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Characterization And Identification Of Novel Regulators Of The Synthesis Of Phospholipids

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CHARACTERIZATION AND IDENTIFICATION OF NOVEL REGULATORS OF
THE SYNTHESIS OF PHOSPHOLIPIDS

by

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Approved by:

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Advisor Date

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DEDICATION

献给我的妻子陆帼蓉和儿子叶心州

献给我的父母和祖父母

To my wife, Guorong Lu, my son, Xinzhou Ye, my parents, sister, and grandparents, with all my love
ACKNOWLEDGMENTS

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Finally, I would like to thank my wife, my parents, my parents-in-law, my grandparents for their unconditional love and numerous supports. I would never make it this far without them.
# TABLE OF CONTENTS

Dedication........................................................................................................................................ ii

Acknowledgements......................................................................................................................... iii

List of Tables..................................................................................................................................... vi

List of Figures..................................................................................................................................... vii

Chapter 1 – **INTRODUCTION** ...................................................................................................... 1

Chapter 2 – **REGULATION OF INOSITOL METABOLISM IS FINE-TUNED BY INOSITOL PYROPHOSPHATES IN SACCHAROMYCES CEREVISAIE**

  Introduction...................................................................................................................................... 14

  Materials and Methods.................................................................................................................... 19

  Results ................................................................................................................................................ 28

  Discussion......................................................................................................................................... 53

Chapter 3 – **DELETION OF THE CARDIOLIPIN-SPECIFIC PHOSPHOLIPASE CLD1 RESCUES GROWTH AND LIFESPAN DEFECTS IN THE TAFAZZIN MUTANT: IMPLICATIONS FOR BARTH SYNDROME**

  Introduction...................................................................................................................................... 59

  Materials and Methods.................................................................................................................... 64

  Results................................................................................................................................................ 71
Discussion...........................................................................................................90

Chapter 4 – INOSITOL BIOSYNTHESIS REGULATES ACTIVATION OF GSK-3A IN NEURONAL CELLS

Introduction.............................................................................................................100

Materials and Methods..........................................................................................105

Results.....................................................................................................................111

Discussion..............................................................................................................126

Chapter 5 – FUTURE DIRECTIONS......................................................................130

References.............................................................................................................138

Abstract ..................................................................................................................173

Autobiographical Statement .....................................................................................175
LIST OF TABLES

Table 1-1  Strains used in Chapter 2  .................................................................20
Table 1-2  Plasmids used in Chapter 2  .........................................................21
Table 1-3  Real-time PCR primers used in Chapter 2  ..............................25
Table 1-4  Mutants that cannot produce 5PP-IP4 exhibit decreased growth on L- .................................................................52
Table 2-1  Strains and plasmids used in Chapter 3  ..................................65
Table 2-2  Real-time PCR primers used in Chapter 3  ..............................68
Table 3-1  Real-time PCR primers used in Chapter 4  ............................108
# LIST OF FIGURES

| Figure 1-1 | Inositol metabolism | .................................................................4 |
| Figure 1-2 | Synthesis of phospholipids | ..........................................................6 |
| Figure 1-3 | CL de novo synthesis and remodeling in *S. cerevisiae* | ..............9 |
| Figure 2-1 | Biosynthetic pathway for inositol pyrophosphates in yeast | ......18 |
| Figure 2-2 | Decreased inositol biosynthesis in *kcs1Δ* is due to downregulation of *INO1* transcription | .........................................................29 |
| Figure 2-3 | Opi1-Ino2-Ino4 is not perturbed in *kcs1Δ* | .................34 |
| Figure 2-4 | 5PP-IP₄ is indispensible for optimal *INO1* transcription | .............38 |
| Figure 2-5 | Basic leucine zipper (bZIP) and inositol pyrophosphate kinase (DINS) domains of Kcs1 are required for *INO1* transcription | .........................................................41 |
| Figure 2-6 | Increased Kcs1 protein levels in I- conditions | .....................44 |
| Figure 2-7 | Kcs1 protein levels in response to exogenous inositol | .....................47 |
| Figure 2-8 | Model of regulation of *INO1* transcription by Kcs1 and inositol pyrophosphates | .................................57 |
| Figure 3-1 | CL de novo synthesis and remodeling in *S. cerevisiae* | ..........61 |
| Figure 3-2 | Deletion of *CLD1* rescues growth and chronological life span defects in *taz1Δ* | .................................73 |
Figure 3-3  Increased *CLD1* expression in the stationary phase is concomitant with increased CL unsaturation ..........................76

Figure 3-4  *CLD1* expression is increased in response to respiration and activated by HAP .................................................................81

Figure 3-5  Overexpression of *CLD1* decreases cell growth, respiration, and mitochondrial aconitase activity .........................87

Figure 3-6  Overexpression of *CLD1* leads to increased ATP and ethanol ..........................................................91

Figure 3-7  Proposed model .............................................................................97

Figure 4-1  Exogenous inositol is not essential for cell proliferation or maintaining inositol homeostasis in SK-N-SH cells ............112

Figure 4-2  Inositol biosynthesis is essential for cell proliferation ............115

Figure 4-3  Exogenous inositol does not regulate transcription of the genes for inositol biosynthesis or uptake .......................120

Figure 4-4  VPA decreases intracellular inositol and increases the inhibitory phosphorylation of GSK-3α .................................124
CHAPTER 1

INTRODUCTION

Lipids are essential building blocks of the cell. Membrane lipids form a phospholipid bilayer surface, separate cells from the outside environment, and enclose compartments of organelles with distinct shapes and functions in the cytoplasm. Phospholipids are the most abundant lipids in cell membranes, and the major phospholipids in the membranes of \textit{S. cerevisiae} and all eukaryotes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL). Phospholipid synthesis is required for membrane biogenesis and is critical for nearly all aspects of cellular activities.

Regulation of the synthesis of phospholipids is a highly coordinated event, which has been well studied in yeast (Carman and Henry, 1999; Greenberg and Lopes, 1996; Henry et al., 2012b). Expression of many of the genes encoding phospholipid synthesis enzymes is controlled via a conserved inositol-responsive upstream activating sequence (UAS\textsubscript{INO}) in their promoters in response to inositol availability. Coordinated regulation of synthesis of phospholipids by inositol underscores the importance of regulation of synthesis of inositol, a precursor of inositol-containing lipids. To explore novel mechanisms underlying regulation of synthesis of phospholipids, I carried out three projects described in this dissertation to address the current knowledge gaps in phospholipid biosynthesis. First, in addition to the well-characterized regulatory circuits controlled by the
trans-acting factors Ino2, Ino4, and Opi1, I discovered that inositol pyrophosphates are novel regulators of synthesis of inositol and phospholipids. This demonstrates a new level of complexity of inositol metabolism and phospholipid synthesis. Second, there are very few reported studies of the regulation of inositol synthesis in human cells despite the importance of inositol. I demonstrated for the first time that decreased inositol biosynthesis leads to increased GSK-3α phosphorylation in neuronal cells. Third, although synthesis of most phospholipids (PC, PE, PS and PI) is responsive to exogenous inositol, the synthesis of CL is an exception, as the genes in the CL biosynthetic pathway do not contain the UAS_{INO} motif. I showed that the CL-specific phospholipase is the most highly regulated gene in the pathway, and that its regulation affects energy dynamics. The findings in this study have significant implications for the life-threatening disorder Barth syndrome. In summary, I used both yeast and mammalian cells for my dissertation research and elucidated three novel molecular mechanisms underlying the regulation of phospholipid synthesis, which identify new directions for potential research in this field.

1. SYNTHESIS OF INOSITOL AND PHOSPHOLIPIDS

1.1 Inositol metabolism

Inositol, a ubiquitous six-carbon cyclitol, is an essential metabolite and a precursor for inositol phosphates, phosphoinositides, and inositol sphingolipids (Bachhawat et al., 1995b). These inositol-containing molecules play crucial roles
in gene expression, signal transduction, lipid signaling, and membrane biogenesis. The regulation of inositol-related signaling modulates various cell functions, such as cell growth, apoptosis, endocytosis, neuronal plasticity, and membrane trafficking (Greenberg and Lopes, 1996; Michell, 2011; York et al., 2001). The involvement of inositol and its derivatives in such essential cellular processes reflects the importance of the regulation of inositol metabolism.

In all eukaryotic organisms, there are three ways to obtain inositol (Fig. 1-1): (i) uptake via inositol transporters, (ii) de novo synthesis of inositol from glucose-6-phosphate (G6P) by inositol-3-phosphate synthase (Ino1), and (iii) turnover of PI, which is hydrolyzed by phospholipase C. Inositol de novo synthesis is carried out in two steps, as shown in Fig. 1-1. Ino1 is the rate-limiting enzyme and catalyzes the isomerization of glucose-6-phosphate (G6P) to inositol-3-phosphate. This process is a three-step reaction, which is well characterized (Eisenberg, 1967; Kindl and Hoffmann-Ostenhof, 1964; Loewus and Kelly, 1962b). The Ino1 protein contains three domains: a central domain containing N- and C-termini that interact with monomers, an NAD-binding domain, and a catalytic domain that binds the substrate G6P (Strausberg et al., 2002). The native Ino1 enzyme is a homotrimer in mammalian cells and a homotetramer in yeast and plant cells (Seelan et al., 2009). The 1.8 kb mammalian Ino1 mRNA is diversely expressed in tissues, including the testes, heart, placenta, pancreas and brain (Guan et al., 2003). Three putative isoforms were reported (Strausberg et al., 2002). The recently identified γ-form of Ino1 expressed in most tissues was
Figure 1-1: **Inositol metabolism.** Inositol can be taken up from the extracellular environment or synthesized *de novo* or via recycling. In *de novo* synthesis, Ino1 catalyzes the rate-limiting step of isomerization of glucose-6-phosphate (G-6-P) to inositol-3-phosphate (I-3-P). Transcription of *INO1* is repressed by Opi1 when inositol is abundant. The inositol pool is used for the synthesis of PI, catalyzed by Pis1. PI is further phosphorylated to phosphatidylinositol phosphates. PI(4,5)P2 (PIP2 in the figure) is hydrolyzed to IP3 and diacylglycerol (DAG, not shown). IP3 can be serially dephosphorylated to recycle inositol or further phosphorylated into inositol polyphosphates by Ipk2 and Ipk1. Kcs1 converts IP5 and IP6 to inositol pyrophosphates.
suggested to be a negative regulator of Ino1 activity (Seelan et al., 2009).

1.2 Regulation of synthesis of inositol and phospholipids

Regulation of synthesis of inositol and phospholipids in *S. cerevisiae* has been well-characterized. Expression of genes involved in their biosynthetic pathways is coordinated and controlled by the conserved motif (UAS\textsubscript{INO}) in their promoter regions (Carman and Henry, 1999; Greenberg and Lopes, 1996; Henry et al., 2012b). Exogenous inositol greatly affects the biosynthesis of inositol and phospholipids. Supplementation with exogenous inositol leads to increased PI synthesis and decreased synthesis of most of other phospholipids (Gaspar et al., 2006b; Loewen et al., 2004). The mechanism underlying this response to exogenous inositol has been characterized (Carman and Henry, 2007b; Loewen et al., 2004). Inositol potently regulates biosynthesis of inositol and phospholipids by controlling transcription of *INO1* and other UAS\textsubscript{INO}-containing genes through the transcriptional repressor Opi1 (Greenberg et al., 1982). When inositol is limiting, Opi1 interacts with the VAMP-associated protein (VAP) Scs2 and phosphatidic acid (PA) on the periphery of the nucleus and remains tethered in the cytoplasm (Loewen et al., 2004). When inositol is abundant, PA levels are consumed for the synthesis of PI. The decrease in PA levels disrupts stability of Opi1 on the periphery of the nucleus, leading to translocation of Opi1 into the nucleus. In the nucleus, Opi1 interacts with the transcriptional activator complex Ino2-Ino4 and represses transcription of *INO1* as well as other genes whose promoters contain the UAS\textsubscript{INO} motif (Loewen, 2012; Loewen et al., 2004).
Figure 1-2: Synthesis of phospholipids. Phospholipids are synthesized from phosphatidic acid (PA). PA is converted to CDP-diacylglycerol (CDP-DAG), which is utilized for synthesis of phosphatidylinositol (PI), cardiolipin (CL), and phosphatidylserine (PS). Phosphatidylethanolamine (PE) is synthesized by decarboxylating PS. Three sequential methylation steps convert PE to PC. PC and PE can also be synthesized via the Kennedy pathway from DAG. The genes highlighted in red contain the UAS\textsubscript{INO} motif in their promoters and are transcriptionally controlled by inositol.
The Ino2-Ino4-Opi1 regulatory mechanism also controls the transcription of phospholipid biosynthetic genes. The trans-acting factors Ino2, Ino4, and Opi1 exert regulatory effects on the cis-acting inositol-responsive upstream activating sequence (UAS_{INO}) (Bachhawat et al., 1995b), which is found in the promoters of more than 30 genes in phospholipid metabolic pathways (Carman and Han, 2011; Chen et al., 2007; Henry et al., 2012a). Coordinated expression of the genes involved in phospholipid synthesis highlights the importance of inositol metabolism in the regulation of membrane biogenesis.

1.3 Importance of inositol metabolism in human health.

Although inositol from diet can cross the blood-brain barrier and enter the cerebrospinal fluid and brain parenchyma, this process is very slow (Aukema, 1994). Inositol levels in the brain primarily depend on PI turnover and de novo synthesis (Williams et al., 2002). The brain maintains a high level of free inositol (5–50 mM), which is about 100 times higher than in the blood and other tissues (Palmano et al., 1977; Sherman et al., 1977; Stokes et al., 1983; Wong et al., 1987). The large inositol pool in the brain may be associated with active inositol-related signaling. Changes in inositol levels may reflect a modulated signal transduction in the control of neurotransmitter systems and neuronal functions. Stable inositol levels are critical for normal brain function, and altered inositol levels in the brain are associated with psychiatric and neurological problems (Shi et al., 2006). For example, levels of inositol are altered in the brains of patients with Down syndrome (Acevedo et al., 1997; Berry et al., 1995), stroke (Rumpel et al., 2003), bipolar disorder (Belmaker et al., 2002; Shimon et al., 1997), and
suicide victims (Shimon et al., 1997). Interestingly, two FDA-approved drugs for the treatment of bipolar disorder, lithium and valproic acid (VPA), have the common biological property of depleting intracellular inositol (Azab et al., 2007; Berridge et al., 1989; Harwood and Agam, 2003; Klein and Melton, 1996; Li and Jope, 2010; Vaden et al., 2001). Although therapeutic actions of these two FDA-approved drugs have not been elucidated, the common effect of inositol depletion resulted from two disparate drugs suggests that this may be therapeutically relevant. Understanding the regulation of inositol metabolism may have important implications for the therapeutic mechanism of drugs in bipolar disorder as well as other disorders in which inositol levels may play a role.

2. CL SYNTHESIS AND FUNCTIONS

2.1 CL de novo synthesis and remodeling

CL is a unique phospholipid that is predominantly synthesized and localized in mitochondrial membranes (Hostetler et al., 1972; Joshi et al., 2009). Following de novo synthesis, CL undergoes remodeling in which acyl chains are exchanged in a deacylation-reacylation cycle. As depicted in Fig. 1-3, the committed step of CL synthesis is catalyzed by Pgs1, in which CDP-DAG and glycerol-3-phosphate are converted to phosphatidylglycerolphosphate (PGP) (Chang et al., 1998a), which is dephosphorylated to phosphatidylglycerol (PG) by the PGP phosphatase Gep4 (Kelly and Greenberg, 1990; Osman et al., 2010). CL synthase (Crd1) catalyzes
Figure 1-3: CL de novo synthesis and remodeling in *S. cerevisiae*. Pgs1 catalyzes the committed step of CL synthesis by converting CDP-DAG to phosphatidylglycerolphosphate (PGP), which is dephosphorylated to phosphatidylglycerol (PG) by the *GEP4*-encoded PGP phosphatase. CL synthase, encoded by *CRD1*, condenses PG and CDP-DAG to form CL. CL synthesized de novo has primarily saturated acyl chains (CLsat). CLsat is deacylated by the CL-specific phospholipase Cld1 to monolysocardiolipin (MLCL), which is reacylated by tafazzin (the *TAZ1* gene product) to CL containing more unsaturated acyl chains (CLunsat).
the final step of CL de novo synthesis by condensing PG and CDP-DAG to form CL with primarily saturated acyl chains (Chang et al., 1998b; Jiang et al., 1997; Tamai and Greenberg, 1990; Tuller et al., 1998). Following the de novo synthesis of CL on the matrix side of the inner mitochondrial membrane, CL undergoes remodeling in which acyl chains are exchanged. In this process, CL is deacylated to monolysocardiolipin (MLCL) by the CL-specific phospholipase Cld1 on the matrix side of the inner mitochondrial membrane (Baile et al., 2013; Beranek et al., 2009). MLCL is reacylated by the transacylase Taz1 in the mitochondrial periphery (Brandner et al., 2005; Claypool et al., 2008a; Gu et al., 2004; Testet et al., 2005). Remodeled CL has more unsaturated acyl chains than CL synthesized de novo (Beranek et al., 2009; Gu et al., 2004; Vaz et al., 2003; Xu et al., 2003).

2.2 CL cellular functions and related human disorders

As the signature lipid of mitochondria, CL comprises about 15% of total mitochondrial phospholipids (Jakovcic et al., 1971) and interacts with a wide range of mitochondrial proteins (Claypool, 2009; Klingenberg, 2009; Schlame and Ren, 2009; Schlame et al., 2000), including the ADP/ATP carrier (Beyer and Klingenberg, 1985; Claypool et al., 2008b) and respiratory complexes (Eble et al., 1990; Lange et al., 2001; Palsdottir et al., 2003; Shinzawa-Itoh et al., 2007). CL-protein interactions stabilize respiratory chain supercomplexes (Pfeiffer et al., 2003; Zhang et al., 2002) and promote supramolecular associations between the ADP/ATP carrier and respiratory supercomplexes (Claypool et al., 2008b). Therefore, it is not surprising that mitochondrial respiration and energy
production are highly correlated with CL biosynthesis (Claypool et al., 2008b; Gohil et al., 2004; Jiang et al., 2000). Interestingly, CL deficiency also leads to deficiencies in diverse cellular functions other than mitochondrial bioenergetics, including mitochondrial dynamics (DeVay et al., 2009; Joshi et al., 2012), mitochondrial protein import (Gebert et al., 2009; Jiang et al., 2000), cell wall biogenesis (Zhong et al., 2005; Zhong et al., 2007), vacuolar function and morphology (Chen et al., 2008), cell cycle (Chen et al., 2010), aging (Zhou et al., 2009), and apoptosis (Gonzalvez and Gottlieb, 2007; Houtkooper and Vaz, 2008; Schug and Gottlieb, 2009).

