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Novel Regulatory Mechanisms Of Inositol Biosynthesis In Saccharomyces Cerevisiae And Mammalian Cells, And Implications For The Mechanism Underlying Vpa-Induced Glucose 6-Phosphate Depletion

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NOVEL REGULATORY MECHANISMS OF INOSITOL BIOSYNTHESIS IN *SACCHAROMYCES CEREVISIAE* AND MAMMALIAN CELLS, AND IMPLICATIONS FOR THE MECHANISM UNDERLYING VPA-INDUCED GLUCOSE 6-PHOSPHATE DEPLETION

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DEDICATION

谨以此书献给我的妻子冯轲，女儿诗阳和我的父母

Gratefully to my wife Ke Feng, my daughter Sheyung Yu, and my parents, without whom this dissertation would never be completed.

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CHAPTER 1 INTRODUCTION

Parts of this chapter have been published in Yu, W., and Greenberg, M. L. (2016) Inositol depletion, GSK3 inhibition and bipolar disorder. *Future Neurology*. (In press)

Bipolar disorder (BD) is a severe psychiatric illness affecting about 2% of the world population. BD patients suffer from recurring cycles of mania and depression, which greatly hamper interpersonal relationships and career success. The mortality rate of BD patients is 15-20% higher than that of the general population (1). Approximately 15% of BD patients commit suicide (2). Lithium and valproic acid (VPA) are among the most widely used and best-studied mood stabilizers (3,4). However, these and other major anti-bipolar therapies cause serious side-effects and have limited efficacy (5). Thus, there is a great demand for more effective anti-bipolar drugs. Efforts to develop new treatments for BD are hampered by the lack of knowledge of the therapeutic mechanisms of the current drugs. Several hypotheses have been proposed to elucidate the mechanisms underlying the mood-stabilizing effects of the drugs. In this review, we focus on the controversies and connections characterizing two current hypotheses of the therapeutic mechanisms of lithium and VPA – inositol depletion and GSK3 inhibition – and suggest that the two mechanisms may be related.

1. Inositol depletion hypothesis

1.1. Inositol metabolism

Myo-inositol is the precursor of all inositol lipids and inositol phosphates. Eukaryotic cells obtain inositol by three routes. Inositol is taken up from the surrounding environment by inositol transporters (6,7). In the absence of exogenous inositol, it is synthesized *de novo* from glucose-6-phosphate (G6P) in a two-step reaction (Fig. 1-1). G6P is first converted to inositol-3-phosphate by *myo*-inositol-3-phosphate synthase

(MIPS), which is encoded by *ISYNA1* and *INO1* in human and yeast cells, respectively (8-11). The second step is the conversion of inositol-3-phosphate to inositol, which is catalyzed by inositol monophosphatase (IMPase) (12). Inositol is also obtained by recycling inositol phosphates (13). The levels of inositol in brain are significantly higher than in blood and other tissues (14), suggesting that high levels of inositol are critical for normal brain function. Although brain cells can take up inositol from the blood, uptake is slowed by the blood-brain barrier (15,16), suggesting that inositol *de novo* synthesis and the recycling of inositol phosphates are the main sources of inositol in brain (17).

Inositol is an essential substrate for the synthesis of phosphatidylinositol (PI), from which are derived the phosphatidylinositol phosphates. Seven known phosphatidylinositol phosphates are derived from PI, including PI(3)P, PI(4)P, PI(5)P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (18). Phosphoinositides are signaling molecules that mediate cell growth, proliferation, apoptosis, insulin action and many other cellular events (19). It is not surprising, therefore, that perturbation of phosphoinositide metabolism is associated with many disorders (18). Upon receptor mediated activation of phospholipase C (PLC), $PI(4,5)P_2$ is cleaved to form inositol-1,4,5-triphosphates (IP_3) and 1,2-diacylglycerol (DAG) (20). IP_3 can be recycled to *myo*-inositol by a series of dephosphorylations catalyzed by inositol polyphosphate phosphatase and IMPase (21). Alternatively, IP_3 can be phosphorylated sequentially to form IP_4, IP_5, and IP_6 by inositol phosphate kinases (13,21). These molecules convey signals for a variety of cellular processes, although the functions of inositol phosphates are not fully understood (22,23). Inositol phosphates can be further phosphorylated on existing phosphate groups to form pyrophosphates (24,25), whose functions are

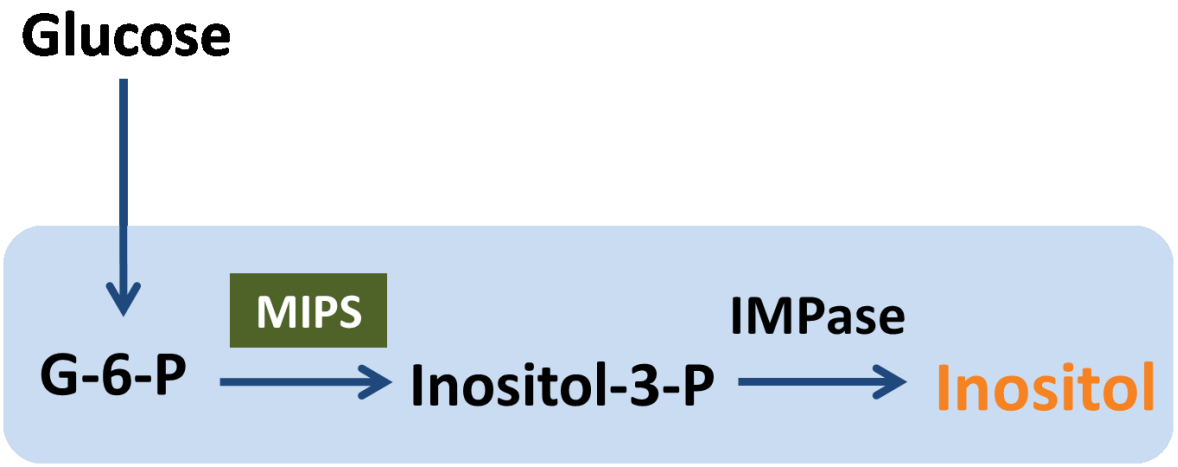

Figure 1-1. The inositol *de novo* synthesis pathway. Inositol is synthesized in a two-step reaction. Glucose 6-phosphate (G-6-P) is converted to inositol-3-phosphate (I-3-P) by *myo*-inositol-3-phosphate synthase (MIPS), which is the rate-limiting enzyme of inositol synthesis. At the second step, I-3-P is dephosphorylated by inositol monophosphatase (IMPase) to generate *myo*-inositol.

involved in the regulation of gene expression, vesicular tracking and DNA repair (26-28).

Many inositol-containing molecules function as metabolic sensors that regulate neuronal function and neurotransmission (13). For example, IP_3 is a second messenger that activates the release of calcium from cellular storage (20). Calcium signaling regulates neuronal differentiation, apoptosis, and exocytosis (29). Many receptors in the central nervous system activate PLC-dependent cleavage of PIP_2 and increase IP_3/calcium release (30). Perturbation of intracellular inositol metabolism has been associated with bipolar disorder, Alzheimer's disease, diabetes and cancer (18). Therefore, maintaining stable inositol homeostasis is critical for normal cellular function (13,31).

1.2. Altered inositol levels in BD

A correlation has been observed between BD and altered levels of inositol in brain. Altered *myo*-inositol and phosphoinositide levels have been observed in brains of living BD patients using magnetic resonance spectroscopy (32-34). Higher *myo*-inositol signals were detected in brains of BD patients during the manic phase (35). Conversely, significantly lower levels of *myo*-inositol were identified in the frontal cortex of BD patients during the depressive phase (36). Frontal cortex samples from postmortem BD patients also exhibited decreased *myo*-inositol levels (37). Furthermore, *myo*-inositol levels were reduced in cerebrospinal fluid obtained from affective depression patients (38). Interestingly, dietary supplementation of inositol (12g/day for 4 weeks in one study) led to significant efficacy for the treatment of depression (39,40). Inositol also alleviated depression in animal models (41,42). These studies suggest that abnormal brain inositol levels may play a role in mood disorders.

1.3. VPA and lithium inhibit inositol synthesis

Despite the fact that lithium has been used for more than 60 years for the treatment of BD, the therapeutic mechanism of the drug remains unknown (4). Similarly, the mechanism underlying VPA efficacy is not understood (43). Lithium was shown to be an uncompetitive inhibitor of IMPase, which catalyzes the conversion of inositol-3-phosphate to *myo*-inositol (44-46). Berridge and co-workers hypothesized that inhibition of inositol synthesis by lithium leads to decreased PI synthesis and subsequent attenuation of PI signaling (46). These pivotal studies laid the foundation for the inositol depletion hypothesis as a potential therapeutic mechanism of action of lithium. In support of the hypothesis, studies in animal models suggested that the mood-stabilizing effect of lithium is correlated with inhibition of inositol synthesis. Lithium reduced *myo*-inositol levels in rat brain (47). Inositol levels in rat cerebral cortex decreased 30% by 6 h after lithium injection, and the reduction of inositol persisted for 24 h. In addition, VPA and lithium treatment led to a reduced intracellular concentration of IP_3 (17,48,49). Lithium-induced inositol depletion resulted in reduction of PIP_3 (49,50). Inositol deficient diet augmented the effect of lithium in behavioral studies (51). These studies support the hypothesis that mood-stabilizing drugs suppress PI signaling via affecting inositol metabolism. Inositol depletion also affects other cellular functions that are associated with psychiatric illness. Inositol depletion and VPA treatment altered $PI(3,5)P_2$ homeostasis and perturbed vacuolar ATPase function in yeast; while similar studies have not yet been carried out in mammalian cells, these functions are important for neurotransmission (52). VPA and lithium prompted synapse formation between hippocampal neurons, which could be reversed by pretreatment with exogenous inositol

(53). Inositol depletion resulted in defective craniofacial development and brain function in a mouse model (54).

Understanding how inositol synthesis is regulated is of obvious importance to elucidating drug-related mechanisms of inositol depletion. Surprisingly, regulation of inositol synthesis in mammalian cells has not been well-studied. In contrast, inositol synthesis has been well characterized in the yeast *Saccharomyces cerevisiae* (55-57). In this yeast, both lithium and VPA were shown to inhibit inositol synthesis. Lithium reduces intracellular inositol levels in yeast, as in human cells, by inhibiting inositol monophosphatase (58,59). Interestingly, VPA was also shown to perturb inositol metabolism in yeast (59). VPA depletes inositol by a different mechanism from that of lithium. Vaden *et al.* first discovered that VPA causes decreased levels of intracellular inositol-3-phosphate and inositol in yeast (59), consistent with inhibition of MIPS, which catalyzes the synthesis of inositol-3-phosphate from G6P. Indeed, VPA was shown to cause a 35% decrease of MIPS enzymatic activity *in vivo* at a drug concentration used therapeutically (0.6 mM). Subsequent studies showed that VPA also inhibited human MIPS expressed in yeast cells (60). In contrast to direct inhibition of IMPase by lithium, inhibition of MIPS activity is indirect and not observed *in vitro*. Indirect inhibition of MIPS by VPA is also observed in human brain (61). Consistent with inositol starvation, chronic VPA treatment significantly decreased PI synthesis and increased CDP-DAG levels in yeast (62).

Both yeast and human MIPS are phosphoproteins, and phosphorylation of MIPS has been shown to regulate activity of both enzymes (63,64). Three phosphorylation sites were identified and mapped to Ser-184, Ser-296 and Ser-374 in yeast MIPS and

the corresponding sites Ser-177, Ser-279, and Ser-357 in human MIPS. VPA was shown to increase phosphorylation of yeast MIPS (64). The simultaneous mutation of both Ser-184 and Ser-374 to Ala resulted in a four-fold increase in MIPS enzyme activity and decreased sensitivity of cells to VPA (64). Although inhibition of MIPS by VPA is indirect, VPA directly or indirectly affects protein kinase A (PKA), AKT (also known as protein kinase B), glycogen synthase kinase-3 (GSK3), and protein kinase C (PKC) signaling pathways (65-67). Therefore, it is plausible that inhibition of MIPS by VPA may be an indirect outcome of affecting these kinases.

VPA-mediated perturbation of inositol metabolism was also reported in animal studies. VPA and lithium caused similar levels of inositol depletion in rat brain (68). Acute VPA treatment reduced inositol levels in mouse frontal cortex tissue (61). Inositol reversed the inhibitory effects of VPA and lithium on the collapse of sensory neuron growth cones and the increase in growth cone area in rat ganglia cells (17). These studies indicate that inositol depletion is a common outcome of structurally disparate anti-bipolar drugs.

1.4. Inositol depletion and PKC

PKC, a target of lithium and VPA that is associated with BD, is affected by inositol. PKC comprises a family of serine/threonine kinases that are ubiquitous in mammalian tissues (69). It is highly enriched in brain, where its activity affects numerous cellular processes, including neurotransmission, secretion, cell proliferation and localization of extracellular receptors (70). Several PKC isoforms are activated by DAG, the signaling molecule generated from the cleavage of PIP_2 (71). Numerous studies associate PKC with the pathophysiology and treatment of BD. Serotonin-

induced PKC translocation was altered in platelets obtained from BD patients during the manic phase (72). The ratio of membrane-bound to cytosolic PKC activities was shown to be elevated in BD patients and decreased after lithium treatment (72). Alteration of PKC levels and activities were also reported in a study of postmortem BD brain (73). Furthermore, a genome-wide association study identified diacylglycerol kinase η (DGKH) as a risk gene in the etiology of BD (74). DGKH catalyzes the metabolism of DAG to phosphatidic acid. Because DAG is a necessary cofactor for many isoforms of PKC, DGKH is thought to attenuate PKC (75). A study of post-mortem brain tissue demonstrated increased expression of DGKH in the prefrontal cortex of BD patients (76), suggesting that altered expression of *DGKH* is involved in the pathogenesis of BD. Taken together, these findings suggest a close association between perturbation of the PKC pathway and BD.

Interestingly, both lithium and VPA were shown to reduce PKC levels (77). Rats chronically treated with lithium exhibited a decrease in membrane-associated PKC α in hippocampus (78). The co-administration of *myo*-inositol reversed the decrease in PKC α and ε levels in hippocampus samples of rats treated with lithium (79). Chronic treatment with VPA also reduced levels of PKC α and ε in rat cells (80). Tamoxifen, a strong inhibitor of PKC widely used for the treatment of breast cancer, exhibited significant efficacy in reducing manic symptoms, although it was not effective for BD patients during the depression phase (81).

In summary, perturbation of PKC activity is closely associated with the etiology of BD. It is tempting to speculate that down-regulation of PKC by lithium and VPA induces

inositol depletion, which may exert therapeutic effects by altering downstream signaling pathways.

1.5. Discrepancies of the inositol depletion hypothesis

Some studies do not support the inositol depletion hypothesis. First, reduction of mouse brain inositol levels alone did not lead to mood stabilization (82). Mice heterozygous for the null allele of the *myo*-inositol transporter 1 (SMIT1) gene exhibited a 33-37% decrease in inositol in brain tissue, which was greater than the 22-25% decrease observed in lithium treated mouse brain. However, only lithium treated mice, but not *SMIT+/-* mice, exhibited a significant decrease in immobility in the forced swim test (82,83). A second argument against the inositol depletion hypothesis is that inositol depletion does not necessarily lead to decreased PI signaling. Phosphatidylinositol levels were compared in WT and *SMIT -/-* mouse fetal brain. Although *SMIT -/-* mice exhibited a 92% decrease in intracellular inositol, phosphatidylinositol levels were not significantly changed (84). However, it is possible that 8% of WT levels of inositol remaining in *SMIT -/-* mice may be sufficient to accommodate normal PI signaling. Third, it is not clear how inositol depletion can effectively treat both mania and depression. Studies by Cheng *et al*. suggested a mechanism whereby VPA and other mood stabilizing drugs may exert their dual action by maintaining stable PI signaling (85). Inositol depletion may attenuate PI signaling in manic patients, while VPA-induced inhibition of prolyl oligopeptidase may increase PI signaling in depressed patients (85).

In summary, while many common outcomes of anti-bipolar drugs are well explained by inositol depletion, some studies are not consistent with the hypothesis.

2. GSK3 inhibition hypothesis

2.1. GSK3 activation is inhibited by lithium

In 1996, Klein and Melton reported that lithium is an inhibitor of GSK3β in *Xenopus* (86). Subsequent studies showed that lithium inhibited GSK3β in *Drosophila,* cultured mammalian cells, and rat brain (87-93). Lithium can directly inhibit GSK3 by competing with the co-factor magnesium for binding to the enzyme (94), and/or by prompting inhibitory serine phosphorylation through multiple mechanisms, including PKA, *phosphatidylinositol-3 kinase* (PI3K)/AKT, PKC pathways, and autoregulation of GSK3 (95-98). These findings led to the hypothesis that GSK3 inhibition may be the therapeutic mechanism of mood-stabilizing drugs.

Glycogen synthase kinase-3 (GSK3) was first identified as a protein kinase that phosphorylates and inactivates glycogen synthase (99,100). As a serine/threonine kinase, GSK3 is involved in the regulation of many cellular functions that affect cell fate determination, cell survival, and signal transduction (101-103). Two isoforms of GSK3 exist in mammalian cells, GSK3α and GSK3β (104,105). These isoforms exhibit 98% identity in the amino acid sequences of their kinase domains (105). GSK3 activity is regulated by the PI3K/AKT pathway, which phosphorylates GSK3α and GSK3β on serine-21 and serine-9, respectively, inhibiting their enzymatic activities (106,107). GSK3β, the predominant form in brain, regulates more than 40 proteins in many cell signaling pathways, some of which play a role in BD as well as Alzheimer's disease and cancer (102,103,108,109).

To determine if GSK3β inhibition may account for the therapeutic effect of mood-stabilizing drugs, behavioral efficacies of reduction of intracellular GSK3β and lithium treatment were compared (110). Mice chronically treated with lithium exhibited

significantly decreased immobility in the forced swim test, a positive anti-depressive effect (111). Interestingly, mice with heterozygous deletion of *GSK3β*, in which GSK3β levels are significantly decreased, also exhibited decreased immobility time in the forced swim test, as well as other behavior changes similar to those seen in lithium treated mice (110). Behavior changes observed in lithium treated and *GSK3β +/-* mice were reversed by overexpression of GSK3β, indicating that GSK3β is a target of lithium (112). Consistent with inhibition of GSK3 as a therapeutic mechanism, mood-stabilizing effects were reported for many GSK3 inhibitors. Rodents treated with GSK3 inhibitors AR-A014418 and L803–mts exhibited reduced immobility time in the forced swim test (113-115). In addition to the anti-depressive effect, GSK3 inhibitors also reduced manic behaviors in animal studies. A variety of GSK3 inhibitors reduced hyperactivity in mouse models of mania (114,116). In addition, mice with heterozygous deletion of GSK3β also exhibited attenuated amphetamine-induced hyperactivity (116). These findings suggest that reduced GSK3 activity contributes to alleviation of manic behavior in animal models.

2.2. Inhibition of GSK3 by VPA

VPA was shown to inhibit GSK3β in some (117-120) but not all studies (121). Chen and co-workers demonstrated that VPA inhibits GSK3 in both *in vivo* and *in vitro* assays (117). The activities of GSK3α and GSK3β, assayed *in vitro* by incorporation of ^{32}P into cAMP response element-binding *protein* (CREB) phosphopeptides, decreased in the presence of VPA in a concentration dependent manner. *In vivo* assays using β-catenin degradation as an indicator of GSK3β activity showed significant increases in β-catenin levels in cytosol and nucleus of VPA-treated neuronal cells, consistent with GSK3 inhibition (117). Phosphorylation of tau protein by human GSK3β was also

decreased in the presence of VPA in a concentration dependent manner (119). Furthermore, VPA treatment inhibited phosphorylation of microtubule-associated protein 1B (Map-1B) by GSK3β *in vivo* in neuronal cells (118). However, in contrast to the finding of Chen and co-workers, VPA did not inhibit GSK3β *in vitro* in the study of Hall and co-workers, suggesting that inhibition of GSK3β by VPA may be indirect (118). The inhibitory effect of VPA on GSK3β was further supported by the finding of increased levels of GSK3β serine-9 phosphorylation in VPA-treated human neuroblastoma cells (122). In addition, similar to lithium, VPA exerted behavioral effects as a result of disrupting the AKT/GSK3 signaling pathway (123).

While the findings described above suggest that VPA inhibits GSK3β, this outcome in not universally supported. In the report of Phiel and co-workers, VPA did not affect the phosphorylation of tau protein by GSK3β in neuronal cells or the *in vitro* phosphorylation of glycogen synthase peptide-2 (121). In addition, increased β-catenin levels were observed in dorsal root ganglia cultures treated with lithium but not VPA (17). Similar results were obtained in other studies (124,125). The variety of model systems and methodologies used in these studies likely contributes to the discrepancy in results, and further investigation is necessary to understand the effect of VPA on GSK3.

2.3. HDAC inhibition and GSK3 activity

Therapeutic concentrations of VPA have been shown to inhibit histone deacetylase (HDAC) (126) (127,128). Interestingly, HDAC inhibitors exhibit anti-depressive effects in mouse models of depression (129,130). HDAC inhibition greatly affects the transcription profile and alters cellular signaling, consequences that could

potentially account for the therapeutic effects of VPA (131). For example, long-term treatment with the HDAC inhibitor Cpd-60 led to significantly increased levels of *Sgk1* expression in mouse brain (130). Up-regulation of *Sgk1* was also reported in rodents treated with lithium and other anti-depressants (132,133). *Sgk1* encodes a protein kinase that phosphorylates and inhibits GSK3β (134). As discussed previously, the activity of GSK3 is negatively regulated by the AKT signaling pathway. VPA increased the activation-associated phosphorylation of Akt and the inhibition-associated phosphorylation of GSK3β (122). Two other HDAC inhibitors, TSA and sodium butyrate, also mimicked the effect of VPA in activating Akt and inhibiting GSK3β, suggesting that HDAC inhibition promotes GSK3β inhibition via the AKT pathway (122). Although the discrepancy remains as to whether VPA directly affects GSK3 activity, as discussed above, these findings suggest that VPA indirectly inhibits GSK3β by inhibiting HDAC, which may underlie the anti-depressant effects of the drug.

