# REGULATION OF YEAST CHOLINE KINASE BY ZINC DEPLETION AND THE YEAST PHOSPHATIDATE PHOSPHATASE BY ZINC DEPLETION AND GROWTH

by

ANIBAL SOTO-CARDALDA

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Written under the direction of

Dr. George M. Carman

and approved by

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New Brunswick, New Jersey

October, 2010

# ABSTRACT OF THE DISSERTATION

Title

By ANIBAL SOTO-CARDALDA

Dissertation Director:

Dr. George M. Carman

In the yeast *Saccharomyces cerevisiae*, the *CKI1*-encoded choline kinase catalyzes the committed step in the synthesis of phosphatidylcholine via the CDP-choline branch of the Kennedy pathway. Analysis of a $P_{CKI1}$-*lacZ* reporter gene revealed that *CKI1* expression was regulated by intracellular levels of the essential mineral zinc. Zinc depletion resulted in a concentration-dependent induction of *CKI1* expression. This regulation was mediated by the zinc-sensing and zinc-inducible transcriptional activator Zap1p. A purified Zap1p probe interacted with two putative $UAS_{ZRE}$ sequences (ZRE1 and ZRE2) in the *CKI1* promoter. Mutations of ZRE1 and ZRE2 to a nonconsensus $UAS_{ZRE}$ attenuated the induction of *CKI1* expression in response to zinc depletion. A $UAS_{INO}$ element in the *CKI1* promoter was responsible for stimulating *CKI1* expression, but this element was not involved in the regulation by zinc depletion. The induction of *CKI1* expression in zinc-depleted cells translated into increased choline kinase activity *in vitro* and *in vivo*, and an increase in phosphatidylcholine synthesis via the Kennedy pathway.

The yeast *PAH1*-encoded PA phosphatase (PAP) catalyzes the dephosphorylation of PA to yield DAG and Pi. The DAG produced by the PAP reaction is utilized for the synthesis of TAG and the synthesis of PE and PC via the Kennedy pathway. Analysis of the $P_{PAH1}$-*lacZ* reporter gene demonstrated that *PAH1* expression was regulated by intracellular levels of zinc. The induction of the *PAH1* gene in response to zinc depletion was dependent on the concentration of zinc. PAP regulation by zinc depletion was mediated by the zinc responsive transcription factor Zap1p. Purified Zap1p interacted with three putative $UAS_{ZRE}$ sequences located in the *PAH1* promoter. The Zap1p mediated induction in *PAH1* expression in response to zinc depletion resulted in an increase in PAP activity *in vitro*. The induction in PAP activity in zinc-depleted cells translated into an increase in DAG and TAG levels. The *pah1*Δ mutation resulted in the decrease of PC synthesis via the Kennedy pathway in response to zinc depletion.

The regulation of the yeast *PAH1*-encoded PAP by growth was examined. The DAG produced in the PAP reaction is converted into the storage lipid TAG throughout growth. As cells progress from the exponential to the stationary phase of growth the PAP activity is induced, resulting in an increased in DAG and TAG levels. The induction in PAP activity coincided with a decrease in PAP protein levels. PAP protein is degraded via the ubiquitin-proteasome pathway as cells progress throughout growth.

# DEDICATION

- To my mom, for always being my teacher in life. Faith can really move mountains.
- To my dad, for showing me the value of hard work and perseverance.
- To my brother and sister, for make me feel back at home each time they ask me their famous question: “what do millonaires do to enjoy themselves?” Now I know the answer- surround yourself with your beloved family.
- To my nephews and nieces, you are my source of energy.
- To Mami Gorda, for always taking care of me. No matter how old I get, I will always be your Papito.

Los amo con todo mi corazón.

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Last but not least, to God for your infinite blessings.

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| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| CDP-DAG | cytidine diphosphate diacylglycerol |
| CMP | cytidine monophosphate |
| CTP | cytidine triphosphate |
| DAG | diacylglycerol |
| DGK | diacylglycerol kinase |
| DGPP | diacylglycerol pyrophosphate |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis (β-aminoethyl ester) N, N, N', N'-tetraacetic acid |
| HAD | haloacid dehalogenase |
| Ins | inositol |
| kDa | kilo Daltons |
| LPP | lipid phosphate phosphatase |
| MG132 | Z-Leu-Leu-Leu-al |
| NEM | N-ethylmaleimide |
| PA | phosphatidic acid |
| PAP | phosphatidate phosphatase |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PI | phosphatidylinositol |

| | |
|---|---|
| $P_i$ | inorganic phosphate |
| PS | phosphatidylserine |
| SDS | sodium dodecyl sulfate |
| TAG | triacylglycerol |
| $UAS_{INO}$ | upstream activating sequence, inositol responsive element |
| $UAS_{ZRE}$ | upstream activating sequence, zinc responsive element |
| UTP | uridine triphosphate |

# INTRODUCTION

The structural and crucial functions of phospholipids are highly conserved from prokaryotes to the most complex of eukaryotes, humans. The yeast *Saccharomyces cerevisiae* is used as a model system for the synthesis of phospholipids. Almost all the phospholipid biosynthetic genes in *S. cerevisiae* have been cloned and characterized, and mutations in these genes have been isolated (1-8). Our current understanding of phospholipid metabolism and its regulation has been facilitated by the characterization of the wild type genes and mutations involved in these biosynthetic pathways and encoded enzymes. The metabolic mechanisms of phospholipid synthesis in yeast are similar to those of higher eukaryotes. Moreover, yeast can be easily grown in large quantities, genetic manipulations are easily accomplished, the entire genome has been completely sequenced, and multiple databases are available (9), making it a very attractive model system.

## Phospholipids

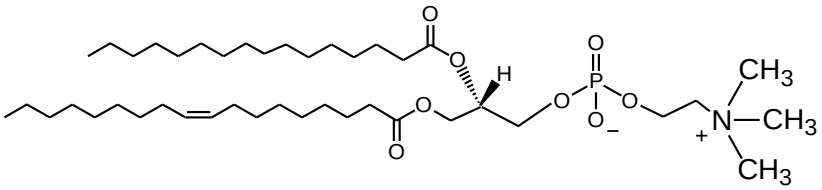
Phospholipids are key components of the architecture and configuration of the cellular barriers, the membranes (10). The importance of phospholipids is sometimes overlooked, even by well-known scientists, who once thought the sole function of phospholipids was limited to being structural building blocks of cell membranes (11). This notion is completely inaccurate, since phospholipids can also serve as precursors of macromolecules, act as molecular chaperones, serve as protein modifications required for membrane association, and are reservoirs of lipid signaling molecules (12-20). Phospholipids consist of a glycerol backbone and two fatty acids attached to the sn-1 and

sn-2 position through an ester linkage and a head group attached in the sn-3 position by a phosphodiester linkage (Fig. 1). The most common fatty acids esterified to the glycerophosphate backbone of yeast phospholipids include palmitic acid, palmitoleic acid, stearic acid and oleic acid (21-23). The most abundant phospholipids in *S. cerevisiae* are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) (Fig. 1) (1-3, 6, 23). Mitochondrial membranes also contain phosphatidylglycerol and cardiolipin (1-3, 6, 23).

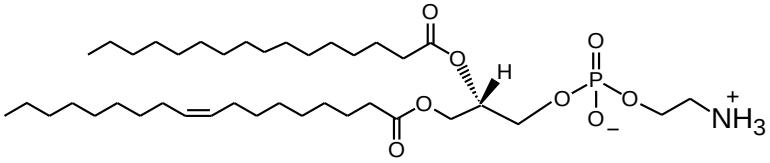
## Phospholipid Biosynthetic Pathways

Phospholipid synthesis in *S. cerevisiae* occurs by two complementary pathways, the CDP-DAG pathway and the Kennedy pathway (Fig. 2) (1, 2, 6, 24, 25). The CDP-DAG pathway uses phosphatidate (PA) as a precursor for the synthesis of PS, PE and PC (Fig. 2). The CDP-DAG synthase encoded by the *CDS1* gene (26) catalyzes the formation of the energy-rich liponucleotide intermediate CDP-DAG from PA and CTP (27). CDP-DAG donates its phosphatidyl moiety to serine for the synthesis of PS by the action of the *PSS1*/*CHO1*-encoded PS synthase (28-31). PS is decarboxylated to form PE by a reaction catalyzed by the *PSD1*-encoded (32, 33) and *PSD2*-encoded (34) PS decarboxylase enzymes. PC is synthesized from PE by a three-step methylation reaction catalyzed by two enzymes (35). The first methylation is catalyzed by the *PEM1*/*CHO2*-encoded PE methyltransferase (36, 37) and the last two methylations are catalyzed by the *PEM2*/*OPI3*-encoded phospholipid methyltransferase (36, 38). The other branch of the CDP-DAG pathway mediates the synthesis of PI from CDP-DAG by displacing CMP with inositol in a reaction catalyzed by the *PIS1*-encoded PS synthase (39, 40). The

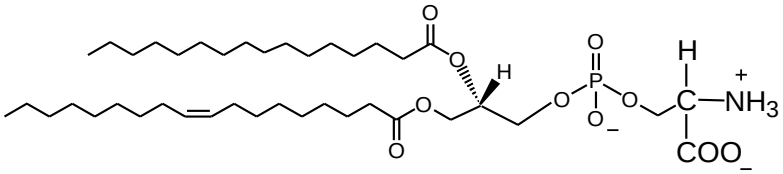
**Figure. 1. Major phospholipids in *S. cerevisiae***



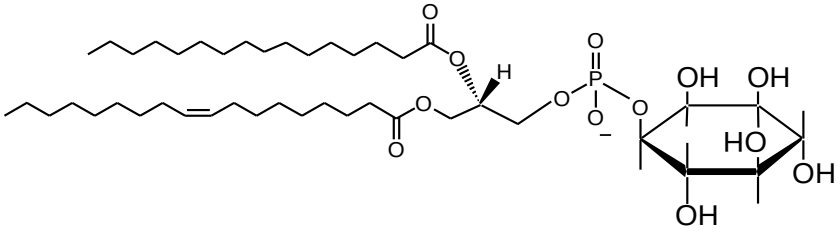
Phosphatidylcholine
(PC)



Phosphatidylethanolamine
(PE)

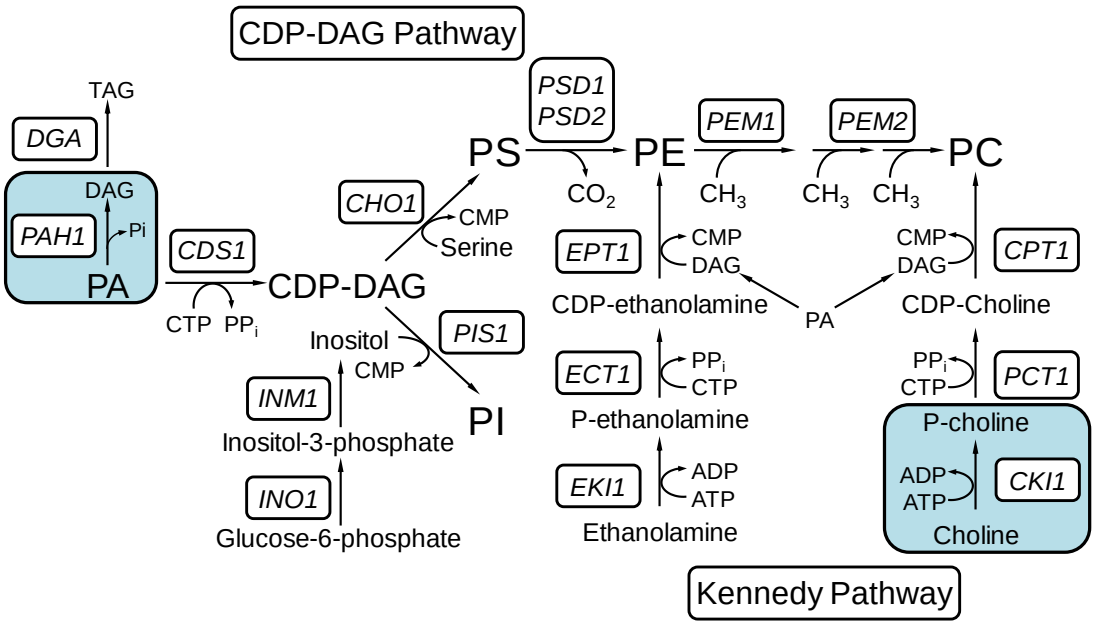


Phosphatidylserine
(PS)



Phosphatidylinositol
(PI)

**Figure. 2. Phospholipid synthesis in *S. cerevisiae*.** The pathways shown for the synthesis of phospholipids include the relevant steps discussed throughout the dissertation. The genes encoding enzymes responsible for the reactions in the pathways are indicated in the figure. The reaction catalyzed by the *CKI1*-encoded choline kinase and the *PAH1*-encoded phosphatidate phosphatase enzymes are highlighted in the boxes. *DAG*, diacylglycerol; *P-choline*, phosphocholine; *P-ethanolamine*, phosphoethanolamine.


CDP-DAG Pathway
TAG
DGA
DAG
PAH1
Pi
PA
CDS1
CTP
PPi
CDP-DAG
CHO1
CMP
Serine
PS
PSD1
PSD2
CO2
PE
PEM1
CH3
PEM2
CH3
PC
EPT1
CMP
DAG
CDP-ethanolamine
PA
CMP
DAG
CPT1
CDP-Choline
PIS1
Inositol
CMP
PI
INM1
Inositol-3-phosphate
INO1
Glucose-6-phosphate
ECT1
PPi
CTP
P-ethanolamine
EKI1
ADP
ATP
Ethanolamine
PPi
CTP
PCT1
P-choline
ADP
ATP
CKI1
Choline
Kennedy Pathway

inositol used for this reaction is derived from glucose-6-phosphate via the reactions catalyzed by the *INO1*-encoded inositol-3-phosphate synthase (41, 42, 42) and the *INM1*-encoded inositol-3-phosphate phosphatase (43).

Cells synthesize PE and PC via the CDP-ethanolamine and CDP-choline branch of the Kennedy pathway in the presence of exogenous ethanolamine or choline, respectively (Fig. 2) (1, 6). Ethanolamine and choline are phosphorylated by the *EKI1*-encoded ethanolamine kinase (44) and by the *CKI1*-encoded choline kinase (45) enzymes using ATP to form phosphoethanolamine and phosphocholine, respectively. The *ECT1*-encoded phosphoethanolamine cytidylyltransferase (46) and the *PCT1/CCT1*-encoded phosphocholine cytidylyltransferase (47) enzymes utilize CTP to activate phosphoethanolamine and phosphocholine to form CDP-ethanolamine and CDP-choline, respectively. DAG reacts with CDP-ethanolamine and CDP-choline to form PE and PC, respectively, by reactions catalyzed by the *EPT1*-encoded ethanolamine phosphotransferase (48, 49, 49) and the *CPT1*-encoded choline phosphotransferase (50, 51, 51) enzymes. The CTP utilized for the synthesis of CDP-DAG, CDP-ethanolamine and CDP-choline comes from UTP through the reaction catalyzed by the *URA7*- (52) and *URA8*-encoded (53) CTP synthetase enzymes. The DAG utilized for the synthesis of PE and PC by the CDP-ethanolamine and CDP-choline branch comes from the dephosphorylation of PA catalyzed by the *PAH1*-encoded PA phosphatase (54). The CDP-ethanolamine and CDP-choline pathways were thought to function only as salvage pathways to the CDP-DAG pathway; however, it is now known that PE and PC can be synthezised even in the absence of exogenous ethanolamine and choline (55-57). The PC and PE synthesized by the CDP-DAG pathway are hydrolyzed by phospholipase D to

produce PA, choline and ethanolamine, respectively (44, 58). The free choline and ethanolamine is recycled via the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway for the synthesis of PC and PE, respectively (44, 58).

## Gene Expression Regulation of Phospholipid Synthesis

The regulation of phospholipid synthesis in *S. cerevisiae* is complex and is controlled at both the transcriptional and post-translational level (1-4). Several factors influence the expression of genes encoding enzymes of both the CDP-DAG and Kennedy pathways. These include water soluble phospholipid precursors (e.g. inositol), lipids, growth phase, and nutrient availability (e.g. zinc) (1-6, 59-61). Several genes encoding phospholipid biosynthetic enzymes are regulated by the presence or absence of inositol. During inositol supplementation gene expression is repressed, while in the absence of inositol maximal gene expression is observed. Regulation of phospholipid synthesis in response to inositol requires PC synthesis. Choline supplementation enhances the repression effects of inositol supplementation (1, 2, 4-6). Regulation of gene expression by inositol is dependent on the Ino2p (62), Ino4p (63) and Opi1p (64) transcription factors, and a *cis*-element in the promoter region of target genes known as upstream activating sequence inositol responsive element ($UAS_{INO}$) (65-68). In the absence of inositol, a heterodimer complex formed by Ino2p and Ino4p recognizes and binds the $UAS_{INO}$ element (CATGTGAAAT), activating the expression of phospholipid biosynthetic genes (Fig. 3) (69). In the presence of inositol, the negative regulator Opi1p binds Ino2p and inhibits gene expression (Fig. 3) (69). *ino2*Δ and *ino4*Δ mutants show low levels of expression of $UAS_{INO}$ containing genes and require inositol for growth. On

the other hand, *opi1*Δ mutants show high levels of gene expression and produce excessive amounts of inositol that is excreted to the growth medium (1, 4-6). In the absence of inositol, Opi1p is tethered to the nuclear/ER membrane by the integral membrane protein Scs2p (Fig. 3) (70). This interaction is stabilized by the binding of Opi1p to PA (71). A *scs2*Δ mutation or the consumption of the PA pool results in the discharge of Opi1p from the nuclear/ER membrane and it translocates to the nucleus, where it binds Ino2p and inhibits the expression of $UAS_{INO}$ containing genes (72-75).

## Regulation of Phospholipid Synthesis by Phosphorylation

A key mechanism of post-tranlational regulation is phosphorylation, by which a protein kinase covalently attaches a phosphate molecule to a target protein (76-79). The phosphorylation status (phosphorylated or dephosphorylated) of a protein affect its enzymatic activity, cellular localization, protein turnover or interaction with other molecules (80, 81). In *S. cerevisiae*, several phospholipid biosynthetic enzymes are regulated by phosphorylation (80, 82). The two major kinases responsible for the regulation of phospholipid biosynthesis are protein kinase A and protein kinase C (83). Protein kinase A regulates cell growth, progression through the cell cycle, and development in response to various nutrients (84). Protein kinase C regulates cell cycle progression and cell wall formation (85, 86).

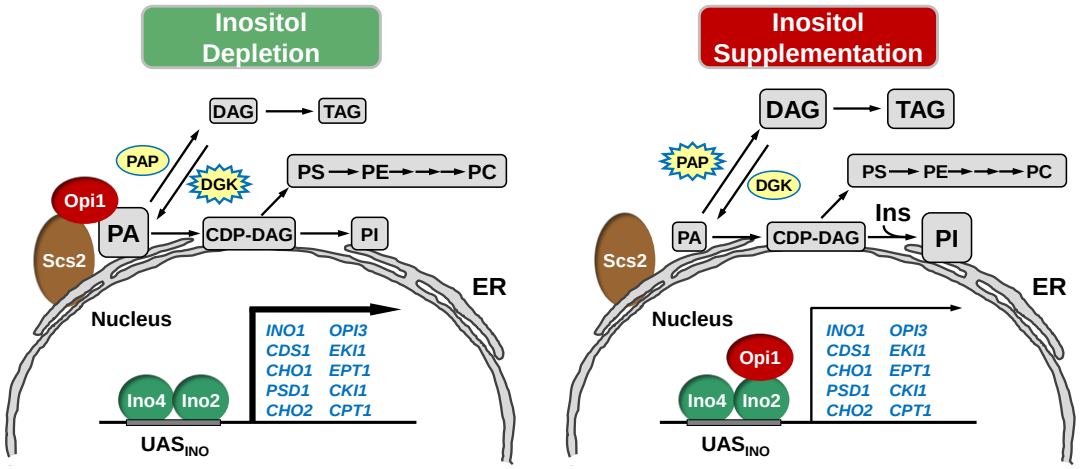
The enzymatic activities of the CDP-DAG and Kennedy pathways are regulated by phosphorylation (80). The *URA7*-encoded CTP synthase is phosphorylated by both protein kinase A and C, resulting in an increase in enzymatic activity (87-89). PS synthase is phosphorylated by protein kinase A resulting in a decrease in its activity (90),

a decrease in the levels of PE and PC, and an increase in the levels of PI (91). Opi1p (64), a transcriptional regulator of the expression of phospholipid-synthesizing enzymes is also regulated by its phosphorylation state (92-94). It is phosphorylated at $Ser^{31}$ and $Ser^{251}$ by protein kinase A stimulating its repressor function (93) and at $Ser^{26}$ by protein kinase C attenuating its function (92). In addition, Opi1p function is stimulated by the phosphorylation at $Ser^{10}$ by casein kinase II (94). Choline kinase is phosphorylated by protein kinase A at $Ser^{30}$ and $Ser^{85}$ (95) and by protein kinase C at $Ser^{25}$ and $Ser^{30}$ (96), resulting in an increase in its catalytic activity. The phosphorylation status of the *PAH1*-encoded PA phosphatase controls the transcriptional expression of phospholipid biosynthetic genes (82, 97). PA phosphatase activity is reduced by phosphorylation by the cyclin-dependent kinase Cdc28p (97, 98), and induced by its dephosphorylation by the Nem1p/Spo7p phosphatase (82). In addition, current work from our laboratory has shown that PAP is also a target of phosphorylation by both protein kinase A and C (unpublished data).

## Zinc Homeostasis in *Saccharomices cerevisiae*

Zinc is an essential nutrient required for the growth and metabolism of *S. cerevisiae* and higher eukaryotes (99). Zinc serves as a cofactor for hundreds of enzymes (100) and a structural component of many transcriptional factors (101). It is known that over 300 enzymes require zinc to function (102), and its estimated that around 1% of the genes in the human genome encode enzymes containing zinc finger motifs (103). In humans, zinc deficiency has detrimental effects on appetite, cognitive functions, embryonic development, epithelial integrity, and immune functions (104). In rats, zinc

**Figure. 3. Regulation of phospholipid synthesis by inositol in *S. cerevisiae*.** (Left) In the absence of inositol, Ino2p and Ino4p bind to the $UAS_{INO}$ element inducing gene expression (indicated by thick arrow). DG kinase (DGK) activity is induced resulting in the formation and accumulation of PA. Opi1p is tethered to the nuclear/ER membrane by interaction with Scs2p and PA. (Right) During inositol supplementation, the pool of PA is consumed for the synthesis of PI, and the induction in PAP activity and formation of DAG, which result in the destabilization of the Opi1p/Scs2p interaction and the subsequent translocation of Opi1p to the nucleus, where it binds to Ino2p and attenuates the expression of $UAS_{INO}$ containing genes.

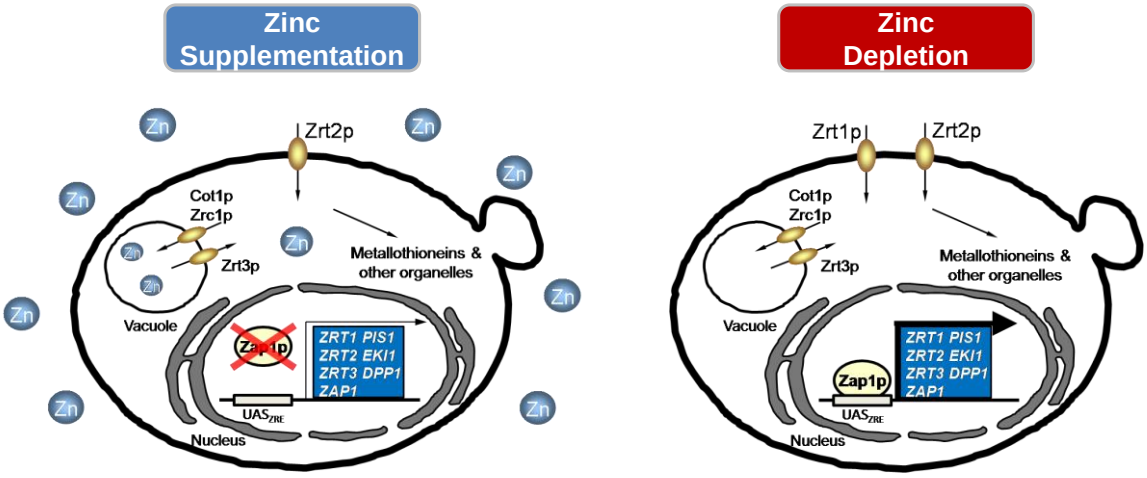

Inositol Depletion
DAG
TAG
PAP
DGK
PS
PE
PC
Opi1
PA
CDP-DAG
PI
Scs2
ER
Nucleus
INO1 OPI3
CDS1 EKI1
CHO1 EPT1
PSD1 CKI1
CHO2 CPT1
Ino4
Ino2
UAS$_{INO}$
Inositol Supplementation
DAG
TAG
PAP
DGK
PS
PE
PC
Ins
PA
CDP-DAG
PI
Scs2
ER
Nucleus
Opi1
INO1 OPI3
CDS1 EKI1
CHO1 EPT1
PSD1 CKI1
CHO2 CPT1
Ino4
Ino2
UAS$_{INO}$

deficiency has been linked to oxidative damage to DNA, lipids and proteins (105) and a decrease in the overall phospholipid content (106-108). Zinc toxicity can also cause detrimental effects on the cell, highlighting that maintaining optimal levels of zinc is crucial for cell survival (99).

Zinc homeostasis is controlled by how much zinc is transported into the cell and how much is excreted. In *S. cerevisiae*, the influx of zinc is principally controlled by the plasma membrane zinc transporters Zrt1p (109), Zrt2p (110) and Fet4p (111). Zinc is taken in and out of the vacuole by the action of the vacuole membrane zinc transporters Zrc1p (112, 113) and Cot1p (54), and Zrt3p (43), respectively (Fig. 4).