A deficiency in CL reacylation resulting from mutations in tafazzin leads to the life-threatening disorder Barth syndrome (BTHS). Although BTHS is the only known disorder genetically linked to CL, perturbation of CL content is also observed in other cardiac pathologies, such as diabetic cardiomyopathy, heart failure, and ischemia/reperfusion injury (Chicco and Sparagna, 2007; Han et al., 2007; Lesnefsky et al., 2009). Because CL mediates a plethora of cellular activities and is associated with many human diseases, understanding how CL synthesis is regulated will benefit our fundamental knowledge of CL functions and shed light on potential therapeutic targets of diseases associated with CL deficiency.

3. PROJECT OUTLINE

The goal of the research described in this dissertation is to characterize and identify novel regulators of the synthesis of phospholipids. To explore
mechanisms underlying the regulation of phospholipid synthesis, I have carried out the following studies:

The studies described in Chapter 2 show that inositol pyrophosphates regulate inositol biosynthesis. I found that inositol pyrophosphate-deficient cells exhibit inositol auxotrophy, decreased intracellular inositol, and decreased phosphatidylinositol due to defective transcription of \textit{INO1}, which encodes the rate-limiting enzyme inositol-3-phosphate synthase, \textit{Ino1}. This is the first demonstration that inositol pyrophosphates are required for synthesis of inositol and phospholipids.

In Chapter 3, I described the effects of \textit{CLD1} deletion on tafazzin-deficient yeast cells. The \textit{cld1Δ} mutant has decreased unsaturated CL, but the CL/MLCL ratio is similar to that of wild type cells. I showed that deletion of \textit{CLD1} rescues growth and lifespan defects in the tafazzin mutant. This suggests that tafazzin deficiency is caused by the decreased CL/MLCL ratio, not by a deficiency in unsaturated CL. I further showed that \textit{CLD1} expression is increased during respiratory growth and regulated by the HAP transcriptional activation complex. Overexpression of \textit{CLD1} leads to decreased mitochondrial respiration and growth and instability of mitochondrial DNA. These findings have implications for BTHS, a mitochondrial disease resulting from mutations in human tafazzin.

In Chapter 4, I described the consequences of perturbation of inositol biosynthesis in neuronal cells. Using an inositol-depleting drug VPA, I found that depletion of intracellular inositol was concomitant with GSK3α phosphorylation.
Using shRNAs targeting the human \textit{INO1} gene to knock down human \textit{INO1} expression in a neural cell line, I determined that GSK3\(\alpha\) phosphorylation was elevated in the \textit{INO1} knockdown cells. Interestingly, GSK3\(\beta\) phosphorylation was not altered. We conclude that perturbation of inositol biosynthesis in neural cells leads to phosphorylation of GSK3\(\alpha\).

While the studies described in the above chapters showed new regulators and novel findings in phospholipid biosynthesis, many interesting questions remain for future research. These potential projects are summarized in Chapter 5.
CHAPTER 2

REGULATION OF INOSITOL METABOLISM IS FINE-TUNED BY INOSITOL PYROPHOSPHATES IN SACCHAROMYCES CEREVISIAE

The work described in this chapter has been published in the Journal of Biological Chemistry 288: 24898-24908, 2013.

INTRODUCTION

Inositol, a ubiquitous six-carbon cyclitol, is an essential metabolite and a precursor of inositol phosphates, phosphoinositides, and sphingolipids (Carman and Han, 2011; Henry et al., 2012a). These inositol-containing molecules play crucial roles in gene expression (Shen et al., 2003; Steger et al., 2003), signal transduction (Strahl and Thorner, 2007), lipid signaling (Kutateladze, 2010), and membrane biogenesis (van Meer et al., 2008a). The regulation of inositol-related signaling modulates various cell functions, such as cell growth, apoptosis, endocytosis, neuronal plasticity, and membrane trafficking (Greenberg and Lopes, 1996; Henry et al., 2012a; York et al., 2001). The involvement of inositol and its derivatives in such essential cellular processes reflects the importance of the regulation of inositol metabolism.

In eukaryotes, inositol can be obtained from exogenous inositol via inositol transporters and from the de novo synthesis of inositol from glucose. Inositol biosynthesis is carried out in two steps, of which the Ino1-catalyzed conversion of glucose-6-phosphate to inositol-3-phosphate is rate-limiting (Loewus and Kelly, 1962a). In Saccharomyces cerevisiae, exogenous inositol potently controls
inositol biosynthesis by regulating *INO1* transcription through the transcriptional repressor Opi1 (Greenberg et al., 1982). In the absence of exogenous inositol, Opi1 is sequestered on the periphery of the nucleus by interaction with the VAMP-associated protein (VAP) Scs2 and with phosphatidic acid (PA) (Loewen et al., 2004). In response to exogenous inositol, PA levels are depleted as PA is utilized in the synthesis of phosphatidylinositol (PI). This results in the rapid translocation of Opi1 to the nucleus, where it inhibits the basic helix-loop-helix (bHLH) transcriptional activator complex Ino2-Ino4 and represses *INO1* transcription (Loewen, 2012; Loewen et al., 2004). This regulatory mechanism also controls the transcription of phospholipid biosynthetic genes. The trans-acting factors Ino2, Ino4, and Opi1 exert regulatory effects on the *cis*-acting inositol-responsive upstream activating sequence (UAS\textsubscript{INO}) (Bachhawat et al., 1995b), which is found in the promoters of more than 30 genes in phospholipid metabolic pathways (Carman and Han, 2011; Chen et al., 2007; Henry et al., 2012a). Coordinated expression of the genes involved in phospholipid synthesis highlights the importance of inositol metabolism in the regulation of membrane biogenesis.

Inositol depletion is an outcome of treatment with mood-stabilizers lithium and valproate due to the inhibition of different steps in the biosynthesis of inositol (Allison et al., 1980; Hallcher and Sherman, 1980; Ju and Greenberg, 2003; Shaltiel et al., 2004a). To gain insight into mechanisms of inositol regulation, we carried out a targeted screen of yeast mutants carrying deletions in genes with possible roles in inositol metabolism to identify mutants that were sensitive to
valproate. One gene identified in this manner is **KCS1**, which encodes inositol pyrophosphate kinase, which catalyzes the synthesis of inositol pyrophosphates. This finding suggested that inositol pyrophosphates may function in the regulation of inositol metabolism.

Inositol pyrophosphates are ubiquitous in mammalian and yeast cells (Barker et al.; Bennett et al.) and have diverse roles in stress response (Dubois et al., 2002a), vesicle trafficking (Saiardi et al., 2002), vacuolar biogenesis (Dubois et al.), telomere maintenance (Saiardi et al., 2005), and energy dynamics (Szikgyarto et al., 2011b). Naturally occurring inositol pyrophosphates are produced from two classes of evolutionarily conserved enzymes that utilize substrates inositol pentakisphosphate (IP$_5$) or inositol hexakis-phosphate (IP$_6$) (Barker et al.). As shown in Fig. 2-1 (see Abbreviations for inositol polyphosphates), Ipk2 and Ipk1 sequentially add a phosphate to distinct sites of the hydroxyl group of the inositol ring. Kcs1 (IP6K in mammals) catalyzes the addition of pyrophosphates to the 5-hydroxyl of IP$_5$ and IP$_6$, generating 5PP-IP$_4$ and 5PP-IP$_5$ (5-IP$_7$). Vip1 (PPIP5K or IP7K in mammals) catalyzes the addition of pyrophosphates to the 1-hydroxyl of IP$_6$, generating 1-IP$_7$ (Lin et al., 2009; Mulugu et al., 2007; Wang et al., 2012). In *S. cerevisiae*, Vip1-produced 1-IP$_7$ is known to regulate phosphate homeostasis by disrupting the Pho80-Pho85-Pho81 complex (Lee et al., 2007). In response to starvation for phosphates, increased 1-IP$_7$ causes inactivation of the kinase complex Pho80-Pho85 (Lee et al., 2007). This leads to activation of the transcription factor Pho4 (Komeili and O'Shea, 1999) and upregulation of *PHO5* and *PHO84*, which scavenge phosphates.
(Carroll and O'Shea, 2002; Wykoff and O'Shea, 2001). Interestingly, recruitment of Ino80 to PHO5 and PHO84 promoters requires the production of IP₄/IP₅ by Ipk2 (Steger et al., 2003), suggesting that inositol polyphosphates play a role in Ino80-mediated chromatin remodeling.

In the current study, we report that inositol pyrophosphates carry out a novel function in the regulation of inositol metabolism. To elucidate the mechanism whereby inositol pyrophosphates regulate inositol synthesis, as suggested by the kcs1Δ phenotype, we determined the effects of disruption of inositol pyrophosphate synthesis on inositol homeostasis. Our findings suggest that inositol pyrophosphates synthesized from IP₅ by Kcs1 are required for the optimal transcription of INO1, but not for activity of the Opi1-Ino2-Ino4 regulatory complex. Moreover, the Kcs1 protein levels are dynamically altered by addition or removal of exogenous inositol, suggesting that rapid turnover of inositol pyrophosphates generated by Kcs1 regulates inositol synthesis. We propose a model in which regulation of Kcs1-catalyzed synthesis of 5PP-IP₄ modulates INO1 transcription.
Figure 2-1: Biosynthetic pathway for inositol pyrophosphates in yeast.

IP$_3$ generated from hydrolysis of PIP$_2$ by Plc1 is the precursor for the synthesis of inositol poly-/pyro- phosphates. Ipk2 catalyzes the synthesis of IP$_4$ and IP$_5$, and Ipk1 catalyzes the synthesis of IP$_6$. Kcs1 can use IP$_5$ or IP$_6$ as substrates. Kcs1 catalyzes the conversion of IP$_5$ to 5PP-IP$_4$ and further to (PP)$_2$-IP$_3$ (not shown), and the conversion of IP$_6$ to 5-IP$_7$. Vip1 catalyzes the synthesis of 1-IP$_7$, and Kcs1 and Vip1 together catalyze the synthesis 1,5-IP$_8$.

The open circles indicate an axial hydroxyl groups that are not phosphorylated. The closed dark circles represent phosphate groups and the closed grey circles β–phosphates. Inositol polyphosphate nomenclature is described in a review article (Bennett et al., 2006b) and summarized in Abbreviations.
MATERIALS AND METHODS

Yeast strains, plasmids and growth media

The yeast *S. cerevisiae* strains used in this study are listed in Table 1-1. Wild type (WT) strain with the GFP-HIS3MX6 cassette integrated at the carboxy terminal end of the *KCS1* open reading frame is obtained from the Yeast-GFP Clone Collection (Invitrogen). Single deletion mutants with the GFP tag and double mutants were obtained by tetrad dissection. Synthetic complete (SC) medium contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), uracil (20 mg/liter), yeast nitrogen base without amino acids (Difco), all the essential components of DIFCO vitamin (inositol-free), 0.2% ammonium sulfate, and glucose (2%). Inositol was supplemented separately where indicated. Synthetic dropout media contained all ingredients mentioned above except for the amino acid used as a selectable marker, and were used to culture strains containing a plasmid. Synthetic complete or dropout medium containing 75 μM inositol is denoted as I+, while medium lacking inositol is denoted I-.

The plasmids used in this study are listed in Table 1-2. The plasmids pFL38, pFV198, pFV217, and pFV241 (Dubois et al., 2002b) are gifts from Dr. Evelyne Dubois, and the UAS<sub>INO</sub> reporter plasmid (Loewen et al., 2004) is a gift from Dr. Christopher Loewen. All the plasmids were amplified and extracted using
<table>
<thead>
<tr>
<th>Strain Description</th>
<th>Genotype Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
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<td>Invitrogen</td>
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<td>Invitrogen</td>
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</tr>
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<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OPi1-GFP-HIS3 Ste2pr-LEU2</td>
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</tr>
<tr>
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<tr>
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<td>Invitrogen</td>
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Table 1-2  plasmids used in this study

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<tr>
<td>pFL38</td>
<td>CEN, URA3</td>
<td>(Dubois et al., 2002b)</td>
</tr>
<tr>
<td>pFV198</td>
<td>pFL38, &lt;i&gt;KCS1&lt;/i&gt;&lt;sup&gt;L1L2→AA&lt;/sup&gt;</td>
<td>(Dubois et al., 2002b)</td>
</tr>
<tr>
<td>pFV217</td>
<td>pFL38, &lt;i&gt;KCS1&lt;/i&gt;&lt;sup&gt;SLL→AAA&lt;/sup&gt;</td>
<td>(Dubois et al., 2002b)</td>
</tr>
<tr>
<td>pFV241</td>
<td>pFL38, &lt;i&gt;KCS1&lt;/i&gt;</td>
<td>(Dubois et al., 2002b)</td>
</tr>
</tbody>
</table>
standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol (Chen et al., 1992).

**Measurement of intracellular inositol**

Intracellular inositol was measured as described previously (Ju and Greenberg, 2003) with minor modifications. Briefly, cells were harvested at 4°C by centrifugation, washed once with ice-cold water, and resuspended in ice-cold 7.5% perchloric acid. Each sample was lysed by vortexing with acid washed glass beads for 10 minutes at 30 second intervals, alternating with 30 seconds incubation on ice. Perchloric acid was removed by titration to pH 7.0 with ice-cold 10 M potassium hydroxide. The cell extracts were clarified by centrifugation for 5 min at 2000 g at 4°C. The supernatants were collected, and intracellular inositol was measured by enzyme-coupled fluorescence assay (Maslanski and Busa, 1990). Inositol content (pmol) was normalized to units of $A_{550}$.

**Determination of phosphatidylinositol (PI) by thin layer chromatography (TLC)**

Yeast cells were grown to the mid-logarithmic growth phases ($A_{550} = 1.0$) at 30°C. Cells were then washed once with ice-cold water, and total lipids were extracted with chloroform: methanol (2:1) (v/v) as described previously (Schneiter and Daum, 2006). The extracted lipids were applied onto silica gel plates (Partsil®...
K6F 60 Å, Whatman) pre-treated with 1.8% boric acid and separated in the one-dimension solvent system chloroform /triethylamine/ethanol/water (30:35:35:7) as described previously (Vaden et al., 2005). Phospholipids were visualized by carbonization at 120 °C for 10 min after dipping plates into 3.2% H₂SO₄ and 0.5% MnCl₂ and subsequent staining with iodine vapor. Stained silica plates were quantified using ImageJ software (National Institutes of Health). Total PI levels in each strain were normalized to total PC levels.

**Spotting assay**

Cells were pre-cultured in I+ to the mid-logarithmic growth phase at 30°C, counted using a hemocytometer, and washed with sterile water. 3 μL aliquots of a series of 10-fold dilutions were spotted onto I+ or I- plates and incubated for 3 days at the indicated temperatures.

**Real-Time quantitative PCR (RT-qPCR) analysis**

Cells were grown to the indicated growth phase and immediately harvested at 4°C. Total RNA was extracted using hot phenol (Ausubel et al., 1994), and purified using the RNeasy Mini Plus kit (QIAGEN, Valencia, CA). Complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s manuals. RT-qPCR reactions were performed in a 20 μL volume using Brilliant III Ultra-Faster SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA).
Triplicates were included for each reaction. The primers for RT-qPCR are listed in Table 1-3. RNA levels were normalized to \textit{ACT1}. Relative values of mRNA transcripts are shown as fold change relative to indicated controls. Primer sets were validated according to Methods and Applications Guide from Agilent Technologies. Optimal primer concentrations were determined, and primer specificity of a single product monitored by a melt curve following the amplification reaction. All the primers were validated by measurement of PCR reaction efficiency. All the primers used in this study have calculated reaction efficiency between 95-105%.

\textit{Quantification of INO1 expression}

\textbf{RT-qPCR analysis.} Cells were pre-grown in I+ to the mid-logarithmic phase and inoculated into fresh I+ medium at A_{550} of 0.05. When the A_{550} reached 0.5, cells were harvested by centrifugation at 3500 rpm for 3 minutes at 30°C, washed with prewarmed I- or I+, and resuspended to fresh I- or I+, respectively. Samples were harvested for RT-qPCR analysis at the indicated times by centrifugation at 3500 rpm for 3 min at 4°C. Cells grown in I+ to an A_{550} of 0.5 were collected at 4°C and used as the 0 hour time point.

\textbf{β-galactosidase reporter assay.} WT and mutant cells that were transformed with the UAS\textsubscript{INO} reporter plasmid were precultured in I+ to the mid-logarithmic growth phase (A_{550} of 0.5-0.8), washed with pre-warmed I-, and resuspended in
### Table 1-3  Real-time PCR primers used in this study.

<table>
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<tr>
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<th>Primers</th>
<th>Sequence (5' to 3')</th>
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<td></td>
<td>Reverse</td>
<td>GCCCAAATCGATTCTAAAA</td>
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<tr>
<td>INO1</td>
<td>Forward</td>
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<td></td>
<td>Reverse</td>
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<tr>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
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<td>Forward</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>PDA1</td>
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<td></td>
<td>Reverse</td>
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<tr>
<td></td>
<td>Reverse</td>
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fresh I-. After continuous growth for 4 hours, cells were harvested, and β-galactosidase was assayed as described previously (Fu and Xiao, 2006; Loewen et al., 2004).

**SDS-PAGE and Western blot analysis**

Cells grown to the indicated growth phase were harvested at 4°C and subjected to mechanical breakage at 4°C with acid washed glass beads in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40, 2 mM EDTA, and 1x protease inhibitor cocktail (Roche). Protein extracts were clarified twice by 5 min-centrifugation at 13,000 g at 4°C to remove cell debris and glass beads. Protein concentration was determined using the BCA™ protein assay (Pierce Protein), with bovine serum albumin as the standard. Extracts containing 50 μg protein were boiled with protein gel sample buffer, separated on 8% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with antibodies (1:3,000 anti-GFP; 1:3,000 anti-tubulin; 1:5,000 appropriate secondary antibodies conjugated with HPR), and visualized using ECL Plus substrate (Pierce Protein), with α-tubulin as the loading control. ImageJ software was used to quantify the intensities of bands.
Visualization of Opi1p-GFP using fluorescence microscopy

To visualize the localization of Opi1p-GFP in WT and kcs1Δ cells, fluorescence microscopy was performed using an Olympus BX41 epi-fluorescence microscope. Images were acquired using an Olympus Q-Color3 digitally charge coupled device camera operated by QCapture2 software. All pictures were taken at 1,000X.
RESULTS

Deletion of KCS1 results in decreased inositol biosynthesis.