2.4. Neurotrophic effects of GSK3 inhibition

Many studies have reported a loss of neuronal and glial cells in the brains of BD patients (135-138), possibly due to increased apoptosis (139). GSK3 is a pro-apoptotic enzyme, the activation of which facilitates apoptosis (140,141). Therefore, GSK3 inhibition may exert its neurotrophic and anti-bipolar effects by decreasing apoptosis of neuronal cells in the brain. The reduction of intracellular GSK3β levels was shown to protect neurons from amyloid-β induced neurotoxicity (142), while overexpression of GSK3β induced caspase-3 dependent apoptosis in mouse neuronal cells (143). Granulocyte-colony stimulating factor (G-CSF) is a neuroprotective growth factor that antagonizes apoptosis by inhibiting GSK3β (144). The connection between altered

GSK3 activity and apoptosis was further supported by the finding that several selective GSK3 inhibitors and anti-bipolar drugs, including VPA and lithium, also provided significant protection from apoptotic cell death (67,145-147). In a mouse model, VPA was shown to exert an anti-apoptotic effect by up-regulating B-cell lymphoma 2 (Bcl-2) expression (148). VPA and lithium increased intracellular Bcl-2 levels in human neuronal cells (149). VPA also inhibited apoptosis in human endothelial cells by preventing Bcl-2 ubiquitination (150). Furthermore, lithium prompted neural precursor cell proliferation via GSK-3β-NF-AT (nuclear factor of activated T cells) signaling (151). GSK3 inhibition exhibited neuroprotective effects against excitotoxicity (152). These studies suggest that VPA and lithium may exert their therapeutic effects by promoting survival and proliferation of neuronal cells as a consequence of GSK3 inhibition.

In addition to promoting anti-apoptotic signaling, inhibition of GSK3β leads to activation of the Wnt pathway and up-regulation of β-catenin (153-155). As a transcription factor, β-catenin plays an important role in regulating neuronal connectivity, which is critical for diverse neuronal functions (156). Some evidence suggests that increased intracellular β-catenin is a potential therapeutic strategy of BD treatment. L803-mts, a selective GSK3β inhibitor with anti-depressive efficacy, caused elevated β-catenin expression in mouse hippocampus (113). Overexpression of β-catenin in mouse brain and lithium treatment induced similar behavior changes, including decreased immobility time in the forced swim test (157). Furthermore, overexpression of β-catenin inhibited amphetamine-induced hyperlocomotion, mimicking the anti-manic effect of lithium. A recent study using induced pluripotent stem cell (iPSC) lines derived from BD patients indicated abnormal neurogenesis and expression of genes that are critical for

Wnt signaling (158). The proliferation defect in BD-iPSC cells was rescued by GSK3 inhibition. Together, these findings suggest that the therapeutic efficacy of GSK3β inhibition in BD may occur by upregulating β-catenin. Targeting Wnt/β-catenin signaling may be a promising strategy for BD treatment.

In summary, GSK3 may play a pivotal role in the therapeutic mechanisms of bipolar disorder therapy. Altered GSK3 activity and protein levels were observed in BD patients. Decreasing GSK3 by genetic ablation or treatment with inhibitors mimics the mood-stabilizing effect of anti-bipolar drugs in animal behavior studies. In addition, several studies reported that VPA inhibits GSK3 enzymatic activity. These findings suggest that GSK3 inhibition, similar to inositol depletion, is a common effect of structurally disparate mood-stabilizing drugs.

3. A unified model of inositol depletion and GSK3 inhibition

While inositol depletion and GSK3 inhibition may appear to be unrelated, we suggest that they may constitute components of a single mechanism. Studies have shown that GSK3 is required for optimal inositol biosynthesis in yeast (159). Yeast cells lacking *GSK3* (*gsk3⊿* cells) exhibit multiple features of inositol depletion: intracellular inositol levels in *gsk3⊿* are 70% lower than in WT cells; the growth rate of *gsk3⊿* cells in I^- medium is significantly slower than that of WT cells; and the mutant exhibits decreased MIPS enzymatic activity (159). These findings indicate that *GSK3* is required for optimal inositol homeostasis in yeast. As discussed above, MIPS activity is regulated by phosphorylation. Interestingly, a potential GSK3 phosphorylation site has been identified in yeast MIPS (Fig. 1-2). Mutation of this residue results in alteration of MIPS enzymatic activity, suggesting a potential regulatory mechanism of MIPS by GSK3

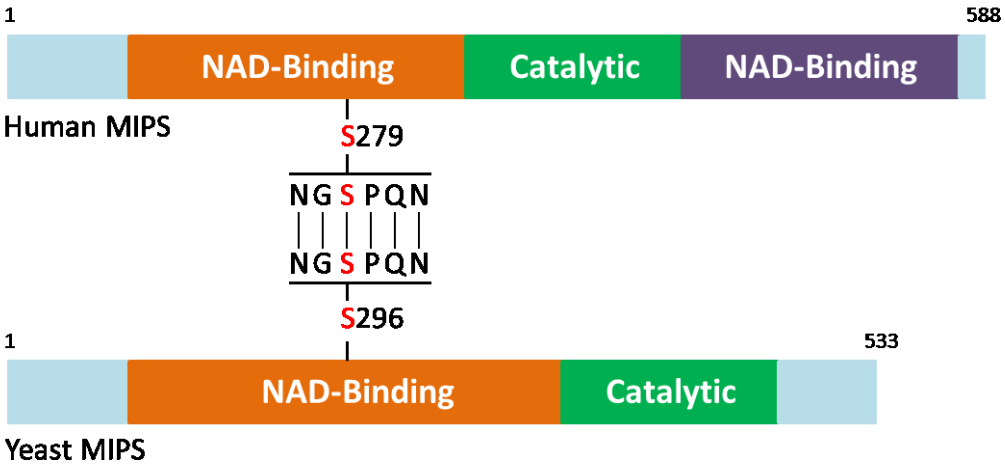

Figure 1-2. A potential GSK3 phosphorylation site in MIPS. Both human and yeast MIPS contain a potential GSK3 phosphorylation site (serine-279 of human MIPS and serine-296 of yeast MIPS) within a six-amino acid region of identity (64).

through phosphorylation (64). Strikingly, a sequence identical to the putative yeast GSK3 phosphorylation site is present in human MIPS (64). Mutation of this site in the human enzyme affected MIPS activity similar to the yeast mutant enzyme. We speculate that regulation of MIPS activity by phosphorylation of this site may be a conserved mechanism of regulation of inositol synthesis. Interestingly, inositol synthesis in neuronal cells was shown to affect GSK3 activity. Knockdown of the *ISYNA1* gene, which encodes MIPS in mammalian cells, led to inactivation of GSK3α by increasing inhibitory phosphorylation of Serine-21 (160), suggesting that GSK3 and inositol synthesis may be coordinately regulated.

GSK3 may also regulate inositol synthesis by affecting metabolism of glucose 6-phosphate (G6P), which is the substrate for inositol *de novo* synthesis. GSK3 controls the conversion of glucose to glycogen by regulating glycogen synthase activity (99). The inhibition of GSK3 in hepatic cells reduces expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, which regulate gluconeogenesis (161). In addition, expression of phosphoglucomutase 2 (PGM2), which catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate (162), requires GSK3 activity (163). Interestingly, lithium inhibits PGM2 (164), whereby it may also affect intracellular G6P production. These findings suggest that GSK3 may regulate inositol synthesis by controlling the availability of G6P. It will be of great importance to determine if VPA, lithium and GSK3 inhibition affect the rate of glucose uptake and G6P production.

The model shown in Fig. 1-3 unifies both inositol depletion and GSK3 inhibition in the following hypothesis. VPA induces inositol depletion by decreasing MIPS activity through inhibition of GSK3. As a major component of intracellular signaling molecules,

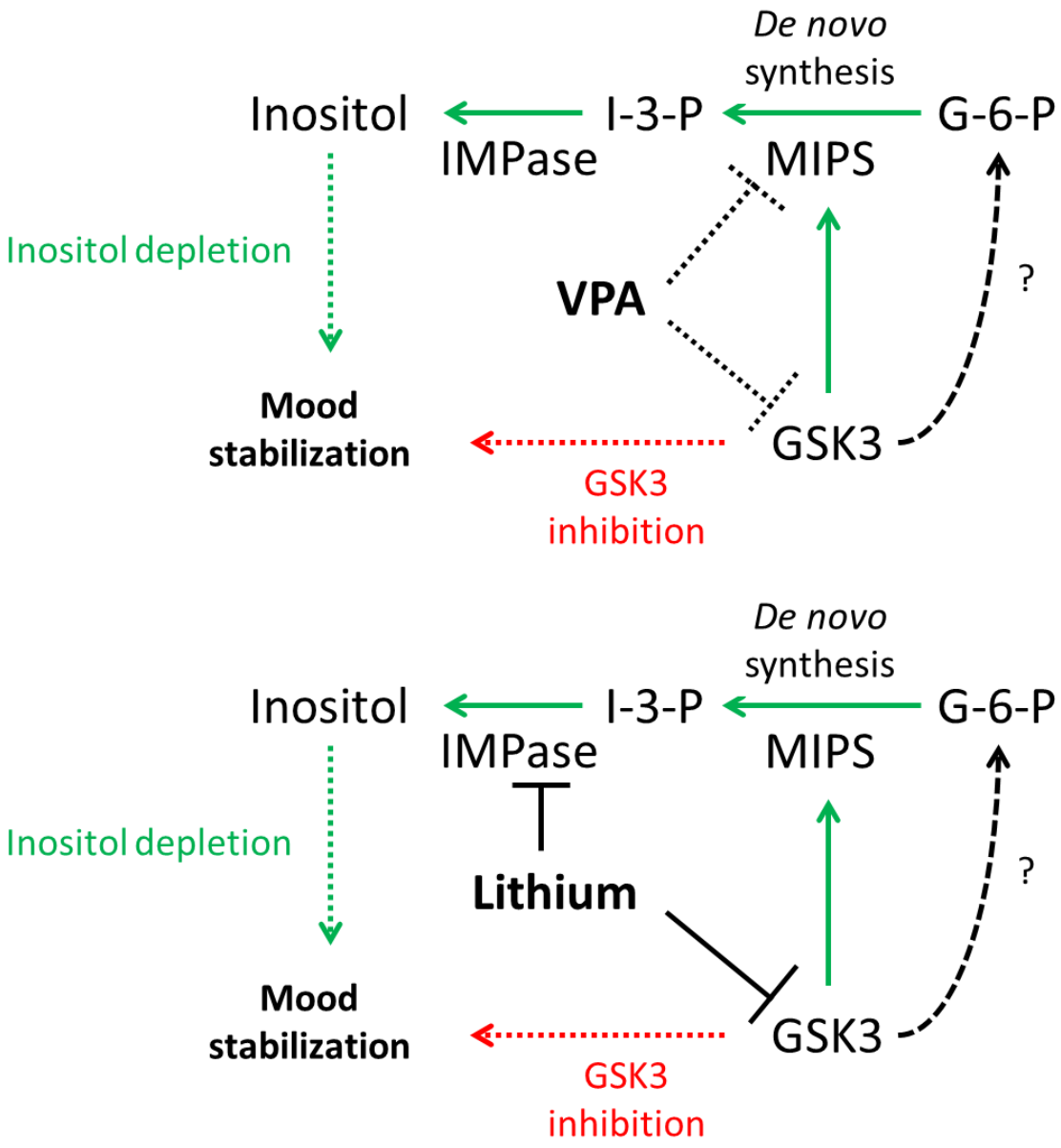

Figure 1-3. Model: Dual effects of VPA and lithium on inositol depletion and GSK3 inhibition contribute to mood stabilization. GSK3 is required for optimal *de novo* synthesis of inositol in yeast. VPA (upper panel) indirectly inhibits MIPS, the rate limiting enzyme of inositol *de novo* synthesis, possibly by inhibiting GSK3, thereby reducing intracellular inositol. Lithium (lower panel) depletes inositol by inhibiting IMPase, and inhibits GSK3 by multiple mechanisms. In addition to inhibition of MIPS activity, GSK3 inhibition may also affect metabolism of G6P, the substrate for inositol *de novo* synthesis. Inositol depletion leads to perturbation of numerous cellular functions, some of which are associated with mood stabilization. Inhibition of GSK3 affects cells in numerous ways, some of which are neurotrophic and may contribute to mood stabilization.

inositol is involved in the regulation of PI synthesis, protein secretion and many other cellular functions (18,19,165). Alteration of inositol metabolism affects expression of hundreds of genes and causes numerous cellular consequences (31,166), among which are those that may lead to mood stabilization. VPA-induced GSK3 inhibition also exerts neurotrophic effects by reducing apoptosis of neuronal cells through up-regulation of anti-apoptotic factors, and by up-regulation of β-catenin. Lithium also causes dual effects of GSK3 inhibition as well as inositol depletion by inhibition of IMPase. The inter-relationship between inositol depletion and GSK3 inhibition may contribute to the therapeutic effects of VPA and lithium.

4. Conclusion

Although the therapeutic mechanisms of VPA and lithium are not understood, both inositol depletion and GSK3 inhibition are common outcomes of treatments by these structurally dissimilar drugs and may play a role in their therapeutic effects. We speculate that VPA- and lithium-induced GSK3 inhibition may inhibit MIPS enzymatic activity by mediating the inhibitory phosphorylation of MIPS, the rate limiting enzyme of *de novo* inositol synthesis, resulting in depletion of intracellular inositol.

Project outline

The goal of the studies presented in this thesis was to elucidate new mechanisms of regulation of inositol metabolism, and to understand the physiological effects of inositol-depleting compounds. Three independent projects focusing on the regulation of inositol synthesis are described in the following chapters. The interrelationships of these projects are demonstrated in Figure 1-4.

The studies in Chapter 2 demonstrate that Mck1 is the yeast GSK3β homolog

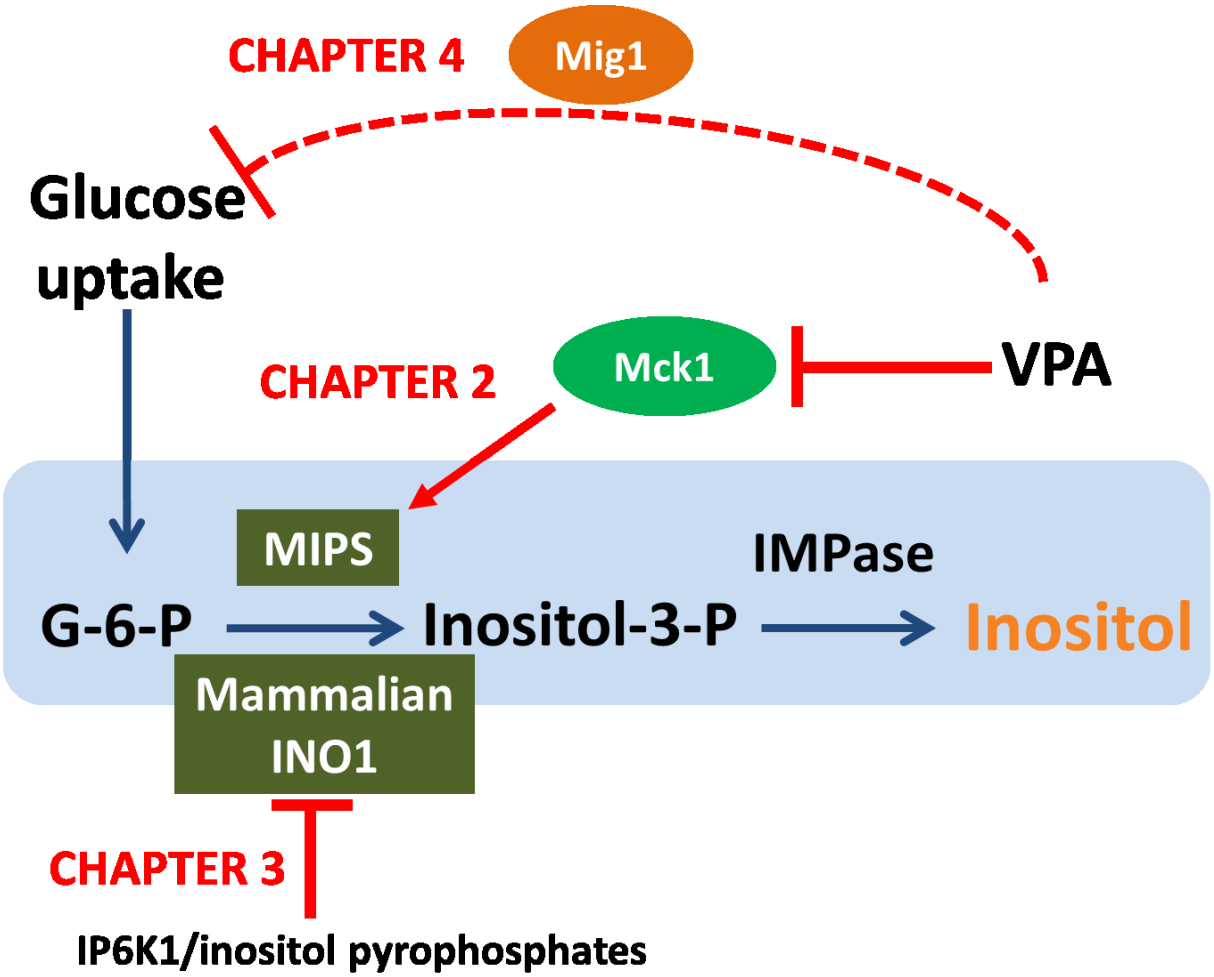

Figure 1-4. Project outline. Chapter 2 demonstrates that Mck1 regulates the rate of inositol synthesis and mediates VPA-induced MIPS inhibition. Chapter 3 characterizes IP6K1 as a novel negative regulator of inositol synthesis in mammalian cells. IP6K1 inhibits expression of the mammalian MIPS-encoding gene *Isyna1* (*mIno1*). Studies in Chapter 4 show that VPA depletes intracellular G-6-P, possibly by inhibiting expression of hexose transporter genes. VPA induces Mig1 nuclear translocation, which represses hexose transporter gene expression.

required for optimal synthesis of inositol. *mck1*Δ exhibited multiple features of inositol depletion, which could be rescued by supplementation of inositol. Intracellular inositol levels and MIPS activity were also decreased in *mck1*Δ cells. VPA requires Mck1 to inhibit MIPS enzymatic activity. Taken together, Mck1 mediates VPA-induced MIPS inhibition, which thereby leads to inositol depletion.

In Chapter 3, I identified and characterized IP6K1 as a novel regulator of inositol synthesis in mammalian cells. *Ip6k1* ablation led to profound changes in DNA methylation and expression of *mIno1*, which encodes the rate-limiting enzyme inositol-3-phosphate synthase. Interestingly, IP6K1 preferentially bound to the phospholipid phosphatidic acid, and this binding was required for IP6K1 nuclear localization and the regulation of *mINO1* transcription. This is the first demonstration of IP6K1 as a novel negative regulator of inositol synthesis in mammalian cells.

Chapter 4 describes the effect of VPA on G-6-P metabolism and expression of hexose transporter genes. This study demonstrated that chronic VPA treatment depletes intracellular G-6-P. VPA also inhibits expression of *HXT2*, *HXT3*, *HXT4*, and *HXT4*, which encode glucose transporters, possibly by activating Mig1-mediated transcription repression. These findings suggest that VPA depletes intracellular G-6-P, at least partially, by decreasing glucose uptake. Therefore, VPA-induced perturbation of G-6-P metabolism may affect downstream pathways, including inositol synthesis and glycolysis.

While this dissertation describes exciting findings on the regulation of inositol synthesis, many interesting questions remain. These questions are presented in Chapter 5 as topics for future study.

CHAPTER 2 MCK1 REGULATES THE RATE OF INOSITOL SYNTHESIS BY INCREASING *MYO*-INOSITOL-3-PHOSPHATE SYNTHASE (MIPS) ACTIVITY IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

Myo-inositol is the precursor of all inositol compounds, including phosphoinositides, inositol phosphates, inositol sphingolipids, and glycosylphosphatidylinositols. Inositol compounds are critical for many key cellular processes (20,27,167,168), and are essential for the viability of eukaryotes. The pivotal role of inositol is underscored by the link between perturbation of inositol metabolism and human neurological disorders(18). In addition, inositol depletion was proposed as the therapeutic effect of valproic acid (VPA) and lithium. Therefore, elucidating how inositol synthesis is regulated is of obvious importance to understanding cell function and the pathologies underlying many illnesses.

The *de novo* synthesis of inositol has been well characterized in the yeast *Saccharomyces cerevisiae*. Inositol is synthesized *de novo* from glucose-6-phosphate (G-6-P) in a two-step reaction. G-6-P is first converted to inositol-3-phosphate by the rate-limiting enzyme *myo*-inositol-3-phosphate synthase (MIPS), which is encoded by *INO1* (8-10,169,170). Then inositol-3-phosphate is dephosphorylated to *myo*-inositol (12). Inositol synthesis is tightly regulated at the level of transcription of *INO1* in response to extracellular inositol levels (171,172). Furthermore, both yeast and human MIPS are phosphoproteins, whose activities are regulated by phosphorylation (63,64). Yeast cells treated with VPA at concentrations used therapeutically exhibited decreased MIPS activity (59), and the inhibition of MIPS activity was indirect and not observed *in vitro* (60). VPA induced MIPS inhibition was also reported in human brain tissue (61).

VPA has also been shown to increase phosphorylation of MIPS (64). Therefore, VPA possibly inhibits MIPS via an as yet unidentified kinase.

A clue to the mechanism underlying VPA-induced MIPS inhibition came from the finding that yeast *GSK3* homologs are required for optimal *de novo* synthesis of inositol. Yeast *gsk3*Δ cells exhibited multiple features of inositol depletion, including decreased growth in I^- media, decreased intracellular inositol levels, and increased sensitivity to the inositol-depleting drug VPA (159). As a serine/threonine kinase, GSK3 is involved in the regulation of many cellular functions (101-103). Two isoforms of GSK3 exist in mammalian cells, GSK3α and GSK3β (104,105). These isoforms exhibit 98% identity in the amino acid sequences of their kinase domains (105). GSK3β, the predominant form in the brain, regulates more than 40 proteins in many cell signaling pathways, some of which play a role in BD as well as Alzheimer's disease and cancer (102,103,108,109). The inhibition of GSK3β activity contributes to alleviation of BD symptoms in animal models (110,113-116). Interestingly, a potential GSK3 phosphorylation site was identified in both yeast and human MIPS, suggesting that GSK3 regulates MIPS phosphorylation (64). Furthermore, VPA has been shown to inhibit GSK3 in several studies (117-120). Therefore, GSK3 possibly mediates VPA-induced MIPS inhibition, which leads to inositol depletion.

