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## Two Regulatory Aspects of INO1 Transcription in Yeast

Tschen-wei Chang  
*University of Massachusetts Amherst*

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**TWO REGULATORY ASPECTS OF *INO1* TRANSCRIPTION IN YEAST**

A Thesis Presented

by

TSCHEN-WEI CHANG

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

MASTER OF SCIENCE

February 2015

Department of microbiology

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Approved as to style and content by:

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John M. Lopes, Chair

---

Kevin Griffith, Member

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Yasu Morita, Member

---

John M. Lopes, Department Head Department  
of Microbiology

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## ABSTRACT

### TWO REGULATORY ASPECTS OF *INO1* TRANSCRIPTION IN YEAST

February, 2015

TSCHEN-WEI CHANG, B.S., CHUNG SHAN MEDICAL UNIVERSITY,  
TAICHUNG, TAIWAN

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by Professor John M. Lopes

The long term goal of this study is to understand the mechanisms of transcriptional regulation in the brewer's yeast - *Saccharomyces cerevisiae*. This study is focused on understanding the mechanisms of expression control of a phospholipid biosynthetic gene, *INO1*. This study also includes investigation into transcriptional regulation of a gene in tandem upstream of *INO1*, called *SNA3*.

For more than three decades, *INO1* expression has been used as a model for transcription studies. *INO1* is repressed under growth conditions with inositol and derepressed by two transcription activators, Ino2 and Ino4, when the environment lacks inositol. More recently it was shown that coordination of the centromeric binding factor, Cbf1, with Ino2 and Ino4 is required for efficient derepression of *INO1*. Transcription of the *INO1* adjacent *SNA3* gene is also influenced by inositol. It was shown that *INO1* and *SNA3* are co-regulated by Cbf1, Ino2 and Ino4 but the complex mechanism of regulation of these two genes is not yet fully understood.

A separate aspect of *INO1* expression is that it is growth phase regulated. Under inositol depleted conditions, the expression of *INO1* increases during log phase and decreases during stationary phase. Most genes in yeast are believed to be expressed at a constant level through all growth phases. It is unclear how *INO1* growth phase regulation takes place.

The first part of my work focused on exploring the mechanism through which Cbf1, Ino2 and Ino4 control the inositol-mediated regulation of *INO1* and *SNA3*. This included determining the necessity of the Cbf1 binding site for Ino2 and Ino4 binding, as well as for the inositol mediated regulation of *INO1* and *SNA3*. The second part of my work focused on understanding the growth phase regulation of *INO1*. This includes examining the expression of *INO1* in individual cells in a growing population.

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Overview

Transcriptional regulation is fundamental for proper functioning of cells and for life. Research on the mechanisms that control transcription will help us understand how complex life is made possible. Several model organisms that share high gene functional similarity with humans have been applied in transcriptional studies. Among them, the yeast, *Saccharomyces cerevisiae*, is the most facile and versatile model system for examining eukaryotic gene function and regulation. The complete genome sequence of this rapidly replicating eukaryote has been available to the public since its release in 1996. The ease of culturing and genetic manipulation made *S. cerevisiae* an even more valuable model.

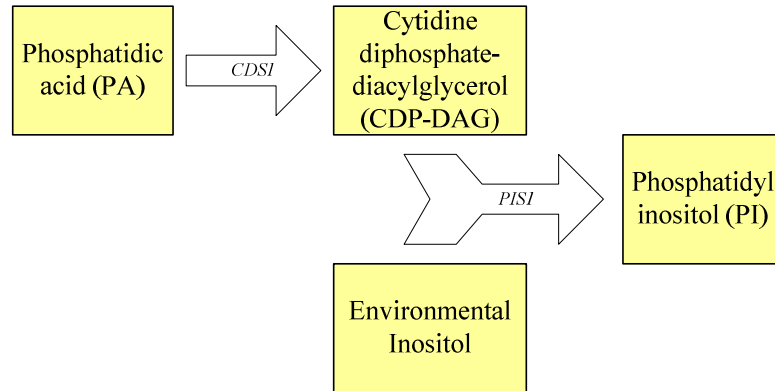
One of the target areas for transcriptional research is phospholipid synthesis. Phospholipids are major components of cellular membranes and are also essential for the regulation of a diverse set of cellular processes, including signaling, cell division, differentiation and development (Allen et al., 1988; Block and Pletscher, 1988; Majerus et al., 1988). Altered regulation of phospholipid biosynthetic genes is often associated with cellular dysfunction (Greenberg and Lopes, 1996). Considering the importance of phospholipids, it is not surprising that phospholipid synthesis is highly regulated and strongly conserved in eukaryotes – from yeast to human. For decades, phospholipid synthesis has been studied in *S. cerevisiae* and the information collected has been an invaluable foundation for understanding this process in higher eukaryotes (Carman and Henry, 1999; Carman and Zeimet, 1996; Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998).

This study is focused on understanding two novel aspects of transcriptional regulation in *S. cerevisiae* that involve the phospholipid biosynthetic gene *INO1* (Chapter 2 and Chapter 3 in this thesis). *INO1* encodes inositol-3-phosphate synthase (IPS) that is required for the *de novo* synthesis of inositol phosphates and inositol-containing phospholipids, like phosphatidylinositol (PI) and its derivatives (Donahue and Henry, 1981; Klig and Henry, 1984) (Fig. 1.1). Deletion of *INO1* results in auxotrophy for inositol. Inositol auxotrophy is a hallmark of defects in the transcription machinery because transcription of *INO1* is exquisitely sensitive to perturbations in the transcription machinery.

When exogenous inositol is present (I+), PA is converted into CDP-DAG, and PI synthase (encoded by *PIS1*) condenses CDP-DAG and inositol to make PI (Fig. 1.1 A). *INO1* is repressed under this condition. However, when inositol is not supplied exogenously to the cell (I-), it can be synthesized *de novo* from G6P via the function of *INO1* (Culbertson et al., 1976; Lopez et al., 1999; Murray and Greenberg, 1997) (Fig. 1.1 B).

Not surprisingly, *INO1* transcription is regulated by the presence of inositol (Carman and Han, 2009; Chen et al., 2007; Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998). Our current understanding of the mechanism of regulation of *INO1* transcription is summarized in Fig. 1.1 and Fig. 1.2. Under I+ conditions, PA is utilized rapidly to make PI. The drop of PA levels in the cytoplasm leads to the release of a PA-binding repressor, Opi1p, from the endoplasmic reticulum (ER). Opi1p immediately translocates into the nucleus and prevents *INO1* transcription by interacting with Ino2, an essential activator for *INO1* expression (Greenberg et al., 1982a; Kaadige and Lopes, 2006; Loewen et al., 2004) (Fig 1.2 A). Under I- conditions, PI synthesis is limited and PA levels are elevated. The repressor Opi1 binds PA and is retained in the ER, and *INO1* transcription is activated (Fig 1.2 B).

A.



B.

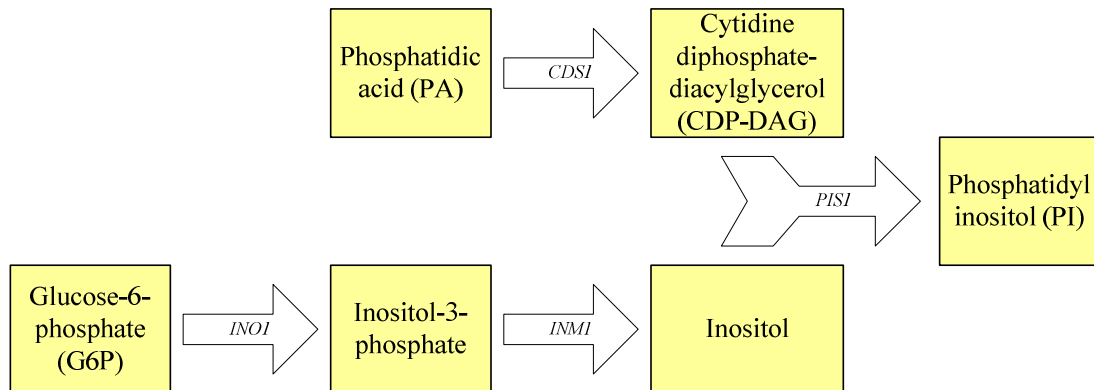
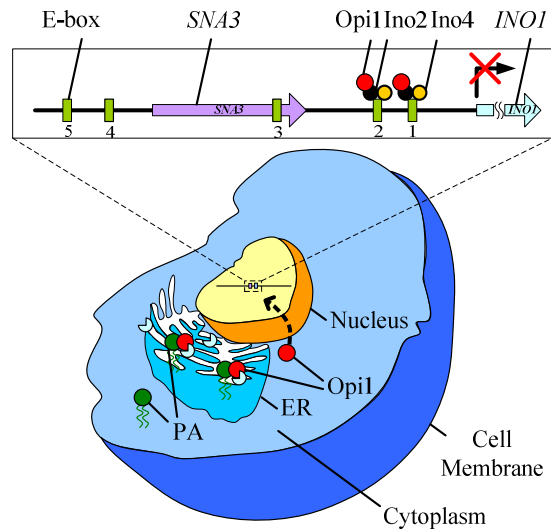


Figure. 1.1. Yeast PI biosynthetic pathway.

PI can be synthesized (A) directly from exogenously supplied inositol or (B) from glucose-6-phosphate. Biosynthetic genes are noted in italics.

A.



B.

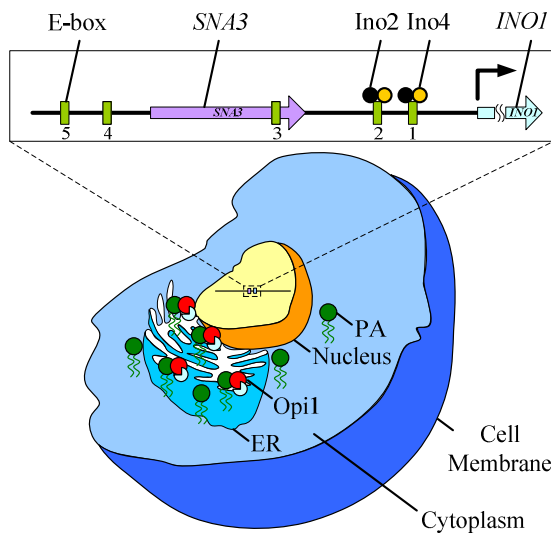


Figure. 1.2. Inositol mediated *INO1* regulation.

Transcriptional regulation of *INO1* (A) When exogenous inositol is supplied, PA levels drop, freeing Opi1 from the endoplasmic reticulum (ER). Opi1 enters the nucleus and inhibits *INO1* expression by binding to Ino2. (B) When the environment lacks inositol, PA levels accumulate and the PA-Opi1 complex is bound to the ER, preventing Opi1 from translocating. *INO1* transcription is turned on by the activators Ino2 and Ino4.

Complete derepression of *INO1* requires three basic helix loop helix (bHLH) transcription activators Ino2, Ino4, and Cbf1 (Ambroziak and Henry, 1994; Lopes and Henry, 1991; Nikoloff and Henry, 1994; Shetty and Lopes, 2010). Ino2 and Ino4 bind as a heterodimer to two Upstream Activation Sequences (E-boxes 1 and 2) in the *INO1* promoter (Koipally et al., 1996), whereas Cbf1 binds to a region farther upstream of *INO1*, a region that spans the ORF and the promoter of a gene in tandem upstream of *INO1*, called *SNA3* (Fig 1.2). The binding of Ino2/Ino4 and Cbf1 are interdependent (Shetty and Lopes, 2010). Interestingly, *SNA3* displays the same inositol-dependent transcriptional regulation as *INO1*. The transcription factors Cbf1, Ino2, and Ino4 have also been shown to regulate *SNA3* expression, although they function in repression in the presence of inositol rather than activation in the absence of inositol as is the case with *INO1* (Shetty and Lopes, 2010).

*INO1* regulation is not only responsive to the availability of environmental inositol, but also to the growth phases. The transcription of *INO1* in a cell culture has been reported to continuously increase during exponential phase, reach the maximum when the culture hits stationary phase, and decrease during stationary phase (Lamping et al., 1994; Robinson et al., 1996). The activity of the *INO1* product, IPS, shows a similar pattern (Culbertson et al., 1976). However, the amount of IPS in the culture does not decrease in stationary phase presumably because of the stability of IPS (Homann et al., 1987).



## 1.2 Organization

The two aspects of transcriptional regulation that this study is focused on are “The inositol mediated co-regulation of *INO1* and *SNA3*” (Chapter 2) and “The growth phase regulation of *INO1*” (Chapter 3). Chapter 4 will summarize the work and provide future directions.

### 1.2.1 The inositol mediated co-regulation of *INO1* and *SNA3*

Since *SNA3* and *INO1* display similar inositol mediated transcriptional regulation and share the transcription factors Ino2, Ino4, and Cbf1, our lab proposed that *SNA3* and *INO1* transcription are co-regulated (Shetty and Lopes, 2010). Studies in our lab showed that, while Cbf1 binding mapped to regions spanning the *SNA3* ORF and promoter, Ino2 and Ino4 only bind to E-boxes 1 and 2 located within the *INO1* promoter (Shetty and Lopes, 2010). These studies also showed that the inositol-mediated regulation of *SNA3* is not a function of its upstream sequences. We suggest a novel transcriptional mechanism in yeast – the first case of transcriptional regulation of a gene from sequences downstream of that gene. Our goal in Chapter 2 is to gain understanding of the *INO1-SNA3* co-regulation mechanism. We will approach this goal by answering the following questions. Question 1: Where exactly does Cbf1 bind? Published data from our lab suggested that Cbf1 binds to a region which covers three Upstream Activation Sequences (UAS): E-boxes 3, 4, and 5 (Shetty and Lopes, 2010). Here we will determine the Cbf1 binding site(s) and the necessity of the E-boxes 4 and 5 for Ino2 and Ino4 binding. E-box mutants will be generated in the genome and the binding of the three bHLH transcription factors will be examined by a CHIP-qPCR method. Question 2: How do E-boxes 4 and 5 affect *INO1* and *SNA3* expression? No current information informs how these two E-boxes located within the *SNA3* promoter participate in the inositol mediated regulation of *INO1* and

*SNA3*. The transcription of the two genes will be examined in E-box 4 and E-box 5 mutants using qRT-PCR. Question 3: Is the inositol mediated regulation of *INO1* and *SNA3* evolutionary conserved within the *Saccharomyces* genus? *INO1* and *SNA3* expression in I+ and I- media will be examined in three *Saccharomyces* species (in addition to *S. cerevisiae*) by qRT-PCR.

### **1.2.2 The growth phase regulation of *INO1***

*INO1*, as well as many other phospholipid biosynthetic genes, was reported to be growth phase regulated (Culbertson et al., 1976; Homann et al., 1987; Lamping et al., 1994; Robinson et al., 1996). In other words, if we record the amount of *INO1* transcript normalized for the transcript level of a housekeeping gene over the course of time, we will observe a curve instead of a flat line. The expression of *INO1* varies with growth phases. Researchers interested in growth phase regulation have looked into *INO1* transcript levels (Lamping et al., 1994; Robinson et al., 1996), protein levels (Homann et al., 1987), as well as enzymatic activity (Culbertson et al., 1976). However, the mechanism of the *INO1* growth phase regulation remains obscure and is a subject of our study. In Chapter 3, we will seek answers to the following question: Is the growth phase regulation of *INO1* an effect of equal participation of all cells or is population dependent? Previous studies examined *INO1* growth phase regulation on a population scale. Our work will investigate the reaction of individual cells in the culture to growth phase changes. Experiments combine qRT-PCR and fluorescence microscopy.

## CHAPTER 2

### THE INOSITOL MEDIATED CO-REGULATION OF *INO1* AND *SNA3*

#### 2.1 Introduction

*INO1* (Inositol-3-phosphate synthase) is a structural gene essential for the de novo synthesis of PI from G6P. In *S. cerevisiae*, the transcription of *INO1* is regulated by inositol (Ambroziak and Henry, 1994; Hirsch and Henry, 1986; Lopes and Henry, 1991; Nikoloff and Henry, 1994). The expression of *INO1* is repressed in the presence of inositol and derepressed when inositol becomes limiting. It was previously reported that an upstream tandem transcript (0.6 Kb) displayed similar regulation (Hirsch and Henry, 1986). The transcript had no known function but has since been named *SNA3*.

The inositol mediated regulation of *INO1* and *SNA3* share at least some of the major players although these players possess opposite functions in the regulation of the two genes (Hirsch and Henry, 1986). These main characters include the transcription factors Ino2, Ino4, Cbf1 and the transcription repressor protein Opi1. The regulation, however, requires not only the effort of these *trans*-acting factors, but also *cis*-acting DNA sequences – two E-boxes in the intercistronic region. An E-box is a transcription factor binding site where the specific sequence of DNA, CANNTG, is recognized by bHLH proteins that can bind to it and activate transcription of the gene.