To identify potential regulators of inositol biosynthesis, we carried out a targeted screen for the growth of mutants hypersensitive to the inositol depleting drug valproate. Yeast mutants carrying deletions in genes with reported functions in inositol metabolism (Saccharomyces Genome Database (SGD)) were grown on I+ or I- plates. We screened 26 deletion mutants in categories expected to affect inositol metabolism, including inositol polyphosphate kinases, protein kinases and protein phosphatases, vacuolar proteins, and ER membrane proteins. Deletion mutants that exhibited defective growth on I- were further tested for growth on medium supplemented with valproate. One of the mutants identified in this screen was kcs1Δ. Inositol auxotrophy of kcs1Δ was also reported in genome-wide studies of inositol auxotrophy (Villa-García et al., 2011; Young et al., 2010). To further investigate the role of KCS1 in the regulation of inositol metabolism, we analyzed the growth of the kcs1Δ mutant. As seen in Fig. 2-2A, kcs1Δ cells showed an extended lag phase when inoculated into I+ medium compared to isogenic WT cells. Importantly, they did not significantly grow in I- medium. Furthermore, growth of the mutant was diminished relative to that of WT cells at elevated temperatures, even in the presence of inositol (Fig. 2-2B). Consistent with inositol auxotrophy, intracellular inositol levels in kcs1Δ were reduced to less than 30% of WT levels (Fig. 2-2C), and PI were about 42% of
Fig. 2-2

A

![Graph showing A$_{550}$ vs. Time (hours)]

- WT +
- WT -
- kcs1Δ +
- kcs1Δ -

B

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- WT
- kcs1Δ
Fig. 2-2

**C**

Inositol (pmol)

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<th></th>
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<th>vip1Δ</th>
<th>kcs1Δvip1Δ</th>
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**D**

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Figure 2-2: Decreased inositol biosynthesis in kcs1Δ is due to downregulation of INO1 transcription. (A) Growth curves for WT and isogenic kcs1Δ, vip1Δ, and kcs1Δvip1Δ cells grown in I+ and I-. Cells were inoculated in I+ or I- at initial A550 of 0.05, and A550 was measured at indicated times. The growth curves shown in the figure are representative of three experiments.
Figure 2-2 (continued): (B) Serial ten-fold dilutions of WT and kcs1Δ cells were spotted on synthetic complete medium without or with supplementation of inositol. Plates were incubated at indicated temperatures for 3 days. The figure shows a representative experiment that has been reproduced three times. (C) Cells were grown in synthetic complete medium containing 5 μM inositol and harvested in the logarithmic phase (A_{550} ≤ 1.0) or the stationary phase (A_{550} ≥ 2.0). Intracellular inositol was measured as described under “Experimental Procedures.” The average values and standard deviation of at least three independent experiments are shown. (D) PI levels were assayed using the TLC method described under “Experimental procedures”. Cells were grown and harvested at the logarithmic phase in the same condition as for intracellular inositol assay. The figure is representative of two independent experiments. (E) INO1 expression in WT and kcs1Δ cells was determined using RT-qPCR as described under “Experimental Procedures”. Cell pellets were collected for assaying before (0 hour) and after (0.5, 1, and 2 hours) removal of inositol. Values were normalized to the internal control ACT1. INO1 transcripts normalized to ACT1 are represented as fold change relative to WT INO1 levels at 0 hour. (F) Transcripts of PDA1, RDN18, TAF10, TDH3, TFC1, UBC6, and SPT15 and INO1 were assayed by RT-qPCR in WT and kcs1Δ cells 2 hours after removal of inositol. The values of gene transcription in WT normalized to ACT1 are represented as fold change relative to the values in kcs1Δ. ACT1 is used as the internal control. The data shown in (E) and (F) are the average of at least three experiments ± standard deviation.
WT (Fig. 2-2D). Inositol biosynthesis is activated in WT cells in inositol-deficient medium by dramatically upregulating \textit{INO1} transcription (Henry et al., 2012a). However, upregulation of \textit{INO1} mRNA was not observed in \textit{kcs1Δ} (Fig. 2-2E), suggesting that transcription of \textit{INO1} is defective in the mutant. We addressed the possibility that defective \textit{INO1} transcription resulted from a global repression of transcription by comparing mRNA expression of a variety of genes in WT and \textit{kcs1Δ}, including genes in glycolysis (\textit{PDA1, TDH3}), basal transcription (\textit{TAF10, TFC1, SPT15}), and protein processing (\textit{RDN18, UBC6}). None of these genes exhibited decreased expression in \textit{kcs1Δ} (Fig. 2-2F). Taken together, these studies suggested that decreased \textit{INO1} transcription in \textit{kcs1Δ} diminishes biosynthesis of inositol and PI, leading to inositol auxotrophy.

\textit{Decreased inositol biosynthesis in kcs1Δ is not due to perturbation of the UAS_{INO} regulatory complex Opi1-Ino2-Ino4.}

The native promoter of \textit{INO1} contains the UAS_{INO} element that is widely found in the promoter regions of many genes, including genes involved in phospholipid metabolism (Bachhawat et al., 1995b). As shown in Fig. 2-3A, transcription of genes containing the UAS_{INO} element is activated by the Ino2-Ino4 heterodimer interacting with the UAS_{INO}-containing promoter, and repressed by the interaction of Opi1 with Ino2 (Ambroziak and Henry, 1994; Bachhawat et al., 1995a; Henry et al., 2012a; Schwank et al., 1995). Among genes regulated in this manner, \textit{INO1} is the most responsive (Henry et al., 2012a; Loewen, 2012). In the absence of inositol, localization of Opi1 on the ER is stabilized by interaction with Scs2 and PA (Loewen et al., 2004). In response to exogenous inositol, Opi1 is
Fig. 2-3
Fig. 2-3

C

INO2/ACT1 normalized to WT I+

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<thead>
<tr>
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<th>I-</th>
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<tr>
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<tr>
<td>kcs1Δ</td>
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D

INO4/ACT1 normalized to WT

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<th>I+</th>
<th>I-</th>
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</thead>
<tbody>
<tr>
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<td>0.8</td>
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<tr>
<td>kcs1Δ</td>
<td>0.8</td>
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</table>
**Figure 2-3: Opi1-Ino2-Ino4 is not perturbed in kcs1Δ.** (A) The regulatory mechanism of \textit{INO1} transcription. Ino2 and Ino4 are activators of \textit{INO1} transcription. Scs2 stabilizes the negative regulator Opi1 on the ER membrane with phosphatidic acid (PA). Upon addition of inositol, synthesis of phosphatidylinositol (PI) rapidly consumes PA, releasing Opi1p, which translocates to the nucleus and represses \textit{INO1} expression. (B) WT or \textit{kcs1Δ} cells with GFP-tagged Opi1 were cultured in I+ to the mid-logarithmic phase (A550 of 0.5), washed with pre-warmed I-, and resuspended in prewarmed and fresh I-. After growing for 2 hours in I-, cells were examined under a fluorescence microscope. To compare the localization of Opi1-GFP on the periphery of the nucleus and in the nucleus, 75 μM inositol was added to WT or \textit{kcs1Δ} cells in I-. After addition of inositol for 5 minutes, cells were observed under the fluorescence microscope. The figure is representative of six independent experiments. (C) \textit{INO2} and (D) \textit{INO4} transcripts were assayed in WT and \textit{kcs1Δ} cells cultured in the conditions described in Fig. 2E. The data shown in (C) and (D) are the average of three experiments ± standard deviation.
translocated to the nucleus, inhibiting \textit{INO1} transcription (Carman and Henry, 2007a; Loewen et al., 2004). We addressed the possibility that decreased transcription of \textit{INO1} in \textit{kcs1Δ} is caused by retention of the transcription repressor Opi1 in the nucleus. As shown in Fig. 2-3B, in I- conditions, GFP-tagged Opi1 locates on the nuclear rim in \textit{kcs1Δ} as observed in WT cells, indicating that the translocation of Opi1 is not perturbed in \textit{kcs1Δ}. Therefore, \textit{KCS1} does not regulate \textit{INO1} transcription by affecting the localization of Opi1.

We further investigated if the \textit{INO1} transcription defect in \textit{kcs1Δ} was caused by perturbation of the transcriptional activators Ino2 and Ino4. \textit{INO2} is known to be upregulated in I-, while \textit{INO4} is constitutively expressed in both I+ and I- (Ashburner and Lopes, 1995). While \textit{INO2} transcripts were decreased in \textit{kcs1Δ} relative to WT cells, expression in I- was greater than in I+ in both strains (Fig. 2-3C), indicating that decreased \textit{INO1} expression in \textit{kcs1Δ} is not due to an inability to upregulate \textit{INO2}. Levels of the constitutively expressed \textit{INO4} were not significantly diminished in \textit{kcs1Δ} (Fig. 2-3D). These experiments suggest that decreased transcription of \textit{INO1} in \textit{kcs1Δ} is most likely not due to decreased availability of Ino2 and Ino4, although levels of \textit{INO2} transcription were somewhat decreased relative to WT.

\textit{KCS1 is required for optimal INO1 transcription.}

As mentioned, \textit{OPI1} is a transcriptional repressor of \textit{INO1}, and deletion of \textit{OPI1} leads to overproduction of inositol (Greenberg et al., 1982). Not surprisingly, deletion of \textit{OPI1} restored growth of \textit{kcs1Δ} on I- at 30°C and 37°C (Fig. 2-4A).
Serial ten-fold dilutions of WT, kcs1Δ, opi1Δ, kcs1Δopi1Δ, ipk1Δ, kcs1Δipk1Δ, ipk2Δ, kcs1Δipk2Δ, vip1Δ, and kcs1Δvip1Δ cells were spotted on l- and l+ plates, which were incubated at 30°C or 37°C for 3 days. The figure shown is representative of three experiments. (B) WT and isogenic mutants were transformed with a UAS\textsubscript{INO}-lacZ reporter plasmid. Cells were precultured in l+ to the mid-logarithmic phase (A550 of 0.5-0.8), then pelleted, washed with prewarmed l-, and resuspended in prewarmed and fresh l-. After the shift, cells were continuously cultured for 4 hours. β-galactosidase activity was measured as described under “Experimental Procedures.” The data shown in (B) are the average of six experiments ± standard deviation.
Interestingly, deletion of *OPI1* also alleviated the growth defect of *kcs1Δ* on I+ at 30°C (Fig. 2-4A), suggesting that the Opi1-controlled repression of other genes may also be deleterious to the growth of *kcs1Δ*. Deletion of *OPI1* in *kcs1Δ* restored PI levels (Fig. 2-2D). Relatively higher PI levels in *opi1Δ* than WT were most likely due to overproduction of inositol in *opi1Δ*. To determine if *INO1* transcription is also restored in *kcs1Δopi1Δ*, we analyzed *INO1* expression in the double deletion mutant transformed with the *INO1*-lacZ reporter. Surprisingly, although deletion of *OPI1* increased *INO1*-lacZ expression in *kcs1Δ*, expression in *kcs1Δopi1Δ* was only 20-30% of that in WT and *opi1Δ* cells (Fig. 2-4B), suggesting that *KCS1* is required for optimal *INO1* transcription.

*Both bZIP and DINS domains of Kcs1 are required for INO1 transcription.*

As depicted in Fig. 2-5A, Kcs1 has two functional domains (Huang and Symington, 1995; Saiardi et al., 2000): the kinase domain (also referred to diphosphoinositol polyphosphate synthase, DINS) (Saiardi et al., 2000; Saiardi et al., 1999) and two bZIP domains containing four leucine heptad repeats (Dubois et al., 2002a; Huang and Symington, 1995). Plasmids containing the full length *KCS1* or *KCS1* with site mutations in each functional domain were constructed and characterized previously (Dubois et al., 2002a) (Fig. 2-5A). To determine if these domains are required for *INO1* transcription, we assayed growth and *INO1* expression in *kcs1Δ* cells transformed with these plasmids. In contrast to the full length *KCS1* (*p*KCS1), the kinase-mutated *KCS1* (*p*KCS1<sub>SLL→AAA</sub>) did not rescue inositol auxotrophy or restore *INO1* transcription in *kcs1Δ* (Fig. 2-5B and 2-5C). It has been demonstrated that synthesis of inositol pyrophosphates 5-IP<sub>7</sub> and
Fig. 2-5

A

KCS1  

KCS1^{L1L2\rightarrow AA}  

KCS1^{SLL\rightarrow AAA}  

bZIP  

DINS  

L1 L2  

SLL  

L1 L2  

AAA  

B

I-  

I+  

pEV  

pkcs1^{L1L2\rightarrow AA}  

kcs1\Delta  

pkcs1^{SLL\rightarrow AAA}  

pKCS1  

C

INOH1/ACT1  

WT+peV  

kcs1\Delta+peV  

kcs1\Delta+pkcs1^{L1L2\rightarrow AA}  

kcs1\Delta+pkcs1^{SLL\rightarrow AAA}  

kcs1\Delta+pKCS1  

I+  

I-
**Figure 2-5: Basic leucine zipper (bZIP) and inositol pyrophosphate kinase (DINS) domains of Kcs1 are required for INO1 transcription.** (A) Diagram of the bZIP and the DINS functional domains of Kcs1 indicating site mutations disrupting individual domains. (B) Serial ten-fold dilutions of *kcs1Δ* cells carrying either empty vector (pURA3), mutated bZIP domain (p*kcs1*^{L-L2} AA), mutated DINS domain (p*kcs1*^{S-LL} AAA), or WT *KCS1* were spotted on I- or I+ plates. Plates were incubated at 30°C for 3 days. The figure shown is representative of three experiments. (C) Cells harboring the empty vector (pEV), WT *KCS1*, or mutated *KCS1* were cultured in I+ to the mid-logarithmic phase (A550 of 0.5), pelleted, washed with prewarmed I+ or I-, and resuspended in fresh prewarmed I+ or I-. After the shift, cells were grown for 2 hours. *INO1* mRNA was quantified using RT-qPCR as described in “Experimental Procedures.” The data shown in (C) are the average of three experiments ± standard deviation.
5PP-IP$_4$ is virtually eliminated by mutation of the kinase domain (Dubois et al., 2002a). Therefore, Kcs1 kinase activity, which catalyzes the synthesis of inositol pyrophosphates, is required for inositol biosynthesis as well as optimal INO1 transcription. Previous studies also indicated that site mutations in the bZIP domain did not affect the generation of inositol pyrophosphates (Dubois et al., 2002a). Unexpectedly, kcs1Δ cells containing the bZIP-mutated KCS1 exhibited decreased growth on I-, which was rescued by inositol (Fig. 2-5B). Consistent with the defective growth on I-, the strain also exhibited a 50% decrease in INO1 expression compared to WT (Fig. 2-5C). Therefore, both the bZIP and the kinase domains of Kcs1 are required for INO1 transcription.

**Kcs1 protein modulates INO1 transcription.**

To gain insight into how KCS1 modulates INO1 transcription, we measured protein levels of GFP-tagged Kcs1 in WT and opi1Δ cells that were grown in I+ or I-. Two bands were detected by anti-GFP, most likely corresponding to full-length and truncated Kcs1 proteins, as reported previously (Nishizawa et al., 2008). WT cells cultured in I- (Fig. 2-6A), conditions in which INO1 transcription is increased, exhibited elevated levels of Kcs1 protein compared to WT cells cultured in I+. In addition, both Kcs1 protein and INO1 transcription levels were decreased at elevated temperature compared to those observed at 30˚C (Figs. 2-6A and 2-6B). Interestingly, decreased Kcs1 protein levels in I+ relative to I- were not observed in opi1Δ cells (Fig. 2-6A), indicating that OPI1 is required to regulate Kcs1 protein in response to inositol.
**Fig. 2-6**

A

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<tr>
<th></th>
<th>WT</th>
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<th>opI1Δ</th>
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<td></td>
<td>30°C</td>
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<td>Exposure time</td>
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**Kcs1-GFP**

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<thead>
<tr>
<th></th>
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<th>37°C</th>
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<tr>
<td></td>
<td>1.0</td>
<td>0.04</td>
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<td></td>
<td>0.55</td>
<td>3.2</td>
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<tr>
<td></td>
<td>2.7</td>
<td>3.2</td>
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</table>

**α-Tubulin**

|       | 0.17 | 0.25 |

Normalized Kcs1-GFP

|       | 1.0  | 2.1  |

B

**INO1-LacZ expression**

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<thead>
<tr>
<th></th>
<th>Miller Unit</th>
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<tr>
<td>30°C</td>
<td>4.0</td>
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<tr>
<td>37°C</td>
<td>1.0</td>
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Figure 2-6: Increased Kcs1 protein levels in I- conditions. (A) Cell lysates were prepared from WT or isogenic opi1Δ cells containing GFP-tagged Kcs1. Cells were cultured in I+ or I- to the mid-logarithmic phase (A₅₅₀ of 0.5) at 30°C or 37°C as indicated. Anti-GFP antibody was used to detect Kcs1-GFP protein levels using Western blot analysis. 50 μg of total protein was loaded for each sample, and α-tubulin was used as an internal control. The levels of full-length Kcs1 protein (upper band) were quantified using ImageJ software (lower panel). The figure shown is representative of three experiments. (B) INO1 derepression at higher temperature was measured in WT cells transformed with the UASINO-lacZ reporter plasmid. Cells were precultured in I+ at 30°C or 37°C to the mid-logarithmic phase (A₅₅₀ of 0.5-0.8), then pelleted, washed with I- that was prewarmed to 30°C or 37°C, and resuspended in prewarmed and fresh I-. After the shift, cells were continuously cultured at 30°C or 37°C for 4 hours. β-galactosidase activity was measured as described under “Experimental Procedures.” The data shown in (B) are the average of six experiments ± standard deviation.
In order to determine if Kcs1 protein levels respond specifically to inositol, we observed the effects on Kcs1 protein of shifting cells from I+ to fresh I- medium conditions that increase INO1 expression. WT cells were grown in I+ to the mid-logarithmic phase (A550 of 0.5), then shifted to prewarmed I+ or I- medium, and harvested for analysis of Kcs1 protein levels and INO1 expression. As shown in Fig. 2-7A, by 2 hours after the shift to I-, levels of the full length Kcs1 protein increased more than 10-fold. Levels decrease after 4 hours, and Kcs1 was not detected at 6 hours. This pattern is consistent with the pattern of INO1 expression (Fig. 2-7B), which peaked at 2 hours and was significantly diminished at 6 hours. Kcs1 protein was not increased significantly in cells shifted to fresh I+ medium (Fig. 2-7A). These findings indicated that Kcs1 protein levels and INO1 transcription levels are regulated similarly in WT cells in response to exogenous inositol. In contrast to WT cells, opi1Δ cells did not exhibit an increase in Kcs1 protein in response to inositol (Fig. 2-7A), indicating that Opi1 regulates Kcs1 protein levels. Interestingly, in spite of the dramatic increase in Kcs1 protein in response to the shift from I+ to I-, transcription of KCS1 was not altered (Fig. 2-7C).

