1-1-2010

Identification Of Deletion Mutants Of Inositol Kinases And Phosphatases Hypersensitive To Valproate

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IDENTIFICATION OF DELETION MUTANTS OF INOSITOL KINASES AND PHOSPHATASES HYPERSENSITIVE TO VALPROATE

by

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THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

In partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2010

MAJOR: Biological Sciences

Approved by:

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Advisor Date

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DEDICATION

This thesis is dedicated to my parents

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Miriam Greenberg for her support, guidance and patience in this accomplishment.

I would also like to thank my committee members Dr. Lori Pile and Dr. Karan Beningo for their patience and support.

I would like to express my appreciation to all my labmates for their help and wonderful friendship.

Finally I wish to thank Adrian Vasquez for his encouragement and great companionship.

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CHAPTER ONE

INTRODUCTION

Significance

Bipolar disorder (BD) is a chronic psychiatric illness affecting at least 1% of the world population. It is characterized by fluctuations between manic and depressed mood states. With an attempted suicide rate of 25-50% (Jamison, 2000), it is the psychiatric condition most commonly associated with suicide. *The Global Burden of Disease* report documented BD as the sixth greatest cause of death or disability globally (Narrow et al., 2002). Approximately 10.3% of all costs of global biomedical illnesses are attributed to BD (Andreasen, 2001). Patients with BD generally experience high rates of relapse, chronicity, residual symptoms, cognitive and functional impairment, as well as psychosocial disability leading to damaged relationships and poor job or school performance. A number of medications are used to treat BD. However, none of the drugs prescribed to treat BD are completely effective, and the development of more effective drugs with fewer side-effects is hampered by the lack of knowledge of the therapeutic mechanism of action of these drugs and the molecular mechanism underlying BD. Therefore, to improve the treatment of BD, it is important to understand the therapeutic mechanisms of action of current drugs.

Treatments for BD

Medications known as “mood stabilizers” such as lithium, valproate (VPA) and lamotrigine are used to treat BD. Atypical antipsychotics have also been

used, but most research has focused on lithium and VPA. VPA, the subject of this research, is a widely prescribed and established anticonvulsant that was approved by the FDA in 1995 as a mood stabilizer to treat BD. It is a simple branched-chain fatty acid (2-propylpentanoic acid; 2-propylvaleric acid; di-n-propylacetic acid) that was found to be an anticonvulsant in 1962 during a screen for compounds having anti-seizure activity (as reviewed by Henry, 2003). In spite of the wide usage of VPA, many side effects have been identified. The clinical side effects of VPA include sedation, gastrointestinal symptoms, tremor and a benign increase in hepatic transaminase levels, dyspepsia, weight gain, dysphoria, fatigue, dizziness, drowsiness, hair loss, headache and nausea (reviewed by Kostrouchova et al., 2007). When used in early pregnancy, VPA causes a 3-fold increased risk of congenital anomalies such as neural tube defects resulting in spina bifida (Lammer et al., 1987; Koren and Kennedy, 1999; Koren et al., 2006). Another major drawback of VPA is that 20%–40% of patients fail to respond satisfactorily. All of these negative effects highlight a great need to develop more effective drugs to treat BD. However, the molecular mechanism of the therapeutic action of VPA in BD is still unknown. A known target of VPA is inositol metabolism (O'Donnell et al., 2000; Vaden et al., 2001; Shaltiel et al., 2004; Williams et al., 2002). Decreased inositol also results from treatment with lithium. The work in this dissertation focuses on the effects of VPA on inositol metabolism.

Biosynthesis and importance of inositol

Inositol is an essential molecule ubiquitously found in all biological systems. It is a six carbon cyclitol that exists in nine possible isomeric forms (*myo, L- chiro, D-chiro, scyllo, neo, cis, epi, allo, and muco*). Of these, *myo*-inositol is physiologically the most common and important isomer (Shi et al., 2005). Inositol biosynthesis occurs in two steps. In the first step, glucose-6-phosphate is converted to L-*myo*-inositol-3-phosphate (MIP) by 1D-*myo*-inositol-3 phosphate synthase (MIPS) (Donahue and Henry, 1981). *Myo*-inositol monophosphatase (IMPase) is the second enzyme in the pathway, which converts MIP into free *myo*-inositol (Majumder et al., 1997). In yeast, MIPS is encoded by *INO1* while *INM1* encodes IMPase. Phosphorylation of the inositol ring at one or more positions generates numerous phosphoinositides (PIs) and inositol phosphates (IPs) (Fig 1).

Inositol plays an important role in several cellular processes, including protein secretion, growth regulation, signal transduction, transcription regulation, and membrane biogenesis (Carman and Henry, 1999; Lohia et al., 1999; Fisher et al., 2002). A number of genes show altered expression in response to inositol (Nikoloff and Henry, 1991; Paultauf et al., 1992; Lopes et al., 1991; Bailis et al., 1992). These include genes that affect the processes of lipid biosynthesis, protein folding and secretion, vacuole fusion, and others (Jesch et al., 2005).

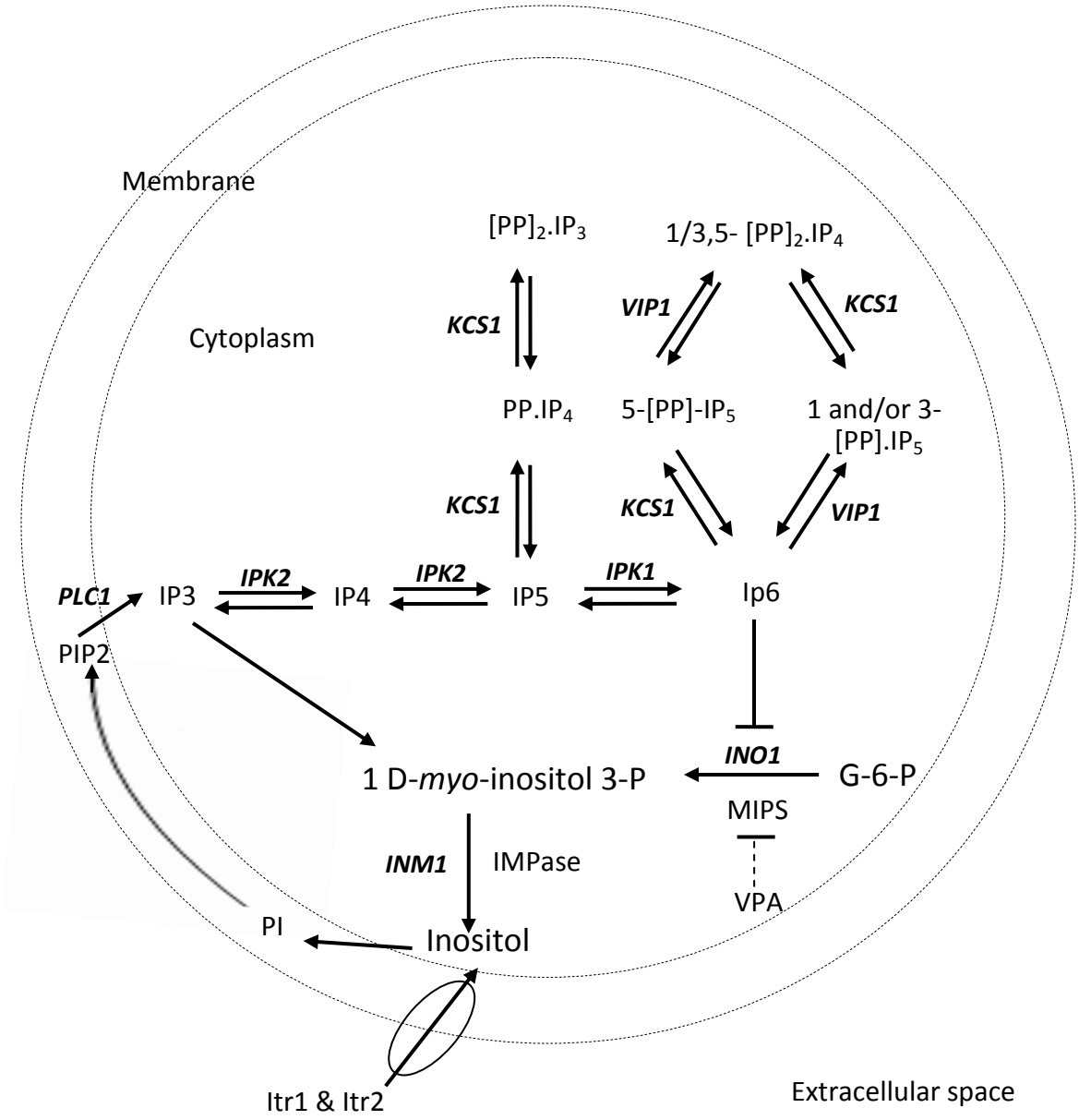

Figure 1: Inositol metabolism: Yeast obtain inositol in three ways: uptake of inositol from extracellular fluid by inositol transporters (Itr1p and Itr2p), turnover of inositol containing phospholipids, and *de novo* synthesis. Inositol biosynthesis is a two-step pathway: 1D-*myo*-inositol-3 phosphate synthase (MIPS) converts glucose-6-phosphate to inositol-3-phosphate (MIP), which is converted to free *myo*-inositol by *myo*-inositol monophosphatase (IMPase). Inositol is converted to phosphatidylinositol, then to other phosphoinositides (PIs) and inositol phosphates (IPs) in the PI cycle. VPA causes inositol depletion by indirect inhibition of MIPS. IP6 inhibits *INO1* expression. **Current Hypothesis:** Loss of *KCS1* and *VIP1* lead to inositol depletion, causing sensitivity to VPA.

Moreover, perturbation of inositol metabolism is linked to several neuropsychiatric disorders such as BD, Parkinson's disorder, and Alzheimer's disease (Silverstone et al., 2002; LaFerla, 2002). All of these factors emphasize the importance of understanding the effect of VPA on inositol metabolism to elucidate its therapeutic mechanism of action.