Zinc homeostasis is tightly regulated at the transcriptional level. In zinc limited conditions, the expression of the high affinity zinc transporter Zrt1p is induced to maximize the uptake of zinc; whereas in response to zinc supplementation, the expression of the *ZRT1* gene is blocked and the Zrt1p is rapidly ubiquitinated and degraded in the vacuole (109, 114, 115). In zinc limiting conditions the zinc sensor and zinc activating transcription factor Zap1p is activated, and it binds to an upstream activating sequence zinc responsive element ($UAS_{ZRE}$) located in the promoter region of *ZRT1* and other zinc responsive genes, inducing their expression (Fig. 4) (109, 116, 117). Zap1p is a 99 kDa protein (880 amino acids) composed of three functional domains (Fig. 5), two activation domains (118) and a DNA binding domain (119, 120). The two activation domains (AD1 and AD2) are located at the amino and carboxy-terminal half, respectively, serve as zinc sensors, regulating Zap1p activity (121, 122). These two activation domains are regulated independently, and both are inhibited by binding of zinc (121, 122), which results in the inactivation of Zap1p activity. Thus, the activation domains serve as zinc sensors for

**Figure. 4. Regulation of zinc homeostasis in *S. cerevisiae*.** The influx of zinc into the cell is mediated by Zrt1p, Zrt2p and Fet4p. Zrc1p and Cot1p mediate the influx of zinc into the vacuole, where it is stored. The efflux of zinc from the vacuole to the cytosol is mediated by Zrt3p. In the cytoplasm, zinc can be either bound to methallothioneins or transported into different organelles. In zinc supplemented cells, the transcriptional activity of Zap1p is inhibited, resulting in the basal expression (indicated by the thin arrow) of ZRE containing genes (highlighted in the blue box). In zinc depleted cells the transcriptional activity of Zap1p is induced, resulting in the maximal expression (indicated by the thick arrow) of $UAS_{ZRE}$ containing genes.

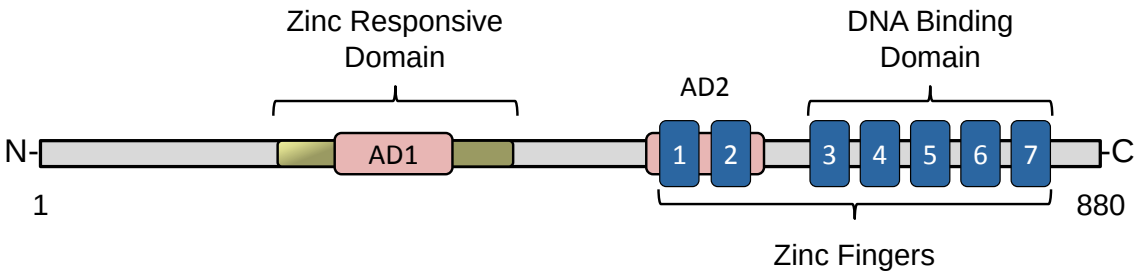

Zinc Supplementation
Zinc Depletion
Zrt2p
Zrt1p
Cot1p
Zrc1p
Zrt3p
Metallothioneins & other organelles
Vacuole
ZRT1 PIS1
ZRT2 EKI1
ZRT3 DPP1
ZAP1
Zap1p
UAS$_{ZRE}$
Nucleus
Zn

controlling the activation of Zap1p in response to intracellular zinc levels. The DNA binding domain composed of five $C_2H_2$ zinc fingers (ZF3 – ZF7) is localized at the C-terminal region of the protein (Fig. 5) (119), and it is required for the recognition and binding of Zap1p to the ZRE sequences localized in the promoter region of zinc responsive genes. The ZRE sequence is composed of 11 bases with a consensus sequence of 5'-ACCTTNAAGGT-3'. Zap1p recognizes variations in the ZRE consensus sequence, resulting in different magnitudes of induction among zinc inducible genes (123, 124).

## Regulation of Phospholipid Synthesis by Zinc Depletion

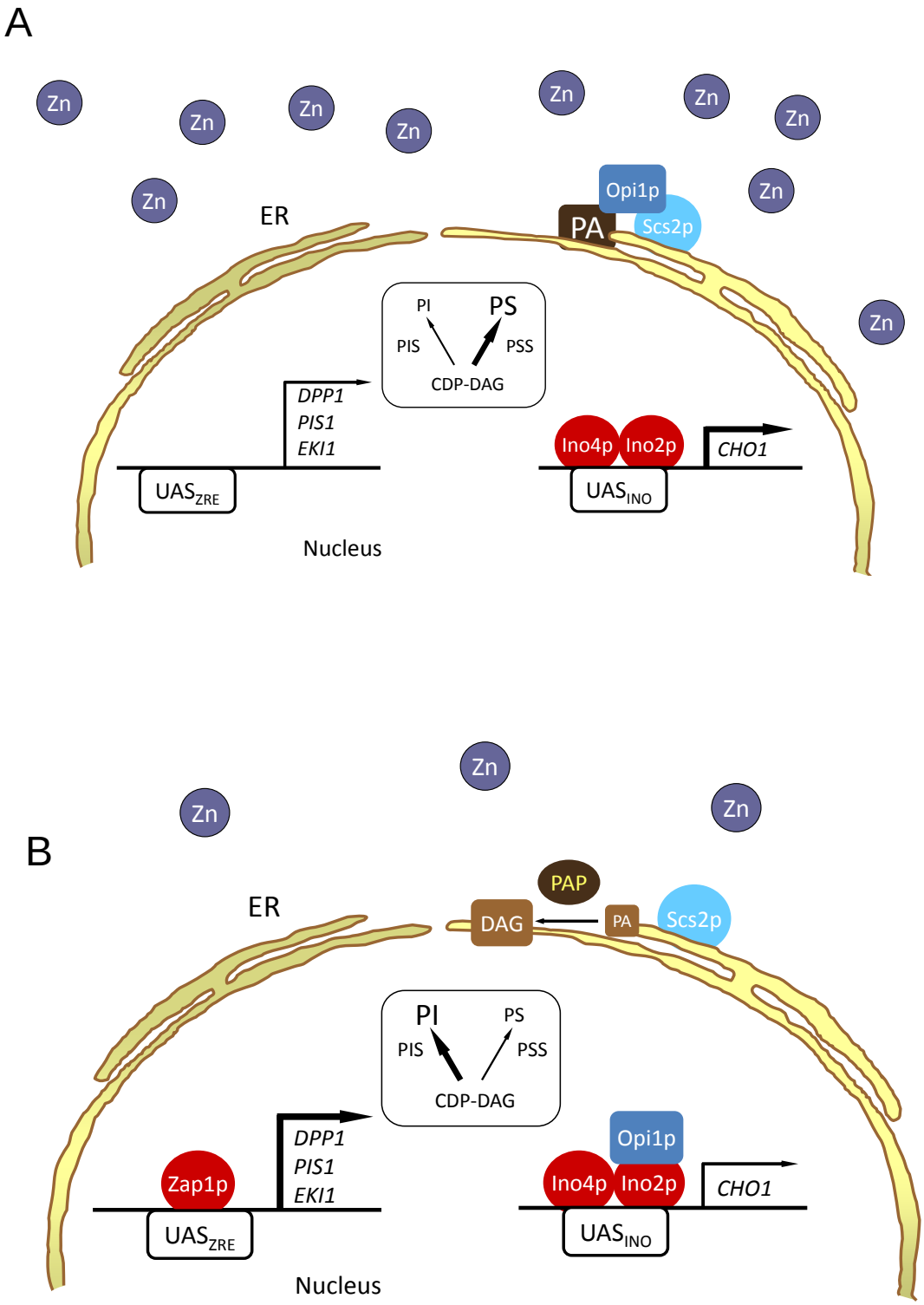
PC is synthesized from the phospholipid precursor PA by the complementary CDP-DAG and Kennedy (CDP-choline branch) pathways (Fig. 2) (6, 69). In zinc depleted cells, the activity of the CDP-DAG pathway enzymes PS synthase, PS decarboxylase, and the PE and phospholipid methyltransferases are reduced (61). On the other hand, zinc depletion causes the induction of PI synthase activity (61, 124). The regulation of the PS and PI synthase enzymes in response to zinc depletion occurs at the level of transcription (61, 124) (Fig. 6). The repression of *CHO1*-encoded PS synthase in response to zinc depletion is mediated by Opi1p (61), whereas the induction of *PIS1*-encoded PI synthase is mediated by Zap1p (Fig. 6) (124). Opi1p attenuates transcription of *CHO1* (and other $UAS_{INO}$-containing genes in the pathway) by interaction with Ino2p, a component of the transcriptional activator Ino2p-Ino4p complex that binds to a $UAS_{INO}$ element in its promoter (69, 125). Zap1p induces transcription of *PIS1* by interaction with $UAS_{ZRE}$ sequences in the *PIS1* promoter (124). The transcriptional regulation of the PS synthase and PI synthase enzymes translates into a decrease in the cellular PE content

**Figure. 5. Zap1p protein domain structures.** The diagram shows the position of the two zinc activation domains (AD1 and AD2), the zinc response domain (ZRD), the $C_2H_2$ zinc fingers (1-7) and the DNA binding domain formed by zinc fingers 3-7.


Zinc Responsive
Domain
DNA Binding
Domain
AD2
N-
AD1
1
2
3
4
5
6
7
-C
1
880
Zinc Fingers

and an increase in the cellular PI content, respectively (61). The reciprocal regulation of these two enzymes is part of an overall mechanism by which the synthesis of PI is coordinately regulated with the synthesis of phospholipids through the CDP-DAG pathway (1, 2, 125). Despite the fact that the activity of the CDP-DAG pathway is repressed in response to zinc depletion, this growth condition does not have a significant effect on the cellular PC content (61). In addition to *PIS1*, the induction in gene expression of the *DPP1* and *EKI1* genes in response to zinc depletion is dependent on Zap1p (60, 123). *DPP1* is one of the most highly induced genes in response to zinc depletion (126). It encodes diacylglycerol pyrophosphate (DGPP) phosphatase localized in the vacuole that catalyzes the dephosphorylation of DGPP to yield PA and then dephosphorylates PA to produce DAG (60, 127-130). The induction of the *DPP1* gene under zinc starvation results in a decrease in DGPP and PA levels (59). The induction of *EKI1* in zinc starved cells results in an increase in the PC formed from the PE synthesized via the CDP-ethanolamine pathway (60, 123). This indicates that under zinc starvation the Kennedy pathway compensates for the reduction in the synthesis of PC through the CDP-DAG pathway.

**Figure. 6. Regulation of phospholipid synthesis in response to zinc availability.** (A) In zinc supplemented cells (denoted by numerous zinc atoms) *PIS1*, *DPP1* and *EKI1* (left) and *CHO1* (right) are expressed to a certain extent. Maximum expression of *CHO1* (indicated by thick arrow) is dependent on the interaction of the Ino2p–Ino4p complex with the $UAS_{INO}$ element in the gene promoter. Under this growth condition, Opi1p is associated with the nuclear/ER membrane through interactions with PA and Scs2p. (B) During zinc limiting conditions (depicted as a reduced number of zinc atoms), the Zap1p transcription factor is induced and binds to the $UAS_{ZRE}$ in the *PIS1*, *DPP1* and *EKI1* gene promoters to increase transcription (indicated by the thick arrow). In zinc limiting conditions (indicated by the thin arrow) transcription of *CHO1* is attenuated by Opi1p binding to Ino2p. Dissociation of Opi1p from the nuclear/ER membrane and its translocation into the nucleus are caused by a decrease in PA concentration. An increase in PAP activity may be responsible for the decrease in PA concentration and the consumption of PA for PI synthesis.


A
Zn
ER
Opi1p
PA
Scs2p
PI
PS
PIS
PSS
CDP-DAG
DPP1
PIS1
EKI1
Ino4p
Ino2p
CHO1
UAS$_{ZRE}$
UAS$_{INO}$
Nucleus
B
PAP
DAG
PA
Scs2p
Zap1p

## Choline Kinase

Choline kinase (ATP:choline phosphotransferase; EC 2.7.1.32) is a cytosolic enzyme that catalyzes the transfer of the γ-phosphate from ATP to choline to form phosphocholine and ADP (Fig.7) (131). Choline kinase catalyzes the committed step in the CDP-choline branch of the Kennedy pathway for the synthesis of PC (Fig. 2) (3, 131, 131). This enzyme plays a central role in the synthesis of PC via the Kennedy pathway and in the overall metabolism of phospholipids in *S. cerevisiae* and higher eukaryotes (132).

Choline kinase has been purified from rat kidney and rat liver (133, 134). Using a yeast mutant defective in choline kinase the structural gene (*CKI1*) from *S. cerevisiae* has been isolated by genetic complementation (45). Attempts for protein purification from yeast have been ineffective (135) due to endogenous proteases in yeast (131, 136).(131) The *CKI1* gene has been expressed in Sf-9 insect cells and the enzyme has been purified to homogeneity from the cytosolic fraction by standard chromatography methods (137). The molecular mass of the purified choline kinase (73 kDa) is in agreement with the expected size (66.3 kDa) (137). Its optimal enzymatic activity is dependent of $Mg^{+2}$ ions at pH 9.5 and a temperature of 30°C. It is allosterically regulated with respect to ATP and ADP (137). The choline kinase protein contains a conserved phosphotransferase and a choline kinase motif, required for its enzymatic function (Fig. 8) (138-140). An active choline kinase can be found as a homo-dimer, homo-tetramer or homo-octamer depending on the ATP levels (137). The *CKI1* gene is not essential as *cki1*Δ mutant cells synthesize PC via the CDP-DAG pathway (Fig. 2). Choline kinase and the CDP-choline pathway play a vital role when the CDP-DAG pathway is defective. Cells unable to

**Figure. 7. Choline kinase reaction.** Choline is phosphorylated using ATP as the phosphate donor to form phosphocholine (P-choline).

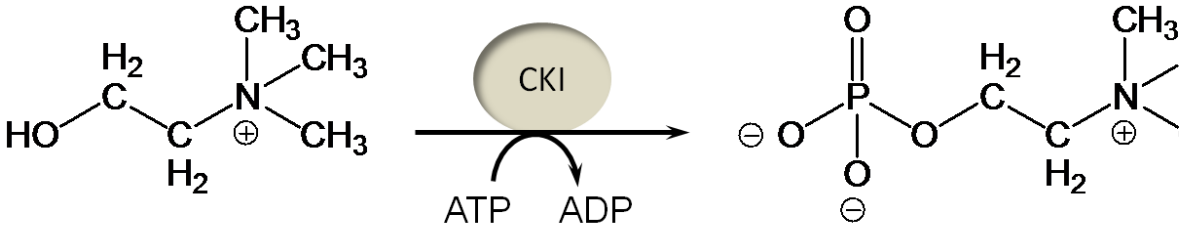
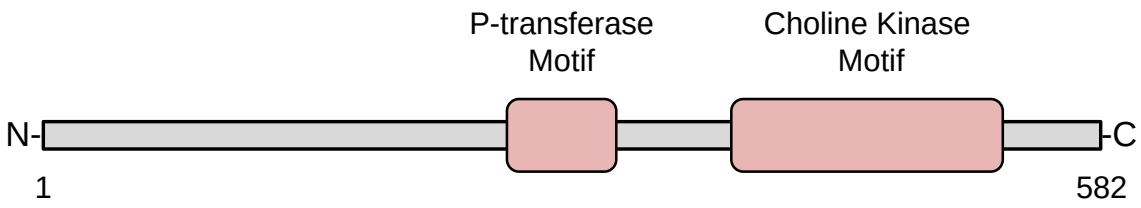

CKI
ATP
ADP
Choline
Mg2+
P-Choline

**Figure. 8. Choline kinase protein domain structures.** The diagram shows the position of the phosphotransferase (P-transferase) and choline kinase motifs.


P-transferase
Motif
Choline Kinase
Motif
N-
-C
1
582

synthesize PS, PE and PC via the CDP-DAG pathway required choline supplementation to grow (e.g. *cho1*Δ, *cho2*Δ *opi3*Δ) (1, 2, 6).

In yeast, choline kinase is regulated at the genetic and biochemical levels. As mentioned above, choline kinase is phosphorylated on multiple serine residues by both protein kinases A and C resulting in an increase in choline kinase activity and PC synthesis via the CDP-choline pathway (96, 141). *CKI1* expression is regulated by growth phase and by water-soluble phospholipid precursors (142). Maximal levels of *CKI1* mRNA and choline kinase are observed at the exponential phase of growth and decline as cells enter the stationary phase of growth (142). Choline kinase is regulated in choline kinase expression is repressed in response to inositol/choline supplementation and induced in their absence; parallel to other phospholipid biosynthetic genes (142).

In mammals, choline kinase exists in three forms encoded by two genes, *Chk*$\alpha$ and *Chk*$\beta$ (143-145). The *Chk*$\alpha$ has two splice variants, CKα1 and CKα2 that differ in 18 additional amino acids found in the CKα2 isoform (143). All isoforms seem to be expressed in all mouse tissues, with the testes and liver showing the highest CKα expression, and the heart and the liver showing the highest CKβ expression (146). Similar to yeast, active mammalian choline kinase is found as a dimer, showing different choline kinase activity based on the isoform combination with α/α being the more active (143). Mice mutant for CKα die at embryonic day 3 (144), while CKβ mutant mice suffer muscular dystrophy and neonatal bone deformities (144), highlighting the importance of choline kinase.

Several genes that participate in essential metabolic pathways are altered during the carcinogenic process, and choline kinase is one of those cancer-related genes (147-

149). Increase in activity and misregulation of choline kinase is a frequent event found in a variety of human tumors such as lung, colorectal, prostate and human breast carcinomas (147-149). Significant differences have been detected in choline phospholipid metabolites of malignant vs. benign breast lesions (150). The aberrant accumulation of PC metabolites (e.g. phosphocholine) and the dramatic increase in both choline kinase mRNA and activity observed in epithelial ovarian cancer provides a tool for cancer diagnosis and treatment (151). Based on its relationship with cancer propagation and tumor cells, the synthesis of inhibitory drugs against choline kinase and the understanding of its regulation constitute an excellent approach for the development of treatment against cancer.

## Phosphatidate Phosphatase

Phosphatidate phosphatase (PAP) (3-*sn*-phosphatidate phosphohydrolase; EC 3. 1. 3. 4) catalyzes the dephosphorylation of PA to yield DAG and $P_i$ (Fig. 9) (152). PAP activity was first described in 1957 in chicken liver by the laboratory of Eugene P. Kennedy (152). In 1984, PAP activity was described in yeast by Hosaka and Yamashita (153). In 1989, Lin and Carman (154) purified and characterized a $Mg^{2+}$-dependent PAP from yeast. It was not until 2006 that Han and coworkers (54) identified the gene encoding the enzyme and name it *PAH1* (phosphatidic acid phosphohydrolase).

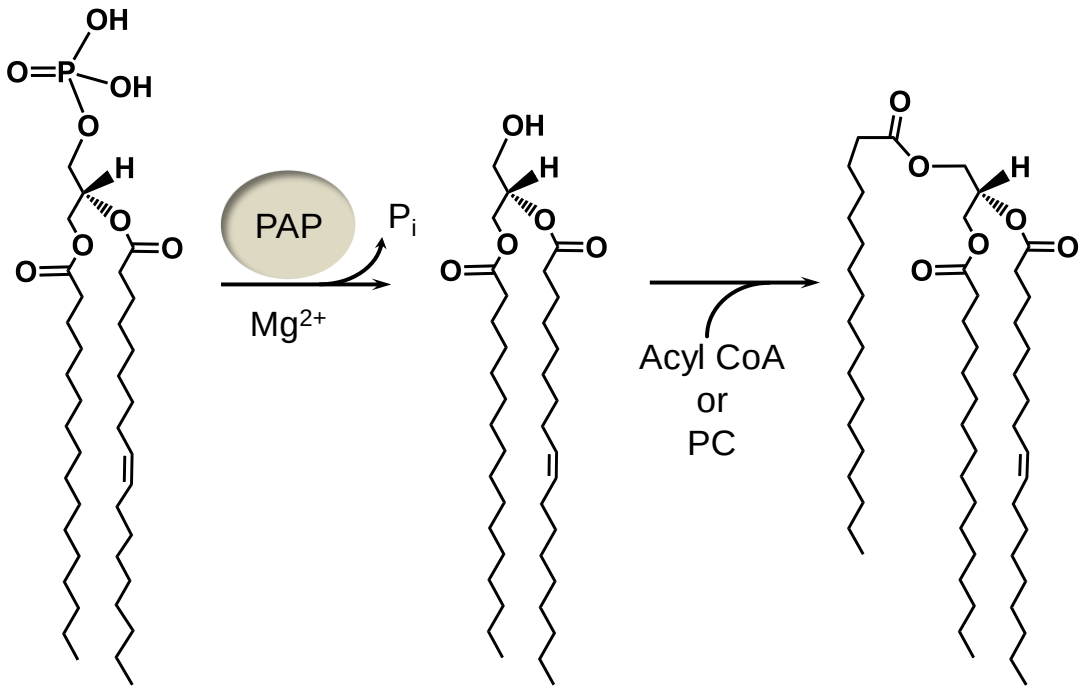
PA phosphatases were first classified in two categories based on their dependency on $Mg^{2+}$ ions for their catalytic activity (155). Type 1 (PAP) are $Mg^{2+}$-dependent, and type 2 (lipid phosphate phosphatase, LPP) are $Mg^{2+}$-independent. PAP enzymes use PA as their sole substrate, whereas LPP enzymes use a broad range of substrates (PA, DGPP,

lyso-PA, sphingoid base phosphate and isoprenoid phosphate (127, 128, 156-158). In yeast, the majority of the PAP activity is encoded by the *PAH1* gene; however the *pah1*Δ mutant retains some PAP activity suggesting the existence of one or more unknown genes that encode PAP enzymes (54).

The PAP activity encoded by *PAH1* requires a DxDxT catalytic motif characteristic of $Mg^{2+}$-dependent phosphatase enzymes. The first aspartate residue is required for the phosphatase reaction (Fig. 10) (159, 160). This catalytic motif is localized within a haloacid dehalogenase (HAD)-like domain localized in the middle of the protein (Fig. 10) (159, 160). PAP also possesses a NLIP domain at the N-terminal region, and a mutation in a conserved glycine residue disrupts PAP activity (Fig. 10) (161, 162).

The *PAH1*-encoded PAP can be found both in the cytosol and as a membrane bound protein (54). The membrane bound PAP is salt extractable, indicating that the enzyme associates with the membrane as a peripheral membrane protein (42, 54). This enzyme plays a critical role by supplying the DAG required for the synthesis of TAG and for the synthesis of phospholipids via the Kennedy pathway (Fig. 2) (Fig. 9) (54). The DAG produced by the dephosphorylation of PA catalyzed by PAP is preferentially channeled for the synthesis of phospholipids during the exponential phase of growth when cells are grown in a nutrient rich environment (54, 163, 164). As cells are starved for nutrients they cease mitotic division and arrest in the G1 phase of the cell cycle (151). This phase of growth shows a decrease in growth rate, resistance to environmental stress, thickening of the cell wall, ability to survive long periods of starvation and accumulation of glycogen (151). In addition, as cells enter the stationary phase of growth, DAG is

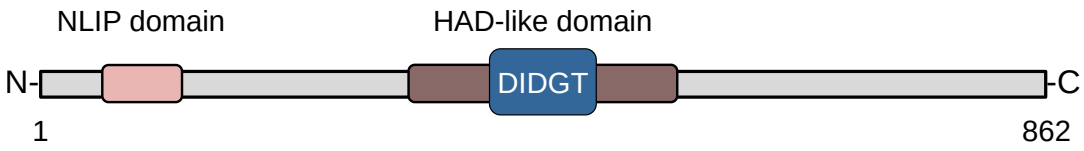
**Figure. 9. Role of PAP in TAG synthesis.** PAP catalyzes the dephosphorylation of PA to yield DAG and $P_i$. DAG is acylated by a reaction catalyzed by a DAG acyltransferase (*DGA1*, *LRO1*) to yield TAG.



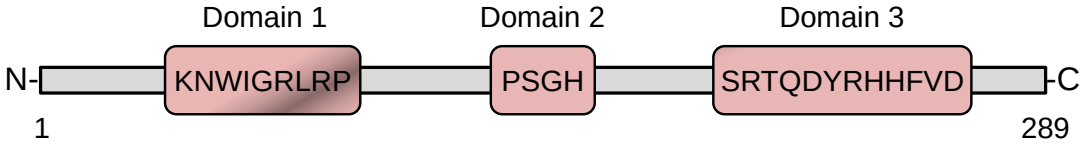

Phosphatidate (PA) | Diacylglycerol (DAG) | Triacylglycerol (TAG)

**Figure. 10. Domain structures of *PAH1*-encoded PAP (Pah1p) and *DPP1*-and *LPP1*-encoded LPP (Dpp1p and Lpp1p).** The diagram shows the position of their catalytic motifs.

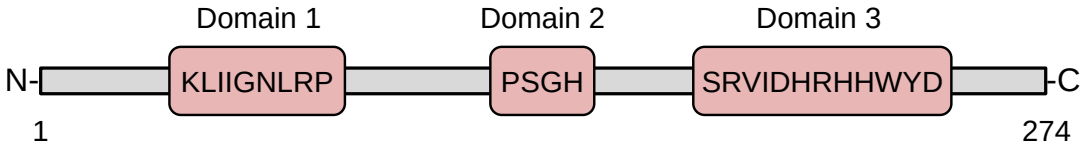
Pah1p




Dpp1p




Lpp1p



primarily utilized for the synthesis of TAG (54, 163, 164).

Almost all LPP activity in yeast is encoded by the *DPP1* and *LPP1* genes (128, 156). Dpp1p (33.5 kDa) and Lpp1p (31.5 kDa) are integral membrane proteins with six transmembrane domains localized in the vacuole and the Golgi, respectively (59, 60, 165). The Dpp1p and Lpp1p catalytic motif is composed of three lipid phosphatase domains, KXXXXXXRP (domain 1), PSGH (domain 2), and SRXXXXXHXXXD (domain 3), characteristic of a superfamily of LPP enzymes (Fig. 10) (166-168). Both Dpp1p- and Lpp1p-encoded LPP activities have been shown to be involved primarily in lipid signaling and not in phospholipid synthesis (129). In plants, DGPP has been shown to function as a signaling molecule in response to osmotic stress and pathogens (169).

