3.4) and CHO1-encoded PSS3 (EC 2.7.8.8) are two of the most highly regulated enzymes in lipid metabolism. The PAP reaction is the committed step for the synthesis of the neutral lipid TAG, whereas the PSS reaction is the committed step in the CDP–DAG pathway for the de novo synthesis of the major membrane phospholipids PC and PE. The DAG derived from the PAP reaction is also used in the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway for the synthesis of PC and PE, respectively, when cells are defective in PSS or other enzymes in the CDP–DAG pathway are supplemented with choline or ethanolamine (1, 2). For catalytic function, both Cho1 and Pah1 are required to associate with the membrane where their phospholipid substrates reside. Cho1 is an integral membrane enzyme in the ER (3–6), whereas Pah1 is a peripheral membrane enzyme that translocates from the cytosol to the nuclear/ER membrane (7–9).

Analyses of the pah1Δ and cho1Δ mutants lacking Pah1 and Cho1, respectively, have shed light on the importance of PAP and PSS activities in lipid metabolism and cell physiology. The pah1Δ mutation increases the level of the PAP substrate PA but decreases the levels of the PAP product DAG and its derivative TAG (10–12). The lack of Pah1 causes a variety of phenotypes that include the derepression of phospholipid synthesis genes (e.g. INO1, OP13, and INO2), the increase of phospholipid synthesis, the expansion of the nuclear/ER membrane, the susceptibility to fatty acid-induced toxicity, and the reduction of lipid droplet formation (10–14). The impact of the Pah1 deficiency on overall cell physiology is further exemplified by the fact that pah1Δ mutant cells cannot grow on non-fermentable carbon sources (i.e. respiratory deficiency) (10, 15) as well as at elevated temperatures (10, 13, 15). The mutant cells are hypersensitive to oxidative stress, have a shortened chronological life span (16), and exhibit defects in cell wall integrity (17, 18) and vacuole morphology. This work was supported, in whole or in part, by National Institutes of Health Grant GM028140. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: Dept. of Food Science, Rutgers University, 61 Dudley Rd., New Brunswick, NJ 08901. Tel.: 848-932-0267; E-mail: gcarman@rutgers.edu.
2 In this paper, Saccharomyces cerevisiae is used interchangeably with yeast.
3 The abbreviations used are: PAP, phosphatidate phosphatase; PSS, phosphatidylserine synthase; DGK, diacylglycerol kinase; DAG, diacylglycerol; TAG, triacylglycerol; PA, phosphatidate, PS, phosphatidylserine, PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; ER, endoplasmic reticulum; SC, synthetic complete; UAS, upstream activating sequence; UASINO, inositol-sensitive upstream activation sequence.
4 The PAP encoded by PAH1 differs from the lipid phosphate phosphatase enzymes encoded by APP1 (83, 84), DPP1 (85), and LPP1 (86), which dephosphorylate a broad spectrum of substrates (e.g. PA, lyso-PA, DAG pyrophosphate, and isoprenoid pyrophosphate) and are not involved in de novo lipid synthesis.
5 The S. cerevisiae PSS differs from the enzyme from Gram-negative bacteria (e.g. E. coli), which catalyzes its CDP–DAG–dependent reaction via a metal cofactor–independent ping-pong reaction mechanism (87), or the enzyme from mammalian cells, which catalyzes an exchange reaction between PC or PE and serine (88).
ole fusion (19). Some of the \textit{pah1\Delta} phenotypes require the function of \textit{Dgk1} \((12, 14, 20)\), the CTP-dependent DGK that phosphorylates DAG to form PA (Fig. 1). The \textit{cho1\Delta} mutant lacks the ability to synthesize PS \((21, 22)\) and thus requires the supplementation of choline or ethanolamine to synthesize PC or PE by the Kennedy pathway \((1, 2)\). Studies with cells lacking Cho1 have revealed that PS is required for protein kinase C function \((23, 24)\), tryptophan transport \((25)\), vacuole function and morphogenesis \((26)\), and direction of endocytic proteins to the plasma membrane \((27)\).

\textit{Cho1} and \textit{Pah1} are regulated for their functions in lipid metabolism by genetic mechanisms. The expression of \textit{CHO1} is elevated in the exponential phase when cells are grown in the absence of the phospholipid precursor molecules inositol, choline, ethanolamine, and serine \((28–32)\) as well as in the presence of the essential nutrient zinc \((33)\). In contrast, the \textit{CHO1} expression is reduced by inositol supplementation in the exponential phase, and this regulation is enhanced by the addition of choline, ethanolamine, or serine in the growth medium \((28–32)\). The gene expression is also reduced by zinc depletion from the growth medium \((33)\) or when cells progress from the exponential to the stationary phase of growth \((34, 35)\). These regulations of the \textit{CHO1} expression are mediated by the Henry regulatory circuit, which involves the \textit{Ino2–Ino4} complex that drives transcriptional activation through its binding to the UAS\textit{INO} element and the PA-regulated repressor \textit{Opi1}, which inhibits the function of the activator complex through its interaction with \textit{Ino2} \((1, 2, 36)\). The \textit{CHO1} transcriptional regulation plays a role in the partitioning of CDP–DAG for the synthesis of PI and PS and the PS-derived synthesis of PE and PC in the CDP–DAG pathway \((1, 2, 36)\).

The expression of \textit{PAH1} is also regulated by some of the same growth conditions that regulate the \textit{CHO1} expression, but with an opposite effect \((37–39)\). The transcriptional regulation of \textit{PAH1} involves the transcription factors \textit{Ino2}, \textit{Ino4}, \textit{Op1}, \textit{Gis1}, and \textit{Rph1} (for inositol and growth phase regulation) as well as the transcription factor \textit{Zap1} (for zinc-mediated regulation) \((37–39)\). The induction of the \textit{PAH1} transcription in the stationary phase or in response to zinc depletion correlates with the elevation of PAP activity \((37, 38)\). On the one hand, the induced expression of PAP activity in zinc-replete stationary phase cells is responsible for increased synthesis and accumulation of TAG that occurs at the expense of phospholipid synthesis \((37)\). On the other hand, the induced expression of PAP activity in zinc-depleted exponential phase cells is responsible for increased synthesis of PC via the CDP–choline branch of the Kennedy pathway \((38)\).

