Regulation of Inositol Metabolism Is Fine-tuned by Inositol Pyrophosphatases in *Saccharomyces cerevisiae* **

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**Background:** Regulation of inositol metabolism is crucial for cellular functions.

**Results:** Inositol pyrophosphate-deficient cells exhibit defective inositol biosynthesis. Protein levels of the inositol pyrophosphate biosynthetic enzyme Kcs1 are dynamically altered in response to inositol.

**Conclusion:** *INO1* transcription and inositol biosynthesis are regulated by modulation of inositol pyrophosphate synthesis.

**Significance:** Inositol pyrophosphatases are novel regulators of biosynthesis of inositol and inositol phospholipids.

Although inositol pyrophosphatases have diverse roles in phosphate signaling and other important cellular processes, little is known about their functions in the biosynthesis of inositol and phospholipids. Here, we show that *KCS1*, which encodes an inositol pyrophosphate kinase, is a regulator of inositol metabolism. Deletion of *KCS1*, which blocks synthesis of inositol pyrophosphates on the 5-hydroxyl of the inositol ring, causes inositol auxotrophy and decreased intracellular inositol and phosphatidylinositol. These defects are caused by a profound decrease in transcription of *INO1*, which encodes myo-inositol-3-phosphate synthase. Expression of genes that function in glycolysis, fatty acid oxidation, and protein processing is not affected in *kcs1*. Deletion of *INO1*, the *INO1* transcription repressor, does not fully rescue *INO1* expression in *kcs1*. Both the inositol pyrophosphate kinase and the basic leucine zipper domains of *KCS1* are required for *ino1* expression. Kcs1 is regulated in response to inositol, as Kcs1 protein levels are increased in response to inositol depletion. The Kcs1-catalyzed production of inositol pyrophosphates from inositol pentakiphosphosphate but not inositol hexakisphosphate is indispensable for optimal *ino1* transcription. We conclude that *INO1* transcription is fine-tuned by the synthesis of inositol pyrophosphates, and we propose a model in which modulation of Kcs1 controls *INO1* transcription by regulating synthesis of inositol pyrophosphates.

Inositol, a ubiquitous six-carbon cyclitol, is an essential metabolite and a precursor of inositol phosphates, phosphoinositides, and sphingolipids (1, 2). These inositol-containing molecules play crucial roles in gene expression (3, 4), signal transduction (5), lipid signaling (6), and membrane biogenesis (7). The regulation of inositol-related signaling modulates various cell functions, such as cell growth, apoptosis, endocytosis, neuronal plasticity, and membrane trafficking (2, 8, 9). 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 (10). In *Saccharomyces cerevisiae*, exogenous inositol potently controls inositol biosynthesis by regulating *INO1* transcription through the transcriptional repressor Opi1 (11). In the absence of exogenous inositol, Opi1 is sequestered on the periphery of the nucleus by interaction with the vesicle-associated membrane protein-associated protein Ssc2 and with phosphatidic acid (PA) 3 (12). 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 transcriptional activator complex Ino2-Ino4 and represses *INO1* transcription (12, 13). 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 (UASINO) (14), which is found in the promoters of more than 30 genes in phospholipid metabolic pathways (1, 2, 15). 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 (16–19). 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, *KCS1*, encodes inositol pyrophosphate kinase, which catalyzes

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*The abbreviations used are: PA, phosphatidic acid; PI, phosphatidylinositol; bZIP, basic leucine zipper; DINS, diphosphoinositol polyphosphate synthase; UASINO, inositol-responsive upstream-activating sequence; IP<sub>7</sub>, inositol hexakisphosphate; IP<sub>6</sub>, inositol pentakisphosphate; IP<sub>5</sub>, inositol tetraisphosphate; IP<sub>4</sub>, inositol trisphosphate; qPCR, quantitative PCR; IP<sub>3</sub>, inositol 1,4,5-trisphosphate.*

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Interestingly, recruitment of Ino80 to produce 1-IP7 is known to regulate phosphate homeostasis by activation of the kinase complex Pho80-Pho85 (29). This leads to removal of exogenous inositol, suggesting that rapid turnover of inositol pyrophosphates generated from IP5 by Kcs1 regulates inositol phosphate synthesis on inositol homeostasis. Our findings suggested that inositol pyrophosphates may function in the regulation of Kcs1-catalyzed synthesis of 5PP-IP4 modulates synthesis. We propose a model in which regulation of Kcs1-catalyzed synthesis of 5PP-IP4, modules INO1 transcription.