Four genes encode yeast *GSK3* homologs, *MCK1*, *MDS1*, *MRK1* and *YGK3* (173). The yeast *gsk3*Δ strain is a quadruple null mutant in which all four genes are knocked out. The functions of these genes are not well understood. More importantly, it is not clear which gene is involved in the regulation of yeast inositol synthesis.

In this report, we demonstrate that *MCK1* encodes the yeast *GSK3* homolog required for the optimal synthesis of inositol. *mck1*Δ cells exhibited decreased growth in the presence of VPA, which was rescued by the supplementation of inositol. The intracellular inositol levels, MIPS activity, and *in vivo* rate of inositol synthesis were decreased in *mck1*Δ cells. VPA treatment did not reduce MIPS activity in *mck1*Δ cells, indicating that Mck1 mediates VPA-induced MIPS inhibition. Interestingly, the triple-null mutant (*mds*Δ*1mrk1*Δ*ygk3*Δ) did not exhibit any feature of inositol depletion, suggesting that inositol depletion observed in *gsk3*Δ cells is due to ablation of the *MCK1* gene. This study demonstrates for the first time that the yeast *GSK3* homolog Mck1 regulates inositol synthesis by increasing MIPS activity.

MATERIALS AND METHODS

Yeast strains

All strains used in this study were in the W303 background. The WT strain was crossed with the *gsk3⊿* strain for the generation of haploid spores. The isogenic *gsk3* mutant strains used in this study were selected from haploid spores. The *GSK3* genotype of each mutant strain generated in this study was confirmed by PCR. To determine MIPS enzymatic activity, I constructed yeast strains that harbor a gene encoding His-Xpress tagged MIPS in their genomes. The *INO1* gene in each strain was first knocked out with a KanMX cassette, which was replaced by an N-terminal His-Xpress tagged *INO1* cassette cloned from pRD-*INO1* plasmid, which was generously provided by Dr. Rania Deranieh (64). The *HA-MCK1* strain, which expresses a C-terminal HA tagged Mck1, was constructed for the purpose of protein purification. The

stop codon of the *MCK1* gene was replaced by a sequence that coding for the HA tag and a KanMX cassette.

Growth media

Yeast cells were grown at 30°C or 37°C in synthetic complete (SC) medium, which contained glucose (2% w/v), adenine (20 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), methionine (20 mg/liter), tryptophan (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), threonine (300 mg/liter), ammonium sulfate (0.2% w/v), inositol-free Difco vitamin mix, vitamin-free yeast base, plus agar (2% w/v) for solid medium. Inositol (75 μM) and VPA (1 mM) were added separately as indicated.

Measurement of intracellular inositol and G-6-P levels

Intracellular inositol levels were determined using the method of Maslanski and Busa with modification (174). In brief, yeast cells were lysed in dH_2O containing 1X protease inhibitor by vortexing with acid-washed glass beads at 4°C. Cell extracts were mixed with 7.5% perchloric acid and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were collected and titrated with ice cold KOH to pH 7. Samples were clarified by centrifugation and loaded onto columns containing 1 ml AG 1-X8 resin/H_20 (1:1) mixture. Inositol was eluted with 5 ml dH_2O, eluates were dried in an oven at 70°C and stored at -80°C. Prior to assay, samples were dissolved in dH_2O. Inositol content in samples was measured as described previously (62).

To determine intracellular G-6-P levels, yeast cells were washed twice with ice-cold dH_2O and lysed in dH_2O containing 1X protease inhibitor by vortexing with acid-washed glass beads at 4°C. Cell extracts were mixed with 1 ml ice cold MeOH:$CHCl_3$ (2:1), vortexed, and stored at -20°C for 2 h. Samples were then mixed with extraction

solution (50% MeOH, 4 mM tricine pH 5.4) and centrifuged at 18,000 g for 10 min. The upper phase was collected and kept on ice. The lower chloroform phase was extracted again with extraction solution. Upper phases from both extractions were combined, dried with a speedvac, and stored at -80°C. Prior to assay, samples were dissolved in dH_2O. G-6-P content in samples was measured by the enzyme-coupled fluorescence assay developed by Zhu *et al*. with modification (175).

MIPS activity assay

Cells expressing His-Xpress tagged MIPS were grown to the early stationary phase and lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 0.6 M sorbitol, 0.3 M NaCl, 1X protease inhibitor and 1X phosphatase inhibitor) by vortexing with acid-washed glass beads at 4°C. MIPS protein was purified from cell extracts using PureProteome™ Nickel Magnetic Bead System (Millipore). Purified MIPS protein was dialyzed (1 mM Tris acetate pH 8.0, 0.05 M dithiothreitol , 0.025X protease inhibitor and 0.1X phosphatase inhibitor) and concentrated with Amicon Ultra-0.5 Centrifugal Filter system (Millipore). MIPS protein concentration was determined by Bradford assay. Enzymatic activity of 3 µg purified MIPS was determined by enzyme-coupled colorimetric assay (176).

***In vitro* phosphorylation of MIPS**

HA-Mck1 was purified from the *HA-MCK1* strain by immunoprecipitation. His-tagged MIPS was purified as described previously. 4 µg MIPS was incubated with 5 µg HA-Mck1 in reaction buffer (20 mM Tris-Cl pH7.5, 200 mM ATP, 1.5 µCi ^{32}P-ATP, 10 mM $MgCl_2$, 5 mM DTT) for 30 min in a 1.5 ml tube. 10 µM solution from the reaction tube was spotted on a piece of P81 filter paper, which was washed three times in 100

ml 0.75% phosphoric acid. MIPS phosphorylation levels were determined by autoradiography.

Measurement of the rate of inositol *de novo* synthesis *in vivo*

Cells were grown in SC I^+ medium to the mid log phase, washed twice with dH_2O, transferred to SC I^- medium, and incubated for 1 h or 3 h. [U-^{13}C]glucose was added to a final concentration of 0.2%. After 15 min, cells were harvested and lysed in dH_2O containing 1X protease inhibitor by vortexing with acid-washed glass beads at 4°C. Soluble protein in cell extract was reduced by filtration using the Amicon Ultra-0.5 Centrifugal Filter system (Millipore). ^{13}C labeled I-3-P and inositol in samples were determined by LC-MS.

Table 2-1. Strains used in this study.

Strain	Genotype	Source
W303	*MATa his3, leu2, ura3, trp1, ade2*	Andoh T. (173)
*mck1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mck1::TRP1*	This study
*mrk1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mrk1*	This study
*mds1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mds1::HIS3*	This study
*ygk3*Δ	*MATa his3, leu2, ura3, trp1, ade2, ygk3::LEU2*	This study
*gsk3*Δ	*MATa his3, leu2, ura3, trp1, ade2, mck1::TRP1, mrk1, mds1::HIS3, ygk3::LEU2*	Andoh T. (173)
WY234	*MATa his3, leu2, ura3, trp1, ade2, mrk1, mds1::HIS3, ygk3::LEU2*	This study
His-Xpress WT	*MATa his3, leu2, ura3, trp1, ade2, INO1-HIS-XPRESS*	This study
His-Xpress *mck1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mck1::TRP1, INO1-HIS-XPRESS*	This study
His-Xpress *mrk1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mrk1, INO1-HIS-XPRESS*	This study
His-Xpress *mds1*Δ	*MATα his3, leu2, ura3, trp1, ade2, mds1::HIS3, INO1-HIS-XPRESS*	This study
His-Xpress *ygk3*Δ	*MATa his3, leu2, ura3, trp1, ade2, ygk3::LEU2, INO1-HIS-XPRESS*	This study
His-Xpress *gsk3*Δ	*MATa his3, leu2, ura3, trp1, ade2, mck1::TRP1, mrk1, mds1::HIS3, ygk3::LEU2, INO1-HIS-XPRESS*	This study
His-Xpress WY234	*MATa his3, leu2, ura3, trp1, ade2, mrk1, mds1::HIS3, ygk3::LEU2, INO1-HIS-XPRESS*	This study
HA-MCK1	*MATa his3, leu2, ura3, trp1, ade2, MCK1-HA*	This study

RESULTS

mck1⊿ and *gsk3⊿* cells exhibit similar features of inositol depletion

Our lab has previously shown that *GSK3* genes are required for optimal inositol biosynthesis in yeast (159). To identify the yeast *GSK3* gene involved in the regulation of inositol synthesis, I constructed single, double and triple *gsk3* mutants that are isogenic to the *gsk3*Δ quadruple mutant. Consistent with our previous study, the growth of *gsk3⊿* cells was greatly decreased at elevated temperatures (159). Among four *gsk3* single mutants, *mck1⊿* was the only one that exhibited decreased growth at elevated temperatures (Fig. 2-1). At 37°C, *mck1⊿* cells grew slightly slower than WT, while the other three single mutants grew at a rate similar to WT. The decreased growth of *mck1⊿* was further exacerbated at 38°C. Interestingly, the *mrk1⊿mds1⊿ygk3⊿* triple mutant, which retained *MCK1* in the genome, grew slightly better than WT at 38°C. These findings suggest that *MCK1* is required for optimal growth in I^- conditions at elevated temperatures. Interestingly, the triple deletion of *MRK1*, *MDS1*, and *YGK1* genes benefited cell growth at elevated temperature in I^- conditions.

VPA inhibits the growth of inositol auxotrophic strains by depleting intracellular inositol. We determined VPA sensitivity of *gsk3* mutants. Both *mck1⊿* and *gsk3⊿* cells exhibited decreased growth in the presence of VPA, which was rescued by supplementation of inositol (Fig. 2-1). All other mutants grew as well as WT, suggesting that deletion of the *MCK1* gene accounts for VPA sensitivity.

To determine the effects on inositol metabolism of deleting each *GSK3* gene, intracellular inositol levels were measured in *gsk3* mutants. Consistent with previous

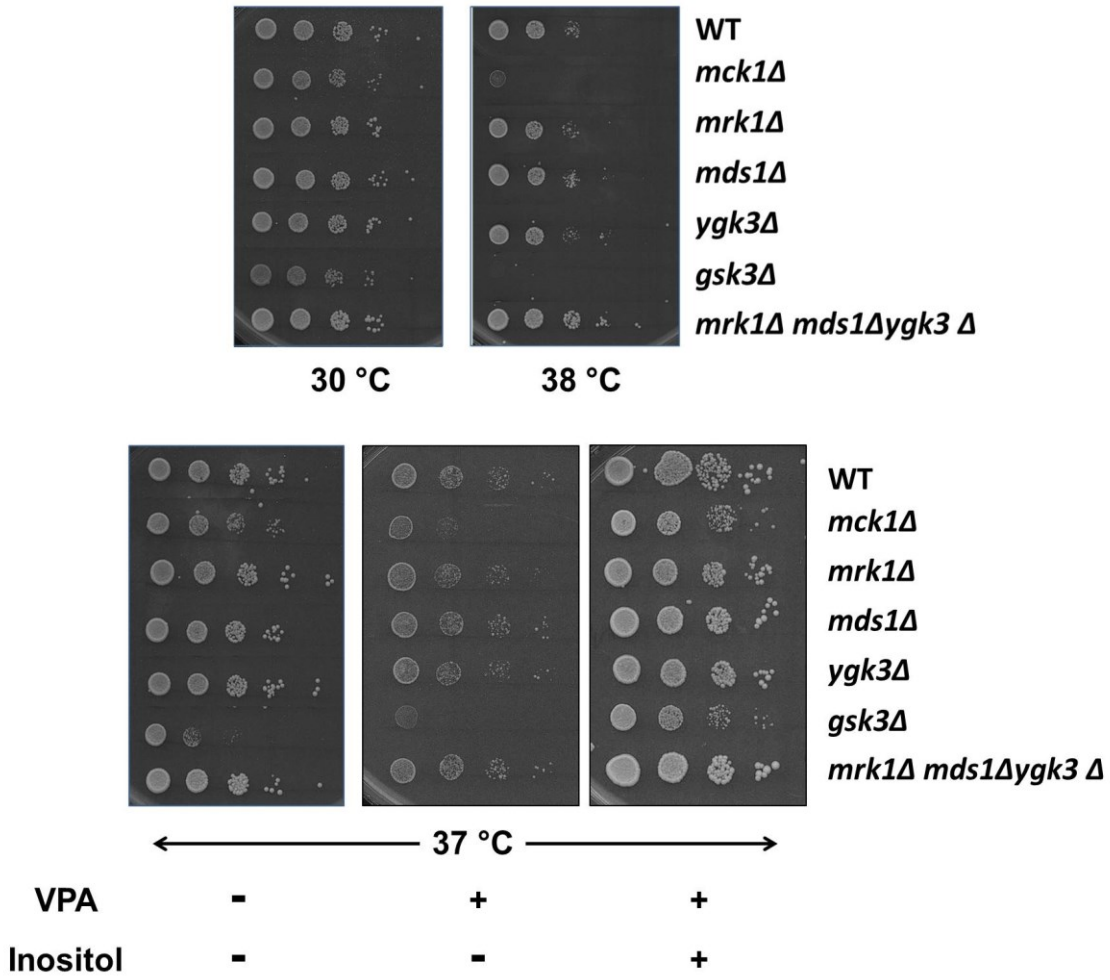

Figure 2-1. *mck1Δ* and *gsk3Δ* cells exhibit similar phenotypes. WT and *gsk3* mutant cells were serially diluted and spotted on SC plates supplemented with VPA (1 mM) and inositol (75 μM) as indicated. Plates were incubated at the indicated temperatures for 3 days. *mck1Δ* and *gsk3Δ* cells exhibit decreased growth at elevated temperatures and in the presence of VPA.

studies (159), inositol levels were significantly decreased in *gsk3Δ* cells (Fig. 2-2). The *mck1Δ* strain was the only *gsk3* mutant that exhibited decreased intracellular inositol levels compared to WT. A nearly 50% drop in intracellular inositol was observed in *mck1Δ* cells, similar to the 60% decrease seen in *gsk3Δ* cells. The other three single mutants exhibited levels of inositol similar to WT. Interestingly, inositol levels were 40% higher in the *mrk1Δmds1Δygk3Δ* triple mutant, suggesting that one or more of these genes inhibits inositol synthesis. In summary, deletion of *MCK1* causes multiple features of inositol depletion, which can account for the defects observed in *gsk3Δ* cells.

Mck1 regulates MIPS activity

We hypothesized that decreased intracellular inositol levels in *mck1Δ* cells resulted from decreased activity of MIPS, the rate-limiting enzyme of inositol synthesis. To test this, I purified MIPS from cells expressing His-Xpress tagged protein. Compared to WT, a 50% decrease in MIPS activity was observed in *mck1Δ* cells (Fig. 2-3). The other three *gsk3* single mutants and the *mrk1Δmds1Δygk3Δ* triple mutant exhibited WT MIPS activity. I further studied the effects of VPA on MIPS activity in WT, *mck1Δ* and *gsk3Δ* cells. As shown in Fig. 2-4, VPA caused more than a 40% decrease in MIPS activity in WT cells. However, VPA treatment did not significantly decrease MIPS activity in *mck1Δ* and *gsk3Δ* cells. These findings suggest that *MCK1* is the only *GSK3* gene required for normal MIPS activity, and that Mck1 mediates VPA-induced MIPS inhibition. Inositol depletion in *mck1Δ* cells possibly resulted from MIPS inhibition. However, Mck1 did not phosphorylate MIPS *in vitro* (Fig. 2-5), suggesting that Mck1 indirectly regulates

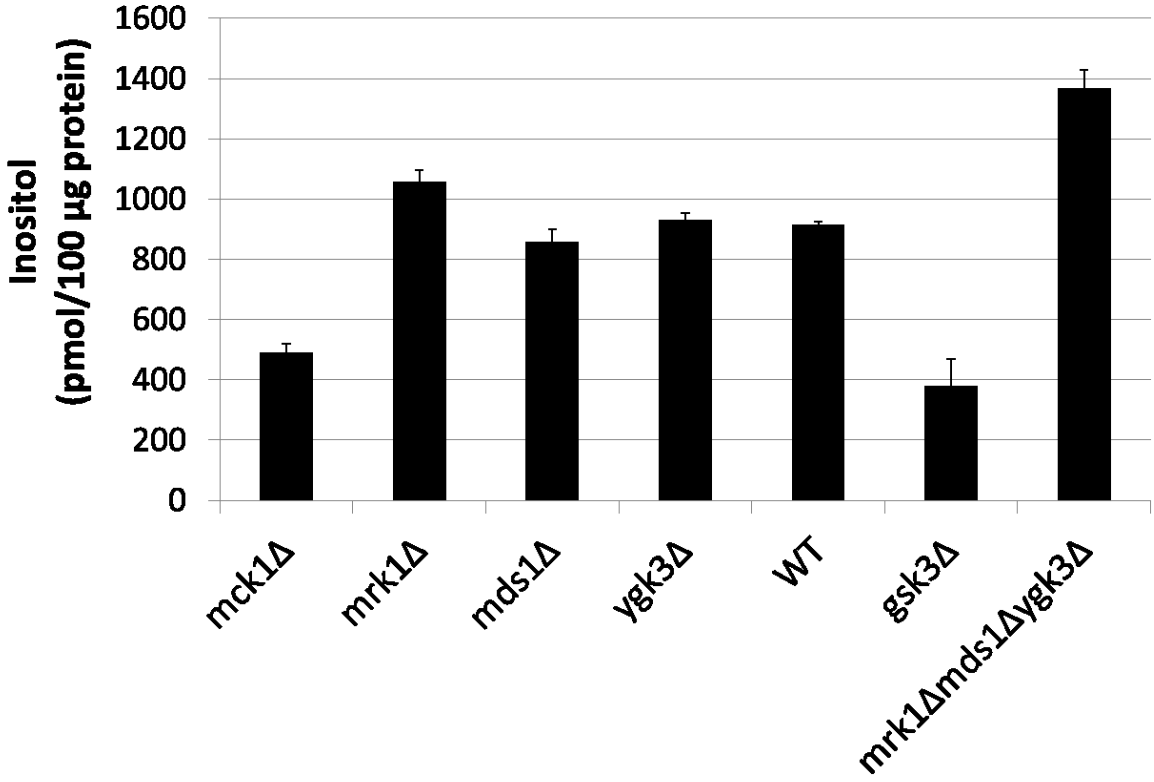

Figure 2-2. Decreased intracellular inositol levels in *mck1Δ* and *gsk3Δ* cells. Cells were grown in SC medium to the early stationary phase. Intracellular inositol levels were determined as described in "*Materials and Methods*". Values shown are mean ± SEM (n=9).

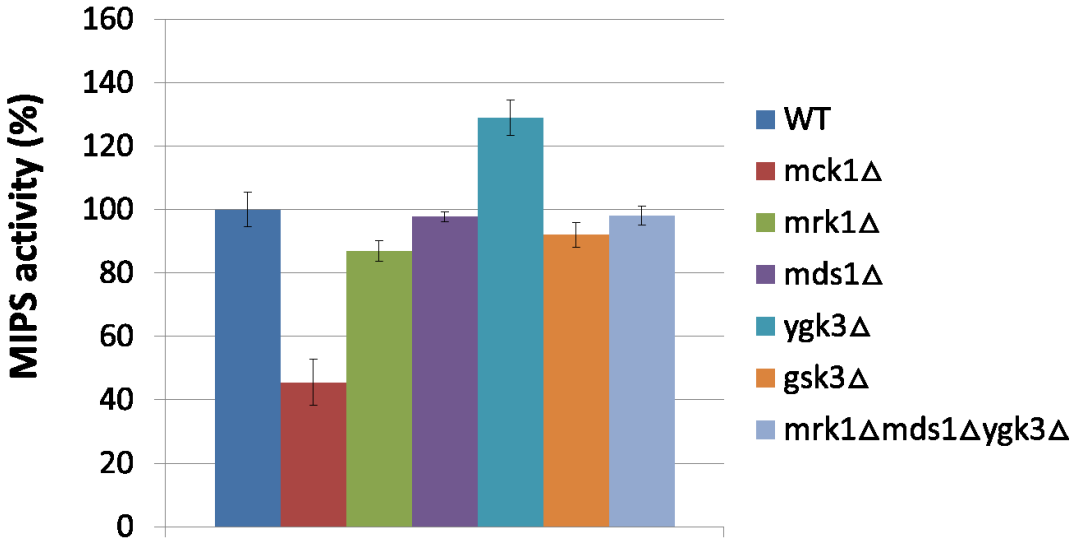

Figure 2-3. MIPS activity is decreased in *mck1Δ* cells. Cells expressing His-Xpress tagged MIPS were grown in SC medium to the early stationary phase. Enzymatic activity of MIPS protein purified from cell extracts was determined as described in "*Materials and Methods*". Values shown are mean ± SEM (n=6).

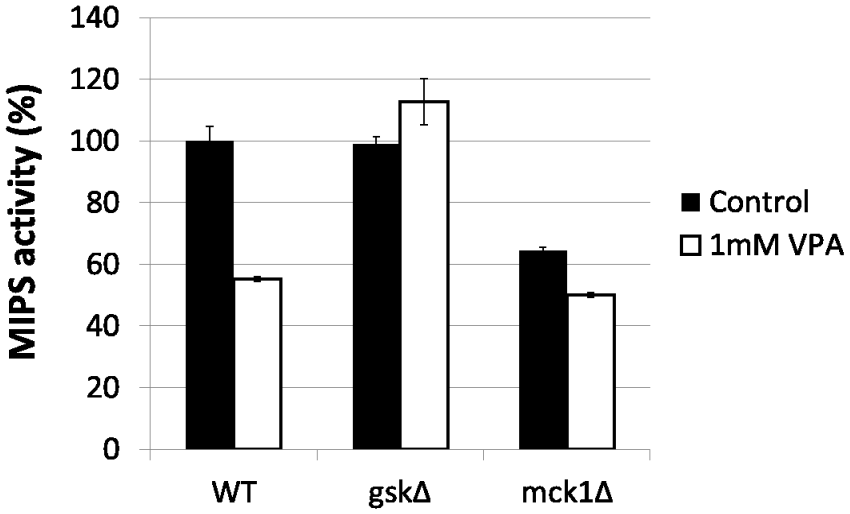

Figure 2-4. VPA does not decrease MIPS activity in *mck1Δ* and *gsk3Δ* cells.
Cells expressing His-Xpress tagged MIPS were grown in SC medium to the mid log phase and treated with 1 mM VPA for 3 h. MIPS activities were determined as described in "*Materials and Methods*". Values shown are mean ± SEM (n=6).