In reciprocal experiments, we assayed Kcs1 protein levels in cells shifted from I- to I+ (Fig. 2-7D). WT cells were precultured in I- to the mid-logarithmic phase (A550 of 0.5), inositol was then added, and cells were harvested for analysis of Kcs1 protein levels at the indicated times. In control cells (I-), Kcs1 protein exhibited a steady decrease after 1 hour and was reduced to less than 10% of the initial level within 4 hours. In cells supplemented with inositol, the decrease in
Fig. 2-7

A

WT

\[ \text{Kcs1p-GFP} \]
\[ \alpha\text{-tubulin} \]

\( \gamma pf1\Delta \)

\[ \text{Kcs1p-GFP} \]
\[ \alpha\text{-tubulin} \]

B

\( \text{INO1} \)

C

\( \text{KCS1} \)

WT

\[ \text{Kcs1-GFP relative to the control} \]

\( \gamma pf1\Delta \)

\[ \text{Kcs1-GFP relative to the control} \]

\[ \text{time (hours)} \]

\[ \text{time (hours)} \]
Figure 2-7: Kcs1 protein levels in response to exogenous inositol. (A) WT and isogenic *opi1Δ* cells were precultured in I+ to the mid-logarithmic phase (A$_{550}$ of 0.5), washed with prewarmed I+ or I-, and resuspended in prewarmed I+ or I- medium. Cells were grown for the indicated times, and Kcs1-GFP protein levels were assayed as described in Fig. 6. Kcs1-GFP levels are normalized to the level of each individual strain at time 0. The figure shown is representative of two independent experiments. (B) *INO1* and (C) *KCS1* transcription levels in response to the same shift to I+ or I- were assayed using RT-qPCR as described under “Experimental Procedures”. The data shown in (B) and (C) are the average of three experiments ± standard deviation. (D) WT cells were precultured in I- to the mid-logarithmic phase (A$_{550}$ of 0.5), and inositol was added as indicated. Growth curves are depicted in the upper panel. Cells were grown for the indicated times, and Kcs1-GFP protein levels were assayed as described in Fig. 6 and shown in the lower panel. The figure shown is representative of three experiments.
Kcs1 protein levels was greater than in I- controls. The decrease in Kcs1 protein is consistent with the well-established rapid decrease in INO1 transcription observed in response to inositol (Gaspar et al., 2006a; Loewen et al., 2004). Taken together, these experiments indicate that Kcs1 protein, but not the transcription of KCS1, is regulated in response to exogenous inositol, and this modulation of Kcs1 protein requires Opi1.

Inositol pyrophosphates 5PP-IP4 synthesized from IP5 by Kcs1 are required for INO1 transcription.

The findings that Kcs1 protein is required for INO1 expression and that levels of INO1 transcription correspond to levels of Kcs1 protein suggest that Kcs1-catalyzed synthesis of inositol pyrophosphates regulates INO1 expression. We analyzed well-characterized inositol pyro-phosphate mutants to determine which inositol pyrophosphates are responsible for the regulation of INO1 transcription.

The biosynthetic pathways for generating soluble inositol polyphosphates are depicted in Fig. 2-1. Hydrolysis of PIP2 by Plc1 provides IP3 as a precursor for the synthesis of inositol polyphosphates. Ipk2 catalyzes the synthesis of IP4 and IP5, and Ipk1 catalyzes the synthesis of IP6. Kcs1 catalyzes the pyrophosphorylation of IP5 to 5PP-IP4 and further to (PP)2-IP3 (not shown), and IP6 to 5-IP7. (Draskovic et al., 2008; Onnebo and Saiardi, 2009; Saiardi et al., 1999). Vip1 catalyzes the synthesis of inositol pyrophosphates at the 1- hydroxyl site of the inositol ring (Lin et al., 2009; Mulugu et al., 2007; Wang et al., 2012). To assess which inositol poly- and/or pyro- phosphates are involved in the regulation of inositol biosynthesis, we assayed inositol auxotrophy and INO1
expression in all the single and double mutants shown in Table 1-4. Inositol poly-/pyro-phosphates synthesized by the WT and deletion strains shown in Table 1-4 have been characterized previously by high performance liquid chromatography (HPLC) (Dubois et al., 2002a; Onnebo and Saiardi, 2009; Saiardi et al., 2002; York et al., 2005). As seen in Figs. 2-4A and 2-4B, ipk1Δ did not exhibit growth defects on I- plates, while deletion of KCS1 and/or IPK2 caused inositol auxotrophy consistent with severe defects in INO1-lacZ expression. Deletion of KCS1 in ipk1Δ, which additionally depletes inositol pyrophosphates synthesized from IP₅, led to inositol auxotrophy. Consistent with this, INO1-lacZ expression was greatly reduced in kcs1Δipk1Δ compared to both WT and ipk1Δ. These findings suggest that Kcs1-generated 5PP-IP₄ is required for optimal inositol biosynthesis.

Inositol defects resulted from deletion of VIP1 were less severe than defects observed in kcs1Δ. Intracellular inositol was decreased by 20% in vip1Δ but 70% in kcs1Δ compared to WT (Fig. 2-2C), and INO1-lacZ expression was decreased about 50% in vip1Δ but almost not detected in kcs1Δ (Fig. 2-4B). The severe inositol defects in kcs1Δ, but not in vip1Δ, led to inositol auxotrophy. The double mutant kcs1Δvip1Δ has severe inositol defects as an inositol auxotroph. It exhibited a 60-80% decrease in intracellular inositol (Fig. 2-2C) and greatly decreased INO1-lacZ expression (Fig. 2-4B). Therefore, we conclude that kcs1Δ is epistatic to vip1Δ with respect to inositol biosynthesis.
Table 1-4

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<th>IP^8</th>
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Legend:  
+ Indicates increased growth on L- 

No published data are available.
DISCUSSION

This is the first demonstration that Kcs1, which catalyzes the synthesis of inositol pyrophosphates, regulates inositol biosynthesis by controlling INO1 expression. We report that: 1) kcs1Δ cells exhibit reduced intracellular inositol and PI, decreased INO1 expression, and decreased growth on inositol-free media; 2) disruption of either functional domain of Kcs1 protein causes inositol deficiency; 3) Kcs1 protein, but not transcription, is regulated in response to inositol; and 4) deletion of KCS1, but not IPK1, causes inositol deficiency, suggesting that synthesis of inositol pyrophosphates from IP₅ but not IP₆ is necessary for inositol synthesis. Based on these findings, we propose a model in which Kcs1-catalyzed synthesis of inositol pyrophosphates modulates INO1 transcription.

Inositol pyrophosphate deficient kcs1Δ cells exhibited defective inositol metabolism. Deletion of KCS1 led to an extended lag phase and nearly no growth in I- (Fig. 2-2A). Consistent with this, intracellular inositol in kcs1Δ cells was decreased to less than 30% of WT (Fig. 2-2C), while PI was decreased to about 42% of WT. In response to inositol depletion, kcs1Δ cells displayed severely reduced INO1 derepression compared to WT cells (Fig. 2-2E). We conclude that the inositol defects in kcs1Δ are caused by defective INO1 transcription.

Disruption of either of the two functional domains DINS/kinase and bZIP of Kcs1 resulted in defective inositol biosynthesis (Fig. 2-5). Although site mutations in either domain resulted in defective INO1 expression and inositol auxotrophy, the
mutated bZIP domain led to relatively mild defects in \textit{INO1} transcription compared to the mutated kinase domain (Fig. 2-5C). Disruption of the bZIP domain in \textit{KCS1} does not reduce the production of inositol pyrophosphates (Dubois et al., 2002a). Interestingly, the bZIP domain of \textit{KCS1} shares homology with the bZIP domain of \textit{Opi1} (White et al., 1991), as seen in the sequence alignment (Fig. 2-8A). The role of the bZIP domain has not been characterized in either protein. We speculate that the bZIP domain of \textit{Opi1} and \textit{Kcs1} may share binding sites, and that \textit{INO1} transcription may be regulated by the bZIP domains which mediate spatial localization of the proteins to the vicinity of the chromosomal regions where \textit{INO1} is located.

Our findings indicate that \textit{Kcs1} protein, but not transcription, is regulated in response to inositol. A novel mechanism underlying the regulation of \textit{KCS1} transcription in response to phosphate signals was identified previously (Nishizawa et al., 2008). Pho4-mediated transcription of the antisense and intragenic RNAs in \textit{KCS1} leads to the production of truncated \textit{Kcs1} protein and down-regulation of \textit{Kcs1} kinase activity (Nishizawa et al., 2008). This mechanism of regulation of phosphate signaling involves a positive feedback loop, in which species of the mRNAs and proteins of \textit{KCS1} are regulated by transcription of the antisense and intragenic RNAs. In contrast to Pho4-mediated regulation of \textit{KCS1}, the \textit{KCS1} mRNA levels did not change in response to inositol (Fig. 2-7C), and the full length and truncated \textit{Kcs1} proteins were similarly increased in I- (Fig. 2-7A) and decreased in I+ (Fig. 2-7D). These findings suggest a different mechanism underlying regulation of \textit{Kcs1} protein in inositol biosynthesis.
compared to phosphate signaling. We speculate that Kcs1 protein may be controlled by translation or posttranslational modification, and/or stability of Kcs1 protein.

Analysis of inositol pyrophosphate mutants indicates that inositol pyrophosphates synthesized from IP$_5$ but not IP$_6$ are the most likely regulators of inositol biosynthesis. As summarized in Table 1-4, ipk1Δ, which lacks IP$_6$ and IP$_7$, did not exhibit inositol defects, while kcs1Δipk1Δ, which lacks 5PP-IP$_4$, IP$_6$ and IP$_7$, exhibited severe inositol defects. These findings suggest that 5PP-IP$_4$, synthesized from IP$_5$, is required for inositol biosynthesis. However, we cannot completely rule out the possibility that 5-IP$_7$ is required for inositol biosynthesis. Indeed, deletion of ipk1Δ caused only about a 30% decrease in INO1 expression (Fig. 2-4B), consistent with the findings of Wu and co-workers (Shen et al., 2003). Therefore, 5PP-IP$_4$ is sufficient for inositol regulation, but IP$_7$ also contributes to regulation. This is consistent with the moderate inositol defects observed in vip1Δ. Due to the difficulty of constructing a strain that can generate 5PP-IP$_4$ and IP$_6$, but not IP$_7$, it is difficult to elucidate the specific role of IP$_7$ in regulating INO1 transcription.

Interestingly, deletion of PLC1, the gene encoding phospholipase C that hydrolyses PIP$_2$ and generates IP$_3$ as precursors for inositol poly-/pyrophosphates, exhibited elevated INO1 expression (Demczuk et al., 2008; Rupwate et al., 2012). It is likely that regulation of INO1 gene expression and inositol biosynthesis is coordinated with PLC activation in addition to the negative feedback curcuit in response to exogenous inositol. However, deletion of PLC1 is
lethal in some genetic backgrounds (Yoko-o et al., 1993). This complicates our understanding of the regulation of *INO1* expression by *PLC1*. Interestingly, inositol polyphosphates IP$_5$ and IP$_6$, produced from phosphorylation of IP$_3$ have roles in Ino80-mediated chromatin remodeling, a process also required for *INO1* expression (Shen et al., 2003; Steger et al., 2003). Regulation of *INO1* expression by synthesis of inositol pyrophosphates from IP$_5$ and IP$_6$ will further complicate the regulation of *INO1* expression as altered levels of IP$_5$ and IP$_6$ may affect chromatin structure. We propose a model, depicted in Fig. 2-8, in which optimal *INO1* transcription is modulated by the synthesis of inositol pyrophosphate, 5PP-IP$_4$ (derived from IP$_5$). Under derepressing conditions (I-), Opi1 is excluded from the nucleus (Henry et al., 2012a; Loewen et al., 2004), while Kcs1 protein levels are increased (Fig. 2-7A). Increased Kcs1 protein accelerates production of 5PP-IP$_4$, which is required for optimal *INO1* expression. Nuclear Opi1 most likely decreases Kcs1 protein as increased Kcs1 was observed in *opi1Δ* and in I- (during which Opi1 is excluded from the nucleus). Consistent with this, under repressing conditions (I+), Kcs1 is rapidly decreased (Fig. 2-7D), most likely due to Opi1 translocation into the nucleus where it represses *INO1* expression (Henry et al., 2012a; Loewen et al., 2004) and decreases Kcs1 protein. Kcs1 and Opi1 may compete for a common binding site via the bZIP domain in the nucleus. Therefore, Opi1-dependent modulation of Kcs1 protein allows one or the other to interact with the common sites of specific nuclear proteins required for *INO1* transcription in the nucleus, leading to repression or transcription of *INO1*, respectively. In this scenario, Kcs1 protein
Figure 2-8: Model of regulation of \textit{INO1} transcription by Kcs1 and inositol pyrophosphates. (A) Alignment of the bZIP domains in Opi1 and Kcs1. (B) Model depicting regulation of \textit{INO1} transcription by modulation of Kcs1 protein. Under derepressing conditions (I-, left panel), Opi1 is excluded from the nucleus. Increased Kcs1 protein facilitates synthesis of 5PP-IP$_5$ from IP$_5$, leading to optimal transcription of \textit{INO1}. Under repressing conditions (I+, right panel), Opi1 is present in the nucleus where it represses Kcs1 and \textit{INO1} expression.
levels control \textit{INO1} transcription by regulating the synthesis of inositol pyrophosphates. We speculate that 5PP-IP4 may be required to recruit transcriptional activators to the \textit{INO1} promoter region or stabilize the interaction among those activators.

In conclusion, we identified a novel mechanism whereby inositol biosynthesis is regulated by modulation of Kcs1 protein and suggested a model in which Kcs1-catalyzed synthesis of inositol pyrophosphates regulates \textit{INO1} transcription.
CHAPTER 3

DELETION OF THE CARDIOLIPIN-SPECIFIC PHOSPHOLIPASE CLD1
RESCUES GROWTH AND LIFESPAN DEFECTS IN THE TAFAZZIN MUTANT:
IMPLICATIONS FOR BARTH SYNDROME

The work described in this chapter has been published in the Journal of Biological Chemistry 289: 3114-3125, 2014.

INTRODUCTION

Cardiolipin (CL) is a unique phospholipid that is predominant in mitochondrial membranes (Hostetler et al., 1972; Joshi et al., 2009). Unlike other membrane phospholipids, it contains two phosphatidyl moieties, four acyl chains and two negative charges (Lecocq and Ballou, 1964; Pangborn, 1947). As the signature lipid of mitochondria, it comprises about 15% of total mitochondrial phospholipids (Jakovcic et al., 1971) and interacts with a wide range of mitochondrial proteins (Claypool, 2009; Klingenberg, 2009; Schlame and Ren, 2009; Schlame et al., 2000), including the ADP/ATP carrier (Beyer and Klingenberg, 1985; Claypool et al., 2008b) and respiratory complexes (Eble et al., 1990; Lange et al., 2001; Palsdottir et al., 2003; Shinzawa-Itoh et al., 2007). CL-protein interactions stabilize respiratory chain supercomplexes (Pfeiffer et al., 2003; Zhang et al., 2002) and promote supramolecular associations between the ADP/ATP carrier...
and respiratory supercomplexes (Claypool et al., 2008b). Therefore, it is not surprising that mitochondrial respiration and energy production are highly correlated with CL biosynthesis (Claypool et al., 2008b; Gohil et al., 2004; Jiang et al., 2000). Interestingly, CL deficiency also leads to deficiencies in diverse cellular functions other than mitochondrial bioenergetics, including mitochondrial dynamics (DeVay et al., 2009; Joshi et al., 2012), mitochondrial protein import (Gebert et al., 2009; Jiang et al., 2000), cell wall biogenesis (Zhong et al., 2005; Zhong et al., 2007), vacuolar function and morphology (Chen et al., 2008), cell cycle (Chen et al., 2010), aging (Zhou et al., 2009), and apoptosis (Gonzalvez and Gottlieb, 2007; Houtkooper and Vaz, 2008; Schug and Gottlieb, 2009). As CL is engaged in a plethora of cellular activities, the regulation of CL synthesis is crucially important.

The synthesis of CL is well-characterized in Saccharomyces cerevisiae. As seen in Fig.3-1, Pgs1 catalyzes the committed step of CL synthesis by converting CDP-DAG and glycerol-3-phosphate to phosphatidylglycerolphosphate (PGP) (Chang et al., 1998a), which is dephosphorylated to phosphatidylglycerol (PG) by the PGP phosphatase Gep4 (Kelly and Greenberg, 1990; Osman et al., 2010). CL synthase (Crd1) catalyzes the final step of de novo CL synthesis by condensing PG and CDP-DAG to form CL with primarily saturated acyl chains (Chang et al., 1998b; Jiang et al., 1997; Tamai and Greenberg, 1990; Tuller et al., 1998). Following the de novo synthesis of CL on the matrix side of the inner
Figure 3-1: CL de novo synthesis and remodeling in S. cerevisiae. Pgs1 catalyzes the committed step of CL synthesis by converting CDP-DAG to phosphatidylglycerolphosphate (PGP), which is dephosphorylated to phosphatidylglycerol (PG) by the GEP4-encoded PGP phosphatase. CL synthase, encoded by CRD1, condenses PG and CDP-DAG to form CL. CL synthesized de novo has primarily saturated acyl chains (CL_sat). CL_sat is deacylated by the CL-specific phospholipase Cld1 to monolysocardiolipin (MLCL), which is reacylated by tafazzin (the TAZ1 gene product) to CL containing more unsaturated acyl chains (CL_unsat).
mitochondrial membrane, CL undergoes remodeling in which acyl chains are exchanged. In this process, CL is deacylated to monolysocardiolipin (MLCL) by the CL-specific lipase Cld1 on the matrix side of the inner mitochondrial membrane (Baile et al., 2013; Beranek et al., 2009). MLCL is reacylated by the transacylase Taz1 in the mitochondrial periphery (Brandner et al., 2005; Claypool et al., 2008a; Gu et al., 2004; Testet et al., 2005). Remodeled CL has more unsaturated acyl chains than CL synthesized de novo (Beranek et al., 2009; Gu et al., 2004; Vaz et al., 2003; Xu et al., 2003). Although the CL remodeling genes and enzymes have been identified in yeast, the function of CL remodeling and mechanisms underlying its regulation are not understood.