The loss of \textit{Pah1} and its PAP activity (\textit{e.g. \textit{pah1\Delta}} mutation) increases the level of PA and its utilization for the synthesis of membrane phospholipids \((10, 37, 40)\), raising a question whether the expression of \textit{Cho1} and its PSS activity is regulated through cell growth. Here, we showed that the \textit{pah1\Delta} mutation results in the induction of \textit{Cho1} and PSS activity in a growth-dependent manner from the exponential to the stationary phase and that the elevation of the \textit{CHO1} expression is controlled through the UAS\textit{INO} element in the promoter. The mutation in the core consensus of UAS\textit{INO} abolished the induction of \textit{Cho1} and PSS activity, resulting in the suppression of the \textit{pah1\Delta} phenotypes in lipid synthesis, nuclear/ER membrane morphology, and lipid droplet formation. Collectively, this work advances the understanding of how PAP activity regulates phospholipid synthesis through the transcriptional regulation of the PSS enzyme.

\textbf{Results}

\textit{The \textit{pah1\Delta} mutation induces the expression of \textit{Cho1} and PSS activity}

We examined the effect of growth phase on the expression of \textit{CHO1} and its encoded PSS activity in wild type and \textit{pah1\Delta} mutant cells (Fig. 2). In this work, the level of \textit{Cho1} was examined by immunoblotting to analyze the \textit{CHO1} expression.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Lipid synthesis in yeast. The pathways shown for the synthesis of lipids include the relevant steps discussed in this work. A more comprehensive figure for the synthesis of TAG and membrane phospholipids via the CDP–DAG and Kennedy pathways may be found in Ref. 2. The CDP–DAG pathway of phospholipid synthesis is highlighted in blue, whereas the Kennedy pathway is shown in gray to indicate its minor role in phospholipid synthesis in cells grown without choline (Cho) or ethanolamine (Etn). The reactions catalyzed by the \textit{CHO1}-encoded PSS, \textit{PAH1}-encoded PAP, and \textit{DGK1}-encoded DGK are indicated.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{The \textit{pah1\Delta} mutation induces the expression of \textit{Cho1} and PSS activity. Wild type (\textit{W303-1A}) and the \textit{pah1\Delta} mutant were grown at 30°C in SC medium to the exponential (E) and stationary (S) phases. Cell extracts were prepared and assayed for the expression of \textit{Cho1} by immunoblot analysis with anti-\textit{Cho1} antibody (A) or for PSS activity (B). The immunoblot in A is representative of two independent experiments, whereas the data in B are means \(\pm S.D.\) (error bars) from triplicate determinations of two independent experiments. The positions of the 30-kDa (i.e. phosphorylated) and 27-kDa (i.e. unphosphorylated) forms of \textit{Cho1} are indicated in A.}
\end{figure}
PAP controls expression of PSS for phospholipid synthesis

Figure 3. Induced expression of Cho1 in the pah1Δ mutant is mediated by the UASINO element in the CHO1 promoter. A and B, the pah1Δ mutant was transformed with pRS415, pGH440 (pRS415 + CHO1 with 1,000-bp promoter), or its derivative with the indicated promoter truncation. C, the pah1Δ mutant was transformed with pRS415, pGH440 (CHO1), or its derivative with the UASINO mutation (cho1). The transformants were grown at 30 °C in 5C-Leu medium to the exponential (E) and stationary (S) phases. Cell extracts were prepared and subjected to immunoblot analysis with anti-Cho1 antibody. The immunoblots in the figure are representative of two independent experiments.

because of the caveat that mRNA abundance does not necessarily correlate with protein abundance. The anti-Cho1 antibody recognizes two forms of Cho1 that differ in their electrophoretic mobility (41). The 30-kDa form represents Cho1 phosphorylated by protein kinase A at Ser-46 and Ser-47, whereas the 27-kDa form represents the unphosphorylated form of the enzyme (41). In wild-type cells, the levels of Cho1 (Fig. 2A) and its PSS activity (Fig. 2B) were reduced in the stationary phase when compared with the exponential phase. As described previously (34), PSS activity was 3.6-fold lower in the stationary phase than in the exponential phase. Compared with wild type, the pah1Δ mutant had 2.2-fold higher PSS activity in the exponential phase. In addition, the pah1Δ mutant showed a 2.4-fold induction of the enzyme activity from the exponential to the stationary phase. Consequently, in the stationary phase, the PSS activity of the pah1Δ mutant was 19-fold higher than the enzyme activity of wild type. The elevation of PSS activity in the pah1Δ mutant correlated with the increase of the Cho1 level. These results indicate that the induced expression of Cho1 and PSS activity in the pah1Δ mutant is up-regulated during growth from the exponential to the stationary phase.

Induced expression of Cho1 and PSS activity in the pah1Δ mutant is mediated by the CHO1 UASINO element

The up-regulation of the Cho1 level in the pah1Δ mutant suggested that the enzyme is controlled at the level of its gene expression. The CHO1 promoter contains a UASINO element as well as other putative regulatory elements. To identify the relevant element by an unbiased approach, we constructed CHO1 alleles that contain different lengths of the promoter by 5’-nested deletion and analyzed their expression on a low-copy plasmid in the pah1Δ mutant. We reasoned that if the plasmid-borne CHO1 allele contains a UAS, its expression would be up-regulated like the endogenous expression of Cho1 in the pah1Δ mutant. We did not analyze the plasmid-borne CHO1 alleles in the cho1Δ pah1Δ mutant background. Supplementation of choline or ethanolamine, which is required for viability of cells containing the cho1Δ mutation, alters the regulation of phospholipid synthesis (21, 22). Accordingly, pah1Δ cells transformed with the CHO1 allele were grown to the exponential and stationary phases and were examined for the Cho1 level by immunoblot analysis (Fig. 3). Compared with the pah1Δ mutant that expresses the endogenous CHO1 (i.e. vector control), its transformants containing the CHO1 allele with the 200-bp or longer promoter exhibited higher levels of Cho1 in both the exponential and the stationary phases of growth (Fig. 3A). However, the increase of the Cho1 level was not shown by the expression of the CHO1 allele with the 100-bp or shorter promoter, indicating that the UAS element is located between −200 and −100 of the CHO1 promoter. Further truncation analysis of the 100-bp region showed that the Cho1 level of the pah1Δ mutant was increased by the CHO1 allele with the 170-bp or longer promoter, but not with the 150-bp promoter (Fig. 3B), indicating that the UAS element affecting expression is located between −170 and −150. Analysis of the 20-bp region of the CHO1 promoter revealed that it contains the UASINO element (−163 to −154).