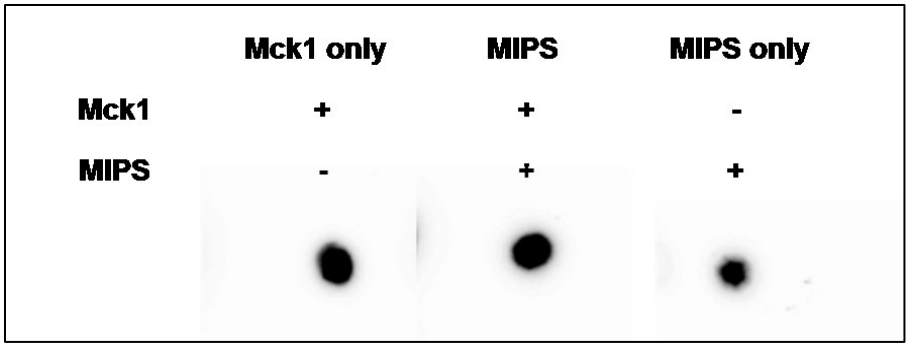

Figure 2-5. Mck1 does not phosphorylate MIPS *in vitro*. HA tagged Mck1 was purified from *HA-MCK1* cells by immunoprecipitation. His-Xpress tagged MIPS was purified as described previously. Mck1 and MIPS were incubated in reaction buffer with ^{32}P-ATP. MIPS phosphorylation was determined by autoradiography.

MIPS phosphorylation, or that Mck1 requires co-factors to target MIPS in physiological conditions. Another possibility is that the HA tag disrupts Mck1 conformation, and results in catalytic inactivity.

Surprisingly, WT and *gsk3Δ* cells exhibited similar levels of MIPS activity. However, the *in vitro* MIPS assay does not reflect the rate of inositol synthesis in yeast cells in physiological conditions. Therefore, I developed an assay to determine the rate of inositol synthesis *in vivo*.

Mck1 regulates rate of inositol *de novo* synthesis

In collaboration with Dr. Krishna Rao Maddipati, WSU Lipidomics Core director, I developed a novel method to measure the rate of inositol synthesis *in vivo*. In brief, cells grown in I^+ medium were washed, transferred to I^- medium, and grown for 1-3 h. [U-^{13}C]glucose was added to the media to allow synthesis of ^{13}C labeled inositol-3-phosphate and inositol, which were detected by LC-MS. As seen in Fig. 2-6*A*, *mck1Δ* and *gsk3Δ* cells exhibited significantly lower levels of [U-^{13}C]inositol-3-phosphate compared to WT cells after 1 h of labeling. The differences in inositol-3-phosphate levels reached a maximum at 2 h. WT, *mck1Δ* and *gsk3Δ* cells exhibited similar levels of [U-^{13}C]inositol at the 1 h time point (Fig. 2-6*B*). At 3 h, levels of [U-^{13}C]inositol were significantly decreased in *mck1Δ* and *gsk3Δ* cells. However, *mck1Δ* and WT cells exhibited similar levels of [U-^{13}C]inositol. The reason for this is not clear. These results indicate that Mck1 is required for optimal rate of inositol synthesis *in vivo*.

Mck1 possibly regulates MIPS by controlling glucose 6-phosphate availability

Mck1 may regulate MIPS activity by at least three mechanisms, including regulation of MIPS phosphorylation, MIPS protein level, and controlling the availability of

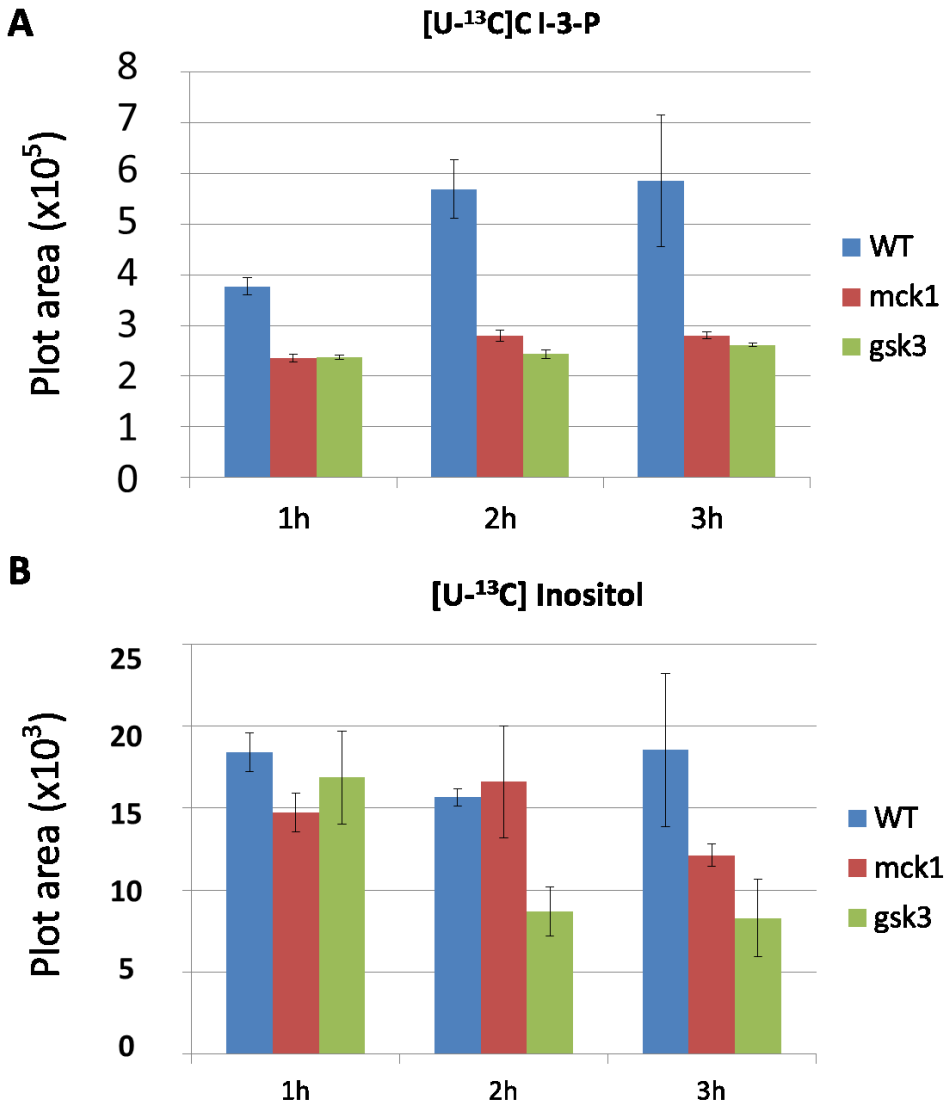

Figure 2-6. Rates of inositol-3-phosphate and inositol synthesis are decreased in *gsk3Δ* and *mck1Δ* cells. Cells were cultured in SC medium supplemented with 75 μM inositol to the mid log phase and transferred to SC I^- media for 1-3 h. [U-^{13}C]glucose was then added at a final concentration of 0.2% and cells were incubated for 15 min. Levels of ^{13}C labeled I-3-P (*A*) and inositol (*B*) in cell extracts were determined by LC-MS. Values shown are mean ± SEM (n=6).

glucose 6-phosphate (G-6-P), the substrate of MIPS. As described previously, Mck1 does not phosphorylate MIPS *in vitro*. In addition, MIPS levels are not decreased in *mck1⊿* and *gsk3⊿* cells (data not shown). To address the third possibility, we measured intracellular G-6-P levels in *mck1⊿* and *gsk3⊿* cells. Compared to WT, the intracellular G-6-P levels were 80% and 70% lower in *gsk3⊿* and *mck1⊿* cells, respectively (Fig. 2-7). In addition, intracellular G-6-P levels were 75% lower in WT cells treated with VPA (shown and discussed in Chapter 4). These results suggest that Mck1 regulates MIPS by controlling G-6-P levels, and that VPA-induced inositol depletion is possibly a combined outcome of both G-6-P depletion and MIPS inhibition.

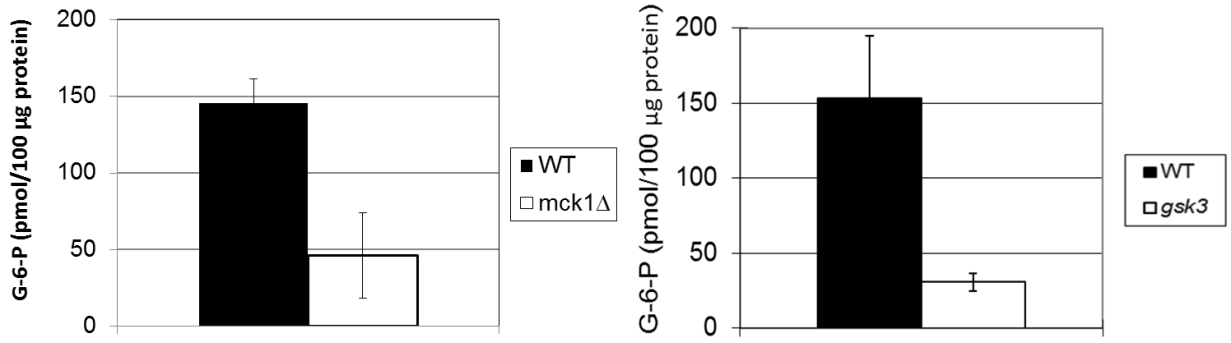

Figure 2-7. Intracellular G-6-P levels are decreased in *mck1Δ* and *gsk3Δ* cells. Cells were grown in SC medium to the early stationary phase. Intracellular G-6-P levels were determined as described in "*Materials and Methods*". Values shown are mean ± SEM (n≥8).

DISCUSSION

The current study discovered for the first time that the yeast *GSK3* gene *MCK1* regulates inositol synthesis. Major findings of this study are as follows. 1) Mck1 is required for optimal intracellular inositol levels and MIPS activity. 2) VPA inhibits MIPS enzymatic activity via Mck1. 3) The rate of inositol synthesis is decreased in *mck1Δ* and *gsk3Δ* cells *in vivo*. 4) *mck1Δ* and *gsk3Δ* cells exhibit decreased intracellular G-6-P levels. These findings identify Mck1 as a regulator of inositol *de novo* synthesis in yeast.

Among the four single mutant strains *mck1Δ*, *mrk1Δ*, *mds1Δ,* and *ygk3Δ*, only *mck1Δ* cells exhibited decreased growth at elevated temperatures, as observed in *gsk3Δ* cells (Fig. 2-1), while *mrk1Δ*, *mds1Δ* and *ygk3Δ* cells grew as WT. In addition, *mck1Δ* and *gsk3Δ* exhibit severely decreased growth in the presence of VPA. The other three *gsk3* single mutants grew normally compared to WT. The addition of inositol to growth media rescued sensitivities to VPA in *mck1Δ* and *gsk3Δ* cells. VPA has been shown to deplete intracellular inositol and inhibits the growth of inositol auxotrophs (60,64,159). These findings suggest that *mck1Δ* cells are defective in inositol synthesis. Therefore, exogenous inositol must be provided to maintain normal levels of inositol in these cells. In fact, the intracellular inositol levels in *mck1Δ* cells decreased to levels similar to those of *gsk3Δ* cells (Fig. 2-2). The other three single mutants, *mrk1Δ*, *mds1Δ* and *ygk3Δ,* exhibited normal inositol levels. In addition, the simultaneous deletion of *MRK1*, *MDS1* and *YGK3* did not result in any features of inositol depletion, suggesting that only *MCK1* was required for optimal inositol levels.

MIPS purified from *mck1Δ* cells exhibited decreased enzymatic activity (Fig. 2-3), suggesting that *MCK1* is required for optimal MIPS activity. MIPS is a phosphoprotein,

and the phosphorylation of MIPS has been reported to regulate its enzymatic activity (63,64). Yeast MIPS contains a potential GSK3 phosphorylation site, Ser-296. Phosphorylation of this site up-regulates MIPS activity (64), which is consistent with our finding that Mck1 positively regulates MIPS activity. On the other hand, phosphorylation of MIPS Ser-184, which is a potential PKA phosphorylation site, downregulates MIPS activity (64). Interestingly, Mck1 has been reported to inhibit PKA activity in yeast (177). Therefore, *MCK1* ablation may contribute to increased phosphorylation on Ser-184. These findings suggest that MIPS inhibition in *mck1⊿* cells results from decreased activating phosphorylation of Ser-296 and increased inhibitory phosphorylation of Ser-184. However, Mck1 did not phosphorylate MIPS *in vitro* (Fig. 2-5), suggesting that Mck1 may need co-factors to phosphorylate MIPS *in vivo*, or that the effect of Mck1 is indirect, e.g., via a kinase activated by Mck1. It is also possible that the HA tag inhibits Mck1 catalytic activity. VPA has been shown to inhibit mammalian GSK3 in several studies (117-120). As a yeast *GSK3* homolog, Mck1 is a possible target of VPA. In support of this hypothesis, VPA did not inhibit MIPS activity in *mck1⊿* and *gsk3⊿* cells (Fig. 2-4). PKA, AKT, and PKC signaling pathways are also affected by VPA treatment (65-67). Further studies are required to determine if these pathways mediate VPA-induced MIPS inhibition. Taken together, these studies indicate that Mck1 mediates VPA-induced MIPS inhibition by regulating MIPS phosphorylation.

We determined rates of inositol synthesis under physiological conditions in WT, *mck1⊿* and *gsk3⊿* cells. As shown in Fig. 2-6, rates of synthesis of inositol-3-phosphate and inositol were decreased in *mck1⊿* cells, which is consistent with decreased MIPS activity and decreased inositol levels observed in *mck1⊿* cells. Therefore, the

decreased rate of inositol synthesis in *mck1⊿* cells is caused, at least in part, by decreased MIPS activity. Surprisingly, rates of I-3-P and inositol synthesis *in vivo*, but not MIPS activity determined *in vitro*, were decreased in *gsk3⊿* cells, which suggested the involvement of a second level of regulation of inositol synthesis. Previous studies have shown that the glycolytic intermediate dihydroxyacetone phosphate (DHAP) is a strong inhibitor of MIPS (178,179). The accumulation of intracellular DHAP leads to inositol auxotrophy (178), suggesting a link between perturbation of glycolysis and inhibition of inositol *de novo* synthesis. Furthermore, Mck1 negatively regulates the activity of pyruvate kinase (177,180), which catalyzes the final and irreversible step of glycolysis in yeast (181). The levels of pyruvate kinase influence the glycolytic flux in yeast cells (182), which suggests that increased pyruvate kinase activity in *mck1⊿* cells may increase the flux of G-6-P into glycolysis. In fact, the intracellular G-6-P levels were significantly decreased in *mck1⊿* and *gsk3⊿* cells (Fig. 2-7). The limited availability of G-6-P may contribute to the decreased rates of inositol synthesis in *mck1⊿* and *gsk3⊿* cells. It is possible that the regulation of inositol *de novo* synthesis is regulated at the level of G-6-P flux.

This study demonstrates for the first time that the yeast *GSK3* homolog *MCK1* is required for optimal rate of inositol synthesis. *mck1⊿* cells exhibit multiple features of inositol depletion and decreased intracellular G-6-P levels. In addition, Mck1 mediates VPA-induced MIPS inhibition. Future studies should focus on the elucidation of potential mechanisms by which Mck1 regulates MIPS activity.

CHAPTER 3 INOSITOL HEXAKISPHOSPHATE KINASE 1 (IP6K1) REGULATES INOSITOL SYNTHESIS IN MAMMALIAN CELLSINTRODUCTION

The work described in this chapter has been published in Yu, W., Ye, C., and Greenberg, M. L. (2016) Inositol Hexakisphosphate Kinase 1 (IP6K1) Regulates Inositol Synthesis in Mammalian Cells. *The Journal of biological chemistry* 291, 10437-10444 (183) .

INTRODUCTION

Inositol is a ubiquitous six-carbon cyclitol that is essential for viability of eukaryotic cells. *Myo*-inositol, physiologically the most important stereoisomer of inositol, is the precursor of all inositol compounds, including phosphoinositides, inositol phosphates, inositol sphingolipids, and glycosylphosphatidylinositols. Inositol compounds are essential for gene expression (167), trafficking (27), signal transduction (20) and membrane biogenesis (168). The crucial role of inositol is underscored by the link between perturbation of inositol metabolism and human disorders (18). Therefore, elucidating the mechanisms underlying the control of inositol homeostasis is expected to have important implications for understanding cell function and the pathologies underlying many illnesses.

While cellular inositol can be obtained from the extracellular environment or by recycling inositol lipids (7,23), the *de novo* synthesis of inositol is essential for inositol homeostasis and cellular proliferation when environmental inositol is limiting (160,184). Inositol synthesis is a two-step reaction in which glucose-6-phosphate (G6P), the branch point metabolite for glycolysis, the pentose phosphate pathway, and inositol synthesis, is converted to inositol-3-phosphate, which is dephosphorylated to inositol. The first and rate-limiting step of inositol synthesis is catalyzed by inositol-3-phosphate synthase (8-10,169,170). Significantly high levels of inositol are found in brain tissue, which has limited access to exogenous inositol (14), suggesting that *de novo* synthesis

of inositol is critical for normal brain function. Importantly, alterations in inositol metabolism have been associated with bipolar disorder and Alzheimer's disease (18,37,185).

Despite its importance, little is known about the regulation of inositol synthesis in mammals. In contrast, the regulation of inositol synthesis has been well-characterized in the budding yeast *Saccharomyces cerevisiae*. Inositol synthesis in yeast is regulated at the level of transcription of *INO1* (171,172), the gene that codes for inositol-3-phosphate synthase, and by phosphorylation of the Ino1 protein (64). *INO1* expression is controlled by the transcriptional repressor Opi1 in response to inositol and phosphatidic acid (PA) levels (56). Opi1 is stabilized by physically interacting with PA on the endoplasmic reticulum (ER) membrane. When inositol is limiting, PA levels are increased, Opi1 remains in the ER, and *INO1* transcription is derepressed to increase inositol synthesis. When inositol levels are abundant, PA is rapidly consumed for phosphatidylinositol synthesis, Opi1 is released from the ER and translocates into the nucleus, where it represses *INO1* transcription resulting in decreased inositol synthesis. We have recently demonstrated that transcription of *INO1* in yeast is regulated by the synthesis of inositol pyrophosphates, as *INO1* transcription requires the Kcs1-catalyzed synthesis of diphosphoinositol tetrakisphosphate (PP-IP_4) (167).

In contrast to inositol synthesis in yeast, transcription of *Isyna1* (the mammalian homolog of yeast *INO1,* which we refer as *mINO1* in this report), is not regulated in response to inositol (160,186). However, in the current study, we demonstrate for the first time that inositol synthesis in mouse embryonic fibroblast (MEF) cells is regulated by inositol pyrophosphate. Knockout of inositol hexakisphosphate kinase IP6K1 [which

catalyzes the formation of pyrophosphate at position five of inositol pentakisphosphate /inositol hexakisphosphate to generate 5PP-IP_4/5PP-IP_5 (IP_7), respectively (187)], led to increased transcription and altered DNA methylation of *mINO1.* This resulted in increased levels of mIno1 protein and increased intracellular inositol. Thus, in contrast to positive regulation of *INO1* in yeast, inositol pyrophosphate negatively regulates *mINO1* transcription. Intriguingly, similar to the yeast transcriptional repressor Opi1, IP6K1 protein bound with high affinity to PA. Deletion of the PA-binding sequence abrogated both nuclear localization of IP6K1 and repression of *mINO1* transcription. Our findings suggest a model whereby inositol pyrophosphate negatively regulates inositol synthesis by PA-facilitated entry into the nucleus and repression of *mINO1* transcription.

MATERIALS AND METHODS

Cell lines, yeast strains, and growth conditions

Wild type, IP6K1-KO, IP6K1-KO +pMX-IP6K1 and IP6K1-KO +pMX-EV MEF cell lines (kindly provided by Dr. Anutosh Chakraborty, Scripps Research Institute), were grown in DMEM (Gibco) containing 10% FBS (Hyclone) and penicillin (100 units/m)/ streptomycin (100 µg/ml) (Invitrogen) (188). IP6K1-KO +pMX-IP6K1 and IP6K1-KO +pMX-EV cells were grown in this media supplemented with 4µg/ml blasticidin (Sigma). The yeast *S. cerevisiae kcs1*∆ strain was obtained from Yeast Deletion Clones (Invitrogen). Yeast cells were grown at 30°C or 37°C in synthetic complete (SC) medium, which contained glucose (2% w/v) and necessary supplements including adenine (20 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), methionine (20 mg/liter), tryptophan (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), threonine (300

mg/liter), ammonium sulfate (0.2% w/v), inositol-free Difco vitamin mix, vitamin-free yeast base, plus agar (2% w/v) for solid medium. Inositol (75 μM) was added separately as indicated.

Real-time PCR

MEF cells were grown in DMEM containing 10% FBS. RNA was extracted from cells by using RNeasy plus Mini Kit (QIAGEN), and was converted to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-PCR was performed using Brilliant III Ultra-Fast SYBR green qPCR master mix (Agilent) and MX3000p qPCR System (Agilent). mRNA levels were normalized to *GAPDH*.

Immunoblotting

Cells were harvested, washed with ice-cold PBS and lysed by vortexing in lysis buffer (50 mM Tris-HCL, 125 mM NaCl, 1% NP40, 2 mM EDTA and 1X protease inhibitor) for 30 min at 4°C. Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo). Cell extract corresponding to 30 μg protein was analyzed by SDS-PAGE on a 10% gel. Immunoblotting was performed using primary antibodies against mIno1 (ISYNA1) (sc-134687, rabbit, Santa Cruz), IP6K1 (GTX103949, rabbit, Gene Tex), β-actin (sc-69879, mouse, Santa Cruz) and corresponding secondary antibodies.