In an environment where inositol is absent (I-), *INO1* transcription is derepressed (Ambroziak and Henry, 1994; Hirsch and Henry, 1986; Lopes and Henry, 1991; Nikoloff and Henry, 1994). PA levels increase under I- conditions and PA binds to the repressor protein Opi1. This results in the binding of Opi1 to an ER integral membrane protein, Scs2, and thus Opi1 is kept outside of the nucleus (Loewen et al., 2003, 2004). The two bHLH transcription factors

Ino2p and Ino4p form heterodimers and bind to E-boxes 1 and 2, which are located in the *INO1* promoter region (Fig. 2.1) (Ambroziak and Henry, 1994; Koipally et al., 1996). The binding of the Ino2/Ino4 heterodimers leads to recruitment of the TATA binding protein and the activation of transcription (Lo et al., 2005). Ino2, Ino4 and their binding sites E-boxes 1 and 2 are essential for *INO1* derepression. More recently, it was shown that complete derepression of *INO1* also requires the Cbf1p bHLH protein that binds to regions upstream of the *INO1* promoter, encompassing the *SNA3* gene and its promoter (Shetty and Lopes, 2010). These regions cover three potential bHLH transcription factor binding sites: the E-boxes 3, 4, and 5. It has not yet been examined, however, where exactly Cbf1 binds. Ino2, Ino4, and E-boxes 1 and 2 are essential for *INO1* activation, while the absence of Cbf1 causes a dramatic decrease of *INO1* expression when compared to WT (Shetty and Lopes, 2010).

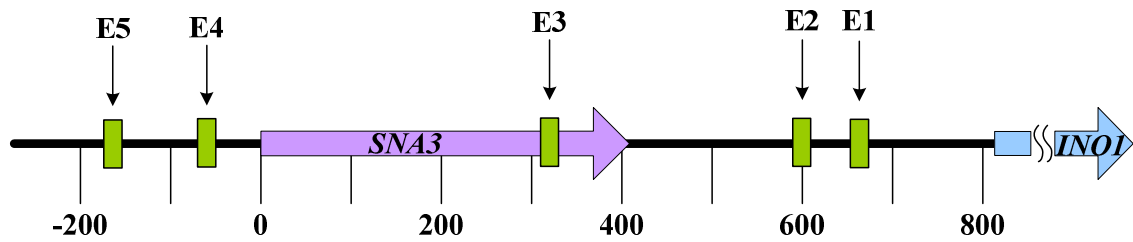
When grown in an environment containing inositol (I+), the *INO1* gene is repressed (Brickner and Walter, 2004; Hancock et al., 2006; Kaadige and Lopes, 2003, 2006; Kagiwada and Hashimoto, 2007; Loewen et al., 2004). Under I+ conditions, the associated decrease in PA levels results in release of Opi1p from the ER. Opi1 travels into the nucleus where it interacts with Ino2 and prevents *INO1* transcription (Gardenour et al., 2004; Heyken et al., 2005; Loewen et al., 2004; Wagner et al., 2001). In a recent study, it was shown that the binding of Ino2 and Ino4 to the *INO1* promoter region and the binding of Cbf1 to the regions spanning the *SNA3* promoter and the *SNA3* ORF are interdependent (Shetty and Lopes, 2010).

It was demonstrated in early *S. cerevisiae* studies that when regulatory sequences were inserted downstream of the transcription start site or within the gene, they become inactive and lose their ability to regulate transcription (Guarente and Hoar, 1984; Struhl, 1984). Thus, it has long been taken for granted that *S. cerevisiae* transcriptional regulation can only involve upstream activation sequences (UAS) that are present within 500bp upstream of the gene but

not downstream of the gene (Struhl, 1989). However, a computational study published in 2000 suggested that tandem adjacent genes in *S. cerevisiae* exhibit a high degree of correlation in gene expression and that only one of the promoters contains the relevant UAS element (Cohen et al., 2000). The results of this study revealed the possibility of regulation from a downstream sequence.

A more recent study showed evidence that, while Ino2 and Ino4 regulate *SNA3* expression, they do not bind upstream of the *SNA3* gene, and that the tandem *SNA3-INO1* genes are subject to regulation from the intergenic region (Shetty and Lopes, 2010). While the expression pattern of *INO1* and *SNA3* is identical, it is achieved by opposing mechanisms. Contrary to *INO1*, *SNA3* transcription is derepressed in the absence of *cis*- sequences in the *SNA3-INO1* intergenic region or the *trans*- activators of *INO1* acting through this intergenic region (Shetty and Lopes, 2010). The detailed mechanism of *SNA3* regulation is not yet understood.

In this chapter, we will examine the importance of E-boxes 4 and 5 to Cbf1 (Fig 2.1), Ino2 and Ino4 binding, as well as the importance of these *cis*- sequences to *INO1* and *SNA3* regulation. We will also look into the *INO1* and *SNA3* transcript levels under I+ and I- conditions in the *Saccharomyces* species: *S. bayanus*, *S. mikatae*, and *S. castellii* (Fig. 2.2). Homologues of *INO1* and *SNA3* can be found in several species of the *Saccharomyces* genus. It is unclear whether *INO1* and *SNA3* expression is also controlled in response to exterior inositol concentration in these organisms.



**E1= CACATG (+662 to +667)**  
**E2= CACATG (+597 to +602)**  
**E3= CACATG (+322 to +327)**  
**E4= CACATG (-72 to -67)**  
**E5= CACATG (-179 to -174)**  
**SNA3= +1 to +402**  
**INO1= +839 to +2440**  
**Numbering is relative to SNA3 ORF**  
**E-boxes are labeled as E**

**Figure. 2.1. Regulatory sequences of the SNA3 and INO1 loci.**

**E-boxes are labeled as E.**

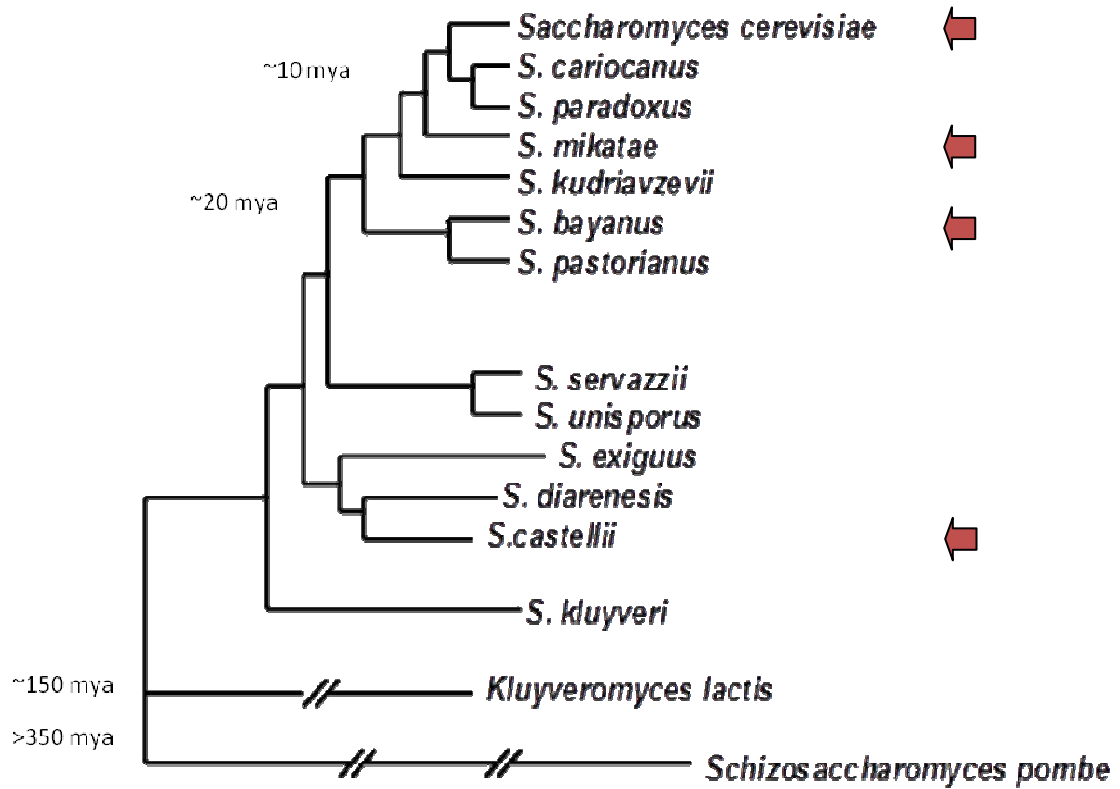


Figure. 2.2. Phylogenetic relationships of selected yeast species.

Arrows indicate *Saccharomyces* species used in this study. Mya stands for million years ago.  
 This figure is reproduced from  
<http://www.ucdenver.edu/academics/colleges/medicalschool/programs/Molbio/faculty/JohnstonM/Pages/JohnstonM.aspx>.

## 2.2 Materials and Methods

### 2.2.1 Strains, media, and growth conditions

The *S. cerevisiae* strain used in this study was BY4742 (MAT $\alpha$  his3-1 leu2-0 lys2-0 ura3-0) (Brachmann et al., 1998). Strains with genomic TAP-tagged *INO2*, *INO4*, and *CBF1* were purchased from Open Biosystems (Huntsville, Alabama, USA) (Ghaemmaghami et al., 2003).

Yeast mutant strains created for this study contain specific point mutations in E-box 4 or E-box 5 in the *SNA3* promoter region. The specific *SNA3* promoter element was replaced with restriction sites to generate mutant alleles in either WT and/or TAP-tagged strains. The mutant alleles were created using a two-step process (Gray et al., 2004). First, an *SNA3* promoter::*URA3* strain was generated by replacing 245 base pairs of the *SNA3* promoter with the *URA3* gene under the control of its own promoter. The *URA3* cassette was flanked by 45 base pairs of DNA homologous to the *SNA3* promoter and was amplified from *YEp357R* (Myers et al., 1986) using the *SNA3* -200to-245\_25bp*URA3* F and *SNA3* +1to+45\_25bp*URA3* R primer pair (Table 2.1). The *SNA3* promoter::*URA3* strain was then used to generate strains carrying *SNA3* promoter mutant alleles by transforming with PCR products created with the E4 mutation primers, the E5 mutation primers (Table 2.1), and the *pGEM-T:SNA3* -310 to +100 plasmid (below) and selecting on 5'FOA medium.

The *Saccharomyces* species strains (generously provided by Dr. Mark Johnston, Washington Univ. School of Medicine, St. Louis, MO) used in this study were: *S. bayanus* (623-6C), *S. mikatae* (IFO 1815), and *S. castellii* (NRRL Y-12630) (Cliften et al., 2003). *Saccharomyces* genomic sequences were obtained from the Gene/Sequence Resources option and the Fungal BLAST option of the *Saccharomyces* Genome Database (SGD) ([www.yeastgenome.org](http://www.yeastgenome.org); R64-1-1 version).



Yeast cultures were grown at 30°C in a complete synthetic medium containing 2% glucose (w/v), but lacking inositol (I-), choline (Kelly and Greenberg, 1990), and uracil (in case of reporters). Where indicated, 75 µM inositol (I+) and 1 mM choline was added.

Plasmid-containing *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) were grown at 37°C in Luria–Bertani broth with 50 µg/ml ampicillin. Yeast were transformed using the Lithium acetate based one-step method (Chen et al., 1992). Genomic DNA was extracted using a Zymo Yeast DNA extraction kit (Zymo Research, Orange, CA).

### **2.2.2 Plasmid construction**

The TA plasmid *pGEM-T* was ligated with the PCR product of the *SNA3*-310to+100 primer pair (Table 2.1), covering the -310 bp to +100 bp region of the *SNA3* gene, and was named *pGEM-T:SNA3* -310 to +100.

E-boxes 4 and 5 in the *SNA3* promoter (positioned -67 to -72 and -174 to -179, respectively) were mutagenized using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). *pGEM-T:SNA3* -310 to +100 was used for this mutagenesis. E4 mutation primers and E5 mutation primers (Table 2.1) were used to create the single E-box mutants.

### **2.2.3 RNA Analysis**

RNA was isolated from yeast using a glass bead disruption and hot acid phenol method (Collart and Oliviero, 2001), subjected to DNase digestion using Promega RQ1 RNase-Free DNase (Madison, WI), and purified using a ZYMO RNA clean and concentrator kit (Orange, CA). RNA (1µg) was used to synthesize cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). For quantification, cDNA was diluted 1:10 and qPCR was performed as previously described with 500 nM primer concentrations (Jani and Lopes, 2008). *INO1*, *SNA3*, and *TCM1*

transcripts from *S. cerevisiae* were quantified using the *INO1*+1019to+1226, *SNA3*+23to+191 and *TCM1*+794to+945 primer pairs, respectively (Table 2.1). *INO1*, *SNA3*, and *TCM1* transcripts from *S. bayanus*, *S. mikatae*, and *S. castellii* were quantified using the *INO1* qPCR, *SNA3* qPCR, and *TCM1* qPCR primer pairs listed in Table 2.2.

#### **2.2.4 Chromatin Immunoprecipitation (ChIP) assays**

ChIP assays were performed as previously described (Aparicio et al., 2004) with some modifications. Cells were fixed with formaldehyde for 15 minutes. Lysis was performed on a multivortexer using glass beads. The cell extract was sonicated using a model 100 Sonic Dismembrator with a Branson 250 Microtip Sonicator (Fisher Scientific, Pittsburgh, PA) at 50% duty cycle with a power of 6. Sonication was performed 20 x 20 sec with at least 1 min on ice between pulses to fractionate DNA to ~300 bp. Immunoprecipitations were performed by incubating 800 µl chromatin with 40 µl IgG sepharose beads for 1 hr on a nutator at room temperature. Beads were washed twice each with FA lysis buffer, FA lysis buffer containing 500 mM NaCl and ChIP wash buffer followed by a wash with TE buffer. Protein-DNA complexes were eluted from the beads by incubating the beads in ChIP elution buffer for 10 minutes at 65°C followed by TE buffer. The supernatants from the two steps were combined and treated with 25 µg RNase A (Invitrogen, Carlsbad, CA) and incubated for 15 min at 37°C. DNA was eluted by incubating the supernatant at 65°C O/N with 100 µg Proteinase K (Invitrogen, Carlsbad, CA) and 0.1% SDS. DNA was purified using a Zymo ChIP DNA Clean and Concentrator kit (Orange, CA). For qPCR analysis, ChIP DNA and Input DNA were diluted 1:10 and 1:100, respectively. qPCR analysis was performed as previously described (Jani and Lopes, 2008). Primers used for qPCR analysis are listed in Table 2.1.

**Table 2.1: Oligonucleotides used in this study - A**

Oligo		Sequence
SNA3 -200to-245 _25bpURA3	F	5'-GTGGGCATATAGTTTTTCGCTATGTATATAGCGGATGAGTGC GTTT AGCTTTTCAATTC AATTCATCATT-3'
SNA3 +1to+45 _25bpURA3	R	5'-ATAGCTCATTTCGATGGTCATGGTCATTAATATGGTCTCTGTCCATTT AGTTTTGCTGGCCGCATCTTCTC-3'
E4 mutation primer	F	5'-GGAAAGAGAGTACAGCACGAATGAGCGGGCGCCAGGAAAGGGGC TAGGTTAAAAAATA-3'
E4 mutation primer	R	5'-TATTTTTTAACCTAGCCCTTTCCTGGGCGCCGCTCATTCTGTGCTGT ACTCTCTTCC-3'
E5 mutation primer	F	5'-GTATATAGCGGATGAGTGC GTTGGGACTATTCTTTAATGAGG CCTATGAAGGCTCGTTTTCTTTTTTTTT-3'
E5 mutation primer	R	5'-AAAAAAAAAGAAAACGAGCCTTCATAGGCCTCATTAAAGAAATA GTAATCCCAACGCACTCATCCGCTATATAC-3'
SNA3-310to+100	F	5'-TTACATTCACCCTACAACAT-3'
SNA3-310to+100	R	5'-CTGGGGGAATGAAAACCGCC-3'
INO1+1019to+1226	F	5'-GTATTAACCGGTCTCCATTGC -3'
INO1+1019to+1226	R	5'-CCGACGGGCTTCATATATTTG-3'
SNA3 +23to+191	F	5'-ACCATGACCATCGAATGAGC-3'
SNA3 +23to+191	R	5'-TGAATGATTGCTGGGAAGAA-3'
TCM1+794to+945	F	5'-CCAGAGCTGGTCAAAGAGGT -3'
TCM1+794to+945	R	5'-ACCGTAGTGGACGAAACCAC-3'
ChIP fragment A	F	5'-CTTCATCCTTCTTCCCAGAATATTGAAC-3'
ChIP fragment A	R	5'-GACGAAAGCTCCAATTTATATACGTCTC-3'
ChIP fragment B	F	5'-ATTGCCTTTTCTTCGTTCC-3'
ChIP fragment B	R	5'-CATTCAACACTTTCGATTCC-3'
ChIP fragment C	F	5'-CCCTGCAGAGGAATCTCAAG-3'
ChIP fragment C	R	5'-CACTAAGTACGGCCGGAAGA-3'
ChIP fragment D	F	5'-TAATTTAGAAATGGACAGAGACCA-3'
ChIP fragment D	R	5'-GTATCCCTGTTGAACATACCCTTA-3'
ChIP fragment E	F	5'-ACGTGATGAAGGCTCGTTTT-3'
ChIP fragment E	R	5'-TGGTTGTTTGCTTTCTGCTG-3'
ChIP fragment F	F	5'-TCCTCTTGTGTGGGACGAT-3'
ChIP fragment F	R	5'-TCAATGCAACGCTTACTGC-3'