To determine whether the UASINO element is required for the induction of CHO1 expression in the pah1Δ mutant, we examined the effect of its mutation (CATGTGAAAG → TTTTTAAAG, mutations underlined) (42) in the plasmid-borne CHO1. Compared with the wild type allele, the mutant allele of CHO1 lacking the consensus sequence of UASINO did not significantly increase the Cho1 level in the pah1Δ mutant (Fig. 3C). To further confirm this result, the UASINO mutation was introduced to the chromosomal CHO1 of wild type and the pah1Δ mutant. Immunoblot analysis (Fig. 4A) showed that the cho1 mutant had a significant reduction of the Cho1 level in the exponential phase. Compared with the pah1Δ mutant, the cho1 pah1Δ mutant showed a great reduction of the Cho1 level in the exponential and stationary phases (Fig. 4A). In fact, its Cho1 level was almost identical to that of the cho1 mutant. Analysis of the CHO1 allele with 1,000-bp promoter, or its derivative with the UASINO mutation (cho1). The transformants were grown at 30 °C in 5C-Leu medium to the exponential (E) and stationary (S) phases. Cell extracts were prepared and subjected to immunoblot analysis with anti-Cho1 antibody. The immunoblots in the figure are representative of two independent experiments.
lated) and 27-kDa (i.e. unphosphorylated) forms of Cho1 are indicated in A.

Effects of the CHO1 UASINO mutation on lipid composition of the pah1Δ mutant

We explored the effects of the CHO1 UASINO mutation on the changes of lipid composition imparted by the pah1Δ mutation. Yeast cells were grown in SC medium containing [2-14C]acetate for steady-state labeling of lipids (43, 44). The radiolabeled lipids were extracted from exponential and stationary phase cells and analyzed by TLC for total lipids and their derivatives containing the chromosomal CHO1 UASINO, mutation (cho1) were grown at 30 °C in SC medium to the exponential (A) and stationary (B) phases. The data in B are means ± S.D. (error bars) from triplicate determinations of two independent experiments. The positions of the 30-kDa (i.e. phosphorylated) and 27-kDa (i.e. unphosphorylated) forms of Cho1 are indicated in A.

Effects of the CHO1 UASINO mutation on nuclear/ER membrane morphology, lipid droplet formation, and temperature sensitivity of the pah1Δ mutant

The pah1Δ mutant has an irregularly shaped nucleus with the expansion of the membrane that is attributed to the increase of phospholipid synthesis (11, 13). Because the CHO1 UASINO mutation exerts a suppressive effect on the pah1Δ-mediated increase of phospholipid synthesis, we examined the nuclear/ER membrane morphology of the cho1 pah1Δ mutant (Fig. 7A). The expression of the Sec63-GFP fusion protein was used to mark the nuclear/ER membrane in the cell (13). Compared with wild type, the cho1 mutant showed little difference in the nuclear/ER membrane morphology and contained a round nucleus. As described previously (13), most of the pah1Δ cells showed the irregular, expanded nuclear/ER morphology. In contrast, the cho1 pah1Δ cells (93%; Fig. 7B) had the rounded-shaped nucleus in the exponential phase, indicating that the CHO1 UASINO mutation suppressed the nuclear/ER membrane phenotype of the pah1Δ mutant. The suppressive effect was less strong in the stationary phase, and the number of cho1 pah1Δ cells containing a round nucleus was reduced by 53%.

**Figure 4.** The inductions of Cho1 and PSS activity in the pah1Δ mutant are abolished by the CHO1 UASINO mutation. Wild type, the pah1Δ mutant, and their derivatives containing the chromosomal CHO1 UASINO, mutation (cho1) were grown at 30 °C in SC medium to the exponential (A) and stationary (B) phases. Cell extracts were prepared and assayed for the expression of Cho1 by immunoblot analysis with anti-Cho1 antibody (A) or for PSS activity (B). The immunoblot in A is representative of two independent experiments, whereas the data in B are means ± S.D. (error bars) from triplicate determinations of two independent experiments. The positions of the 30-kDa (i.e. phosphorylated) and 27-kDa (i.e. unphosphorylated) forms of Cho1 are indicated in A.

**Figure 5.** Effect of the CHO1 UASINO mutation on lipid composition in the pah1Δ mutant. Wild type, the pah1Δ mutant, and their derivatives containing the chromosomal CHO1 UASINO, mutation (cho1) were grown at 30 °C in SC medium to the exponential (A) and stationary (B) phases of growth in the presence of [2-14C]acetate (1 μCi/ml). Lipids were extracted and separated by the one-dimensional thin-layer chromatography system for neutral lipids, and the images were subjected to ImageQuant analysis. The data are reported as counts/min found in a particular lipid per 10^6 cells. The amount of label found in the lipids from the stationary phase cultures was reduced relative to that found from the exponential phase because of the turnover of lipids during growth and incorporation of the label into other metabolites. The data are means ± S.D. (error bars) from triplicate determinations. Erg, ergosterol; ErgE, ergosterol ester; FA, fatty acid; PL, phospholipids.
**PAP controls expression of PSS for phospholipid synthesis**

We also examined the mutants for the formation of lipid droplets by staining with the fluorescent dye BODIPY 493/503 (Fig. 8A). The wild-type and cho1 cells had a similar number of lipid droplets and showed a ~1.5-fold increase in the organelle number from the exponential to the stationary phase (Fig. 8B). Compared with wild type, the pah1Δ mutant showed a significant reduction (2- and 4.5-fold, respectively) of lipid droplet formation in the exponential and stationary phases. In addition, its lipid droplet formation was not increased in the stationary phase. This phenotype of the pah1Δ mutant was suppressed by the CHO1 UASINO mutation, and the cho1 pah1Δ mutant had a number of lipid droplets similar to that of the wild type in the exponential phase. In the stationary phase, the cho1 pah1Δ mutant showed a reduction in lipid droplet formation, but its lipid droplet number was still higher than that of wild type. This result indicates that, in the stationary phase of growth, the CHO1 UASINO mutation has a partial suppressive effect on the defect of the pah1Δ mutant in lipid droplet formation.

The lack of growth at the elevated temperature (e.g. 37 °C) is characteristic of the pah1Δ mutant (10, 13, 15). Unlike the phenotypes described above, temperature sensitivity of the pah1Δ mutant in the exponential and stationary phases was not suppressed by the CHO1 UASINO mutation (Fig. 9).

**Figure 6. Effect of the CHO1 UASINO mutation on phospholipid composition in the pah1Δ mutant.** Wild type, the pah1Δ mutant, and their derivatives containing the chromosomal CHO1 UASINO mutation (cho1) were grown at 30 °C in SC medium to the exponential (A) and stationary (B) phases of growth in the presence of [2-14C]acetate (1 μCi/ml). Lipids were extracted and separated by the one-dimensional thin-layer chromatography system for phospholipids, and the images were subjected to ImageQuant analysis. The amount of label found in the phospholipids from the stationary phase cul-

**Figure 7. Effect of the CHO1 UASINO mutation on nuclear/ER membrane morphology in the pah1Δ mutant.** Wild type, the pah1Δ mutant, and their derivatives containing the chromosomal CHO1 UASINO mutation (cho1) were transformed with YCplac111-SEC63-GFP and grown at 30 °C in SC-Leu medium to the exponential and stationary phases. A, the fluorescence signal of the GFP-tagged ER marker Sec63 was visualized by fluorescence microscopy. The images shown are representative of multiple fields of view. White bar, 2 μm. B, the percentage of cells with round nuclear/ER membrane morphology was determined from >3 fields of view (≥150 cells). The data are averages ± S.D. (error bars).