Inositol metabolism and VPA

Evidence shows that VPA interferes with inositol metabolism in yeast (O'Donnell et al., 2000; Shaltiel et al., 2004; Williams et al., 2002). VPA decreases *de novo* synthesis of inositol by indirectly inhibiting MIPS activity *in vivo* in yeast (Vaden et al., 2001; Ju et al., 2004). Yeast cells grown in the presence of VPA exhibited decreased intracellular inositol and an increase in the expression of the structural gene *INO1* and the regulatory gene *INO2*, consistent with inositol depletion (Vaden et al., 2001). Some evidence suggests that VPA also interferes with inositol metabolism in mammals. VPA was found to decrease activity of human MIPS expressed in yeast cells (Ju et al., 2004) and activity of MIPS in crude homogenates of human postmortem prefrontal cortex (Shaltiel et al., 2004). Further, VPA reduced brain inositol levels in a magnetic resonance spectroscopy study in rats (O'Donnel et al., 2000). Another study showed that VPA decreased growth cone collapse and increased growth cone area of rat sensory neurons in culture. These effects were eliminated by supplementation with inositol (Williams et al., 2002). All of these studies support that VPA depletes inositol levels and affects inositol metabolism.

IPs are important molecules in inositol metabolism

IP3, produced by the cleavage of phosphatidylinositol bisphosphate (PIP2) by PI-specific phospholipase C (PLC), is further phosphorylated to synthesize other IPs (Berridge and Irvine, 1989; Majerus, 1992). These IPs include inositol tetrakisphosphate (IP4), inositol pentakisphosphate (IP5), and inositol hexakisphosphate (IP6) that are generated through the subsequent action of several classes of evolutionarily conserved inositol phosphate kinases (IPKs) (Irvine and Schell, 2001; York, 2006; Communi et al., 1994; Shears, 2004). IPs have been shown to convey signals for a variety of hormones, growth factors, and neurotransmitters (Berridge, 1993; Berridge and Irvine, 1989). Among all IPs, IP3 and IP6 are the most highly studied in relation to human disease.

IP3 is synthesized by the PLC catalyzed hydrolysis of PIP2 (Strahl and Thorner, 2007). IP3 is phosphorylated to IP4 and, IP4 is further phosphorylated to IP5 by inositol polyphosphate multikinase (IPK2). IP5-kinase (IPK1) phosphorylates IP5 to generate IP6 (York et al., 1999), and IP6 is further phosphorylated to IP7 and IP8 by Kcs1p and Vip1p, hexakisphosphate kinases (IP6Ks) (Luo et al., 2003; Huh et al., 2003). IP3 plays an essential role as a second messenger in the IP3/Ca^{2+} signal transduction pathway that is responsible for modulating the activity of numerous cellular processes. IP6 is the most abundant inositol phosphate and is found in all mammalian cells. It exhibits strong antioxidant properties (Hawkins et al., 1993) and causes inhibition of Fe^{3+}-catalyzed hydroxyl-radical formation. Other functions of IP6 include inhibition of

protein phosphatases (Larsson et al., 1997) and activation of PKC (Efanov et al., 1997). Anticancer properties have been reported (Fox and Eberl, 2002; Vucenik and Shamsuddin, 2003). IP6 is also found in the yeast nucleus and modulates mRNA transport out of the nucleus (York et al., 1999).

An important effect of IP6 on inositol metabolism is the inhibition of nucleosome mobilization of the INO80 complex that positively regulates *INO1* expression (Ford et al., 2007; Shen et al., 2003). However, the effect of IP6 on *INO1* expression has not been studied. The *de novo* synthesis of inositol and IPs are characterized in the greatest detail in the yeast *Saccharomyces cerevisiae*, and very little is known about inositol metabolism in human cells (reviewed in Azab et al., 2008). Therefore, yeast is an ideal model system to carry out research to understand the effect of VPA on inositol metabolism and IPs.

Yeast as a model system

The baker's yeast *S. cerevisiae* has been used as a eukaryotic model organism to study the effect of VPA on inositol metabolism (Ju and Greenberg, 2003; Ju et al., 2004; Vaden et al., 2001). Yeast can be easily used for genetic, molecular, and biochemical approaches. The complete sequence of the yeast genome and the disruption mutants of all nonessential genes are available. Many of the genes that encode components of the PI pathway have been cloned in yeast, and regulation of inositol metabolism in yeast is well characterized at a molecular level (Greenberg and Lopes, 1996).

A high degree of conservation of function between yeast and higher eukaryotes indicates that the yeast system can be utilized to understand complex eukaryotic cellular processes. Conservation of function with respect to inositol metabolism has been demonstrated from yeast to humans, and homologs of the yeast genes *INO1* and *INM1* are present in the human genome. Human MIPS and yeast *INO1* exhibited 56% amino acid similarity, and human IMPA1 and yeast *INM1* shared 43% similarity (www.ncbi.nlm.nih.gov). In addition, the expression of human MIPS complements the inositol auxotrophy of yeast *ino1* null mutants (Ju et al., 2004). It has also been shown that human MIPS is inhibited by VPA (Ju et al., 2004; Shaltiel et al., 2004). O' Brien et al., (1990) showed that at least 40% of the genes responsible for heritable diseases in humans have counterparts in yeast. *S. cerevisiae* has been successfully used as a model for understanding pathogenic mechanisms in Huntington's disease (HD) and Parkinson's disease (PD) (Willingham et al., 2003). These studies demonstrate that yeast is a powerful system that can be utilized to study the effect of VPA on IPs and inositol depletion.

Project outline

In yeast, VPA causes inositol depletion by indirectly inhibiting MIPS encoded by *INO1* (Vaden et al., 2001; Ju et al., 2004). Factors that regulate *INO1* expression in yeast include the transcriptional activators Ino2p/ Ino4p (Koipally et al., 1996; Schwank et al., 1995), intracellular inositol levels (Heyken et al., 2005) and chromatin remodeling complexes (Shen et al., 2000). The

INO80 chromatin remodeling complex positively regulates chromatin remodeling at the yeast *INO1* promoter (Ford et al., 2007). In yeast, IP6 inhibits the activity of the INO80 complex (Shen et al., 2003), suggesting that increased IP6 may lead to decreased *INO1* expression. *KCS1* and *VIP1* convert IP6 to IP7 (Luo et al., 2003; Lin et al., 2009), and loss of *KCS1* and *VIP1* leads to increased intracellular IP6 levels (Dubois et al., 2002; Mulugu et al., 2007). Therefore, I hypothesized that perturbation of these pathways may inhibit inositol synthesis and increased VPA sensitivity.

To test the hypothesis, I screened deletion mutants of all genes reported to affect inositol metabolism for inositol auxotrophy. *kcs1*Δ was identified as an inositol auxotroph in this screen. I further characterized *kcs1*Δ with respect to inositol metabolism and VPA sensitivity. My results showed that loss of *KCS1* caused VPA sensitivity due to inositol depletion. Because *kcs1*Δ causes increased IP6 levels, I tested other mutants in the IP pathway to determine if other mutants that perturb IP6 cause inositol auxotrophy. Of the mutants tested, (*ipk1*Δ*, ipk2*Δ*, plc1*Δ and *vip1*Δ), *vip1*Δ, which increases IP6 exhibited partial inositol auxotrophy and VPA sensitivity in contrast to the other mutants, which most likely do not affect IP6 levels. In summary, the loss of *KCS1* or *VIP1* exacerbates VPA induced inositol depletion. As discussed above, about 50% of patients with BD do not respond to VPA (Dilsaver at al., 1993). The current study may be relevant to understanding the genetic factors that contribute to responsiveness to this drug.

CHAPTER TWO

MATERIALS AND METHODS

Materials

All chemicals and reagents used were reagent grade or better. Amino acids, *myo*-inositol and VPA were purchased from Sigma. Vitamin supplements were purchased from Difco. Glucose was purchased from Fisher Scientific. Agar was bought from US Biological (Swampscott, MA). Chloroform, methanol, ethanol, acid phenol (pH 4.3) and Tris-HCl were bought from Fisher. RNeasy Mini Kit was purchased from Qiagen. Transcriptor first strand synthesis kit and Dna free kit were purchased from Roche and Ambion. Brilliant SYBR green QPCR and 8X optical strip tubes were purchased from Stratagene.

Growth media

Synthetic complete (SC) medium with inositol (I+SC) contained glucose (2% w/v), necessary amino acids: adenine, arginine, histidine, methionine, tryptophan (20 mg/L), lysine (200 mg/L), uracil (40 mg/L), and leucine (60 mg/L), inositol (75μM), ammonium sulphate (0.2% w/v), vitamins (Culbertson and Henry, 1975) and the components of vitamin-free yeast base: boric acid (200 μg/l), calcium chloride (80 μg/l), magnesium sulfate (0.2 g/l), potassium iodide (40 μg/l), potassium phosphate monobasic (0.4 g/l), sodium molybdate (80 μg/l), and zinc sulfate (160 μg/l), plus agar (2% w/v) for solid medium. Synthetic complete medium without inositol (I-SC) contained all of the above mentioned ingredients except inositol. Synthetic minimal (SM) medium with inositol (I+SM) contained all

the necessary ingredients as mentioned for I+SC except adenine, arginine and lysine. Synthetic minimal medium without inositol (I-SM) contained all of the components in I+SM except inositol. Complex medium (YPD) contained glucose (2% w/v), bacto-peptone (2% w/v), agarose (2%w/v), and yeast extract (1% w/v Agar (2% w/v) was added to the above mentioned medium for solid medium. Cells were grown at 30°C unless otherwise indicated.

Yeast strains

All the deletion mutants were derived from the deletion collection and were isogenic with BY4741 (*his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*) but carried the deletion of the specific gene. The list of mutants is given in Table1.

Growth conditions

To compare growth patterns, cells were initially grown in liquid YPD medium and inoculated to an A_{550} of 0.01 in SM and SC liquid media with or without inositol. To assay inositol-less death, liquid cultures were inoculated from cultures grown in the presence of 75 μM inositol for 24 h.