**Table 2.2: Oligonucleotides used in this study - B**

<b>Oligo</b>		<b>Sequence</b>
S. bayanus INO1 qPCR	F	GTATCAAGCCCGTCTCCATTGC
S. bayanus INO1 qPCR	R	CCCACGGGCTTCATGTATTTG
S. bayanus SNA3 qPCR	F	CGAGCAAGACCGAATGAGATA
S. bayanus SNA3 qPCR	R	TGGATGATAGCTGGGAAGAA
S. bayanus TCM1 qPCR	F	CCAGAGCTGGTCAAAGAGGT
S. bayanus TCM1 qPCR	R	ACCGTAGTGGACGAAACCACC
S. mikatae INO1 qPCR	F	GTATCAAACCTGTCTCCATCGC
S. mikatae INO1 qPCR	R	CCAACGGGTTTCATATACTTT
S. mikatae SNA3 qPCR	F	AACACAGAATGAGATACTCC
S. mikatae SNA3 qPCR	R	TGAATAATGGCAGGGAAGAA
S. mikatae TCM1 qPCR	F	CCAGAGCTGGTCAAAGAGGT
S. mikatae TCM1 qPCR	R	ACCGTAGTGAACAAAACCACC
S. castellii INO1 qPCR	F	GCATTAGACCAGTATCCATCGC
S. castellii INO1 qPCR	R	CCGACGGCGGGCATGTATTTA
S. castellii SNA3 qPCR	F	ACCATGACCATCGAATA
S. castellii SNA3 qPCR	R	TGAACGGTTGCTGGGAAGAA
S. castellii TCM1 qPCR	F	CTAGAGCTGGTCAAAGAGGT
S. castellii TCM1 qPCR	R	ACCGTAGTGGACGAAACCACC

## 2.3 Results

### 2.3.1 E-box 5 is a Cbf1 binding site, E-box 4 regulates Ino4 binding

The inositol mediated regulation of the tandem gene pair *INO1* and *SNA3* is regulated by common transcription regulators (Shetty and Lopes, 2010). These include three bHLH proteins: Ino2, Ino4, and Cbf1. Ino2 and Ino4 are known to only bind the E boxes 1 and 2 located within the *SNA3-INO1* intergenic region for both *INO1* and *SNA3* regulation. Previous genome wide regulator binding studies suggested E-box 5 as a preferred binding site for Cbf1p (Ferreiro et al., 2004; Harbison et al., 2004). However, this suggestion is based on E-box 5 being a close match to the consensus Cbf1 binding site because the ChIP study was not designed to distinguish between E box 4 and E box 5. Furthermore, recent ChIP assays showed that Cbf1p binds multiple regions across the *SNA3* promoter and ORF region (Shetty and Lopes, 2010). It is unclear at the moment, however, where exactly Cbf1 binds and whether the E-boxes within the *SNA3* promoter and ORF region play a regulatory role for *INO1* and *SNA3*.

To understand the importance of the *SNA3* promoter region E-boxes to the binding of the known regulators, we generated E-box 4 and E-box 5 mutant strains harboring TAP-tagged Ino2, Ino4 and Cbf1. These mutant strains, along with the cognate wild type strains were grown under I+ and I- conditions. ChIP-qPCR analysis was performed to quantify the binding of the transcription factors within fragments A-F spanning the *SNA3* promoter-*SNA3* ORF-*INO1* promoter region (Fig. 2.3 A).

Since the ChIP-qPCR data needs to be normalized for sources of variability, including amount of chromatin, efficiency of immunoprecipitation, and DNA recovery, we analyzed our ChIP-qPCR data relative to input as this includes normalization for both background levels and input chromatin going into the ChIP. The results are plotted in Fig. 2.3, with the fragments A-F on the X-axis and the ChIP/INPUT ratio on the Y-axis.

Consistent with published data (Shetty and Lopes, 2010), under both I+ and I- growth conditions, Cbf1 binding in the wild type based Cbf1 TAP-tagged strain occurs at fragments C-E with significantly strong binding at fragment E under the I- condition (Fig. 2.3 B). This suggests that Cbf1 binds to the E-box(es) within this region in response to a decrease in exogenous inositol.

Cbf1 binding in the E-box 4 mutant Cbf1-TAP tagged strain show characteristics similar to what was observed in wild type, with binding at fragments C-E under both I+ and I- conditions and remarkable binding at E under the I- condition (Fig. 2.3 C). From this piece of data, we can assume that Cbf1 does not bind to the *SNA3* promoter region via E-box 4.

When the binding was examined in the E-box 5 mutant Cbf1-TAP tagged strain, there was again no recognizable Cbf1 binding at fragments A, B, and F, but notably, under the I- condition, the binding at fragments C and D were lowered and the binding at fragment E showed a 3 fold drop compared with the wild type condition (Fig. 2.3 D). Since the mutation of E-box 5 results in loss of Cbf1 binding, we identified E-box 5 as the Cbf1 binding site within the region examined. This conclusion is supported by predictions made in previous studies (Harbison et al., 2004; Kent et al., 2004).

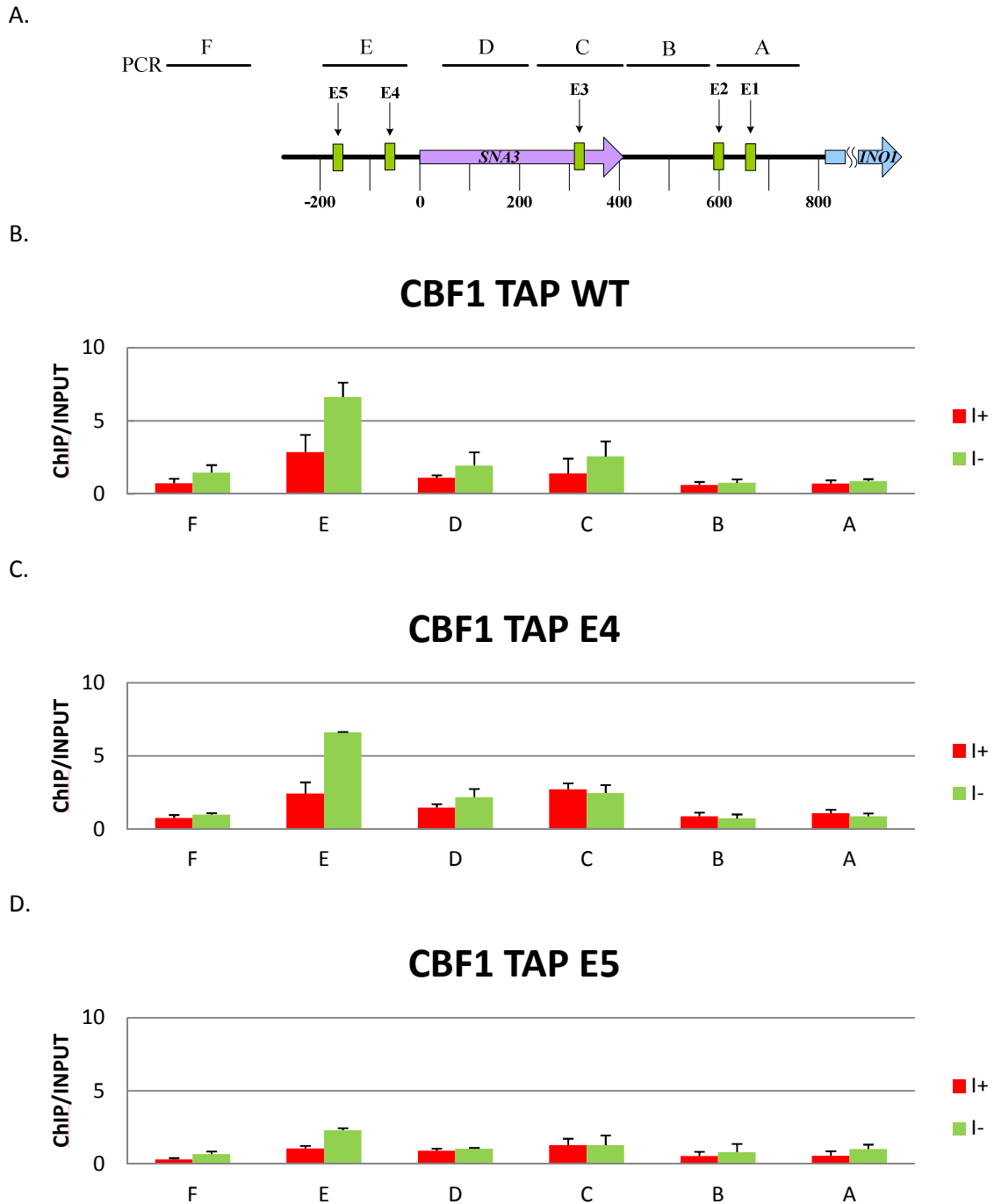


Figure. 2.3. Cbf1 binds to E-box 5.

(A) Schematic showing primer positions (A to F) and E-boxes in the *SNA3* promoter, *SNA3* ORF, and the the *INO1-SNA3* intergenic region. (B-D) ChIP analysis performed using wild type or E-box mutant Cbf1 TAP-tagged strains grown under I+ and I- conditions. The data represent means and standard errors of the means from at least three different experiments. E-boxes are labeled as E.

We were next interested to determine if mutating E-boxes 4 and 5 affected Ino2 and Ino4 binding. The two transcription factors only bind to E-boxes 1 and 2 within our region of interest. However, it was also shown that the binding of the two proteins is dependent on the presence of Cbf1 (Shetty and Lopes, 2010). It is possible that the binding site of Cbf1 is also required for proper Ino2/Ino4 binding. It may also be possible that there are other unknown transcription factors that bind E-box 4 and somehow interact with the known regulators of *INO1* and *SNA3*.

As expected, Ino2 binding in the wild type based Ino2-TAP tagged strain appeared within the *SNA3-INO1* intergenic region and was elevated in the I- conditions (Fig. 2.4 B), which is consistent with previously published data (Shetty and Lopes, 2010). Unfortunately, Ino2 binding was also observed further upstream in the *SNA3* ORF which is inconsistent with published results (Fig 2.4B). This may be due to poor sonication in this area. The Ino2 binding pattern in the E-box 4 and E-box 5 mutant strains do not show recognizable differences (Fig. 2.4 C and D), suggesting that both E-boxes do not have effects on Ino2 binding. However, it is not possible to draw conclusions because of the lack of consistency with published studies.

In the Ino4-TAP tagged wild type strain, binding was also evident within the *SNA3-INO1* intergenic region where Ino4 was reported to bind the E-boxes 1 and 2 and was elevated under I- conditions (Fig. 2.5 B). When compared with wild type, the binding of Ino4 in the E-box 4 mutant strain showed a generally lower binding at fragments A and B and no obvious effect of inositol (Fig. 2.5 C). Thus, E-box 4, located within the *SNA3* promoter region, is somehow regulating the binding of Ino4 to its binding sites within the downstream *SNA3-INO1* intergenic region. The mutation at E-box 5 did not appear to have much of an effect on binding of Ino4 although there might have been a slight increase in binding (Fig. 2.5 D).



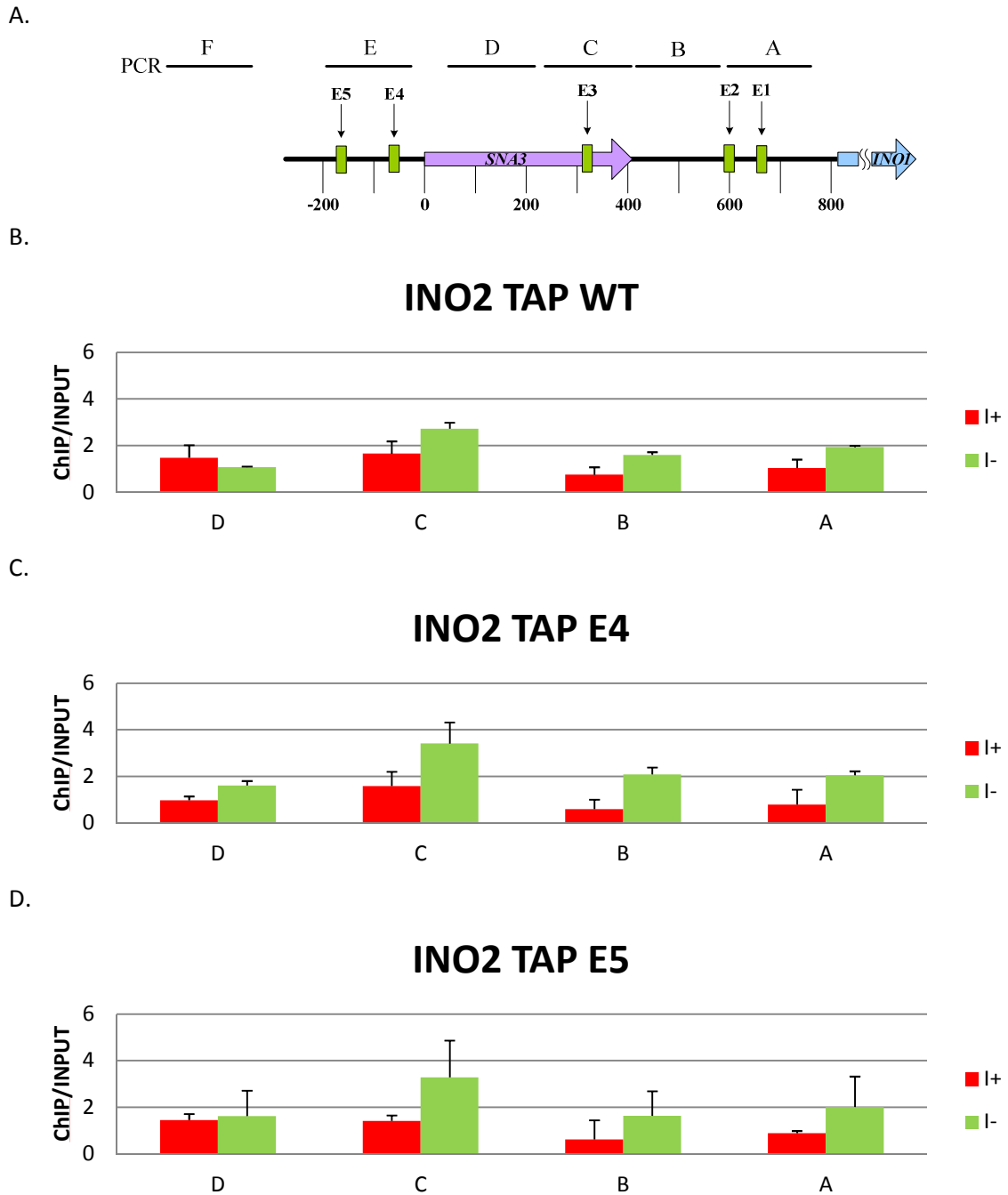


Figure. 2.4. E-boxes 4 and 5 do not appear to affect Ino2 binding to the INO1-SNA3 intergenic region.

(A) Schematic showing primer positions (A to F) and E-boxes in the *SNA3* promoter, *SNA3* ORF, and the *INO1-SNA3* intergenic region. (B-D) ChIP analysis performed using wild type or E-box mutant Ino2 TAP-tagged strains grown under I+ and I- conditions. The data represent means and standard errors of the means from at least three different experiments. E-boxes are labeled as E.

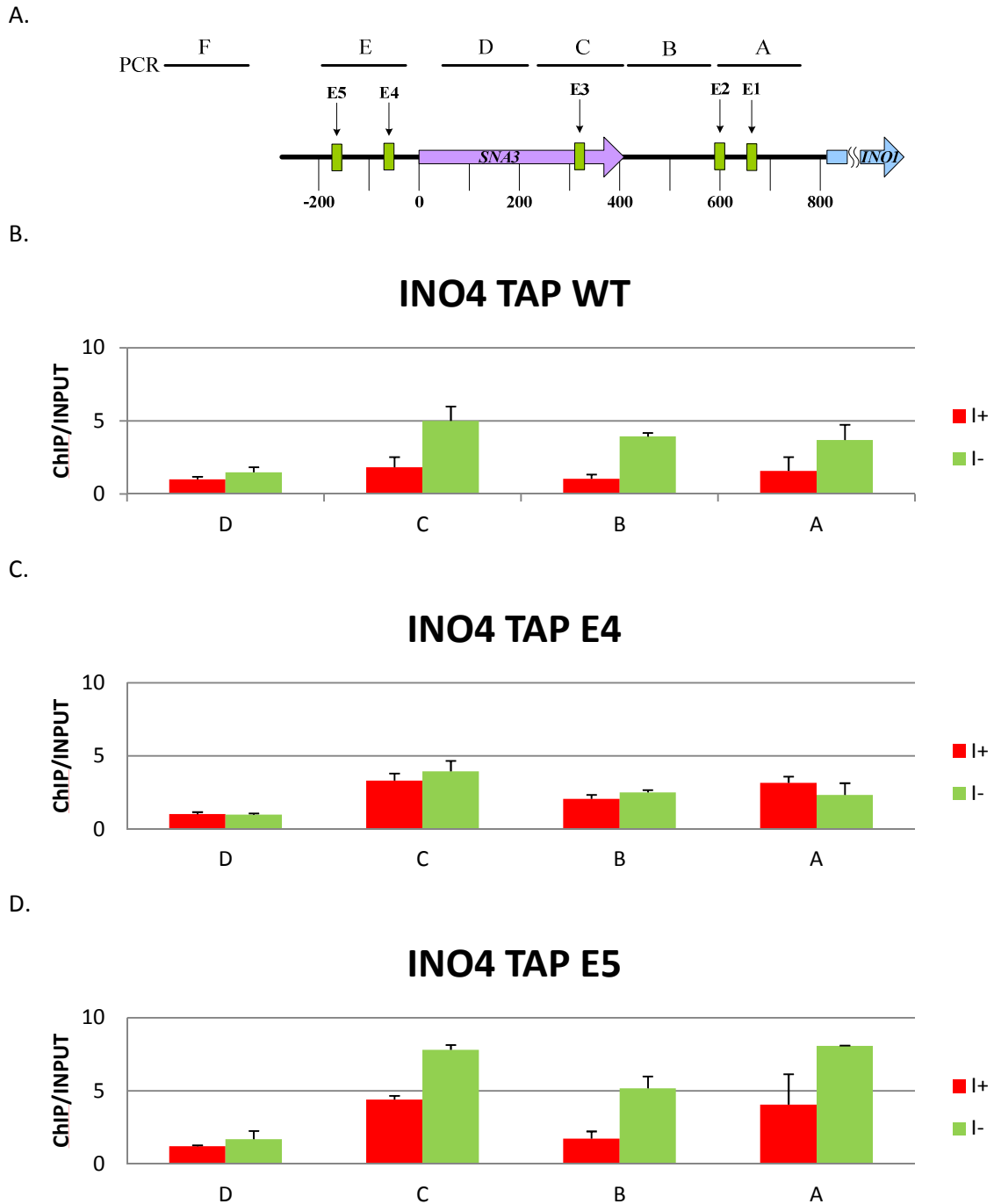


Figure. 2.5. E-box 4 regulates binding of Ino4 at the downstream *INO1-SNA3* intergenic region.