**Effect of the dgk1Δ mutation on the induced expression of Cho1 and PSS activity of the pah1Δ mutant**

The lack of Dgk1 (i.e. the dgk1Δ mutation) suppresses the pah1Δ phenotypes (e.g. increased phospholipid synthesis, nuclear/ER membrane expansion, and induced INO1 expres-

Because both PAP and DGK control the PA level (10, 20), we examined the effect of the dgk1Δ mutation on the PA content in the stationary phase (Fig. 10C). We also examined the effect of the dgk1Δ mutation on temperature sensitivity exhibited by the pah1Δ
PAP controls expression of PSS for phospholipid synthesis

Figure 8. Effect of the CHO1 UASINO mutation on lipid droplet formation in the pah1Δ mutant. Wild type, the pah1Δ mutant, and their derivatives containing the chromosomal CHO1 UASINO mutation (cho1) were grown at 30°C in SC medium to saturation. Serial dilutions (1:10) of the cells were spotted (5 and 37°C. The data are representative of three independent experiments. The conversion of PA to DAG and CDP–DAG appears to be a precursor of CDP–DAG used as the substrate of PSS (Fig. 1).

Discussion

PAP and PSS catalyze reactions in the overlapping pathways of lipid biosynthesis (Fig. 1). The enzyme reactions are linked to the lipid intermediate PA, which is the substrate of PAP and the precursor of CDP–DAG used as the substrate of PSS (Fig. 1). The conversion of PA to DAG and CDP–DAG appears to be a major regulatory step that bifurcates the lipid biosynthetic pathway into two branches (i.e. TAG synthesis and phospholipid synthesis) (10, 12, 37). The changes in PAP activity are directly correlated with TAG synthesis but are inversely correlated with phospholipid synthesis during cell growth (37). Accordingly, yeast cells have a lower PAP activity in the exponential phase when the rate of phospholipid synthesis is high, whereas they have a higher PAP activity in the stationary phase when the rate of phospholipid synthesis is low (37). The elevation of phospholipid synthesis in the pah1Δ mutant, which causes the expansion of the nuclear/ER membrane, is related to the increased availability of the precursor PA by the defect of its conversion to DAG as well as by the increased expression of phospholipid synthesis genes (10, 12, 13). We showed in this study that the expression of CHO1-encoded PSS, which catalyzes the committed step in the CDP–DAG pathway of de novo phospholipid synthesis, is highly induced in the pah1Δ mutant and that the enzyme induction is increased in the stationary phase when the CHO1 expression in wild type is reduced. That the induction of CHO1 expression in the pah1Δ mutant was mediated through the UASINO element supports the notion that the elevation of the PA level reduces the Opi1-mediated transcriptional repression of the UASINO-containing genes as per the Henry regulatory circuit (Fig. 11). PA is known to sequester Opi1 at the nuclear/ER membrane (2, 45), and the phospholipid synthesis genes (e.g. INO1, INO2, and OPI3) previously known to be induced by the pah1Δ mutation (13) contain the UASINO element (2, 46, 47).

The CHO1 UASINO mutation in the pah1Δ mutant abolished the up-regulation of the Cho1 level and PSS activity, suppressing the increase of phospholipid synthesis and nuclear/ER membrane as well as the decrease of TAG synthesis and lipid droplet formation. These observations highlight the role of the CHO1-encoded PSS in the synthesis of membrane phospholipids and the role of the PAH1-encoded PAP in the negative regulation of the CHO1 expression. The effects of the CHO1 UASINO mutation on the pah1Δ phenotypes were pronounced in the exponential phase of growth when PSS activity is expressed at its highest level (34). The partial suppression of the pah1Δ phenotypes in the stationary phase suggests that the higher availability of PA may compensate for the reduction of the PSS level.

Although the PA level of the pah1Δ mutant was reduced by the CHO1 UASINO mutation in the stationary phase, it was still higher than that of wild type cells (Fig. 6B). This difference in the PA content would be expected to attenuate the Opi1 function. As indicated above, it has been known that transcription of other UASINO-containing phospholipid synthesis genes (e.g. INO1 and OPI3) is induced in the pah1Δ mutant (13, 48). Although it is unknown whether transcriptional induction of these genes leads to the increased expression and function of their encoded proteins, the lack of total suppression of PA content, especially in the stationary phase, is consistent with an increase in phospholipid methyltransferase activity encoded by OPI3. Phospholipid synthesis genes (e.g. CKII (49), CPT1 (50), EKI1 (51), and EPT1 (52)) in the Kennedy pathway are also subject to transcriptional regulation through the UASINO element, but their encoded activities are not expected to play a major role in the transcriptional induction of CHO1.
major role in this regulation because growth medium was not supplemented with choline and/or ethanolamine (1, 2).

The increase of the PI content in the pah1Δ mutant was not suppressed by the CHO1 UAS\textsubscript{NO} mutation in the exponential or stationary phase of growth. Whereas the expression of PIS1-encoded PI synthase is not regulated through Opi1 (28, 53), the INO1 gene encoding the inositol-3-phosphate synthase, which is crucial for the synthesis of inositol and thus for PI synthesis (54), is regulated by Opi1 (28, 55). Thus, the induced expression of INO1 in response to the pah1Δ mutation (13) is consistent with the increased availability of inositol for PI synthesis. However, if the induced expression of INO1 is translated to an increase in the level and activity of inositol-3-phosphate synthase, it would not be massive because pah1Δ mutant cells do not excrete inositol (10).

The pah1Δ mutation also causes a dramatic increase in the amounts of fatty acids and ergosterol ester, primarily in the stationary phase of growth (10) (Fig. 5). Although the mecha-
nism for these changes is unclear, the elevated levels of the lipids were almost reduced to the levels of wild type by alleviating the induced expression of PSS activity. We hypothesize that the PA content, as regulated by the pah1Δ and cho1 pah1Δ mutations, is the basis for the changes in the levels of fatty acids and ergosterol ester. This assertion is supported by the fact that fatty acid synthesis, as mediated by the ACC1, FAS1, and FAS2 genes, is under the control of the Henry regulatory circuit (56, 57) and that the ARE1 and ARE2 genes, which encode the acyl-CoA sterol acyltransferase enzymes, contain a putative UASINO element in their promoters. Accordingly, additional studies are warranted to address the expression regulation of those genes.