Table 1: Deletion mutants screened for inositol auxotrophy (as described in the *S. cerevisiae* Genomic Data base)

Group	**Deletion mutant**	**Protein encoded by the gene**	**Function of the protein** (Cited directly from SGD)
Kinases	*kcs1*Δ	IP5/IP6/IP7 kinase	Generates high energy inositol pyrophosphates required for cellular processes such as vacuolar biogenesis, stress response and telomere maintenance
	*ipk1*Δ	IP5 kinase	Required for synthesis of IP6
	*ipk2*Δ	Inositol polyphosphate multikinase	Sequentially phosphorylates IP3 to form IP5; also has diphosphoinositol polyphosphate synthase activity
	*vip1*Δ	IP6/IP7 kinase	Generates IP7 required for phosphate signaling; likely involved in cortical actin cytoskeleton function

	*ire1*Δ	Serine-threonine kinase	Mediates the unfolded protein response (UPR) by regulating Hac1p synthesis
	*tos3*Δ	Protein kinase	Involved in phosphorylation and activation of Snf1p; functionally orthologous to LKB1, a mammalian kinase associated with Peutz-Jeghers cancer-susceptibility syndrome
	*ypk1*Δ	Protein serine/threonine/tyrosine kinase	Involved in control of chromosome segregation and in regulating entry into meiosis; related to mammalian glycogen synthase kinases of the GSK-3 family
	*rck1*Δ	Protein kinase	Involved in the response to oxidative stress; identified as suppressor of *S. pombe* cell cycle checkpoint mutations

	*lsb6*Δ	Type II phosphatidylinositol 4-kinase	Binds Las17p, which is a homolog of human Wiskott-Aldrich Syndrome protein involved in actin patch assembly and actin polymerization
Phosphatases	*ddp1*Δ	Polyphosphate phosphatase	Hydrolyzes diphosphorylated IPs and diadenosine polyphosphates; has high specificity for diadenosine hexa- and pentaphosphates
	*ymr1*Δ	Phosphatidylinositol 3-phosphate (PI3P) phosphatase	Involved in various protein sorting pathways, including CVT targeting and endosome to vacuole transport

	*dpp1*Δ	Diacylglycerol pyrophosphate (DGPP) phosphatase	Zinc-regulated vacuolar membrane-associated lipid phosphatase, dephosphorylates DGPP to phosphatidate (PA) and PI, then PA to diacylglycerol; involved in lipid signaling and cell metabolism
Vacuolar proteins	*vac1*Δ	Multivalent adaptor protein	Facilitates vesicle-mediated vacuolar protein sorting by ensuring high-fidelity vesicle docking and fusion
	*vac7*Δ	Integral vacuolar membrane protein	Involved in vacuole inheritance and morphology; activates Fab1p kinase activity under basal conditions and also after hyperosmotic shock
	*avt4*Δ	Vacuolar transporter	Exports large neutral amino acids from the vacuole

	*vac14*Δ	Protein involved in regulated synthesis of PtdIns(3,5)P(2)	Involved in control of trafficking of some proteins to the vacuole lumen via the MVB, and in maintenance of vacuole size and acidity; interacts with Fig4p; activator of Fab1p
Membrane proteins	*csg2*Δ	Endoplasmic reticulum (ER)membrane protein	Required for mannosylation of inositolphosphorylceramide and for growth at high calcium concentrations
	*git1*Δ	Plasma membrane permease	Mediates uptake of glycerophosphoinositol and glycerophosphocholine as sources of the nutrients inositol and phosphate; expression and transport rate are regulated by phosphate and inositol

	*scs2*Δ	Integral ER membrane protein	Regulates phospholipid metabolism via an interaction with the FFAT motif of Opi1p, also involved in telomeric silencing
Transferases	*cpt1*Δ	Cholinephosphotransferase	Required for phosphatidylcholine biosynthesis and for inositol-dependent regulation of EPT1 transcription
	*ipt1*Δ	Inositolphosphotransferase	Involved in synthesis of mannose-(inositol-P)2-ceramide (M(IP)2C), the most abundant sphingolipid , can mutate to resistance to the antifungals syringomycin E and DmAMP1 and to K. lactis zymocin

Other mutants	*ubx6*Δ	UBX (ubiquitin regulatory X) domain-containing protein	Interacts with Cdc48p. Transcription of *UBX6* is repressed when cells are grown in media containing inositol and choline
	*mtd1*Δ	NAD-dependent 5,10-methylenetetrahydrafolate dehydrogenase	plays a catalytic role in oxidation of cytoplasmic one-carbon units; expression is regulated by Bas1p and Bas2p, repressed by adenine, and may be induced by inositol and choline
	*opi10*Δ	Over producer of inositol	Involved in phospholipid biosynthesis
	*ctr1*Δ	Choline/ethanolamine transporter	Involved in uptake of nitrogen mustard and the uptake of glycine betaine during hypersaline stress

	*hor7*Δ	Protein of unknown function	Overexpression suppresses Ca^{2+} sensitivity of mutants lacking inositol phosphorylceramide mannosyltransferases Csg1p and Csh1p; transcription is induced under hyperosmotic stress
	*tep1*Δ	Homolog of human tumor suppressor gene PTEN/MMAC1/TEP1	Has lipid phosphatase activity and is linked to the phosphatidylinositol signaling pathway; plays a role in normal sporulation

The cells were harvested by low speed centrifugation at room temperature, washed twice with inositol-free (I-) medium, and reinoculated in I+ or I-media to an A_{550} of approximately 0.1 at time zero. Cells were grown at 30^0C in a rotary shaker at 200 rpm. Cell number was calculated by microscopic counting using a hemocytometer every two hours. To determine viable cells, serial dilutions were plated on YPD and colonies were counted.

Determining hypersensitivity to VPA

Cells were grown in liquid SM and SC media with or without inositol to the logarithmic phase of growth, at which time different concentrations of VPA were added as indicated. A_{550} was measured every six hours. Cells were also plated on liquid SM and SC media with the same concentrations of inositol and VPA as used in the liquid cultures. The concentrations of VPA are given in mM.

Real-time PCR

Cells were grown to the early stationary phase in the presence or absence of VPA (0.6 mM) and were harvested by centrifugation at 3500 rpm for 10 min. Total RNA was isolated using the hot phenol extraction method (Elion and Warner, 1984). 50 µl of total RNA was passed through a Qiagen gDNA column to remove genomic DNA. The sample was then applied to a Qiagen RNeasy spin column to enrich for mRNA. cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche Biochem) according to the manufacturer's protocol. Real-time PCR reactions were performed in a 25 µl volume using Brilliant SYBR Green QPCR Master Mix (Stratagene) in 8X optical strip tubes

from Stratagene. cDNA for each sample was synthesized in duplicate and each cDNA was amplified in triplicate during the RT-PCR reaction. The primers used in real-time PCR are listed in Table 2. *ACT1* was used as the internal control and the RNA level of *INO1* was normalized to *ACT1* levels in all samples. The protocol for the RT-PCR reaction is as follows: denature at 95°C for 10 min followed by 40 cycles consisting of 60 s at 95°C and 60 s at 57°C.

Table 2: The real-time PCR primers used in this study

Gene (bp)	Primers	Sequence	Product length
INO1	Forward	5'-AGTCTGTTCTGGCCCAGTTCTTAG-3'	210
	Reverse	5'-CGGTTATGGCCACCTAACATCAAC-3'	
ACT1	Forward	5'-TCGTGCTGTCTTCCCATCTATCG-3'	218
	Reverse	5'CGAATTGAGAGTTGCCCCAGAAG-3'	

CHAPTER THREE

RESULTS

Screen of deletion mutants to identify inositol auxotrophs

Deletion mutants of genes that affect inositol metabolism (Table 1) were screened for inositol auxotrophy. Cells were plated on both YPD and SM plates in the presence and absence of inositol and incubated at 30^{o}C. Three potential inositol auxotrophs were identified, *kcs1*Δ (Fig 2-B), *vac7*Δ (Fig 2-A) and *vac14*Δ (Fig 2-C). Growth of *kcs1*Δ but not the others was restored by inositol. Therefore, *kcs1*Δ was subjected to further analysis.

Inositol auxotrophy of *kcs1*Δ

The growth of *kcs1*Δ was compared to that of the MIPS mutant *ino1*Δ. Isogenic WT, *kcs1*Δ and *ino1*Δ cells were grown in liquid SM at 30^{o}C in the presence and absence of inositol. Both *kcs1*Δ and *ino1*Δ exhibited a marked decrease in growth compared to the WT in liquid or solid I-SM medium (Fig 3 and 4). Growth was restored by supplementation of inositol. A more stringent test of inositol auxotrophy, inositol-less death, was compared in both *ino1*Δ and *kcs1*Δ. Cells of *ino1*Δ and *kcs1*Δ were grown in I+ SM medium and immediately shifted to I-SM and I+SM media.

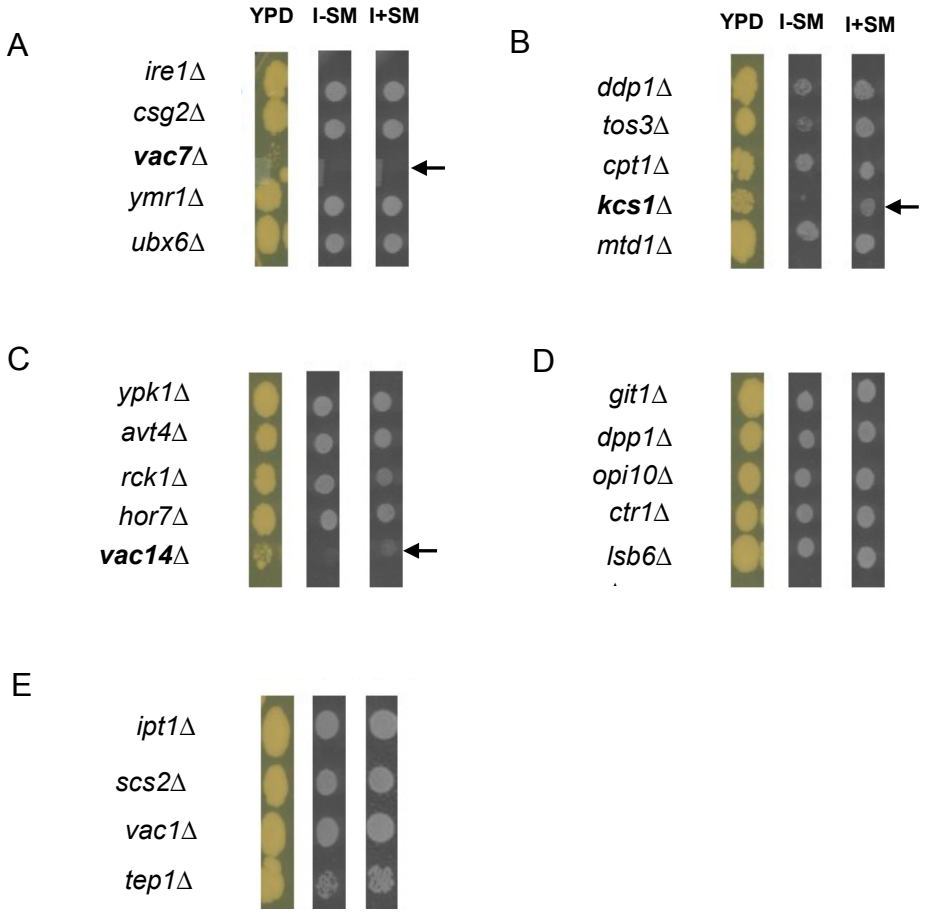

Figure 2: *kcs1Δ* is an inositol auxotroph

Wild type (WT) and deletion mutant cells were grown to the logarithmic phase in liquid YPD. Cell number was determined using a haemocytometer slide, and 10^4 cells/ml were spotted on YPD and SM plates in the presence or absence of inositol. The plates were incubated at 30^{o}C for 4 days. Arrow (←) indicates the pattern of growth of inositol auxotrophs.