Measurement of intracellular inositol and G-6-P levels in MEF cells

Intracellular inositol levels were determined as described before using the method of Maslanski and Busa (174). In brief, MEF cells were lysed in lysis buffer (50 mM Tris-HCL, 125mM $NaCl_2$, 1% NP40 and 2mM EDTA) at 4°C. Cell extracts were mixed with 7.5% perchloric acid and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were collected and titrated with ice cold KOH to pH 7. Samples were

clarified by centrifugation and loaded onto columns containing 1 ml AG 1-X8 resin/H_20 (1:1) mixture. Inositol was eluted with 5 ml dH_2O, eluates were dried in an oven at 70°C and stored at -80°C. Prior to assay, samples were dissolved in dH_2O. Inositol content in samples was measured as described previously (62).

To determine intracellular G-6-P levels, MEF cells were washed twice with ice cold PBS. Cell pellets were mixed with 1 ml ice cold MeOH:$CHCL_3$ (2:1), vortexed and stored at -20°C for 2 h. Samples were then mixed with extraction solution (50% MeOH, 4 mM tricine pH 5.4) and centrifuged at 18,000 g for 10 min. The upper phase was collected and kept on ice. The lower chloroform phase was extracted again with extraction solution. Upper phases from both extractions were combined, dried with a speedvac, and stored at -80°C. Prior to assay, samples were dissolved in dH_2O. G-6-P content in samples was measured by the enzyme-coupled fluorescence assay developed by Zhu *et al*. with modification (175).

Protein lipid overlay assay

pGEX-6P-2 plasmids harboring IP6K1 variants were constructed using a Q5 Site-Directed Mutagenesis Kit (NEB) with pGEX-6P-2-IP6K1 (from Dr. Anutosh Chakraborty) as template. pGEX-6P-2-IP6K1 and corresponding mutants were transformed into BL21 (DE3) pLysS cells for IPTG-induced overexpression. *E. coli* cells were lysed by French press to obtain cell extracts. IP6K1 proteins were then purified from *E.coli* cell extracts using a GST spin purification kit (Life Technology). A protein lipid overlay assay was performed according to the protocol of Dowler and co-workers with modification (189). In brief, lyophilized lipids were dissolved in a 1:1 solution of methanol and chloroform to make 1 mM stocks. Lipids were diluted to concentrations ranging from 500 to 5 μM in a

2:1:0.8 solution of methanol:chloroform:water. Then 1 µl lipid samples from each dilution were spotted on nitrocellulose membranes. After drying for 1 h at room temperature, membranes were incubated in blocking buffer (50 mM Tris-HCL pH 7.5, 150 mM $NaCl_2$, 0.1% Tween 20 and 2 mg/ml BSA) with gentle rocking for 1 h at room temperature. Membranes were incubated overnight at 4°C with gentle rocking in 10 ml blocking buffer containing 25 µg purified IP6K1 proteins. IP6K1 proteins bound to lipid drops on the membrane were detected by immunoblotting using primary antibodies against IP6K1 (GTX103949, rabbit, Gene Tex) and corresponding secondary antibodies.

Fluorescence microscopy

pEGFP-C1 plasmids harboring IP6K1 variants were constructed using a Q5 Site-Directed Mutagenesis Kit (NEB) with pEGFP-C1-IP6K1 (from Dr. Anutosh Chakraborty) as template. IP6K1-KO cells were transfected with pEGFP-C1-IP6K1 plasmid and corresponding mutants using Lipofectamine 2000 (Life technology). Fluorescence microscopy was performed using an Olympus BX41 fluorescence microscope. Images were acquired using an Olympus Q-Color3 camera operated by QCapture2 software. All pictures were taken at 400X at room temperature.

Determination of DNA methylation levels

Genomic DNA samples extracted from WT and IP6K1-KO cells using a Wizard Genomic DNA Purification Kit (Promega) were subjected to DNA bisulfite conversion using an EZ DNA Methylation-Gold Kit (Zymo Research), and sequencing for the determination of methylation levels.

Table 3-1. RT-PCR primers used in this study

Gene	Sequence
mINO1 (*Isyna1*)	CCTGTAGTGAATGCCCTCTTC CGCTCCATCTTGTGCTCTAATA
Gapdh1	AACAGCAACTCCCACTCTTC CCTGTTGCTGTAGCCGTATT

RESULTS

IP6K1 rescues inositol deficiency in the yeast *kcs1Δ* mutant

We have previously shown that Kcs1-catalyzed inositol pyrophosphate synthesis is required for optimal transcription of *INO1* in yeast (167). To determine if mouse IP6K1 can supply the function of yeast Kcs1, we expressed IP6K1 in the yeast *kcs1*Δ mutant. As seen in Fig. 3-1, *kcs1*Δ mutant cells expressing the wild type IP6K1 gene grew normally, while the kinase-dead IP6K1 ($IP6K1^{KD}$) gene (28) failed to support growth of the *kcs1*Δ mutant. Therefore, mouse IP6K1 is the functional homolog of yeast Kcs1, which can rescue defective inositol synthesis in yeast by restoring inositol pyrophosphate synthesis.

IP6K1 is a negative regulator of inositol synthesis in MEF cells

To ascertain if IP6K1 regulates mammalian inositol synthesis, we determined the effects of knocking out IP6K1 (IP6K1-KO). Surprisingly, IP6K1-KO cells exhibited a 5-fold increase in *mINO1* mRNA levels (Fig. 3-2*A*). Consistent with increased transcription, mIno1 protein levels were also increased in IP6K1-KO cells (Fig. 3-2*B*), and inositol levels were 75% higher in the IP6K1-KO cells than in WT cells (Fig. 3-2*C*). Levels of intracellular G-6-P, the substrate for inositol synthesis, decreased correspondingly (Fig. 3-2*D*). Taken together, these findings indicate that IP6K1 is a negative regulator of inositol synthesis.

IP6K1 regulates *mINO1* DNA methylation

As seen in Fig. 3-2*B*, two mIno1 proteins were expressed in IP6K1-KO cells. A previous study reported that alternative splicing of *mINO1* results in α, β, and γ mRNA transcripts, which generate protein isoforms (190). The α transcript is the full-length

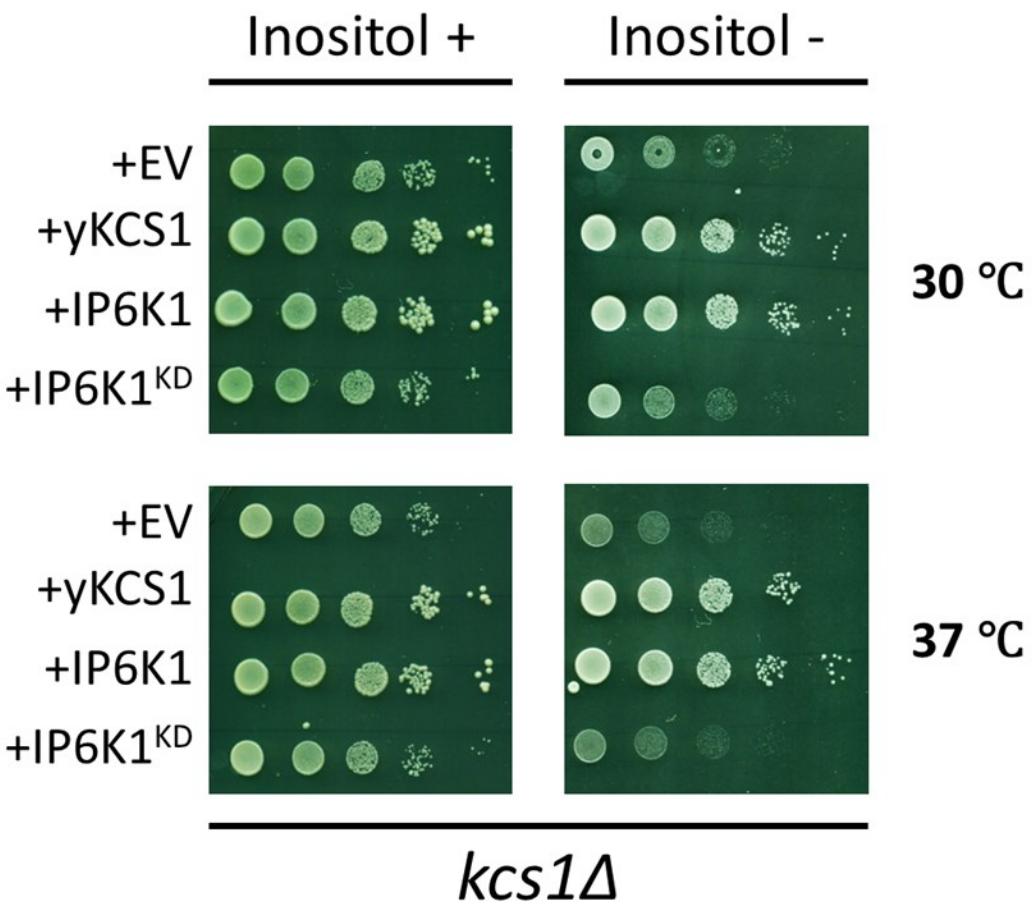

Figure 3-1. IP6K1 rescues *kcs1*Δ inositol auxotrophy. Serial dilutions of yeast *kcs1*Δ cells transformed with indicated plasmids were spotted on synthetic complete Ura$^-$ medium in the presence or absence of 75 μM inositol. Plates were incubated at the indicated temperatures for 3 days.

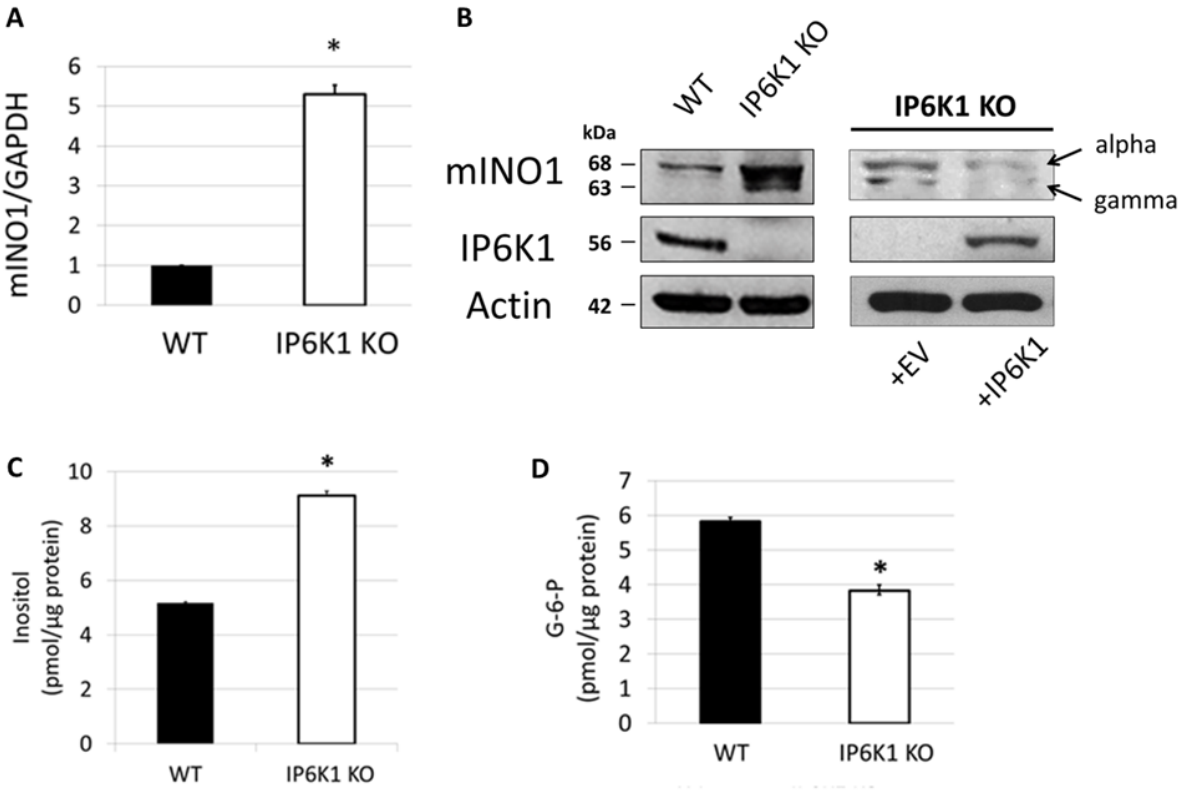

Figure 3-2. Inositol synthesis is up-regulated in IP6K1-KO cells. *A*, *mINO1* mRNA levels are increased in IP6K1-KO cells. *mINO1* mRNA levels were determined by RT-PCR (n=4) (*p<0.05). *B*, mIno1 protein levels were profoundly increased in IP6K1-KO cells (left). The expression of IP6K1 in IP6K1-KO cells restored mIno1α and mIno1γ levels (right). 30 µg total cell extract from each sample were subjected to Western blot analysis using 10% SDS-PAGE gel. *C*, IP6K1-KO cells exhibited increased intracellular inositol levels. Intracellular inositol levels in MEF cells were determined as described in "*Experimental procedures*" (n=6) (*p<0.05). *D*, Intracellular G6P levels were decreased in IP6K1-KO cells. Intracellular G6P levels in MEF cells were determined by enzyme-coupled fluorescence assay as described in "*Experimental procedures*" (n=6) (*p<0.05). Values are mean ± SEM. Statistical significance was determined by unpaired *t* test.

mRNA, the β mRNA contains a truncated exon 2, and the γ mRNA is transcribed from a second ATG site within the unremoved intron 4. To determine which isoforms are expressed in IP6K1-KO cells, we compared cDNA from WT and IP6K1-KO cells by PCR analysis, using specific primers that distinguished among these mRNAs. Both the α and γ mRNAs were present in the IP6K1-KO cells, but only the α mRNA was detected in WT cells (data not shown). These findings indicated that isoforms observed in Fig. 3-2B are translated from the α (upper band) and γ (lower band) splicing forms.

Previous studies indicated that *in vitro* methylation of the human *INO1* promoter significantly decreased reporter gene expression (191). To determine if altered DNA methylation could account for the mIno1 isoforms observed in IP6KO cells, we analyzed methylation of *mINO1* DNA using DNA bisulfite conversion of WT and IP6K1-KO genomic DNA followed by probing with primers targeting the sequence from – 286 to +160, which is enriched in CpG islands (Fig. 3-3). In the *mINO1* promoter region, most of the CpG sites exhibited a similar, but slightly decreased, pattern of methylation in IP6K1-KO cells compared to WT cells. However, the CpG sites between the first and second ATGs exhibited markedly less methylation in IP6K1-KO cells. These findings suggest that IP6K1 may negatively regulate *mINO1* transcription by increasing the methylation of *mINO1* DNA.

IP6K1 requires PA-binding for nuclear localization and repression of mINO1 transcription

As discussed above, negative regulation of *INO1* transcription in yeast is mediated by the Opi1 transcriptional repressor. While homologs of Opi1 have not been identified in mammalian cells, we considered the possibility that IP6K1 may exhibit functional similarity to Opi1. Opi1 is retained in the cytoplasm by binding to PA, and

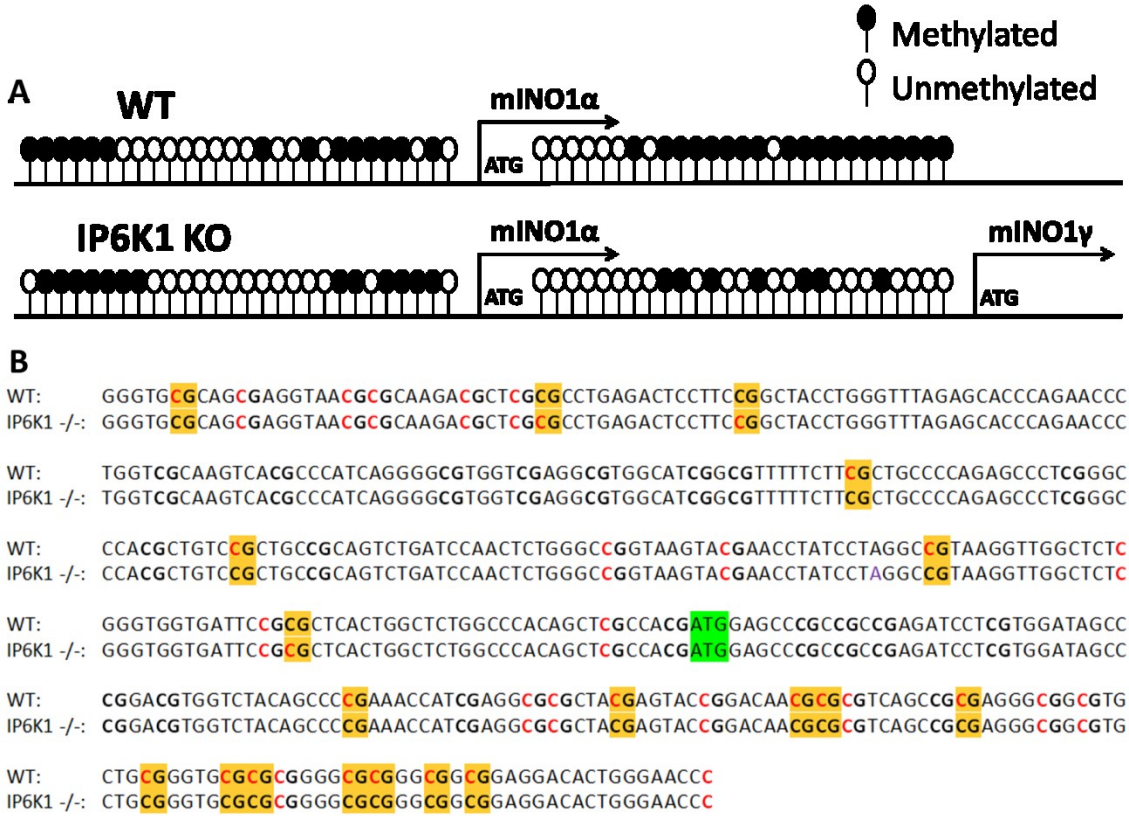

Figure 3-3. Methylation pattern of *mINO1* DNA is altered in IP6K1-KO cells. *A*, DNA methylation levels in MEF cells were determined as described in "*Experimental procedures*". CpG islands are depicted as balloons. Methylated cytosines are filled, and unmethylated cytosines are unfilled. *B*, Raw data of *mINO1* DNA methylation experiment. CpG islands are depicted in bold. Methylated cytosines are depicted in red, whereas unmethylated cytosines are depicted in black. CpG islands with altered methylation in IP6K1-KO cells are highlighted with yellow shade, and the start codon is highlighted with green shade.

translocates to the nucleus when PA is limiting. As shown in the protein lipid overlay assay (Fig. 3-4*A*), purified recombinant IP6K1 displayed a markedly high affinity to PA, and only weak binding to other phospholipids.

To determine the functional significance of PA-binding, we constructed the IP6K1 deletions shown in Fig. 3-5 and determined the ability of truncated proteins to bind PA. Deletion of Q2 but not the other sequences greatly diminished PA-binding activity of IP6K1 (Fig. 3-4*B*). The Q2 domain alone was sufficient for PA-binding (Fig. 3-4*D*). We then determined the effect of Q2 deletion on localization of IP6K1. IP6K1-KO cells expressing GFP-tagged wild type IP6K1 exhibited intense fluorescence in the nucleus in about 75% of cells (Fig. 3-6). Deletion of the Q2 domain resulted in decreased nuclear localization in more than 60% of cells. Interestingly, IP6K1-KO cells expressing GFP-Q2Δ exhibited increased *mINO1* mRNA levels compared to cells expressing wild type IP6K1 (Fig. 3-7). These results indicate that interaction with PA facilitates IP6K1 nuclear localization and is required by IP6K1 to repress *mINO1* expression.

Protein BLAST sequence alignment identified a small region of sequence homology between the Q3 domain of IP6K1 and yeast Opi1, especially in the region of positively charged residues that are critical to the PA-binding activity of the yeast protein (Fig. 3-5). The IP6K1 region containing these sequences was designated HOPA (*H*omology to *O*pi1 *PA*-binding). To determine the function of the HOPA domain, we constructed an IP6K1 mutant in which this region is deleted (HOPAΔ). The HOPAΔ protein exhibited normal PA-binding activity (Fig. 3-4*C*) as expected, as PA-binding is conferred by the Q2 domain. Strikingly, however, deletion of the HOPA domain resulted in exclusion of IP6K1 from the nucleus in more than 70% of cells (Fig. 3-6). Consistent

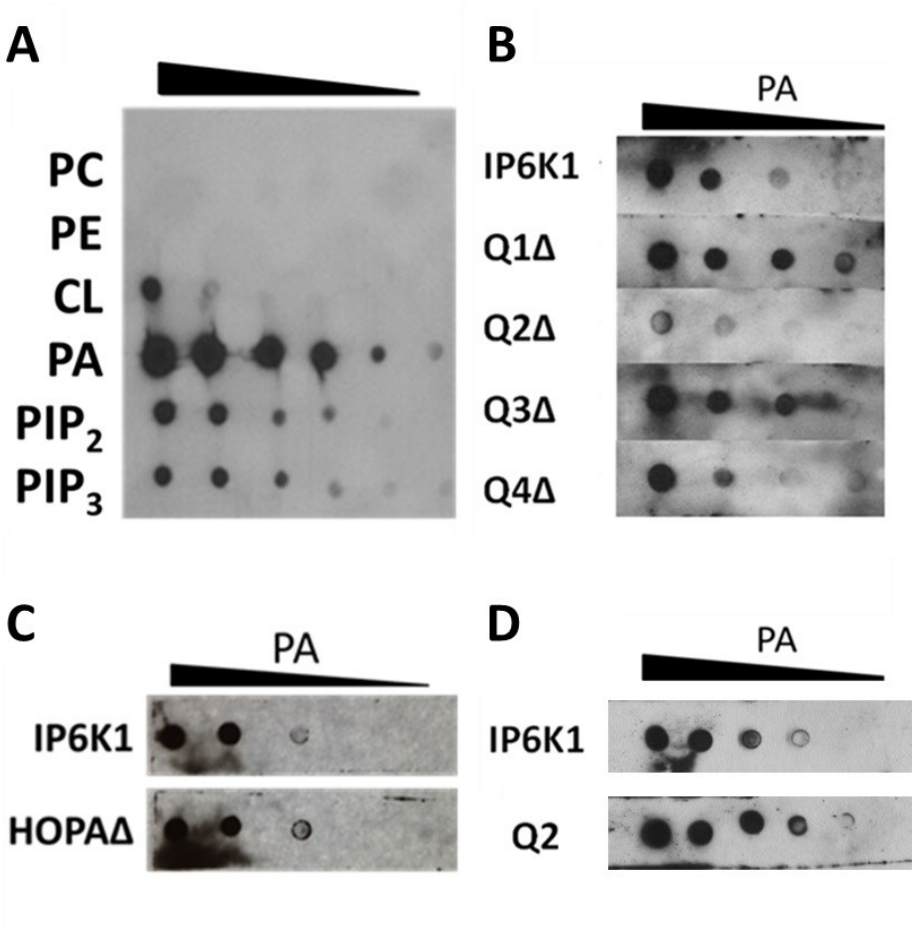

Figure 3-4. IP6K1 binds preferentially to phosphatidic acid (PA). *A*, IP6K1 protein was purified from *E. coli* cells expressing the IP6K1 gene on the pGEX-6-P2 overexpression vector. Serial dilutions of the indicated lipids were spotted on a nitrocellulose membrane, which was incubated overnight in buffer containing 25 µg of IP6K1 protein. Interactions between IP6K1 and lipids were determined by immunoblotting. *B*, Deletion of the Q2 domain of IP6K1 decreased binding to PA. *C*, Deletion of the HOPA domain of IP6K1 did not affect binding to PA. *D*, Q2 domain of IP6K1 binds to PA.