**Inositol Pyrophosphates Regulate Inositol Synthesis**

**TABLE 1**

| Strains used in this study |
|-----------------------------|
| BY4741 MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Invitrogen |
| BY4742 MAT β his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Invitrogen |
| kcs1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δ:KanMX6 Invitrogen |
| vip1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vip1Δ:KanMX6 Invitrogen |
| ipk1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ipk1Δ:KanMX6 Invitrogen |
| ipk2Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ipk2Δ:KanMX6 This study |
| opi1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 opi1Δ:KanMX6 Invitrogen |
| kcs1Δvип1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δvип1Δ:KanMX6 This study |
| kcs1Δopi1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δopi1Δ:KanMX6 This study |
| kcs1Δipk1Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δipk1Δ:KanMX6 This study |
| kcs1Δipk2Δ MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δipk2Δ:KanMX6 This study |
| WT Opi1-GFP MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 12 |
| kcs1Δ Opi1-GFP MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kcs1Δ:Opi1-GFP-HIS3 Ste2pr::LEU2 This study |
| WT KCS1-GFP MAT β his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 KCS1-GFP-HIS3 Invitrogen |
| opi1Δ KCS1-GFP MAT β his3Δ1 leu2Δ0 ura3Δ0 opi1Δ:KanMX6 KCS1-GFP-HIS3 This study |

**TABLE 2**

| Plasmids used in this study |
|-----------------------------|
| pTLA16ILIZ UAS<sub>NsiI</sub> reporter plasmid 12 |
| pFL38 CEN, URA3 22 |
| pFV198 pFL38, KCS1<sup>L1L2</sup>− 22 |
| pFV217 pFL38, KCS1<sup>NL4</sup>− 22 |
| pFV241 pFL38, KCS1 22 |

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmids, and Growth Media**

The yeast *S. cerevisiae* strains used in this study are listed in Table 1. Wild type (WT) strain with the GFP-HIS3MX6 cassette integrated at the carboxyl-terminal end of the *KCS1* open reading frame was 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+, whereas medium lacking inositol is denoted I−.

The plasmids used in this study are listed in Table 2. The plasmids pFL38, pFV198, pFV217, and pFV241 (22) were gifts from Dr. Evelyne Dubois, and the UAS<sub>NsiI</sub> reporter plasmid (12) was a gift from Dr. Christopher Loewen. All the plasmids...
Inositol Pyrophosphates Regulate Inositol Synthesis

were amplified and extracted using standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol (33).

Measurement of Intracellular Inositol

Intracellular inositol was measured as described previously (19) 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 min at 30-s intervals, alternating with a 30-s 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 at 5 min at 2000 rpm for 5 min at 4 °C. Cells grown in I− or I0.5–0.8, 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 A550 of 0.5 were collected at 4 °C and used as the 0-h time point.

β-Galactosidase Reporter Assay—WT and mutant cells that were transformed with the UASINO1 reporter plasmid were pre-cultured in I− to the mid-logarithmic growth phase (A550 of 0.5–0.8), washed with prewarmed I−, and resuspended in fresh I−. After continuous growth for 4 h, cells were harvested, and β-galactosidase was assayed as described previously (12, 38).

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% Nonidet P-40, 2 mM EDTA, and 1X protease inhibitor mixture (Roche Applied Science). Protein extracts were clarified twice by 5 min of centrifugation at 13,000 × g at 4 °C to remove cell debris and glass beads. Protein concentration was determined using the BCA™ protein assay kit (Pierce Protein), with secondary antibodies conjugated with HPR) and visualized using ECL Plus substrate (Pierce Protein), with α-tubulin as the loading control. Image software was used to quantify the intensities of bands.