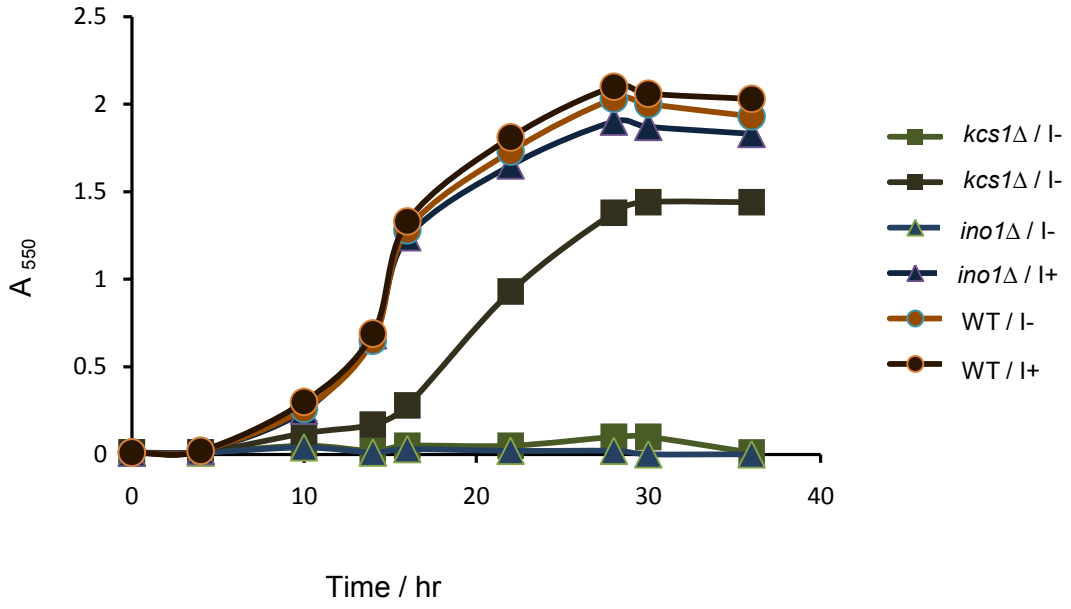

Figure 3: Decreased growth of *kcs1*Δ in the absence of inositol

WT, *kcs1*Δ *and ino1*Δ cells were grown in liquid SM at 30°C, in the presence and absence of inositol. Growth was followed by measuring A_{550}. The data are representative of two independent experiments in which duplicate samples were measured.

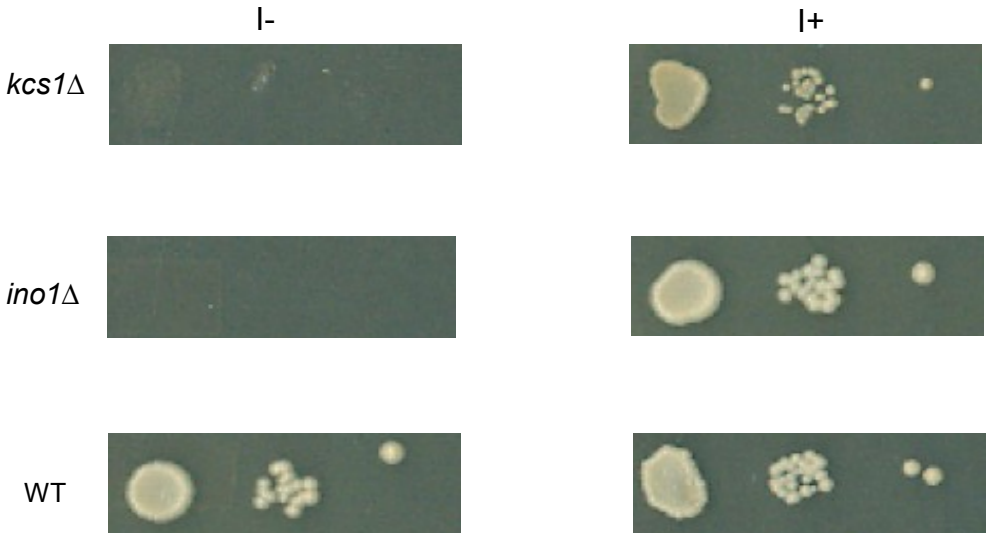

Figure 4: Decreased growth of *kcs1*Δ in the absence of inositol

WT and *kcs1Δ* cells were grown in liquid YPD for 24 hrs at 30^{o}C. Cells were washed twice and cell number was determined using a haemocytometer slide and serially diluted cells were spotted (10^3 cells/ml, 10^2cells/ml, 10^1 cells/ml) on SC plates in the presence or absence of inositol (75 μM). The plates were incubated at 30^{o}C for 4 days.

Viable cells were measured at the times indicated. As seen in Fig. 5, both mutants exhibited inositol-less death, although *kcs1*Δ was slightly less sensitive to inositol starvation compared to *ino1*Δ. Taken together, these experiments indicate that *kcs1*Δ is an inositol auxotroph.

Determination of VPA hypersensitivity of *kcs1*Δ

Because *kcs1*Δ is an inositol auxotroph, I hypothesized that the mutant is hypersensitive to VPA. To test the VPA sensitivity of *kcs1*Δ, growth of *kcs1*Δ was compared to that of WT in the presence of VPA. First, cells were plated on I+SM in the presence of 0-0.6 mM VPA. Growth was monitored for 3-4 days. As shown in Fig 6, the growth of *kcs1*Δ was more sensitive to VPA than that of WT. To further analyze the growth of *kcs1*Δ in the presence of VPA, cells were grown in liquid media to the logarithmic phase of growth, at which time VPA (range 0 - 0.6 mM) was added. The results obtained in the growth curves showed a gradual decrease in growth with increased concentrations of VPA in liquid cultures (Fig 7). It is clearly seen that the growth of *kcs1*Δ is more sensitive to VPA than that of WT at each concentration of VPA tested.

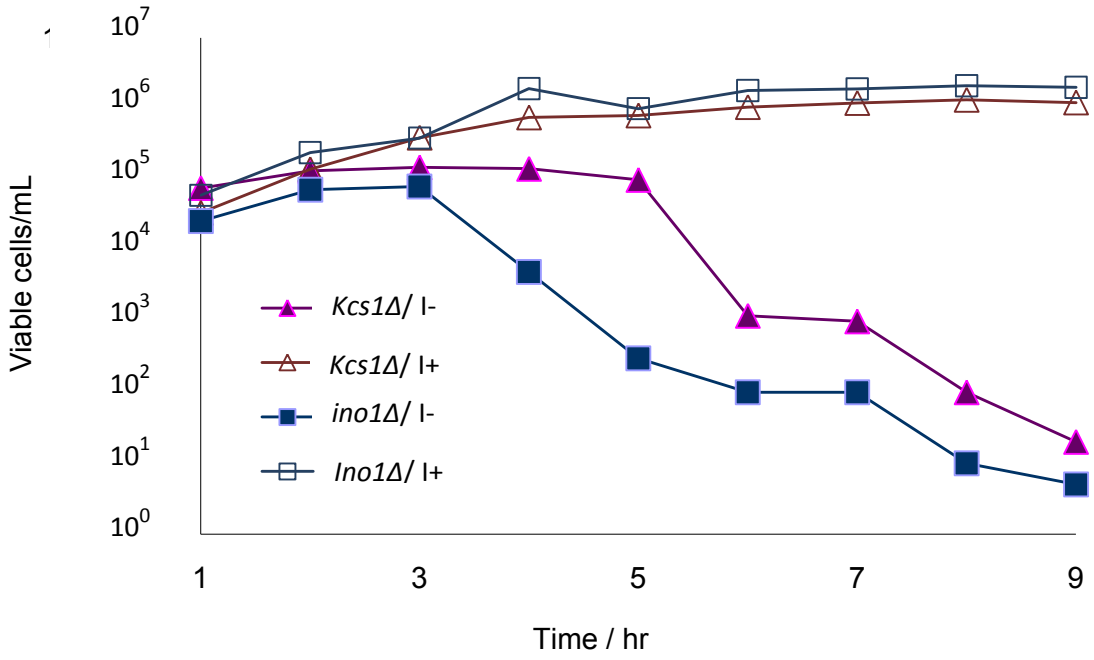

Figure 5: *kcs1*Δ undergoes inositol-less death

Cells of *ino1*Δ and *kcs1*Δ were grown overnight in I+ SM media at 30°C. Cells were harvested, washed twice with I-SM medium, and immediately shifted to I+ or I-media at an A_{550} of approximately 0.1 at time zero. Cells were harvested at the indicated times, serially diluted, plated on YPD plates, and incubated at 30°C for 9 h. The number of viable cells was determined as described in Materials and Methods.