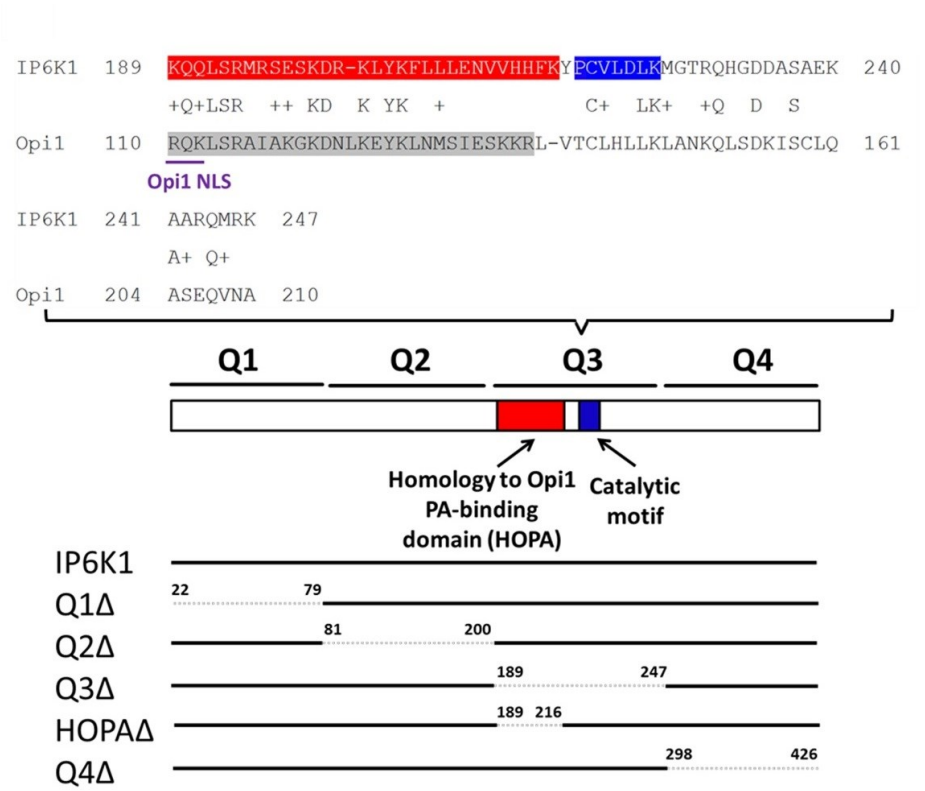

Figure 3-5. IP6K1 exhibits sequence homology to yeast Opi1 (upper panel). The IP6K1 HOPA domain that exhibits homology to the PA-binding domain of yeast Opi1 is highlighted in red. The catalytic motif of IP6K1 is highlighted in blue. The PA-binding domain of yeast Opi1 is highlighted in gray. The nuclear localization signal (NLS) of yeast Opi1 is underlined in purple. IP6K1 deletion mutants were constructed by site-directed mutagenesis according to the schematic figure (lower panel). Residues deleted are indicated by numbers above the bar.

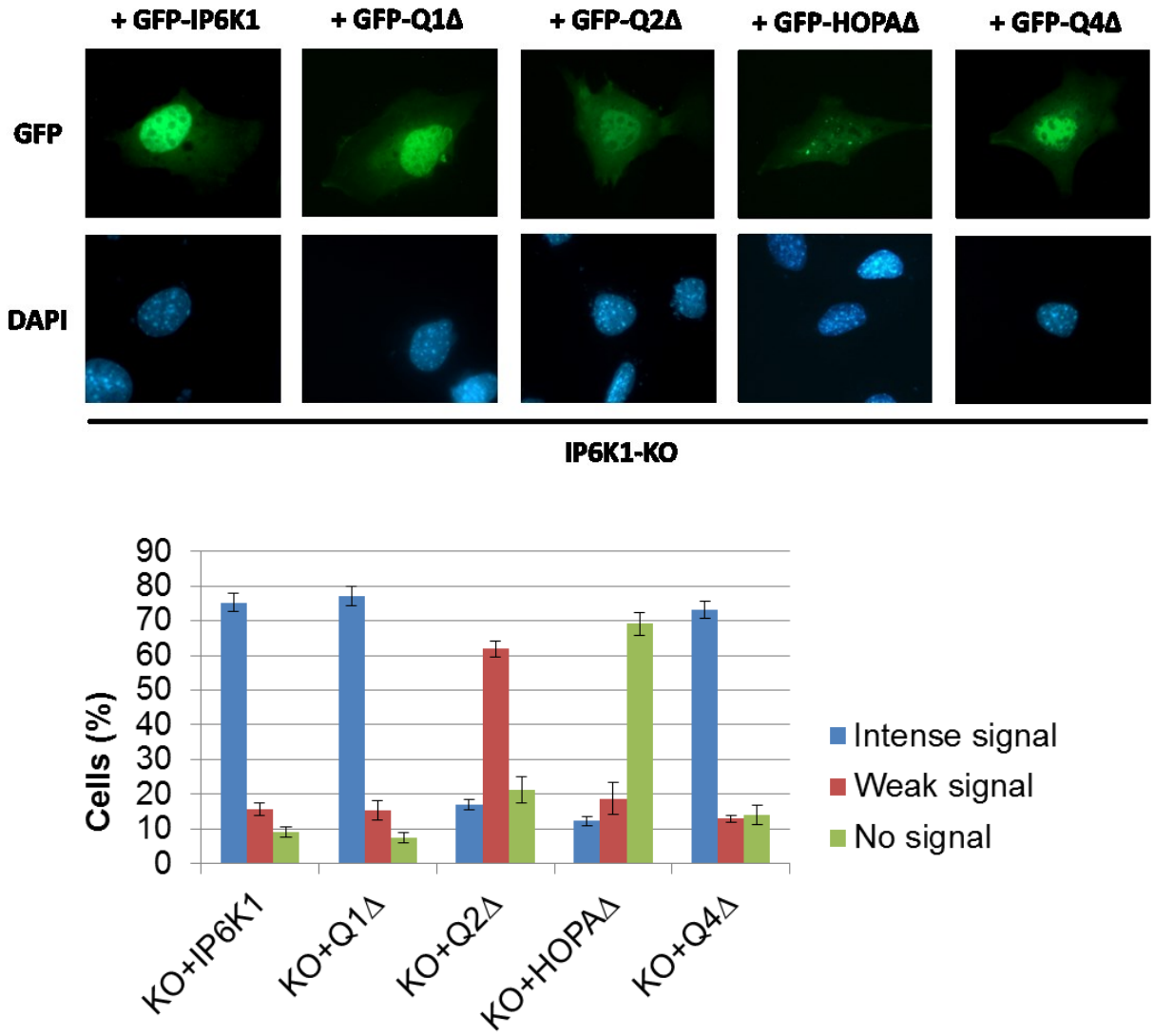

Figure 3-6. PA-binding is required for nuclear localization of IP6K1. IP6K1-KO cells were transfected with plasmids harboring GFP-tagged WT or mutant IP6K1. Intracellular localization of IP6K1 (upper panel) was determined by fluorescence microscopy. The number of cells showing each phenotype (lower panel) was determined in three independent experiments (n > 150). Values are mean ± SEM.

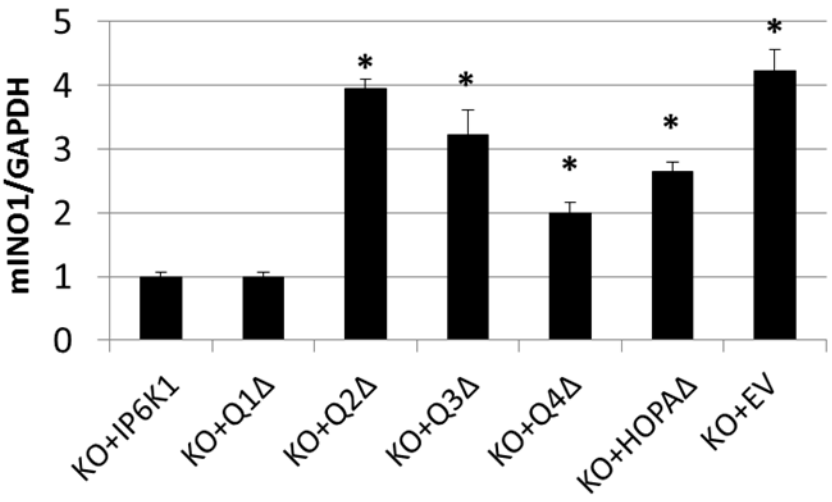

Figure 3-7. PA-binding to IP6K1 is required for repression of *mINO1* transcription. IP6K1-KO cells were transfected with plasmids harboring the indicated GFP-tagged IP6K1 mutants. The *mINO1* mRNA levels were determined by RT-PCR (2 independent experiments with 2 replicates) (*$p<0.05$). Values are mean ± SEM. Statistical significance comparing transcription levels to KO+IP6K1 control was determined by unpaired *t* test.

with this finding, cNLS Mapper predicts a potential nuclear localization signal (NLS) in the HOPA domain. Interestingly, the NLS of yeast Opi1 is in the homologous region (Fig. 3-5). Expression of HOPAΔ in IP6K1-KO cells resulted in increased *mINO1* mRNA levels relative to cells expressing WT IP6K1 (Fig. 3-7). The catalytic motif of IP6K1 also lies in the Q3 domain (192,193). Expression of Q3Δ, which lacks both the HOPA domain and the catalytic motif, also led to increased *mINO1* mRNA levels in IP6K1-KO cells (Fig. 3-7).

The Q1Δ deletion did not affect nuclear localization (Fig. 3-6) or *mINO1* mRNA levels. Although the Q4Δ deletion did not affect nuclear localization (Fig. 3-6), it resulted in increased *mINO1* transcription (Fig. 3-7). The Q4 domain contains the ATP-binding motif (192,193), which is critical for IP6K1 catalytic activity (28,194,195). Increased *mINO1* transcription in IP6K1-KO cells expressing Q4Δ indicates that IP6K1 requires catalytic activity to suppress *mINO1* expression. Together, these findings indicate that binding of PA is required for localization of IP6K1 to the nucleus, where the ATP-binding dependent synthesis of inositol pyrophosphate represses *mINO1* transcription.

DISCUSSION

Despite the essential role of inositol in cell function, the mechanisms that regulate inositol synthesis in mammalian cells have not been characterized. The current study demonstrates for the first time that *de novo* synthesis of inositol in MEF cells is regulated by inositol hexakisphosphate kinase 1 (IP6K1), which mediates transcriptional control of the gene (*mINO1*) coding for the rate-limiting enzyme of inositol synthesis. Our findings indicate that IP6K1 alters methylation and negatively regulates

transcription of *mINO1,* and that binding of IP6K1 to PA is essential for nuclear localization of IP6K1 and repression of transcription.

IP6K1-KO cells exhibited increased expression of *mINO1* (Fig. 3-2*A*), which was accompanied by increased mIno1 protein and inositol levels (Fig. 3-2*B*,*C*). These findings indicate that IP6K1 is a negative regulator of *mINO1* transcription. Increased *mINO1* expression in IP6K1-KO cells is most likely due to decreased DNA methylation (Fig. 3-3), which is associated with silencing of gene expression (196). Seelan *et al.* investigated the effects of methylation of the *INO1* promoter on transcription of *INO1* in human neuroblastoma cells transfected with plasmids carrying *in vitro* methylated *INO1* (191). They determined that the methylated *INO1* promoter led to significantly less transcription than the non-methylated control. Our finding that IP6K1-KO cells exhibited both increased *mINO1* transcription and decreased DNA methylation is the first *in vivo* demonstration of regulation of *mINO1* by *in vivo* methylation.

Two lines of evidence support a role for regulation of methylation by inositol pyrophosphate. First, IP_7 (the product of IP6K1) was shown to inhibit Akt (197), which negatively regulates methylation of imprinted genes (198). Therefore, the deficiency of IP_7 in IP6K1-KO cells may lead to decreased *mINO1* methylation as a result of increased Akt signaling. Second, IP6K1 associates with chromatin and controls histone demethylation by regulating the demethylase Jumonji domain-containing 2C (JMJD2C), which catalyzes the removal of trimethyl groups from lysines 9 and 36 of histone H3 (26). IP6K1-KO cells exhibited decreased levels of histone H3 lysine 9 trimethylation (H3K9me3) and increased transcription of JMJD2C-regulated genes. Interestingly, DNA regions associated with H3K9me3 exhibited increased methylation (199), while mutation

of H3K3 led to decreased DNA methylation (200). In this light, IP6K1 may regulate *mINO1* methylation indirectly by regulating histone modification.

IP6K1 exhibits dual localization in the cytoplasm and nucleus (Fig. 3-6). The probable NLS of IP6K1 was localized to the Q3 domain, the deletion of which led to exclusion of IP6K1 from the nucleus (Fig. 3-6). Binding of the IP6K1 Q2 domain to PA is essential for translocation of the protein to the nucleus and repression of *mINO1* transcription (Figs. 3-6, 3-7). Binding to PA has been shown to promote nuclear localization of transcription factor WER in *Arabidopsis* (201,202). Inhibition of phospholipase D (PLD), resulting in decreased conversion of phosphatidylcholine (PC) to PA, suppresses nuclear localization of WER (202). Nuclear association of PLD has been reported both in mammals and plants (203,204). Furthermore, PA synthesis has been demonstrated in the nuclear envelope of mammalian cells (205,206), and PA has been detected in the nucleus (207). To our knowledge, the current study is the first to report the dependence on binding to PA for nuclear localization of a mammalian protein.

The catalytic function of IP6K1 is necessary for repression of *mINO1* transcription, as deletion of the Q4 domain resulted in increased *mINO1* expression (Fig. 3-7). The “SLL” ATP-binding motif, which is highly conserved in the inositol polyphosphate kinase (IPK) family, is localized in the Q4 domain (192,193), and mutation of the ATP-binding site impairs catalysis (28,194). Therefore, the synthesis of inositol pyrophosphate is required for repression of *mINO1* transcription.

Surprisingly, while Kcs1 and IP6K1 carry out the same enzymatic function, they regulate *INO1* expression in an opposite manner in yeast and mouse cells. *INO1*

expression is positively regulated in yeast by Kcs1 (167) but negatively regulated by IP6K1 in mouse cells (Fig. 3-2*A*). In fact, the observed negative regulation of *mINO1* expression by IP6K1 more closely resembles that of the yeast transcriptional repressor Opi1. Both Opi1 and IP6K1 bind to PA, translocate from the cytoplasm to the nucleus, and repress *INO1* transcription. However, while PA-binding retains Opi1 in the cytoplasm, PA-binding mediates IP6K1 nuclear translocation (Fig. 3-6). In yeast, PA levels indirectly reflect inositol synthesis, which regulates *INO1* expression. As inositol does not regulate *INO1* expression in mammalian cells (9, 22), we speculate that PA levels may reflect the state of cellular energy metabolism, which would affect inositol synthesis as a result of competition for the common substrate G-6-P.

Inositol pyrophosphate regulation is an intriguing area of signaling research. Shears suggested that inositol pyrophosphates are metabolic messengers that respond to the cellular energy demand (208). In support of this concept, perturbing the energy balance in mammalian cells leads to decreased synthesis of inositol pyrophosphates (209). Interestingly, inositol pyrophosphate deficiency in yeast *kcs1*Δ cells leads to dysfunctional mitochondria with greatly reduced respiratory capacity (210). To compensate, glycolysis is increased to generate ATP. G-6-P is the branch point of inositol *de novo* synthesis and glycolysis. We observed decreased intracellular G-6-P levels in IP6K1-KO cells (Fig. 3-2*D*), which may be caused, at least in part, by increased inositol synthesis. Therefore, IP6K1 may repress *mINO1* expression to maintain inositol synthesis at a relatively low level, reserving G-6-P for glycolysis.

Our findings suggest the following model (Fig. 3-8). Translocation of IP6K1 to the nucleus is facilitated by interaction with PA. In the nucleus, IP6K1 associates with

chromatin and synthesizes IP_7/PP-IP_4. IP_7/PP-IP_4 inhibits transcription of *mINO1* by increasing methylation of *mINO1* DNA, inhibiting the recruitment of transcription factors to the promoter region of *mINO1,* or perturbing assembly of the transcription complex. This study is the first to describe the molecular control of *de novo* synthesis of inositol in mammalian cells, and suggests that inositol synthesis and cellular energy demand are coordinately controlled. These findings have important implications for understanding essential cellular functions that are dependent on inositol, and human disorders that result from perturbation of inositol homeostasis.

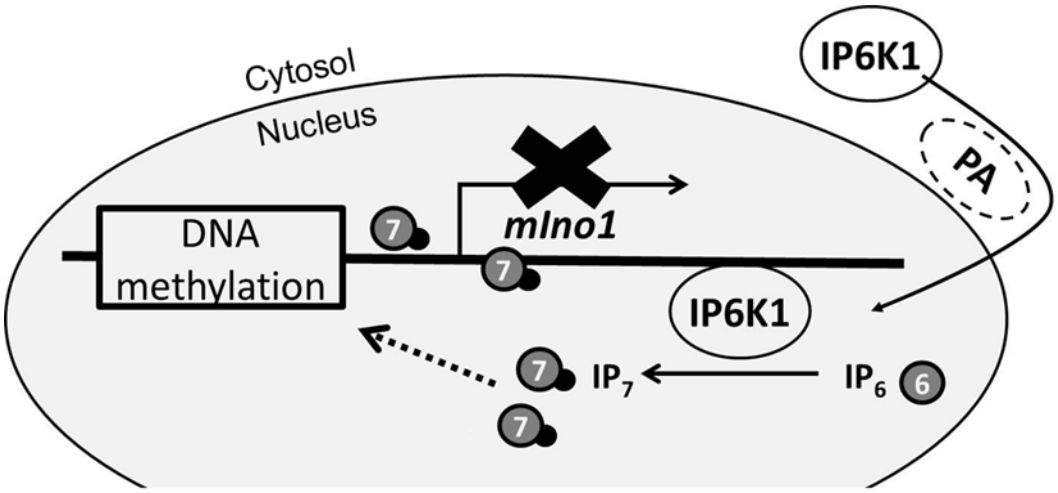

Figure 3-8. Model of regulation of *mINO1* transcription by IP6K1. PA-binding facilitates localization of IP6K1 to the nucleus, where it associates with chromatin and synthesizes IP_7/PP-IP_4. IP_7/PP-IP_4 represses *mINO1* expression by promoting methylation of *mINO1* DNA and/or inhibiting the transcription apparatus.

CHAPTER 4 VPA INHIBITS EXPRESSION OF HEXOSE TRANSPORTER GENES *HXT2* AND *HXT4* VIA TRANSCRIPTION REPRESSOR MIG1

INTRODUCTION

VPA has been used for decades for the treatment of bipolar disorder. However, the therapeutic mechanism underlying this drug is not well understood, which hinders the development of novel anti-bipolar drugs with improved efficacy. Among many biochemical outcomes of VPA treatment, VPA-induced inositol depletion has been proposed as one potential mechanism (31). In *Saccharomyces cerevisiae* cells, VPA depletes intracellular inositol by inhibiting *myo*-inositol-3-phosphate synthase (MIPS) (59,60,64), which catalyzes the rate-limiting step of inositol *de novo* synthesis (8-10,169,170). In addition, VPA was shown to upregulate expression of glycolysis genes in yeast (unpublished data of Shyamala Jadhav). Interestingly, MIPS activity is greatly inhibited by the glycolysis intermediate dihydroxyacetone phosphate (DHAP) (178,179). Yeast mutants that accumulate intracellular DHAP are inositol auxotrophs (178). These findings suggest that inositol *de novo* synthesis and glycolysis are intrinsically coordinated. As both glycolysis and inositol synthesis pathways utilize glucose 6-phosphate (G-6-P) as the common substrate, VPA-induced perturbation of glycolysis and inositol synthesis may both result from changes in G-6-P metabolism.

Glucose is the preferred carbon source for most eukaryotic cells. To adapt to fluctuation of glucose in the environment, yeast cells developed mechanisms to sense and respond to glucose availability. The first step in the regulation of glucose metabolism is glucose uptake, which is regulated by the transcription of hexose transporter (*HXT*) genes (211). Twenty genes that encode proteins in the hexose transporter family have been identified, including *HXT1-HXT17* , *SNF3*, *RGT2*, and

GAL2 (212). Snf3 and Rgt2 are glucose sensors, which regulate the expression of *HXT* genes but do not transport glucose (213,214). Only *HXT1-HXT7* are known to express functional hexose transporters (215). *HXT1* and *HXT3* encode low-affinity glucose transporters (212,216). Their expression is induced by high levels of glucose (217). *HXT2*, *HXT6*, and *HXT7* encode high-affinity transporters, while *HXT4* encodes intermediate-affinity transporter (216). Transcription of *HXT2* and *HXT4* is induced by low levels of glucose (218) and repressed by high levels of glucose (217,219-221). *HXT6* and *HXT7* transcription is also repressed in high glucose (222).