The *S. cerevisiae PAH1*-encoded PAP enzyme shows homology with mammalian lipin 1, 2 and 3 (54, 161). The human *LPIN1* gene encodes a protein involved in fat metabolism, but its molecular function was unknown until 2006 (170-173). The *LPIN*1-encoded lipin 1 was identified by positional cloning of the mutation that causes fatty liver dystrophy in the *fld* mouse (161). Mice lacking the *Lpin1* gene showed lipodystrophy, insulin resitance, peripheral neuropathy and neonatal fatty liver (161). The identification of human lipin as a PAP homolog facilitated the identification of all mammalian lipins as PAP enzymes. Lipin 2 and 3 were identified based on sequence homology to lipin 1 (161). Lipin 1, 2 and 3 share a 60% amino acid sequence similarity, but show a different tissue expression pattern. Lipin 1 shows its highest expression in the adipose tissue, skeletal muscle and testes, and it is also expressed in the liver, heart, brain and kidney (161). Lipin 2 is mainly expressed in the liver, kidney, brain and lung, while lipin 3 is expressed solely in the liver (174). Mutations in the *LPIN1* gene have been identified

from patients suffering from muscle pain, weakness and myoglobinuria, a condition characterized by the presence of myoglobin in the urine, usually associated with rhabdomyolysis (175). In addition, *LPIN2* mutations have been shown to cause Majeed syndrome, a condition characterized by chronic recurrent multifocal osteomyelitis, congenital dyserythropoietic anemia and neutrophilic dermatosis (176, 177).

# HYPOTHESIS

Zinc starvation results in the decrease in CDP-DAG pathway enzymes (e.g., PS synthase, PS decarboxylase, PE methyltransferase and phospholipid methyltransferase), a decrease in PE content, and an increase in PI content. Yet under these growth conditions, the cellular content of PC remains unchanged. In this dissertation, we addressed the hypothesis that the CDP-choline branch of the Kennedy pathway was induced by zinc depletion to compensate for the down-regulation of PC synthesis via the CDP-DAG pathway. We also examined the hypothesis that the *PAH1*-encoded PA phosphatase was also induced by zinc depletion because the enzyme supplies the DAG needed for PC synthesis via the Kennedy pathway. The PA phosphatase enzyme also provides the DAG needed to synthesize TAG, and we examined the hypothesis that the expression of PA phosphatase activity correlates with the synthesis of TAG during the growth of yeast.

# EXPERIMENTAL PROCEDURES

## Materials

All chemicals were reagent grade. Growth medium supplies and yeast nitrogen base lacking zinc sulfate were purchased from Difco and BIO 101, respectively. Clontech was the supplier of the Yeastmaker$^{TM}$ transformation kit. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Sigma was the source of ampicillin, aprotinin, ATP, benzamidine, bovine serum albumin, choline, phosphocholine, CDP-choline, diethyl ether, dithiothreitol, ethylene glycol-bis (β-aminoethyl ester) N, N, N', N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), glycerol, glycine, hexane, IGEPAL CA-630, imidazole-HCl, leupeptin, magnesium chloride, magnesium sulfate, MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal), N-ethylmaleimide (NEM), Octyl-β-D-lucopyranoside, O-nitrophenyl β-D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, potassium chloride, reinickate salt, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sodium phosphate, sucrose, Tris, Triton X-100 and β-mercaptoethanol. Fisher was the supplier of chloroform, methanol, hydrochloric acid, acetic acid and 0.5 mm glass beads. Avanti Polar Lipids was the source of lipids. Silica gel 60 thin layer chromatography plates were from EM Science. Bio-Rad was the supplier of protein assay reagents, electrophoretic reagents, acrylamide solutions, immunochemical reagents, the DNA size ladder used for agarose gel electrophoresis, and protein molecular mass standards for SDS-PAGE. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Genosys Biotechnology, Inc. prepared oligonucleotides for PCR and electrophoretic mobility shift assays. New

England BioLabs was the source of modifying enzymes, recombinant Vent DNA polymerase, restriction endonucleases, nucleotides and NEBlot kit. DNA gel extraction and plasmid DNA purification kits were purchased from Qiagen, Inc., and carrier DNA for yeast transformation from Clontech. GE Healthcare supplied the ProbeQuant G-50 columns, polyvinylidene difluoride paper, and the enhanced chemifluorescence Western blotting detection kit. BioSynthesis Inc. supplied rabbit anti-PAP antibodies prepared against a mixture of peptides found at the N- (residues 130-147) and C- (residues 778-794) terminal portions of the protein. Alkaline phosphatase-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies, and mouse anti-PGK antibodies were from Thermo Scientific and Invitrogen, respectively. Liqui-Nox detergent was from Alconox, Inc.

## Strains and Growth Conditions

The strains used in this work are listed in Table I. Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete medium (178) containing 2% glucose at 30 °C. The appropriate amino acids of synthetic complete medium were omitted for selection purposes. Zinc-free medium was synthetic complete medium (178) prepared with yeast nitrogen base lacking zinc sulfate. To deplete internal stores of zinc (60), the following routine was followed; cells were first grown for 24 h in synthetic complete medium containing 1.5 μM zinc sulfate; saturated cultures were diluted into zinc-free medium at an initial density of 1 x $10^6$ cells/ml, and grown for 24 h; cultures were then diluted back to 1 x $10^6$ cells/ml and grown in zinc-free medium containing 0 or 1.5 μM zinc sulfate. The growth medium for the inositol auxotrophic *ino2*Δ and *ino4*Δ mutants (179) was supplemented with 75 μM inositol.

Plasmid maintenance and amplification were performed in *Escherichia coli* strain DH5α. *E. coli* cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μg/ml) was added to bacterial cultures that carried plasmids. For growth on plates, yeast and bacterial media were supplemented with 2% and 1.5% agar, respectively. Yeast cell numbers in liquid medium were determined spectrophotometrically at an absorbance of 600 nm. Exponential phase cells were harvested at a density of 1.5 x $10^7$ cells/ml. Glassware was washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water to remove zinc contamination.

## DNA Manipulations, PCR, and Site-directed Mutagenesis

Standard methods were used for isolation and manipulation of DNA (180). PCR reactions were optimized as described previously (181). Site-specific mutations in plasmids were generated with the QuikChange site-directed mutagenesis kit. All mutations were confirmed by DNA sequencing.

## Plasmid Constructions

The plasmids used in this work are listed in Table II. The pKSK11 plasmid contains the *CKI1* gene promoter fused to the coding sequence of the *lacZ* gene of *E. coli*. This plasmid was constructed by replacing the *CRD1* gene promoter in pSD90 (a plasmid based on YEp357R) with the *CKI1* gene promoter sequence at the SphI/KpnI sites. The *CKI1* promoter was obtained by PCR using the primers shown on Table III and by using strain W303-1A genomic DNA as the template. The PCR primer used in the forward

direction corresponds to -308 bp from the start codon, and the primer used in the reverse direction corresponds to + 23 bp from the start codon. The correct orientation of the *CKI1* promoter was confirmed by restriction enzyme digestion.

Plasmid pCK-$UAS_{INO}$ is a derivative of pKSK11, in which the $UAS_{INO}$ element (1) sequence (5'TATTCACAT-3') in the *CKI1* promoter was mutated to a nonconsensus sequence 5'-TATTTTTTTT-3'. Mutagenesis was performed with the QuikChange site-directed mutagenesis kit using plasmid pKSK11 as the template and the mutagenic primers presented on Table IV. Plasmids pCK-ZRE1 and pCK-ZRE2 were also derivatives of pKSK11, in which the putative $UAS_{ZRE}$ element sequences ZRE1 and ZRE2 in the *CKI1* promoter were mutated to the nonconsensus sequence 5'-AAAAAAAAAA-3' using the primers shown on Table V. Plasmid pCK-ZRE1,2 is a derivative of pKSK11 where both ZRE1 and ZRE2 were mutated to the previous nonconsensus sequence. pCK-ZRE1,2 was constructed by PCR amplification of plasmid pCK-ZRE1 with primers used for the construction pCK-ZRE2. After PCR amplifications, all samples were digested with DpnI to eliminate the template DNA. The plasmids were amplified in *E. coli*, purified, and sequenced to confirm the mutations in the *CKI1* promoter. Transformation of yeast (182, 183) and bacteria (180) with plasmids were performed as described previously.

The pFP1 plasmid contains the *PAH1* gene promoter fused to the coding sequence of the *lacZ* gene of *E. coli*. This plasmid was constructed by replacing the *DPP1* gene promoter in pJO2 (a plasmid based on YEp357R) with the *PAH1*gene promoter sequence at the KpnI/EcoRI site. The *PAH1* promoter was obtained by PCR using the primers shown on Table III and by using strain W303-1A genomic DNA as the template. The

PCR primer used in the forward direction corresponds to -1000 bp from the start codon, and the primer used in the reverse direction corresponds to +3 bp from the start codon. The correct orientation of the *PAH1* promoter was confirmed by restriction enzyme digestion.

## Electrophoretic Mobility Shift Assay

Double-stranded oligonucleotides used in the electromobility shift assays are presented in Table VI. They were prepared by annealing 25 μM complementary single-stranded oligonucleotides in a reaction mixture (0.1 ml) containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The annealing reactions were incubated for 5 min at 100 $^{o}$C in a heat block, and kept in the heat block for an additional 2 h after it had been turned off. The annealed oligonucleotides (100 pmol), which had a 5' overhanging end, were labeled with [$\gamma$-$^{32}$P]dATP (400-800 Ci/mmol) and Klenow fragment (5 units) for 30 min at room temperature. The labeled oligonucleotides were separated from unincorporated nucleotides by gel filtration using ProbeQuant G-50 spin columns. Purified recombinant GST-Zap1p$^{687–880}$ (124) was incubated with 1 pmol of radiolabeled DNA probe (2.0 x $10^5$ cpm/pmol) for 15 min at room temperature in a total volume of 10 μl. The reaction buffer contained 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dI-dC)•poly(dI-dC), 0.2 mg/ml bovine serum albumin, 0.04% IGEPAL CA-630, and 10 % glycerol. Following incubation, the reaction mixture was resolved on a 6 % polyacrylamide gel (1.5-mm thickness) in 0.5X Tris-borate-EDTA buffer at 100 V for 45 min. Gels were dried onto blotting paper, and the radioactive signals were visualized by phosphorimaging analysis.

TABLE I

Strains used in this study

| Strain | Genotype or relevant characteristics | Source or Ref |
|---|---|---|
| *E. coli* | | |
| DH5α | F$^-$ φ80*dlacZ*ΔM15 Δ(*lacZYA-arg*F) U169 *deo*R *rec*A1 *end*A1 *hd*R17($r_k^-$ $m_k^+$) *pho*A *sup*E44 l$^-$*thi*-1 *gyr*A96 *rel*A1 | (180) |
| *S. cerevisiae* | | |
| W303-1A | *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* | (184) |
| DY1457 | *MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52* | (185) |
| ZHY6 | *MAT***a** *ade6 can1-100oc his3 leu2 ura3 zap1Δ::TRP1* | (185) |
| ZHY3 | *MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 zrt1Δ::LEU2 zrt2Δ::HIS3* | (186) |
| SH304 | *MAT***a** *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 opi1Δ::LEU2* | S. A. Henry |
| SH303 | *MAT***a** *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino2Δ::TRP1* | S. A. Henry |
| SH307 | *MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino4Δ::LEU2* | S. A. Henry |
| GHY57 | *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 pah1Δ::URA3* | (54) |
| TBY1 | *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 dpp1Δ::TRP1/Kan$^r$ lpp1Δ::HIS3/Kan$^r$* | (156) |
| GHY58 | *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 dpp1Δ::TRP1/Kan$^r$ lpp1Δ::HIS3/Kan$^r$ pah1Δ::URA3* | (54) |
| *pdr5*Δ | *MAT***a** *his3-1 leu2-0 met15-0 ura3-0 pdr5Δ::KanMX* | Invitrogen |

TABLE II

Plasmids used in this study

| Plasmid | Relevant characteristics | Source or Ref. |
|---|---|---|
| pSD90 | $P_{CRD1}$-*lacZ* reporter gene containing the *CRD1* promoter with *URA3* | W. Dowhan |
| pKSK11 | $P_{CKI1}$-*lacZ* reporter gene containing the *CKI1* promoter with *URA3* | (187) |
| pCK-ZRE1 | Derivative of pKSK11 with mutations in ZRE1 | (187) |
| pCK-ZRE2 | Derivative of pKSK11 with mutations in ZRE2 | (187) |
| pCK-ZRE1, 2 | Derivative of pKSK11 with mutations in ZRE1 and ZRE2 | (187) |
| pCK-$UAS_{INO}$ | Derivative of pKSK11 with mutations in the $UAS_{INO}$ element | (187) |
| pDg2 | $P_{CYC1\text{-}ZRE}$-*lacZ* reporter gene containing the $UAS_{ZRE}$ element with *URA3* | (116) |
| pFP1 | $P_{PAH1}$-*lacZ* reporter gene containing the *PAH1* promoter with *URA3* | F. Pascual |
| YEp351 | Multicopy *E. coli*/yeast shuttle vector with *LEU2* | (188) |
| pGH312 | $PAH1^{HA}$ gene inserted into YEp351 | (54) |

TABLE III

Primers used to construct the pKSK11 and pFP1plasmid

| Oligonucleotide | Sequence |
|---|---|
| *CKI1*-lacZ F | 5’- TCAGCATGCCTGCAGATATGAATTCCATAGG-3’ |
| *CKI1*-lacZ R | 5’- CGAGGTACCCCTGGACGTGATTCTTGTAC-3’ |
| *PAH1*-lacZ F | 5’- GCGGTACCTAGAGTCCAAACTCAACAGCC -3’ |
| *PAH1*-lacZ R | 5’- GCCGGAATTCATAATCGACCGATGTGTC -3’ |

TABLE IV

Primers used to construct the pCK-$UAS_{INO}$ plasmid

| Oligonucleotide | Sequence |
|---|---|
| CK-$UAS_{INO}$ F | 5’- CTTGTTCTTTGTTCTTTATGGTATAATATTTTTTTTGTGCT CTACCGTTTTTCTTGTCGGCCCAGC- 3’ |
| CK-$UAS_{INO}$ R | 5’- GCTGGGCCGACAAGAAAAACGGTAGAGCACAAAAAAAA TATTATACCATAAAGAACAAAGAACAAG -3’ |

TABLE V

Primers used to construct the pCK-ZRE1, pCK-ZRE2 and pCK-ZRE1, 2 plasmids

| Oligonucleotide | Sequence |
|---|---|
| CK-ZRE1 F | 5'-CTAAGCGATTGGTAACCAAAAAAAAAAAAGAACACCACCAAC-3' |
| CK-ZRE1 R | 5'- GTTGGTGGTGTTCTTTTTTTTTTTTGGTTACCAATCGCTTAC-3' |
| CK-ZRE2 F | 5'-CAGATCGTTCTCTTGTTCTTTGAAAAAAAAAAAATAATATTCACA TGGTGCTCTAC -3 |
| CK-ZRE2 R | 5'-GTAGAGCACCATGTGAATATTATTTTTTTTTTTCAAAGAACAAG AGAACGATCTG -3' |

TABLE VI

Oligonucleotides Used for Electrophoretic Mobility Shift Assay

| Element | Annealed oligonucleotides* |
|---|---|
| *CKI1* ZRE1 | 5'- GTAACCTCCTTCACTTTAGAaca -3' |
| | 3'- CATTGGAGGAAGTGAAATCTTGA -5' |
| *CKI1* ZRE2 | 5'- TCTTTGTTCTTTATGGTATAata -3' |
| | 3'- AGAAACAAGAAATACCATATTAT -5' |
| *CKI1* ZRE3 | 5'- GGTTAAATCTCGAAGAGACAgaa -3' |
| | 3'- CCAATTTAGAGCTTCTCTGTCTT -5' |
| *PAH1* ZRE1 | 5'- GGTCTACCAATCTGAAGGTGTGTTagg -3' |
| | 3'- ccaGATGGTTAGACTTCCACACAATCC -5' |
| *PAH1* ZRE2 | 5'- AGCAGCGCACGGTGAGGGTAGAAGgaa -3' |
| | 5'- tcgTCGCGTGCCACTCCCATCTTCCTT -5' |
| *PAH1* ZRE3 | 5'-ACAGTTTTACCTTCTAAGAAACATaca -3' |
| | 3'-tgtCAAAATGGAAGATTCTTTGTATGT -5' |

* Underlined sequences are putative ZRE sites. The lower case letters indicate the nucleotides filled with the Klenow fragment.

## Preparation of Cell Extracts, Cytosolic Fractions and Protein Determination

All steps were performed at 4 °C. Yeast cells were disrupted with glass beads with a Mini BeadBeater-16 (Biospec Products) in 50 mM Tris-maleate buffer (pH 7.0) containing 1 mM EDTA, 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 pepstatin (189). Glass beads and cell debris were removed by centrifugation at 1,500 x g for 10 min, and the supernatant was used as the cell extract. The cell extract was centrifuged at 100,000 x g for 1 hour to separate the cytosolic from the membrane fraction. Protein concentration was determined by the Coomassie Blue dye-binding assay of Bradford (190) using bovine serum albumin as the standard.

## Choline Kinase Activity Assay

Choline kinase activity was measured in a reaction mixture that contained 67 mM glycine-NaOH buffer (pH 9.5), 5 mM [*methyl*-$^{14}$C]choline (2,000 cpm/nmol), 5 mM ATP, 10 mM $MgSO_4$, 1.3 mM dithiothreitol, and enzyme in a final volume of 60 µl (137). The radiolabeled product phosphocholine was separated from the radiolabeled substrate by precipitation of choline as a reineckate salt and quantified by liquid scintillation counting. (191). A unit of choline kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphocholine/min. All enzyme assays were conducted in triplicate, and the average standard deviation of the assays was ± 5%. Enzyme reactions were linear with time and protein concentration.

## Preparation of $^{32}$P-Labeled PA

$^{32}$P-labeled PA was synthesized from DAG and [γ-$^{32}$P]ATP using purified *E. coli* DAG kinase (192). Labeled PA was synthesized in the presence of 1 mM cardiolipin, 50 mM imidazole (pH 6.6), 50 mM octyl-β-D-glucopyranoside, 50 mM sodium chloride, 12.5 mM magnesium chloride, 1 mM EGTA, 10 mM β-mercaptoethanol, 5 mM ATP, 0.25 mg DAG, [γ-$^{32}$P]ATP (3000Ci/mmol) and DAG kinase in a final volume of 0.1 ml at 30°C for 1 hour. The reaction was stopped with 0.5 ml acidic methanol, 1 ml chloroform and 1 ml $MgCl_2$. The aqueous and chloroform soluble phases were separated by centrifugation. The chloroform phase was dried *in vacuo*, resuspended in chloroform and separated by thin-layer chromatography using the solvent system chloroform/methanol/water (65:25:4, v/v) (193). The PA was purified from the silica and solubilized with 2 mM PA on 20 mM Triton X-100.

## PA Phosphatase Activity Assay

PAP activity was measured by following the release of water-soluble $^{32}P_i$ from chloroform-soluble [$^{32}$P]PA for 20 min at 30 °C in a total reaction volume of 0.1 ml (193). For measurement of PAP activity, the reaction mixture contained 50 mm Tris-HCl buffer (pH 7.5), 0.2 mM [$^{32}$P]PA (10,000-12,000 cpm/nmol), 2 mM Triton X-100, 5 mm $MgCl_2$ and enzyme protein to a final volume of 0.1 ml. Because the unknown PAP is sensitive to treatment with NEM, the PAP activity assay was done in the presence and absence of 25 mM NEM to distinguish between these two PAP activities. A unit of PAP activity was defined as the amount of enzyme that catalyzed the dephosphorylation of 1 nmol of PA/min. Specific activity was defined as units/mg of protein. All enzyme assays

were conducted in triplicate, and the average standard deviation of the assays was ± 5%. Enzyme reactions were linear with time and protein concentration.

### β-galactosidase Activity Assay

β-galactosidase activity was measured for 5 min at room temperature by following the release of *O*-nitrophenol from *O*-nitrophenyl β-D-galactopyranoside at 410 nm. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 3 mM *O*-nitrophenyl-β-D-galactopyranoside, and enzyme in a total volume of 0.1 ml. A unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol *O*-nitrophenol/min. Specific activity was defined as units/mg of protein. All enzyme assays were conducted in triplicate, and the average standard deviation of the assays was ± 5%. Enzyme reactions were linear with time and protein concentration.

### Labeling and Analysis of CDP-Choline Pathway Intermediates and PC

Exponential phase cells were labeled for five to six generations with 10 μM [*methyl*-$^{14}$C]choline (0.2 μCi/ml). The CDP-choline pathway intermediates and phosphatidylcholine were extracted from whole cells by a chloroform/methanol/water extraction, followed by the separation of the aqueous and chloroform phases (194). The aqueous and chloroform phases that contained the CDP-choline pathway intermediates and PC, respectively, were dried *in vacuo* and then dissolved in 100 μl of methanol: water (1:1, v/v) and 100 μl of chloroform, respectively. The CDP-choline pathway intermediates and PC were subjected to thin-layer chromatography (TLC) on silica gel

plates using the solvent systems methanol/0.6% sodium chloride/ammonium hydroxide (10:10:1, v/v) and chloroform/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v), respectively (195). The positions of the labeled compounds on silica gel plates were determined by phosphorimaging and compared with standards. The amount of each labeled compound was determined by liquid scintillation counting and normalized based on cell number.

## Labeling and Analysis of Neutral Lipids

Steady-state labeling of neutral lipids with [2-$^{14}$C]acetate was performed as described previously (196). Total lipids were extracted by following the method of Bligh and Dyer (194). Neutral lipids were analyzed by one-dimensional TLC in silica-gel 60 plates using the solvent system hexane/ diethyl ether/ glacial acetic acid (40:10:1, v/v) (197). The position of the labeled lipids on the TLC plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis. The relative amount of each labeled lipid was analyzed using the ImageQuant software.

## Data Analyses

The Student's *t*-test (SigmaPlot software) was used to determine statistical significance, and $p$ values $< 0.05$ were taken as a significant difference.

# RESULTS

## PART I

*Regulation of the Saccharomyces cerevisiae CKI1-encoded Choline Kinase in Response to Zinc Depletion*

### Effect of Zinc Depletion on the Expression of *CKI1*

In this and in subsequent experiments, cells were grown in medium that lacked inositol supplementation to obviate the regulatory effects that inositol has on phospholipid metabolism (2, 4, 69). Cells were grown in a defined zinc-free medium that contained 1.5 µM zinc sulfate. This concentration of zinc was equivalent to that found in standard synthetic growth media (178). To show the detrimental effect of zinc deprivation on the growth of the yeast *Saccharomyces cerevisiae*, wild type cells were grown in the absence and presence of zinc and the growth was monitored for 48 hours at a wavelength of 650 nm using a Thermo Max Microplate reader (Fig. 11). The absence of zinc from the growth media resulted in a 3.5-fold reduction in the growth of zinc-depleted cells when compared to cell grown in the presence of zinc (Fig. 11). This result demonstrates the importance of zinc as a nutrient for the growth of yeast.

In this and subsequent experiments cells were harvested at the exponential phase of growth to obviate the depletion of other nutrients as cells enter the stationary phase of growth and the regulatory effects that has on phospholipid metabolism. To confirm that our growth conditions depleted the intracellular level of zinc, we made use of a $P_{CYC1\text{-}ZRE}$-*lacZ* reporter gene assay that is sensitive to the labile pool of intracellular zinc (43, 116). In this assay, when the total intracellular concentration of zinc is limiting (~10 pmol/$10^6$

cells), the transcriptional activity of Zap1p is up-regulated, and this results in elevated levels of β-galactosidase activity (43, 117). As described previously (43), the β-galactosidase activity of wild type zinc-depleted cells was greatly induced when compared with cells that were grown with zinc (Fig. 12).

The effect of zinc depletion on the expression of the *CKI1* gene was examined by use of a $P_{CKI1}$-*lacZ* reporter gene. This reporter gene was constructed by fusing the *CKI1* promoter in frame with the coding sequence of the *E. coli lacZ* gene. Thus, the expression of β-galactosidase activity was dependent on transcription driven by the *CKI1* promoter. Wild type cells bearing the $P_{CKI1}$-*lacZ* reporter gene were grown to the exponential phase in the absence or presence of increasing concentrations of zinc. Following growth, cell extracts were prepared and used for the assay of β-galactosidase activity. The depletion of zinc from the growth medium resulted in a concentration-dependent increase in *CKI1* expression (Fig. 13). The β-galactosidase activity in zinc-depleted cells was 7-fold greater than the activity found in cells grown with 1.5 μM zinc. *CKI1* expression was not further affected by 5 μM zinc.

