

# The Transcriptional Activator Ino2p Dissociates from the Yeast *INM1* Promoter in Induction

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Mood stabilizers lithium and valproates are widely used in the treatment of bipolar disorder. It has been shown that these drugs can affect the inositol monophosphatase activity and thus the inositol *de novo* biosynthesis. However, the molecular mechanism of this action has thus far been vague. As such, characterizing the regulation of the gene encoding inositol monophosphatase at the molecular level can help to understand the bipolar disorder. As the model organism, the inositol monophosphatase is encoded by *INM1* in *Saccharomyces cerevisiae*. In this study, we showed, using real-time reverse transcriptase polymerase chain reaction analysis, that *INM1* is expressed in the presence of inositol, suggesting that the presence of inositol is required for *INM1* transcriptional activation. We also demonstrated, using chromatin immunoprecipitation, that Ino2p is present at the promoter under uninduced conditions. Upon induction, Ino2p dissociates from the *INM1* promoter. Furthermore, chromatin remodelers Ino80p and Snf2p are recruited to *INM1* promoter upon induction as well as histone acetylases Gcn5p and Esa1p. Altogether, we have provided the evidence which describes how the transcriptional activator and coactivators participate in *INM1* activation.

## Introduction

**B**IPOLAR DISORDER IS A NEUROLOGICAL condition which always leads to cyclic and extreme changes in mood, and it is the most common serious neurological disorder with estimates of its worldwide occurrence of about 4%. Due to the recurrent mood swings from depression to mania or from depression to euthymic, bipolar disorder has the highest suicidal risk among all psychiatric disorders. It is estimated that ~10–20% of patients commit suicide over the course of their illness. As such, bipolar disorder imposes a great burden on both patients and their families (Ludtmann *et al.*, 2011; Geddes and Miklowitz, 2013; Yerevanian and Choi, 2013).

Mood stabilizers play a very important role in the treatment of bipolar disorder. Two conventional anti-bipolar drugs, lithium and valproic acid (VPA) have been widely used in the treatment of bipolar disorder. However, the response rate of monotherapy with these drugs can only yield the optimum results in about one-third of the patients (Rybakowski *et al.*, 2001; Gould *et al.*, 2007). Because of such poor response rates, research into bipolar disorder remains a hugely complex undertaking.

It has been demonstrated that lithium treatment can regulate the attenuation of inositol signaling and phosphoinositol signaling (Berridge *et al.*, 1989; King *et al.*, 2009). The therapeutic role of VPA has also been associated with the

reduction of *de novo* inositol synthesis and phosphoinositol depletion (Berridge *et al.*, 1989; Agam *et al.*, 2002; Eickholt *et al.*, 2005; Shaltiel *et al.*, 2007; Shimshoni *et al.*, 2007). As such, it has been proposed that inhibition of inositol monophosphatase (IMPase) by lithium results in depletion of brain inositol, an increase in inositol-3-phosphate, downregulation of the phosphoinositide signaling cycle, and subsequent dampening of overactive neurotransmission (Berridge, 1985; Berridge *et al.*, 1989). However, little is known about the molecular mechanism of this regulation. Therefore, it is imperative to identify and characterize the changes in cell signaling caused by the lithium and VPA treatments. Furthermore, the efficacy of lithium and VPA treatments can be understood through the study of the molecular mechanism of the therapeutic actions. As a result, we can understand the cause of bipolar disorder.

As an excellent model system, *Saccharomyces cerevisiae* has been used to study the molecular mechanism of inositol biosynthesis mediated by the VPA and lithium therapeutic effect. It has been reported that VPA, like lithium, causes inositol depletion in yeast (Vaden *et al.*, 2001). The depletion of inositol might suggest the decrease in activity of IMPase, which is encoded by *INM1* in yeast. IMPase catalyze the conversion of inositol-1-phosphate to inositol. Although the regulation of *INM1* has been studied at the cellular level to evaluate the effect of lithium and VPA in *INM1* expression (Murray and Greenberg, 2000; Agam

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*et al.*, 2002; Shaldubina *et al.*, 2002; Shamir *et al.*, 2003), the molecular mechanism of *INM1* expression has not been established. As such, we were interested in understanding the molecular mechanism of *INM1* expression. In this study, we performed biochemical analysis to examine how the transcriptional activator and transcriptional coactivators are involved in *INM1* activation.