(A) Schematic showing primer positions (A to F) and E-boxes in the *SNA3* promoter, *SNA3* ORF, and the *INO1-SNA3* intergenic region. (B-D) ChIP analysis performed using wild type or E-box mutant Ino4 TAP-tagged strains grown under I+ and I- conditions. The data represent means and standard errors of the means from at least three different experiments. E-boxes are labeled as E.

### 2.3.2 E-box 4 plays a role in inositol mediated *SNA3* regulation

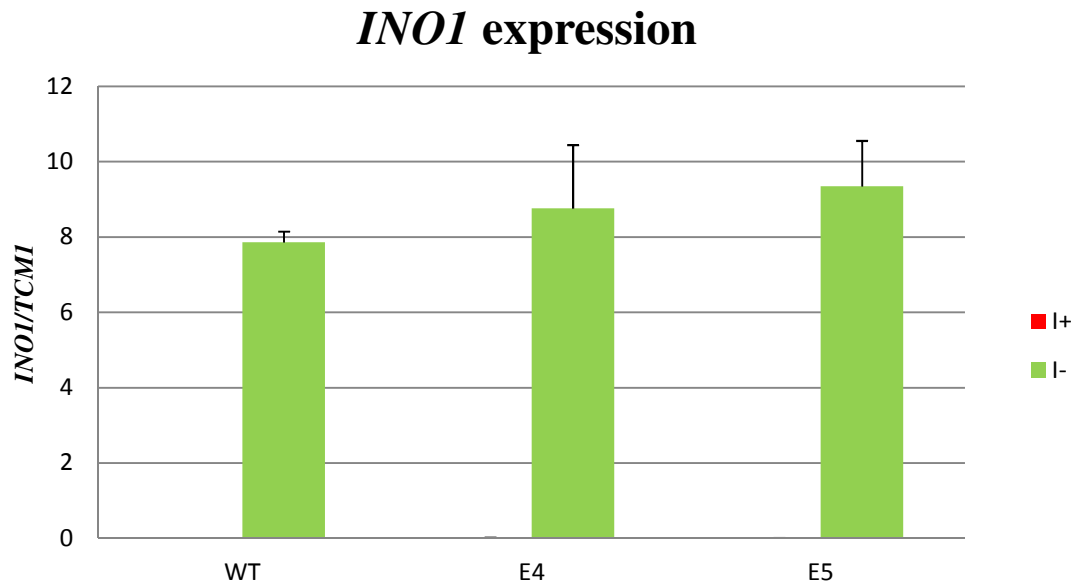
*Cis*- regulatory elements have been reported to control *INO1* and *SNA3* expression: E-boxes 1 and 2 are essential for *INO1* derepression and an E-box 2 mutation results in *SNA3* derepression. Hence, having observed the impact of E-box 4 and E-box 5 mutations on the binding of the *INO1* and *SNA3* regulators Cbf1 and Ino4, we were interested in understanding the effect of these E-boxes on the inositol-mediated regulation of *INO1* and *SNA3*.

To look at the expression of these genes, we generated wild type based E-box 4 and E-box 5 mutants applying the same method we used for generating the E-box mutants in the TAP tagged strains and carried out qRT-PCR for wild type, E-box 4 and E-box 5 mutant strains grown under I+ and I- conditions. The expression values of each of the two genes were normalized to a constitutively expressed ribosomal protein gene, *TCM1*.

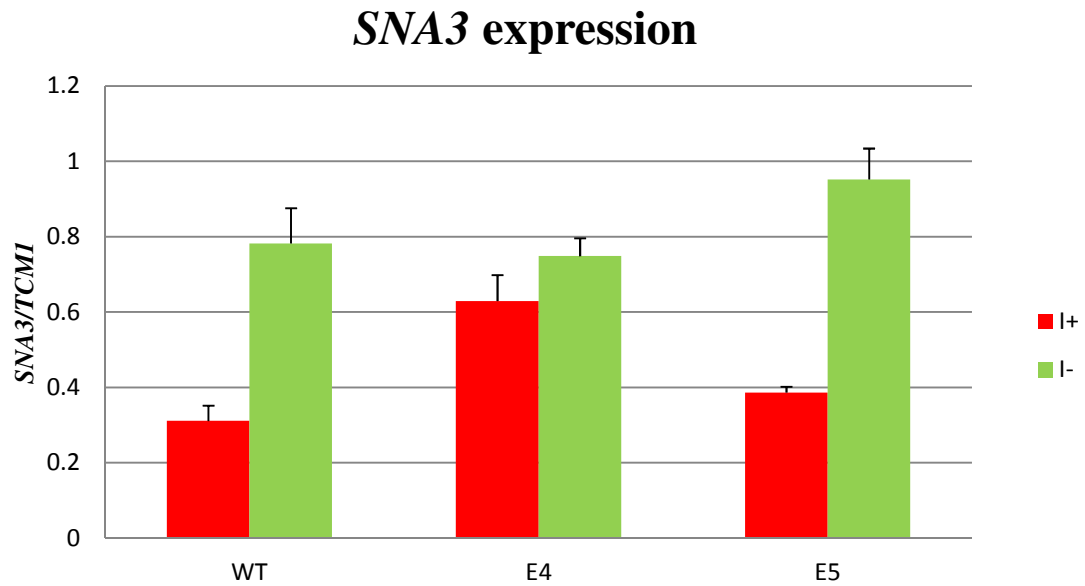
The data clearly showed that *INO1* transcripts were not affected by any of the mutations, whether looking at the I+ or I- growth condition: *INO1* expression is >900 fold up regulated under derepressing conditions in all three strains (Fig. 2.6 A). We concluded that both E-boxes 4 and 5 have no significant effect on *INO1* regulation.

When compared with the I+ condition, *SNA3* expression of the wild type strain showed a 2.5 fold increase when grown in media lacking inositol (Fig. 2.6 B), supporting previous studies (Hirsch and Henry, 1986; Shetty and Lopes, 2010). The mutation at E-box 5 did not seem to have altered the regulation of *SNA3* (Fig. 2.6 B). It is surprising that E-box 5, a binding site of Cbf1, is dispensable for *SNA3* regulation. Interestingly, however, we observed a 2 fold increase of *SNA3* expression in the E-box 4 mutant strain under repressing conditions, whereas the transcript level under derepressing conditions did not show a big difference compared to wild type (Fig. 2.6 B). This data indicates a repressing role of E-box 4 on *SNA3* when inositol is present in the environment.

A.



B.



**Figure. 2.6. Quantitative analysis of *INO1* and *SNA3* mRNA in wild type, E-box 4, and E-box 5 mutants.**

**The bar graphs represent the ratio of *INO1* or *SNA3* to *TCM1*. (A) *INO1* transcript levels. (B) *SNA3* transcript levels. The data represent means and standard errors of the means from at least three different experiments. E-boxes are labeled as E.**

### 2.3.3 The inositol mediated regulation of *INO1*, but not *SNA3*, is evolutionary conserved

Several genomes of the *Saccharomyces* genus have been sequenced and utilized in comparative studies. Species including *S. bayanus*, *S. mikatae*, and *S. kudriavzevii* are closely related and physiologically similar to *S. cerevisiae*. They are capable of forming stable diploids with each other (Barnett, 1992). The *Saccharomyces* species including *S. castellii* and *S. kluyveri* are more physiologically different and in most cases do not form stable diploids with *S. cerevisiae* (Petersen et al., 1999).

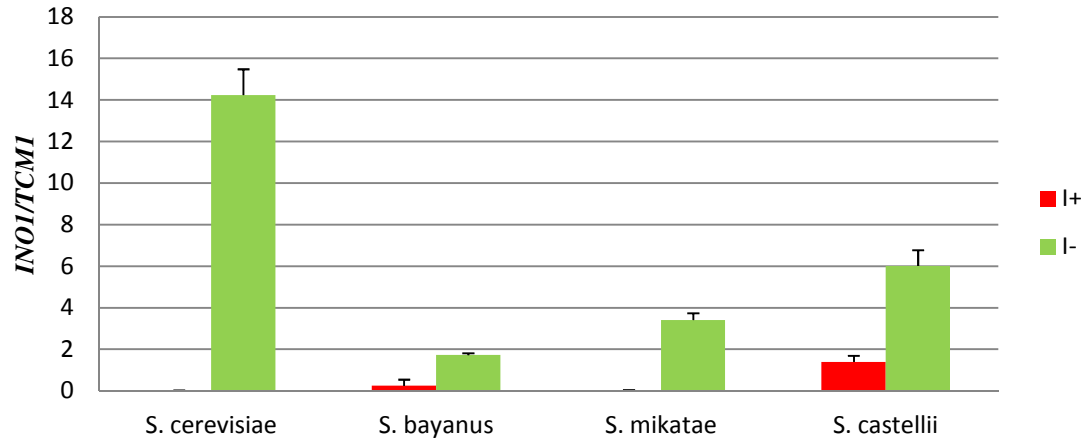
To understand if inositol mediated regulation of *INO1* and *SNA3* is evolutionary conserved within the *Saccharomyces* species, we identified *INO1* and *SNA3* homologues in *S. bayanus*, *S. mikatae*, and *S. castellii* using sequences obtained from the Gene/Sequence Resources option and the Fungal BLAST option of the *Saccharomyces* Genome Database (SGD). Primers specific to each species were designed for these genes and a constitutively expressed ribosomal protein gene, *TCM1*, and qRT-PCR was carried out. The expression values of *INO1* and *SNA3* were normalized to *TCM1*.

*INO1* expression in *S. cerevisiae* showed a 700 fold increase when cultured without inositol, compared to cultures grown with inositol (Fig. 2.7 A). A 7 fold up regulation of *INO1* was also triggered by the lack of inositol in *S. bayanus* (Fig. 2.7 A). In *S. mikatae*, the level of *INO1* expression in I- media was 100 times the expression in I+ media (Fig. 2.7 A). *INO1* transcription in *S. castellii* was enhanced by 5 fold in response to the lack of inositol (Fig. 2.7 A). The data clearly showed that the inositol mediated *INO1* transcription regulation is evolutionary conserved within the *Saccharomyces* genus.

*S. cerevisiae* *SNA3* was 2 fold up regulated under I- conditions as previously reported, however *SNA3* expression in the other three species did not respond to the absence of inositol (Fig. 2.7 B). *SNA3* regulation in the species examined is not controlled by inositol.

A.

### *INO1* expression in various *Saccharomyces* species



B.

### *SNA3* expression in various *Saccharomyces* species

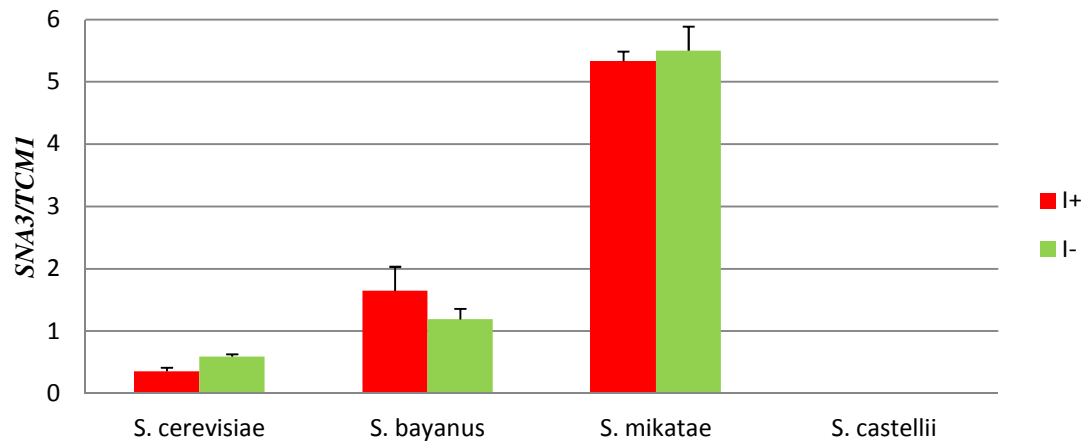


Figure. 2.7. Quantitative analysis of *INO1* and *SNA3* mRNA in the *Saccharomyces* genus.

(A) *INO1* transcript levels are depicted. (B) *SNA3* transcript levels are depicted. The data are the average of at least three independent experiments. Error bars represent the standard error of the mean value.

## 2.4 Discussion

In this chapter, we have examined the importance of E-boxes 4 and 5 to Cbf1, Ino2 and Ino4 binding, as well as the importance of these *cis*- sequences to *INO1* and *SNA3* regulation. A summary of previous studies and our new findings is presented in Fig. 2.8.

While Cbf1 preferably binds to fragment E within the *SNA3* promoter in wild type and E-box 4 mutant strains, we observed a dramatic drop of this binding when a mutation was induced at E-box 5. We identified E-box 5 as a binding site for Cbf1 (A in Fig. 2.8). We found that E-box 4, located in the *SNA3* promoter region, is capable of controlling the binding of Ino4 to its binding sites within the *SNA3-INO1* intergenic region, in response to exterior inositol (B in Fig. 2.8). When *SNA3* expression was examined in the E-box mutant strains, E-box 4 mutation resulted in an increase of *SNA3* expression under repressing conditions (I+), indicating that E-box 4 normally represses *SNA3* when inositol is present (C in Fig. 2.8). *SNA3* expression in E-box 5 mutants was not distinguishable from wild type.

We also looked into regulation of *INO1* and *SNA3* transcription in the *Saccharomyces* species, *S. bayanus*, *S. mikatae*, and *S. castellii* and found that inositol affected expression of *INO1*, but not *SNA3*.

It was previously stated that, while *INO1* and *SNA3* transcription are both up regulated under I- conditions and repressed under I+ conditions, the shared transcription factors Ino2, Ino4, and Cbf1 up regulate *INO1* expression (a in Fig. 2.8) but suppress *SNA3* expression (b and c in Fig. 2.8) in response to low inositol concentrations. Ino2, Ino4, and Cbf1 binding have been reported to be interdependent (d in Fig. 2.8). The E-boxes 1 and 2 are known to be required for *INO1* derepression (e in Fig. 2.8), while E-box 2 represses *SNA3* (f in Fig. 2.8).

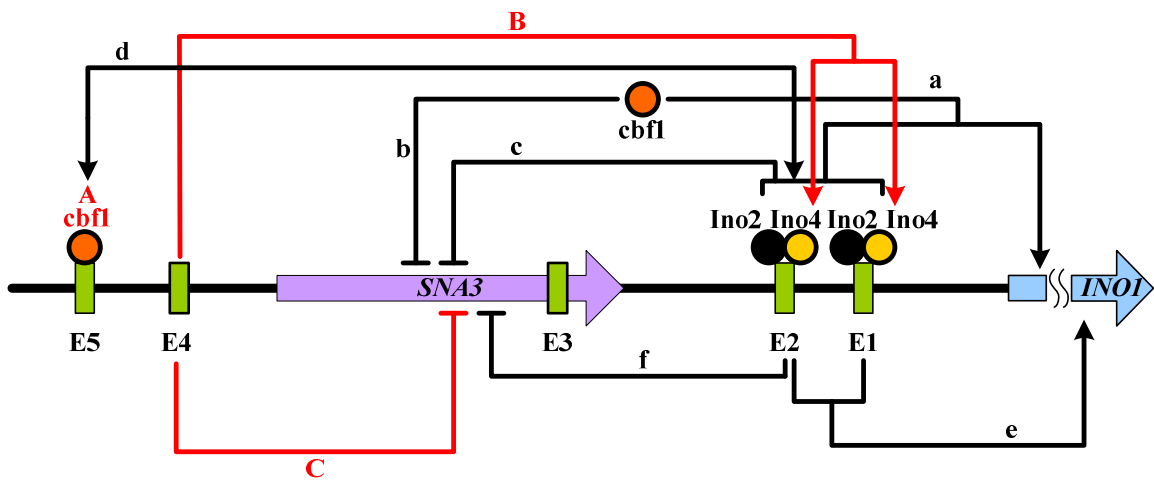


Figure. 2.8. Schematic summary of previous studies and our new findings on SNA3 and INO1 co-regulation.