The repression of *HXT2* and *HXT4* under high glucose conditions is mediated by the Snf1-Mig1 pathway (211,223,224). Mig1 is a zinc-finger protein that binds to DNA and recruits co-repressors Ssn6 and Tup1 to repress gene expression (225). Snf1, the yeast homolog of mammalian AMP-activated kinase, is activated under low glucose conditions. Active Snf1 phosphorylates Mig1 on multiple serine residues (226-228). Phosphorylated Mig1 translocates from the nucleus to the cytoplasm to allow expression of glucose-repressible genes, including *HXT2* and *HXT4* (229). In high glucose, both Mig1 and Snf1 are dephosphorylated by the Reg1-Gly7 protein phosphatase complex, which allows Mig1 to enter the nucleus and restore glucose repression (230,231).

In this study, I discovered that VPA decreased expression of hexose transporter genes and induced Mig1 nuclear translocation. VPA treated cells exhibited decreased expression of *HXT2*, *HXT4*, *HXT6*, and *HXT7*. The ablation of *MIG1* or *REG1* alleviated VPA-induced inhibition of *HXT2* and *HXT4* expression. Furthermore, VPA triggered Mig1 nuclear translocation under non-repressive conditions. This study shows for the

first time that VPA inhibits expression of hexose transporter genes via Mig1. Decreased expression of hexose transporter genes may account for VPA-induced G-6-P depletion.

MATERIALS AND METHODS

Yeast strains

All strains used in this study were in the W303 background. *mig1Δ* and *reg1Δ* strains were constructed by gene knockout using KanMX and ble (phleomycin resistance gene) cassettes, respectively. The *MIG1-GFP:KanMX NRD1-RFP:HghMX* strain was kindly provided by Dr. Stefan Hohmann from University of Gothenburg, Sweden (232).

Growth media

Yeast cells were grown at 30°C in synthetic complete (SC) medium, which contained glucose (2% w/v) and necessary supplements including adenine (20 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), methionine (20 mg/liter), tryptophan (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), threonine (300 mg/liter), ammonium sulfate (0.2% w/v), inositol-free Difco vitamin mix, and vitamin-free yeast base. VPA (1 mM) was added separately as indicated.

Hexokinase activity assay

WT cells were grown in SC medium to the mid log phase and treated with 1 mM VPA for 3 h. Cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$) by vortexing with acid-washed glass beads at 4°C in the presence of 1X protease inhibitor and 1X phosphatase inhibitor. Cell extract was mixed with reaction buffer (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1 mM ATP, 0.5 mM NAD+ and 2 mM glucose). Reactions

were started by adding 1unit/ml glucose-6-phosphate dehydrogenase. Reaction rates were determined by monitoring NADH production at 340 nM in 15 min (233).

Pyruvate kinase assay

WT cells were grown in SC medium to the mid log phase and treated with 1 mM VPA for 3 h, then lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$) by vortexing with acid-washed glass beads at 4°C in the presence of 1X protease inhibitor and 1X phosphatase inhibitor. Cell extract was mixed with reaction buffer (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1 mM ADP, 0.4 mM NADH, 1 unit/mL lactate dehydrogenase and 1 mM phosphoenolpyruvate). Reaction rates were determined by monitoring NADH consumption at 340 nM for 15 min (233).

Measurement of intracellular G-6-P levels

To determine intracellular G-6-P levels, yeast cells were washed twice with ice-cold dH_2O, and lysed in dH_2O containing 1X protease inhibitor by vortexing with acid-washed glass beads at 4°C. Cell extracts were mixed with 1 ml ice cold MeOH:$CHCL_3$ (2:1), vortexed and stored at -20°C for 2 h. Samples were then mixed with extraction solution (50% MeOH, 4 mM tricine pH 5.4) and centrifuged at 18,000 g for 10 min. The upper phase was collected and kept on ice. The lower chloroform phase was extracted again with extraction solution. Upper phases from both extractions were combined, dried with a speedvac, and stored at -80°C. Prior to assay, samples were dissolved in dH_2O. G-6-P content in samples was measured by the enzyme-coupled fluorescence assay developed by Zhu *et al*. with modification (175).

Real-time PCR

Yeast mRNA was extracted from cells using the RNeasy plus Mini Kit (QIAGEN), and was converted to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-PCR was performed using Brilliant III Ultra-Fast SYBR green qPCR master mix (Agilent) and MX3000p qPCR System (Agilent). mRNA levels were normalized to *TCM1* or *ALG9* as indicated.

Fluorescence microscopy

MIG1-GFP:KanMX NRD1-RFP:HghMX cells were grown in SC medium to the early stationary phase, and treated with 1 mM VPA. Fluorescence microscopy was performed using an Olympus BX41 fluorescence microscope. Images were acquired using an Olympus Q-Color3 camera operated by QCapture2 software. All pictures were taken at 400X at room temperature.

Table 4-1. Strains used in this study.

Strain	Genotype	Source
W303	*MATa his3, leu2, ura3, trp1, ade2*	Andoh T. (173)
*mig1*Δ	*MATa his3, leu2, ura3, trp1, ade2, mig1::KanMX*	This study
*reg1*Δ	*MATa his3, leu2, ura3, trp1, ade2, reg1::ble*	This study
MIG1-GFP NRD1-RFP	*MATa his3, leu2, ura3, trp1, ade2,*	Hohmann S. (232)

Table 4-2. RT-PCR primers used in this study.

Gene	Sequence
HXT1	TGTCGGTGGTATCACAGTTT CAAGGTGCCTCTCATTTCAC
HXT2	GTGTCTATTGGGTGGTTCTG GCCTTGGAAGATGGTTGAT
HXT3	CCCAGACCATCCATTCATTC GGCTTACCAGTGAACAACTC
HXT4	GGCTGTTTGGTCTTTTCTTAC AGCGTTGTAGTCAGTACCT
HXT6	CTTTGGAAGAAGTCAACACC CTTATCATCGTGAGCCATTTC
HXT7	CTTTGGAAGAAGTCAACACC CTTGTCATCGTGAGTCATTTC
HXK1	TACCTCCTACCCAGCAAGAA AGAGTGGTCTTGACACCAAAG
HXK2	CTGTGAATACGGTTCCTTCG GTTTGTTGGCCTGGTCTT
GLK1	CTCAAGCACTTGCCTACTAC CAAGAACATCCCTGAGACAC
TCM1	CCACGTCACGGTCATTTAG GTCATACCAGCCTTGTAACC
ALG9	TGGTCTTCTTCCAGGTGAT GGGCGACGATGTCAATAAA

RESULTS

Chronic VPA treatment decreases intracellular G-6-P levels

A microarray analysis carried out by my lab member, Shyamala Jadhav, revealed upregulation of glycolysis genes after chronic treatment with VPA, suggesting that VPA impacts energy metabolism. I conducted a time course study of intracellular G-6-P levels in cells exposed to VPA. As seen in Fig. 4-1, after an initial increase at 30 min, G-6-P levels decreased with chronic exposure to VPA. Hexokinases catalyze the synthesis of G-6-P from glucose (234). VPA did not inhibit hexokinases activities *in vitro* (Fig. 4-2), suggesting that VPA does not inhibit G-6-P synthesis. Pyruvate kinase catalyzes the irreversible conversion of phosphoenolpyruvate to pyruvate, which is one of the rate-limiting and final step of glycolysis (234). Pyruvate kinase activity was not affected by VPA (Fig. 4-2). As activities of enzymes involved in G-6-P synthesis and glycolysis are not affected by VPA, the intracellular G-6-P depletion may result from decreased glucose uptake.

VPA decreases expression of hexose transporter genes

I determined effects of VPA on expression of hexose transporter genes. Surprisingly, VPA treatment resulted in significantly decreased mRNA levels of *HXT2*, *HXT4*, *HXT6*, and *HXT7* (Fig. 4-3). The expression of *HXT1* and *HXT3*, which encode low affinity glucose transporters, was not affected by VPA (data not shown). VPA has been reported to be inhibitor of histone deacetylase (HDAC), which regulates gene expression by deacetylating histones. To determine if changes in transporter gene

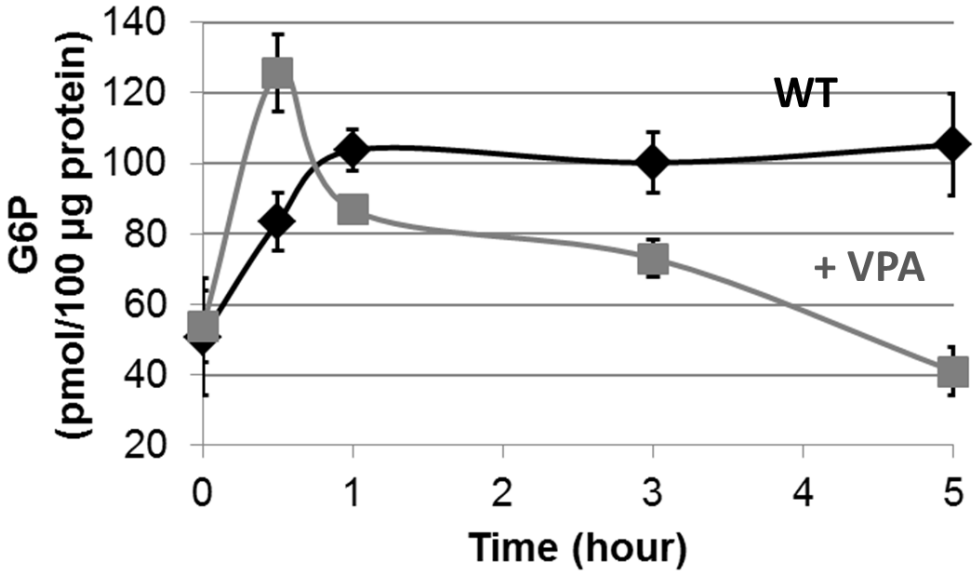

Figure 4-1. VPA depletes intracellular glucose 6-phosphate levels. Cells were grown in SC medium to the early log phase and treated with 1 mM VPA. Intracellular inositol levels were determined as described in "*Materials and Methods*". Data shown are representative of three independent experiments. Values shown are mean ± SEM.

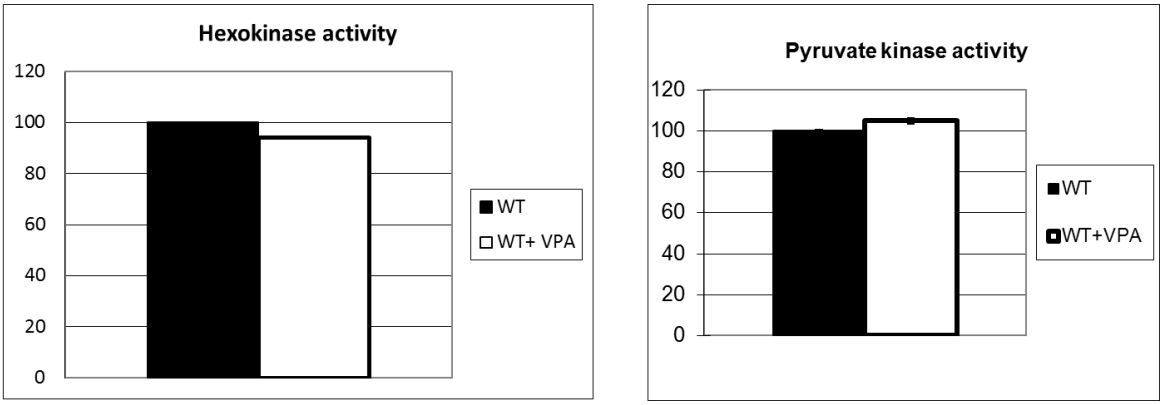

Figure 4-2. VPA does not affect hexokinase (HK) and pyruvate kinase (PK) activities *in vitro*. Yeast cells were grown in SC medium to the early log phase and treated with 1 mM VPA. HK and PK activities were determined as described in "*Materials and Methods*". Values shown are mean ± SEM. Date shown are representative of three independent HK assays and two independent PK assays.

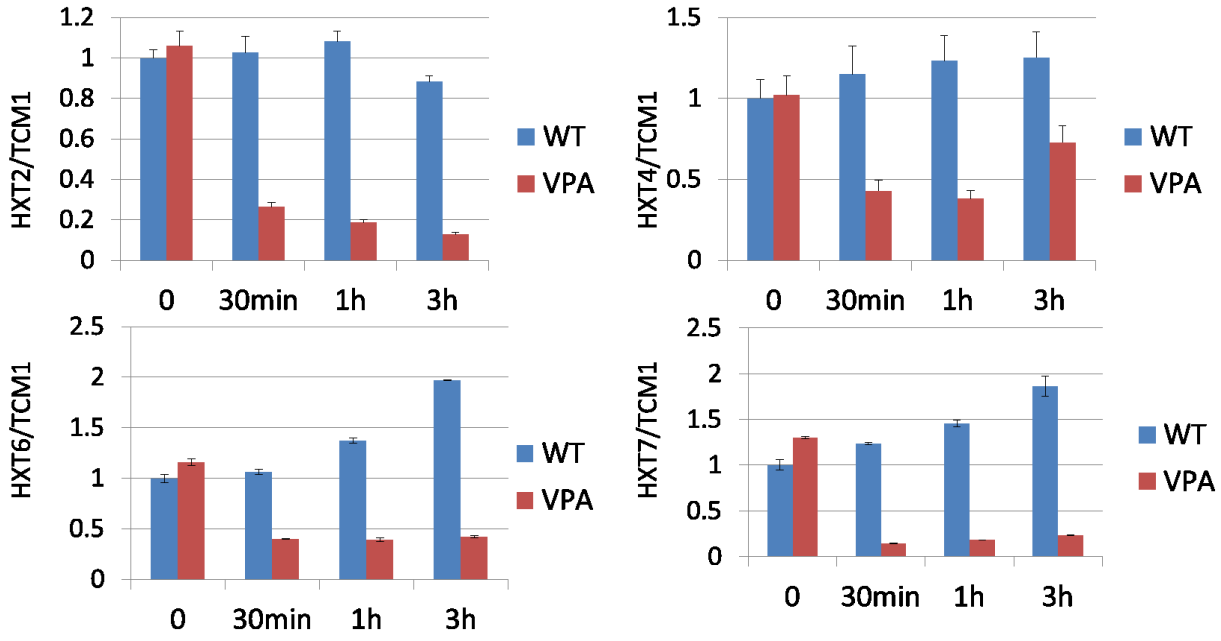

Figure 4-3. VPA inhibits expression of *HXT2*, *HXT4*, *HXT6*, and *HXT7*. Yeast cells were grown in SC medium to the early log phase and treated with 1 mM VPA. mRNA levels of hexose transporter genes were determined by RT-PCR.

expression result from HDAC inhibition, we determined mRNA levels of *HXT2* and *HXT4* in yeast cells treated with TSA, a potent HDAC inhibitor. As seen in Fig. 4-4, TSA treatment did not inhibit *HXT2* and *HXT4* expression, suggesting that VPA did not affect *HXT2* and *HXT4* expression by HDAC inhibition.

VPA-induced decrease in hexose transporter gene expression is Mig1 dependent

The expression of *HXT2* and *HXT4* is repressed by Mig1 (211,223,224). The ablation of *MIG1* delayed the inhibition of *HXT2* and *HXT4* expression in VPA-treated cells (Fig. 4-5), suggesting that Mig1 mediated the effect of VPA on expression of hexose transporter genes. The intracellular localization of Mig1 is determined by its phosphorylation status. Under high glucose conditions, Mig1 is dephosphorylated by the Reg1-Glc7 complex, which leads to Mig1 nuclear translocation to repress *HXT2* and *HXT4* expression (225,230). Under glucose-limited conditions, Mig1 is phosphorylated by Snf1, and therefore excluded from the nucleus to allow expression of *HXT2* and *HXT4* (226). We determined the effect of VPA on Mig1 nucleocytoplasmic distribution in *MIG1-GFP:KanMX NRD1-RFP:HghMX* cells, which express GFP-tagged Mig1 and RFP-tagged nuclear protein Nrd1 (232). Surprisingly, VPA triggered Mig1 nuclear translocation at the early stationary phase (Fig. 4-6), when glucose was depleted in the media. Mig1 nuclear translocation persisted for up to 3 h after VPA treatment. These findings suggest that VPA inhibits *HXT2* and *HXT4* expression by promoting Mig1 mediated transcription repression.

VPA-induced repression of hexose transporter gene expression is Mig1 dependent

As discussed above, Mig1 dephosphorylation by the Reg1-Glc7 complex is

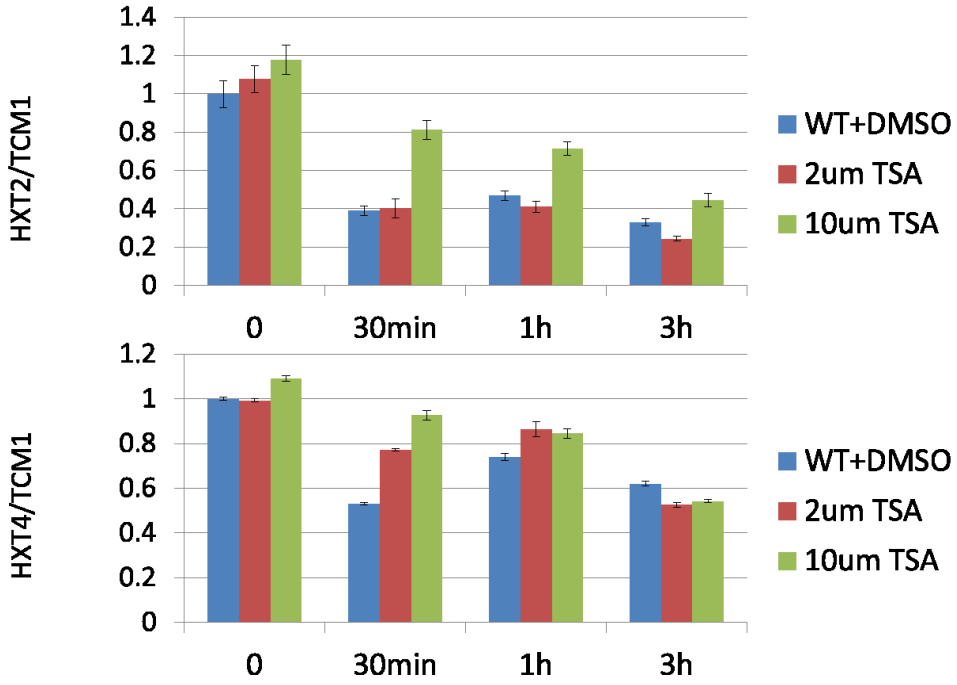

Figure 4-4. Histone deacetylase inhibition does not inhibit *HXT2* or *HXT4* expression. Cells were grown in SC medium to the early log phase and treated with 2 μM or 10 μM TSA. mRNA levels of hexose transporter genes were determined by RT-PCR.

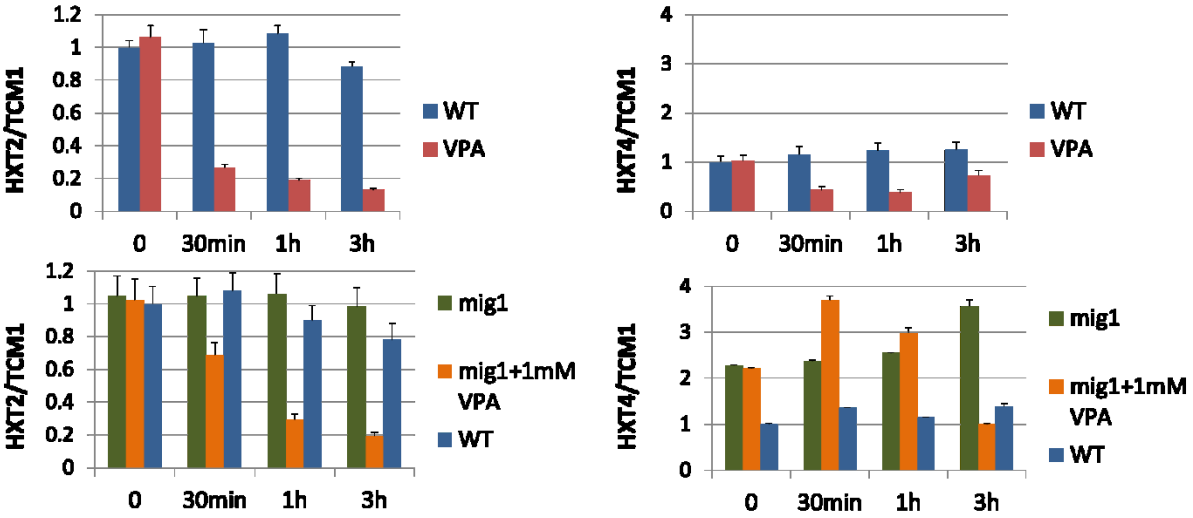

Figure 4-5. VPA-induced inhibition of *HXT2* and *HXT4* expression is delayed in *mig1*∆ cells. WT and *mig1*∆ cells were grown in SC medium to the early log phase and treated with 1 mM VPA. mRNA levels of hexose transporter genes were determined by RT-PCR.