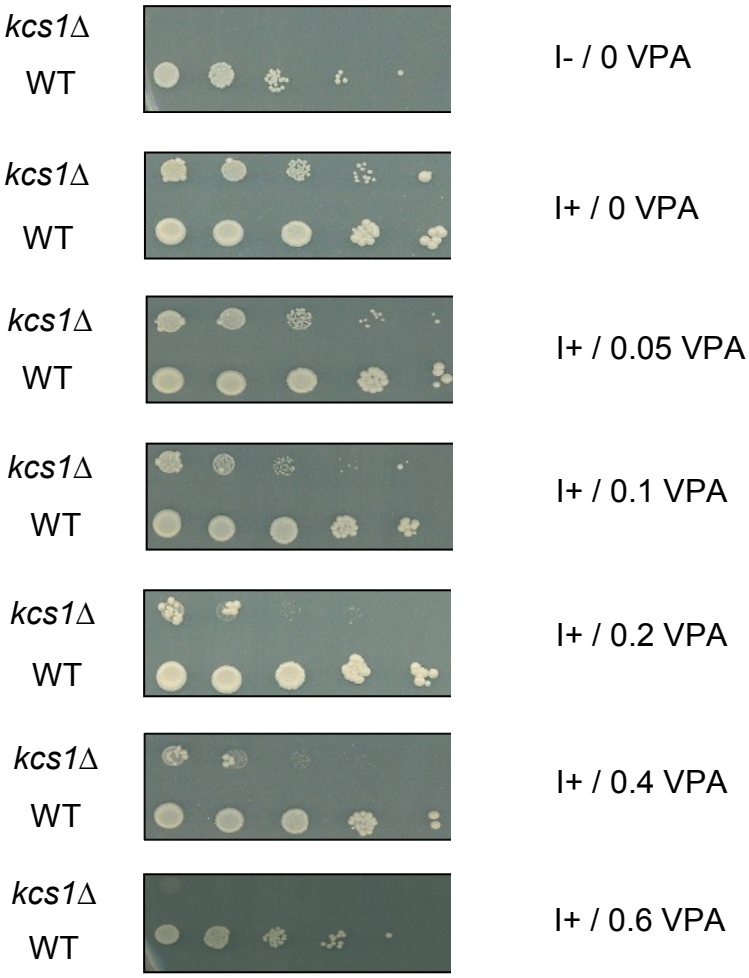

Figure 6: *kcs1Δ* is hypersensitive to VPA

WT and *kcs1Δ* cells were grown in liquid YPD for 24 hrs at 30^{o}C. Cells were washed twice, cell number was determined using a haemocytometer slide, and serially diluted cells were spotted (10^5 cells/ml 10^4 cells/ml 10^3 cells/ml, 10^2 cells/ml and 10^1 cells/ml) on SM plates in the presence or absence of inositol (75μM) and with different concentrations of VPA (mM) added as indicated. The plates were incubated at 30^{o}C for 4 days.

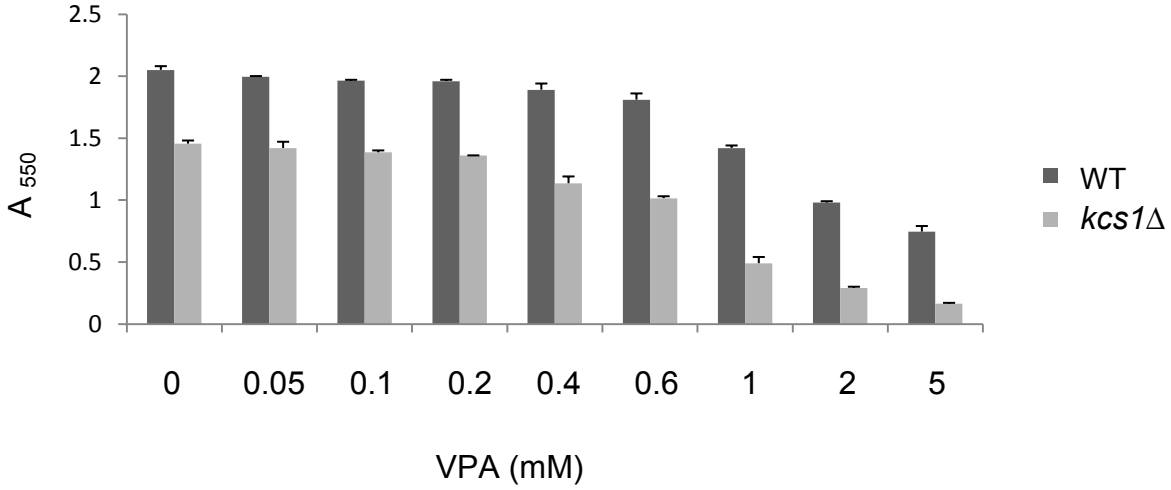

Figure 7: *kcs1Δ* is hypersensitive to VPA

WT and *kcs1*Δ cells were grown in liquid I+SM at 30°C, in the presence of the indicated concentrations of VPA. A_{550} of the cultures in the stationary phase was determined. The data are representative of two independent experiments in which duplicate samples were measured.

Quantification of *INO1* expression in *kcs1*Δ

As discussed above, *INO1* expression is positively regulated by the INO80 complex, which rearranges the nucleosomes at the *INO1* promoter (Ford et al., 2007). Shen et al (2003) showed that IP6 inhibits INO80-induced nucleosome mobilization by inhibiting its ATPase activity. As Kcs1p converts IP6 to IP7 (Luo et al., 2003), I hypothesized that loss of *KCS1* is expected to lead to increased IP6 levels and the subsequent down-regulation of *INO1* expression, which could explain the inositol auxotrophy of the *kcs1*Δ deletion mutant. To test this possibility, *INO1* expression levels were measured by real time-PCR in I+ (75μM) medium, as *kcs1*Δ does not grow in the absence of inositol. As shown in Fig 8, *INO1* expression in *kcs1*Δ showed a 5-fold decrease compared to that of WT in the stationary phase.

Interestingly, although VPA caused increased *INO1* expression in response to inositol depletion in WT (Fig 9-A), consistent with previous studies (Vaden et al., 2001), *kcs1*Δ did not show a significant increase in *INO1* expression in the presence of VPA (Fig 9-B). Therefore, the inability of *kcs1*Δ to increase *INO1* expression in response to inositol depletion might explain the sensitivity of the mutant to VPA.

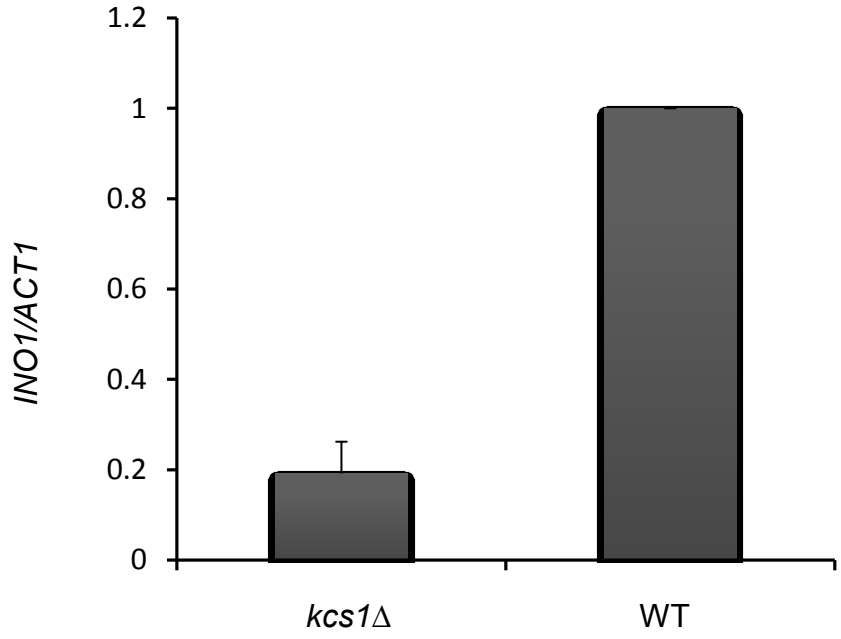

Figure 8: Loss of *KCS1* causes decreased *INO1* expression

WT and *kcs1Δ* cells were grown in I+SM medium at 30^{o}C to the stationary phase. Total RNA was extracted and *INO1* mRNA levels were measured by real-time PCR. *INO1* expression levels were normalized to the levels of the internal control gene *ACT1*. The fold change of *INO1* expression was calculated with respect to WT. Data are representative of two independent experiments in duplicate samples.

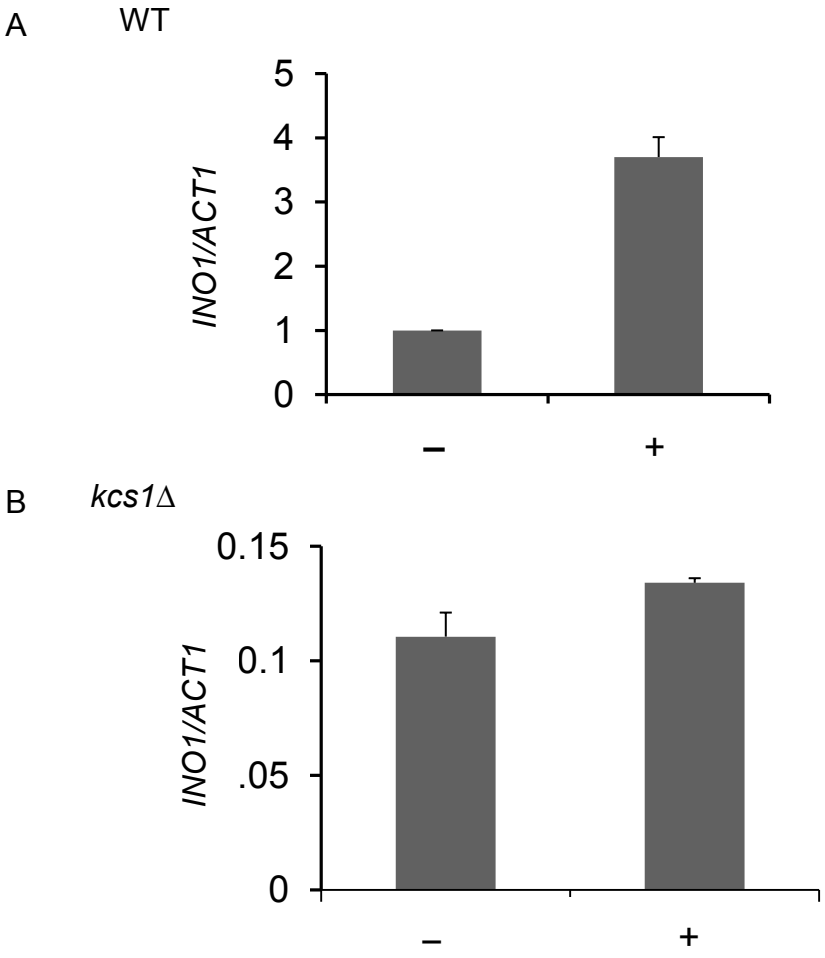

Figure 9: VPA does not increase *INO1* expression in *kcs1*Δ

WT (A) and *kcs1Δ* (B) cells were grown in I+SM medium in the presence (+) or absence (-) of 0.6 mM VPA at 30^{o}C to the stationary phase. Total RNA was extracted and *INO1* mRNA levels were measured by real-time PCR. *INO1* expression levels were normalized to the levels of the internal control gene *ACT1*. Data are representative of two independent experiments in duplicate samples.