## Effect of the *zrt1*Δ *zrt2*Δ Mutation on the Zinc-mediated Regulation of *CKI1* Expression

To confirm that the zinc-mediated regulation of *CKI1* expression was due to the intracellular level of zinc, $P_{CKI1}$-*lacZ* reporter gene activity was examined in a *zrt1*Δ *zrt2*Δ double mutant grown in the presence of zinc. This mutant lacks both the high (Zrt1p) and low (Zrt2p) affinity plasma membrane zinc transporters and contains a low cytoplasmic level of zinc even when the growth medium contains a standard amount of

**Figure. 11. Effect of zinc depletion on the growth of the yeast *S. cerevisiae*.** The growth of wild type cells grown in the absence or presence of 1.5 μM $ZnSO_4$ at 30°C was monitored at 650 nm using a microplate reader. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

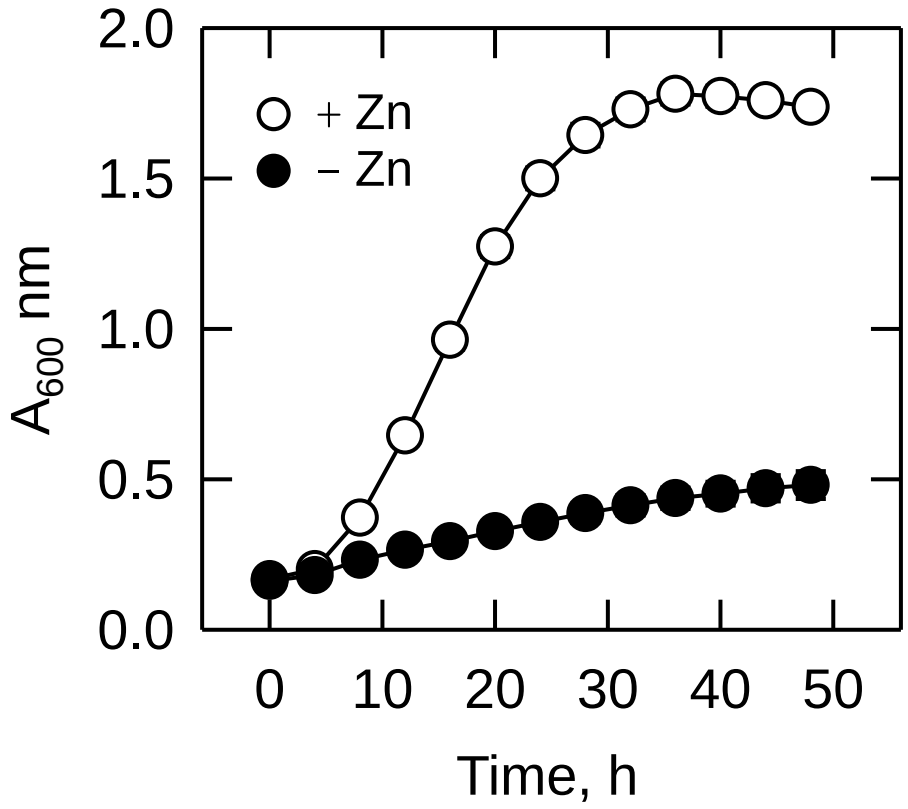

2.0
1.5
1.0
0.5
0.0
$A_{600}$ nm
+ Zn
– Zn
0
10
20
30
40
50
Time, h

**Figure. 12. Effect of zinc depletion on the expression of β-galactosidase activity in wild type cells bearing the $P_{CYC1\text{-}ZRE}$-*lacZ* reporter gene.** Wild type cells bearing the $P_{CYC1\text{-}ZRE}$-*lacZ* reporter plasmid pDg2 were grown to the exponential phase of growth in the presence of indicated concentrations of $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments $\pm$ S.D.

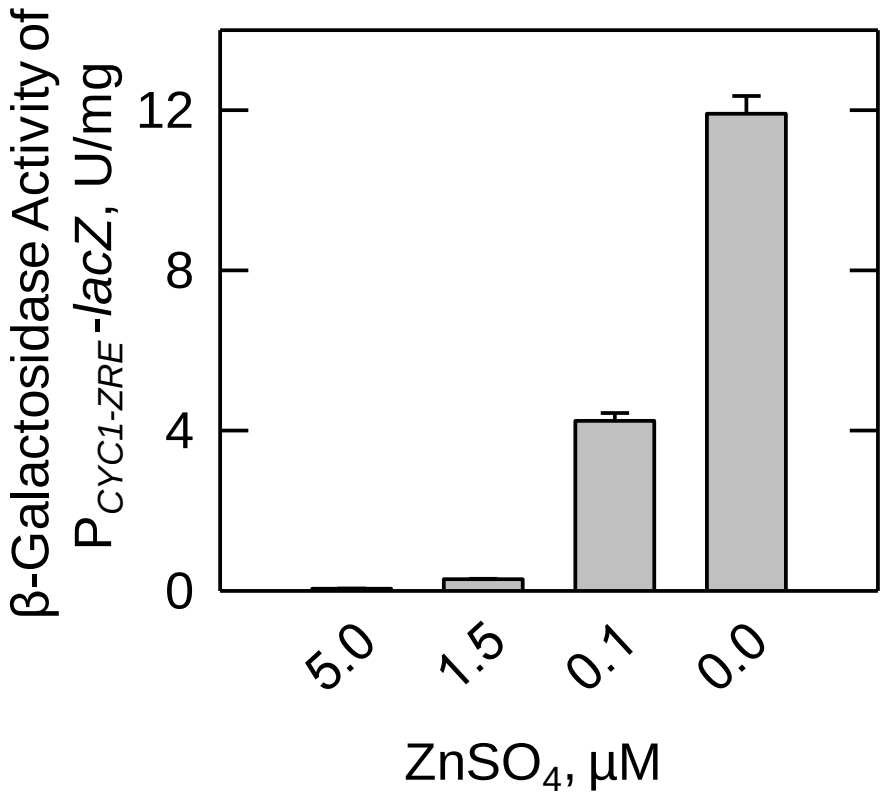
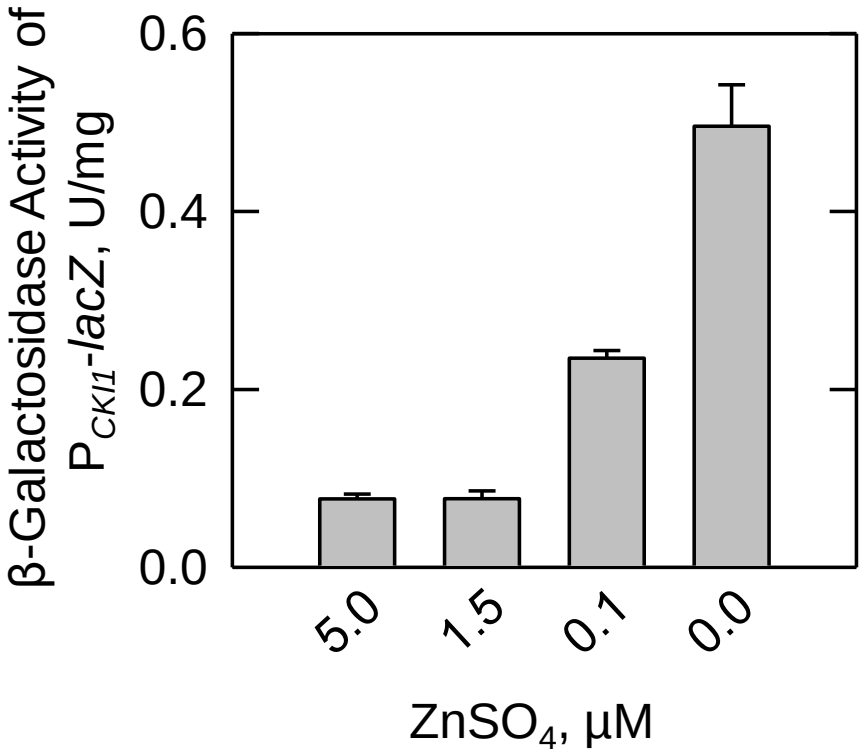

β-Galactosidase Activity of
P CYC1-ZRE -lacZ, U/mg
12
8
4
0
5.0
1.5
0.1
0.0
ZnSO4, μM

**Figure. 13. Effect of zinc depletion on the expression of β-galactosidase activity in wild type cells bearing the $P_{CKI1}$-*lacZ* reporter gene.** Wild type cells bearing the $P_{CKI1}$-*lacZ* reporter plasmid pKSK11 were grown to the exponential phase of growth in the presence of indicated concentrations of $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments $\pm$ S.D.


β-Galactosidase Activity of
P$_{CKI1}$-*lacZ*, U/mg
0.6
0.4
0.2
0.0
5.0
1.5
0.1
0.0
ZnSO$_4$, μM

zinc (109, 110). The *zrt1*Δ *zrt2*Δ double mutant exhibited a high level of β-galactosidase activity that was similar to that shown for wild type cells grown without zinc (Fig. 14). Thus, the induction of *CKI1* expression was mediated by a low intracellular level of zinc.

**Effect of the *zap1*Δ Mutation on the Zinc-mediated Regulation of *CKI1* Expression**

The *CKI1* promoter contains putative $UAS_{ZRE}$ sequences (Fig. 16A) that are potential binding sites for the Zap1p transcription factor (116). Accordingly, we questioned whether the induction of *CKI1* expression by zinc depletion was dependent on Zap1p function. To address this question, the $P_{CKI1}$-*lacZ* reporter gene activity was examined in *zap1*Δ mutant cells that were grown in the presence and absence of zinc. The β-galactosidase activity of *zap1*Δ mutant cells grown with zinc was similar to the activity found in wild type cells grown with zinc (Fig. 15). However, when cells were grown without zinc, the β-galactosidase activity in the *zap1*Δ mutant was 4.4-fold lower than that found in the wild type control (Fig. 15). Thus, the *zap1*Δ mutation attenuated the induction of *CKI1* expression when cells were depleted for zinc.

**Binding of Zap1p to Putative $UAS_{ZRE}$ Sequences in the *CKI1* Promoter**

There are three putative $UAS_{ZRE}$ sequences in the *CKI1* promoter for Zap1p binding (Fig. 16A). The percent identities of ZRE1, ZRE2, and ZRE3 to the consensus $UAS_{ZRE}$ sequence (ACCTTNAAGGT) (116) are 64, 73, and 64%, respectively. We questioned whether Zap1p would bind to labeled oligonucleotides containing these sequences using the electrophoretic mobility shift assay. Purified recombinant GST-$Zap1p^{687-880}$ (amino acids 687-880 of the Zap1p binding domain (119)) interacted with ZRE1 and ZRE2, but not with ZRE3 (Fig. 16B). The interaction of GST-$Zap1p^{687-880}$ with ZRE2 was 2.5-fold

**Figure. 14. Effect of *zrt1*Δ *zrt2*Δ mutations on the expression of the $P_{CKI1}$-*lacZ* reporter gene.** Wild type (WT) and *zrt1*Δ *zrt2*Δ mutant cells bearing the $P_{CKI1}$-*lacZ* reporter plasmid pKSK11 were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments $\pm$ S.D.

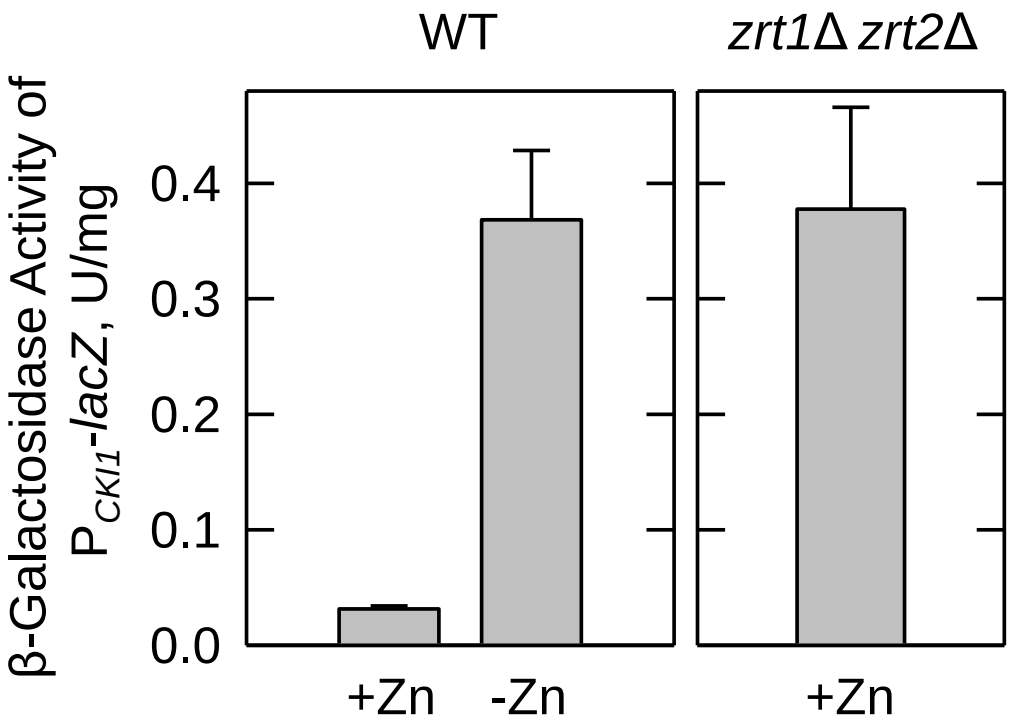
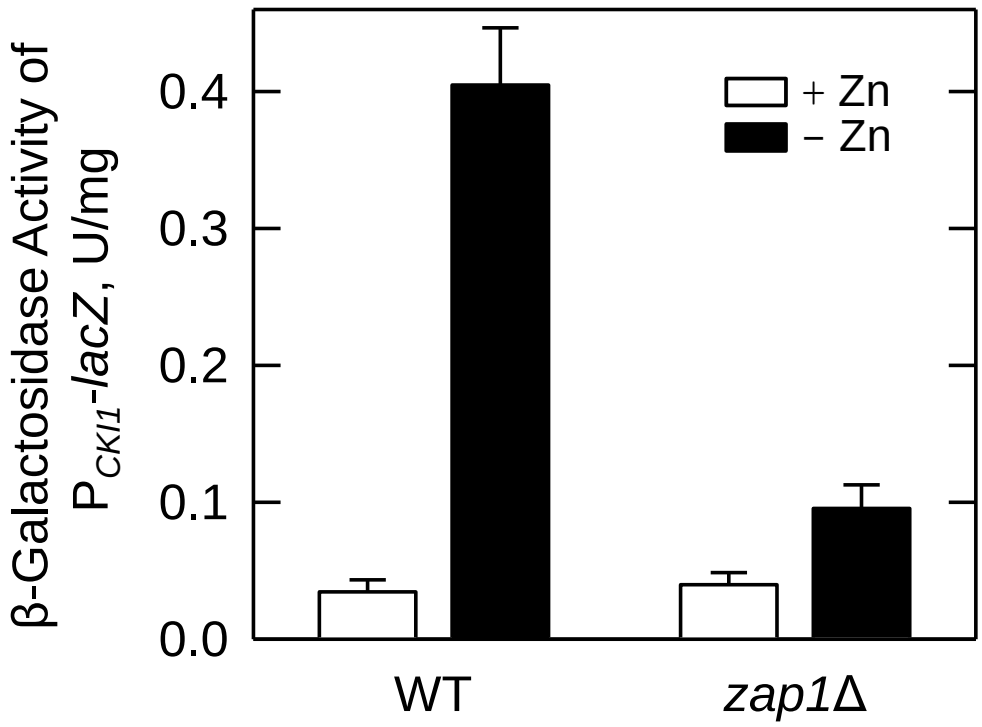

WT
zrt1Δ zrt2Δ
β-Galactosidase Activity of
P_CKI1-lacZ, U/mg
0.0
0.1
0.2
0.3
0.4
+Zn
-Zn
+Zn

**Figure. 15. Effect of the *zap1*Δ mutation on the expression of the $P_{CKI1}$-*lacZ* reporter gene.** Wild type (WT) and *zap1*Δ mutant cells bearing the $P_{CKI1}$-*lacZ* reporter plasmid pKSK11 were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

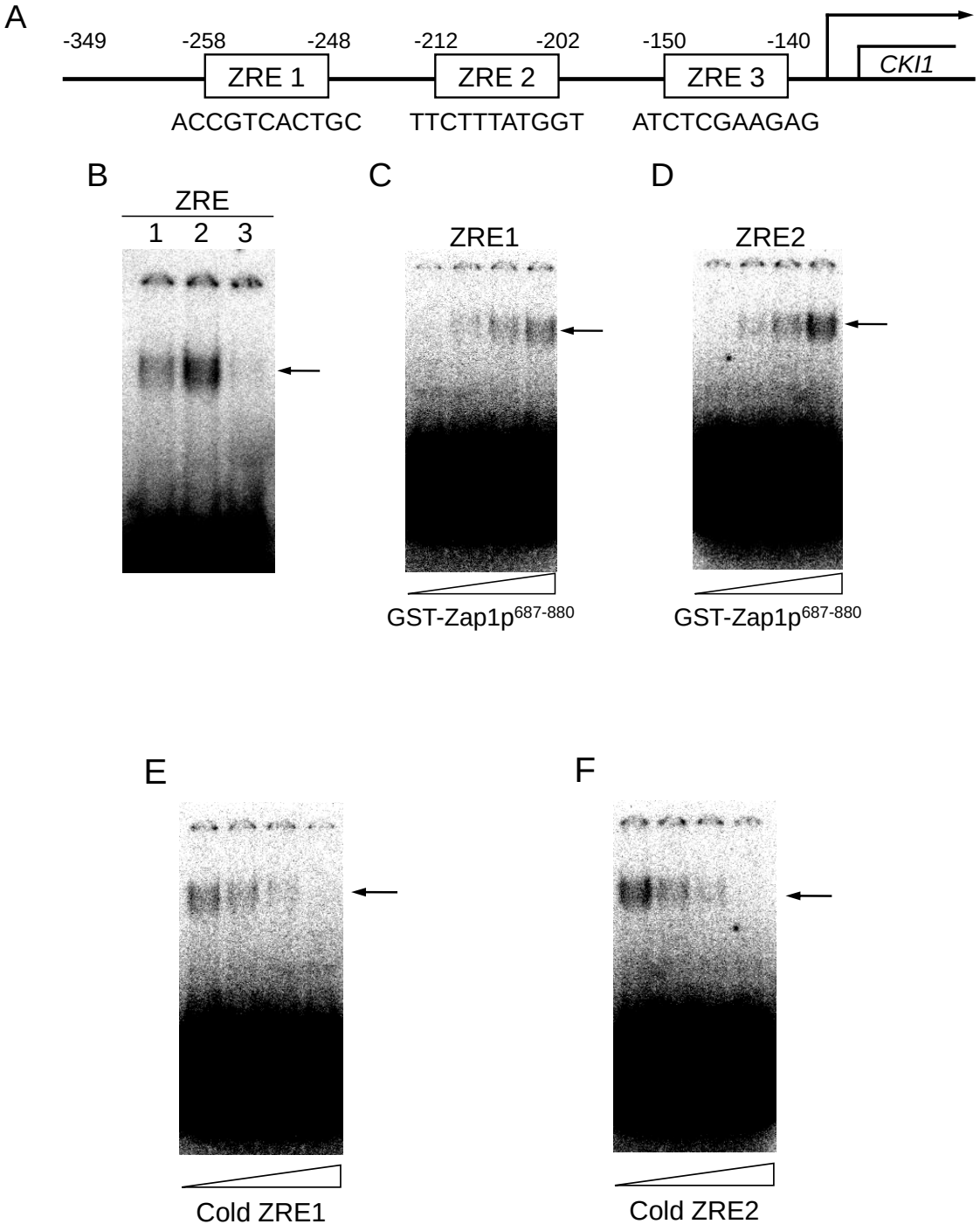

β-Galactosidase Activity of
P$_{CKI1}$-*lacZ*, U/mg
0.0
0.1
0.2
0.3
0.4
+ Zn
– Zn
WT
*zap1*Δ

greater when compared with ZRE1. The specificity of GST-Zap1p$^{687-880}$ binding to ZRE1 and ZRE2 was examined further. The formation of the GST-Zap1p$^{687-880}$-ZRE1 and GST-Zap1p$^{687-880}$-ZRE2 complexes was dependent on the concentration of GST-Zap1p$^{687-880}$ (Fig. 16C and 16D, respectively). Moreover, unlabeled ZRE1 and ZRE2 probes competed with their labeled probe counterparts for GST- Zap1p$^{687-880}$ interactions (Fig 16E and 16F, respectively), and mutations in ZRE1 and ZRE2 to a nonconsensus $UAS_{ZRE}$ sequence abolished interactions with GST-Zap1p$^{687-880}$ (data not shown).

## Effects of ZRE1 and ZRE2 Mutations on the Zinc-mediated Regulation of *CKI1* Expression

We sought evidence that the induction of *CKI1* in response to zinc depletion was dependent on the $UAS_{ZRE}$ sequences in the *CKI1* promoter. The ZRE1 and ZRE2 sequences within the $P_{CKI1}$-*lacZ* reporter gene were mutated to the nonconsensus $UAS_{ZRE}$ sequence 5'-AAAAAAAAAAA-3'. Cells bearing the wild type or mutant $P_{CKI1}$-*lacZ* reporter genes were grown in the presence and absence of zinc; cell extracts were prepared and assayed for β-galactosidase activity. The ZRE1 and ZRE2 mutations caused the attenuation of *CKI1* induction in response to zinc depletion (Fig. 17). The β-galactosidase activity of zinc-depleted cells bearing the $P_{CKI1}$-*lacZ* reporter gene with the ZRE1 and ZRE2 mutations was 1.6- and 3.2-fold lower, respectively, when compared with cells bearing the control reporter gene. These results supported the conclusion that the zinc-mediated regulation of *CKI1* expression was dependent on ZRE1 and ZRE2. These results were consistent with the electrophoretic mobility shift assays that indicated

**Figure. 16. Interactions of GST-Zap1p$^{687-880}$ with putative ZRE sequences in the *CKI1* promoter. (**A), the locations and sequences of the putative ZRE sites in the *CKI1* promoter are shown in the figure. (B), samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.0 x $10^5$ cpm/pmol) with sequences for ZRE1 (*lane 1*), ZRE2 (*lane 2*), and ZRE3 (*lane 3*), in the *CKI1* promoter were incubated with 0.5 μg of purified recombinant GST-Zap1p$^{687-880}$. The ZRE1 (C) and ZRE2 (D) radiolabeled probes were incubated with 0, 0.15, 0.3 and 0.5 μg of recombinant GST-Zap1p$^{687-880}$. (E) and (F), GST-Zap1p$^{687-880}$ was incubated with 0, 25, 50 and 100 pmol of unlabeled oligonucleotide with the sequences for ZRE1 and ZRE2, respectively. Interaction of GST-Zap1p$^{687-880}$ with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6 % polyacrylamide gel. The data shown are representative of two independent experiments. The arrow indicates the position of the GST- Zap1p$^{687-880}$-ZRE complex.

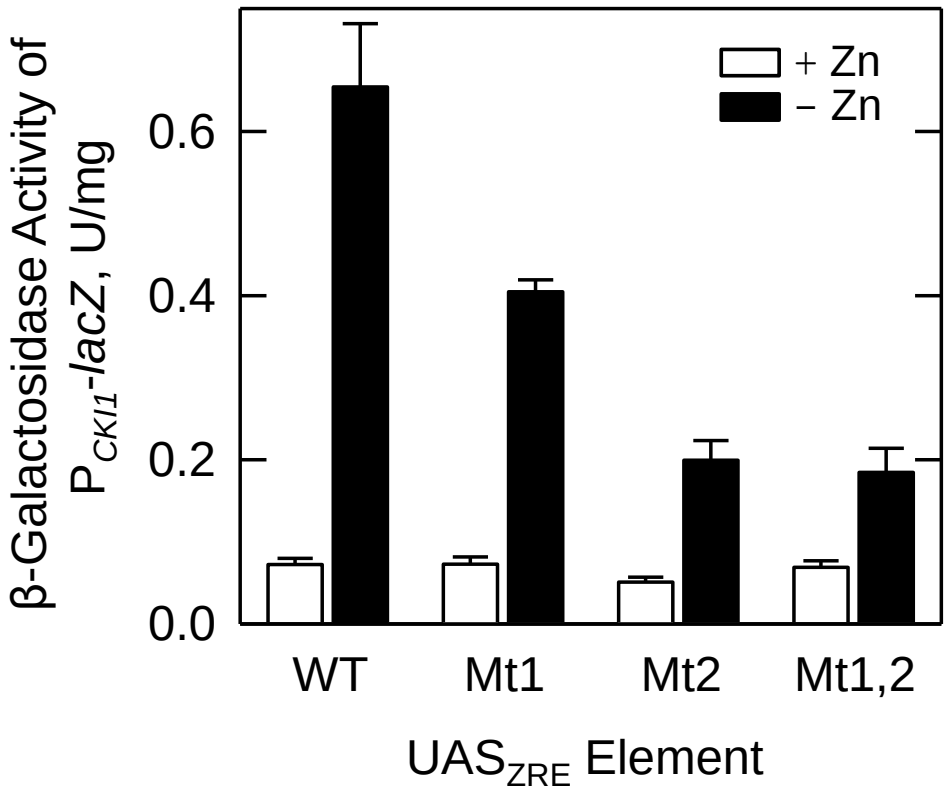

A
-349
-258
-248
-212
-202
-150
-140
ZRE 1
ZRE 2
ZRE 3
CKI1
ACCGTCACTGC
TTCTTTATGGT
ATCTCGAAGAG
B
ZRE
1 2 3
C
ZRE1
GST-Zap1p687-880
D
ZRE2
GST-Zap1p687-880
E
Cold ZRE1
F
Cold ZRE2

that the regulation was primarily governed by ZRE2. We considered the possibility that the full extent of induction was dependent on interaction of Zap1p with ZRE1 and ZRE2 together. However, the effects of the ZRE1 and ZRE2 mutations on the zinc-mediated regulation of *CKI1* expression were not additive or synergistic. The β-galactosidase activity in zinc-depleted cells bearing the reporter gene with the ZRE1 and ZRE2 mutations made in combination was the same as that found in cells bearing the reporter gene with the ZRE2 mutation alone (Fig. 17).