| Gene   | Primers | Sequence (5′ to 3′) |
|--------|---------|---------------------|
| ACT1   | Forward | TTCCGTTGATGGTTATTCA |
|        | Reverse | GCACAATACTGGTCTCATA |
| INO1   | Forward | CAGCTTGTCGACAAAAAG |
|        | Reverse | ATAGGGCTCAATGGAACCC |
| INO2   | Forward | TCTACAGGCTTATCCGAGA |
|        | Reverse | ATTTGCTATCTCCACAGAGG |
| INO4   | Forward | AGCACTTGGTCTCAGAGCCTCA |
|        | Reverse | GTCCATACCCGAGCTCCCA |
| PDA1   | Forward | ATTTAGGTTAGAGAGCCGCTT |
|        | Reverse | AGGTCACCCTCCCTGCAGG |
| RDN18  | Forward | CTTCCGTATGGTTATTCC |
|        | Reverse | GCCCTTCTGCTGAGACCC |
| TAF10  | Forward | AGCACAGCCTGCTGAGAAT |
|        | Reverse | AGCCACAGCTGCTGAGAAT |
| TDH3   | Forward | AGCACTTGGTCTCAGAGCCTCA |
|        | Reverse | TACCATGATTAGACCCGCT |
| TFC1   | Forward | AAACTCCGCTCACTCTGAGT |
|        | Reverse | TCCCTCTGCTGAGACCC |
| UBC6   | Forward | AGCACTTGGTCTCAGAGCCTCA |
|        | Reverse | TACCATGATTAGACCCGCT |
| SPT15  | Forward | GCTATACCAGGGGTATCGAGA |
|        | Reverse | TACATGGAAGATTGGGCC |

PCR efficiency. All the primers used in this study have calculated reaction efficiency between 95 and 105%.

Quantification of INO1 Expression

RT-qPCR Analysis—Cells were pregrown in I+ to the mid-logarithmic phase and inoculated into fresh I+ medium at A550 of 0.05. When the A550 reached 0.5, cells were harvested by centrifugation at 3500 rpm for 3 min 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 A550 of 0.5 were collected at 4 °C and used as the 0-h time point.

β-Galactosidase Reporter Assay—WT and mutant cells that were transformed with the UASINO1 reporter plasmid were pre-cultured in I− to the mid-logarithmic growth phase (A550 of 0.5–0.8), washed with prewarmed I−, and resuspended in fresh I−. After continuous growth for 4 h, cells were harvested, and β-galactosidase was assayed as described previously (12, 38).
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 ×1000.

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) were grown on I− plates. We screened 26 deletion mutants in the categories expected to affect inositol metabolism, including inositol polyphosphate kinases, protein kinases and protein phosphatases, vacuolar proteins, and endoplasmic reticulum 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 (39, 40). 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. 2A, kcs1Δ cells showed an extended lag phase when inoculated into I+ medium compared with 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. 2B). Consistent with inositol auxotrophy, intracellular inositol levels in kcs1Δ were reduced to less than 30% of WT levels (Fig. 2C), and PI were about 42% of WT (Fig. 2D).

Inositol biosynthesis is activated in WT cells in inositol-deficient medium by dramatically up-regulating INO1 transcription (2). However, up-regulation of INO1 mRNA was not observed in kcs1Δ (Fig. 2E), suggesting that transcription of INO1 is defective in the mutant. We addressed the possibility that defective INO1 transcription resulted from a global repression of transcription by comparing mRNA expression of a variety of genes in WT and kcs1Δ, including genes in glycolysis (PDA1 and TDH3), basal transcription (TAF10, TFC1, and SPT15), and protein processing (RDN18 and UBC6). None of these genes exhibited decreased expression in kcs1Δ (Fig. 2F). Taken together, these studies suggested that decreased INO1 transcription in kcs1Δ diminishes biosynthesis of inositol and PI, leading to inositol auxotrophy.