The lack of the DGK1-encoded DGK suppresses the pah1Δ phenotypes that are ascribed to the increase of the PA level (20, 58), which include the induced expression of UASINO-containing genes and increased phospholipid content, nuclear/ER membrane expansion, decreased lipid droplet formation, and reduced chronological life span (10, 12–14, 16, 20, 59). As discussed above, we consider that the increase of the PA level is responsible for induced expression of Cho1 and PSS activity in the pah1Δ mutant. In further support of this, we showed that the dgk1Δ mutation suppressed the pah1Δ mutant for its induced Cho1 expression and PSS activity, but only in the stationary phase of growth with a partial effect. Thus, the PA-mediated regulation of the CHO1 expression is more complex than was expected.

Of the pah1Δ phenotypes examined in this study, temperature sensitivity was not suppressed by the CHO1 UASINO mutation or by the dgk1Δ mutation. These observations support the notion that the inability of the pah1Δ mutant to grow at the elevated temperature is not related to a change in the PA level but rather to other changes (e.g. decrease in DAG production) caused by the loss of PAP activity. Additional pah1Δ phenotypes that are not affected by the dgk1Δ mutation include the decrease of TAG synthesis, sensitivity to fatty acid toxicity (12), and vacuole fragmentation (60).

In addition to the genetic regulation discussed above, the PAP and PSS activities of Pah1 and Cho1, respectively, are biochemically regulated by the substrates and products of their reactions. The PSS substrate CDP-DAG stimulates PAP activity by a mechanism that increases its affinity for PA (61), whereas the PAP substrate PA stimulates PSS activity by a mechanism that increases its affinity for CDP-DAG (62). Moreover, the PAP product DAG inhibits PSS activity by a non-competitive mechanism (62). The two enzymes are also regulated by phosphorylation. Protein kinase A phosphorylates Pah1 (63) and Cho1 (41, 64) and inhibits their PAP and PSS, activities, respectively. For Cho1, the posttranslational modification stabilizes its abundance in the cell for the net effect of stimulating the synthesis of PS (41). The phosphorylation-mediated regulations of the two enzymes are more complex and are discussed elsewhere (1). In addition, the regulations of Pah1 and Cho1 are interrelated with the metabolism of nucleotides and sphingolipids (1, 2, 65).

In summary, the work reported here advances our understanding of how the PAP enzyme reaction regulates phospholipid synthesis through the transcriptional regulation of the PSS enzyme. Clearly, the long-term genetic regulations, coupled with the short-term biochemical regulations of the PAP and PSS enzymes are critical to controlling the balance between the synthesis of TAG for stasis and the synthesis of membrane phospholipids for cell growth.

**Experimental procedures**

**Materials**

All chemicals were reagent grade or better. Culture medium components were from BD Difco. DNA purification kits were from Qiagen. Restriction endonucleases, modifying enzymes, and DNA polymerases were from New England Biolabs. DNA size standards (1 Kb Plus DNA ladder) were from Invitrogen. Oligonucleotides, nucleotides, protease inhibitors, Triton X-100, Ponceau S stain, Aerococcus viridans glycerol-3-phosphate oxidase, horseradish peroxidase, and bovine serum albumin were from Sigma-Aldrich. Carrier DNA for yeast transformation was from Clontech. Bradford protein assay reagent, protein size standards, and electrophoretic reagents were from Bio-Rad. PVDF membrane and the enhanced chemiluminescence substrate for Western blotting were from GE Healthcare. Alkaline phosphatase–conjugated goat anti-rabbit IgG antibodies (product 31340, lot NJ178812), alkaline phosphatase–conjugated goat anti-mouse IgG antibodies (product 31322, lot PB1815636), Triton X-100 (Surfact-Amps, product 28314), Ampex Red, and BODIPY 493/503 were from Thermo Fisher Scientific. Pseudomonas sp. lipoprotein lipase was from Wako. Radiochemicals were from PerkinElmer Life Sciences. Phospholipids were from Avanti Polar Lipids. Liquid scintillation mixtures were from National Diagnostics. Silica gel 60 TLC plates were from EMD Millipore, and LK5D partisil silica gel TLC plates were from Whatman.

**Strains and growth conditions**

The strains used in this work are listed in Table 1. Yeast cells were grown at 30 °C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SC medium containing 2% glucose (66). For selection of yeast cells containing plasmids, appropriate amino acids were omitted from SC medium. Escherichia coli DH5α was used for plasmid maintenance and amplification and was grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4). E. coli transformants containing plasmids were selected by ampicillin (100 µg/ml) resistance. Solid media for the growth of E. coli and yeast contained agar at a concentration of 1.5 and 2%, respectively. Cell numbers in liquid cultures were estimated spectrophotometrically by measuring absorbance at 600 nm.

**DNA manipulations**

Standard methods were used for the isolation of plasmid and yeast genomic DNA and for the manipulation of DNA with restriction enzymes, DNA ligase, and modifying enzymes (66, 67). PCRs were optimized as described by Innis and Gelfand (68). Yeast and E. coli transformations were performed by standard protocols. DNA sequencing reactions were performed according to the dideoxy method with Taq DNA polymerase and analyzed by automated DNA sequencer (service provided by GENEWIZ, South Plainfield, NJ).
Plasmid constructions

The plasmids used in this work are listed in Table 2. pGH340 was constructed from pRS416 at the XbaI and HindIII sites by insertion of PAH1 DNA fragments that were released from pGH315 by digestion with XbaI/BglII (2.002 kb) and BglII/HindIII. pGH440 was constructed from pRS415 at the SacI and HindIII sites by insertion of 2.3-kb pGH340 by replacing the SacI-BsaBI fragment of CTC. The promoter truncation derivatives of pGH440 were produced from genomic DNA of yeast strain W303-1A by PCR from genomic DNA of yeast strain W303-1A (forward primer, ATCAAGCTTATTGATGCCATGAAAAC- (869 bp) of CHOI with that containing the UASINO mutation (CACATG → AAAAAA, −159 to −154) in the CHOI promoter. pGH440 was produced by replacing the BstZ17I-NdeI fragment of CHOI with the URA3 gene.