### Effects of *opi1*Δ, *ino2*Δ, and *ino4*Δ Mutations, and a $UAS_{INO}$ Element Mutation in the *CKI1* Promoter on the Zinc-mediated Regulation of *CKI1* Expression

The *CKI1* promoter contains a $UAS_{INO}$ element (-189 to -197) that is four bases downstream of ZRE2. The $UAS_{INO}$ element is a binding site for the transcriptional activator Ino2p-Ino4p complex (198) that is responsible for maximum expression of phospholipid synthesis $UAS_{INO}$-containing genes (2, 4, 5, 69). Expression of the $UAS_{INO}$-containing genes is balanced by the Opi1p repressor (64), which attenuates transcription through its interaction with Ino2p (2, 4, 5, 69, 199). Given the close proximity of ZRE2 with the $UAS_{INO}$ element in the *CKI1* promoter, we questioned whether the functions of Opi1p, Ino2p, or Ino4p affected the zinc-mediated regulation of *CKI1* expression. As expected, the expression of the $P_{CKI1}$-*lacZ* reporter gene was elevated (2.6-fold) in the *opi1*Δ mutant and reduced (5-fold) in the *ino2*Δ and *ino4*Δ mutants (Fig. 18A). Thus, Ino2p and Ino4p played a positive role in *CKI1* expression and Opi1p played a negative role in expression. The *opi1*Δ mutation did not have a major effect on the induced expression of the $P_{CKI1}$-*lacZ* reporter gene in response to zinc depletion (Fig. 18A), indicating that Opi1p did not play a role in the zinc-mediated

**Figure. 17. Effects of mutations in ZRE1 and ZRE2 in the *CKI1* promoter on the expression of the $P_{CKI1}$-*lacZ* reporter gene in response to zinc depletion.** Wild type cells bearing the $P_{CKI1}$-lacZ reporter plasmids pKSK11 (*WT*), pCK-ZRE1 (*Mt1*, mutation of ZRE1), pCK-ZRE2 (*Mt2*, mutation of ZRE2), and pCK-ZRE1,2 (*Mt1*,*2*, mutations of ZRE1 and ZRE2) were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.


β-Galactosidase Activity of
P_CKI1-lacZ, U/mg
0.0
0.2
0.4
0.6
+ Zn
– Zn
WT
Mt1
Mt2
Mt1,2
UAS_ZRE Element

regulation of *CKI1*. However, the depletion of zinc from the growth medium of the *ino2*Δ and *ino4*Δ mutants did not result in the induction of the $P_{CKI1}$-*lacZ* reporter gene (Fig. 18A). This indicated that Ino2p and Ino4p might play a role in the zinc-mediated regulation of *CKI1*.

The inositol auxotrophic *ino2*Δ and *ino4*Δ mutants grew very slow in synthetic medium in spite of the supplementation of the medium with 75 μM inositol. The depletion of zinc from this growth medium further reduced the growth of the mutants. We were concerned that the repressive effects of inositol supplementation on *CKI1* expression (136) and the added stress of zinc depletion on the growth of the mutants may have affected the zinc-mediated regulation of *CKI1* expression. Accordingly, we utilized another approach to examine whether the $UAS_{INO}$ element played a role in the zinc-mediated regulation of *CKI1*. The $UAS_{INO}$ element within the $P_{CKI1}$-*lacZ* reporter gene was mutated to a nonconsensus sequence (5'-TATTTTTTTT-3'), and the plasmid was expressed in wild type cells that were grown in the presence and absence of zinc. The β-galactosidase activity of cells bearing the mutant reporter gene was reduced (9.5-fold with zinc and 5-fold without zinc) when compared with the activity of cells bearing the wild type reporter gene (Fig. 18B). This result was consistent with the notion that the $UAS_{INO}$ element was required for maximum expression of *CKI1*. The depletion of zinc from the growth medium resulted in a 10-fold induction of β-galactosidase activity driven by the reporter gene with the $UAS_{INO}$ mutation (Fig. 18B). These results supported the conclusion that the $UAS_{INO}$ element was not involved with the zinc-mediated regulation of *CKI1*.

## Effect of Zinc Depletion on Choline Kinase Activity, and on the Incorporation of Choline into PC via the Kennedy Pathway

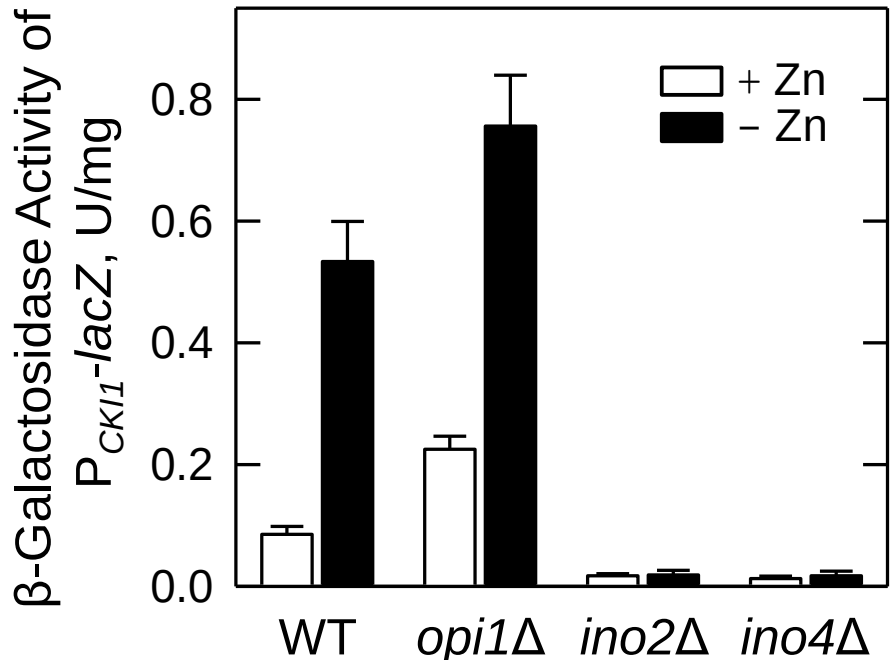
We sought evidence that the transcriptional regulation of *CKI1* in response to zinc depletion translated into changes in choline kinase activity. Wild type cells were grown in the presence and absence of zinc, cell extracts were prepared, and choline kinase activity was measured by following the incorporation of [*methyl*-$^{14}$C]choline into phosphocholine. The specific activity of choline kinase in zinc-depleted cells was 2.7-fold greater when compared with the activity from cells grown with zinc (Fig. 19). This level of induced choline kinase activity is not as great as that observed for the induction of $P_{CKI1}$-*lacZ* reporter gene activity (Fig. 15). This difference may be attributed to the stable nature of *lacZ*-fusion proteins that are not subject to proteolytic turnover when expressed in yeast (200). We also examined the expression of choline kinase activity in *zap1*Δ mutant cells. This analysis showed that the induction of choline kinase activity that occurred in wild type cells in response to zinc depletion was precluded by the *zap1*Δ mutation (Fig. 19). These results provided further evidence for the role of Zap1p in the regulation of *CKI1* expression in response to zinc.

We questioned whether the induction of choline kinase activity by zinc depletion was reflected *in vivo*. To address this question, zinc-depleted cells were labeled to steady-state with [*methyl*-$^{14}$C]choline followed by the extraction and analysis of phosphocholine by TLC. The amount of label incorporated into phosphocholine in zinc-depleted cells was 2-fold greater when compared with cells grown in the presence of zinc (Fig. 20). The choline label was also incorporated into CDP-choline and the end product of the Kennedy pathway PC. While zinc depletion did not affect the steady-state level of

CDP-choline, it caused a 1.8-fold increase in the level of PC (Fig. 20). In addition, zinc depletion caused a 4-fold increase in the cellular content of free choline (Fig. 20).

**Figure. 18. Effects of the *opi1*Δ, *ino2*Δ, and *ino4*Δ mutations, and a mutation in the $UAS_{INO}$ element in the *CKI1* promoter on the expression of the $P_{CKI1}$-*lacZ* reporter gene in response to zinc depletion.** Wild type (*WT*), *opi1*Δ, *ino2*Δ, and *ino4*Δ cells bearing the $P_{CKI1}$-*lacZ* reporter plasmid pKSK11 (A), and wild type cells bearing the $P_{CKI1}$-*lacZ* reporter plasmids pKSK11 (*WT*) and pCK-$UAS_{INO}$ (*Mt,* mutation of $UAS_{INO}$) (B) were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. The growth medium for the *ino2*Δ and *ino4*Δ mutants was supplemented with 75 μM inositol. Cell extracts were prepared and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

A


β-Galactosidase Activity of
P$_{CKI1}$-lacZ, U/mg
0.0
0.2
0.4
0.6
0.8
+ Zn
– Zn
WT
opi1Δ
ino2Δ
ino4Δ

B

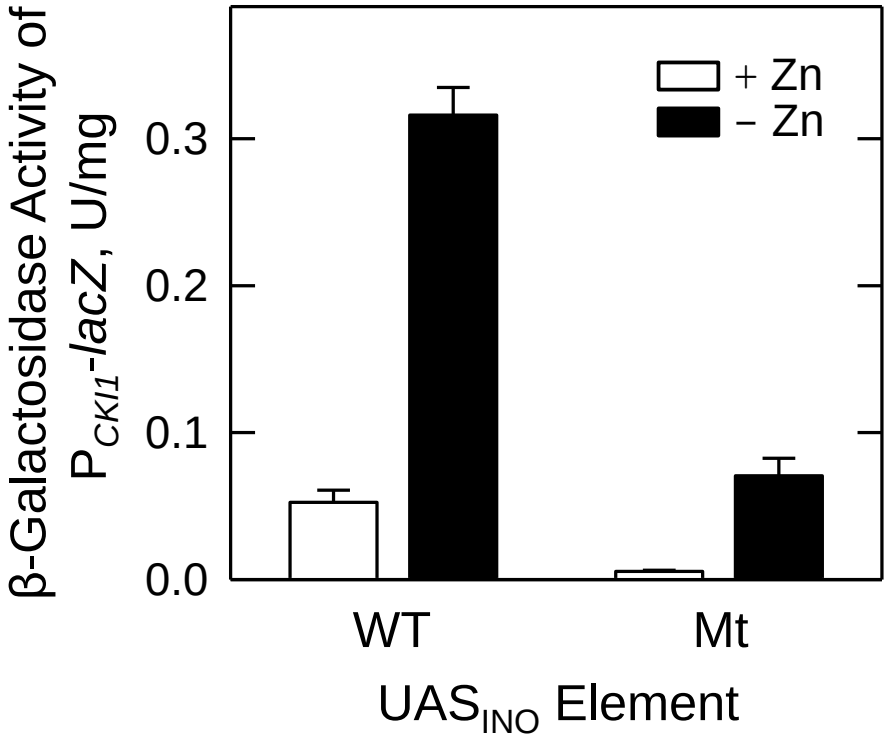

β-Galactosidase Activity of
P$_{CKI1}$-lacZ, U/mg
0.0
0.1
0.2
0.3
+ Zn
– Zn
WT
Mt
UAS$_{INO}$ Element

**Figure. 19. Effect of *zap1*Δ mutation on choline kinase activity in response to zinc depletion.** Wild type (*WT*) and *zap1*Δ mutant cells were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed for choline kinase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

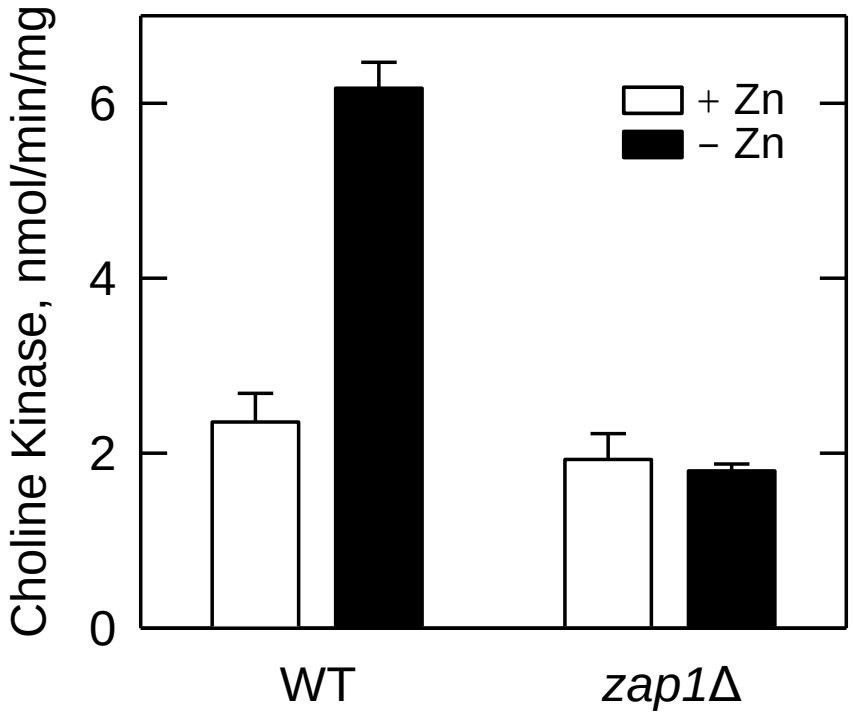
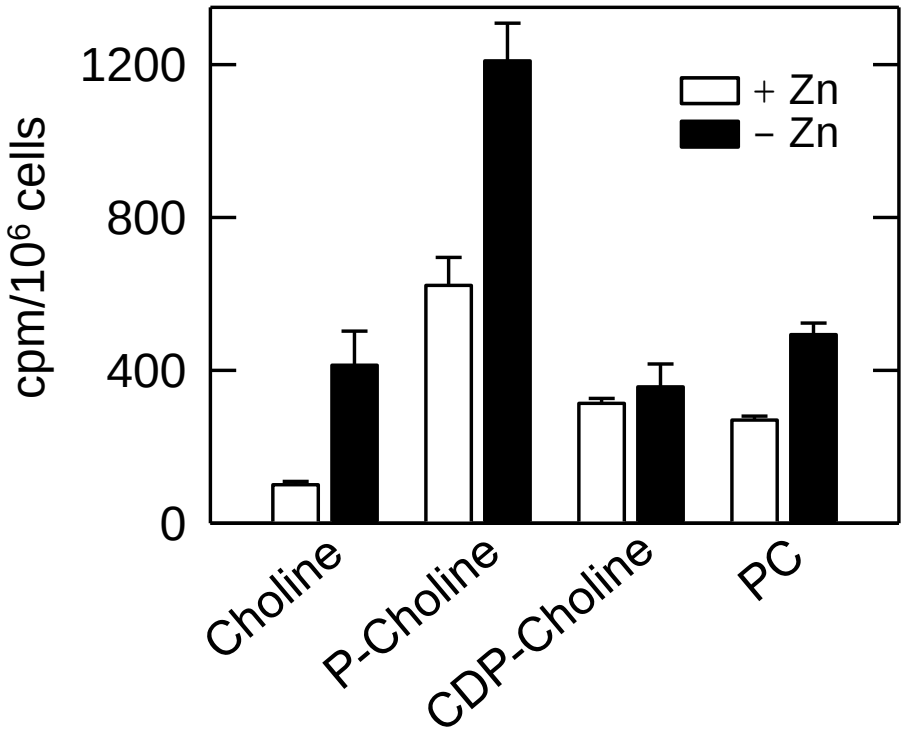

Choline Kinase, nmol/min/mg
0
2
4
6
+ Zn
– Zn
WT
zap1Δ

**Figure. 20. Effect of zinc depletion on the composition of the CDP-choline pathway intermediates and on PC.** Wild type cells were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. The cells were labeled for five to six generations with [*methyl*-$^{14}$C]choline (0.2 μCi/ml). The CDP-choline pathway intermediates and PC were extracted and analyzed by thin-layer chromatography. The values reported were the average of four separate experiments ± S.D.


1200
800
400
0
cpm/10^6 cells
+ Zn
– Zn
Choline
P-Choline
CDP-Choline
PC

# PART II

*Regulation of the Saccharomyces cerevisiae PAH1-encoded Magnesium Dependent Phosphatidate Phosphatase in Response to Zinc Depletion*

## Effect of Zinc Depletion on the Expression of *PAH1*

The effect of zinc depletion on the *PAH1*-encoded PAP was examined. As mentioned above, for this experiments and subsequent experiments, cells were grown in medium that lacks inositol supplementation to prevent the regulatory effects of inositol on phospholipid biosynthetic genes (2, 4, 69). Cells were grown in zinc-free medium that contained 1.5 μM zinc sulfate. The regulation of the *PAH1*-encoded PAP in response to zinc depletion was examined by using the $P_{PAH1}$-*lacZ* plasmid that was constructed by fusing 1000 bp of the *PAH1* promoter in frame with the *E. coli lacZ* coding sequence. The expression of β-galactosidase activity is driven by the *PAH1* promoter. Wild type cell expressing the $P_{PAH1}$-*lacZ* plasmid were grown to the exponential phase of growth in the absence or presence of increasing concentrations of zinc. After growth, cells were collected and cell extracts were prepared and assayed for β-galactosidase activity. The depletion of zinc results in a concentration-dependent increase in the *PAH1* gene expression (Fig. 21). The depletion of zinc from the media resulted in a 10-fold induction in the expression of the *PAH1* gene as compared to the expression found in cells grown in media containing 1.5 μM zinc.

## Effect of the *zrt1*Δ *zrt2*Δ Mutation on the Zinc-mediated Regulation of *PAH1* Expression

To determined wheather the induction the *PAH1* gene expression in response to zinc depletion was due to low cytoplasmic levels of zinc, we made use of the *zrt1*Δ *zrt2*Δ mutant that lack both zinc membrane transporters. As describe above, this mutant strain shows low intracellular levels of zinc even when grown in the presence of zinc (109, 110). The *zrt1*Δ *zrt2*Δ mutant strain expressing the $P_{PAH1}$-*lacZ* reporter plasmid grown in the presence of zinc shows a level of β-galactosidase activity comparable to the wild type cells grown in the absence of zinc (Fig 22). The results confirm that the *PAH1* gene expression is regulated in response to low intracellular levels of zinc.

## Effect of the *zap1*Δ Mutation on the Zinc-mediated Regulation of *PAH1* Expression

The *PAH1* promoter contains three $UAS_{ZRE}$ motifs (Fig. 24A) that are putative binding sites for the zinc responsive transcription factor, Zap1p (116). To determine whether the regulation of the *PAH1* gene in response to zinc depletion was mediated by Zap1p, we expressed the $P_{PAH1}$-*lacZ* in a *zap1*Δ mutant strain. The β-galactosidase activity in *zap1*Δ grown in the presence of zinc was higher than in wild type cells grown in the presence of zinc (Fig. 23). However, when cells were grown in the absence of zinc, the β-galactosidase activity in *zap1*Δ was ~2.5-fold lower when compared to the wild type control (Fig. 23). Thus a *zap1*Δ mutation causes a drop in the *PAH1* expression, suggesting that Zap1p plays a role in the regulation of PAP in response to zinc depletion.

**Figure. 21. Effect of zinc depletion on the expression of β-galactosidase activity in wild type cells bearing the $P_{PAH1}$-*lacZ* reporter gene.** Wild type cells bearing the $P_{PAH1}$-*lacZ* reporter plasmid pFP1 were grown to the exponential phase of growth in the presence of indicated concentrations of $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments $\pm$ S.D.

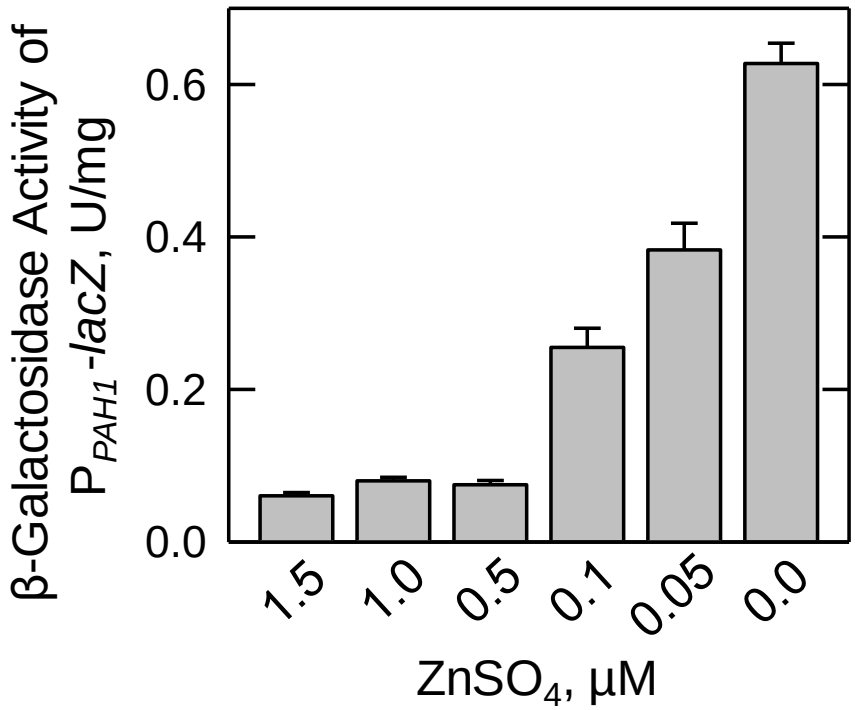

β-Galactosidase Activity of
P*PAH1*-*lacZ*, U/mg
0.0
0.2
0.4
0.6
1.5
1.0
0.5
0.1
0.05
0.0
ZnSO4, μM

**Figure. 22. Effect of *zrt1*Δ *zrt2*Δ mutations on the expression of the $P_{PAH1}$-*lacZ* reporter gene.** Wild type (*WT*) and *zrt1*Δ *zrt2*Δ mutant cells bearing the $P_{PAH1}$-*lacZ* reporter plasmid pFP1 were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

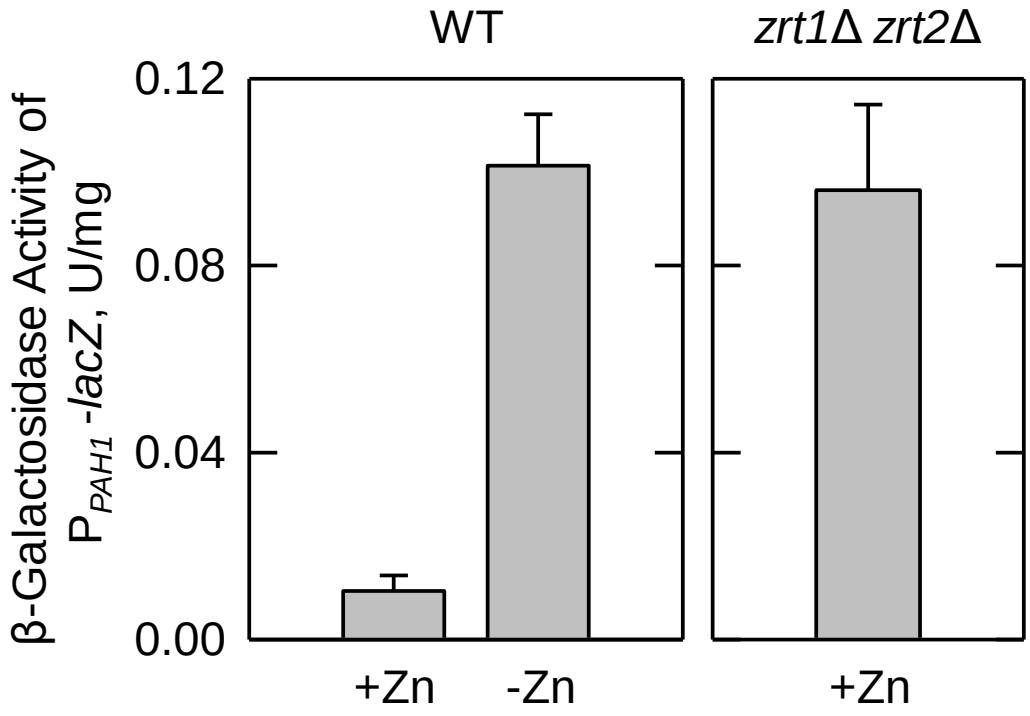
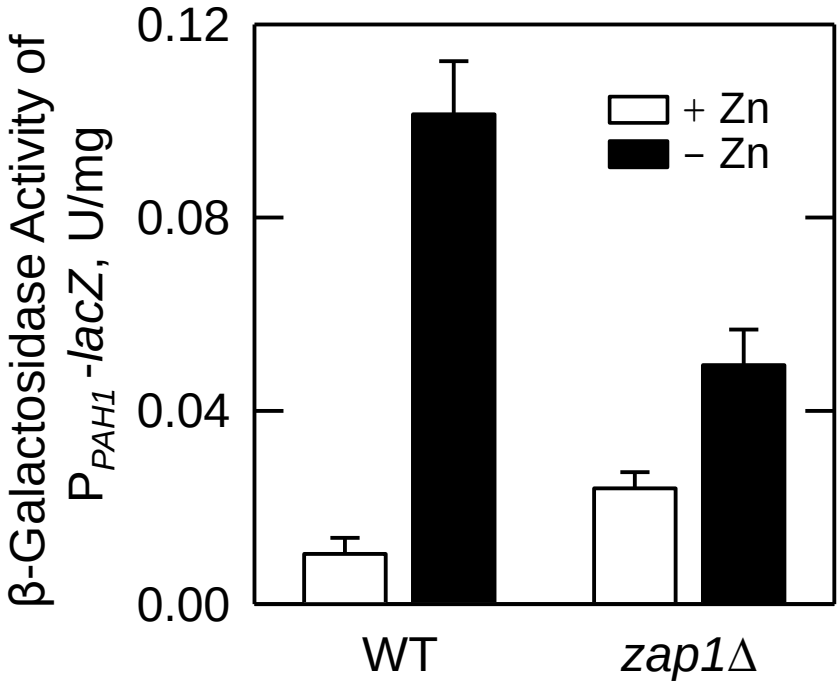

WT
zrt1Δ zrt2Δ
β-Galactosidase Activity of
P PAH1 -lacZ, U/mg
0.12
0.08
0.04
0.00
+Zn
-Zn
+Zn

**Figure. 23. Effect of the *zap1*Δ mutation on the expression of the $P_{PAH1}$-*lacZ* reporter gene.** Wild type (*WT*) and *zap1*Δ mutant cells bearing the $P_{PAH1}$-*lacZ* reporter plasmid pFP1 were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

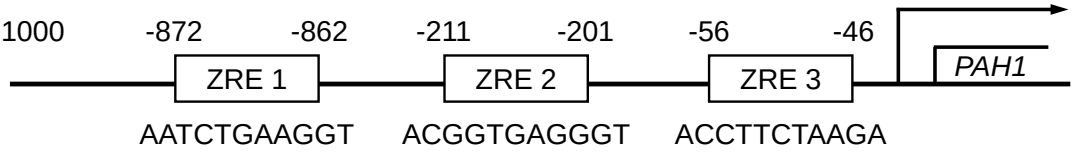

β-Galactosidase Activity of
P PAH1 -lacZ, U/mg
0.12
0.08
0.04
0.00
+ Zn
– Zn
WT
zap1Δ

**Binding of Zap1p to Putative $UAS_{ZRE}$ Sequences in the *PAH1* Promoter**

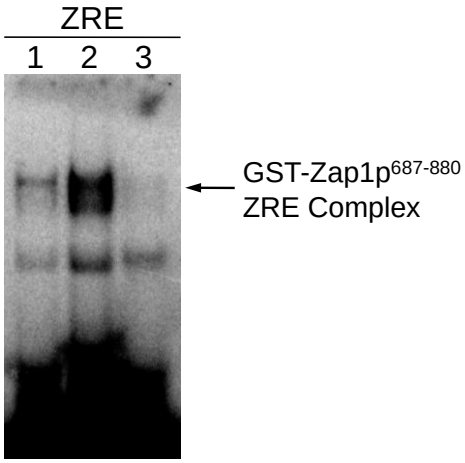
There are three putative $UAS_{ZRE}$ sites in the *PAH1* promoter for the binding of Zap1p (Fig. 24A). These sites (ZRE1, ZRE2 and ZRE3) are located at positions -872, -211 and -56, respectively, in reference to the gene start site and all three putative sites have a 64% identity with the $UAS_{ZRE}$ consensus sequence (ACCTTNAAGGT) (116). To examine whether Zap1p binds to labeled oligonucleotides containing the sequence of these three putative $UAS_{ZRE}$ sites, we used the electrophoretic mobility shift assay. Purified recombinant GST-$Zap1p^{687-880}$ (Zap1p binding domain (119)) interacts with all three ZREs at some extent (Fig. 24B). The interaction of GST-$Zap1p^{687-880}$ with ZRE2 was 3-folds and 5-folds higher when compared with ZRE1 and ZRE3, respectively. The formation of the GST-$Zap1p^{687-880}$ and *PAH1* $UAS_{ZRE}$ complexes was examined in more detail. Labeled oligonucleotides were incubated with increasing amounts of GST-$Zap1p^{687-880}$ and the complex was separated from free labeled oligonuceoltides by electrophoresis. The formation of the GST-$Zap1p^{687-880}$complex with all three ZREs was dependent on the concentration of GST-$Zap1p^{687-880}$ (Fig. 24C). The specificity of the complex formation was examined further. Unlabeled ZRE1, ZRE2 and ZRE3 oligonucleotides competed with their labeled counterparts for the binding of GST-$Zap1p^{687-880}$ (Fig. 24D). In addition, mutations in ZRE1, ZRE2 and ZRE3 to a nonconsensus (AAAAAAAAAAA) $UAS_{ZRE}$ sequence abolish the binding of GST-$Zap1p^{687-880}$ (data not shown). Mutations in ZRE1, ZRE2 and ZRE3 to the consensus sequence (ACCTTNAAGGT (116)) $UAS_{ZRE}$ results in an increase in the interaction with GST-$Zap1p^{687-880}$ that was 11-, 5- and 11-fold higher, respectively, when compared to the wild type sequences (data not shown).