## Materials and Methods

### Yeast strains and growth conditions

Both wild type (WT; *INO2-FLAG*; *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ino2Δ::pESC-URA-INO2FLAG*) and *ino2Δ* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ino2Δ*) yeast strains were used in this study. The pESC-URA-INO2FLAG PLASMID was constructed as described below. The *INO2 ORF* was cloned into *NotI* and *SpeI* sites of pESC-URA plasmid so the *INO2 ORF* carries the C-terminal FLAG (#217454; Agilent). The resulting pESC-URA-INO2FLAG was confirmed by the sequencing. Subsequently, the pESC-URA-INO2FLAG was introduced into *ino2Δ* strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ino2Δ*) and the resulting WT (*INO2-FLAG*) strain was confirmed by Southern blot analysis and polymerase chain reaction (PCR). The yeast culture was grown at 30°C in either synthetic complete media without uracil (SC-ura, #114410622; MPBIO) or synthetic complete media (SC, #114400022; MPBIO), containing 2% glucose (w/v) with 100 μM *myo*-inositol or without *myo*-inositol for WT (*INO2-FLAG*) and *ino2Δ* cells, respectively.

### RNA preparation and real-time reverse transcriptase PCR analysis

The total RNA preparation, first strand cDNA synthesis and real-time reverse transcriptase PCR (qRT-PCR) analysis was performed as described previously (Esposito *et al.*, 2010; Wimalarathna *et al.*, 2011, 2012). Briefly, 250 mL cells were harvested and resuspended in 400 μL lysis solution (10 mM Tris HCl pH 7.5, 10 mM EDTA, 0.5% SDS) followed by acid phenol (pH 4.3) extraction. After 1 h incubation at 65°C, the mixture was subjected to centrifugation, and the aqueous phase was subjected to chloroform extraction, ethanol precipitation, and was resuspended in 50 μL DEPC-treated H<sub>2</sub>O.

Equal amounts (10 μg) of total RNA were treated with RNase-free DNase (cat#79254; Qiagen) at 37°C for 1 h, and purified by phenol/chloroform (3:1) extraction and ethanol precipitation. One microgram of pure RNA was used in the SYBR GreenER Two-Step qRT-PCR kit (cat#11765-100; Invitrogen) for the first strand cDNA synthesis and real-time PCR reaction preparation as described in the manufacturer's manual. All experiments were repeated three times, and in each experiment, PCR reactions were done in triplicate in a 7500 sequence detection system (Applied Biosystems). All qRT-PCR primers are listed in the Supplementary Materials and Methods (Supplementary Data are available online at [www.liebertpub.com/dna](http://www.liebertpub.com/dna)).

The target DNA sequence quantities were estimated from the threshold amplification cycle number ( $C_T$ ) using the Sequence Detection System software (Applied Biosystems). Each DNA quantity was normalized to the *ACT1* DNA quantity by taking the difference between each gene's  $C_T$  and *ACT1*'s  $C_T$  value, which is a  $\Delta C_T$  value. Each relative

RNA fold change was calculated with the following formula:  $2^{(-\Delta C_T)}$  (Esposito *et al.*, 2010; Wimalarathna *et al.*, 2011, 2012).

### ChIP and real-time PCR analysis

The preparation of cross-linked chromatin immunoprecipitation (IP) procedures, and the real-time PCR analyses are as described previously (Esposito *et al.*, 2010; Wimalarathna *et al.*, 2012). All real-time PCR primers, TaqMan MGB probe and the antibody used in the study are listed in the Supplementary Materials and Methods. Input DNA was obtained before IP, whereas mock DNA was obtained without antibody treatment. Mock DNA and all ChIP-derived DNA were precleared with Salmon Sperm DNA/Protein A agarose (50%) to reduce nonspecific background. All experiments were repeated three times, and in each experiment, PCR reactions were done in triplicate.