Decreased Inositol Biosynthesis in kcs1Δ is Not Because of Perturbation of the UASINO Regulatory Complex Opi1-Ino2-Ino4—The native promoter of INO1 contains the UASINO element that is widely found in the promoter regions of many genes, including genes involved in phospholipid metabolism (14). As shown in Fig. 3A, transcription of genes containing the
**Inositol Pyrophosphates Regulate Inositol Synthesis**

**FIGURE 3. Opi1-Ino2-Ino4 is not perturbed in kcs1Δ.** A, regulatory mechanism of INO1 transcription. Ino2 and Ino4 are activators of INO1 transcription. Scs2 stabilizes the negative regulator Opi1 on the endoplasmic reticulum membrane with PA. Upon addition of inositol, synthesis of PI rapidly consumes PA, releasing Opi1p, which translocates to the nucleus and represses INO1 expression. B, WT or kcs1Δ cells with GFP-tagged Opi1 were cultured in I− to the mid-logarithmic phase (A<sub>550</sub> of 0.5), washed with prewarmed I−, and resuspended in prewarmed and fresh I−. After growing for 2 h 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% of 0.5), washed with prewarmed I−, and resuspended in prewarmed and fresh I−. After growing for 2 h in I−, cells were examined under the fluorescence microscope. The figure is representative of six independent experiments. INO2 (C) and INO4 (D) transcripts were assayed in WT and kcs1Δ cells cultured in the conditions described in Fig. 2E. The data shown in C and D are the average of three experiments ± S.D.

**UAS<sub>INO</sub> element** is activated by the Ino2-Ino4 heterodimer interacting with the UAS<sub>INO</sub>-containing promoter and repressed by the interaction of Opi1 with Ino2 (2, 14, 41, 43). Among genes regulated in this manner, INO1 is the most responsive (2, 13). In the absence of inositol, localization of Opi1 on the endoplasmic reticulum is stabilized by interaction with Scs2 and PA (12). In response to exogenous inositol, Opi1 is translocated to the nucleus, inhibiting INO1 transcription (12, 44). We addressed the possibility that decreased transcription of INO1 in kcs1Δ is caused by retention of the transcription repressor Opi1 in the nucleus. As shown in Fig. 3B, in I− conditions, GFP-tagged Opi1 locates on the nuclear rim in kcs1Δ as observed in WT cells, indicating that the translocation of Opi1 is not perturbed in kcs1Δ. Therefore, **KCS1** does not regulate INO1 transcription by affecting the localization of Opi1.

We further investigated if the INO1 transcription defect in kcs1Δ was caused by perturbation of the transcriptional activators Ino2 and Ino4. INO2 is known to be up-regulated in I−, whereas INO4 is constitutively expressed in both I+ and I− (45). Although INO2 transcripts were decreased in kcs1Δ relative to WT cells, expression in I− was greater than in I+ in both strains (Fig. 3C), indicating that decreased INO1 expression in kcs1Δ is not due to an inability to up-regulate INO2. Levels of the constitutively expressed INO4 were not significantly diminished in kcs1Δ (Fig. 3D). These experiments suggest that decreased transcription of INO1 in kcs1Δ is most likely not due to decreased availability of Ino2 and Ino4, although levels of INO2 transcription were somewhat decreased relative to WT.

**KCS1 Is Required for Optimal INO1 Transcription**—As mentioned, **OPII** is a transcriptional repressor of INO1, and deletion of **OPII** leads to overproduction of inositol (11). Not surprisingly, deletion of **OPII** restored growth of kcs1Δ on I− at 30 and 37 °C (Fig. 4A). Interestingly, deletion of **OPII** also alleviated the growth defect of kcs1Δ on I+ at 30 °C (Fig. 4A), suggesting that the Opi1-controlled repression of other genes may also be deleterious to the growth of kcs1Δ. Deletion of **OPII** in kcs1Δ restored PI levels (Fig. 2D). Relatively higher PI levels in opi1Δ than WT were most likely due to overproduction of inositol in opi1Δ. To determine whether INO1 transcription is also restored in kcs1Δ opi1Δ, we analyzed INO1 expression in the double deletion mutant transformed with the INO1-lacZ reporter. Surprisingly, although deletion of **OPII**
increased \textit{INO1-lacZ} expression in \textit{kcs1Δ}, expression in \textit{kcs1Δopi1Δ} was only 20–30\% of that in WT and \textit{opi1Δ} cells (Fig. 4B), suggesting that \textit{KCS1} is required for optimal \textit{INO1} transcription.