**Figure. 24. Interactions of GST-Zap1p$^{687-880}$ with putative ZRE sequences in the *PAH1* promoter.** (A) The locations and sequences of the putative ZRE sites in the *PAH1* promoter. (B) Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.0 x $10^5$ cpm/pmol) with sequences for ZRE1 (*lane 1*), ZRE2 (*lane 2*), and ZRE3 (*lane 3*), in the *PAH1* promoter were incubated with 0.5 μg of purified recombinant GST-Zap1p$^{687-880}$. (C) The ZRE1, ZRE2 and ZRE3 radiolabeled probes were incubated with 0, 0.15, 0.3 and 0.5 μg of recombinant GST-Zap1p$^{687-880}$. (D) GST-Zap1p$^{687-880}$ was incubated with 0, 25, 50 and 100 pmol of unlabeled oligonucleotide with the sequences for ZRE1, ZRE2 and ZRE3, respectively. Interaction of GST-Zap1p$^{687-880}$ with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6 % polyacrylamide gel. The data shown are representative of two independent experiments. The arrow indicates the position of the GST- Zap1p$^{687-880}$-ZRE complex.

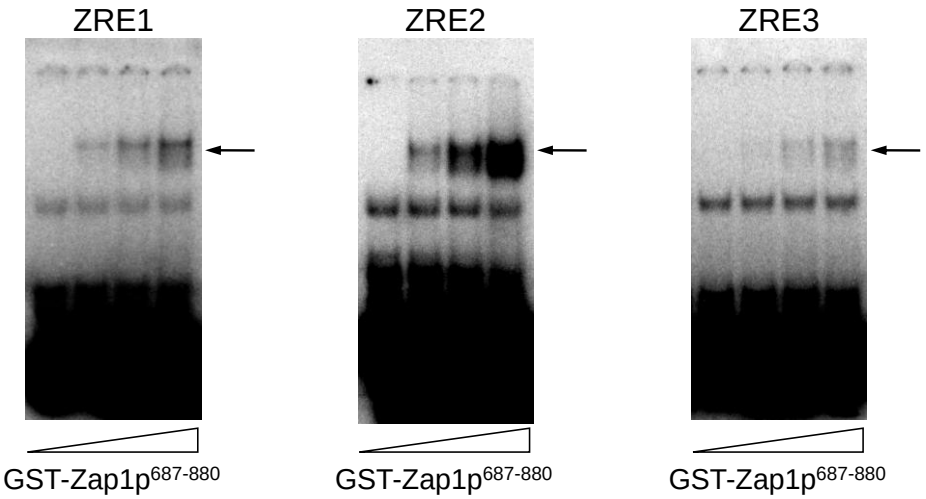
A


-1000
-872
-862
-211
-201
-56
-46
ZRE 1
ZRE 2
ZRE 3
PAH1
AATCTGAAGGT
ACGGTGAGGGT
ACCTTCTAAGA

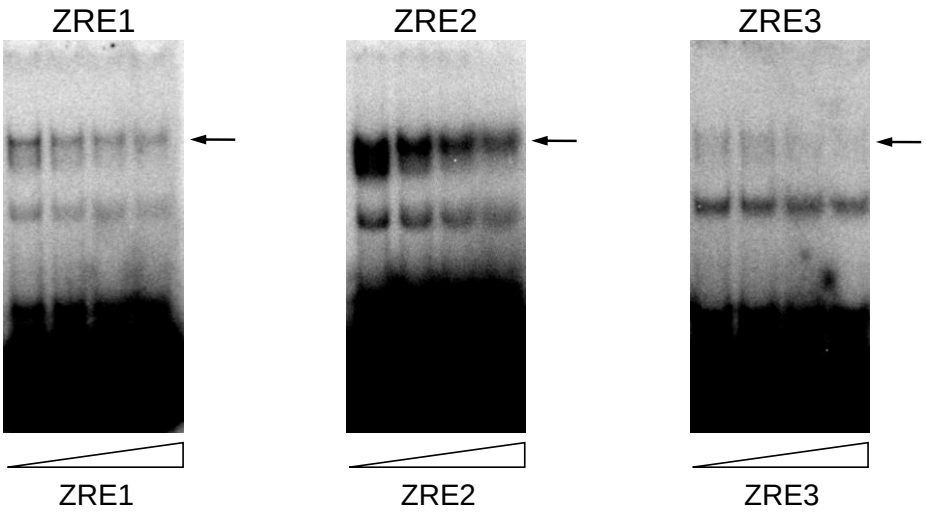
B


ZRE
1 2 3
GST-Zap1p687-880
ZRE Complex

C


ZRE1
ZRE2
ZRE3
GST-Zap1p687-880
GST-Zap1p687-880
GST-Zap1p687-880

D

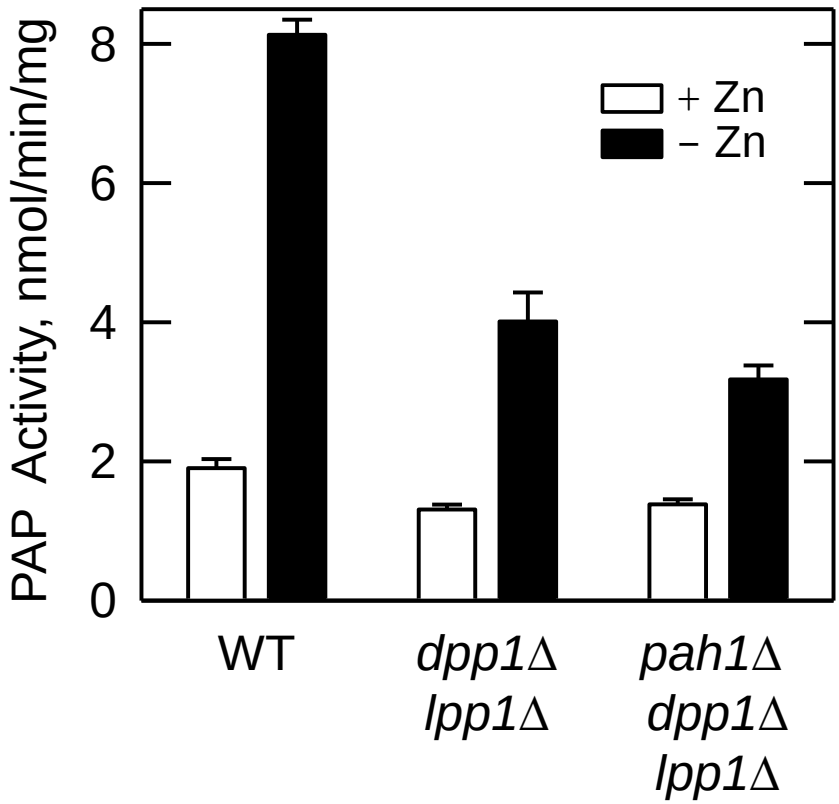

ZRE1
ZRE2
ZRE3
ZRE1
ZRE2
ZRE3

**Effect of zinc depletion on PA phosphatase activity**

We next analyze if the induction of *PAH1* gene expression in response to zinc depletion translated to an increase on PAP activity. To be able to differentiate between the PAP and LPP activity we made use of a *dpp1*Δ *lpp1*Δ double mutant strain that lacks both LPP activities and the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant strain that lacks all known PAP and LPP activities (54, 156). Wild type, *dpp1*Δ *lpp1*Δ and *pah1*Δ *dpp1*Δ *lpp1*Δ mutant cells were grown in the presence and absence of zinc to the exponential phase of growth. Cell extracts were prepared and PA phosphatase activity was measured by following the release of inorganic phosphate from $^{32}$P-labeled PA. In this and subsequent experiments, all PAP assays were done in the presence of 5 mM $MgCl_2$.

Wild type cells grown in the absence of zinc show a 4.3-fold induction in PAP activity when compared to wild type cells grown in the presence of zinc (Fig. 25). This shows that the overall PAP activity (PAP and LPP) is induced in response to zinc depletion. The *dpp1*Δ *lpp1*Δ double mutant cells grown in the absence of zinc show a 49% reduction in PAP activity when compared to wild type cells grown in the absence of zinc, and a 3.1-fold induction in PAP activity when compared to *dpp1*Δ *lpp1*Δ double mutant cells grown in the presence of zinc (Fig. 25). This result showed that PAP activity is induced in response to zinc depletion. The *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant cells grown in the absence of zinc show a 39% reduction in PAP activity when compared to wild type cells grown in the absence of zinc and a 2.3-fold induction in PAP activity when compared to the triple mutant cells grown in the presence of zinc (Fig. 25). The PAP activity still present in the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant cells is attributed to an unknown PAP (54).

**Figure. 25. Effect of zinc depletion on total PAP activity.** Wild type (*WT*), *dpp1*Δ *lpp1*Δ and *pah1*Δ *dpp1*Δ *lpp1*Δ mutant cells were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cell extracts were prepared and assayed for PA phosphatase activity in the presence of 5 mM $MgCl_2$. Each data point represents the average of triplicate enzyme determinations from two independent experiments ± S.D.

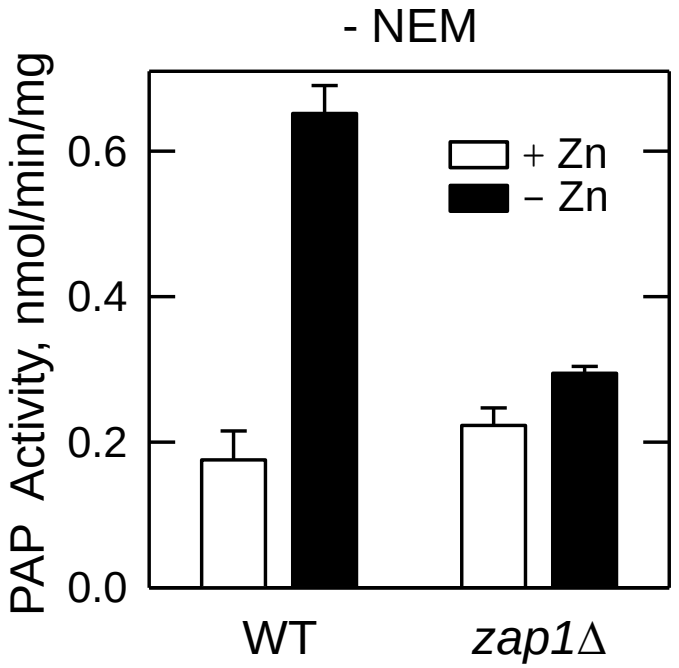

PAP Activity, nmol/min/mg
0
2
4
6
8
+ Zn
– Zn
WT
dpp1Δ lpp1Δ
pah1Δ dpp1Δ lpp1Δ

**Effect of the *zap1*Δ Mutation on PA Phosphatase activity in response to zinc depletion**

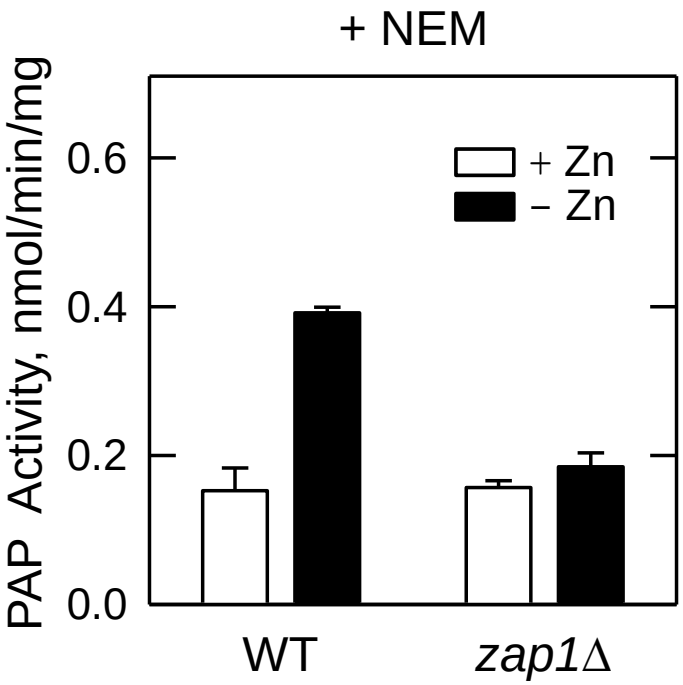
To further investigate the contribution of Zap1p in the regulation of *PAH1* in response to zinc depletion, we grew wild type and *zap1*Δ cells in the presence and absence of zinc. Cytosolic fractions were prepared and assayed for PAP activity. The reasons for using the cytosolic fraction and not total cell extracts for the PAP assay include: (1) to eliminate any contribution of the LPP activity from Dpp1p and Lpp1p; (2) because zinc depletion resulted in a Zap1p-dependent induction in expression of the *DPP1*-encoded DGPP phosphatase and (3) due to the unavailability of a triple mutant strain that lacks the zinc responsive transcription factor gene, *ZAP1* and the two LPP genes, *DPP1* and *LPP1* (60). Zinc depletion resulted in a 3.7-fold increase in PAP activity in wild type cells when compared to cells grown in the presence of zinc. The PAP activity was similar from wild type and *zap1*Δ mutant cells grown in the presence of zinc. But when grown in the absence of zinc, the *zap1*Δ showed 2-fold lower PAP activity than wild type (Fig. 26A). As mentioned above, there is still some PAP activity present in the *pah1*Δ *dpp1*Δ *lpp1*Δ mutant that lacks all known PA phosphatase enzymes, and this activity is sensitive to the thioreactive agent NEM. To exclude PAP activity encoded by the unknown PAP enzyme, the cytosolic fractions were assayed for PAP activity in the presence of NEM at a final reaction concentration of 20 mM. The treatment of NEM resulted in an overall reduction in PAP activity (Fig. 26B). The cytosolic fraction of wild type cells grown in the absence of zinc showed a 2.6-fold induction in PAP activity when compared to cells grown in the presence of zinc (Fig. 26B). The PAP activity in the cytosolic fraction from *zap1*Δ grown

**Figure. 26. Effect of *zap1*Δ mutation on PAP activity in response to zinc depletion.** Wild type (*WT*) and *zap1*Δ mutant cells were grown to the exponential phase of growth in the absence and presence of 1.5 μM $ZnSO_4$. Cytosolic fractions were prepared by ultracentrifugation at 100,000 xg for 1 h and assayed for PAP activity (5 mM $MgCl_2$) in the absence (A) and presence (B) of 20 mM NEM. Each data point represents the average of triplicate enzyme determinations from two independent experiments $\pm$ S.D.

A


- NEM
PAP Activity, nmol/min/mg
0.0
0.2
0.4
0.6
+ Zn
– Zn
WT
zap1Δ

B


+ NEM
PAP Activity, nmol/min/mg
0.0
0.2
0.4
0.6
+ Zn
– Zn
WT
zap1Δ

in the absence of zinc was 2-fold lower when compared to wild type cells grown in the absence of zinc (Fig. 26B). These results demonstrate that Zap1p mediated the *PAH1* gene expression in response to zinc depletion resulting in an increase in PAP activity. Mutational analysis of the $UAS_{ZRE}$ in the $P_{PAH1}$-*lacZ* reporter gene was not pursued as we did for the regulation of the *CKI1*-encoded choline kinase by zinc depletion (187). All data support the notion that the *PAH1* expression and PAP activity regulation in response to zinc depletion is mediated by Zap1p through interaction with the ZRE sequences located in the promoter region of the *PAH1* gene promoter.

**Effect of Zinc Depletion on Neutral Lipid Composition**

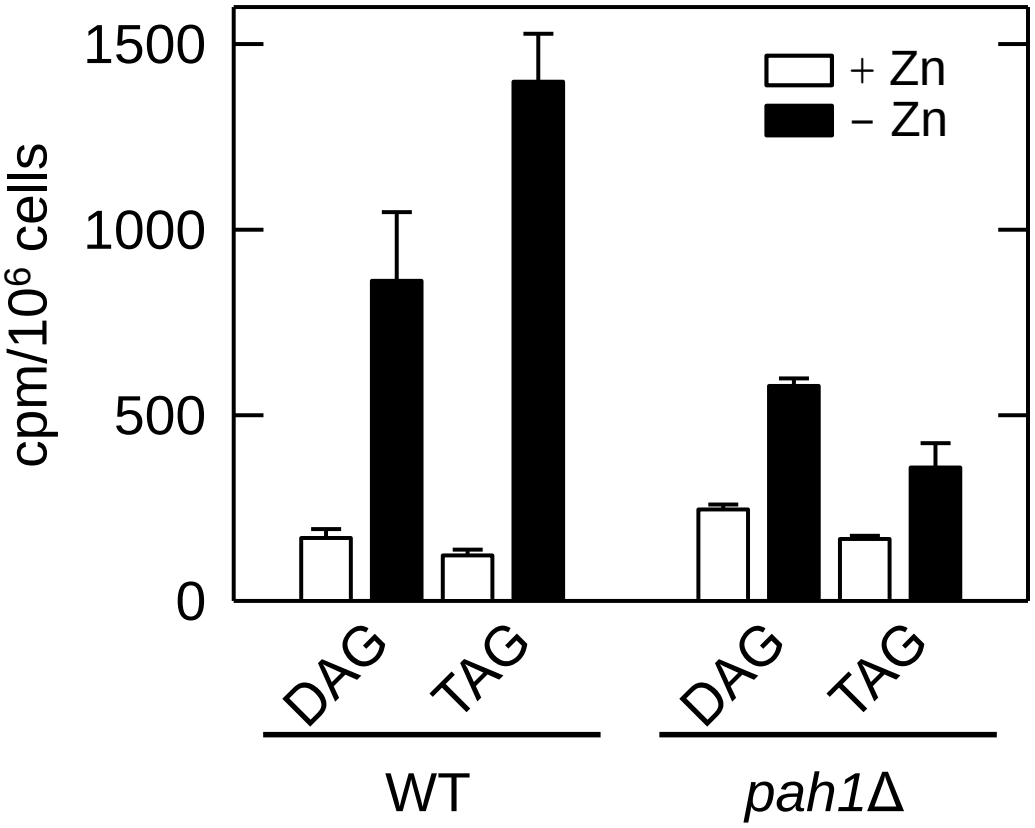
The DAG derived from the PAP reaction may be used for the synthesis of the storage lipid, TAG (54) and for the synthesis of PC via the Kennedy pathway (201). To examine the effects of zinc depletion on the synthesis of DAG and TAG, cells were labeled with [2-$^{14}$C]acetate grown in the presence and absence of zinc, followed by lipid extraction and analysis of neutral lipids by thin-layer chromatography. Wild type cells grown in the absence of zinc showed a 4-fold induction in DAG synthesis when compared to wild type cells supplemented with zinc (Fig. 27). In addition we wanted to investigate the role of PAP in the synthesis of DAG and TAG in zinc depleted cells. In *pah1*Δ mutant cells, zinc depletion resulted in a 33% reduction in DAG when compared to wild type cells grown in the absence of zinc (Fig. 27). In zinc supplemented cells the level of DAG did not show any significant difference between wild type and *pah1*Δ mutant cells (Fig. 27). In wild type cells zinc depletion results in an 11.3-fold induction in the levels of TAG as compared to wild type cells supplemented with zinc (Fig. 27). In

zinc depleted cells the *pah1*Δ showed a 74% reduction in TAG levels as compared to wild type cells grown in zinc depleted media (Fig. 27). Similar to the DAG levels observed in zinc supplemented cells, the levels of TAG did not show any significant difference between wild type and the *pah1*Δ mutant (Fig. 27). These results suggested that yeast cells cope with the depletion of zinc by accumulating DAG and TAG, similar to what is observed as cells enter to the stationary phase of growth (164).

## The Role of PAP on the Synthesis of PC via the Kennedy Pathway in Response to Zinc Depletion

In yeast, zinc depletion leads to the down-regulation of the CDP-DAG pathway resulting in a decrease in the PS and PE levels, an increase in the PI levels, but PC levels remain unchanged (61). Recent work has showed that the CDP-ethanolamine and CDP-choline branches of the Kennedy pathway are up-regulated in response to zinc depletion (123, 187). The increase in PC synthesis via the Kennedy pathway in response to zinc depletion compensates for the diminish in PC synthesis by the CDP-DAG pathway (123, 187). In recent work, Han and coworkers identified the *PAH1*-encoded PAP enzyme as the one responsible for the synthesis of DAG utilized in the synthesis of PE and PC via the Kennedy pathway (54). In this experiment we investigated the role of PAP in the synthesis of PC via the CDP-choline branch of the Kennedy pathway. Cells were labeled to steady-state with [*methyl*-$^{14}$C]choline followed by extraction and analysis of PC and its intermediates choline, phosphocholine and CDP-choline by TLC. As shown previously, wild type cells starved for zinc showed an increase in choline incorporation, phosphocholine formation and PC synthesis, but no change in CDP-choline level (Fig.

**Figure. 27. Effect of zinc depletion on neutral lipid composition.** Wild type (WT) and *pah1*Δ mutant cells were grown to the exponential phase of growth in the absence (black bars) and presence (white bars) of 1.5 μM $ZnSO_4$. These cells were labeled for five to six generations with [2-$^{14}$C]acetic acid (1.0 μCi/ml). Lipids were extracted and separated by one-dimensional thin-layer chromatography and the images were subjected to ImageQuant analysis. The amount of label per lipid was quantified using a [2-$^{14}$C]acetic acid standard spotted in a thin-layer chromatography and analyzed by ImageQuant and quantified by scintillation counting. Each data point represents the average of three experiments $\pm$ S.D.