Red capital letters indicate findings of this research. Black lower-case letters indicate findings from previous. E-boxes are labeled as E.



Considering additional results from this study, we can conclude that:

1. Although Cbf1 binds to E-box 5 (A in Fig. 2.8), it is functioning through a different mechanism to promote *INO1* expression or to down regulate *SNA3* transcription under I- conditions because a mutation of E-box 5 does not affect the expression of the two genes.
2. Ino4 binding to the *SNA3-INO1* intergenic region is regulated by Cbf1 and E-box4 (d and B in Fig. 2.8). E-box 4 is responsible for the differential binding of Ino4 under I+ and I- conditions. The presence of the transcription factor Cbf1 enhances Ino4 binding.
3. Since E-box 4 represses *SNA3* under I+ conditions (C in Fig. 2.8), it is unlikely that this is directly done via its regulatory role on Ino4 (B in Fig. 2.8), which was reported to represses *SNA3* only under I- conditions (c in Fig. 2.8). It is possible that other transcription factors bind E-box 4 and are responsible for the inhibition of *SNA3* under the repressing condition.
4. The reason why E-box 4 controls Ino4 binding but has no significant effect on *INO1* regulation may be that the Ino4 binding difference caused by this E-box is not great enough to trigger a response in *INO1* expression.
5. Unpublished data from a previous lab member, Dr. Ameet Shetty, made him conclude that the *SNA3* expression is not an effect of the *SNA3* promoter region. In his experiment, he fused either the 400bp or the 1kb of the upstream region of *SNA3* (covering E-boxes 4 and 5) to a *lacZ* reporter gene on a plasmid and analyzed *lacZ* expression by  $\beta$ -gal assays. In our current data, however, we found that E-box 4 represses *SNA3* under I+ condition. It is possible that the different results are caused by the participation of the downstream or the even more upstream regions of *SNA3*.
6. Inositol mediated *INO1* regulation is evolutionarily conserved but *SNA3* regulation is not.

It would be interesting to know which transcription factors recognize E-box 4 and if and how significant their effect is on *SNA3* and *INO1* regulation, as well as the binding of our known regulators of this system. DNA Pull-down assays using E-box 4 probes and Mass Spectrometry may give us an insight into the answers of these questions. To date, only inhibitory elements, either *cis*- or *trans*-, were identified for *SNA3* regulation. A screen for *SNA3* activators would provide us some missing pieces of information needed to understand the *SNA3* regulation as a whole. It also remains unclear whether the E-box 3, located within the *SNA3* ORF, has any regulatory role on *INO1* or *SNA3*. Mutation studies might shed light on this question.

## **2.5 Acknowledgement**

The authors would like to thank Dr. Mark Johnston (Washington Univ. School of Medicine) for providing strains. We especially thank Dr. Aishwarya Swaminathan, Bryan Salas, and Philip Hunt for helpful discussions.

## CHAPTER 3

### THE GROWTH PHASE REGULATION OF *INO1*

#### 3.1 Introduction

Phospholipids are the major component of biological membranes. A living cell is always highly engaged in the synthesis of phospholipids and the assembly of phospholipids into cellular membranes (Homann et al., 1987). There are two highly regulated supply routes of metabolic phospholipid precursors: direct uptake from the environment and biosynthesis by the cell itself (Robinson et al., 1996). The presence of soluble precursors in the media plays a strong regulatory role on the expression of phospholipid biosynthetic enzymes at the level of transcription (Lamping et al., 1994). Inositol is one of the phospholipid precursors that has a regulatory effect on the phospholipid biosynthesis and inositol biosynthesis pathways in *S. cerevisiae* (Culbertson et al., 1976; Paltauf et al., 1992; Robinson et al., 1996). Inositol is used by the cell to make Phosphatidylinositol (PI), an essential membrane phospholipid that takes part in signaling, trafficking, and in many other important cellular processes (Greenberg et al., 1982b; Bae-Lee and Carman, 1984). For a cell to maintain normal functions and continue growth, the continuous synthesis of PI is an important task (Homann et al., 1987). Hence, the pool of inositol has to be sufficient under different growth conditions and in all growth phases.

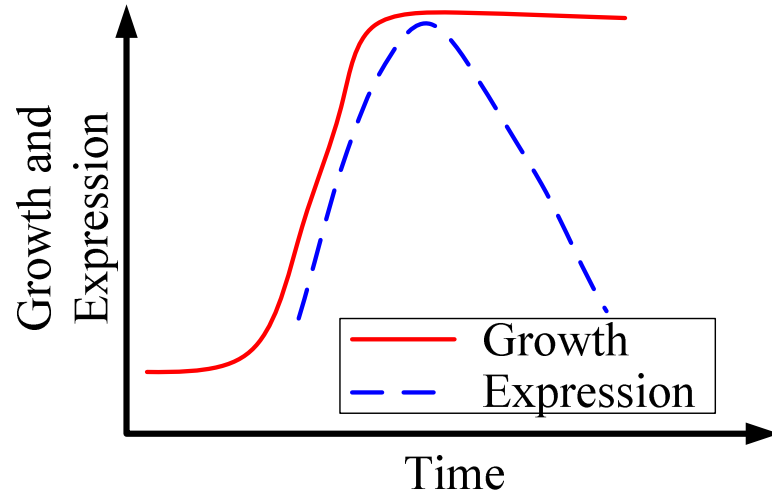
The gene *INO1*, encoding inositol-3-phosphate synthase, responds to changes in environmental inositol concentration. Under conditions where inositol is abundant in the media (I+), *INO1* is repressed. When the inositol provided by the environment is insufficient (I-), *INO1* is derepressed and inositol is synthesized by the cell. In addition to the availability of environmental inositol, Growth phase also affects inositol biosynthetic activities. The regulation of many inositol biosynthetic enzymes varies with growth phases. Specifically, when expression

of these genes or their cognate enzymes is normalized to total protein content or cell number of the course of a growth cycle, a curve is observed (Fig. 3.1 A) instead of a flat line (Fig. 3.1 B).

Growth phase regulation has also been observed for the inositol-regulated *INO1* gene. Under I- conditions, the amount of *INO1* mRNA was reported to increase during exponential growth, reach the maximum as the culture approaches stationary phase, and reduce when the culture is in the stationary phase (Lamping et al., 1994; Robinson et al., 1996) (Fig. 3.1 A). The activity of the *INO1* product is also increased during exponential growth and reduced in stationary phase (Culbertson et al., 1976). However, the subunit levels of the *INO1* product in stationary phase did not show corresponding reduction (Homann et al., 1987). It was proposed that the *INO1* product is inactivated in some way during stationary phase (Homann et al., 1987). The mechanism of the *INO* growth phase regulation is not well understood and is the subject of this work.

In this chapter, we will examine how the different growth phases affect *INO1* expression. Specifically, does the difference in *INO1* expression throughout the growth of a culture occur as a result cellular or population effects?

A.



B.

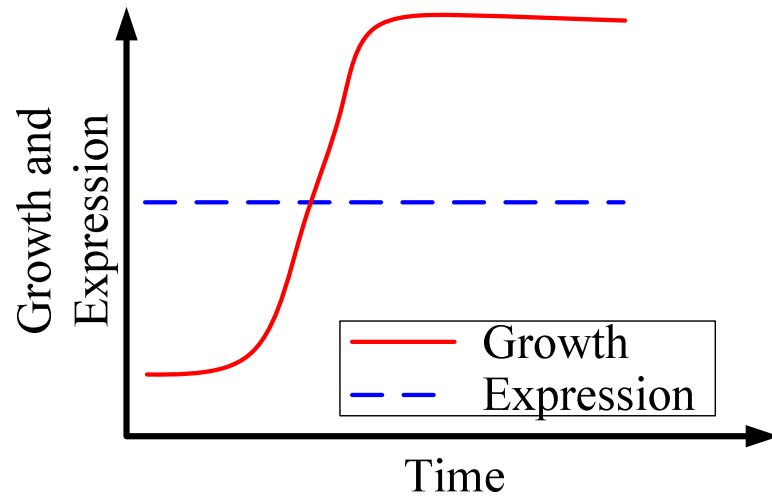


Figure. 3.1. Schematic graphs of gene expression patterns and growth curves.

(A) The expression pattern of a growth phase-regulated gene as a function of the growth curve of the culture. (B) The expression pattern of gene which is not growth phase-regulated.

## 3.2 Materials and Methods

### 3.2.1 Strains, media, and growth conditions

The *S. cerevisiae* strains with the GFP-tagged *INO1* and *TCM1* used in this study were purchased from Invitrogen (Carlsbad, CA) (Huh et al., 2003). The GFP tag is C-terminally positioned in the genome and the parent yeast strain was (ATCC 201388: MATa his3D1 leu2D0 met15D0 ura3D0).

Yeast cultures were grown at 30°C in a complete synthetic medium containing 2% glucose (w/v), 75 µM inositol (I+), and 1 mM choline. Where indicated, 0.7M NaCl was added. When the culture reached the OD<sub>600</sub> of 0.4 (late lag phase), cells were collected, washed with and transferred into the original volume of complete synthetic medium lacking inositol and choline (I-). Following the shift in medium, the OD<sub>600</sub>, *INO1* expression, and *INO1-GFP* fluorescence was examined (for 300 cells) every hour after the shift until the culture was in stationary phase (OD<sub>600</sub> > 0.9).

### 3.2.2 RNA Analysis

At each time point, RNA was isolated from yeast using a glass bead disruption and hot acid phenol method (Collart and Oliviero, 2001), subjected to DNase digestion using Promega RQ1 RNase-Free DNase (Madison, WI), and purified using a ZYMO RNA clean and concentrator kit (Orange, CA). RNA (1µg) was used to synthesize cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). For quantification, cDNA was diluted 1:10 and qPCR was performed as previously described with 500 nM primer concentrations (Jani and Lopes, 2008). *INO1* and *TCM1* transcripts from *S. cerevisiae* were quantified using the *INO1*+1019to+1226 and *TCM1*+794to+945 primer pairs, respectively (Table 3.1).

### 3.2.3 Microscopy and image analysis

At each time point, 3 $\mu$ l of cells were fixed on a media-based 1% low melting agarose pad. A coverslip was then applied on top of the agarose pad. Cells were imaged using a Nikon ECLIPSE E600 microscope, HAMAMATSU ORCA-ER digital camera, and the open lab program 5.5.2 from PerkinElmer (Waltham, Massachusetts) under identical settings: the cells were imaged using a 28 gain, 150-ms exposure, and a 60 $\times$  objective under the Phase filter, whereas a max (255) gain, 750-ms exposure, and a 60 $\times$  objective was used for imaging the same yeast cells under the GFP filter. Cells were manually identified and selected, then recorded by a combination of the MicrobeTracker software (Sliusarenko et al., 2011) and the Matlab R2012a software from Mathworks (Natick, Massachusetts). Background “fake cells” were generated using the same method for later subtraction of background fluorescence. A program, Combined Gui (kindly provided by Dr. Steven Sandler, UMass Amherst Department of Microbiology, Amherst, MA), was used to analyze the fluorescent images in Matlab R2012a. Information regarding the fluorescence intensity of each cell, the number of cells selected and the average fluorescence intensity of the cells selected were acquired. The fluorescence intensity of each cell was normalized to the average fluorescence intensity of the background. At each of the 10 to 11 time points, 300 cells were selected for analysis for each of the two GFP-tagged strains, growing in either I+, I+ 0.7M NaCl, I-, or I- 0.7M NaCl media. Any cell having fluorescence greater than background is considered induced.



**Table 3.1: Oligonucleotides used in this study - C**

<b>Oligo</b>		<b>Sequence</b>
INO1+1019to+1226	F	5'-GTATTAAACCGGTCTCCATTGC -3'
INO1+1019to+1226	R	5'-CCGACGGGCTTCATATATTTG-3'
TCM1+794to+945	F	5'-CCAGAGCTGGTCAAAGAGGT -3'
TCM1+794to+945	R	5'-ACCGTAGTGGACGAAACCAC-3'

### 3.3 Results

#### 3.3.1 The growth phase regulation of *INO1* is cell dependent

##### 3.3.1.1 Growth phase regulation of *INO1* in regular media

When we monitored *INO1* transcription in the *INO1-GFP* strain upon transfer into I-media in the late lag phase and into stationary phase, we found that *INO1-GFP* expression was regulated by growth phase, confirming previous results (Fig. 3.2). More specifically, *INO1-GFP* expression levels started out low after the shift into the derepressing medium and increased throughout the log phase. The expression levels peaked at the beginning of the stationary phase and continuously decreased after the culture continued into stationary phase.

We sought to answer the question: is the difference in *INO1* expression throughout culture growth cell- or population-dependent? To test this, we recorded the fluorescence intensity of the *INO1-GFP* fusion protein in 300 cells each hour after the media shift and until the culture was in stationary phase. If we plot the percentage of fluorescent cells versus time and see a vertical line to 100% fluorescent cells, this would tell us that all the cells in the culture induce *INO1-GFP* simultaneously (Fig. 3.3 A). If we observed a gradual increasing slope, it would indicate that there is an increasing number of fluorescent cells (Fig. 3.3 B).

Our results matched the expected results shown in Fig. 3.3 A (Fig. 3.4) with 99% of cells fluorescing between the 3<sup>rd</sup> and the 4<sup>th</sup> hours after the media shift ( $OD_{600}=0.912$ ). There was no decrease in fluorescence observed once cells entered the stationary phase, which likely reflects the stability of the *INO1-GFP* fusion protein.

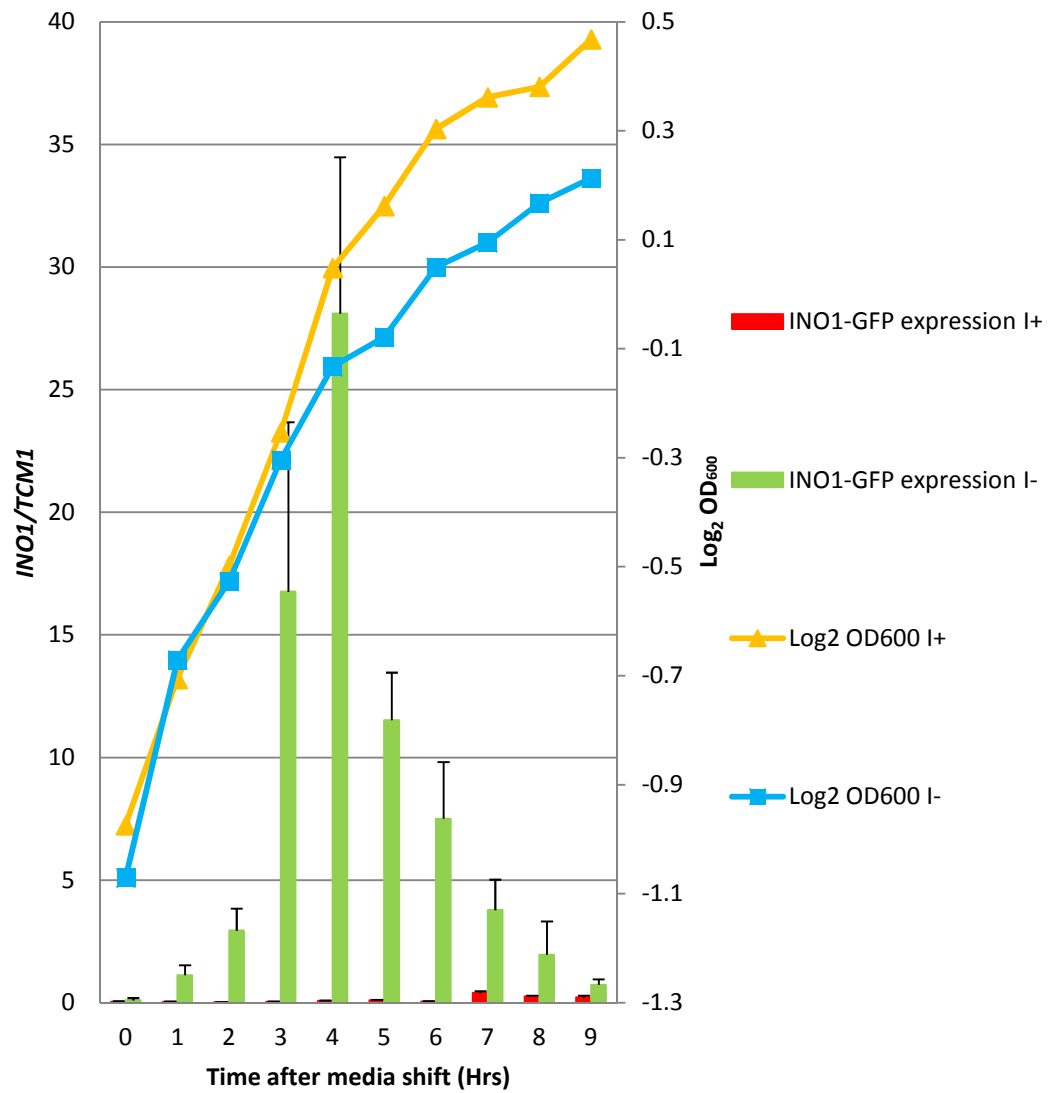
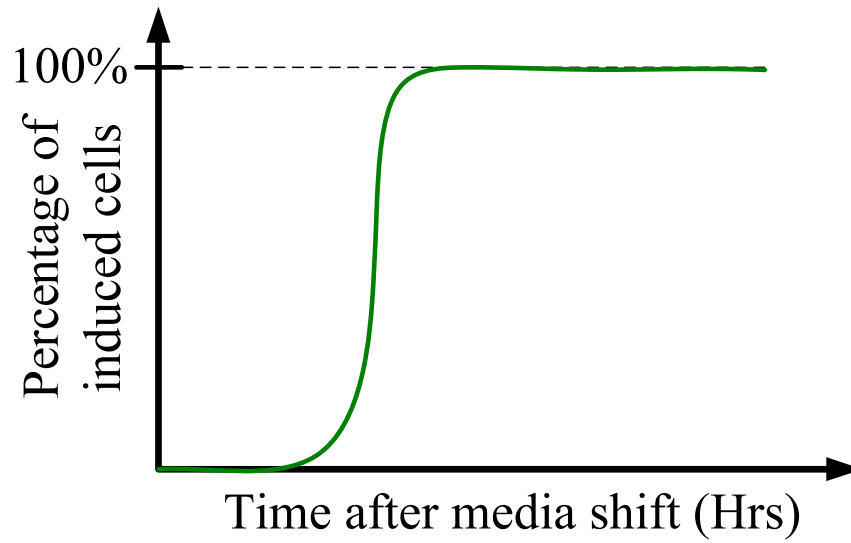


Figure. 3.2. The expression pattern of *INO1* mRNA during growth of a culture.