The importance of CL remodeling is underscored by the X-linked mitochondrial disorder Barth syndrome (BTHS), a cardioskeletal myopathy that results from mutations in the tafazzin gene (the homologue of yeast TAZ1) (Barth et al., 1983; Barth et al., 2004; Barth et al., 1999). Tafazzin deficiency leads to a decrease in the CL/MLCL ratio and a decrease in CL species containing unsaturated fatty acids (Acehan et al., 2011; Gu et al., 2004; Houtkooper et al., 2009; Schlame et al., 2003; Valianpour et al., 2002; Vreken et al., 2000; Xu et al., 2006). Which of these biochemical outcomes leads to the pathology in BTHS is not understood. Genetic inactivation of the CL-specific phospholipase iPLA2-GVIA rescued sterility defects associated with tafazzin deficiency in Drosophila (Malhotra et al.,
2009). The mechanism underlying this rescue is not known. In mammals, CL-specific phospholipases have not been identified, and multiple phospholipases supposedly catalyze the deacylation of CL (Hsu et al., 2013), complicating experiments to elucidate the role of deacylation in mammalian cells. In contrast, CLD1 is the only CL-specific phospholipase in S. cerevisiae (Beranek et al., 2009). The yeast cld1Δ mutant has decreased unsaturated CL compared to wild type cells, but the CL/MLCL ratio is not altered. In this study, we demonstrated for the first time that deletion of CLD1 rescued both respiratory and fermentative growth defects as well as decreased chronological life span in yeast taz1Δ cells. This suggests that deacylation of CL in the absence of tafazzin is deleterious because it leads to a decrease in the CL/MLCL ratio. These findings argue against the current thought that defects in tafazzin deficient cells result from decreased unsaturated CL. We further show that expression of CLD1 is regulated in response to conditions affecting mitochondrial respiration and controlled by the HAP transcriptional activator. Overexpression of CLD1 leads to decreased ATP production from mitochondrial respiration that is compensated by increased glycolysis. Based on these findings, we proposed that transcriptional regulation of CLD1 controls deacylation of CL, and the regulation of this process modulates cellular energy production.
MATERIALS AND METHODS

**Yeast strains, plasmids and growth media**—The yeast *S. cerevisiae* strains and plasmids used in this study are listed in Table 2-1 and Table 2-2. Single deletion mutants were obtained from the yeast knock-out deletion collection (Invitrogen). Double mutants were obtained by tetrad dissection. Parental ρ+ cells were used to generate ρ° derivatives by growing in yeast extract peptone dextrose (YPD) medium containing 20 μg/ml ethidium bromide to the early stationary phase. ρ° strains were confirmed by inability to grow on yeast extract peptone glycerol ethanol (YPGE) medium, the absence of mitochondrial DNA by DAPI staining, and the failure to complement ρ− tester strains for growth on YPGE medium.

To construct a *CLD1*-overexpression plasmid, a 1338-bp sequence containing the entire open reading frame of *CLD1* was amplified from yeast genomic DNA using an EcoRI-tagged forward primer CLD1_EcoRI_F (5’-TATAGAACATGAATTCAAAAGTGAGCTGCAATGAGCA) and an XbaI-tagged reverse primer CLD1_XbaI_R (5’- ATTTTGAGATTCTAGAAAGAAGAAAAAATAGCGGCGA -3’). The PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega). The purified DNA fragments were ligated into pYPGK18 cut with EcoRI and XbaI, downstream of the *PGK1* promoter. All the plasmids were amplified and extracted using standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol.
<table>
<thead>
<tr>
<th>Strains and plasmids used in this study</th>
<th>Strains and plasmids used in this study</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td><strong>BY4742</strong></td>
<td>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Invitrogen</td>
</tr>
<tr>
<td><strong>crd1Δ</strong></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 crd1Δ::KanMX6 Invitrogen</td>
</tr>
<tr>
<td><strong>cld1Δ</strong></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cld1Δ::KanMX6 Invitrogen</td>
</tr>
<tr>
<td><strong>taz1Δ</strong></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 taz1Δ::KanMX6 this study</td>
</tr>
<tr>
<td><strong>cld1Δtaz1 Δ</strong></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cld1Δ::KanMX6 taz1Δ::KanMX6 this study</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td><strong>BY4741 ρ°</strong></td>
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</tr>
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<td>ρ° mutant derived from BY4741 cld1Δtaz1Δ this study</td>
</tr>
<tr>
<td><strong>pYPGK18</strong></td>
<td>2μm, LEU2 (Vaz et al., 2003)</td>
</tr>
<tr>
<td><strong>pYPGK18CLD1</strong></td>
<td>derived from pYPGK18, expression CLD1 from PGK1 promoter this study</td>
</tr>
</tbody>
</table>
Synthetic complete (SC) medium contained adenine (20.25 mg/L), arginine (20 mg/L), histidine (20 mg/L), leucine (60 mg/L), lysine (200 mg/L), methionine (20 mg/L), threonine (300 mg/L), tryptophan (20 mg/L), uracil (20 mg/L), yeast nitrogen base without amino acids (Difco), all the essential components of DIFCO vitamin (inositol-free), 0.2% ammonium sulfate, and glucose (2%). Inositol (75 μM) was supplemented in all media used in this study. Synthetic dropout media contained all ingredients mentioned above except for the amino acid used as a selectable marker, and were used to culture strains containing a plasmid.

**Chronological life span**—Yeast chronological life span is determined by survival of non-dividing cells in a prolonged stationary culture (Fabrizio and Longo, 2003). A standard protocol previously described was followed (Hu et al., 2013) to assess chronological life span. In brief, individual colonies were inoculated in 10 ml of SC glucose medium and incubated overnight. The cultures were then diluted in 50 ml of SC medium, and cells were allowed to grow until saturation. Viable cells were measured every 2 or 3 days by counting colonies that were serially diluted and plated on YPD plates and represented as percentage of cells at day 2. The viability is considered to be 100% at or before day 2.
**Spotting assay**—Cells were pre-cultured in SC medium to the early stationary growth phase at 30°C and washed with sterile water. Three μL aliquots of a series of 10-fold dilutions of 0.5 units of A550 cells were spotted onto indicated plates and incubated at 30°C.

**Real-Time quantitative PCR (RT-qPCR) analysis**—Cells were grown to the indicated growth phase and harvested at 4°C. Total RNA was extracted using hot phenol (Kohrer and Domdey, 1991) and purified using the RNeasy Mini Plus kit (QIAGEN, Valencia, CA). Complementary DNA (cDNA) was synthesized using the First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s manuals. RT-qPCR reactions were performed in a 20 μL volume using Brilliant III Ultra-Faster SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). Triplicates were included for each reaction. The primers for RT-qPCR are listed in Table 2-2. RNA levels were normalized to ACT1. Relative values of mRNA transcripts are shown as fold change relative to indicated controls. Primer sets were validated according to Methods and Applications Guide from Agilent Technologies. Optimal primer concentrations were determined, and primer specificity of a single product monitored by a melt curve following the amplification reaction. All the primers were validated by measurement of PCR reaction efficiency and have calculated reaction efficiencies between 95-105%.
Table 2-2  Real-time PCR primers used in this study.

<table>
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<th>Gene</th>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
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<td><em>ACT1</em></td>
<td>Forward</td>
<td>TCCGGTGATGGTGTTACTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCCAAATCGATTCTCAAAAA</td>
</tr>
<tr>
<td><em>PGS1</em></td>
<td>Forward</td>
<td>TTTGCTCCAACTCACTCGTCTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATTGCAAATGGAGAAAGGTTGC</td>
</tr>
<tr>
<td><em>GEP4</em></td>
<td>Forward</td>
<td>AAAGGCGGTGGTCTTGGATAAGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGGAAACCGCGGTATTGCTAA</td>
</tr>
<tr>
<td><em>CRD1</em></td>
<td>Forward</td>
<td>TGCGGCATAATTCTGGGTAGAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATCCATGCCACACGACCAGATA</td>
</tr>
<tr>
<td><em>CLD1</em></td>
<td>Forward</td>
<td>ACTGGCTTTGGCTTTATGCGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCAGGTACAAGTGATGCGCCTGA</td>
</tr>
<tr>
<td><em>TAZ1</em></td>
<td>Forward</td>
<td>CGAAGCCATCTTGGCTCCATGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAATGGGCGGCTTTGCTTCT</td>
</tr>
<tr>
<td><em>ADH1</em></td>
<td>Forward</td>
<td>GGTCTAGGTTCTTTGGCTGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACCACCGATGGATCTGAATAA</td>
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<tr>
<td><em>ADH2</em></td>
<td>Forward</td>
<td>GTACTGTTGTCCTGGTTGTTCTG</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GTAAGAGCCGACAATGGAGATAG</td>
</tr>
<tr>
<td><em>PGK1</em></td>
<td>Forward</td>
<td>AGGCTTCTGCCCCAGGTTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGCAGTTGTGGCAAGTC</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>Forward</td>
<td>AGTCTTTTGGGTGCGGTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACATTGACGCTGGTGCAAG</td>
</tr>
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</table>

(Szijgyarto et al., 2011a)
**Measurement of respiration**—Cell respiration was analyzed in a closed 500 μL chamber equipped with a micro Clark-type oxygen electrode (Oxygraph plus system, Hansatech) at 30°C. Cells grown to the logarithmic phase were mixed in fresh growing media using a protein concentration of 2 mg/mL following measurements of basal respiration. State 4 and 3 respiration was determined in the presence of 4 μM oligomycin and 5 μM FCCP, respectively. KCN (0.2 mM) was added at the end of the experiment to inhibit cytochrome c oxidase to normalize for (subtract) cytochrome c oxidase independent oxygen consumption. Oxygen consumption was recorded on a computer and analyzed with the Oxygraph plus software. Respiration rates are defined as consumed O₂ (nmol)/min·total protein (mg).

**Determination of ATP concentrations**—Yeast cells were cultured to the logarithmic phase and flash-frozen with liquid nitrogen. ATP levels were determined by the bioluminescence method described previously (Lee et al., 2010).

**Determination of ethanol concentrations**—Yeast cells were cultured in 10-mL growth medium for the indicated times to the logarithmic phase after inoculation at A₅₅₀ of 0.05, and cells were pelleted by a 5-min centrifugation at 3,000 rpm. Supernatants were used to determine ethanol concentrations in the media. An ethanol colorimetric assay kit from BioVision was used to assay ethanol concentrations according to the manufacturer’s manual.
Mitochondrial aconitase activity—Cultures (2L) of yeast cells in the mid-logarithmic phase were harvested for isolation of mitochondria. Mitochondria were isolated as described previously (Diekert et al., 2001). Briefly, spheroplasts generated by zymolyase treatment were ruptured by Dounce homogenization, and mitochondria were obtained by differential centrifugation. Total mitochondrial protein concentration was determined using the BCA protein assay (Pierce Protein). Mitochondrial aconitase activity was determined in mitochondrial extracts (50-μg protein) using an aconitase-isocitrate dehydrogenase-coupled assay, in which NADPH formation was monitored at $A_{340}$ for 1 h (Gardner, 2002).

Determination of CL by mass spectrometry—Total lipid extracts from 10 mg of cells (dry weight) were analyzed by HPLC-MS as described previously (Houtkooper et al., 2006).
RESULTS

Deletion of CLD1 rescues growth and life span defects of the taz1Δ mutant.

Deacylation of CL in the absence of tafazzin leads to a decreased ratio of CL/MLCL and decreased unsaturated CL (Acehan et al., 2011; Gu et al., 2004; Valianpour et al., 2002; Xu et al., 2006), either of which may be responsible for cellular defects in tafazzin-deficient cells. We wished to distinguish between decreased CL/MLCL ratio vs. decreased unsaturated CL as the mechanism underlying the defects in the taz1Δ mutant. Blocking CL deacylation by deletion of CLD1 prevents the decrease in CL/MLCL ratio (Beranek et al., 2009). However, the CL that is synthesized de novo but not remodeled is mostly saturated, in contrast to remodeled CL in wild type (WT) cells, which is mostly unsaturated (Beranek et al., 2009). To determine if the decreased CL/MLCL ratio is responsible for taz1Δ defects, we determined the effects of CLD1 deletion in taz1Δ mutants. Interestingly, deletion of CLD1 rescues the respiratory growth defect of the taz1Δ mutant (Fig. 3-2A). Because mitochondrial respiration varies in strains with different genetic backgrounds (Ocampo et al., 2012) and the presence of polymorphic mitochondrial DNA can contribute to differences in mitochondrial respiration (Dimitrov et al., 2009), we assayed the effects of CLD1 deletion independent of mitochondrial respiration. To do so, we constructed ρ^0 strains (which lack mitochondrial DNA) of the WT and CL mutants. While CL
deficient cells grow normally on glucose (Fig. 3-2A), which can be fermented, growth on glucose is compromised in the mutants if they lack mitochondrial DNA (Fig. 3-2B). Deletion of CLD1 rescued this growth defect (Fig. 3-2B). We predicted that CL deficient cells would exhibit a decreased chronological life span similar to the decreased replicative life span observed in these cells (Zhou et al., 2009). As shown in Fig. 3-2C, both crd1Δ and taz1Δ mutants exhibited a dramatic decrease in chronological life span. Deletion of CLD1 partially rescued the decrease in taz1Δ life span, as the life span of taz1Δcld1Δ was almost similar to that of WT (Fig. 3-2C). The observation that deletion of CLD1 suppresses the defects in taz1Δ indicates that deacylation of CL is deleterious in the absence of tafazzin, and that the decreased CL/MLCL ratio but not decreased CL unsaturation is likely the primary cause of taz1Δ defects.
Fig. 3-2: Deletion of CLD1 rescues growth and chronological life span defects in taz1Δ. (A) Serial 10-fold dilutions of WT, crd1Δ, cld1Δ, taz1Δ, and crd1Δtaz1Δ cells were spotted on synthetic complete medium with 2% glucose or 2% ethanol as carbon sources. Plates were incubated at 30°C for 3 days. (B) Serial 10-fold dilutions of respiration-incompetent (ρ0) cells of the above mutants were spotted on synthetic complete medium with 2% glucose. (C) Chronological life span of WT, crd1Δ, cld1Δ, taz1Δ, and crd1Δtaz1Δ cells was determined as described under “Experimental Procedures.” The data depicted in the figure is a representative of three experiments.
CLD1 expression is highly regulated in response to growth phase, glucose availability and respiratory activity.

The finding that cld1Δ rescued respiratory defects in taz1Δ suggested that CLD1 expression plays a role in respiration. We first compared expression of CL biosynthetic genes, including PGS1, GEP4, CRD1, CLD1, and TAZ1, in logarithmically growing cells (in which energy is generated primarily from glycolysis) and in cells in the stationary phase (during which energy is generated from respiration). Expression of all the CL biosynthetic genes was increased in the stationary phase (Fig. 3-3A). However, while PGS1, GEP4, CRD1, and TAZ1 were increased about 3-5 fold, CLD1 was increased by about 10-fold in the early stationary phase and more than 30-fold in the later stationary phase (Fig. 3-3A). The large increase in CLD1 expression suggests that levels of unsaturated CL may be increased during stationary phase. This was in fact observed (Fig. 3-3B). Specifically, in the C68 cluster, the most unsaturated CL (C68:4, m/z 699.5) was abundant, while a more saturated species (C68:2, m/z 701.5) was less abundant in stationary phase cells. Conversely, the C68:4 CL was much less abundant than C68:2 in logarithmically growing cells. This is also evident in the C60 cluster as the most saturated CL (C60:0, m/z 647.4) was absent from stationary cells but clearly present in logarithmically growing cells. Deletion of CLD1 prevents CL remodeling and leads to decreased unsaturated CL (Beranek et al., 2009). As expected, cld1Δ exhibited a decreased degree of unsaturated CL compared to WT regardless of growth phase (Fig. 3-3C). Interestingly, unsaturated CL levels
Fig. 3-3

Gene expression levels for different conditions (EL, ML, ES, S) with bars representing normalized gene expression levels (GENE/ACT1) for different genes (PGS1, GEP4, CRD1, CLD1, TAZ1).
Fig. 3-3C

C

m/z=684-690

wild type - log

wild type - stationary

cld1D - log

cld1D - stationary

m/z=698-704

wild type - log

wild type - stationary

cld1D - log

cld1D - stationary
Fig. 3-3: Increased \textit{CLD1} expression in the stationary phase is concomitant with increased CL unsaturation. (A) WT cells were grown in SC medium to the early-logarithmic (EL), mid-logarithmic (ML), early-stationary (ES), and stationary (S) growth phases, and \textit{PGS1, GEP4, CRD1, CLD1}, and \textit{TAZ1} expression was quantified by RT-PCR as described under “Experimental Procedures.” Values of each gene were normalized to the internal control \textit{ACT1} and are represented as fold change relative to those in EL. Data shown are mean ± S.E. \( (n = 3) \). (B) Cells grown in YPD in the logarithmic and stationary phases were extracted for CL acyl composition analysis by HPLC-mass spectrometry, described under “Experimental Procedures.” (C) WT and \textit{cld1Δ} cells grown in SC media in the logarithmic and stationary phases were extracted for CL acyl composition analysis by HPLC-mass spectrometry, described under “Experimental Procedures.”
were greater in stationary phase than in log phase \textit{cld1}\textDelta cells. This finding suggests that an as yet unidentified mechanism regulates CL saturation in the absence of Cld1.

Increased \textit{CLD1} expression in the stationary phase, during which glucose is exhausted and cells shift from fermentation to oxidative phosphorylation, suggested that \textit{CLD1} may be transcriptionally regulated in response to glucose availability and the need to respire. To test this prediction, we examined the expression of \textit{CLD1} in response to acute removal of glucose and in respiration-deficient cells (\(\rho^0\) cells). As expected, expression of \textit{CLD1} but not the other CL biosynthetic genes was greatly increased in response to glucose starvation, by 6-fold and 10-fold, during 30-min and 60-min starvation, respectively (Fig. 3-4A). Furthermore, \textit{CLD1} transcription was increased in the stationary phase in \(\rho^+\) cells but not in respiration incompetent \(\rho^0\) cells (Fig. 3-4B). These findings indicate that \textit{CLD1} expression is upregulated during respiratory conditions and in response to glucose deprivation.

Using the \textit{Promoter Database of Saccharomyces cerevisiae} (SCPD) to search for putative regulatory elements in the upstream region of the \textit{CLD1} gene, we identified consensus sequences for Hap2 and Mig1 (Fig. 3-4C), transcription factors that mediate activation of respiratory gene expression and glucose repression, respectively (Nehlin and Ronne, 1990; Pfeifer et al., 1989; Santangelo, 2006). Consistent with this observation, the HAP complex regulates
A

![Graph A](image1)

B

![Graph B](image2)
### Fig. 3-4

**Diagram: CLD1 Gene Region**

```
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<tr>
<th>Gene</th>
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<th>Crick Strand</th>
</tr>
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</tr>
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</tr>
<tr>
<td>MIG1</td>
<td>CTCCACTTTAT</td>
<td>0.82</td>
</tr>
</tbody>
</table>
```

+: Watson strand  
-: Crick strand
Fig. 3-4

D

![Bar chart showing CLD1 expression in stationary phase relative to log phase for WT, hap2Δ, hap3Δ, hap4Δ, and hap5Δ.]