Construction of yeast mutants

The cho1Δ::URA3 mutant was derived from the yeast strain W303-1A by one-step gene replacement (69). The W303-1A strain was transformed with a 2.9-kb cho1Δ::URA3 disruption cassette that was released from pGH442 by digestion with SacI and HindIII, and the resulting transformants were selected on the SC-Ura medium supplemented with 1 mM choline. The cho1Δ mutant, which contains a defective CHOI UASINO element, was derived from the cho1Δ::URA3 mutant by one-step gene replacement. The cho1Δ mutant was transformed with a 2.3-kb CHOI DNA that was released from pGH440m by digestion with SacI and HindIII, and the resulting transformants were selected on 5-fluoroorotic acid–containing medium. The gene replacement in the 5-fluoroorotic acid–resistant transformant was confirmed by DNA sequencing of the PCR-amplified CHOI. The pGH440m cho1Δ mutant was derived from the cho1Δ mutant by one-step gene replacement. The

Table 1

| Strains used in this study | Relevant characteristics | Source/Reference |
|---------------------------|--------------------------|-----------------|
| E. coli DH5α             | F − 80dlacZΔM15Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK− mB−) proA supE444λ thi-1 gyrA96 relA1 | Ref. 67 |
| S. cerevisiae W303-1A    | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Ref. 89 |
| GY75                     | pah1Δ::URA3 derivative of W303-1A | Ref. 10 |
| GY76                     | cho1 (CACATG → AAAAAA, −159 to −154) derivative of W303-1A | This study |
| GY78                     | cho1 (CACATG → AAAAAA, −159 to −154) pah1Δ::URA3 derivative of W303-1A | This study |
| GY70                     | cho1Δ::URA3 derivative of W303-1A | This study |
| RS453                    | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-3 | Ref. 90 |
| SS1026                   | pah1Δ::TRP1 derivative of RS453 | Ref. 13 |
| SS1144                   | dgk1Δ::HIS3 derivative of RS453 | Ref. 20 |
| SS1147                   | dgk1Δ::HIS3 pah1Δ::TRP1 derivative of RS453 | Ref. 20 |

Table 2

| Plasmids used in this study | Relevant characteristics | Source/Reference |
|----------------------------|--------------------------|-----------------|
| pRS415                     | Single-copy E. coli/yeast shuttle vector with LEI2 | Ref. 91 |
| pRS416                     | Single-copy E. coli/yeast shuttle vector with URA3 | Ref. 91 |
| YEp351                     | Multicopy E. coli/yeast shuttle vector with LEI2 | Ref. 92 |
| pGH340                     | pRS415 containing PAH1 at the XbaI/HindIII sites | Ref. 93 |
| pGH342                     | Yeplac111-SEC63-GFP | Ref. 10 |
| pGH440                     | pRS415 containing CHOI with the 1,000-bp promoter at the SacI/HindIII sites | This study |
| pGH440-900                 | pGH440 derivative containing the 900-bp CHOI promoter | This study |
| pGH440-900                 | pGH440 derivative containing the 800-bp CHOI promoter | This study |
| pGH440-700                 | pGH440 derivative containing the 700-bp CHOI promoter | This study |
| pGH440-600                 | pGH440 derivative containing the 600-bp CHOI promoter | This study |
| pGH440-500                 | pGH440 derivative containing the 500-bp CHOI promoter | This study |
| pGH440-400                 | pGH440 derivative containing the 400-bp CHOI promoter | This study |
| pGH440-300                 | pGH440 derivative containing the 300-bp CHOI promoter | This study |
| pGH440-200                 | pGH440 derivative containing the 200-bp CHOI promoter | This study |
| pGH440-100                 | pGH440 derivative containing the 100-bp CHOI promoter | This study |
| pGH440-0                   | pGH440 derivative containing the 0-bp CHOI promoter | This study |
| pGH440-190                 | pGH440 derivative containing the 190-bp CHOI promoter | This study |
| pGH440-170                 | pGH440 derivative containing the 170-bp CHOI promoter | This study |
| pGH440-150                 | pGH440 derivative containing the 150-bp CHOI promoter | This study |
| pGH440m                   | pGH440 with the UASINO mutation (CACATG → AAAAAA, −159 to −154) in the CHOI promoter | This study |
| pGH442                     | pGH440 derivative with cho1Δ::URA3 | Ref. 13 |
| YEpplac111-SEC63-GFP      | SEC63-GFP fusion inserted into the CEN/LEI2 vector | Ref. 13 |
cho1 mutant was transformed with a 3-kb pah1Δ::URA3 disruption cassette that was released from pG317 by digestion with the XbaI and HindIII. The pah1Δ mutation was confirmed from the Ura+ transformants by temperature sensitivity and PCR analysis of gene disruption.

Preparation of cell extracts

All steps were performed at 4 °C. Yeast cell pellets were resuspended in breaking buffer (50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml peptatin). Glass beads (0.5-mm diameter) were added to the cell suspension, and the mixture was vigorously agitated using the Mini-BeadBeater-16 (BioSpec Products). Unbroken cells and glass beads were precipitated by centrifugation at 1,500 × g for 10 min, and the supernatant was transferred to a new tube for use as cell extracts. Protein concentration of cell extracts was estimated by the method of Bradford (70) using bovine serum albumin as the standard.

SDS-PAGE and immunoblot analysis

SDS-PAGE (71) and immunoblotting (72–74) using PVDF membrane were performed as described previously. The samples for immunoblotting were normalized to total protein loading. Ponceau S staining was used to monitor the protein transfer from the polyacrylamide gels to the PVDF membrane. Immunoblot analysis of the protein Cho1 was performed with anti-Cho1 antibody raised against the N-terminal portion of the protein (41) at a concentration of 0.25 μg/ml. Goat anti-rabbit IgG antibody conjugated with alkaline phosphatase was used as a secondary antibody at a dilution of 1:5,000. Immune complexes were detected on immunoblots using the enhanced chemiluminescence Western blotting reagents as described by the manufacturer. Images were acquired by fluorimaging analysis. Immunoblotting signals were in the linear range of detectability.

Enzyme assays

All assays were conducted at 30 °C in a total volume of 0.1 ml. PSS activity was measured by following the incorporation of water-soluble [3-3H]serine (10,000 cpm/nmol) into chloroform-soluble [3-3H]PS (75–77). The enzyme reaction contained 50 mM Tris-HCl (pH 8.0), 0.6 mM MnCl2, 4 mM Triton X-100, 0.2 mM CDP–DAG, and 0.5 mM serine (75–77). The enzyme assays were conducted in triplicate, and the average S.D. of the assays was ±5%. The reactions were linear with time and protein concentration. A unit of PSS activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min.

Radiolabeling and analysis of lipids

The steady-state labeling of lipids with [2-14C]acetate was performed as described previously (43). Lipids were extracted (78) from the radiolabeled cells and then separated by one-dimensional TLC for neutral lipids (79) or phospholipids (80). The resolved lipids were visualized by phosphorimaging and quantified by ImageQuant software using a standard curve of [2-14C]acetate. The identity of radiolabeled lipids was confirmed by comparison with the migration of authentic standards visualized by staining with iodine vapor.