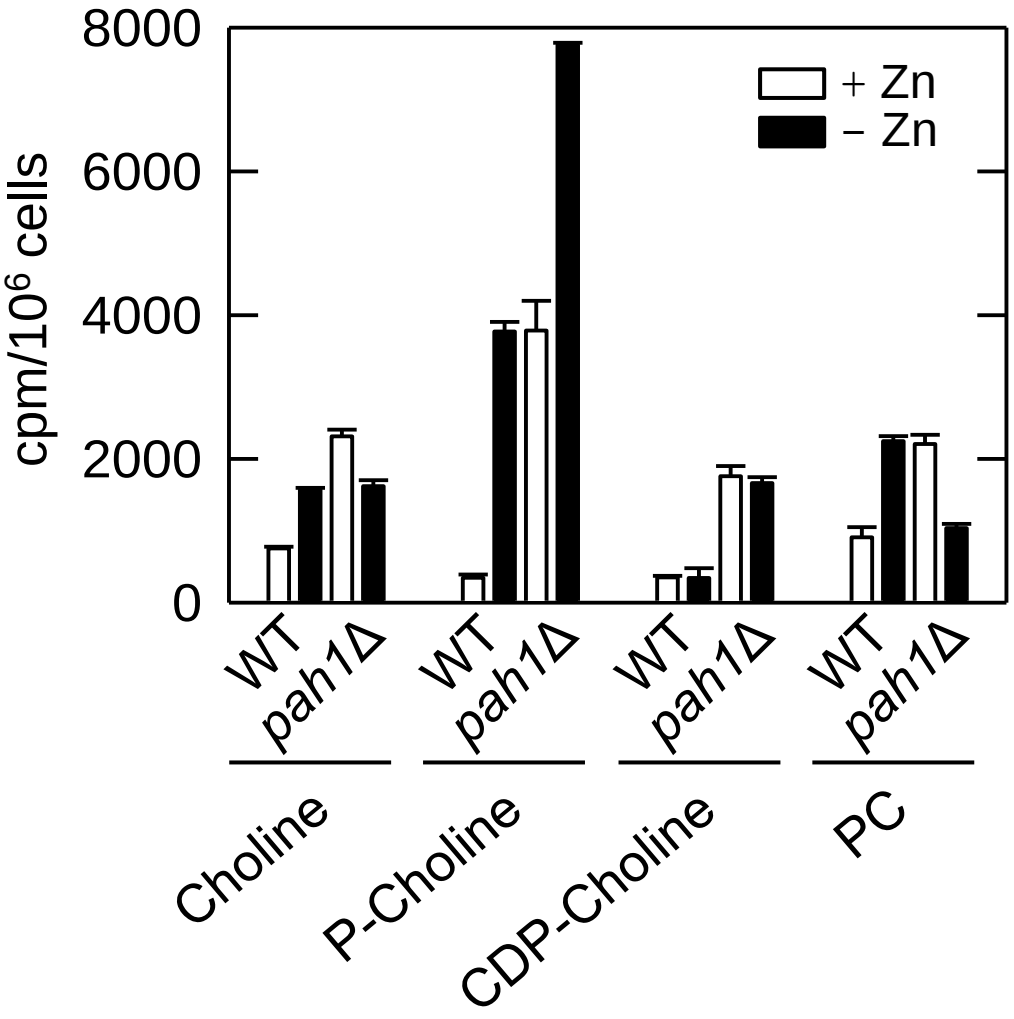

1500
1000
500
0
cpm/10^6 cells
+ Zn
− Zn
DAG
TAG
DAG
TAG
WT
pah1Δ

28) (187). Next we analyzed the effect of *pah1*Δ mutation in the synthesis of PC via the Kennedy pathway in response to zinc depletion. The level of free choline in *pah1*Δ grown in the absence of zinc showed no change compared to wild type cells grown in the absence of zinc. In contrast, a 30% reduction in free choline levels was observed in WT cells when compared to *pah1*Δ mutant cells grown in the presence of zinc (Fig. 28). The *pah1*Δ mutation resulted in an overall induction in phosphocholine formation when compared to wild type cells independent of whether they were grown in the presence or absence of zinc (Fig. 28). The results from this experiment showed that the *pah1*Δ mutation resulted in an accumulation of phosphocholine, probably due to the blockage in PC synthesis via the Kennedy pathway. PAP activity controls the levels of PA, thus controlling the repressive functions of Opi1p over phosphilipid biosynthetic genes (e.g. *CKI1*) (155). Another possibility for the increase in phosphocholine formation is that *pah1*Δ mutation resulted in an increase in *CKI1* expression and choline kinase activity due to the accumulation of PA and tethering of Opi1p to the nuclear/ER membrane, in both the presence and absence of zinc. The *CKI1* expression and choline kinase activity could be examined in the *pah1*Δ mutant to investigate this possibility.

As shown before, the CDP-choline levels remain unchanged in wild type cells grown in the absence of zinc (Fig. 28) (187). The *pah1*Δ mutant grown in the absence of zinc showed a ~4.5-fold increase in CDP-choline levels when compared to control cells grown in the absence of zinc. However, no change was observed with *pah1*Δ cells grown in the presence of zinc (Fig. 28). The *pah1*Δ mutation resulted in a decrease in the supply of DAG, the substrate for the final step for the synthesis of PC via the Kennedy pathway (Fig. 2), resulting in an accumulation of CDP-choline. Finally, we examined the effect of

the *pah1*Δ mutation on the formation of PC in response to zinc depletion (Fig. 28). As shown before, the levels of PC increased when wild type cells were depleted for zinc (Fig. 28) (187). The *pah1*Δ mutant cells showed a 52% decrease in PC levels as compared to wild type cells grown in the absence of zinc (Fig. 28). Surprisingly, *pah1*Δ cells grown in the presence of zinc showed an increase in the PC level comparable to those observed in control cells grown in the absence of zinc, which is 2.4-fold higher than wild type cells grown in the presence of zinc (Fig. 28). These results demonstrated that PAP is required for the synthesis of PC via the Kennedy pathway in response to zinc depletion and mutations in PAP resulted in the misregulation of the choline branch of the Kennedy pathway in response to zinc depletion.

**Figure. 28. The role of PAP on the synthesis of PC via the Kennedy pathway in response to zinc depletion.** Wild type, *pah1*Δ, *dpp1*Δ *lpp1*Δ, and *pah1*Δ *dpp1*Δ *lpp1*Δ cells were grown to the exponential phase of growth in the absence or presence of 1.5 μM $ZnSO_4$. The cells were labeled for five to six generations with [*methyl*-$^{14}$C]choline (0.2 μCi/ml). The CDP-choline pathway intermediates choline, phosphocholine, CDP-choline and PC were extracted and analyzed by thin-layer chromatography. The values reported were the average of four separate experiments ± S.D.


8000
6000
4000
2000
0
cpm/10^6 cells
+ Zn
– Zn
WT
pah1Δ
WT
pah1Δ
WT
pah1Δ
WT
pah1Δ
Choline
P-Choline
CDP-Choline
PC

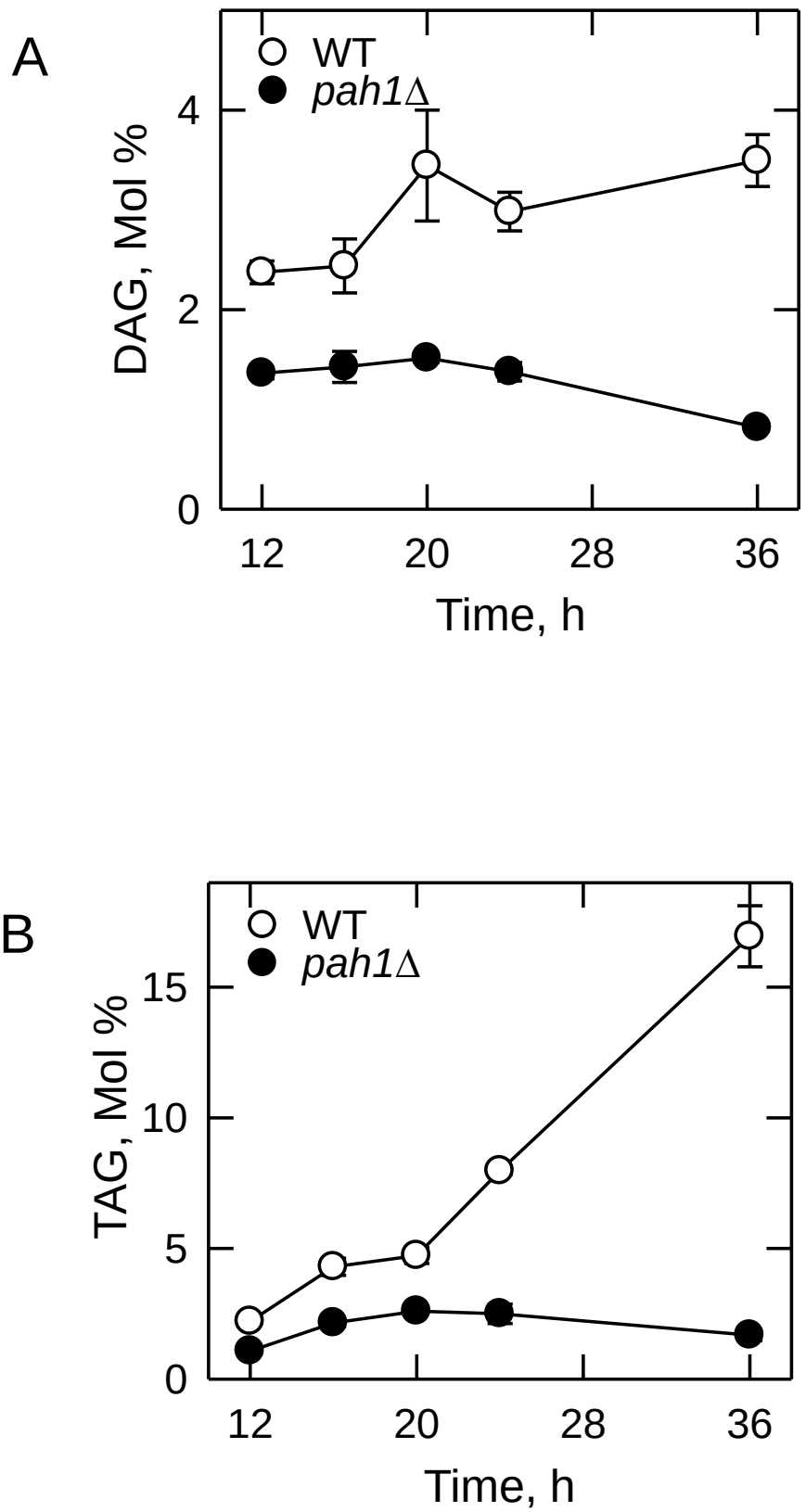
# PART III

*Regulation of the Saccharomyces cerevisiae PAH1-encoded PAP by Growth Phase*

## Effect of Growth on DAG and TAG Content in *pah1*Δ Mutant Cells

Taylor and Parks (164) have shown that TAG is synthesized throughout the exponential phase of growth and that this lipid accumulates in the stationary phase. *In vivo* labeling experiments have shown that TAG is synthesized from PA (164). As described previously (164), the amount of TAG in wild type cells increased as cells progressed from the exponential to stationary phases of growth. Moreover, it has been shown recently that *pah1*Δ mutant cells contain reduced amounts of DAG and the storage lipid TAG (54). The effect of the *pah1*Δ mutation on the TAG content was more drastic during the stationary phase of growth (54). Owing to the fact that PA is the phospholipid precursor of TAG in the *de novo* biosynthetic pathway (Fig. 2) (202), we examined the role of the *PAH1*-encoded PAP enzyme in the synthesis of TAG during growth using a *pah1*Δ mutant (Fig. 29). Wild type and *pah1*Δ mutant cells were incubated with [2-$^{14}$C]acetate and harvested at different times from exponential to the stationary phase of growth, followed by extraction and analysis of neutral lipids by TLC. In wild type cells, the steady state content of TAG in the stationary phase (17 mol %) was 7.7-fold higher than that found (2.2 mol %) in the early exponential phase of growth (Fig. 29). The *pah1*Δ mutant showed a significant decrease in the amounts of TAG throughout growth (Fig. 29). As described previously for stationary phase cells (54), the loss of *PAH1*-encoded PAP activity resulted in a 90 % reduction in TAG content compared to wild type cells. The *pah1*Δ mutation resulted in adecrease in DAG levelsat both the exponential and stationary phase of growth as compared to wild type cells (Fig. 29) (54). These

**Figure. 29. Effect of *pah1*Δ mutation on neutral lipid composition during growth.** Wild type (*WT*) and *pah1*Δ mutant cells were grown in synthetic complete media and labeled with [2-$^{14}$C]acetate (1.0 μCi/ml). Labeled cells were collected at the indicated time intervals and lipids were extracted and separated by one-dimensional thin-layer chromatography. The phosphorimages were analyzed using ImageQuant. The percentage of each individual lipid was normalized to the total $^{14}$C-labeled chloroform fraction. (A) diacylglyceol, (B) triacylglycerol. The values reported were the average of three separate experiments ± S.D.


A
WT
pah1Δ
DAG, Mol %
0
2
4
12
20
28
36
Time, h
B
WT
pah1Δ
TAG, Mol %
0
5
10
15
12
20
28
36
Time, h

results confirmed that TAG is synthesized during the exponential phase and accumulates at the stationary phase of growth and that PAP plays a crucial role as the supplier of DAG for the synthesis of TAG.

**Effect of Growth on PAP Activity**

The work of Hosaka and Yamshita (163) has shown that PAP activity increases as cells progress from the exponential to stationary phase of growth. The increase in PAP activity correlates with the accumulation of TAG that occurs in the stationary phase (163, 164). At the time of these early studies, it was unknown that at least three genes (i.e., *PAH1*, *DPP1*, and *LPP1*) encoding PA and lipid phosphate phosphatase enzymes that utilize PA as substrate to produce DAG (54, 128, 156). In addition, the analysis of the *pah1*Δ *dpp1*Δ *lpp1* Δ triple mutant has revealed that an unknown gene(s) encodes a PAP enzyme (54). Thus, it is unclear which PAP enzyme activity is responsible for the synthesis of TAG during growth.

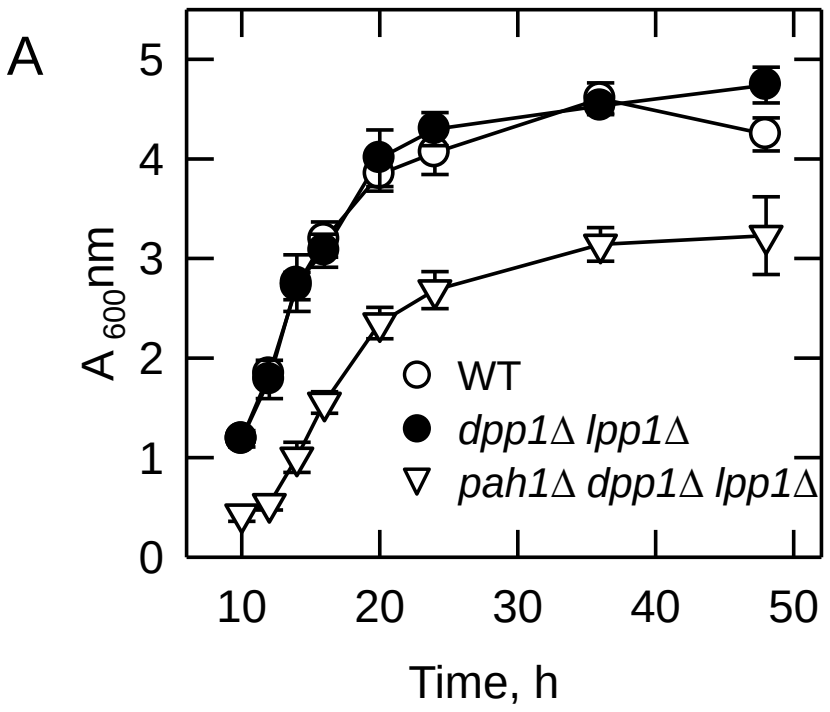
To confirm whether the increase in the levels of DAG and TAG is due to an increase in the *PAH1*-encoded PAP activity, we monitored the growth rate and the levels of PAP activity during growth of wild type and mutant strains. In this experiment we followed the growth rate of wild type, *dpp1*Δ *lpp1*Δ, and *pah1*Δ *dpp1*Δ *lpp1*Δ (Fig. 30A) (54, 156) mutant cells and harvested them at different times from the exponential to the stationary phase of growth. Cell extracts were prepared and assayed for PAP activity. The *dpp1*Δ *lpp1*Δ double mutation did not show any growth defect (128, 156), where as the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant showed slower growth compared to wild type cells (Fig. 30A).

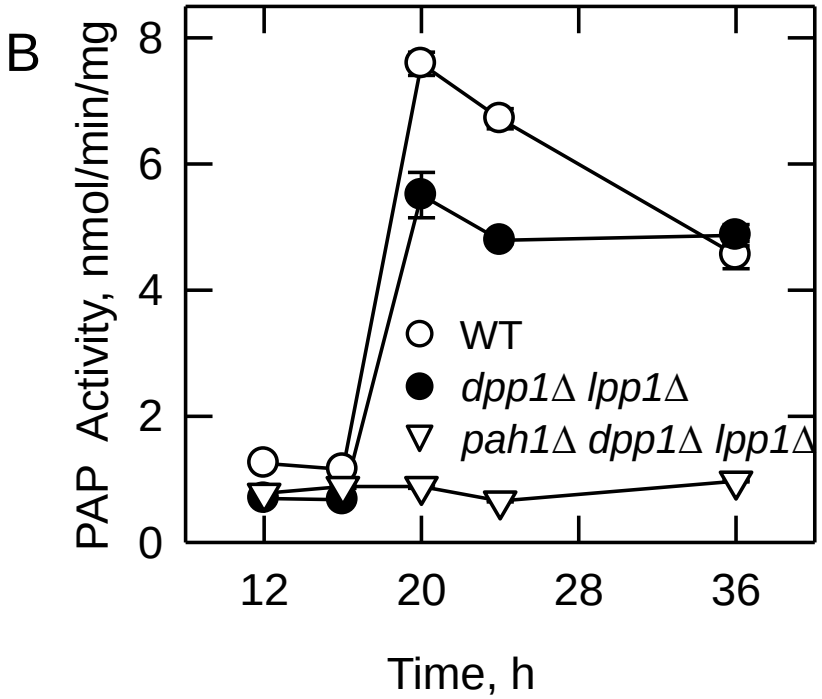
Wild type cells show a 7.2-fold increase in PAP activity when cells were at the late exponential phase of growth (20 h) when compared with the PAP activity exhibited at the early exponential phase of growth (12 h) (Fig. 30B). After wild type cells enter the stationary phase of growth (24 h and 36 h), there was a 8.6 and 39% reduction in total PAP activity, respectively, when compared to the level of PAP activity at 20 h (Fig. 30B). The *dpp1*Δ *lpp1*Δ double mutant cells showed a 31% reduction in PAP activity during the late exponential phase of growth (20 h) when compared to wild type cells and maintained a constant level of activity throughout the stationary phase of growth (Fig. 30B). The unknown PAP activity observed in the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant did not changed throughout growth (Fig. 30B). These results indicated that the *PAH1*-encoded PAP activity is responsible for the synthesis and buildup of TAG throughout growth.

**Effect of Growth Phase on PAP Protein Abundance and Stability**

The abundance of the *PAH1*-encoded PAP protein was measured throughout growth by immunoblotting using antibodies directed against the C-terminal portion of the protein. Immunoblot analysis indicated that the abundance of the PAP protein in wild type cells was relatively low, especially in mid- to late-exponential phase cells (data not shown). Therefore, to observe the PAP protein signal more clearly during growth, the analysis was performed with cell extracts derived from the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant that expressed *PAH1* on the multicopy plasmid pGH312. The immunoblot analysis of cell extracts derived from these cells showed that the PAP protein was highest in the exponential phase and it declined during growth (Fig. 31). Plasmid pGH312

**Figure. 30. Effect of growth on *PAH1*-encoded PAP activity.** (A) The growth of wild type (*WT*), *dpp1*Δ *lpp1*Δ, and *pah1*Δ *dpp1*Δ *lpp1*Δ mutant cells grown in synthetic complete media was monitored by recording their optical density (OD) at $A_{600nm}$ at the indicated times. Each data point represents the average of triplicate determination of cell absorbance. (B) PAP activity from cell collected at the indicated time of growth. Each data point represents the average of triplicate enzyme determinations from two independent experiments $\pm$ S.D.


A
5
4
3
2
1
0
$A_{600}$nm
10
20
30
40
50
Time, h
WT
dpp1Δ lpp1Δ
pah1Δ dpp1Δ lpp1Δ


B
8
6
4
2
0
PAP Activity, nmol/min/mg
12
20
28
36
Time, h
WT
dpp1Δ lpp1Δ
pah1Δ dpp1Δ lpp1Δ

directs the expression of a functional N-terminal HA-tagged PAP enzyme (54). Immunoblot analysis of the cell extracts with anti-HA antibodies showed the same pattern of PAP protein abundance during growth (data not shown). In contrast, the abundance of the *PGK1*-encoded PGK protein was relatively constant throughout growth (Fig. 31). In a complementary experiment, we measured the PAP activity from *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant cells expressing the *PAH1* gene from the pGH312 plasmid or the empty vector (YEp351) (Fig. 32). Expression of the *PAH1* gene resulted in a 15- and 10-fold increased in PAP activity during late exponential and stationary phase of growth, respectively, as compared to cells expressing the empty vector at the same stage of growth (Fig. 32). The expression of *PAH1*-encoded PAP activity followed the same general pattern as that observed in the *dpp1*Δ *lpp1*Δ double mutant (i.e., activity increased as cells progressed into stationary phase) (Fig. 30). These results confirmed that the *PAH1*-encoded PAP activity increases during the transition from exponential to stationary phase, almost at the same point when the PAP protein abundance drop to undetectable levels.

We considered the possibility that the PAP protein was being degraded in the later stages of growth by way of the ubiquitin-proteasome pathway (203, 204). To test this hypothesis, the abundance of the PAP protein was examined in cells grown in the presence of the proteasome inhibitor MG132 (203). For this experiment, the *PAH1* gene was overexpressed in the *pdr5*Δ mutant that is defective in a multidrug efflux pump (205). The *pdr5*Δ mutation prevents efflux of MG132 facilitating the inhibition of the proteasome degradation pathway (203, 206). In the absence of MG132, the PAP protein is degraded in a growth phase dependent manner (Fig. 33). On the other hand, the PAP

protein was stabilized in cells treated with MG132. These results indicated that the degradation of PAP was mediated by the ubiquitin-proteasome pathway as cells progressed into the stationary phase of growth.

To further test the hypothesis that PAP is degraded via the ubiquitin-proteasome pathway as cells progress from exponential to stationary phase, we examine the abundance of PAP in mutants defective in ubiquitin-conjugating enzymes. Protein ubiquitylation is a three step pathway catalyzed by an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and ubiquitin-ligases (E3). First, an E1 enzyme activates an ubiquitin using an ATP molecule. Second, the activated ubiquitin is transferred from the E1 to an E2. Third, the E2 in cooperation with an E3 attachs an ubiquitin molecule into the target protein. In addition to their ubiquitin carrier functions, E2 enzymes also have a role in determined the length and topology of ubiquitin chains (207).

In this experiment, we investigated the role of the ubiquitin-conjugating enzymes Ubc2p (208), Ubc4p (209), Ubc5p (209), and Ubc8p (210) in the degradation of PAP via the ubiquitin-proteasome pathway. The growth of wild type and *ubc2*Δ, *ubc4*Δ, *ubc5*Δ, and *ubc8*Δ mutants was monitored from exponential to the stationary phase of growth. Cells were collected at different stages of growth to examine the PAP protein abundance by Western blotting. The slow growth of these mutants made it difficult to determine the stage of growth of the cultures at the time of harvesting. As a result of this complication, the role of these ubiquitin-conjugating enzymes in the PAP degradation via the ubiquitin-proteasome pathway could not be established. Further experiments need to be performed to identify the mechanism of PAP degradation in response to growth.

**Figure. 31. Effect of growth phase on PAP protein levels.** *pah1*Δ *dpp1*Δ *lpp1*Δ mutant cells expressing the *PAH1* gene from the multicopy plasmid pGH312 were grown in synthetic complete media and cells were collected at the indicated time of growth. Cell extracts were prepared and subjected to immunoblot analysis using anti-PAP and anti-PGK antibodies (loading control). The ratio of PAP to PGK was determined by ImageQuant analysis of the data. The optical density of the culture was recorded and the absorbance at 48 h was arbitrarily set at 100% culture saturation.

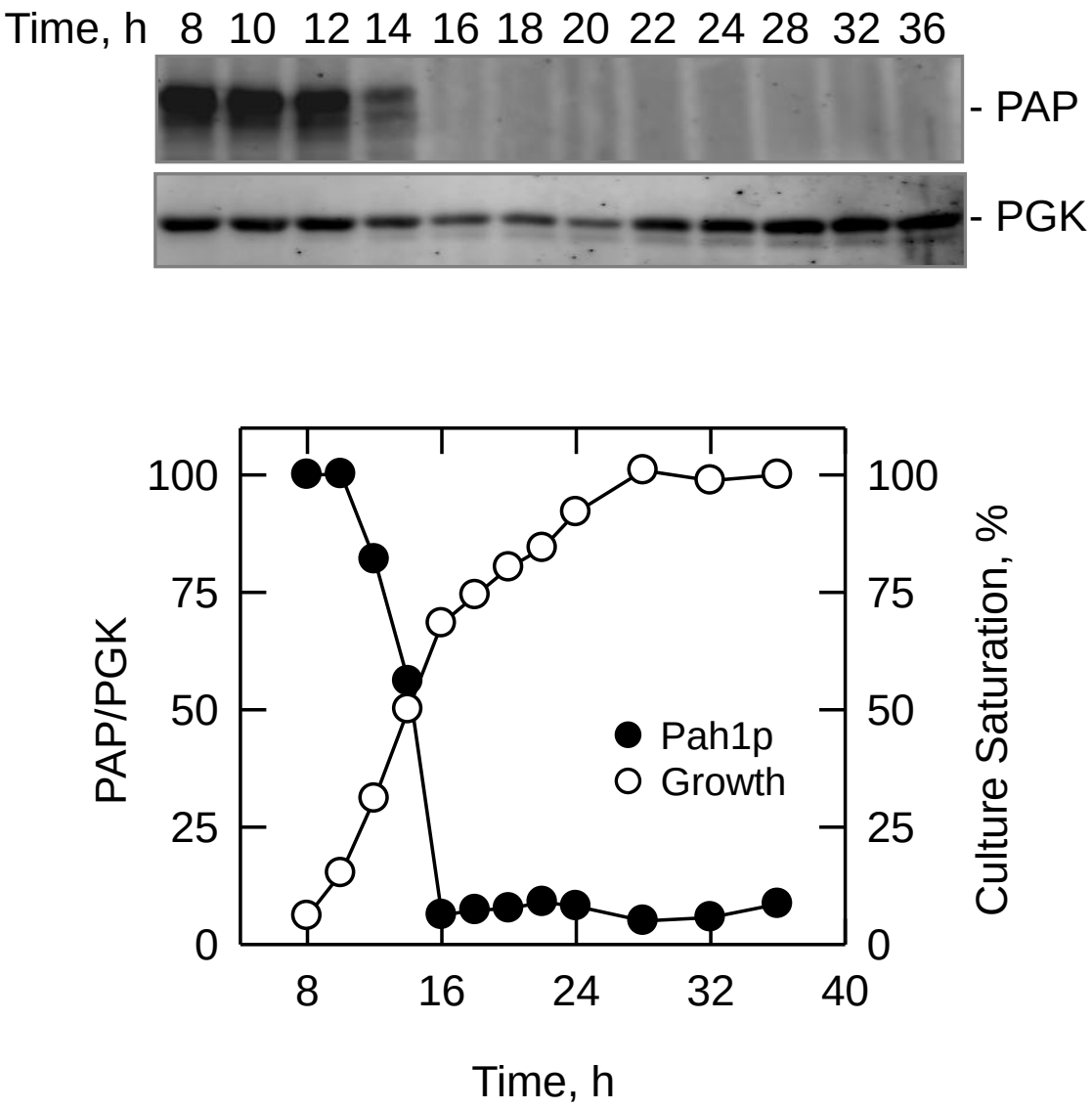

Time, h 8 10 12 14 16 18 20 22 24 28 32 36
- PAP
- PGK
PAP/PGK
Culture Saturation, %
Pah1p
Growth
Time, h
0
25
50
75
100
8
16
24
32
40

**Figure. 32. Effect of growth phase on PAP activity.** *pah1*Δ *dpp1*Δ *lpp1*Δ cells expressing *PAH1* from the multicopy plasmid pGH312 or the empty vector [YEp351] were grown in synthetic complete media and were collected at the indicated time of growth. Cell extracts were prepared and assayed for PAP activity. Each data point represents the average of triplicate enzyme determinations from two independent experiments ± S.D.