E

![Bar chart showing CLD1 expression for WT and mig1Δ.]

Fig. 3-4: **CLD1** expression is increased in response to respiration and activated by HAP. (A) **Glucose limitation.** WT cells harvested in the mid-logarithmic phase were washed with pre-warmed media with or without glucose and resuspended in fresh media with or without glucose for 30-min and 60-min. Analyses of *PGS1, GEP4, CRD1, CLD1*, and *TAZ1* expression was determined using RT-qPCR and normalized to *ACT1*, as described under “Experimental Procedures.” The data represent as fold change relative to expression in media containing glucose and include the mean ± S.E. (*n* = 6). (B) **Respiratory competency.** $\rho^+$ and $\rho^0$ cells were grown to the mid-logarithmic (ML) and early stationary (ES) phases. The values shown represent the fold change relative to expression of WT $\rho^+$ cells in ML, and include mean ± S.E. (*n* = 6). (C) Sequence alignment depicting consensus sequences for Mig1 and Hap2 in the upstream region of the *CLD1* gene using the *Promoter Database of Saccharomyces cerevisiae* (SCPD). 1 indicates a 100% match with putative consensus sequences, and 0.88 and 0.82 indicates one mismatch. (D) **Effect of the HAP complex.** Expression was determined in *hap2Δ, hap3Δ, hap4Δ*, and *hap5Δ* cells grown to the mid-logarithmic phase. Data shown are mean ± S.E. (*n* = 6). (E) **Effect of MIG1.** Expression was determined in WT and *mig1Δ* cells grown to the mid-logarithmic phase. Data shown are mean ± S.E. (*n* = 3).
CLD1. As seen in Fig. 3-4D, CLD1 expression in the stationary phase was greatly reduced in hap2Δ, hap3Δ, hap4Δ, and hap5Δ mutants, indicating that the HAP complex up-regulates CLD1 transcription. Mig1 has been shown to repress gene expression in the presence of glucose (Santangelo, 2006; Schuller, 2003). If Mig1 repressed CLD1 transcription in the presence of glucose, CLD1 transcription would be increased in mig1Δ cells. However, CLD1 transcription in mig1Δ cells was decreased in these conditions (Fig. 3-4E). Thus, Mig1 appears to be a positive regulator of CLD1 expression. This is consistent with reported activator activity of Mig1 (Bu and Schmidt, 1998; Wu and Trumbly, 1998). Taken together, these findings indicate that expression of CLD1 is increased in response to respiration conditions, and this increase is mediated by the HAP and Mig1 transcriptional factors.

Constitutive overexpression of CLD1 leads to decreases in respiration and mitochondrial aconitase activity, and instability of mitochondrial DNA.

As expression of CLD1 is deleterious to tafazzin-deficient cells, we predicted that increased CLD1 expression alters metabolism and perturbs cell growth. Consistent with this, cell growth was decreased when CLD1 was overexpressed (Fig. 3-5A). One possible explanation for this is that increased CLD1 expression perturbs respiration. In support of this, basal respiration in mitochondria from cells that overexpressed CLD1 was about half that of control cells (Fig. 3-5B).
This difference was even more pronounced comparing the maximum respiratory capacity that was achieved by uncoupling the respiratory chain with trifluorocarbonylcyanide phenylhydrazone (FCCP). Therefore, constitutive overexpression of CLD1 decreases mitochondrial respiration.

A possible mechanism to account for decreased respiration in CLD1 overexpressing cells is suggested by the observation that over 60% of cells became cytoplasmic petites. The respiratory growth deficiency of the petites was not complemented by crossing to ρ− tester strains, and mitochondrial DNA was not observed in the petite cells stained with DAPI. As aconitase is required for mitochondrial genome maintenance (Chen et al., 2005), we tested the possibility that aconitase activity might be decreased in cells overexpressing CLD1. In fact, the kinetics of aconitase enzymatic activity in mitochondria from CLD1 overexpressing cells exhibited a 60% decrease compared to cells overexpressing empty vector (Fig. 3-5C). Taken together, these studies indicate that increasing CL deacylation by constitutive overexpression of CLD1 impairs cell growth and respiration and decreases mitochondrial DNA stability, suggesting that deacylation of CL is an important control point for mitochondrial function.

*Increased fermentation compensates for decreased respiration in cells overexpressing CLD1.*

As respiration was decreased in cells overexpressing CLD1, we expected to see a concomitant decrease in ATP synthesis. The contribution of mitochondria to
Fig. 3-5

A

Cell growth (A_{550})

$\text{WT+EV}$

$\text{WT+CLD1}$

hours

0 5 10 15 20 25 30 35 40

B

O2 consumption (nmol/min, mg protein)

$\text{WT+EV}$

$\text{WT+CLD1}$

Basal

oligomycin

FCCP

C

Mitochondrial aconitase activity

Absorbance at 340 nm

0 0.2 0.4 0.6 0.8 1

reaction time (min)

0 10 20 30 40 50 60

Slope=0.0147

Slope=0.0061
Fig. 3-5: Overexpression of *CLD1* decreases cell growth, respiration, and mitochondrial aconitase activity. (A) Growth of WT cells in SC media overexpressing *CLD1* or empty vector (EV). Cells were inoculated at an initial $A_{550}$ of 0.05, and $A_{550}$ was measured at the indicated times. The growth curves shown in the figure are representative of three experiments. (B) Oxygen consumption was measured in logarithmically growing cells using a Clark-type electrode as described under “Experimental Procedures.” Data shown are mean ± S.E. ($n = 3$-6). (C) The kinetics of aconitase enzymatic activity in mitochondria from control and *CLD1* overexpressing cells grown to the logarithmic phase were determined as described under “Experimental Procedures”. Data shown are mean ± S.E. ($n = 3$).
cellular ATP production can be estimated by the decrease in oxygen consumption resulting from the addition of oligomycin, an inhibitor of ATP synthesis. Under basal conditions, the decrease in respiration caused by oligomycin was significantly less in mitochondria from CLD1 overexpressing cells than in controls (Fig. 3-5B), suggesting that mitochondrial ATP synthesis was decreased. Interestingly, however, total ATP levels were actually higher in CLD1 overexpressing cells (Fig. 3-6A). This suggested that cells may compensate for the respiratory loss by increasing ATP generation from fermentation. Consistent with this, ethanol production was significantly higher in CLD1-overexpressing cells than in controls (Fig. 3-6B). To determine if up-regulation of genes in glycolysis/fermentation could account for increased ethanol production, we analyzed expression of GAPDH and PGK1, which encode enzymes that catalyze key steps in glycolysis (glyceraldehyde-3-P dehydrogenase and phosphoglycerate kinase, respectively), as well as ADH1 and ADH2, which encode the fermentation enzyme alcohol dehydrogenase. As seen in Fig. 3-6C, expression of ADH1 and ADH2 was increased 2-fold, which most likely accounts for the increase in ethanol production. Expression of GAPDH and PGK1 was not altered. These findings indicate that overexpression of CLD1 leads to decreased mitochondrial respiration and ATP synthesis, which is compensated by increasing glycolysis.
DISCUSSION

A deficiency in CL reacylation catalyzed by tafazzin is deleterious in eukaryotes (Acehan et al., 2011; Gu et al., 2004; Schlame et al., 2003; Valianpour et al., 2002; Vreken et al., 2000; Xu et al., 2006), most notably in humans where it leads to the life-threatening disorder BTHS (Barth et al., 1983; Barth et al., 2004; Barth et al., 1999). The loss of tafazzin results in perturbation of CL metabolism. Specifically, the CL/MLCL ratio is decreased, as are the levels of unsaturated CL species. Although many studies suggest that the deleterious effects of tafazzin deficiency result from the absence of unsaturated CL (Schlame et al., 2003; Schlame and Ren, 2006; Schlame et al., 2002; Valianpour et al., 2003; Xu et al., 2003), no reports to date have distinguished between decreased unsaturated CL and decreased CL/MLCL as the cause of the cellular defects. In the current study, we addressed this question by characterizing the effects of CLD1 deletion on tafazzin deficient yeast cells. The cld1Δ mutant has decreased unsaturated CL (similar to the taz1Δ mutant), but the CL/MLCL ratio is not decreased. We report that cld1Δ rescues growth and respiration defects of the taz1Δ mutant, indicating that the decreased CL/MLCL ratio, and not decreased unsaturated CL, leads to the defects in tafazzin-deficient cells.
Fig. 3-6

**ADH1**

- EV: 1
- CLD1: 2

**ADH2**

- EV: 1
- CLD1: 2

**GAPDH**

- EV: 1
- CLD1: 1

**PGK1**

- EV: 1
- CLD1: 1
Fig. 3-6: Overexpression of *CLD1* leads to increased ATP and ethanol. (A) ATP levels in logarithmically growing cells overexpressing *CLD1* or empty vector (EV) were determined as described under “Experimental Procedures.” Data shown are mean ± S.E. (*n* = 3). (B) Ethanol concentrations were determined as described under “Experimental Procedures”. Data shown are mean ± S.E. (*n* = 6) (*p*<0.05, **p**<0.01). (C) WT cells overexpressing *CLD1* or EV grown to the logarithmic phase were harvested for mRNA extraction. Expression of *ADH1*, *ADH2*, *GAPDH*, and *PGK1* was determined using RT-qPCR as described under “Experimental Procedures”. Values of each gene were normalized to the internal control *ACT1*. Transcripts normalized to *ACT1* are represented as fold change relative to those in control cells. Data shown are mean ± S.E. (*n* = 3)
Interestingly, the double mutant cld1Δtaz1Δ exhibited defective growth in glycerol/ethanol medium at 37°C as reported in Beranek et al (Beranek et al., 2009). We tested growth of WT, crd1Δ, cld1Δ, taz1Δ, and cld1Δtaz1Δ cells in media containing glucose, glycerol, ethanol, or glycerol/ethanol as carbon sources. Indeed, we found that the double mutant grew poorly compared to WT when glycerol/ethanol was used as carbon source, similar to the observation of Beranek et al. However, in these carbon sources, we did not observe respiratory growth defects in taz1Δ at 30°C. Although taz1Δ exhibited decreased growth in glycerol/ethanol at 37°C, high temperature stress complicates respiration defects. In marked contrast, we observed that taz1Δ cells exhibit a significant respiratory growth defect in ethanol medium at 30°C. Under these conditions, we observed that the double mutant rescued the respiratory defects of taz1Δ. As cld1Δ restores CL levels but not unsaturated CL species in taz1Δ, this finding indicates that rescue of respiratory growth of taz1Δ by cld1Δ results from restoration of CL levels.

While deletion of CLD1 does not appear to affect growth, expression of the gene is deleterious in the absence of reacylation, as taz1Δ cells that have the wild type CLD1 gene are defective, while those carrying the cld1Δ mutation grow normally. To gain insight into the mechanism underlying the deleterious effects of increased CLD1, we characterized growth and mitochondrial function of cells
overexpressing this gene. Interestingly, overexpression of CLD1 resulted in increased ATP levels (Fig. 3-6A) despite a significant reduction in mitochondrial respiration (Fig. 3-5B). Two possibilities may explain this seemingly surprising finding. First, overexpression of CLD1 leads to growth slowdown, therefore less ATP is required and utilized to maintain cellular functions. Second, CLD1 overexpression shifts metabolism from respiration toward glycolysis and fermentation (Fig. 3-7), compensating for defective oxidative phosphorylation. This indicates that regulation of Cld1-mediated deacylation of CL influences energy metabolism by modulating the relative contribution of glycolysis and respiration. CL is an essential component of oxidative phosphorylation complexes. For example, it was identified in the crystal structure of cytochrome c oxidase (Shinzawa-Itoh et al., 2007), the proposed rate-limiting enzyme of the electron transport chain (reviewed in (Huttemann et al., 2012)) and is required for optimal enzyme function and activity (Koshkin and Greenberg, 2000). Therefore, reduced mitochondrial respiration in CLD1 overexpressing cells would be expected if the CL pool is modified. (We hypothesize that during stationary growth, when oxidative phosphorylation is used, CL may be tuned towards increased membrane fluidity or association with the supercomplexes). Furthermore, such alterations lead to mitochondrial DNA instability.

Apparently, there is yet another level of regulation of the CL metabolic pathway intersecting with cytochrome c oxidase regulation. We show here that CLD1 gene regulation is mediated by the Hap2/3/4/5p transcription factor complex (Fig. 3-4E), which is also a crucial regulator of cytochrome c oxidase subunit V
isoforms Va and Vb (Kwast et al., 1999). These isoforms result in an enzyme with higher affinity for oxygen when the substrate is scarce. Furthermore, overexpression of components of the Hap2/3/4/5p complex rescues cytochrome c oxidase deficiencies (Fontanesi et al., 2008). In another example of coordinate control, regulation of COX4 translation requires Pgs1, the enzyme that catalyzes the committed step of CL synthesis (Su and Dowhan, 2006). Taken together these findings suggest an integrated and concerted response to environmental stress that affects the CL pathway and oxidative phosphorylation, both of which are interconnected.

Our findings suggest that increased CLD1 is deleterious to cells because it decreases respiration. However, CLD1 expression was increased during respiratory growth and regulated by the HAP complex (Fig. 3-4), the transcriptional activator that responds to respiratory growth signals. This raises the question of what is the function of the CL remodeling pathway, and the corollary question of why is CLD1 expression increased in response to respiratory conditions. We speculate that the function of CL remodeling is to remediate the deleterious effects of respiration (Fig. 3-7). In support of this possibility, superoxides generated by respiratory complex III cause peroxidation of CL and decreased cytochrome c oxidase activity (Paradies et al., 2000; Paradies et al., 2001; Paradies et al., 1998). Exogenous supplementation of CL, but not peroxidized CL or other phospholipids, rescued both reduced activity of cytochrome c oxidase and increased generation of ROS in reperfused heart (Paradies et al., 2001; Petrosillo et al., 2007). In this light, CL remodeling may be
**Fig. 3-7: Proposed model.** Regulation of Cld1-mediated deacylation of CL influences energy metabolism. *CLD1* expression is upregulated in response to increased respiration. Increased *CLD1* expression modulates the relative contributions of oxidative phosphorylation and glycolysis to cellular energy production. We speculate that the function of CL deacylation, which is increased during respiratory conditions that are known to increase oxidative stress, is to remove peroxidized acyl chains from damaged CL.
a mechanism whereby damaged fatty acyl chains are replaced. Although different approaches have been used, this proposed model is similar to the model described in Baile et al. (Baile et al., 2013), in which they suggested a feedback loop between oxidative phosphorylation and CL remodeling. Specifically, they found that CLD1 expression is regulated by carbon sources, and the activity of Cld1 is increased by dissipating the mitochondrial membrane potential (Baile et al., 2013). They suggested that CL remodeling functions to increase oxidative phosphorylation efficiency and/or replace oxidized CL.

Our findings have implications for understanding the mechanism underlying BTHS. Many studies of BTHS have concluded that the disorder is due to the complete lack of the ‘normal’ unsaturated (tetratinoleoyl, or L4) CL in the heart (Schlame et al., 2003; Schlame and Ren, 2006; Schlame et al., 2002; Valianpour et al., 2003; Xu et al., 2003). However, the current study indicates that in yeast, a total lack of the ‘normal’ unsaturated CL species is not deleterious to cells. The large number of mammalian phospholipases complicates the ability to distinguish between decreased CL/MLCL vs. decreased unsaturated CL in human cells. Gross and co-workers (Kiebish et al., 2013) reported that ablation of phospholipase iPLA2g in the mouse reduced MLCL levels by only ~50% indicating that other phospholipases deacylate CL. Mass spectrometry analysis of phospholipase activity identified at least four phospholipases that deacylate CL in vitro (Hsu et al., 2013). The identification of mammalian CL-specific phospholipases may ultimately enable this question to be addressed.
ACKNOWLEDGMENTS

In this chapter, I designed all the experiments, prepared all the experimental samples, collected the data, and analyzed the results of all the figures. The following people contributed technically to the experiment.

Iliana A. Chatzispyrou, Riekelt H. Houtkooper, and Frédéric M. Vaz did the mass spectrometry analysis of my samples (Figs. 3-3B and C). Icksoo Lee measured oxygen consumption (Fig. 3-5B). Maik Hüttemann measured ATP levels (Fig. 3-6A). Wenjia Lou helped with ethanol measurements (Fig. 3-6B), the aconitase activity assay (Fig. 3-5C), and the construction of the CLD1 overexpression plasmid. Yiran Li helped with real-time PCR experiments (Figs. 3-4D, E and 3-6C). Shuliang Chen did sample preparation (Fig. 3-3B).
INTRODUCTION

Inositol, a six-carbon cyclitol, is an essential metabolite. It serves as the precursor of inositol lipids and inositol phosphates (Carman and Han, 2011; Henry et al., 2012a; Michell, 2008; Michell, 2011), which play crucial roles in gene expression, signal transduction, lipid signaling, vesicle trafficking, and membrane biogenesis (De Camilli et al., 1996; Lemmon, 2003; Majerus and York, 2009; Shen et al., 2003; Steger et al., 2003; van Meer et al., 2008a). Many types of cultured mammalian cells require supplementation of exogenous inositol for growth, and inositol-requiring mammalian cells and mutants of yeast undergo cell death in response to inositol deprivation (Culbertson and Henry, 1975; Eagle, 1955; Eagle et al., 1957; Kao and Puck, 1968; Keith et al., 1977).