*INO1* transcript levels were normalized to *TCM1*. The binary logarithm of the OD<sub>600</sub> readings is presented.

A.



B.

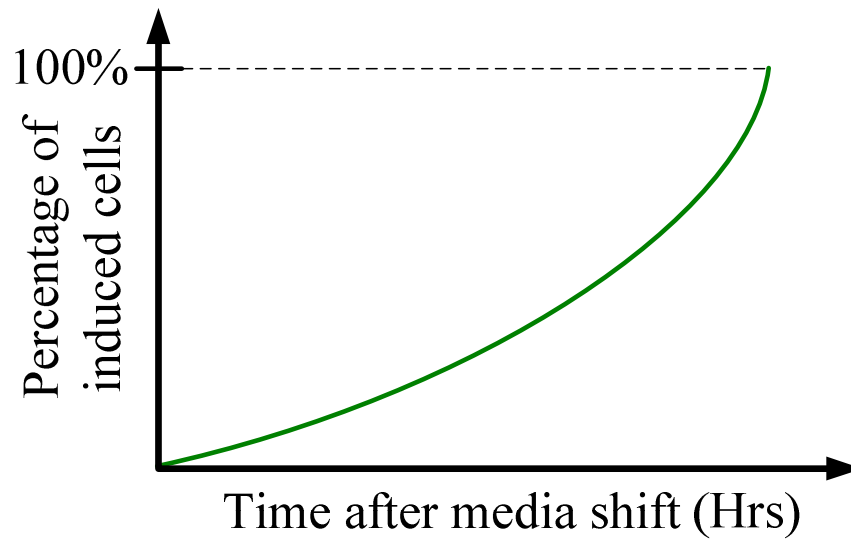
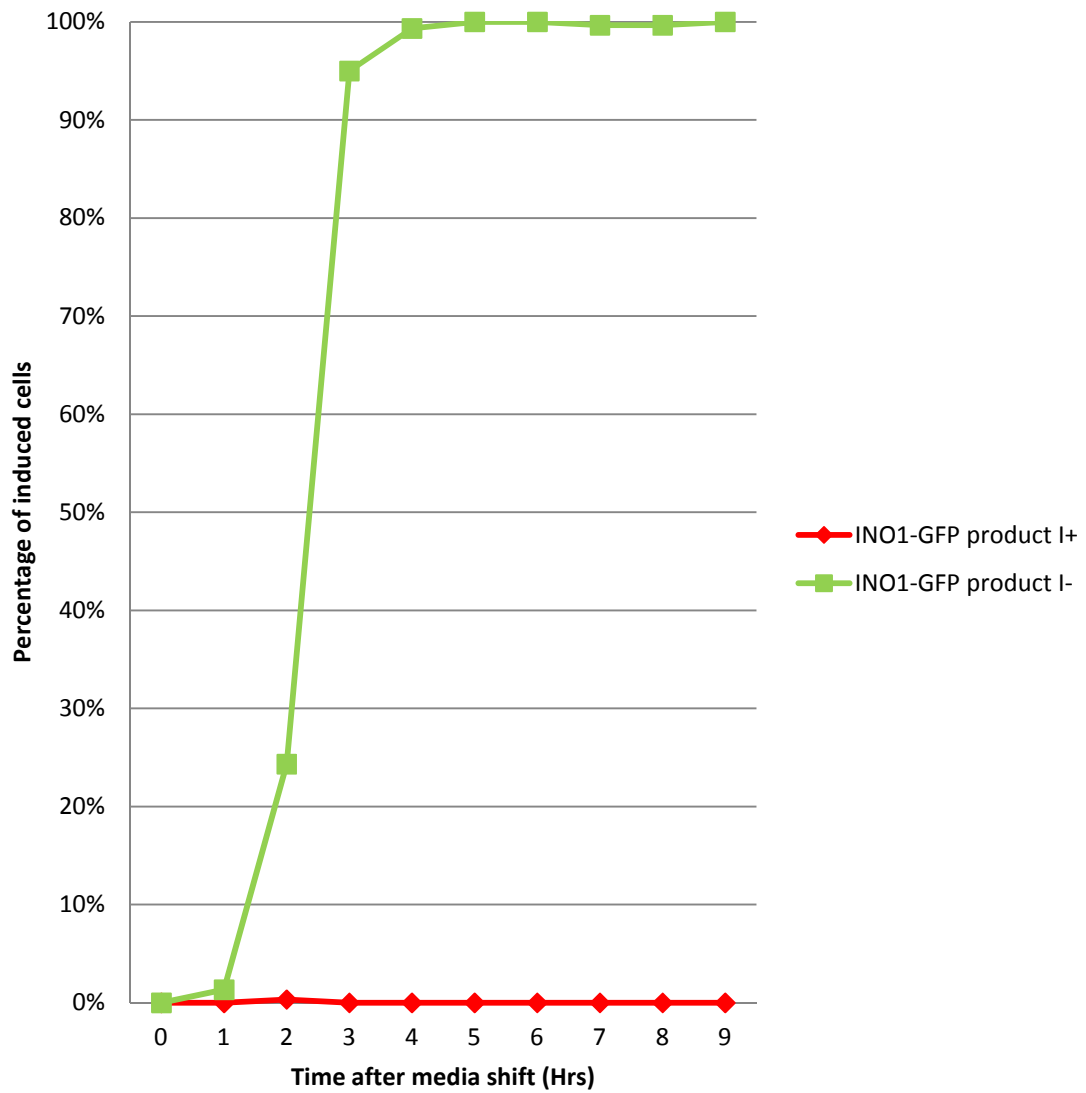


Figure. 3.3. Schematic graphs showing the percentage of fluorescent cells in a culture versus time.

(A) Cell dependent model and (B) population dependent model.



**Figure. 3.4. Percentage of fluorescent *INO1-GFP* cells in the culture versus time.**

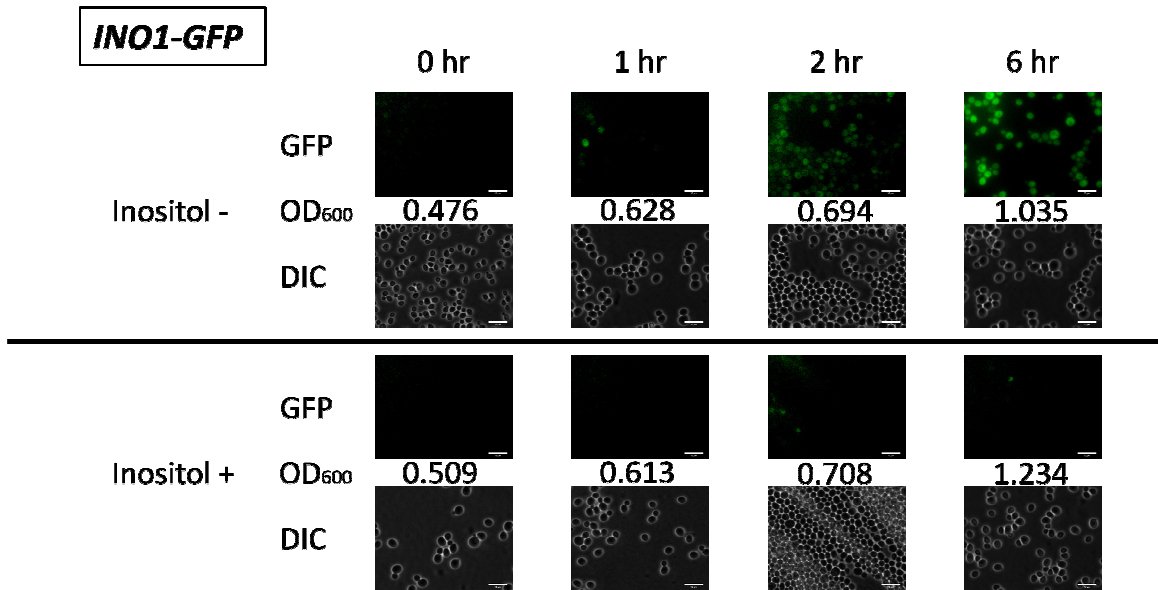
**Cells are considered induced when the fluorescence intensity of the cell is discernable above the average intensity of the background.**

When we examined the *INO1-GFP* cells collected from different growth phases, we were able to quantify the fluorescence intensity in each cell (Fig. 3.5 A). Visual examination suggested a gradual increase in fluorescence intensity throughout the population of cells. This was clearly different from the case of the *TCM1-GFP* control gene where neither the percentage of fluorescing cells nor the intensity of fluorescence was affected by the growth phase (Fig. 3.5 B).

To quantify what we observed and confirm that the growth phase regulation of *INO1* occurs uniformly throughout the entire population, we binned 300 cells according to their fluorescence intensity and plotted the results as a function of time after the media shift. We expected to see one of the two distribution patterns shown in Fig. 3.6. If there is only a small subset of cells that shifts between the different bins while the rest of the cells stay in the same bin (Fig. 3.6 A), we can assume that the changes in the amount of the *INO1-GFP* product in different growth phases is population dependent. Whereas shifting of the entire population will support that the model that growth phase regulation of *INO1* is cell dependent (Fig. 3.6 B).

In our data, there was no significant fluorescence produced by *INO1-GFP* when cells were grown in the repressing media (Fig.3.7 A). Whereas in derepressing media, fluorescence was first recognizable in 95% of the cells at the 3<sup>rd</sup> hour ( $OD_{600}=0.81$ ). In the 4<sup>th</sup> and the 5<sup>th</sup> hour ( $OD_{600}=0.912$  and  $0.947$ , respectively), fluorescence increased reaching a maximum at the 5<sup>th</sup> hour (Fig.3.7 B). The *TCM1-GFP* control strain showed a steady intensity in both I+ and I- media throughout the different growth phases (Fig. 3.7 C and D).

A.



B.

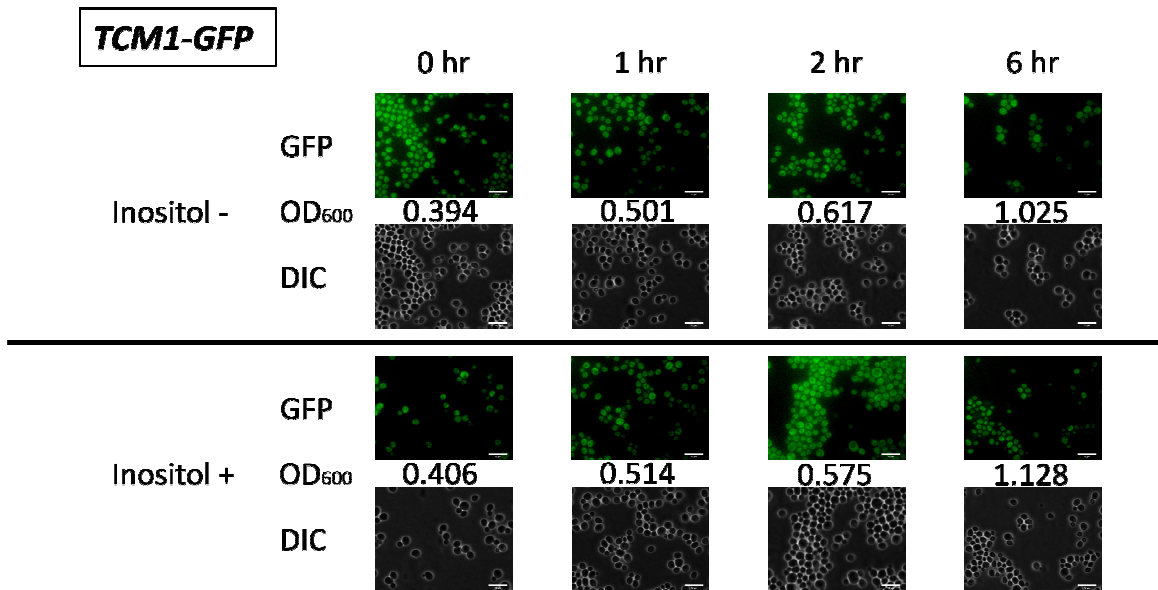
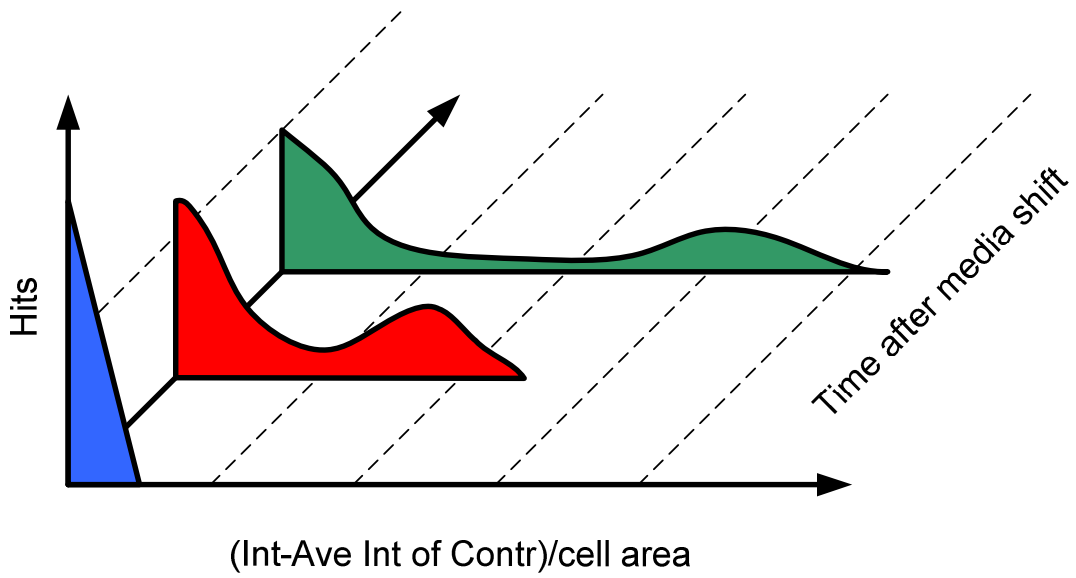


Figure. 3.5. Microscope pictures of *INO1-GFP* and *TCM1-GFP* cells at different time points.

(A) Phase contrast and green fluorescence pictures of *INO1-GFP* cells. (B) Phase contrast and green fluorescent pictures of *TCM1-GFP* cells. All pictures are taken under the 60x objective. Pictures at time 0 were taken directly after the media shift. The O.D.600 levels are shown for each image.

A.



B.

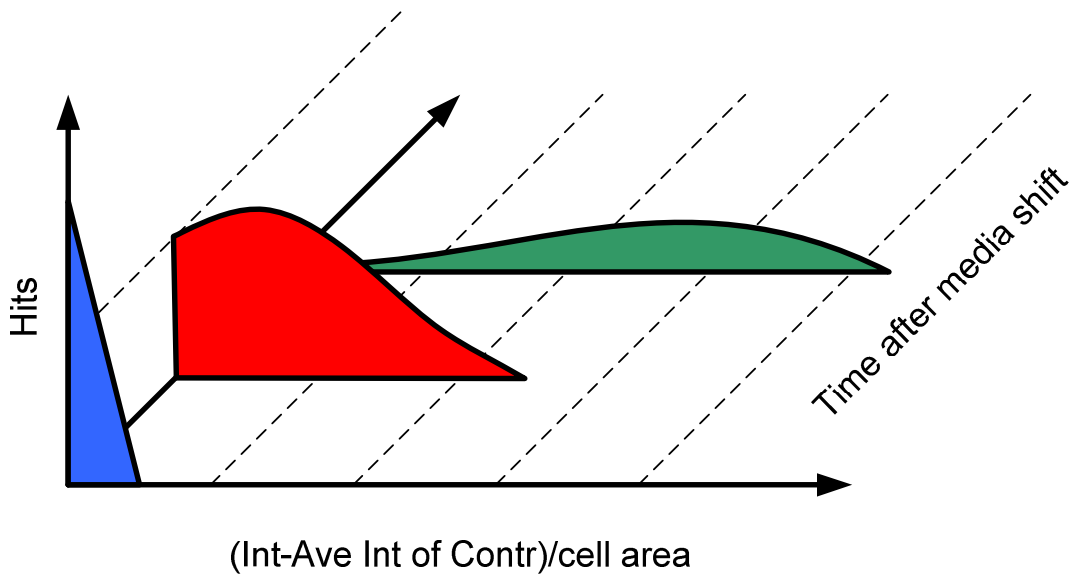


Figure. 3.6. Schematic graphs of fluorescence intensity per area over time.