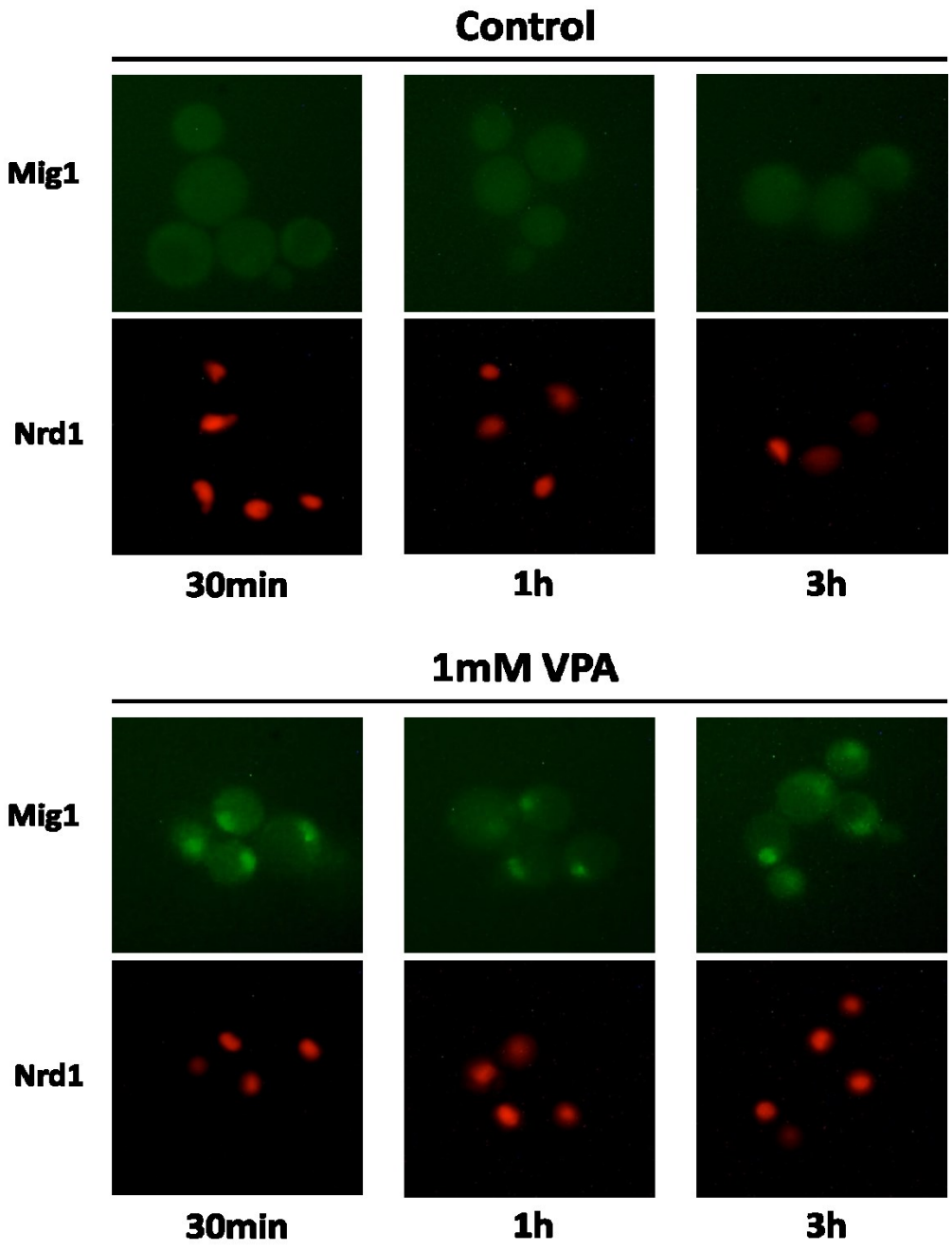

Figure 4-6. VPA triggers Mig1 nuclear translocation under low glucose conditions. *MIG1-GFP:KanMX NRD1-RFP:HghMX* cells were grown in SC medium to the early stationary phase and treated with 1 mM VPA. Mig1 intracellular localization was determined by fluorescence microscopy.

required for Mig1 entry into the nucleus under high glucose conditions. We determined *HXT2* and *HXT4* mRNA1 levels in VPA treated *reg1*∆ cells. VPA did not inhibit *HXT4* expression in *reg1*∆ cells (Fig. 4-7). However, deletion of *REG1* did not restore *HXT2* mRNA levels in VPA-treated cells (data not shown), which suggested that Mig1 may require additional co-factors to repress *HXT2* expression. These findings support the hypothesis that VPA promotes Mig1 dephosphorylation via the Reg1-Gly7 complex to repress expression of hexose transporter genes, and thereby reduces intracellular G-6-P levels.

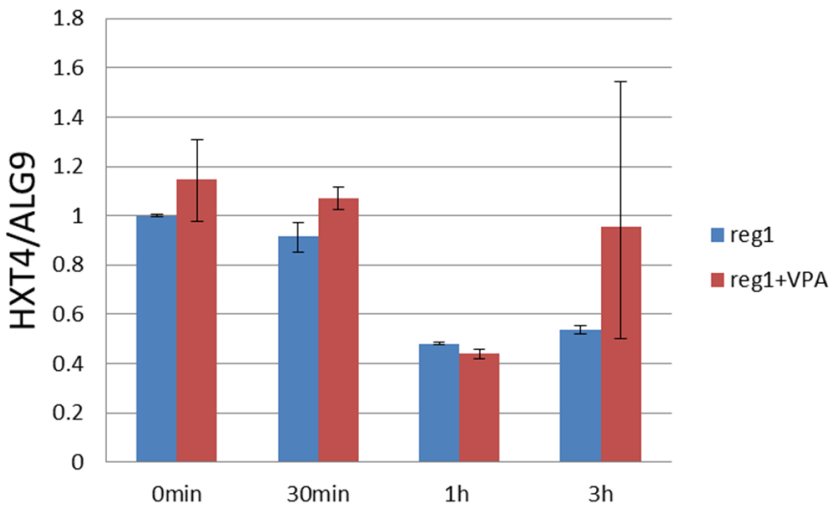

Figure 4-7. Reg1 is required for VPA-induced inhibition of *HXT4* expression. *reg1*Δ cells were grown in SC medium to the early log phase and treated with 1 mM VPA. mRNA levels of *HXT4* were determined by RT-PCR.

DISCUSSION

The current study demonstrates for the first time that VPA depletes intracellular G-6-P levels. Furthermore, VPA promotes Mig1 mediated transcription repression of hexose transporter genes, possibly by activating the Reg1-Glc7 protein phosphatase complex. G-6-P plays a central role in energy metabolism. These findings suggest that VPA-induced cellular effects, including inositol depletion, increased glycolytic gene expression, and increased ethanol production, may result, at least partially, from perturbation of G-6-P metabolism.

Chronic VPA treatment depleted intracellular G-6-P levels (Fig. 4-1). G-6-P is at the branch point of glycolysis, the inositol *de novo* synthesis pathway, the pentose phosphate pathway, and the glycogen synthesis pathway. Therefore, perturbation of G-6-P metabolism is expected to affect these pathways. Glycolysis and inositol synthesis both utilize G-6-P as a substrate. Interestingly, the *Km* of MIPS for G-6-P (1.18 mM) is much higher than that of the glycolytic enzyme phosphoglucoisomerase (0.3 mM), which convert G-6-P to fructose 6-phosphate (235,236). These findings suggest that G-6-P depletion would drive G-6-P flux to glycolysis rather than to inositol synthesis. Consistent with this hypothesis, VPA decreases inositol synthesis (60), upregulates expression of glycolytic genes, and increases ethanol production (personal communication with Shyamala Jadhav and Michael Salsaa). In this light, G-6-P depletion may contribute, at least in part, to VPA-induced inositol depletion.

VPA decreased mRNA levels of *HXT2*, *HXT4*, *HXT6*, and *HXT7* (Fig. 4-3), which encode intermediate-affinity and high-affinity glucose transporters that function optimally at glucose concentrations of 1% or lower (211). Decreased expression of *HXT2* in VPA

treated yeast cells was also observed in a microarray study carried out by my lab member Shyamala Jadhav. The expression of *HXT2*, *HXT4*, *HXT6*, and *HXT7* genes is regulated in response to extracellular glucose concentrations (211). Low glucose (0.1%) induces the expression of *HXT2* and *HXT4*, while high glucose (4%) represses expression of all four genes. The medium used in this study contains 2% glucose. As glucose is gradually consumed, the expression of intermediate-affinity and high-affinity glucose transporters is required by yeast cells to take up glucose efficiently. VPA-induced inhibition of *HXT2*, *HXT4*, *HXT6*, and *HXT7* may decrease glucose uptake and therefore deplete intracellular G-6-P.

Expression of *HXT2* and *HXT4* genes is repressed by the Mig1 repressor under high glucose conditions (211). Deletion of the *MIG1* gene delayed inhibition of *HXT2* and *HXT4* expression after VPA treatment (Fig. 4-5), suggesting that VPA inhibits expression of *HXT2* and *HXT4* via Mig1. The yeast genome contains a *MIG1* homolog, *MIG2*, which has similar but not identical functions in regulating expression of glucose repressible genes (225,237). Therefore, Mig2 may repress transcription of *HXT* genes in response to VPA in *mig1*Δ cells at a slower rate.

Mig1 exhibits dual intracellular localization. Under low glucose conditions, Mig1 is phosphorylated by Snf1 and localizes in the cytosol (226). In high glucose, Mig1 is dephosphorylated by the Reg1-Gly7 protein phosphatase complex, which allows Mig1 to enter the nucleus and repress gene expression (225,230). Interestingly, VPA triggered Mig1 nuclear localization under low glucose conditions (Fig. 4-6), which was consistent with VPA-induced inhibition of *HXT2* and *HXT4* expression. These findings suggest that VPA causes Mig1 dephosphorylation and thereby promotes its

nucleocytoplasmic distribution. Deletion of the *REG1* gene abolished the inhibitory effect of VPA on *HXT4* expression (Fig. 4-7), suggesting that VPA promotes Reg1-Gly7 mediated dephosphorylation of Mig1. In support of this hypothesis, my previous studies have shown that VPA inhibits *HXK1* and *GLK1* expression in 30 min (Fig. 4-8). *HXK1*, *HXK2* and *GLK1* encode glucose kinases that convert glucose to G-6-P (238). In addition, Hxk2 functions as a transcription regulator, which represses expression of *HXK1* and *GLK1* in response to availability of glucose (239). Similar to Mig1, Hxk2 shuttles in and out of the nucleus to regulate gene expression (240). Therefore, the decrease in *HXK1* and *GLK1* mRNA levels in VPA treated cells suggests that VPA triggers nuclear translocation of Hxk2. The nucleocytoplasmic distribution of Hxk2 is determined by the phosphorylation status of the protein. The dephosphorylated Hxk2 is imported into the nucleus, while the phosphorylated form is exported from the nucleus to the cytoplasm (241). Interestingly, the phosphorylation and dephosphorylation of Hxk2 are also carried out by Snf1 kinase and the Reg1-Glc7 protein phosphatase complex (241). These findings suggest that VPA-induced inhibition of *HXK1* and *GLK1* expression may also result from Reg1-Glc7 mediated Hxk2 dephosphorylation.

My study suggests the following model (Fig. 4-9). VPA promotes dephosphorylation of Mig1 via the Reg1-Glc7 complex, and triggers the nuclear translocation of Mig1. In the nucleus, Mig1 represses expression of hexose transporter genes, which leads to decreased glucose uptake. With limited availability of glucose, the synthesis of G-6-P decreases accordingly and results in G-6-P depletion.

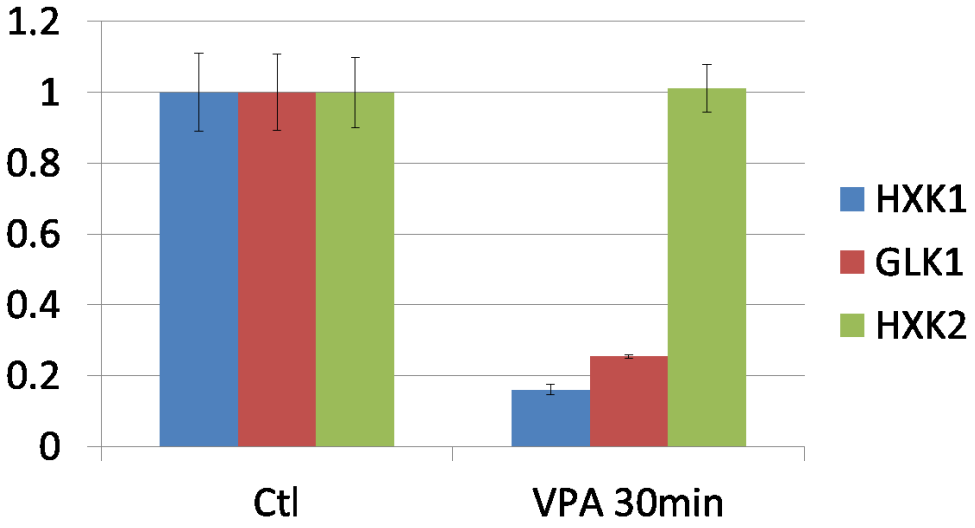

Figure 4-8. VPA decreases *HXK1* and *GLK1* expression in 30 min. WT cells were grown in SC media to the early log phase and treated with 1 mM VPA. mRNA levels of *HXK1*, *HXK2* and *GLK1* were determined by RT-PCR.

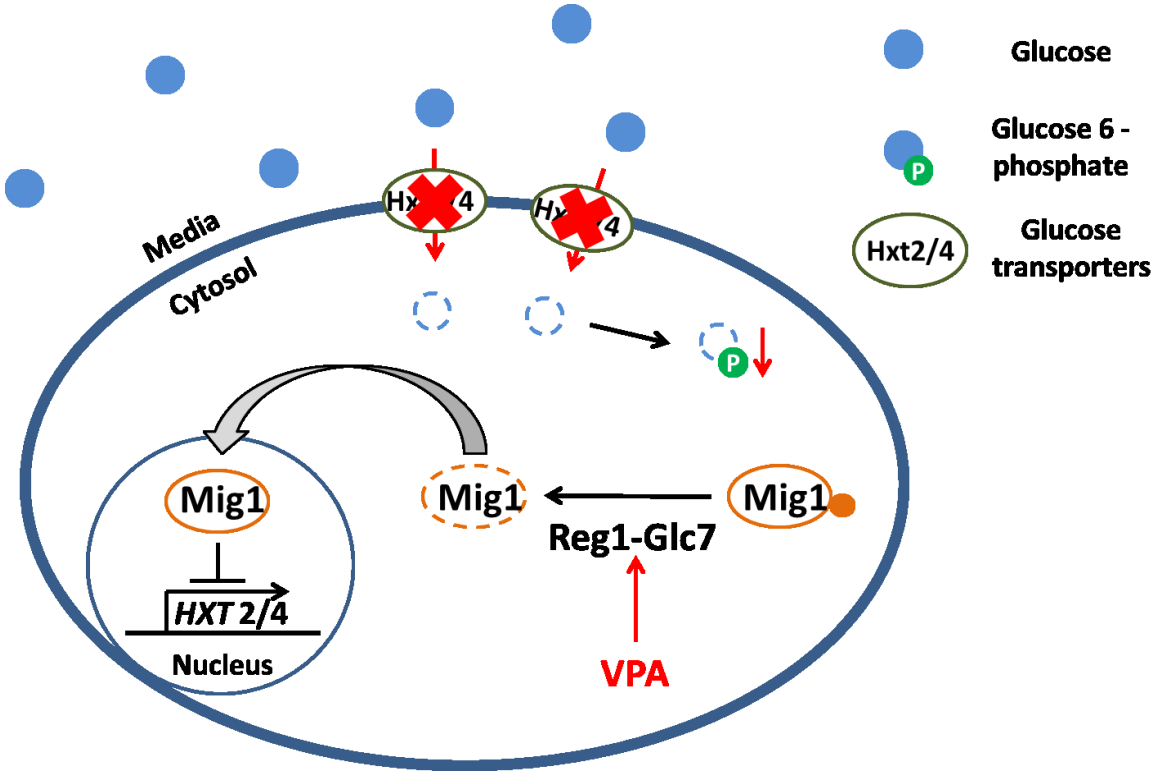

Figure 4-9. Model of VPA-induced glucose 6-phosphate depletion. VPA leads to dephosphorylation of Mig1 via the Reg1-Glc7 complex. Dephosphorylated Mig1 translocates to the nucleus and represses *HXT2/4* transcription. Downregulation of glucose transporter expression decreases glucose uptake. Limited availability of intracellular glucose results in decreased G-6-P synthesis, which contributes, at least in part, to G-6-P depletion.

CHAPTER 5 FUTURE DIRECTIONS

The studies described here identified and characterized novel regulatory mechanisms of inositol biosynthesis, both in yeast and mammalian cells. However, many questions that arose from these studies await answers. The following discussion describes questions that may be developed into research projects for future students.

1. How does Mck1 regulate MIPS activity in response to VPA?

In Chapter 2, I have shown that deletion of *MCK1* decreased MIPS activity (Fig. 2-3). As discussed before, MIPS activity is regulated by phosphorylation (64). In addition, Mck1 has been shown to phosphorylate a protein substrate and therefore regulate its activity (242). However, Mck1 did not phosphorylate MIPS *in vitro* (Fig. 2-5). There are at least two possible explanations. First, the phosphorylation of MIPS by Mck1 may require co-factors or scaffold proteins that target Mck1 to MIPS. The second possibility is that Mck1 does not phosphorylate MIPS directly. Instead, Mck1 regulates a down-stream pathway that phosphorylates MIPS. The mechanism whereby Mck1 regulates MIPS activity remains to be determined.

Mck1 has been shown to mediate VPA-caused MIPS inhibition (Fig. 2-4). However, it is not clear how VPA regulates Mck1. In mammalian cells, Gsk3 activity is regulated at the phosphorylation level by PI3K/Akt pathways (106,107). It is possible that VPA regulates Mck1 by a conserved mechanism in yeast. Further investigation is required to characterize the signaling pathway between VPA and Mck1.

In addition, the simultaneous deletion of *MRK1*, *MDS1*, and *YGK3* results in increased intracellular inositol levels, suggesting that at least one of these genes

downregulates inositol synthesis. It will be interesting to study how these genes affect inositol metabolism.

2. What is the role of inositol pyrophosphate in the regulation of inositol synthesis in mammalian cells?

As discussed in Chapter 3, IP6K1-KO cells exhibited decreased DNA methylation levels at the *mIno1* promoter region (Figure 3-3). How does IP6K1 or its product, inositol pyrophosphate, regulate DNA methylation? I hypothesize that inositol pyrophosphate affects enzymatic activity of DNA methyltransferase. Future work should focus on potential links between existing regulatory mechanisms of DNA methylation and functions of inositol pyrophosphate.

I also showed that IP6K1 negatively regulates *mIno1* expression (Fig. 3-2, 3-7). IP6K1 has been reported to have direct interaction with chromatin (26), which suggests a close association between IP6K1 and DNA. I hypothesize that IP6K1 or its product, inositol pyrophosphate, represses *mIno1* transcription by inhibiting the assembly of the transcription complex. Addressing this hypothesis will benefit the characterization of functions of inositol pyrophosphate in the regulation of inositol synthesis.

3. What is the role of VPA in regulating glucose metabolic flux?

Studies have been reported that VPA inhibits inositol synthesis and increases glycolysis. G-6-P is the common substrate for both pathways. VPA also depletes intracellular G-6-P levels, which is at the branch point of glycolysis, inositol synthesis, the pentose phosphate pathway, and the glycogen synthesis pathway. These findings suggest that many of the plethora of biochemical effects of VPA may result from perturbation of G-6-P metabolism. It is of great importance to determine the effect of

VPA on glucose metabolic flux. The elucidation of this question will greatly benefit the understanding of the therapeutic mechanism of VPA.

Finally, I want to emphasize that we have certainly not finished the exciting journey towards the exploration, identification, and characterization of functions of the pivotal molecule inositol, and the underlying regulatory mechanisms that control its synthesis. It is my privilege if my dissertation sparks new ideas for future students. Together, we push science to a new frontier.

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ABSTRACT

NOVEL REGULATORY MECHANISMS OF INOSITOL BIOSYNTHESIS IN *SACCHAROMYCES CEREVISIAE* AND MAMMALIAN CELLS, AND IMPLICATIONS FOR THE MECHANISM UNDERLYING VPA-INDUCED GLUCOSE 6-PHOSPHATE DEPLETION

by

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August 2016

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Myo-inositol is the precursor of all inositol containing molecules, including inositol phosphates, phosphoinositides and glycosylphosphatidylinositols, which are signaling molecules involved in many critical cellular functions. Perturbation of inositol metabolism has been linked to neurological disorders. Although several widely-used anticonvulsants and mood-stabilizing drugs have been shown to exert inositol depletion effects, the mechanisms of action of the drugs and the role of inositol in these diseases are not understood. Elucidation of the molecular control of inositol synthesis will shed light on the pathologies of inositol related illnesses.

In *Saccharomyces cerevisiae*, deletion of the four glycogen synthase kinase-3 genes, *MCK1*, *MRK1*, *MDS1*, and *YGK3*, resulted in multiple features of inositol depletion. My studies demonstrated that the *MCK1* gene is required for normal inositol homeostasis. *mck1*Δ and *gsk3*Δ (*mck1*Δ*mrk1*Δ*mds1*Δ*ygk3*Δ) cells exhibited similar features of inositol depletion. *MCK1* ablation led to decreased *myo*-inositol-3-phosphate

synthase (MIPS) activity and a decreased rate of inositol *de novo* synthesis. This is the first demonstration that Mck1 controls inositol synthesis by regulating MIPS activity.

While elegant studies have revealed several inositol-regulating mechanisms in yeast, very little is known about regulation of inositol synthesis in mammals. My studies discovered that IP6K1, an inositol hexakisphosphate kinase that catalyzes the synthesis of inositol pyrophosphate, negatively regulates inositol synthesis in mammalian cells. Interestingly, IP6K1 preferentially bound to the phospholipid phosphatidic acid, and this binding was required for IP6K1 nuclear localization and the transcriptional regulation of *Isyna1*, which encodes mammalian MIPS. This is the first demonstration of the molecular control of *de novo* synthesis of inositol in mammalian cells.

VPA depletes intracellular glucose 6-phosphate in yeast cells by an unidentified mechanism. My studies discovered that VPA inhibits expression of hexose transporter genes *HXT2*, *HXT4*, *HXT6*, and *HXT7*. Mig1, a DNA-binding transcription repressor that translocates to the nucleus to repress gene expression under high glucose conditions, is required to inhibit *HXT2* and *HXT4* expression. Interestingly, VPA triggered Mig1 nuclear localization under non-repressive conditions. Furthermore, ablation of *REG1*, which regulates Mig1 translocation, reversed VPA-induced inhibition of *HXT4* expression. These findings suggest that VPA may inhibit glucose uptake by activating Mig1-mediated repression of hexose transporter genes.

AUTOBIOGRAPHICAL STATEMENT

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Publications

Yu, W. and Greenberg, M. L. Mck1 regulates the rate of inositol synthesis by promoting *myo*-inositol-3-phosphate synthase (MIPS) activity in S*accharomyces cerevisiae*. (In preparation)

Yu, W. and Greenberg, M. L. A novel method of measuring the rate of inositol *de novo* synthesis in physiological conditions. (In preparation)

Yu, W. and Greenberg, M. L. VPA depletes intracellular glucose 6-phosphate by repressing the transcription of glucose transporter genes. (In preparation)

Yu, W.*, Ye, C.*, and Greenberg, M. L. (2016) Inositol Hexakisphosphate Kinase 1 (IP6K1) Regulates Inositol Synthesis in Mammalian Cells†. *The Journal of biological chemistry* 291, 10437-10444 (183). (*equal contributors, †selected as a *JBC* paper of the week)

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