Both bZIP and DINS Domains of \textit{Kcs1} Are Required for \textit{INO1} Transcription—As depicted in Fig. 5A, \textit{Kcs1} has two functional domains (46, 47) as follows: the kinase domain (also referred as DINS) (47, 48) and two bZIP domains containing four leucine heptad repeats (22, 46). Plasmids containing the full-length \textit{KCS1} or \textit{Kcs1} with site mutations in each functional domain were constructed and characterized previously (Fig. 5A) (22).

To determine whether these domains are required for \textit{INO1} transcription, we assayed growth and \textit{INO1} expression in \textit{kcs1Δ} cells transformed with these plasmids. In contrast to the full-length \textit{KCS1} (p\textit{KCS1}), the kinase-mutated \textit{Kcs1} (p\textit{KCS1\\textit{L1L2->AAA}}) did not rescue inositol auxotrophy or restore \textit{INO1} transcription in \textit{kcs1Δ} (Fig. 5, B and C). It has been demonstrated that synthesis of inositol pyrophosphates 5-IP\textsubscript{7} and 5PP-IP\textsubscript{4} is virtually eliminated by mutation of the kinase domain (22). Therefore, \textit{Kcs1} kinase activity, which catalyzes the synthesis of inositol pyrophosphates, is required for inositol biosynthesis as well as optimal \textit{INO1} transcription. Previous studies also indicated that site mutations in the bZIP domain did not affect the generation of inositol pyrophosphates (22). Unexpectedly, \textit{kcs1Δ} cells containing the bZIP-mutated \textit{KCS1} exhibited decreased growth on \textit{l−}, which was rescued by inositol (Fig. 5B). Consistent with the defective growth on \textit{l−}, the strain also exhibited a 50\% decrease in \textit{INO1} expression compared with WT (Fig. 5C). Therefore, both the bZIP and the kinase domains of \textit{Kcs1} are required for \textit{INO1} transcription.

\textit{Kcs1} Protein Modulates \textit{INO1} Transcription—To gain insight into how \textit{KCS1} modulates \textit{INO1} transcription, we measured protein levels of GFP-tagged \textit{Kcs1} in WT and \textit{opi1Δ} cells that were grown in \textit{l+} or \textit{l−}. Two bands were detected by
Inositol Pyrophosphates Regulate Inositol Synthesis

A

WT

opiΔ

30°C

37°C

30°C

37°C

Exposure time

INOS1-LacZ expression

30°C

37°C

Miller Units

1

2

3

4

5

α-Tubulin

Kcs1-GFP

inositol

normalized
Kcs1-GFP

FIGURE 6. Increased Kcs1 protein levels in I− conditions. A, cell lysates were prepared from WT or isogenic opiΔ cells containing GFP-tagged Kcs1. Cells were cultured in I− or I− to the mid-logarithmic phase (A550 of 0.5) at 30 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 UASINO1-lacZ reporter plasmid. Cells were precultured in I− at 30 or 37 °C to the mid-logarithmic phase (A550 of 0.5–0.8), then pelleted, washed with I− that was prewarmed to 30 or 37 °C, and resuspended in prewarmed and fresh I−. After the shift, cells were continuously cultured at 30 or 37 °C for 4 h. β-Galactosidase activity was measured as described under “Experimental Procedures.” The data shown in B are the average of six experiments ± S.D.

anti-GFP, most likely corresponding to full-length and truncated Kcs1 proteins, as reported previously (49). WT cells cultured in I− (Fig. 6A), conditions in which INO1 transcription is increased, exhibited elevated levels of Kcs1 protein compared with WT cells cultured in I+. In addition, both Kcs1 protein and INO1 transcription levels were decreased at elevated temperature compared with those observed at 30 °C (Fig. 6, A and B). Interestingly, decreased Kcs1 protein levels in I+ relative to I− were not observed in opiΔ cells (Fig. 6A), indicating that OPI1 is required to regulate Kcs1 protein in response to inositol.

To determine whether Kcs1 protein levels respond specifically to inositol, we observed the effects on Kcs1 protein of shifting cells from I+ to fresh I− medium, which are 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. 7A, by 2 h after the shift to I−, levels of the full-length Kcs1 protein increased more than 10-fold. Levels decrease after 4 h, and Kcs1 was not detected at 6 h. This pattern is consistent with the pattern of INO1 expression (Fig. 7B), which peaked at 2 h and was significantly diminished at 6 h. Kcs1 protein was not increased significantly in cells shifted to fresh I+ medium (Fig. 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, opiΔ cells did not exhibit an increase in Kcs1 protein in response to inositol (Fig. 7A), indicating that Opi1 regulates Kcs1 protein levels. Interestingly, despite the dramatic increase in Kcs1 protein in response to the shift from I+ to I−, transcription of KCS1 was not altered (Fig. 7C).