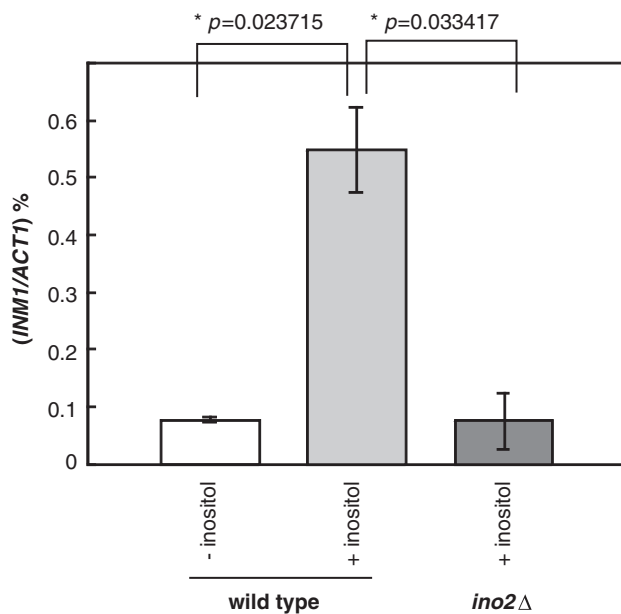
Target DNA sequence quantities were estimated as described below. A corrected  $C_T$  was calculated by subtracting the  $C_T$  value for the corresponding mock (negative control) from the  $C_T$  value for the IP or for the input sample. Subsequently, the (IP/INPUT)% (normalized IP) was calculated for each sample as described below. First of all, the  $\Delta C_T$  value was calculated by subtracting the corrected  $C_T$  value for the IP sample from the corrected  $C_T$  value for the corresponding input DNA to normalize the differences in ChIP aliquots. Second, each IP quantity was then calculated with the following formula:  $2^{(\Delta C_T)} \times 100\%$  to represent (IP/INPUT)  $\times 100\%$  (normalized IP).

## Results

### *INM1* is expressed in the presence of *myo*-inositol

To study how *myo*-inositol affects the *INM1* expression, WT (*INO2-FLAG*) cells were grown at 30°C in SC-ura (synthetic complete media without uracil) containing 2% glucose (w/v) and 10 μM inositol. When the optical density reached 0.9–1.1, cells were harvested and washed twice with SC-ura-ino to remove inositol completely. Subsequently, one half of cells were resuspended in SC-ura with 100 μM inositol and incubated at 30°C for 2 h. On the other hand, the other half of cells were resuspended in SC-ura-ino and incubated at 30°C for 2 h. Subsequently, cells were collected by centrifugation for total RNA preparation followed by qRT-PCR analysis. Our results showed that the relative ratios of *INM1/ACT1* were 0.08 and 0.55 under the absence or the presence of inositol, respectively (Fig. 1). The *INM1* expression increased significantly, which is about seven-fold in the presence of 100 μM inositol. This observation suggests that *INM1* is expressed in the presence of inositol. As such, *INM1* is uninduced in the absence of inositol and it is induced in the presence of inositol.

Previously, it was identified that *INM1* promoter contains the UAS<sub>INO</sub> consensus sequences (Wimalarathna *et al.*, 2012). It has also been demonstrated that the transcriptional activator Ino2p is required to bind to the UAS<sub>INO</sub> element for transcriptional activation of UAS<sub>INO</sub>-containing genes (Schwank *et al.*, 1995). It is instructed to examine whether Ino2p plays a role in *INM1* expression. In this study, the *ino2Δ* cells were grown in the presence of inositol. Our results showed that the expression levels of *INM1* were



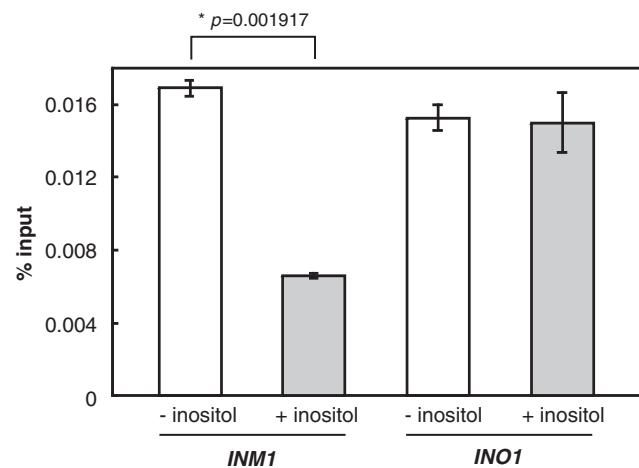
**FIG. 1.** *INM1* is expressed in the presence of inositol. *INM1* and *ACT1* mRNA were detected by qRT-PCR with *INO1* and *ACT1* probes, respectively. Mid-log phase WT (INO2-FLAG) cells or *ino2Δ* cells were grown 2 h at 30°C in synthetic complete media without uracil (SC-ura) or synthetic complete media (SC) containing 2% glucose (w/v) with 100 μM *myo*-inositol or without *myo*-inositol, respectively. qRT-PCR, real-time reverse transcriptase polymerase chain reaction; WT, wild type.

similar to the WT (INO2-FLAG) cells in the absence of inositol, suggesting that *INM1* is not expressed under the inducing conditions in the absence of Ino2p (Fig. 1). Therefore, *INM1* induction was inhibited in *ino2Δ* cells, suggesting that Ino2p plays an important role in *INM1* transcriptional activation.