(A) A model for population-dependent regulation. (B) A model for cell-dependent regulation.



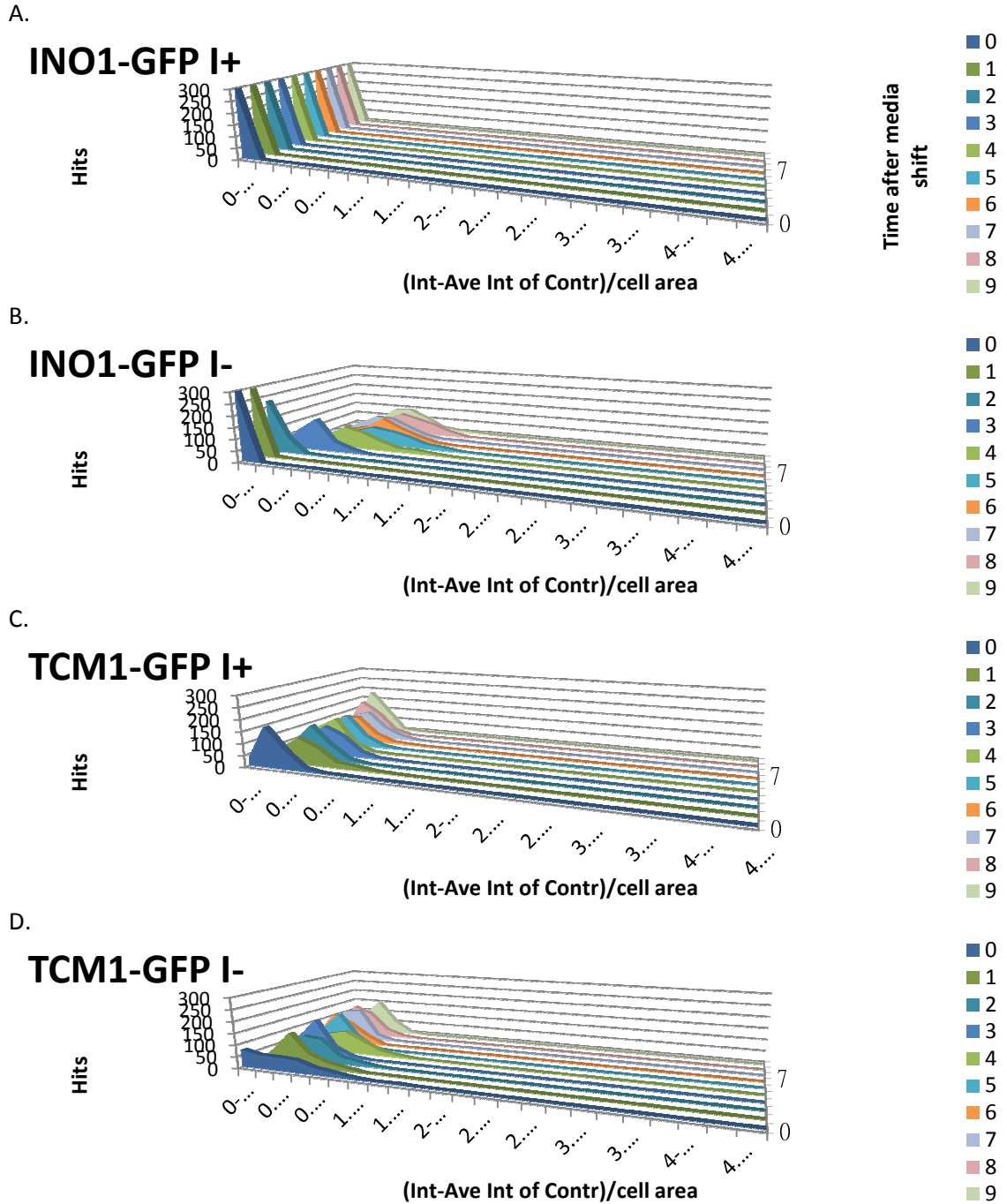
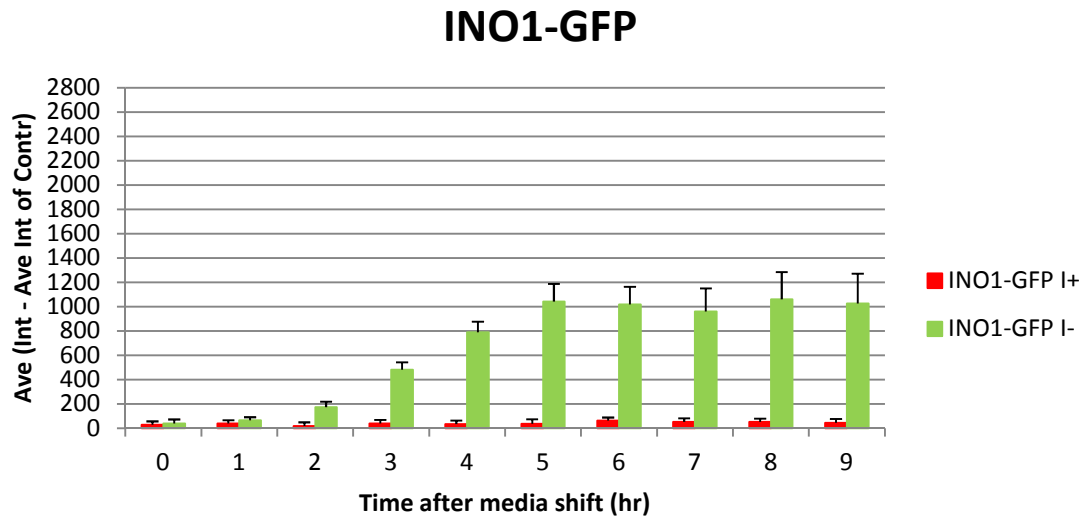


Figure. 3.7. Statistic graphs of fluorescence intensity per area over time.

Statistic graphs of fluorescent intensity of *INO1-GFP* cells shifted into I+ media (A) and I- media (B). Statistic graphs of fluorescent intensity of *TCM1-GFP* cells shifted into I+ media (C) and I- media (D). At each time point, 300 cells were examined per strain per media. Data from time 0 was generated by examining cells collected directly after the media shift.

We also examined the average fluorescence intensity of 300 cells at each hour after the media shift (Fig. 3.8A). The brightness generated by the *INO1-GFP* product increased throughout exponential growth (between the 2<sup>nd</sup> and the 5<sup>th</sup> hour after the media shift. Again, no decrease in brightness was observed in the stationary phase, which may be a function of protein stability. The *TCM1-GFP* control yielded a constant level of fluorescence (Fig. 3.8B).

A.



B.

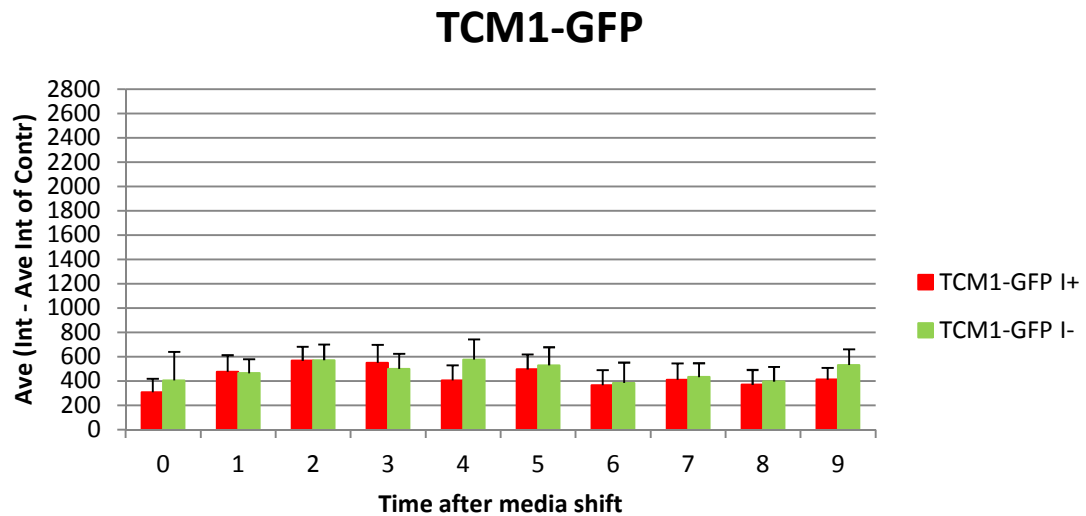


Figure. 3.8. The average fluorescence intensity of the cells examined.

(A) The average fluorescence intensity of *INO1-GFP* cells versus time after the media shift. (B) The average fluorescence intensity of *TCM1-GFP* cells versus time after the media shift. At each time point, 300 cells were examined per strain per media. Data from time 0 was generated by examining cells collected directly after the media shift.

### 3.3.1.2 Growth phase regulation of *INO1* under osmotic shock

It has been previously reported that the expression of another growth phase regulated phospholipid biosynthetic genes, *CHO1*, was altered in response to osmotic shock. The addition of 0.7 M NaCl eliminated the effect of the growth phase on the *CHO1* expression but did not affect the inositol response (Robinson et al., 2000). We were curious if and how osmotic shock affects *INO1* growth phase regulation. In contrast to *CHO1*, we found that the expression of *INO1-GFP* was still growth phase regulated (Fig. 3.8). However, the expression levels observed in the I- osmotic shock media were generally 20 times lower than what we saw in the standard I- media (compare Fig. 3.8 and Fig. 3.2).

Despite the lower transcript levels of *INO1-GFP*, the pattern of cells grown under osmotic shock showing fluorescence was effectively the same as cells grown under normal conditions (compare Fig. 3.9 and Fig. 3.4).

As we previously observed in the cells grown in I+ media, there was no fluorescence generated by the *INO1-GFP* fusion protein in the cells grown under osmotic shock (Fig. 3.6 A and Fig. 3.10 A, respectively). Under osmotic shock conditions, the induction pattern generally matched our observations in the standard media (Fig. 3.10 B and Fig. 3.6 B, respectively). However, the cells in the osmotic shock media generally displayed a broader pattern of fluorescence intensity than the cells from the standard media (compare Fig. 3.10 B and Fig. 3.6 B). This broader distribution increased in brightness throughout the growth of the culture (Fig. 3.10 B). The control strain with *TCM1-GFP* was not affected by the osmotic shock (Fig. 3.10 C and D and Fig. 3.6 C and D).

The average brightness of the cells in osmotic shock I- media followed the same pattern as in standard I- media. However, starting from the 5<sup>th</sup> hour after media shift (OD<sub>600</sub>=0.834), which was the peak of *INO1-GFP* mRNA expression was (Fig. 3.8), the intensity of the osmotically

shocked cells started yielding more intense fluorescence and by the 7<sup>th</sup> hour, the intensity was double that of cells in standard I- media (Fig. 3.11 A and Fig. 3.8 A). The variation observed in Fig. 3.11 A was pronounced because of the wide variety in fluorescence intensity of the cells (Fig 3.10 B).

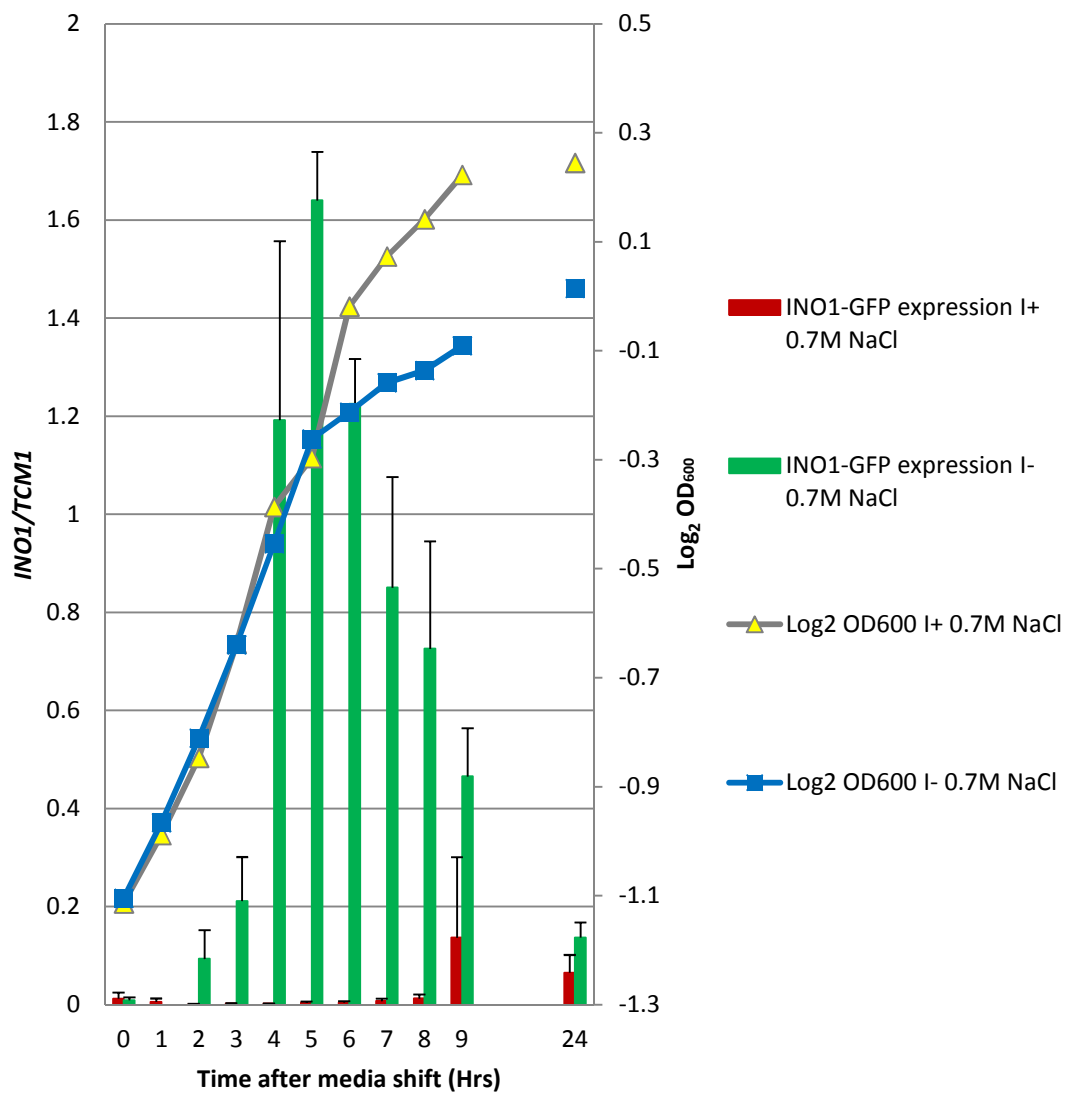
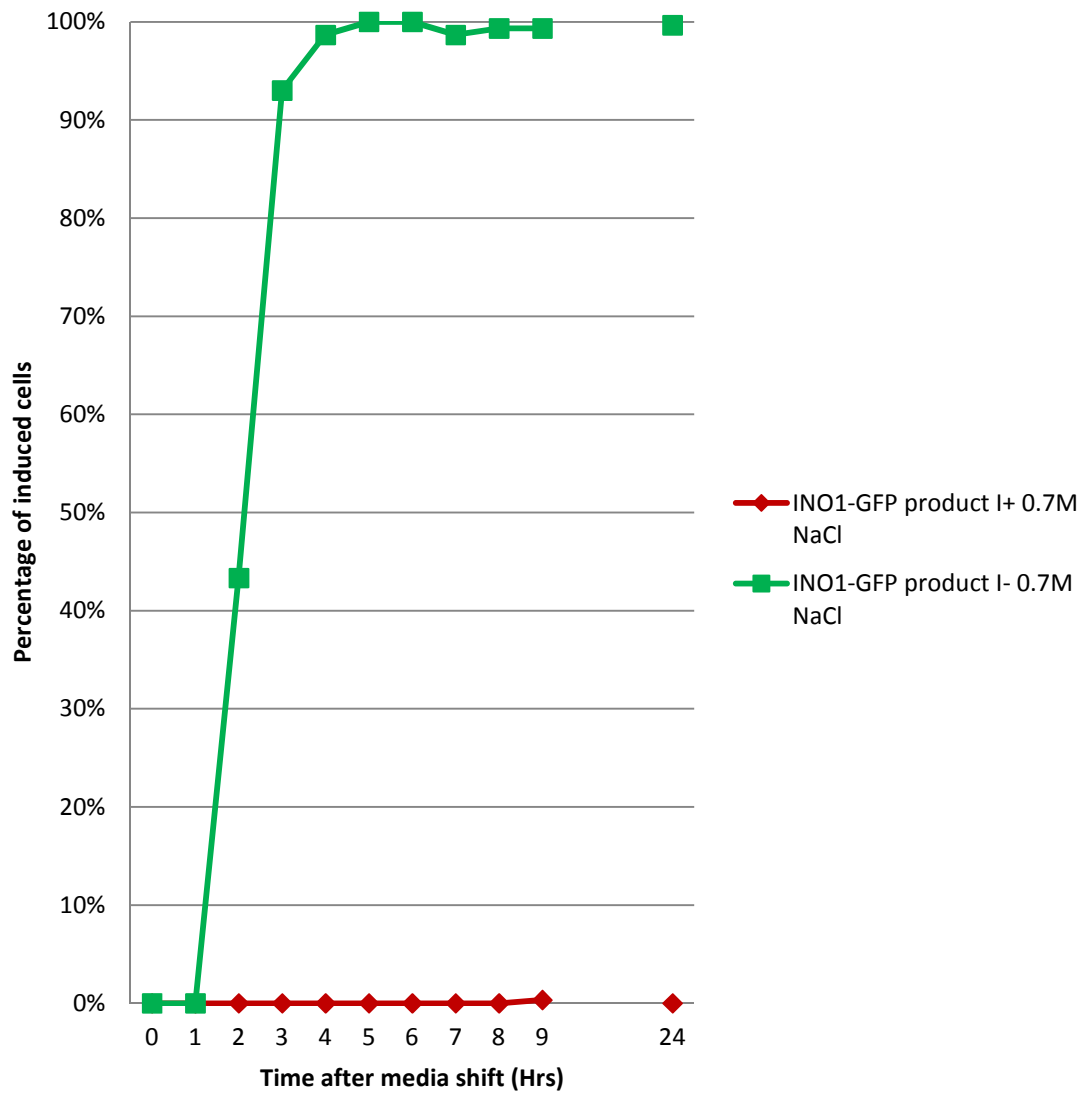


Figure. 3.9. The expression pattern of *INO1* mRNA in osmotic shock media.