Eukaryotic organisms can potentially obtain inositol from the environment, by the \textit{de novo} synthesis of inositol from glucose, or via recycling of inositol by dephosphorylation of inositol phosphates. These processes are orchestrated to maintain intracellular inositol homeostasis. Inositol uptake in yeast (Lai et al., 1995; Lai and McGraw, 1994) and mammals (Wolfson et al., 2000; Wolfson et al., 1998), and inositol biosynthesis in yeast (Henry et al., 2014; Hirsch and Henry,
1986; Loewen et al., 2004) are affected by exogenous inositol. In mammals, inositol uptake is also regulated in response to glucose, pH, osmolality, growth factors, and other stimuli (Di Daniel et al., 2009; Fu et al., 2012; Miyakawa et al., 1999; Novak et al., 1999; Olgemoller et al., 1993; Spizz and Pike, 1992; Uldry et al., 2004; Yorek et al., 1998). Inositol de novo synthesis is a highly conserved pathway that is carried out in two steps, of which the conversion of glucose-6-phosphate to inositol-3-phosphate, catalyzed by the INO1 gene product inositol-3-phosphate synthase, is rate-limiting (Eisenberg, 1967; Kindl and Hoffmann-Ostehof, 1964; Loewus and Kelly, 1962a; Loewus and Kelly, 1962b; Strausberg et al., 2002). The regulation of inositol biosynthesis has been intensively studied in yeasts (Bachhawat et al., 1995b; Carman and Han, 2011; Chen et al., 2007; Henry et al., 2012a; Loewen et al., 2004; Ye et al., 2013). In addition to the transcriptional regulation of INO1 in response to exogenous inositol (Henry et al., 2014; Hirsch and Henry, 1986; Loewen et al., 2004), optimal inositol biosynthesis requires glycogen synthase kinase-3 (GSK-3) (Azab et al., 2007) and inositol pyrophosphates (Ye et al., 2013). Furthermore, Ino1 is posttranslationally regulated by phosphorylation (Deranieh et al., 2013), and enzyme activity is inhibited by the glycolysis intermediate dihydroxyacetone phosphate (DHAP) (Migaud and Frost, 1996; Shi et al., 2005). Mammalian INO1 expression is altered by estrogen, glucose, and lovastatin, and is regulated by the transcription factor E2F1 (Guan et al., 2003; Rivera-Gonzalez et al., 1998; Seelan et al., 2004; Seelan et al., 2011). Highly regulated inositol synthesis underscores the importance of maintaining inositol homeostasis.
The brain maintains a high level of free inositol (5–50 mM), which is about 100 times higher than that in blood and other tissues (Palmano et al., 1977; Sherman et al., 1977; Stokes et al., 1983; Wong et al., 1987). Altered inositol levels in the brain are associated with psychiatric and neurological problems (Seelan et al., 2009; Shi et al., 2006). For example, levels of inositol are altered in the brains of patients with Down’s syndrome (Acevedo et al., 1997; Berry et al., 1995), stroke (Rumpel et al., 2003), bipolar disorder (Belmaker et al., 2002; Shimon et al., 1997), and suicide victims (Shimon et al., 1997). Although dietary inositol can cross the blood-brain barrier and enter the cerebrospinal fluid and brain parenchyma, this process is very slow (Aukema, 1994). Inositol levels in the brain primarily depend on inositol recycling and de novo synthesis (Williams et al., 2002). However, the requirement of brain cells for inositol synthesis and the cellular consequences of perturbation of inositol synthesis in neuronal cells are not well studied.

Lithium, a mood-stabilizer used for the treatment of bipolar disorder, is an uncompetitive inhibitor of inositol monophosphatase and inositol polyphosphatase (Allison and Stewart, 1971; Berridge et al., 1989; Pollack et al., 1994), and causes a decrease in intracellular inositol by blocking inositol recycling and synthesis. The mood-stabilizer valproic acid (VPA) inhibits inositol biosynthesis by indirectly decreasing activity of the rate-limiting enzyme Ino1 (Ju and Greenberg, 2003; Shaltiel et al., 2004b; Vaden et al., 2001). Both drugs decrease cellular inositol and inositol 1,4,5-trisphosphate levels (Eickholt et al., 2005; Shimshoni et al., 2007; Williams et al., 2002), indicating that inositol
depletion may attenuate inositol-dependent signaling. While inositol depletion may be therapeutically relevant (Berridge et al., 1989), another proposed target of mood-stabilizers is GSK-3 (Klein and Melton, 1996; Lucas and Salinas, 1997). The two major isoforms of GSK-3, GSK-3α and GSK-3β, share 85% sequence homology and are encoded by independent genes (Cohen and Frame, 2001). Both isoforms are expressed in the brain and have many regulatory functions in neural systems, including neurogenesis, neuronal structure, synaptic plasticity, and neuronal survival (Hur and Zhou, 2010). Lithium is a competitive inhibitor of GSK-3, and lithium treatment increases the inhibitory phosphorylation of this kinase (Klein and Melton, 1996; Lucas and Salinas, 1997; Ryves and Harwood, 2001; Zhang et al., 2003). Lithium-induced GSK-3 phosphorylation is caused by disrupting the signaling complex of Akt, β-arrestin 2, and protein phosphatase 2A (Beaulieu et al., 2008). Previous studies have not linked lithium-mediated inhibition of GSK-3 to inositol depletion. Interestingly, however, the inositol-depleting drug VPA has also been shown to inhibit GSK-3 activity (Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al., 2005). Common cellular effects of inositol depletion and GSK-3 inhibition in response to lithium and VPA treatment suggest that inositol metabolism and GSK-3 activity may be interdependent. It has not been determined if inositol metabolism affects GSK-3 activity in neuronal cells. Elucidating the interplay between inositol synthesis and GSK-3 in neuronal cells will have important implications for the pathophysiologic basis of a wide range of disorders in which inositol levels play a role.
In the current study, we characterized the role of inositol synthesis in proliferation of SK-N-SH neuroblastoma cells. We found that \textit{INO1} expression is essential for cell proliferation and neurite outgrowth. We further showed that inositol synthesis regulates GSK-3\(\alpha\) activation, as inhibition of inositol synthesis by knocking down \textit{INO1} expression or exposure to VPA led to increased phosphorylation at Ser21 of GSK-3\(\alpha\), which inactivates the kinase. This is the first demonstration in neuronal cells of the importance of inositol \textit{de novo} synthesis, and the first report showing that inositol synthesis affects GSK-3\(\alpha\) activation. These findings have implications for the therapeutic mechanisms of mood-stabilizers and suggest that inositol depletion and GSK-3 activity are intrinsically related.
MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM), Medium 199, and penicillin-streptomycin solution (100X) were purchased from Invitrogen. Fetal bovine serum (FBS) and dialyzed FBS were purchased from Hyclone. Inositol, lithium chloride, valproic acid, glucose-6-phosphate, NAD+, inositol dehydrogenase, and diaphorase were purchased from Sigma. Control shRNA lentiviral particles and INO1 (also named ISYNA1) shRNA lentiviral particles, puromycin dihydrochloride, and polybrene were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The protease inhibitors and phosphatase inhibitors were purchased from Roche.

The rabbit polyclonal IgG against the Ino1 protein (H-300), mouse monoclonal IgG against actin (C-2), and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit monoclonal IgG against phospho-GSK-3α (ser21) (36E9), rabbit monoclonal IgG against GSK-3α (D80E6), rabbit monoclonal IgG against phospho-GSK-3β (Ser9) (D85E12), rabbit monoclonal IgG against GSK-3β (27C10), and anti-rabbit IgG were purchased from Cell Signaling Technology. All the primary antibodies were diluted 1:1000 and secondary antibodies 1:5000 in dilution buffer containing 1X tris-buffered saline (TBS), 0.1% Tween-20 with 5% nonfat dry milk.
Cell culture

SK-N-SH neuroblastoma cells were obtained from ATCC. Cells were regularly cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For experiments to test effects of inositol deficiency, Medium 199 supplemented with 10% dialyzed FBS were used as inositol-deficient media. Inositol was added to this medium where indicated for inositol-rich media. All cells were cultured at 37°C in 95% air and 5% CO₂. Of note, DMEM contains 40 μM inositol, and Medium 199 has 0.28 μM inositol. The dialyzed FBS contains trace amounts of small molecules.

Establishment of stable cell lines

SK-N-SH cells were transduced with lentiviral particles that contain 3 specific constructs targeting human INO1, or with control lentiviral particles containing non-specific scrambled shRNA. Stable cells were isolated in media 24 hours after the transduction with 5 μg/ml puromycin for 2 weeks.

Proliferation assay

Cell proliferation was carried out using a proliferation assay kit (CellTiter 96 AQeous One Solution cell proliferation assay, Promega) and following the manufacturer’s instruction. Cells were inoculated at a concentration of 5,000 cells per well in 96-well plates. On the indicated days, the assay reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)
2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added and incubated at 37°C for 4 hours. Relative cell numbers were quantified by absorbance at 490 nm. The quantity of MTS formazan produced from MTS tetrazolium is directly proportional to the number of living cells (Cory et al., 1991).

**RNA isolation and real-time quantitative PCR (RT-qPCR)**

SK-N-SH cells were cultured in 6-well plates. Total RNA was extracted using the RNeasy Mini Plus kit (QIAGEN, Valencia, CA). Complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s manuals. RT-qPCR reactions were performed in a 20 μL volume using Brilliant III Ultra-Faster SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). Triplicates were included for each reaction. The primers for RT-qPCR are listed in Table 3-1. RNA levels were normalized to succinate dehydrogenase, SDHA. Relative values of mRNA transcripts are shown as fold change relative to indicated controls.

**SDS-PAGE and Western blots**

Cell extracts were obtained by breaking cells in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40, 2 mM EDTA, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail and were clarified twice by 10 min-centrifugation at 13,000 g at 4°C to remove cell debris. Protein concentration was determined using the BCA™ protein assay (Pierce Protein), with bovine serum albumin as the standard. Cell extracts containing 20 μg protein were boiled with protein gel sample buffer, separated on 10% SDS-PAGE, and
### Table 3-1  Real-time PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>Forward</td>
<td>CGAACGTCTTCAGGTGCTTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGAACATCGGAACTGCGAC</td>
</tr>
<tr>
<td>INO1</td>
<td>Forward</td>
<td>CTGCATCGAGAACATCCTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTTCAACATAGGGTAGGTGGC</td>
</tr>
<tr>
<td>SMIT1</td>
<td>Forward</td>
<td>AAGGTGGTGGTTCGAATCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCACAGGATTGTTTTGGGTC</td>
</tr>
<tr>
<td>HMIT</td>
<td>Forward</td>
<td>CATCTGCAGAATGGTTGCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AACTCGCCGAGCTTTAATTG</td>
</tr>
</tbody>
</table>
electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with antibodies and visualized using ECL substrate (Pierce Protein). ImageJ software was used to quantify the intensities of bands.

*Measurement of intracellular inositol*

Intracellular inositol was measured as described previously (Ju and Greenberg, 2003; Ye et al., 2013) with minor modifications. Briefly, cell extracts were obtained in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40, 2 mM EDTA and were clarified twice by 10 min-centrifugation at 13,000 g at 4°C to remove cell debris. Cell extracts containing 50 μg protein were used to measure intracellular inositol. Protein was precipitated using ice-cold 7.5% perchloric acid. After centrifugation, perchloric acid in the supernatants was removed by titration to pH 7.0 with ice-cold 10 M potassium hydroxide. The cell extracts were again clarified by centrifugation for 5 min at 2,000 g at 4°C. The supernatants were collected, and intracellular inositol was measured by enzyme-coupled fluorescence assay (Maslanski and Busa, 1990). Inositol content in cell extracts of 50 μg protein was normalized to the indicated controls.
Measurement of the de novo synthesis of inositol

The de novo synthesis of inositol was measured in crude cell extracts as described previously (Azab et al., 2007; Barnett et al., 1970; Chen and Charalampous, 1966; Shi et al., 2005) with modifications. Crude cell extracts containing 50 μg protein obtained in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40 were used to test the activity of inositol de novo synthesis. The cell extracts were resolved using Amicon 10 KDa-cutoff centrifugal filters and washed with buffer containing 100 mM Tris acetate, pH 7.0, 20 mM NH₄Cl, and 2 mM dithiothreitol. Endogenous inositol, glucose-6-phosphate, and other small molecules were removed during this process. The protein extracts were resuspended in reaction buffer containing 100 mM Tris acetate, pH 7.7, 20 mM NH₄Cl, 2 mM dithiothreitol, 0.8 mM NAD⁺, and 1 mM glucose-6-phosphate, and incubated at 37°C for 1 hour. The reaction was stopped by adding 7.5% perchloric acid. After centrifugation, perchloric acid in the supernatants was removed by titration to pH 7.0 with ice-cold potassium hydroxide. After 5-min centrifugation at 2,000 g, the supernatants were resolved using ion exchange columns filled with AG 1-X8 (200-400 mesh, formate form). The eluate containing inositol was collected and dried in an oven at 70°C. The dried samples were resuspended in inositol assay buffer, and inositol produced from glucose-6-phosphate in this mixture was measured as described above (Ju and Greenberg, 2003; Maslanski and Busa, 1990; Ye et al., 2013).
RESULTS

*Exogenous inositol is not essential for cell proliferation or maintaining inositol homeostasis in SK-N-SH cells.*

Inositol is an essential growth factor that is required for survival and proliferation of many types of cultured cells (Eagle et al., 1957). Inositol deficiency in these cells causes an arrest of cell growth, cytopathogenic defects, and cell death. However, some cells are able to proliferate in inositol-free or inositol-deficient culture media due to active inositol biosynthesis (Eagle et al., 1957). To determine if inositol is essential for SK-N-SH neuronal cells, we assayed cell growth in inositol-deficient media (Medium 199 with 10% dialyzed serum). Medium 199 contains only 0.28 μM inositol, which is significantly less than the minimal requirement (1 μM) reported for most types of cells (Eagle et al., 1957). As seen in Fig. 4-1A, proliferation of cells cultured in the inositol-deficient media was similar to that of cells grown in media supplemented with exogenous inositol. The ability of SK-N-SH cells to grow in inositol-deficient media indicates that the *de novo* synthesis of inositol provides sufficient inositol for cell proliferation. Interestingly, cells cultured in inositol-deficient media exhibited levels of intracellular inositol similar to those of cells grown in media supplemented with 0.5-10 mM inositol (Fig. 4-1B). The homeostatic inositol pool in SK-N-SH cells suggests that inositol biosynthesis may be upregulated in inositol-deficient media.
Fig. 4-1
Fig. 4-1: Exogenous inositol is not essential for cell proliferation or maintaining inositol homeostasis in SK-N-SH cells. (A) SK-N-SH cells were inoculated at a concentration of 5,000 cells per well in 96-well plates at day 0, and cell numbers were estimated by the proliferation assay described under “Materials and methods.” (B) Intracellular inositol levels were assayed in cells cultured in inositol-deficient media without (control) or with inositol supplement (0.5, 1, 5, 10 mM). The data shown in A and B are the average of at least three experiments ± S.D.
Inositol biosynthesis is essential for cell proliferation.

To understand if inositol biosynthesis is required for SK-N-SH cells, we decreased inositol biosynthesis by stably knocking down expression of the gene encoding inositol-3-phosphate synthase, *INO1*, which encodes the rate-limiting enzyme of inositol biosynthesis. Two knockdown lines of SK-N-SH cells were obtained, shRNA-INO1-1 and shRNA-INO1-2, which exhibited 60% and 82% decreases in *INO1* expression, respectively (Fig. 4-2A). As expected, cell proliferation was dependent on the level of *INO1* expression (Fig. 4-2B). Interestingly, neurite outgrowth was remarkably inhibited in cells in which *INO1* expression was decreased (Fig. 4-2C). These findings indicate that *de novo* inositol synthesis is essential for cell proliferation and neurite outgrowth in SK-N-SH cells.

*Exogenous inositol does not regulate transcription of INO1, SMIT1, and HMIT, the genes for inositol biosynthesis or uptake.*

In yeast cells, exogenous inositol modulates the biosynthesis and uptake of inositol by controlling transcription of the inositol biosynthetic gene *INO1* (Henry et al., 2014; Hirsch and Henry, 1986; Loewen et al., 2004) and the genes encoding inositol transporters (Lai et al., 1995; Lai and McGraw, 1994). To ascertain if this is a conserved mechanism regulating inositol metabolism in SK-N-SH cells, we determined the effects of exogenous inositol on mRNA levels of
Fig. 4-2A

Lane
1  scrambled shRNA
2  shRNA_INO1_1
3  shRNA_INO1_2
Fig. 4-2B

The graph shows cell proliferation measured by absorbance at 490 nm ($A_{490}$) for scrambled, shRNA-INO1-1, and shRNA-INO1-2 treatments. The graph indicates a decrease in cell proliferation from scrambled to shRNA-INO1-2 treatments.
Fig. 4-2C

Scrambled control

INO1 knockdown
Fig. 4-2: Inositol biosynthesis is essential for cell proliferation. (A) Western blot analysis of Ino1 and inhibitory phosphorylation levels of GSK-3α (Ser21) and GSK-3β (Ser9). Actin was used as the loading control. Scrambled control and Ino1 knockdown (shRNA_INO1_1 and shRNA_INO1_2) SK-N-SH cells were cultured to about 70% confluence, and cells were refreshed with media containing 10% serum (+serum) or no serum (-serum) for 4 hours. Cells were harvested and lysed for Western blot analysis as described under “Materials and methods.” (B) Cell proliferation was assayed as described in Fig. 4-1A, and cell numbers were estimated 4 days after inoculation. The data shown are the average of four experiments ± S.D. (C) Control and INO1 knockdown cells (about 1X10⁶) were plated in 100-mm dishes and photographed at day 2 using a microscope at 200 X magnification.
*INO1*, Na+/inositol transporter SMIT1, and H+/inositol transporter HMIT in these cells. Cells were initially cultured in inositol-deficient media to deplete inositol. After supplementation with inositol (0.1, 1, 10 mM) for the indicated times, cells were harvested for mRNA analysis. As shown in Fig. 4-3A, mRNA levels of human *INO1* were not affected by the addition of inositol. Consistent with this finding, *INO1* protein levels were also not altered by exogenous inositol (Fig. 4-3B). In addition, the genes encoding inositol transporters were not regulated by inositol, as mRNA levels of SMIT1 (Fig. 4-3C) and HMIT (Fig. 4-3D) were not significantly changed in response to exogenous inositol. Therefore, in contrast to regulation of inositol biosynthesis in yeast cells, the biosynthesis and uptake of inositol were not transcriptionally regulated in response to exogenous inositol in SK-N-SH cells.

*Decreased Ino1 protein levels leads to inactivation of GSK3α.*

Previous studies showed that lithium and VPA inhibit inositol synthesis (Allison and Stewart, 1971; Berridge et al., 1989; Ju and Greenberg, 2003; Pollack et al., 1994; Shaltiel et al., 2004b; Vaden et al., 2001) and GSK-3 activity (Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al., 2005; Klein and Melton, 1996; Lucas and Salinas, 1997). To address the possibility that inositol synthesis affects GSK-3 activity, we measured levels of phosphorylation at Ser21 of GSK-3α and at Ser9 of GSK-3β in SK-N-SH cells as a function of inositol depletion.
Fig. 4-3

A

![Bar graph showing INO1/SDHA expression over time with different inositol concentrations.]