#### *Ino2p* departs from the *INM1* promoter upon induction

As *INM1* expression was inhibited in the absence of Ino2p, we are interested in examining whether Ino2p regulates *INM1* expression directly. ChIP coupled with real-time PCR (qPCR) analysis was performed. WT (INO2-FLAG) cells were grown in SC-ura until mid-log phase. For inducing conditions, cells were harvested and washed twice with a complete synthetic medium without inositol (SC-ura-ino) to remove inositol completely. Subsequently, cells were resuspended in SC-ura with 100 μM inositol and incubated at 30°C for 2 h. For repressing conditions, cells were resuspended in SC-ura-ino and incubated at 30°C for 2 h. Subsequently, cells were fixed and immunoprecipitated with antibodies against FLAG followed by qPCR analysis.

The IP signals were observed at the *INM1* upstream activating sequences (*UAS*). The relative IP value represented the ratio of *INM1* promoter IP DNA normalized to *INM1* input DNA (Esposito *et al.*, 2010; Konarzewska *et al.*, 2012; Wimalarathna *et al.*, 2012). Our results showed that Ino2p IP value was 1.7% under uninduced conditions (Fig. 2). The Ino2p IP value decreased to 0.7% under induced conditions. The decrease is more than 50% and is significant. As a control, we also examined the presence of Ino2p IP at the *INO1* promoter, and our results showed that the quantity of



**FIG. 2.** Ino2p departs from the *INM1* promoter in induction. Real-time PCR of DNA immunoprecipitated with antibodies against Ino2p-FLAG ( $\alpha$ -FLAG) at *INM1* promoter and *INO1* promoter. All experiments were repeated at least three times and, in each experiment, PCR reactions were done in triplicate. The % of input is graphed as mean  $\pm$  standard deviation.

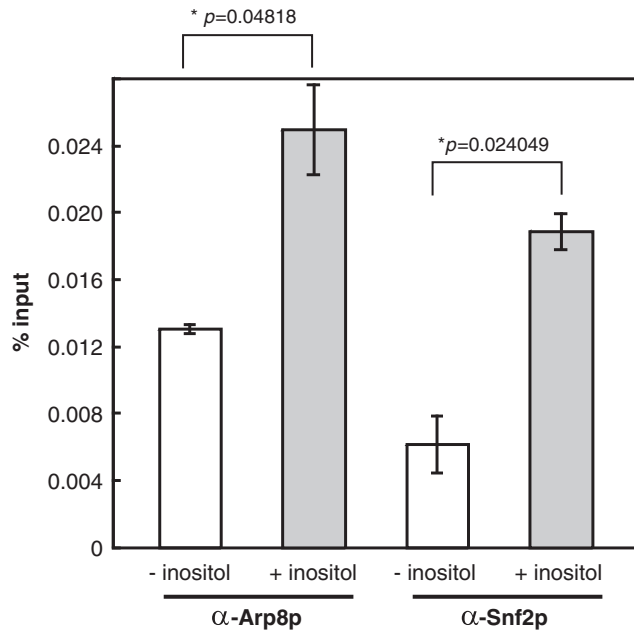
Ino2p remained constant at the *INO1* under both conditions (Fig. 2). The result that Ino2p presents at the *INO1* promoter constitutively is consistent with previous observations (Brickner and Walter, 2004; Dasgupta *et al.*, 2005; Ford *et al.*, 2007; Ford *et al.*, 2008). As such, our result suggests that Ino2p is present at the *INM1* promoter under uninduced conditions, and it departs from the promoter after induction.