In reciprocal experiments, we assayed Kcs1 protein levels in cells shifted from I− to I+ (Fig. 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 h and was reduced to less than 10% of the initial level within 4 h. In cells supplemented with inositol, the decrease in 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 (12, 50). 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 IP3 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 pyrophosphate 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. 1. Hydrolysis of phosphatidylinositol 4,5-bisphosphate by PtdInsP2 provides PtdIns(4,5)P2 as a precursor for the synthesis of inositol polyphosphates. Ipk2 catalyzes the synthesis of PtdIns(4,5)P2 and PtdIns(4,5,6)P3, and Ipk1 catalyzes the synthesis of PtdIns(4,5,6,7)P4. Kcs1 catalyzes the pyrophosphorylation of PtdIns(4,5)P2 to 5PP-IP4 and further to (PP)2-IP3 (not shown) and (PP)2-IP4, to 5-IP5 (48, 51, 52). Vip1 catalyzes the synthesis of inositol pyrophosphates at the 1-hydroxyl site of the inositol ring (26–28). To assess which inositol poly- and/or pyrophosphates 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 4. Inositol poly-/pyrophosphates synthesized by the WT and deletion strains shown in Table 4 have been characterized previously by high performance liquid chromatography (HPLC) (22, 23, 51, 53). As seen in Fig. 4, A and B, ipk1Δ did not exhibit growth defects on I− plates, although 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 IP3, led to inositol auxotrophy. Consistent with this, INO1-lacZ expression was greatly reduced in kcs1Δipk1Δ compared with both WT and ipk1Δ. These find-
**Inositol Pyrophosphates Regulate Inositol Synthesis**

**FIGURE 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_{SS0} 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.

**TABLE 4**

| Mutants that cannot produce SPP-IP₄ exhibit decreased growth on I− | IP₃ | IP₆ | 5-IP₃ | 1-IP₆ | 1,5-IP₆ | 5-PP-IP₄ | Growth on I− |
|---|---|---|---|---|---|---|---|
| WT | + | + | + | + | + | + | + |
| kcs1Δ | + | + | + | + | + | + | + |
| ipk1Δ | + | + | + | + | + | + | + |
| kcs1Δipk1Δ | + | + | + | + | + | + | + |
| ipk2Δ | + | + | + | + | + | + | + |
| kcs1Δipk2Δ | + | + | + | + | + | + | + |
| vip1Δ | + | + | + | + | + | + | + |
| kcs1Δvip1Δ | + | + | + | + | + | + | + |

Source 22, 23, 48, 50 This study

This study demonstrates that Kcs1, which catalyzes the synthesis of inositol pyrophosphates, regulates inositol biosynthesis by controlling INO1 expression. We report the following: 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.

**DISCUSSION**

This study demonstrates that Kcs1, which catalyzes the synthesis of inositol pyrophosphates, regulates inositol biosynthesis by controlling INO1 expression. We report the following: 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. 2A). Consistent with this, intracellular inositol in kcs1Δ cells was decreased to less than 30% of WT (Fig. 2C), whereas PI was decreased to about 42% of WT. In response to inositol depletion, kcs1Δ cells displayed severely reduced INO1 derepression compared with WT cells (Fig. 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. 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 *INO1* transcription compared with the mutated kinase domain (Fig. 5C). Disruption of the bZIP domain in *KCS1* does not reduce the production of inositol pyrophosphates (22). Interestingly, the bZIP domain of *KCS1* shares homology with the bZIP domain of Opi1 (54), as seen in the sequence alignment (Fig. 8A). The role of the bZIP domain has not been characterized in either protein. We speculate that the bZIP domain of Opi1 and Kcs1 may share binding sites and that *INO1* transcription may be regulated by the bZIP domains that mediate spatial localization of the proteins to the vicinity of the chromosomal regions where *INO1* is located.