B

<table>
<thead>
<tr>
<th>Serum</th>
<th>2h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol (1 mM)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- Ino1
- Actin
Fig. 4-3: Exogenous inositol does not regulate transcription of the genes for inositol biosynthesis or uptake. (A) mRNA levels of *INO1* were measured in SK-N-SH cells incubated in the presence of inositol (0, 0.1, 1, 10 mM) for the indicated times (1, 5, and 10 hours) after growth in inositol-deficient media. Values were normalized to the internal control SDHA (succinate dehydrogenase complex, subunit A). *INO1* mRNA levels normalized to SDHA were represented as fold change relative to cells exposed to 0 mM inositol for 1 hour. (B) Western blot analysis of Ino1 protein levels. SK-N-SH cells were cultured to reach about 70% confluence and then were incubated with either serum or inositol for indicated times. Cells were harvested and lysed for Western blot analysis as described under “Experimental procedures.” Actin was used as the loading control. (C) mRNA levels of Na+/inositol transporter SMIT1 and (D) H+/inositol transporter HMIT were measured as described above.
Phosphorylation of these sites inactivates GSK-3 kinase (Cross et al., 1995; Srivastava and Pandey, 1998). As seen in Fig. 4-2A, GSK-3α phosphorylation was increased in response to inhibition of inositol synthesis, while GSK3β was not significantly altered. GSK-3α phosphorylation was dependent on the level of Ino1 knockdown. Thus, an 82% decrease in Ino1 protein in shRNA-INO1-2 cells led to a 2.4-fold increase in GSK-3α phosphorylation, while a 60% decrease in shRNA-INO1-1 led to a 1.5-fold increase. These results indicated that decreasing the de novo synthesis of inositol led to GSK-3α inactivation.

_VPA-induced transient decrease in inositol is associated with GSK-3α inactivation._

We have previously shown that the mood stabilizer VPA inhibits inositol synthesis in both yeast and human cells (Ju and Greenberg, 2003; Shaltiel et al., 2004b; Vaden et al., 2001). If inositol depletion was the cause of inhibitory phosphorylation of GSK-3α as seen in Fig. 4-2A, VPA may be expected to cause a similar effect. To address this possibility, we assayed GSK-3 phosphorylation in cells treated with VPA. As seen in Fig. 4-4A, VPA caused a significant decrease in inositol levels in the first hour of exposure, after which inositol levels were restored. As predicted, GSK-3α phosphorylation was increased in response to VPA (Fig. 4-4B), while GSK-3β phosphorylation was not significantly affected. Interestingly, GSK-3α phosphorylation continued to increase after inositol levels were restored (Figs. 4-4A and 4-4B). It is likely that inositol levels are restored by
recycling of inositol from inositol phosphates, and maintaining inositol homeostasis is associated with the regulation of GSK-3\(\alpha\) phosphorylation.

Fig. 4-4A
Fig. 4-4B: VPA decreases intracellular inositol and increases the inhibitory phosphorylation of GSK-3α. (A) Intracellular inositol levels were measured in SK-N-SH cells after exposure to VPA for the indicated times. (B) Western blot analysis of the protein levels of GSK-3α (Ser21) and GSK-3β (Ser9) and total protein levels of GSK-3α and GSK-3β. Actin was used as the loading control.
DISCUSSION

Despite the importance of inositol, there are very few reported studies of the consequences of inositol depletion in human cells. Here, we report that inositol synthesis is essential for proliferation and neurite outgrowth of SK-N-SH human neuroblastoma cells, and that inhibition of inositol biosynthesis leads to GSK-3α inactivation.

The interplay between inositol biosynthesis and GSK-3 activity reported in the current study has implications for understanding the therapeutic mechanisms of the mood-stabilizers used to treat bipolar disorder. Lithium and VPA are mood-stabilizers with disparate chemical properties. Interestingly, both drugs have been shown to decrease cellular inositol content by blocking inositol biosynthesis, and both drugs also inhibit GSK-3 activity. Consistent with these findings, two prevailing hypotheses for the therapeutic mechanisms of action of these drugs are inositol depletion (Berridge et al., 1989) and GSK-3 inhibition (Klein and Melton, 1996). A potential link between inositol depletion and GSK-3 inhibition has not been previously tested in human cells. While the inositol depleting drug lithium leads to increased phosphorylation of GSK-3β (Beaulieu et al., 2008; Zhang et al., 2003), we showed that VPA causes transient inositol depletion leading to increased phosphorylation of GSK-3α. We further report a direct link between inositol synthesis and GSK-3 activity in neuronal cells. Specifically, inositol depletion induced by knocking down INO1 expression results in GSK-3α inactivation.
Interestingly, while GSK-3α phosphorylation was increased in response to inositol depletion, GSK-3β was not significantly altered. Previous studies have shown that inhibitory phosphorylation and kinase activity of GSK-3 are affected by exposure to lithium and VPA (Beaulieu et al., 2008; Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al., 2005; Kim et al., 2013; Klein and Melton, 1996; Lucas and Salinas, 1997; Phiel et al., 2003; Ryves and Harwood, 2001; Zhang et al., 2003). While some studies characterized the inhibitory effects of the drugs on both GSK-3α and GSK-3β, most have focused on the effects on GSK-3β. This is the first demonstration in SK-N-SH cells that a decrease in inositol biosynthesis led to the preferential inactivation of GSK-3α. Further studies are needed to elucidate the significance of the differential inhibition of GSK-3α and GSK-3β, which do not have identical functions.

The inactivation of GSK-3α in SK-N-SH cells was dependent on the degree of Ino1 knockdown (Fig. 4-2). Interestingly, the degree of inactivation of GSK-3α also correlates with exposure times to VPA (Fig. 4-4B). While inhibition of inositol synthesis potently increases GSK-3α phosphorylation, inositol homeostasis is highly maintained, as intracellular inositol levels were restored upon prolonged exposure to VPA (Fig. 4-4A). It is likely that turnover of phosphatidylinositol and/or recycling of inositol phosphates are increased to compensate for decreased inositol biosynthesis. However, the mechanism whereby inhibition of inositol synthesis causes inactivation of GSK-3α activity (by increasing
phosphorylation of this kinase) remains unclear. Perturbation of inositol homeostasis resulting from disrupted inositol synthesis may modulate PI3K/AKT signaling. We speculate that inhibition of inositol synthesis activates the synthesis of PI3,4,5P3. The synthesis of PI3,4,5P3 is required for recruiting AKT, and the subsequent activation of AKT on the plasma membrane potently inhibits GSK-3 by phosphorylation (Cantley, 2002; Czech, 2003; Di Paolo and De Camilli, 2006).

Surprisingly, exogenous inositol did not regulate inositol biosynthesis or uptake by controlling expression of the inositol biosynthetic gene *INO1* or the genes encoding inositol transporters SMIT1 and HMIT. In contrast, the transcription of *INO1* and the inositol transporter genes in yeast cells is highly regulated in response to exogenous inositol, and inositol synthesis and uptake are modulated by this regulation (Henry et al., 2014; Hirsch and Henry, 1986; Lai et al., 1995; Lai and McGraw, 1994; Loewen et al., 2004). This indicates that neuronal cells have evolved different mechanisms to regulate inositol metabolism. For example, inositol uptake in mammals is regulated by glucose, pH, osmolality, and growth factors (Di Daniel et al., 2009; Fu et al., 2012; Miyakawa et al., 1999; Novak et al., 1999; Olgemoller et al., 1993; Spizz and Pike, 1992; Uldry et al., 2004). While the genes encoding inositol transporters are not transcriptionally regulated in response to exogenous inositol in SK-N-SH cells, the activity of inositol uptake may be controlled by different mechanisms. Inositol synthesis in yeast is also regulated by the synthesis of inositol pyrophosphates (Ye et al., 2013) and the
glycolysis intermediate dihydroxyacetone phosphate (DHAP) (Migaud and Frost, 1996; Shi et al., 2005), and requires GSK-3 (Azab et al., 2007). One or more of these mechanisms identified in yeast may also control inositol biosynthesis in mammalian cells.

In summary, we showed that de novo inositol synthesis catalyzed by Ino1 is required for proliferation of SK-N-SH cells during inositol-deficient conditions and for GSK-3α activation. These findings have implications for understanding the therapeutic mechanisms of the mood-stabilizers used for treatment of bipolar disorder.
The importance of regulation of the synthesis of phospholipids is underscored by crucial roles of phospholipids in cellular functions, most notably in membrane biogenesis and cell signaling. The synthesis of phospholipids is highly regulated in response to cell growth, division, aging, or stress conditions. This regulation potently controls phospholipid composition in cellular membranes and generates specific lipids to relay signals. For example, cardiolipin (CL), the signature phospholipid of mitochondria, comprises about 10-15% of total mitochondrial phospholipids (Jakovcic et al., 1971; van Meer et al., 2008b), the levels of which are correlated with mitochondrial respiration (Claypool et al., 2008b; Gohil et al., 2004; Jiang et al., 2000). Interestingly, the externalization of CL to the outer mitochondrial membrane is a signal for mitophagy in neuronal cells (Chu et al., 2013). Phospholipids are not only structurally important components of cellular membranes, but they also act as signals for organelle homeostasis. The importance of phospholipids is further underscored by their roles in human disorders. For example, mutations in the tafazzin gene, which encodes the transacylase for CL remodeling, lead to the life-threatening disorder Barth syndrome (Barth et al., 1983; Barth et al., 2004; Barth et al., 1999). Therefore, understanding the regulation of synthesis of phospholipids will shed light on our
fundamental knowledge of cell biology as well as human health. Chapters 2 and 3 in this dissertation describe novel mechanisms underlying the regulation of phospholipid synthesis, and Chapter 4 describes the consequence of inhibition of the synthesis of inositol, a precursor of inositol lipids. New questions wait to be addressed. I challenge future students to push the limit of our knowledge of lipid biology by addressing the following questions.

1. **How is inositol pyrophosphate kinase Kcs1 regulated to control inositol biosynthesis?**

In Chapter 2, I showed that the control of \textit{INO1} expression in response to inositol is associated with Kcs1 protein levels (Fig. 2-7). However, \textit{KCS1} mRNA levels are not altered in response to inositol (Fig. 2-7C), suggesting that Kcs1 protein is controlled at the level of translation, post-translational modification, and/or stability of Kcs1 protein. The underlying mechanisms by which Kcs1 protein levels respond to exogenous inositol are unclear, and it remains to be determined if the levels of inositol pyrophosphates are altered in response to exogenous inositol.

I have also shown that the DNA-interacting bZIP domain of the Kcs1 protein is required for \textit{INO1} expression and inositol synthesis (Fig. 2-5), suggesting that Kcs1 may directly interact with the chromatin region of \textit{INO1}. Recently, IP6K1, the mammalian homolog of Kcs1, has been shown to interact with chromatin and
regulate histone methylation (Burton et al., 2013). While regulation of histone modifications influences gene transcription, it is not clear if Kcs1-mediated regulation of INO1 expression involves the interaction of Kcs1 with the chromatin region of the INO1 promoter, and/or methylation of the region.

Further investigations may elucidate a molecular mechanism underlying how inositol pyrophosphates regulate inositol synthesis by modulating INO1 expression.

2. Is the mechanism whereby inositol pyrophosphates regulate inositol biosynthesis conserved in mammals?

Chapter 2 describes a mechanism whereby inositol synthesis is regulated by inositol pyrophosphates in the yeast Saccharomyces cerevisiae. Specifically, Kcs1, which catalyzes synthesis of inositol pyrophosphates, modulates INO1 expression. While this finding demonstrates a new level of complexity of regulation of inositol synthesis, it is unclear if this regulation is conserved from yeast to mammals.

Mammalian Ino1 has four putative mRNA isoforms (α, β, γ, and δ) derived from alternative splicing, of which the α isoform is the full-length Ino1 mRNA (Seelan et al., 2009). These isoforms exhibit tissue specificity (Guan et al., 2003; Seelan et al., 2009). However, regulation of expression of INO1 isoforms has not been rigorously studied in mammals. My preliminary studies (not discussed in Chapter
2) indicate that total Ino1 protein was increased, and the γ isoform was expressed in IP6K1 knockout mouse embryonic fibroblasts (MEF). Consistent with the increase in Ino1 protein, *INO1* mRNA levels were also increased in these cells. Therefore, regulation of *INO1* expression in inositol pyrophosphate-deficient mammalian cells is different from that in yeast cells. We further determined the activity of inositol synthesis in crude cell extracts of wild type and IP6K1 knockout cells. Surprisingly, inositol synthesis was significantly decreased in IP6K1 knockout cells. Therefore, the finding that inositol pyrophosphate deficiency leads to decreased inositol synthesis is conserved from yeast to mammals, although the regulation of *INO1* expression is disparate. It is unclear how inositol synthesis is decreased in IP6K1 knockout cells in spite of increased *INO1* expression. I am currently addressing two questions regarding this regulatory mechanism. First, how does IP6K1 regulate *INO1* transcription? Second, how is Ino1 activity regulated in IP6K1 knockout cells?

Elucidating how inositol synthesis is controlled by inositol pyrophosphates may uncover a novel mechanism underlying the regulation of *INO1* expression in mammals.
3. What is the mechanistic link between inositol biosynthesis and GSK-3 activity?

In Chapter 4, I described a novel interplay between inositol synthesis and GSK-3α phosphorylation in SK-N-SH neuronal cells. Specifically, inhibition of inositol biosynthesis by shRNA-mediated knockdown of INO1 expression or by VPA treatment leads to increased inhibitory phosphorylation of GSK-3α. However, the mechanism whereby inhibition of inositol synthesis causes increased phosphorylation of GSK-3α remains unclear. My current hypothesis is that decreasing inositol synthesis activates PI3K/AKT signaling, which regulates phosphorylation of GSK-3. PI3K kinase converts PI4,5P2 to PI3,4,5P3, which is required for recruiting AKT to the plasma membrane. Activation of AKT on the plasma membrane potently inhibits GSK-3 by phosphorylation (Cantley, 2002; Czech, 2003; Di Paolo and De Camilli, 2006). We speculate that inositol depletion may modulate the synthesis of PI3,4,5P3 by affecting the enzymatic activity or protein levels of PI3K kinase. The mechanism whereby inositol depletion causes GSK-3 inhibition has implications for understanding the therapeutic mechanisms of the mood-stabilizers used for treatment of bipolar disorder.
4. What are the functions of CL remodeling?

In Chapter 3, we have shown that deletion of the CL-specific phospholipase Cld1 rescues growth and lifespan defects in the tafazzin mutant, and that Cld1 regulation in response to mitochondrial respiration modulates energy dynamics. The finding that deletion of Cld1 alleviates the deleterious effects of the loss of tafazzin suggests that CL-specific phospholipases may be potential targets for treating BTHS patients. However, CL-specific phospholipases have not been identified in mammals. We have received funding from the Barth Syndrome Foundation for a project entitled “Identification of human CL phospholipases that are deleterious to tafazzin-deficient cells,” which proposes to identify potential phospholipases that deacylate CL. Characterization of human CL phospholipases may identify potential therapeutic targets for treating BTHS patients.

While our studies showed that regulation of the phospholipase Cld1 responds to mitochondrial respiration and modulates energy dynamics, this raises the question of what is the function of CL remodeling, and the corollary question of why is \( CLD1 \) expression increased in response to respiration. Because superoxides generated from mitochondrial respiration can impair CL functions by peroxidation of CL (Paradies et al., 2000; Paradies et al., 2001; Paradies et al., 1998), we speculate that one possible function of deacylation of CL is to remove
peroxidized CL. In this model, CL remodeling acts as a mechanism whereby damaged fatty acyl chains are replaced. To test this possibility, the specificity of Cld1 enzymatic activity for peroxidized CL can be assayed in vitro. Although the cld1Δ mutant grows normally under respiratory conditions (Fig. 4-2), it is not clear if cld1Δ is sensitive to ROS-inducing reagents, such as H₂O₂. Elucidating the potential role of Cld1 in deacylating peroxidized CL may identify an exciting physiological function of CL remodeling.

While Cld1 regulation may remediate the deleterious effects of respiration by replacing peroxidized CL, the role of CL remodeling is largely not understood. A powerful tool to elucidate function is that of identifying genetic interaction with the gene in question. Synthetic genetic array (SGA) analysis is a yeast-based high-throughput assay to identify genetic interactions, which yields unbiased functional information pertaining to a query strain. I am currently in the process of carrying out an SGA analysis using the cld1Δ mutant as the query strain crossed with the entire genome deletion set. While the remodeling of CL is disrupted in both cld1Δ and taz1Δ mutants, CL levels are not affected in cld1Δ. Therefore, this SGA analysis using cld1Δ as the query strain will hopefully identify genetic interactions that are due to the loss of CL remodeling but not to decreased CL. A complete list of genes that genetically interact with CLD1 will be available from this study. I encourage future students to investigate the function of CL remodeling based on these genetic findings.

While we are approaching the end of this dissertation, we have certainly not finished our discussion of what makes studying the regulation of the synthesis of
inositol and cardiolipin so exciting. No doubt my labmates will enjoy the science in our wonderful lab with unbeatable spirit. If you ever wonder where science will take you, my answer is to enjoy the journey, because science is full of beauty along the way.
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concentrations but increases it at low inositol concentrations. *Brain research*. 855:158-161.


Phospholipids are the most abundant lipids in cell membranes. The synthesis of phospholipids is crucial for cellular membrane biogenesis and nearly all aspects of cellular processes. Understanding the regulation of synthesis of phospholipids is beneficial to our fundamental knowledge of cell biology as well as human health.

Regulation of the synthesis of phospholipids is intensively studied in the yeast *S. cerevisiae*. Most notably, the synthesis of phospholipids is coordinated with the synthesis of inositol, a precursor of inositol-containing lipids, by controlling expression of the genes encoding phospholipid biosynthetic enzymes. In addition to this well-characterized regulatory circuit controlled by the *trans*-acting factors Ino2, Ino4, and Opi1, this dissertation shows that inositol
pyrophosphates are novel regulators of the synthesis of inositol and phosphatidylinositol that control \textit{INO1} expression.

Despite the importance of inositol, there are very few reported studies of the cellular consequences of perturbation of inositol synthesis in human cells. Studies of SK-N-SH neuronal cells in this dissertation demonstrate that inositol biosynthesis is essential for cell proliferation and neurite outgrowth, and inhibition of inositol biosynthesis leads to inactivated GSK-3\(\alpha\), which has many regulatory functions in neural systems. This novel finding bridges two prevailing hypotheses of inositol depletion and GSK-3 inhibition and suggests a unifying hypothesis for the therapeutic mechanisms of action of mood-stabilizing drugs.

Although the synthesis of most phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol) is responsive to inositol, the synthesis of cardiolipin (CL) is an exception. Characterization of the regulation of CL synthesis has unveiled the critical role of CL remodeling via the regulation of the CL-specific phospholipase Cld1. Transcriptional regulation of Cld1-mediated deacylation of CL influences energy metabolism by modulating the relative contribution of glycolysis and respiration to ATP production. Interestingly, \textit{CLD1} expression is responsible for defective growth and respiration in tafazzin-deficient cells. We demonstrate that these underlying defects of tafazzin deficiency are caused by the decreased CL/MLCL ratio, not by a deficiency in unsaturated CL. These findings have significant implications for the life-threatening disorder Barth syndrome.
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