Inositol auxotrophy of *vip1*Δ

The finding that *kcs1*Δ exhibited inositol auxotrophy suggested that perturbation of other IP kinases and /or phosphatases might also lead to inositol auxotrophy, particularly those affecting IP6 levels. Therefore, I analyzed *ipk1*Δ*, ipk2*Δ*, plc1*Δ *and vip1*Δ for this phenotype.

Of these mutants, *vip1*Δ is expected to increase IP6 levels. As shown in Fig 10-A, fewer colonies were observed in *vip1*Δ compared to WT on solid medium in the absence of inositol. The growth of *vip1*Δ was analyzed further in liquid cultures. As seen in Fig 12, growth was decreased in I-, and restored upon supplementation with inositol. Although the loss of *IPK2* did not show a significant effect on growth compared to WT in the absence of inositol on solid medium (Fig 10-B), *ipk2*Δ showed slightly less growth than that of WT in I- liquid medium (11-C). Here, the growth was restored upon supplementation with inositol. However, *ipk1*Δ and *plc1*Δ grew similar to WT in both liquid and solid medium (Fig 10-A, 11-A & B).

VPA sensitivity of *vip1Δ*

As IP6K encoded by *VIP1* converts IP6 into IP7 (York et al., 2005), the loss of *VIP1* might increase intracellular IP6 levels, causing inositol depletion and VPA sensitivity. Growth of *vip1Δ* was compared to that of WT in both liquid and solid media in the presence of VPA. Cells were plated on I- medium in the presence of different concentrations of VPA. Growth was monitored for 3-4 days.

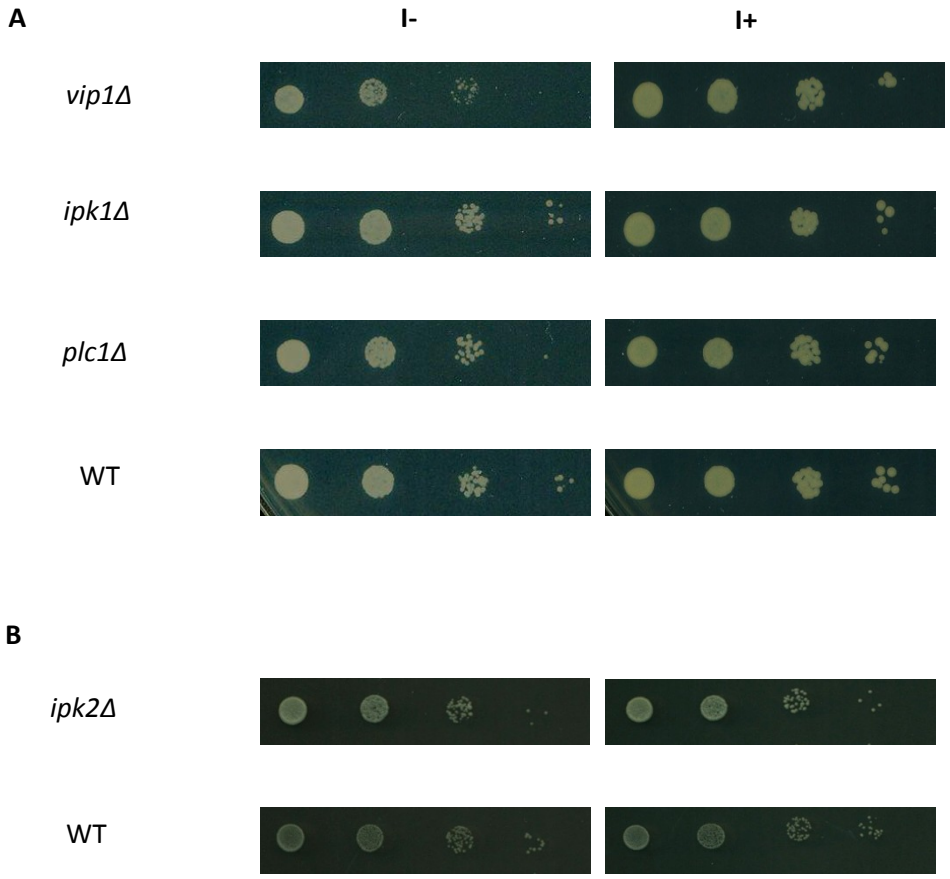

Figure 10: *vip1*Δ shows partial inositol auxotrophy

Isogenic *vip1Δ, ipk1Δ*, *plc1Δ, ipk2Δ* and WT cells were grown in liquid YPD for 24 hrs at 30^{o}C. Cells were washed twice, cell number was determined using a haemocytometer slide, and serially diluted cells (10^5 cells/ml, 10^4 cells/ml, 10^3 cells/ml, 10^2cells/ml, 10^1 cells/ml) were spotted on SM plates in the presence or absence of inositol (I) (A). The mutant *ipk2Δ* was spotted on SC plates (B), because SM medium does not support the growth of this mutant. The plates were incubated for 4 days.

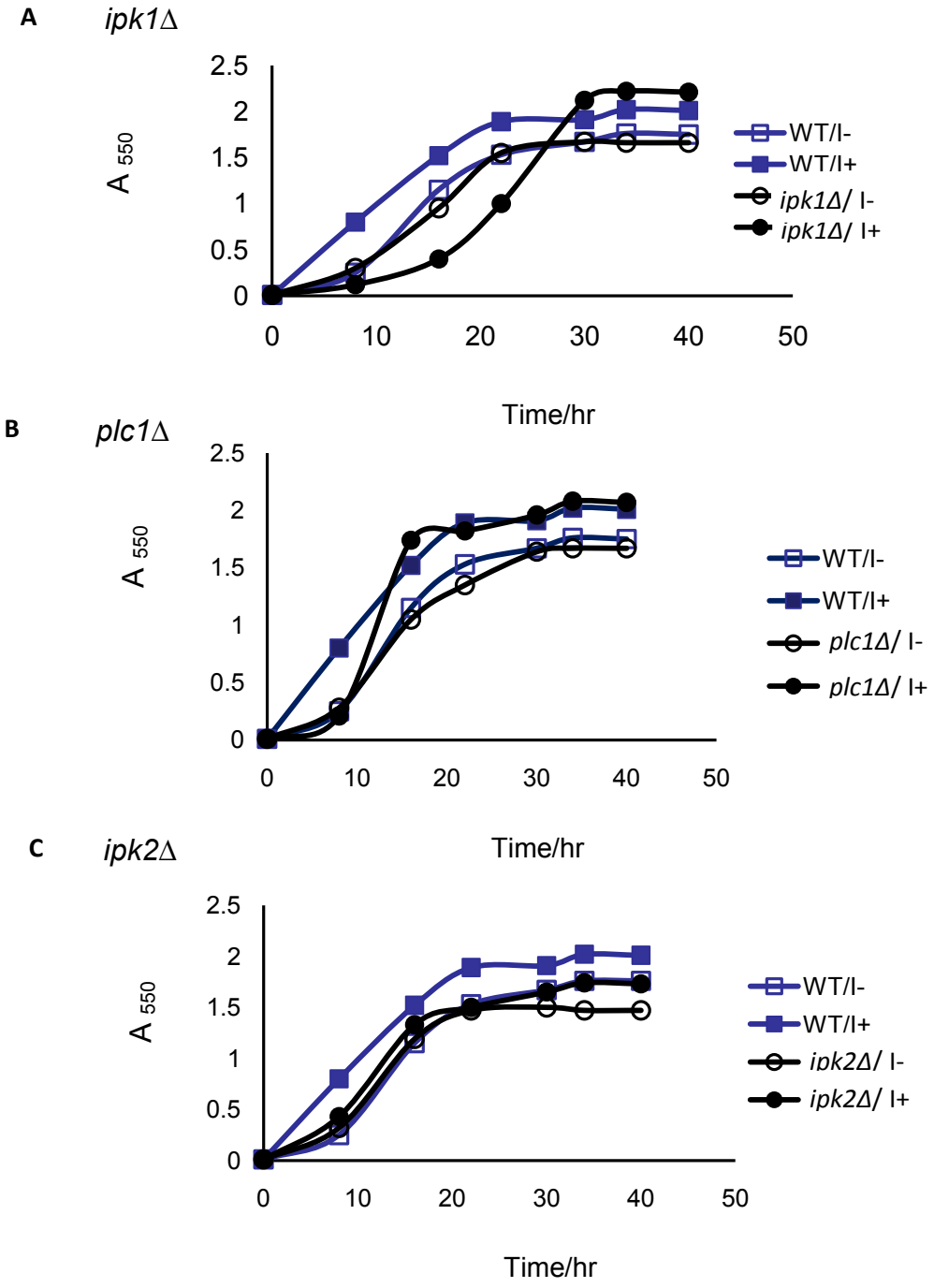

Figure 11: *ipk1Δ*, *ipk2Δ* and *plc1Δ* and are not inositol auxotrophs

Cells of *ipk1Δ* (A) and *plc1Δ* (B) were grown in liquid SM and *ipk2Δ* (C) was grown in liquid SC in the presence and absence of inositol at 30^0C. Growth was followed by measuring A_{550}.

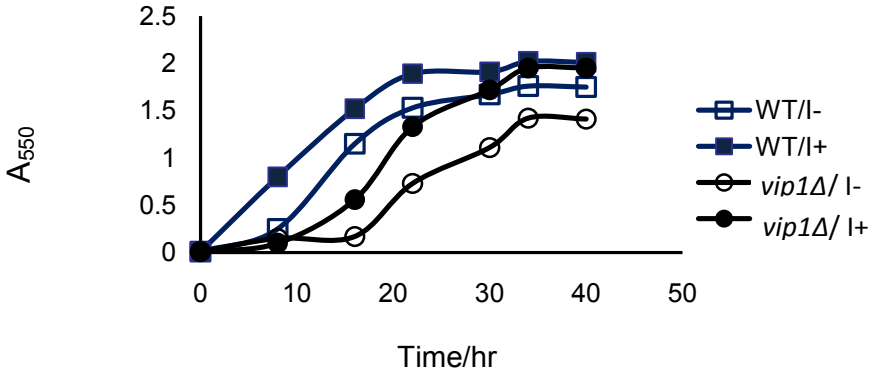

Figure 12: *vip1*△ exhibits decreased growth in I-

vip1Δ was grown in liquid SM in the presence and absence of inositol at 30^0C, and growth was followed by measuring A_{550}.