Our findings indicate that Kcs1 protein, but not transcription, is regulated in response to inositol. A novel mechanism underlying the regulation of *KCS1* transcription in response to phosphate signals was identified previously (49). Pho4-mediated transcription of the antisense and intragenic RNAs in *KCS1* leads to the production of truncated Kcs1 protein and down-regulation of Kcs1 kinase activity (49). This mechanism of regulation of phosphate signaling involves a positive feedback loop, in which species of the mRNAs and proteins of *KCS1* are regulated by transcription of the antisense and intragenic RNAs. In contrast to Pho4-mediated regulation of *KCS1*, the *KCS1* mRNA levels did not change in response to inositol (Fig. 7C), and the full-length and truncated Kcs1 proteins were similarly increased in I− (Fig. 7A) and decreased in I+ (Fig. 7D). These findings suggest a different mechanism underlying regulation of Kcs1 protein in inositol biosynthesis compared with phosphate signaling. We speculate that Kcs1 protein may be controlled by translation or post-translational modification and/or stability of Kcs1 protein.

Analysis of inositol pyrophosphate mutants indicates that inositol pyrophosphates synthesized from IP₅, but not IP₆, are the most likely regulators of inositol biosynthesis. As summarized in Table 4, *ipk1Δ*, which lacks IP₆ and IP₇, did not exhibit inositol defects, whereas *ks1Δipk1Δ*, which lacks 5PP-IP₄, IP₆ and IP₇, exhibited severe inositol defects. These findings suggest that 5PP-IP₄, synthesized from IP₆, is required for inositol biosynthesis. However, we cannot completely rule out the possibility that 5-IP₇ is required for inositol biosynthesis. Indeed, deletion of *ipk1Δ* caused only about a 30% decrease in *INO1* expression (Fig. 4B), consistent with the findings of Wu and co-workers (3). Therefore, 5PP-IP₄ is sufficient for inositol regulation, but IP₇ also contributes to regulation. This is consistent with the moderate inositol defects observed in *vip1Δ*. Because of the difficulty of constructing a strain that can generate 5PP-
IP₄ and IP₆, but not IP₇, it is difficult to elucidate the specific role of IP₇ in regulating INO1 transcription.

Interestingly, deletion of PLC1, the gene encoding phospholipase C that hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates IP₃ as precursors for inositol poly-/pyrophosphates, exhibited elevated INO1 expression (55, 56). It is likely that regulation of INO1 gene expression and inositol biosynthesis is coordinated with phospholipase C activation in addition to the negative feedback circuit in response to exogenous inositol. However, deletion of PLC1 is lethal in some genetic backgrounds (42). This complicates our understanding of the regulation of INO1 expression by PLC1. Interestingly, inositol polyphosphates IP₅ and IP₆, produced from phosphorylation of IP₃, have roles in Ino80-mediated chromatin remodeling, a process also required for INO1 expression (3, 4). Regulation of INO1 expression by synthesis of inositol pyrophosphates from IP₃ and IP₆ will further complicate the regulation of INO1 expression as altered levels of IP₃ and IP₆ may affect chromatin structure. We propose a model, depicted in Fig. 8, in which optimal INO1 transcription is modulated by the synthesis of inositol pyrophosphate, 5PP-IP₄ (derived from IP₃). Under derepressing conditions (1−), Opi1 is excluded from the nucleus (2, 12), although Kcs1 protein levels are increased (Fig. 7A). Increased Kcs1 protein accelerates production of 5PP-IP₄, which is required for optimal INO1 expression. Nuclear Op1, most likely decreases Kcs1 protein as increased Kcs1 was observed in opi1Δ and in 1− (during which Opi1 is excluded from the nucleus). Consistent with this, under repressing conditions (1+), Kcs1 is rapidly decreased (Fig. 7D), most likely due to Op1 translocation into the nucleus where it represses INO1 expression (2, 12) and decreases Kcs1 protein. Kcs1 and Op1 may compete for a common binding site via the bZIP domain in the nucleus. Therefore, Op1-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 levels control INO1 transcription by regulating the synthesis of inositol pyrophosphates. We speculate that 5PP-IP₄ may be required to recruit transcriptional activators to the 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 INO1 transcription.

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