Analysis of PA

PA was analyzed by the fluorometric coupled enzyme assay of Morita et al. (81) with minor modifications (82). The cellular lipids were extracted (78), solubilized with purified Triton X-100 (Surfact-Amps), and digested with lipoprotein lipase. The PA derived from glycerol 3-phosphate was coupled to the formation of resorufin with glycerol-3-phosphate oxidase to produce hydrogen peroxide, which is reduced by peroxidase using Amplex Red.

Microscopy

For nuclear/ER membrane morphology analysis, cells were grown at 30 °C and collected at the exponential and stationary phases of growth. The cells were resuspended in a reduced volume of the same medium. For the analysis of lipid droplets, cells were grown in the same medium and collected at the exponential and stationary phases, stained for 30 min with 2 μM BODIPY 493/503, and washed with phosphate-buffered saline (pH 7.4). The average number of cells with normal nuclear/ER membrane structure (i.e. round- to oval-shaped circle) or the number of lipid droplets per cell was scored from ≥3 fields of view (≥150 cells). The fluorescence images were observed under a microscope (Nikon Eclipse Ni-U) with a long pass green fluorescent protein filter, captured by the DS-Qi2 camera with the imaging software NIS-Elements BR.

Analyses of data

SigmaPlot software was used for the statistical analysis of data. The p values < 0.05 were taken as a significant difference.

Author contributions—G.-S. H. and G. M. C. designed the study, analyzed the results, and prepared the manuscript. G.-S. H. performed the experiments.

References

1. Carman, G. M., and Han, G.-S. (2011) Regulation of phospholipid synthesis in the yeast Saccharomyces cerevisiae. Annu. Rev. Biochem. 80, 859–883
2. Henry, S. A., Kohlwein, S. D., and Carman, G. M. (2012) Metabolism and regulation of glycerolipids in the yeast Saccharomyces cerevisiae. Genetics 190, 317–349
3. Habeler, G., Natter, K., Thallinger, G. G., Crawford, M. E., Kohlwein, S. D., and Trajanoski, Z. (2002) YPL.db: the Yeast Protein Localization database. Nucleic Acids Res. 30, 80–83
4. Kumar, A., Agarwal, S., Heyman, J. A., Matson, S., Heidtman, M., Piccirillo, S., Umansky, L., Drawid, A., Jansen, R., Liu, Y., Cheung, K. H., Miller, P., Gerstein, M., Roeder, G. S., and Snyder, M. (2002) Subcellular localization of the yeast proteome. Genes Dev. 16, 707–719
5. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O’Shea, E. K. (2003) Global analysis of protein localization in budding yeast. Nature 425, 686–691
6. Natter, K., Leitner, P., Faschinger, A., Wolinski, H., McCraith, S., Fields, S., and Kohlwein, S. D. (2005) The spatial organization of lipid synthesis in the yeast Saccharomyces cerevisiae derived from large scale green fluorescent protein tagging and high resolution microscopy. Mol. Cell. Proteomics 4, 662–672
7. Karanasios, E., Han, G.-S., Xu, Z., Carman, G. M., and Siniossoglou, S. (2010) A phosphorylation-regulated amphipathic helix controls the mem-

PAP controls expression of PSS for phospholipid synthesis

J. Biol. Chem. (2017) 292(32) 13230–13242 13239
brane translocation and function of the yeast phosphatidate phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 17539–17544

8. Karanasios, E., Barbosa, A. D., Sembongi, H., Mari, M., Han, G.-S., Reggiori, F., Carman, G. M., and Siniossoglou, S. (2013) Regulation of lipid droplet and membrane biogenesis by the acidic tail of the phosphatidate phosphatase Pah1p. *Mol. Biol. Cell.** 24**, 2124–2133

9. Barbosa, A. D., Sembongi, H., Su, W.-M., Abreu, S., Reggiori, F., Carman, G. M., and Siniossoglou, S. (2015) Lipid partitioning at the nuclear envelope controls membrane biogenesis. *Mol. Biol. Cell.** 26*, 3641–3657

10. Han, G.-S., Wu, W.-L., and Carman, G. M. (2006) *Saccharomyces cerevisiae* lipin homolog is a Mg²⁺-dependent phosphatidate phosphatase enzyme. *J. Biol. Chem.** 281*, 9210–9218

11. Han, G.-S., Siniossoglou, S., and Carman, G. M. (2007) The cellular functions of the yeast lipin homolog Pah1p are dependent on its phosphatidate phosphatase activity. *J. Biol. Chem.** 282*, 37026–37035

12. Fakas, S., Qiu, Y., Dixon, J. L., Han, G.-S., Ruggles, K. V., Garbarino, J., Sturley, S. L., and Carman, G. M. (2011) Phosphatidate phosphatase activity plays a key role in protection against fatty acid-induced toxicity in yeast. *J. Biol. Chem.** 286*, 29074–29085

13. Santos-Rosa, H., Leung, J., Grimsey, N., Peak-Chew, S., and Siniossoglou, J. (2012) PAP controls expression of PSS for phospholipid synthesis. *Genetics** 157*, 1461–1473

14. Barbosa, A. D., Sembongi, H., Su, W.-M., Abreu, S., Reggiori, F., Carman, G. M., and Siniossoglou, S. (2013) Regulation of lipid droplet and membrane biogenesis by the acidic tail of the phosphatidate phosphatase Pah1p. *Mol. Biol. Cell.** 24*, 2124–2133

15. Han, G.-S., Wu, W.-L., and Carman, G. M. (2006) *Saccharomyces cerevisiae* lipin homolog is a Mg²⁺-dependent phosphatidate phosphatase enzyme. *J. Biol. Chem.** 281*, 9210–9218

16. Han, G.-S., Siniossoglou, S., and Carman, G. M. (2007) The cellular functions of the yeast lipin homolog Pah1p are dependent on its phosphatidate phosphatase activity. *J. Biol. Chem.** 282*, 37026–37035

17. Han, G.-S., Siniossoglou, S., and Carman, G. M. (2007) The cellular functions of the yeast lipin homolog Pah1p are dependent on its phosphatidate phosphatase activity. *J. Biol. Chem.** 282*, 37026–37035
PAP controls expression of PSS for phospholipid synthesis

48. O’Hara, L., Han, G.-S., Peak-Chew, S., Grimsey, N., Carman, G. M., and Siniossoglou, S. (2006) Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg2+-dependent phosphatidate phosphatase. J. Biol. Chem. 281, 34357–34358

49. Hosaka, K., Murakami, T., Kodaki, T., Nikawa, J., and Yamashita, S. (1990) Repression of choline kinase by inositol and choline in Saccharomyces cerevisiae. J. Bacteriol. 172, 2005–2012