#### *The recruitment of chromatin remodelers in INM1 induction*

It has been shown that both Ino80p and Snf2p are directly involved in *INO1* expression at the transcriptional levels (Ford *et al.*, 2007; Ford *et al.*, 2008; Konarzewska *et al.*, 2012). As one of the *UAS<sub>INO</sub>*-containing genes, it is instructed to examine whether these two chromatin remodelers also participate in the *INM1* gene expression. ChIP coupled with qPCR analysis was again performed here. Antibodies against both Arp8p subunit of INO80 (Arp8-IP) and Snf2p subunit of SWI/SNF (Snf2-IP) were used in the ChIP. The relative IP value represented the ratio of promoter IP DNA normalized to input DNA. Our results showed that the quantity of Arp8-IP is 1.3% and 2.5% for uninduced and induced WT (INO2-FLAG) cells, respectively (Fig. 3). The increase of INO80 upon induction is about two-folds. For Snf2-IP, the quantity of Snf2p is 0.6% and 1.8% for uninduced and induced WT (INO2-FLAG) cells, respectively. The increase of Snf2p is more than two-folds upon induction. As such, our results demonstrated that both Ino80p and Snf2p remodelers are recruited to *INM1* promoter upon induction and the presence of both remodelers at the *INM1* promoter suggests that they are directly involved in *INM1* expression.

#### *Both Gcn5p and Esa1p histone acetylases are directly involved in INM1 induction*

It has been demonstrated that both Gcn5p and Esa1p are responsible for histone acetylation during *INO1* activation



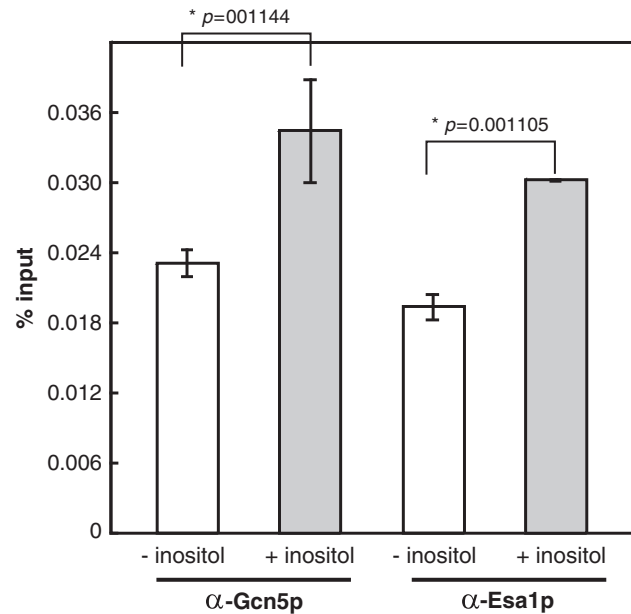
**FIG. 3.** Both INO80 and SWI/SNF chromatin remodelers are recruited to the *INM1* promoter in induction. Real-time PCR of DNA immunoprecipitated with antibodies against INO80 ( $\alpha$ -Arp8p) and SWI/SNF ( $\alpha$ -Snf2p) at *INM1* promoter. Annotation was as described in the legend of Figure 2.

(Esposito *et al.*, 2010; Konarzewska *et al.*, 2012). To examine whether these histone acetylases are also recruited to the *INM1* promoter, WT (INO2-FLAG) strain was used in the ChIP analysis. Antibodies against both Gcn5p (Gcn5-IP) and Esa1p (Esa1-IP) were used in the ChIP. Results showed that the relative IP values for Gcn5-IP were 2.3% and 3.4% of input under repressing and inducing conditions, respectively (Fig. 4). These results suggested that Gcn5p is recruited to the *INM1* promoter upon transcriptional activation. Similar results were also observed for Esa1p, which showed the relative IP values were 1.9% and 3.0% of input under repressing and inducing conditions, respectively (Fig. 4). As such, both histone acetylases are recruited to the *INM1* promoter during induction.

### Discussion

RNA analysis was performed to understand how phospholipid precursor inositol regulates the *INM1* expression. We observed that *INM1* is expressed in the presence of inositol and it is repressed in the absence of inositol (Fig. 1). Previously, it has been shown that *INO1* is repressed in the presence of inositol and it is derepressed in the absence of inositol (Ford *et al.*, 2007; Ford *et al.*, 2008; Wimalaratna *et al.*, 2011). As such, the regulatory pathway of *INM1* is completely opposite from *INO1*. Furthermore, our results showed that *INM1* induction is inhibited in *ino2A* cells, suggesting that Ino2p is required for *INM1* induction.