As shown in Fig 13, *vip1*Δ was hypersensitive to VPA. Growth of mutants, *ipk1*Δ*, ipk2*Δ and *plc1*Δ was similar to WT. In order to further analyze the effects of VPA on growth, cells were grown to the logarithmic phase, at which time VPA (0-0.6 mM) was added. A decrease in growth with increased concentrations of VPA was seen throughout the growth cycle of *vip1*Δ (Fig 14), while *ipk1*Δ*, ipk2*Δ*,* and *plc1*Δ grew similar to WT (Figs 14 and 15).

The loss of *VIP1* causes decreased *INO1* expression

One possible explanation for inositol auxotrophy of *vip1*Δ is decreased expression of *INO1* in the mutant. Consistent with this, *INO1* expression in *vip1*Δ was decreased 2-fold compared to that of WT in the stationary phase (Fig 16-A). In contrast, *plc1*Δ and *ipk2*Δ did not show a significant change in *INO1* expression (Fig 16-A and B). Interestingly, *ipk1*Δ exhibited a significant increase in *INO1* expression compared to WT (Fig16-A). As *IPK1* converts IP5 to IP6, loss of *IPK1* may have caused decreased IP6 levels. As Vip1p also converts IP6 to IP7 (Lin et al., 2009), it is likely that loss of *VIP1* leads to elevated levels of IP6, resulting in inhibition of the INO80 complex (Shen et al., 2003), similar to what was observed for *kcs1*Δ*.*

Figure 13

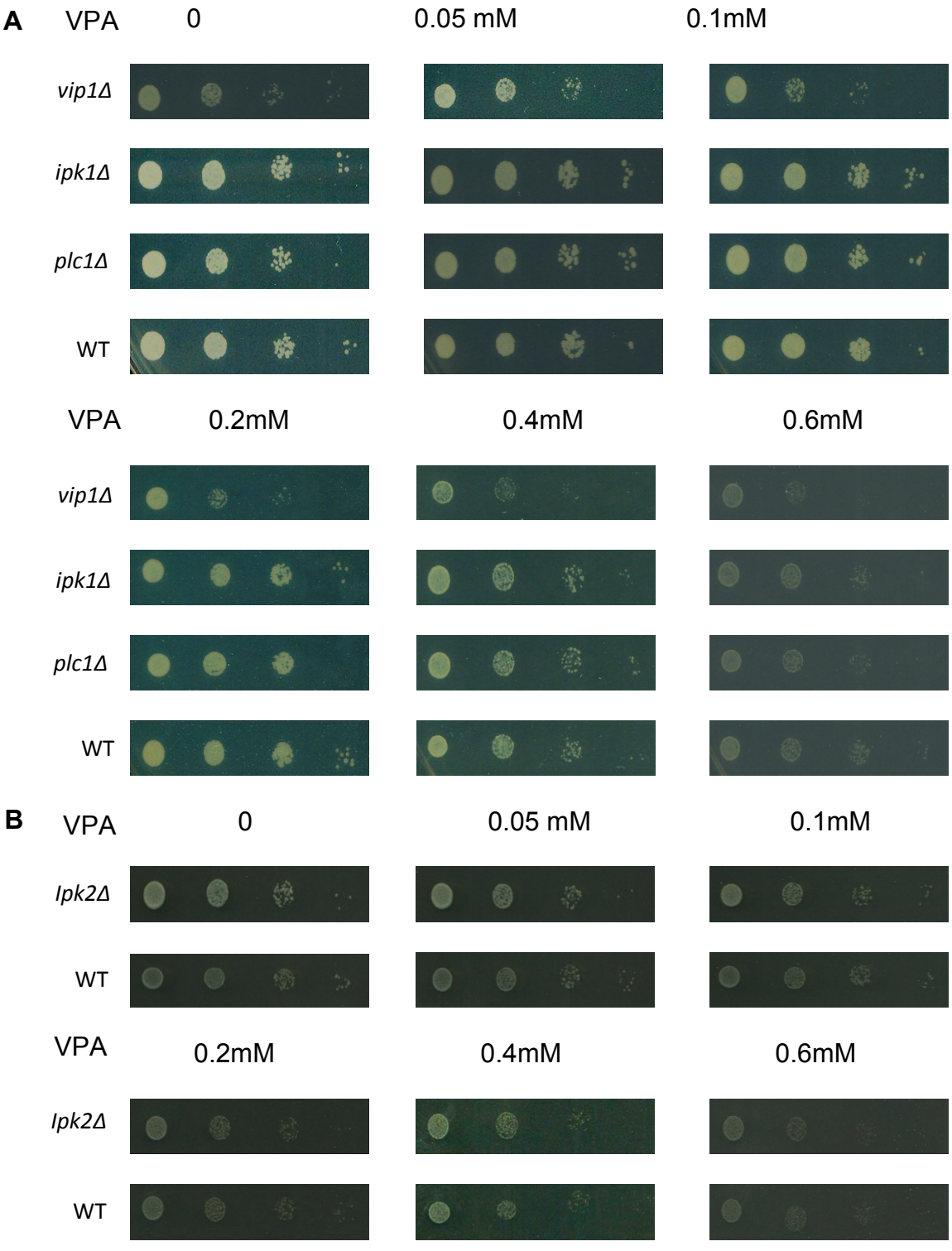
A
VPA
0
0.05 mM
0.1mM
vip1Δ
ipk1Δ
plc1Δ
WT
VPA
0.2mM
0.4mM
0.6mM
vip1Δ
ipk1Δ
plc1Δ
WT
B
VPA
0
0.05 mM
0.1mM
Ipk2Δ
WT
VPA
0.2mM
0.4mM
0.6mM
Ipk2Δ
WT

Figure 13: *vip1Δ* is hypersensitive to VPA

WT, *vip1Δ, ipk1Δ, ipk2*Δ and *plc1Δ* cells were grown in liquid YPD for 24 hrs at 30^{o}C. Cells were washed twice and cell number was determined using a haemocytometer slide. Serially diluted cells (10^5 cells/ml 10^4 cells/ml 10^3 cells/ml, 10^2 cells/ml and 10^1 cells/ml) of WT, *vip1Δ, ipk1Δ* and *plc1*Δ were spotted on SM plates (A) and *ipk2Δ* was spotted on SC plates (B) in the absence of inositol and with different concentrations of VPA as indicated. The plates were incubated for 4 days.

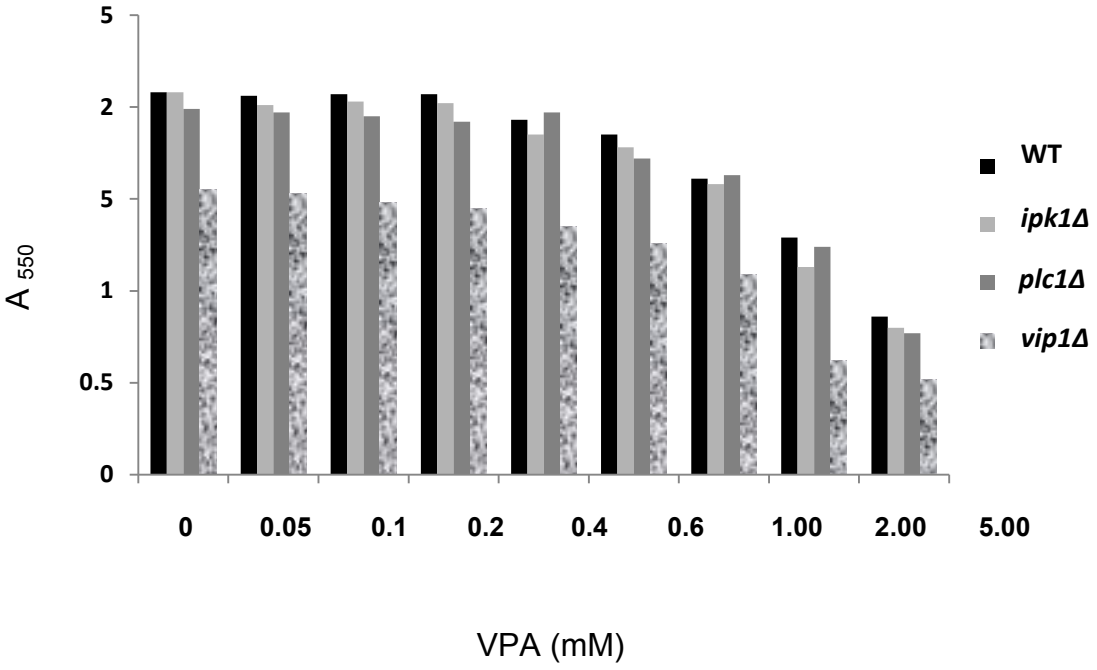

Figure 14: *vip1Δ* is sensitive to VPA

WT, *vip1Δ, ipk1Δ* and *plc1Δ* cells were grown to the stationary phase in liquid I-SM at 30°C, in the presence of the indicated concentrations of VPA. Growth was followed by measuring A_{550}.

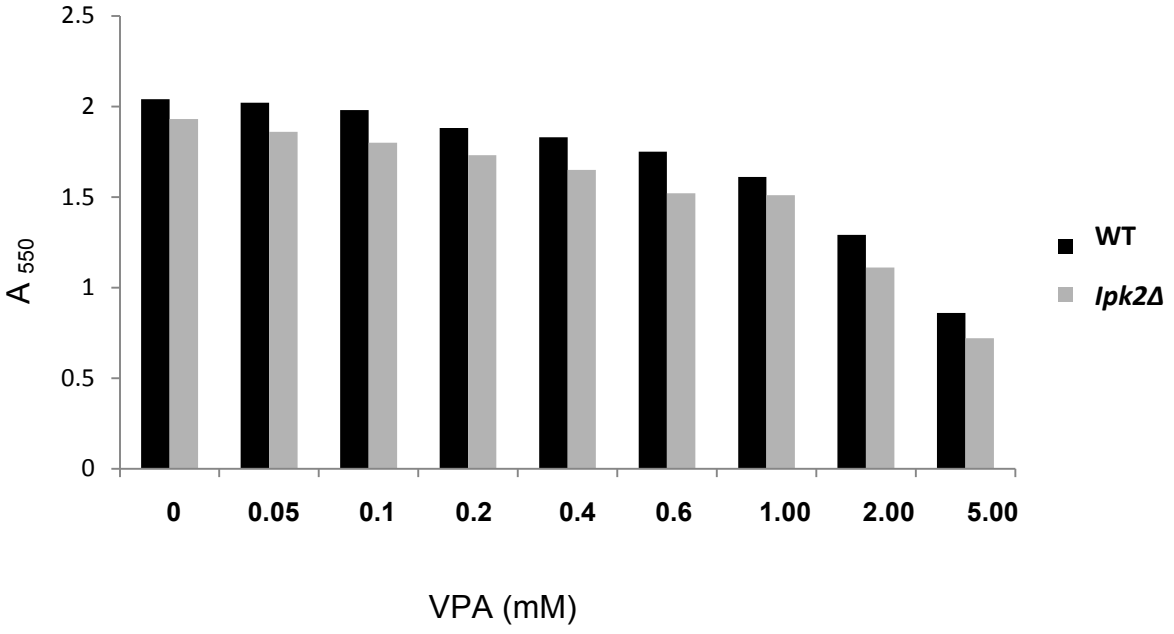

Figure 15: *ipk2Δ* is not sensitive to VPA

WT and *ipk2Δ* cells were grown to the stationary phase in liquid I-SC at 30°C, in the presence of the indicated concentrations of VPA. Growth was followed by measuring A_{550}.