50. Morash, S. C., McM aster, C. R., Hjelmstad, R. H., and Bell, R. M. (1994) Studies employing Saccharomyces cerevisiae cpi1 and cpi2 null mutants implicate the CPT1 gene in coordinate regulation of phospholipid biosynthesis. J. Biol. Chem. 269, 28769–28776

51. Kersting, M. C., Choi, H. S., and Carman, G. M. (2004) Regulation of the yeast EK11-encoded ethanolamine kinase by inositol and choline. J. Biol. Chem. 279, 35353–35359

52. McMaster, C. R., and Bell, R. M. (1994) Phosphatidylcholine biosynthesis via the CDP-choline pathway in Saccharomyces cerevisiae: multiple mechanisms of regulation. J. Biol. Chem. 269, 14776–14783

53. Anderson, M. S., and Lopes, J. M. (1996) Carbon source regulation of gene expression in Saccharomyces cerevisiae. Mol. Cell. Biol. 16, 3320–3328

54. Hasslacher, M., Ivessa, A. S., Paltauf, F., and Koblwein, S. D. (1993) Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism. J. Biol. Chem. 268, 10946–10952

55. Donahue, T. F., and Henry, S. A. (1986) Phosphatidylcholine synthesis in Saccharomyces cerevisiae: characteristics of the enzyme and identification of its structural gene in yeast. J. Biol. Chem. 261, 7077–7085

56. 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

57. Schuster, H. L., Hahn, A., Troster, F., Schweizer, E., and Hahn, A. (1992) characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J. Biol. Chem. 269, 28769–28776

58. Lin, R. C., Dawes, I. W., Brown, A. J., Li, P., Huang, X., Parton, R. G., and Palm, D. B. O. (1994) Membrane-associated phosphatidylserine synthase functions in conjunction with the Pho85p-Pho80p and Cdc28p-Orl1p complexes of regulation. EMBO J. 13, 2923–2932

59. Hansmanasab, A., Han, G.-S., and Carman, G. M. (2017) Tips on the analysis of phospholipid synthesis in vivo. Biochemistry 281, 248–254

60. Chang, Y.-F., and Carman, G. M. (2008) CTP synthetase and its role in phospholipid synthesis in yeast. J. Biol. Chem. 283, 1140–1149

61. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

62. Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. 194, 281–301

63. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W.-I., Voelker, D. R., and Carman, G. M. (2013) Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J. Biol. Chem. 288, 40186–40196

64. Kinney, A. J., and Carman, G. M. (1988) Characterization of the yeast lipin Pah1p/Smp2p Mg2+-dependent phosphatidate phosphatase. J. Biol. Chem. 263, 14331–14338

65. Larson, T. J., and Dowhan, W. (1976) Ribosomal-associated phosphatidylserine phosphatase. J. Biol. Chem. 251, 10857–10862

66. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W.-I., Voelker, D. R., and Carman, G. M. (2013) Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J. Biol. Chem. 288, 40186–40196

67. Chae, M., Han, G.-S., and Carman, G. M. (2012) The Saccharomyces cerevisiae actin patch protein App1p is a phosphatidate phosphatase enzyme. J. Biol. Chem. 287, 5212–5218

68. Binet, R. J., and Hamilton, S., eds) pp. 65–111, IRL Press, New York

69. Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. 194, 281–301

70. Rose, M. D., Winston, F., and Heiter, P. (1989) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

71. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917

72. Van Doff, L., Goil, V. M., Gu, Z., and Greenberg, M. L. (2005) Separation of yeast phospholipids using one-dimensional thin-layer chromatography. Anal. Biochem. 338, 162–164

73. Chae, M., Han, G.-S., and Carman, G. M. (2017) Tips on the analysis of phospholipid acid by the fluorometric coupled enzyme assay. Anal. Biochem. 526, 69–70

74. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917

75. Chae, M., Han, G.-S., and Carman, G. M. (2012) The Saccharomyces cerevisiae actin patch protein App1p is a phosphatidate phosphatase enzyme. J. Biol. Chem. 287, 5212–5218

76. Chae, M., Han, G.-S., and Carman, G. M. (2013) Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J. Biol. Chem. 288, 6427–6437

77. Chae, M., Han, G.-S., and Carman, G. M. (2013) Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J. Biol. Chem. 288, 6427–6437

78. Toke, D. A., Bennett, W. L., Dillon, D. A., Wu, W.-I., Chen, X., Oshiro, J., Voelker, D. R., Fischl, A. S., and Carman, G. M. (1998) Isolation and characterization of the Saccharomyces cerevisiae DPP1 gene encoding for diacylglycerol pyrophosphate phosphatase. J. Biol. Chem. 273, 3278–3284

79. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W.-I., Voelker, D. R., and Carman, G. M. (1998) Isolation and characterization of the Saccharomyces cerevisiae LPP1 gene encoding a Mg2+-dependent phosphatidate phosphatase. J. Biol. Chem. 273, 14331–14338

80. Larson, T. J., and Dowhan, W. (1976) Ribosomal-associated phosphatidylserine synthase from Escherichia coli: purification by substrate-specific elution from phosphocellulose using cytidine 5'-diphospho-1,2-diacyl-sn-glycerol. Biochemistry 15, 5212–5218

81. Vance, J. E. (1998) Eukaryotic lipid-biosynthetic enzymes: the same but not the same. Trends Biochem. Sci. 23, 423–428
PAP controls expression of PSS for phospholipid synthesis

89. Ostrander, D. B., O’Brien, D. J., Gorman, J. A., and Carman, G. M. (1998) Effect of CTP synthetase regulation by CTP on phospholipid synthesis in \textit{Saccharomyces cerevisiae}. \textit{J. Biol. Chem.} \textbf{273}, 18992–19001

90. Wimmer, C., Doye, V., Grandi, P., Nehrbass, U., and Hurt, E. C. (1992) A new subclass of nucleoporins that functionally interact with nuclear pore protein NSP1. \textit{EMBO J.} \textbf{11}, 5051–5061

91. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in \textit{Saccharomyces cerevisiae}. \textit{Genetics} \textbf{122}, 19–27

92. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) Yeast/\textit{E. coli} shuttle vectors with multiple unique restriction sites. \textit{Yeast} \textbf{2}, 163–167

93. Choi, H.-S., Su, W.-M., Morgan, J. M., Han, G.-S., Xu, Z., Karanasios, E., Siniossoglou, S., and Carman, G. M. (2011) Phosphorylation of phosphatidate phosphatase regulates its membrane association and physiological functions in \textit{Saccharomyces cerevisiae}: identification of Ser\textsuperscript{602}, Thr\textsuperscript{723}, and Ser\textsuperscript{744} as the sites phosphorylated by \textit{CDC28} (CDK1)-encoded cyclin-dependent kinase. \textit{J. Biol. Chem.} \textbf{286}, 1486–1498