*INO1* transcript levels were normalized to *TCM1*. The binary logarithm of the  $\text{OD}_{600}$  readings is presented.



**Figure. 3.10. Percentage of fluorescent *INO1-GFP* cells in cultures grown in osmotic shock media versus time.**

**Cells are considered induced when the fluorescence intensity of the cell is discernable above the average intensity of the background.**

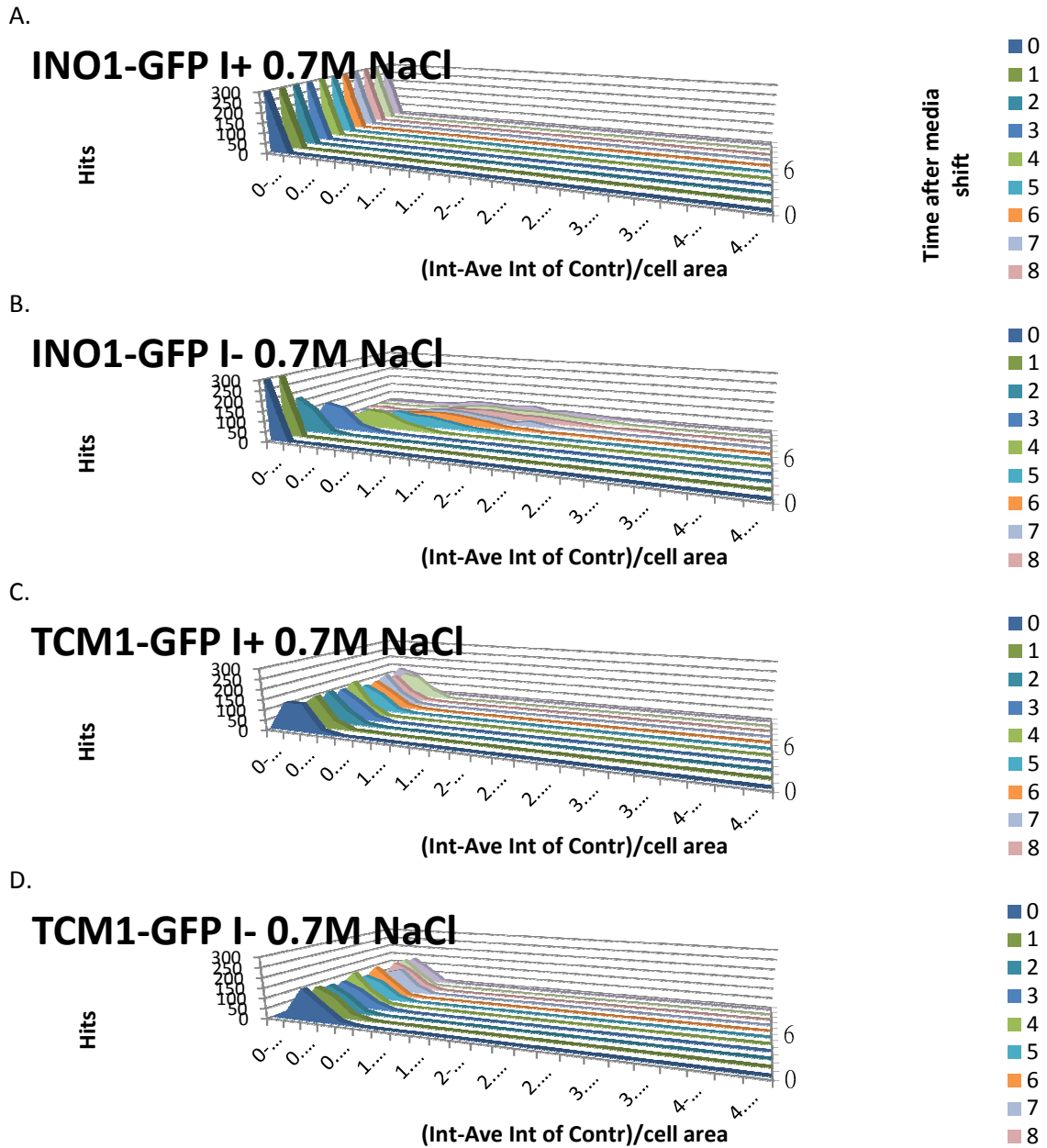
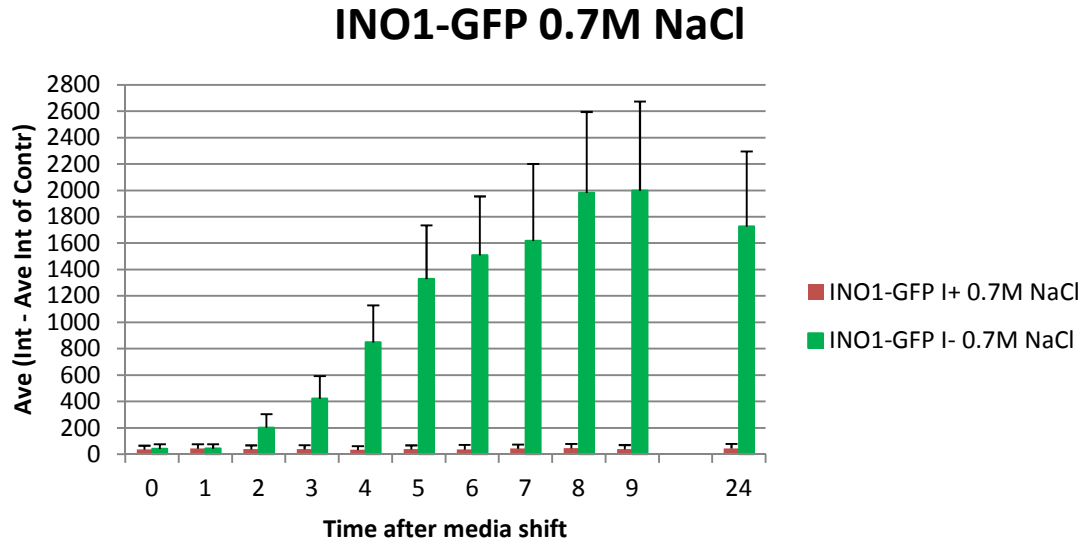


Figure. 3.11. Statistic graphs of fluorescence intensity per area over time.

Statistic graphs of fluorescence intensity of *INO1-GFP* cells shifted into osmotic shock I+ (A) and I- media (B). Statistic graphs of fluorescence intensity of *TCM1-GFP* cells shifted into osmotic shock I+ (C) and I- (D) media. At each time point, 300 cells were examined per strain per media. Data from time 0 was generated by examining cells collected directly after the media shift.



A.



B.

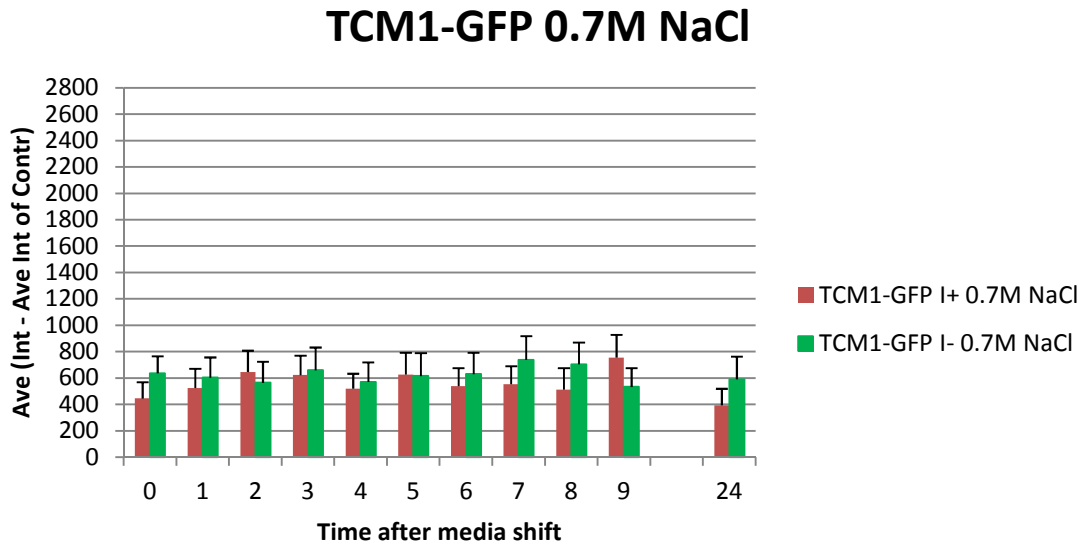


Figure. 3.12. The average fluorescence intensity of the cells examined.

The average fluorescence intensity of *INO1-GFP* (A) and *TCM1-GFP* (B) cells shifted into osmotic shock media. At each time point, 300 cells were examined per strain per media. Data from time 0 was generated by examining cells collected directly after the media shift

### 3.4 Discussion

In this chapter, we examined how cells respond to growth phases in terms of *INO1* regulation. Our data of cells growing in regular I- media agreed with previous reports: the total *INO1* mRNA expression of the culture increased in the exponential phase, reached the maximum when entering the stationary phase ( $OD_{600}=0.912$ ) and decreased during stationary phase. Most individual cells examined in these cultures were involved in contributing to the observed growth phase regulation. At  $OD_{600}=0.912$ , 99% of the cells examined possessed enough fluorescence from *INO1-GFP* to be distinguished from the background. Looking at the fluorescence intensity of each cell, we found that each cell was brighter than as cells progressed through log phase. At  $OD_{600}=0.947$ , which was 1 hour after the peak of *INO1* mRNA expression was observed, all cells showed maximum fluorescence. Surprisingly, the level of fluorescence did not decrease after entering stationary phase. When examining cells cultured in osmotic shock I- media, the pattern of all our observations were generally similar to what we have seen in standard media. However, the *INO1* transcription rate was 20 times lower in osmotic shock media than in standard I- media, with the maximum expression at  $OD_{600}=0.834$ . Although the cultures were started with the same pre-culture and at almost the same concentration ( $OD_{600}=0.456$  and  $0.476$ ), in terms of the doubling time and the expression pattern of *INO1* transcript, cells in the osmotic shock environment reached stationary phase at  $OD_{600}=0.834$ , which happened 1 hour later than what we observed in standard media at  $OD_{600}=0.912$ . All cells examined in osmotic shock fluoresced at  $OD_{600}=0.834$  and in general the intensity was greater and more varied than cells in standard I- media. The average fluorescence intensity of cells grown in osmotic shock I- media at  $OD_{600}=0.896$  was twice that of standard I- grown cells.

It is possible that there is a mechanism that represses *INO1* transcription when the Ino1 protein reaches a certain level in each cell, resulting in the drop of *INO1* transcript levels

observed in the stationary phase. However, we did not observe a decrease in fluorescence as cells entered stationary. Our microscopy data agreed with previous studies that the amount of the Ino1 protein activity increases during log phase and stays elevated in stationary phase. The difference between the patterns of Ino1 protein and the activity of the protein, previously reported, may be due to post-translational modifications of the protein in the stationary phase or simply the protein stability. The elevated fluorescence from *INO1-GFP* in stationary phase could be due to the stability of the GFP or the fusion proteins.

From our data, we understood that the growth phase regulation of the Ino1 protein is not a result of change in the amount of *INO1* expressed by a certain group, but instead, all individual cells in the culture are involved. However, we do not know if the cells respond to the changes in growth phases individually or if there is some communication going on in the culture.

### **3.5 Acknowledgement**

The authors would like to thank Dr. Steven Sandler for generously sharing his microscope and teaching us how to use it. We are deeply grateful to Kathryn Rahlwes for running the qPCR experiments in this chapter and for her company when experiments took long.

## CHAPTER 4

### CONCLUSION

#### 4.1 Summary

This study provides clues for further understanding the mechanism of yeast transcriptional regulation in response to environmental cues. The two aspects of transcriptional regulation that were discussed in this study are “The inositol mediated co-regulation of *INO1* and *SNA3*” and “The growth phase regulation of *INO1*”.

In Chapter 2, we examined the importance of regulatory *cis*- elements in inositol mediated transcription control of the phospholipid biosynthetic gene *INO1* and the gene tandemly upstream of *INO1*, *SNA3*. We also examined the evolutionary conservation of the inositol-dependent *INO1* and *SNA3* regulation in *Saccharomyces* species. Our major findings were: 1) the transcription factor Cbf1 binds to E-box 5, located within the *SNA3* promoter region; 2) E-box 5 is not involved in the inositol mediated transcription regulation of *INO1* and *SNA3*; 3) E-box 4 is involved in the inositol-mediated differential binding of Ino4 to the *INO1*-*SNA3* intergenic region; 4) E-boxes 4 and 5 do not affect *INO1* expression differently under I+ and I- conditions; 5) E-box 4 represses *SNA3* under I+ condition; and 6) inositol-mediated *INO1* regulation is evolutionary conserved but *SNA3* regulation is not conserved.

In Chapter 3, we examined how individual cells in a culture respond to changes in growth phase in terms of *INO1* regulation. Our data showed that: 1) the growth phase regulation of *INO1* is a result of all individual cells in the culture responding to the growth phase; and 2) osmotic shock does not eliminate growth phase regulation of *INO1* as it does with *CHO1*.

## 4.2 Future work

### 4.2.1 Inositol-mediated co-regulation of *INO1* and *SNA3*

We observed a role for E-box 4 in inositol-mediated *SNA3* regulation: when inositol is present (I+), E-box 4 represses *SNA3* expression. To better understand how E-box 4 accomplishes its regulatory role on *SNA3*, transcription factors that bind and act through this *cis*-regulatory element need to be identified. The combination of DNA pull-down assays using E-box 4 as probe and Mass Spectrometry may identify *trans*-acting factors. Mutation studies with the identified factors, in combination with qRT-PCR and ChIP experiments, can provide clues about what and how significant their effect is on *SNA3* regulation, as well as on the binding of our known regulators of this system.

The known *trans*-regulators of *SNA3* – Ino2, Ino4, and Cbf1, as well as the *cis*-acting E-boxes 1, 2, and 4, all play an inhibitory role on *SNA3* expression. Since *SNA3* must somehow be up-regulated under inositol lacking conditions (I-), there must also be transcription activators that are responsible for this response. A possible way of identifying transcription factors that promote *SNA3* expression is to screen through transcription factor mutant strains and search for mutants that lose the ability to up-regulate *SNA3* under I- conditions. Binding sites of putative transcription factors of interest can be identified via ChIP experiments.

We have examined the effect of E-boxes 4 and 5 on the expression of *INO1* and *SNA3*. We have also examined the importance of these E-boxes for the binding of the known regulators of *INO1* and *SNA3*. However, it remains unclear whether E-box 3, located within the *SNA3* ORF, also has a regulatory role on the two genes. To look into this question, the transcript levels of *INO1* and *SNA3* in an E-box 3 mutant strain can be examined.

#### **4.2.2 Growth phase regulation of *INO1***

The level of *INO1* transcription and the level of the *INO1* protein activity increase during exponential phase, reach the maximum at the entry of stationary phase, and decrease during stationary phase. However, the *INO1* protein levels stay high in stationary phase. It is very likely that the different regulatory patterns of the protein level and the protein activity level are a result of post-translational modifications of the protein in the stationary phase. To evaluate this possibility, Mass Spectrometry can be applied to purified exponential phase and stationary phase *INO1* products.

It is clear that every cell in a culture is contributing to the growth phase regulation of *INO1*. However, whether the cells respond to the environment individually or via communication with other cells in the culture remains unknown. Quorum sensing experiments should be carried out to determine if this is the case.

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