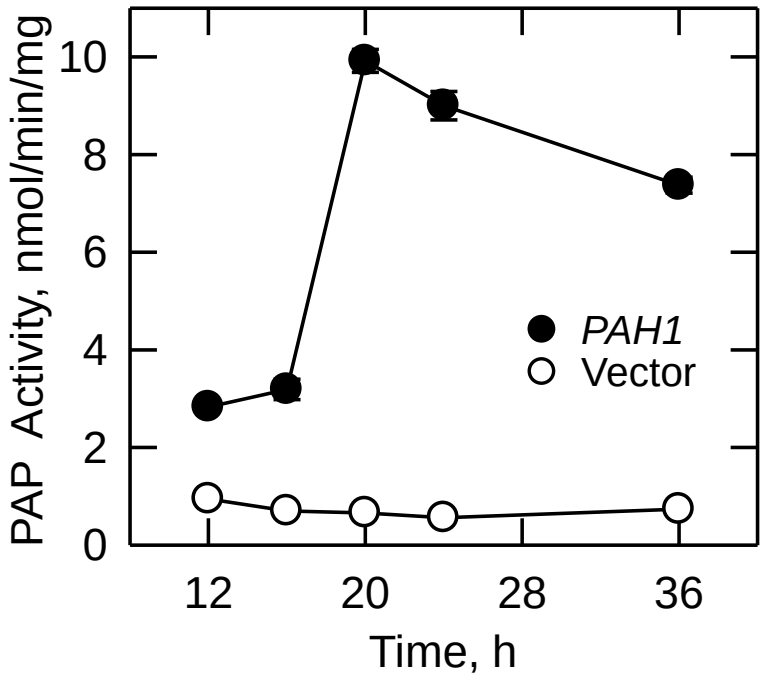
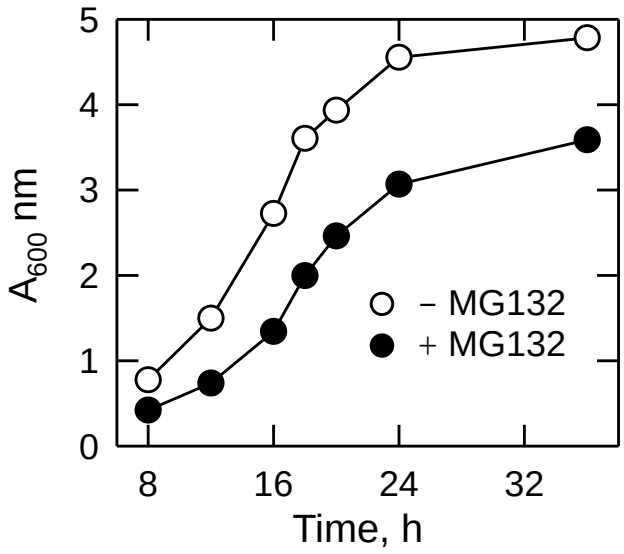
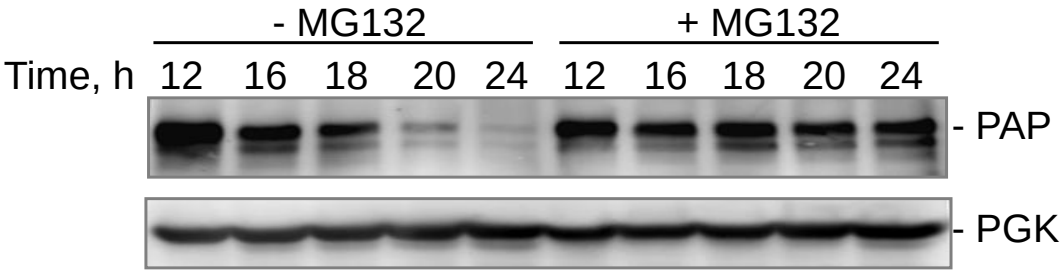

PAP Activity, nmol/min/mg
0
2
4
6
8
10
12
20
28
36
Time, h
PAH1
Vector

**Figure. 33. Effect of MG132 on PAP protein abundance during growth phase.** (A) *pdr5*Δ mutant cells expressing the *PAH1* gene from the multicopy plasmid pGH312 were grown in synthetic complete media in the absence and presence of the proteasomal inhibitor Z-Leu-Leu-Leu-al (MG132) to a final concentration of 50 μM. The growth of these cultures was monitored by recording their absorbance at 600 nm at the indicated times. (B) Cell extracts were prepared and subjected to immunoblot analysis using anti-PAP and anti-PGK antibodies (loading control).

A


5
4
3
2
1
0
$A_{600}$ nm
8
16
24
32
Time, h
– MG132
+ MG132

B


- MG132
+ MG132
Time, h
12
16
18
20
24
12
16
18
20
24
- PAP
- PGK

# DISCUSSION

Zinc is an essential nutrient for the growth and development of eukaryotes (99). In the yeast *Saccharomyces cerevisiae* zinc depletion resulted in a decrease in the CDP-DAG pathway enzymes PS synthase, PS decarboxylase, PE methyltransferase and phospholipid methyltransferase (61). More importantly, zinc depletion resulted in a decrease in PS and PE levels and an increase in PI levels (61). However, PC levels were not affected by zinc depletion (61). These observations led to the hypothesis that the CDP-choline pathway is induced to cope with the reduction in enzymatic activity of the CDP-DAG pathway and maintain the PC unchanged. In this dissertation we investigated the regulation of choline kinase, which catalyzed the committed step in the CDP-choline pathway, and PAP which supplies the DAG utilized in the third and final step of the CDP-choline pathway for the synthesis of PC in response to zinc depletion.

The synthesis of phospholipids is coordinately regulated with the expression of zinc transporters that control zinc homeostasis in *S. cerevisiae* (125). The depletion of zinc from the growth medium results in the induced expression of zinc transporters (e.g., Zrt1p, Zrt2p, Fet4p, and Zrt3p), and their transport functions increase the cytoplasmic levels of zinc (211, 212). At the same time, zinc depletion causes alterations in membrane phospholipid composition (e.g., an increase in PI and decreases in PE, PA, and diacylglycerol pyrophosphate) that are brought by changes in the expression of phospholipid synthesis enzyme activities (59, 61, 123-125). The PC content of cellular membranes is not altered by zinc depletion even though the enzyme activities responsible for its synthesis via the CDP-DAG pathway are repressed (61). In this study, we provided a mechanistic explanation for this observation. The decrease in PC synthesis

via the CDP-DAG pathway was compensated by an increase in PC synthesis via the CDP-choline branch of the Kennedy pathway. Data indicated that the Zap1p-mediated induction of the *CKI1*-encoded choline kinase played an important role in this regulation. This compensatory mechanism complements the Zap1p-mediated induction of the *EKI1*-encoded ethanolamine kinase (Fig. 1) for increased PC synthesis in response to zinc depletion (123).

Analysis of $P_{CKI1}$-*lacZ* reporter gene activity indicated that the expression of *CKI1* was induced in response to zinc depletion, a growth condition that resulted in a limiting intracellular concentration of zinc (43, 117). That a limiting cellular zinc concentration was responsible for this regulation was confirmed by the induced expression of $P_{CKI1}$-*lacZ* reporter gene activity in zinc-supplemented *zrt1*Δ *zrt2*Δ mutant cells that lack the major plasma membrane zinc transporters Zrt1p and Zrt2p (109, 110). The induction of $P_{CKI1}$-*lacZ* reporter gene activity was precluded by the *zap1*Δ mutation. This indicated that *CKI1* expression was mediated by Zap1p, a positive transcription factor that is induced in zinc-depleted cells and repressed in zinc-replete cells (117).

Zap1p interacts with a $UAS_{ZRE}$ in the promoters of several genes to activate transcription when wild type cells are depleted for zinc. These include zinc transporter genes (e.g., *ZRT1*, *ZRT2*, *ZRT3*, *FET4*) that control intracellular stores of zinc (111, 113, 212-214) and phospholipid synthesis genes (e.g., *DPP1*, *PIS1*, *EKI1*) that control membrane phospholipid composition (60, 123, 124). Electrophoretic mobility shift assays indicated that purified GST-$Zap1p^{687-880}$ interacted with two of the three putative $UAS_{ZRE}$ sequences (i.e., ZRE1 and ZRE2) within the *CKI1* promoter. The *in vitro* interactions of GST-$Zap1p^{687-880}$ with ZRE1 and ZRE2 were specific and could be

abolished by mutations to a nonconsensus $UAS_{ZRE}$ sequence. Moreover, mutations of ZRE1 and ZRE2 to a nonconsensus $UAS_{ZRE}$ sequence attenuated the induced expression of the $P_{CKI1}$-*lacZ* reporter gene activity when cells were depleted for zinc.

The Zap1p binding sites (i.e., ZRE1 and ZRE2) in the *CKI1* promoter were not identical (64 and 73% identity, respectively) to the core consensus $UAS_{ZRE}$ that is based on the promoters of *ZRT1*, *ZRT2*, and *ZAP1* (116). It is known that deviations from the core sequence reduce the interaction of Zap1p to the $UAS_{ZRE}$ sequence (123, 124). This provides an explanation as to why the Zap1p-mediated induction of *CKI1* was not as great as that observed for Zap1p-mediated induction of zinc transporter genes (e.g., *ZRT1*, *ZRT2*, *ZRT3*) or for the phospholipid metabolism gene *DPP1* that contain $UAS_{ZRE}$ sequences with high identity to the core sequence (43, 60, 109, 110). We speculate that differences in Zap1p binding efficiency based on the $UAS_{ZRE}$ sequence provide a mechanism to control the relative induction of various genes in response to zinc depletion.

The $UAS_{INO}$ element is found in several phospholipid synthesis genes in *S. cerevisiae* (1, 2, 4-6). It is the binding site for the transcriptional activator complex Ino2p-Ino4p that stimulates transcription of most $UAS_{INO}$-containing genes (2, 4, 5, 69). When exponential-phase wild type cells are supplemented with inositol, or depleted from zinc, the repressor Opi1p interacts with Ino2p to attenuate transcription of $UAS_{INO}$-containing genes such as *CHO1* and *INO1* (69, 125). *CKI1* contains a $UAS_{INO}$, and the work presented here using the $P_{CKI1}$-*lacZ* reporter gene showed that this element was responsible for stimulating *CKI1* expression. However, the $UAS_{INO}$ element was not involved with the regulation of *CKI1* expression in response to zinc depletion.

Choline kinase activity is the functional product of the *CKI1* gene (45, 137). The Zap1p-mediated induction of *CKI1* expression in response to zinc depletion translated into a 2.7-fold increase in choline kinase activity. This conclusion was confirmed by the loss of induced choline kinase activity in *zap1*Δ mutant cells. Moreover, the *in vitro* data showing the zinc-regulated induction of choline kinase activity was mirrored *in vivo*. The incorporation of labeled choline into phosphocholine was elevated by 2-fold in response to zinc depletion. In addition, the increase in *CKI1* expression and choline kinase activity correlated with a 1.8-fold increase in PC content. Thus, the synthesis of PC via the Kennedy pathway was elevated in zinc-depleted cells, and that choline kinase played an important role in this regulation. We also showed that zinc depletion caused an increase in the cellular choline content. This result might reflect an increase in choline transporter activity and/or an increase in the turnover of PC that was synthesized via the Kennedy pathway. Additional work will be required to address these hypotheses.

This work advances our understanding of the regulation of phospholipid synthesis in *S. cerevisiae* by zinc availability, and in particular, the transcriptional regulation of the *CKI1*-encoded choline kinase. The importance of understanding choline kinase regulation is highlighted by the fact that the enzyme in mice is essential for embryonic development (α form (144)) and for normal muscular development (β form (145)). Moreover, unregulated levels of choline kinase play a role in the generation of human tumors by *ras* oncogenes (215-218). By catalyzing the committed step in the Kennedy pathway, choline kinase regulation governs PC content. Whether PC content per se or whether another membrane phospholipid(s) (e.g., PE and PI) regulates zinc transporter function to control zinc homeostasis are important questions that warrant further

investigation.

Recently the *PAH1*-encoded PAP was identified by reverse genetics (54). This enzyme catalyzes the dephosphorylation of PA to yield DAG and $P_i$ (152). The DAG produced by this enzymatic reaction is utilized for the synthesis of TAG and the synthesis of PC and PE via the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway, respectively (1-3, 5, 219). This enzyme contains an HAD-like domain with a catalytic DXDXT motif characteristic of a superfamily of $Mg^{2+}$-dependent phosphatase enzymes (220, 221). In addition, PAP can be found as a cytosolic and peripheral membrane protein (54). This study demonstrated that the *PAH1*-encoded PAP is induced in a Zap1p-dependent manner in response to zinc depletion. The induction in PAP activity resulted in an increase in DAG levels, utilized for the synthesis of PC via the CDP-choline branch of the Kennedy pathway (Fig. 2).

Analysis of a $P_{PAH1}$-*lac*Z reporter gene points out that the expression of the *PAH1* gene was induced in response to zinc depletion. That low intracellular zinc levels induced this regulation was confirmed by the induction in $P_{PAH1}$-*lac*Z reporter expression in *zrt1*Δ *zrt2*Δ mutant cells (lacks the major plasma membrane zinc transporters, Zrt1p and Zrt2p) grown in the presence of zinc (109, 110). Mutation in the zinc responsive transcription factor, Zap1p resulted in a reduction in expression of the $P_{PAH1}$-*lac*Z in response to zinc depletion. The Zap1p dependent induction of the *PAH1* gene translates in an increase in PAP activity in response to zinc depletion. This conclusion was confirmed by the loss of induced PAP activity in *zap1*Δ mutant cells. This demonstrated that *PAH1* induced expression in response to zinc depletion is governed by Zap1p.

Zap1p is composed of two activation domains and a DNA binding domain (118-

120). The two activation domains have the ability to sense intracellular levels of zinc, where high levels inhibit and low levels induce Zap1p activity (121, 122). The DNA binding domain is composed of five zinc fingers that recognize and binds the ZRE sequences in the promoter of zinc responsive genes (119). Electrophoretic mobility shift assays demonstrate that a GST-Zap1p$^{687\text{-}880}$ recognizes and binds all three $UAS_{ZRE}$ sequences (ZRE1, ZRE2 and ZRE3) located in the *PAH1* promoter. The binding affinity of the GST-Zap1p$^{687\text{-}880}$ was different between the *PAH1* $UAS_{ZRE}$ sequences, with ZRE2 showing the strongest and ZRE3 the weakest binding affinity. The interaction of GST-Zap1p$^{687\text{-}880}$ with ZRE1, ZRE2 and ZRE3 were specific and could be abolished by mutations to a nonconsensus $UAS_{ZRE}$ sequence. This demonstrated that Zap1p binds the *PAH1* $UAS_{ZRE}$ sequences inducing the expression and PAP activity in response to zinc depletion.

PAP activity catalyzes the dephosphorylation of PA to yield DAG and Pi. Cells carrying the *pah1*Δ mutation show an accumulation in PA, and diminished levels of DAG and TAG (54). The effect on TAG level is more dramatic as cells enter the stationary phase of growth. Cells enter the stationary phase of growth in response to the lack of essential nutrients. It is characterized by a decrease in growth rate, accumulation of glycogen, resistance to environmental stresses, thickening of the cell wall and the ability to survive starvation (222). Zinc depletion resulted in an increase in the levels of DAG and TAG. Mutation of PAP demonstrated that PAP plays a major role for both the synthesis of DAG and TAG in response to zinc depletion. Thus, zinc depleted cells showed a similar behavior as cells that enter the stationary phase of growth. All together, these data demonstrated the importance of PAP in response to zinc depletion, specifically

in the formation of DAG and TAG.

The product of the PAP reaction, DAG, is utilized for the synthesis of PE and PC via the CDP-ethanolamine and CDP-choline branches of the Kennedy pathway (54). Zinc depletion resulted in an increase in choline incorporation and the formation of phosphocholine, due to an increase in choline kinase activity and PC synthesis (187). The *pah1*Δ mutant showed an increase in choline incorporation. The *pah1*Δ mutation resulted in an increase in phosphocholine formation in cells grown in both the presence and absence of zinc. The reason for an increase in phosphocholine could be attributed to an increase in choline kinase activity as a result of the *pah1*Δ mutation. This hypothesis could be examined by analyzing the *CKI1* expression and choline kinase activity in *pah1*Δ mutant cells grown in the presence and absence of zinc. The *pah1*Δ mutant had an accumulation of CDP-choline, resulting from the decrease in the levels of DAG required for the third and final step for the synthesis of PC via the CDP-choline pathway. Surprisingly, the PC level was higher in *pah1*Δ mutant cells grown in the presence of zinc. One possible reason could be that the *pah1*Δ mutation resulted in the mis-regulation of the CDP-DAG pathway in response to zinc depletion. Additional work will be required to address this hypothesis.

The CDP-DAG pathway is the primary pathway for the synthesis of PC in the yeast *Saccharomyces cerevisiae* (1, 2, 6). During zinc starvation the activity of the CDP-DAG pathway enzymes is down-regulated (61) and the CDP-ethanolamine and CDP-choline pathways are up-regulated to cope the reduction in PC synthesis (123, 187). The *CHO1*-encoded PS synthase catalyze the committed step in the CDP-DAG pathway, forming PS from CDP-DAG and serine (28-31). The *CHO1* gene is down-regulated in

response to zinc depletion in a Zap1p independent manner (61). Instead the regulation of this gene is mediated through the $UAS_{INO}$ element in its promoter and the transcription factors Ino2p, Ino4p and Opi1p (61). Ino2p, Ino4p and Opi1p play an important role in the inositol regulation of phospholipid biosynthesis (1, 2, 4-6). Ino2p (62) and Ino4p (63) function as positive regulators of transcription inducing maximum expression of $UAS_{INO}$ containing genes when inositol is not present, whereas Opi1p (64) serves as a negative regulator inhibiting expression during inositol supplementation. In the absence of inositol supplementation, Opi1p is tethered to the nuclear/ER membrane through interactions with Scs2p (70) and PA (71). Upon inositol supplementation, the PA pool is consumed for the synthesis of CDP-DAG and PI resulting in the translocation of Opi1p from the ER to the nucleus where it binds to Ino2p and inhibits the transcription of $UAS_{INO}$-containing genes (71, 199, 223, 224).

The PA abundance plays a crucial role in Opi1p localization and regulation of phospholipid synthesis (71). The levels of PA are controlled by its *de novo* synthesis from glycerol 3-phosphate, its utilization by the CDP-DAG synthase to form CDP-DAG, its dephosphorylation by PAP to yield DAG and by the hydrolysis of PC by phospholipase D (2). It is possible that accumulation in the levels of PA due to the *pah1*Δ mutation, even in the absence of zinc, maintain Opi1p tethered to the nuclear/ER membrane resulting in the derepression of the CDP-DAG pathway genes (*CHO1*, *PSD1/2*, *CHO2* and *OPI3*). This possibility could be examined by the expression of a $P_{CHO1}$-*lacZ* reporter gene in *pah1*Δ mutant cells grown in the presence and absence of zinc. If the *pah1*Δ mutation results in the derepression of the *CHO1* gene during zinc starvation the results will show no difference in β–galactosidase activity between the two

treatments. In addition, the PS synthase abundance and activity could be examined in *pah1*Δ mutant cells grown in the absence of zinc to confirm this hypothesis. Finally, the phospholipid composition of *pah1*Δ mutant cells during zinc starvation should be examined to corroborate if the derepression of the CDP-DAG pathway restores the levels of PS, PE and PI observed during zinc supplementation. This would demonstrate the crucial role played by PAP in the regulation of PA levels and how this affects the expression of other phospholipid biosynthetic enzymes.

PAP is up-regulated in response to the lack of nutrients, resulting in an increase in DAG and TAG similar to what is observed as cells enter the stationary phase of growth. We investigated the regulation of PAP throughout growth. Stationary phase cells differ from exponential cells in that they stop to multiply, fail to accumulate mass and volume and their metabolism diminishes to a minimum. In addition, as cells transition from the exponential to the stationary phase of growth the synthesis of phospholipids is shifted to TAG synthesis (163, 164). Work by Han and coworkers (54) demonstrated that the *PAH1*-encoded PAP activity produces the DAG utilized for the synthesis of phospholipids and TAG during the exponential and stationary phase of growth, respectively. Work by Taylor and Parks (164) has demonstrated that the synthesis of TAG occurs throughout the exponential phase of growth. Our study confirmed that PAP supplied the DAG required for the synthesis of TAG throughout growth as demonstrated by the reduction in DAG and TAG formation in the *pah1*Δ mutant.

PAP activity increased as cells progressed from the exponential to the stationary phase growth, and this was in parallel with the accumulation of TAG (163, 164). Total PAP activity was induced as cell entered the stationary phase. The *dpp1*Δ *lpp1*Δ double

mutant, lacking all LPP activity, demonstrated that PAP activity was indeed induced as cells enter the stationary phase of growth. The *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant, which lacks all known PAP acitivty, still retains some PAP activity (54). Analysis of the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant suggested that the gene(s) encoding this unknown PAP was not induced during the transition from exponential to stationary phase. To our surprise, the PAP protein level was reduced to undetectable levels as cell transitioned from exponential to stationary phase, at the exact time when PAP activity increased. As cells enter the stationary phase, protein synthesis and degradation are tightly regulated (225). It is predicted that the PAP protein sequence possesses multiple ubiquitination sites. Treatment of *pdr5*Δ mutants with the proteasomal inhibitor MG132 blocked the degradation of PAP, suggesting that PAP was degraded via the ubiquitin-proteasome pathway as cells entered the stationary phase of growth. This may be a mechanism to limit protein activity and cell viability under this challenging condition.

In addition to protein degradation, PAP is regulated by phosphorylation. Phosphorylation of PAP by the cyclin-dependent kinase Cdc28p results in a decrease in PAP activity (97, 98) and its dephosphorylation by the Nem1p/Spo7p phosphatase results in an increase in PAP activity (82). Preliminary data has shown that PAP is a target for phosphorylation for protein kinase A and C. Cdc28p, protein kinase A and C are required for proper progression through the cell-cycle, regulation of growth and development in response to nutrient availability (83-85, 226, 227). This raises the possibility that the increase in PAP observed as cell transition from the exponential to the stationary phase of growth is due to the dephosphorylation of PAP and the reduction in kinase activity observed at this stage of growth. Additional work is required to elucidate the signals and

the mechanism of action for the degradation of PAP and the role of phosphorylation/dephosphorylation on PAP activity in response to growth.

This work demonstrates that the yeast *PAH1*-encoded PAP is regulated in response to zinc depletion in a Zap1p dependent manner. The induction of PAP resulted in an increase in PAP activity which translates in an increase in the levels of DAG and TAG. The DAG produced by the PAP reaction is required for the synthesis of PC via the CDP-choline pathway in response to zinc depletion. In addition, PAP is required for the synthesis of TAG throughout growth. The transition from exponential to the stationary phase of growth resulted in an increase in PAP activity which translates to the rise in DAG and TAG levels. The increase in PAP activity and TAG levels coincides with the proteasomal degradation of PAP.

This work advances our understanding of phospholipid biosynthesis in response to zinc depletion and growth phase, particularly the transcriptional and post-translational regulation of the *PAH1*-encoded PAP enzyme. Our understanding on how PAP is regulated is highlighted by the fact that the yeast PAP shares homology with the human *lipin1* protein (54). Lipin 1 regulates fat metabolism in mammalians cells (161, 170-173). Loss of lipin 1 in mice results in abnormal adipose tissue development, lipodystrophy and insulin resistance and its overexpression results in obesity and insulin sensitivity (161, 170). By catalyzing the pivotal step in TAG synthesis, PAP represents an important candidate for drug development that can regulate the induction (lipid dystrophy) or inhibition (obesity) of enzymatic activity.

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# CURRICULUM VITAE

Aníbal Soto-Cardalda

## Education

2002 B.S. in Biology, Universitiy of Puerto Rico, Rio Piedras Campus, Puerto Rico

2010 Ph.D. in Microbiology and Molecular Genetics, Rutgers University, New Brunswick, New Jersey

## Publications

2008 **Soto, A.** and G.M. Carman. Regulation of the *CKI1*-encoded Choline Kinase by Zinc Depletion. J. Biol. Chem.283: 10079-10088.

2008 T. G. Kinzy, K. H. Chang, J. Davila, J. Defren, L. DeStefano, J. Donovan, A. Esposito, J. Hurley, P. Irizarry-Barreto, R. Roy, **A. Soto**, A. Valentin-Acevedo, R. Ysla and P. R. Copeland. A Birth-to- Death View of mRNA From the RNA Recognition Motif Perspective. *Biochem. and Mol. Bio. Educ.* 36: 1-8.

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