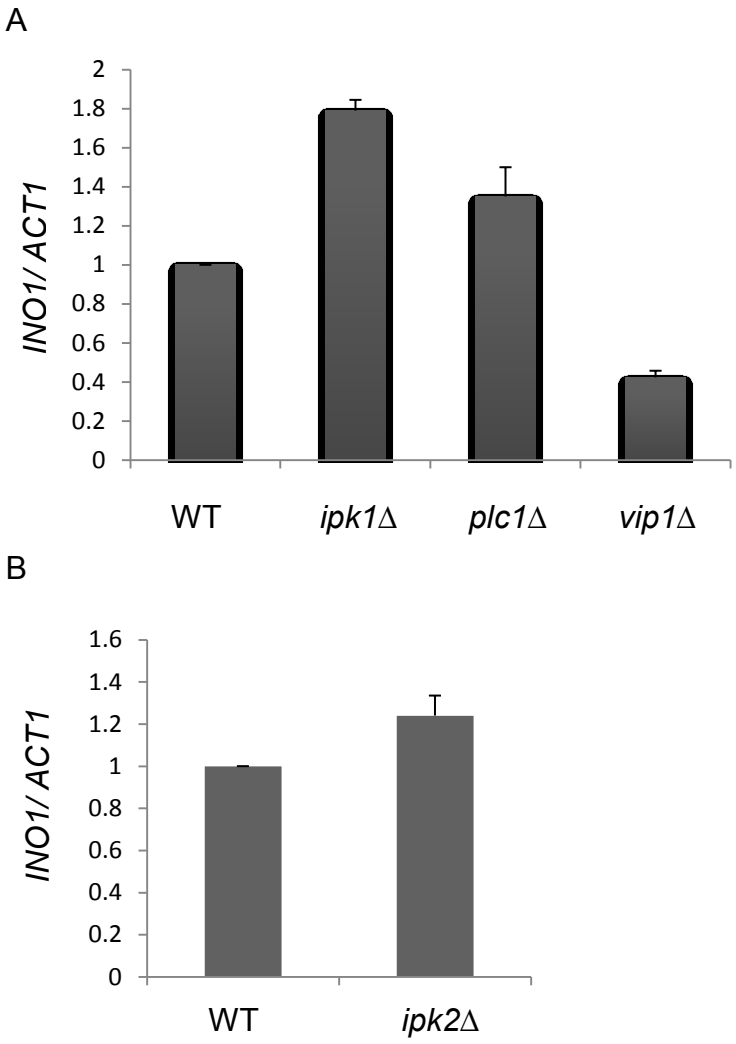

Figure 16: Loss of *VIP1* causes decreased *INO1* expression

vip1Δ, ipk1Δ, *plc1Δ,* and WT (A) were grown in I-SM medium and *ipk21Δ* (B) was grown in I-SC medium at 30^{o}C to the stationary phase. Total RNA was extracted and *INO1* mRNA levels were measured by real-time PCR. *INO1* expression levels were normalized to the levels of the internal control gene *ACT1*. The fold change of *INO1* expression was calculated with respect to WT. Data are representative of two independent experiments in duplicate samples.

CHAPTER FOUR

DISCUSSION

The major finding in this study is that deletion mutants of *KCS1* and *VIPI* genes that affect IP6 synthesis confer inositol auxotrophy and sensitivity to VPA. *KCS1* and *VIPI* are inositol kinases in the PI cycle. Kcs1p phosphorylates IP5 to IP6, IP6 to IP7, and IP7 to IP8 (Luo et al., 2003; Saiardi et al., 2000). Vip1p is also an IP6K, which phosphorylates the 4/6 positions of IP6 and IP7 while Kcs1p phosphorylates the 5 position in IP4, IP6 and IP7. (Lin et al., 2009). Loss of IP6Ks results in pleiotropic cellular defects, including aberrant DNA recombination, abnormal vacuolar morphology, altered gene expression, increased chemotaxis, osmotic stress, altered protein phosphorylation, and decreased telomere length (York 2006, Bennett et al., 2006). The results shown here indicate that *KCS1* and *VIPI* are necessary for inositol biosynthesis, and this may in part explain some of the cellular phenotypes observed in IP6K mutants.

The present study also demonstrates that both *kcs1*Δ and *vip1*Δ mutants are hypersensitive to VPA. As discussed above, our lab has shown that VPA causes inositol depletion, and that inositol supplementation rescues sensitivity to VPA. Therefore, the most likely explanation for VPA sensitivity of these mutants is that loss of *KCS1* or *VIP1* results in a decrease in inositol levels that exacerbates the VPA induced inositol depletion. A key finding in this research is that *kcs1*Δ and *vip1*Δ mutants exhibit decreased *INO1* expression, suggesting that the substrates and/or the products of *KCS1* and *VIP1* regulate *INO1*

expression. As described earlier, increased IP6 due to the loss of *KCS1* and *VIP1* might inhibit *INO1* expression by affecting the chromatin remodeling complex INO80.

In order to determine the relevance of this study to BD, it is important to consider the current knowledge concerning the human homologs of *KCS1*, *VIP1* and the INO80 complex, and how these factors affect the regulation of *INO1* expression. The human homolog of yeast *KCS1* is h-IP6K (Saiardi et al., 1999). Two human *VIP1*-like IP6 kinases have also been cloned and characterized (Fridy et al., 2007). Both of these IP6 kinases convert IP6 to IP7 (Saiardi et al., 1999; Fridy et al., 2007). The route by which animals synthesize IP6 is similar to that used by yeast. In contrast to slime moulds and plants, which synthesize IP6 by stepwise phosphorylation directly from inositol, animals incorporate the first three phosphates while the inositol is in its lipid form. That is, I(1,4,5)P3 formed by PLC catalyzed cleavage of PI-(4,5)P2 is sequentially phosphorylated to IP6. Therefore, human homologs of *KCS1* and *VIP1* may play similar roles in inositol metabolism as the counterparts yeast.

The human INO80 (hINO80) chromatin remodeling contains eight subunits orthologous to yeast, including Ino80 ATPase (Jin et al., 2005), as well as additional metazoan-specific subunits (Kobor et al., 2004; Kusch 2004). The cloning, expression, and functional activity of the hINO80 gene revealed its DNA dependent ATPase and DNA binding activity (Bakshi et al., 2006). There are no studies regarding the role of the hINO80 complex or IP6 in *h-INO1* expression.

However, if the roles of IP6 and hINO80 in inositol biosynthesis are conserved from yeast to humans, we can speculate that mutations in human homologs of *KCS1* and *VIP1* may affect patients' responsiveness and resistance to VPA.

An important unanswered question is if decreased *INO1* expression in the *kcs1*Δ and *vip1*Δ mutants leads to decreased intracellular inositol. It would also be interesting to determine if over expression of *KCS1* and *VIP1* affects sensitivity to VPA. Finally, an analysis of IP6 levels in all of the mutants of IP synthesis would help to elucidate the mechanism underlying altered *INO1* expression in mutants of IP synthesis.

In summary, my data show that *KCS1* and *VIP1* are required for optimal synthesis of inositol, and that loss of these genes leads to sensitivity to VPA. This finding may have implications for responsiveness to VPA. About half of BD patients do not respond to VPA for reasons that have not been identified (Dilsaver at al., 1993). Mutations in genes that affect inositol metabolism, such as *KCS1* and *VIP1,* may affect patients' responsiveness to VPA. Understanding the genetic factors that affect the responsiveness to VPA would contribute greatly to improving treatment efficacy for BD.

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ABSTRACT

IDENTIFICATION OF DELETION MUTANTS OF INOSITOL KINASES AND PHOSPHATASES HYPERSENSITIVE TO VALPROATE

by

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MAY 2010

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Major: Biological Sciences

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Bipolar disorder (BD) is a chronic psychiatric illness affecting at least 1% of the world population. BD is ranked as the sixth greatest cause of death or disability globally. The cause of BD is unknown. Although the anticonvulsant valproate (VPA) is widely used as a mood stabilizer to treat BD, VPA is not completely effective and causes numerous side effects. Hence, it is important to develop more effective drugs with fewer side effects to treat BD. However, drug development is hampered by the lack of knowledge of the therapeutic mechanisms of action of current drugs used to treat BD.

VPA depletes inositol in yeast and mammals. The current study was undertaken to determine whether genes affecting inositol synthesis lead to VPA sensitivity.1D-*myo*-inositol-3 phosphate synthase (MIPS) encoded by *INO1* converts glucose-6-phosphate to L-*myo*-inositol-3-phosphate (MIP) in the *de novo* synthesis of inositol. The activation of *INO1* expression is positively

regulated by the INO80 complex, which is inhibited by IP6. *KCS1* and *VIP1* are inositol hexakisphosphate kinases that convert IP6 to IP7.

I found that *kcs1*Δ is an inositol auxotroph and *vip1*Δ is a partial inositol auxotroph. Both *kcs1*Δ and *vip1*Δ mutants exhibited hypersensitivity to VPA. I also found that loss of *KCS1* and *VIP1* cause decreased *INO1* expression. The results of the study suggested that perturbation of IP synthesis exacerbates VPA induced inositol depletion. These findings have implications for understanding the mechanisms underlying responsiveness or resistance to VPA in bipolar patients.

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