We have shown that the Ino2p is present at the *INM1* promoter in the repressing conditions and a significant amount departs from the *INM1* promoter in the inducing conditions (Fig. 2). It is possible that Ino2p brings in transcriptional coactivators under inducing conditions and then



**FIG. 4.** Both Gcn5p and Esa1p histone acetylases are recruited to the *INM1* promoter in induction. Real-time PCR of DNA immunoprecipitated with antibodies against Gcn5p ( $\alpha$ -Gcn5p) and Esa1p ( $\alpha$ -Esa1p) at *INM1* promoter. Annotation was as described in the legend of Figure 2.

leave the promoter when they complete the activation process. As such, we observed less amount of Ino2p after induction. On the other hand, many studies have demonstrated that Ino2p expression is decreased in the presence of inositol (Ashburner and Lopes, 1995; Eiznhamer *et al.*, 2001). As such, Ino2p occupancy is decreased under the *INM1* inducing conditions because less Ino2p is available in the presence of inositol. Since we observed that Ino2p occupancy remains constant at the *INO1* promoter, the lower Ino2p occupancy at *INM1* promoter might be irrelevant to the Ino2p quantity.

It is possible that the departure of Ino2p from *INM1* promoter is to prevent the binding of the repressor. Many studies have shown that Ino2p constitutively binds to *INO1* promoter (Brickner and Walter, 2004; Dasgupta *et al.*, 2005; Ford *et al.*, 2007; Ford *et al.*, 2008), and the regulation of *INO1* expression depends on the presence of Opi1 repressor in *INO1* promoter. The Opi1p is released from the endoplasmic reticulum (ER) in the presence of inositol. Subsequently, the Opi1 repressor enters the nucleus and interacts with Ino2p to repress *INO1* expression. In the absence of inositol, the Opi1p is anchored by the phosphatidic acid on ER. At this time, Ino2p departs from the *INM1* promoter upon inducing conditions, which is in the presence of inositol. Since Opi1p is released from ER and can interact with Ino2p in the presence of inositol, it is possible that the dissociation of Ino2p from *INM1* promoter is to prevent the negative effect of Opi1p on *INM1* expression.

In an attempt to examine how chromatin remodelers are involved in *INM1* expression, we found that INO80 and SWI/SNF were absent from the *INM1* promoter under uninduced conditions. Both INO80 and SWI/SNF were largely recruited to the *INM1* promoter (Fig. 3). Although this recruitment pattern is different from *INO1* expression, many



studies have shown similar recruitment patterns, including *ARG1*, *CUP1*, and *GALI* (Bryant and Ptashne, 2003; Govind *et al.*, 2005; Wimalarathna *et al.*, 2014). Since we observed the departure of Ino2p from *INM1* promoter upon induction, it is possible that the Ino2p recruits these remodelers before it departs from the promoter. However, our current system cannot examine this possibility. Further biochemical analysis such as kinetic ChIP assay might be able to identify whether Ino2p is required to recruit these remodelers.

Our data also showed that both histone acetylases Gcn5p and Esa1p are recruited to *INM1* promoter upon induction. This observation indicates that both Gcn5p and Esa1p are involved in the *INM1* induction. Recent studies have demonstrated that the recruitment of histone acetylases might not play an important role in the initial activation, but might be important for the departure of chromatin remodelers in the late stage of transcription (Konarzewska *et al.*, 2012). It is not clear whether both histone acetylases are essential for the initial stage of *INM1* activation. As such, further analysis in the recruitment dependency would help to clarify the issue.

In this study, we have defined the inducing conditions for *INM* expression and we have also examined the molecular mechanism of *INM1* induction. It has been shown that the anti-bipolar drugs cause the inositol depletion in yeast (Vaden *et al.*, 2001). In this study, we demonstrated that *INM1* expression is inhibited in the absence of inositol, suggesting that *INM1* expression is inhibited in the presence of anti-bipolar drugs. We also showed both chromatin remodelers and histone acetylases are recruited to *INM1* promoter upon induction. However, further analysis is necessary to uncover the specific role of Ino2p and the interplay within the transcriptional activator, remodelers, and histone modifying enzymes. As such, we will be able to understand the molecular mechanism of *INM1* expression, and understand how anti-bipolar drugs influence its mechanism, which will provide a key insight into the molecular mechanism of bipolar disease.

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### Disclosure Statement

There are no conflicts of interest.

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