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# Protein Degradation Regulates Phospholipid Biosynthetic Gene Expression in Saccharomyces cerevisiae

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# Protein Degradation Regulates Phospholipid Biosynthetic Gene Expression in *Saccharomyces cerevisiae*

A Dissertation Presented

By

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## ABSTRACT

# PROTEIN DEGRADATION REGULATES PHOSPHOLIPID BIOSYNTHETIC GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

FEBRUARY 2019

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Transcriptional regulation of most phospholipid biosynthetic genes in *Saccharomyces cerevisiae* is coordinated by inositol and choline. Inositol affects phosphatidic acid (PA) intracellular levels. Opi1p interacts physically with PA and is the main repressor of the phospholipid biosynthetic genes. It is localized in the endoplasmic reticulum (ER) bound to the ER membrane protein Scs2p. When PA levels drop, Opi1p is translocated into the nucleus repressing most phospholipid biosynthetic genes. The *OPI1* locus was identified in a screen looking for **o**ver**p**roduction and excretion of **i**nositol (Opi$^-$). Opi$^-$ mutants are generally associated with a defect in repression of the phospholipid biosynthetic genes. Using a conditional shut-off library we conducted a screen that identified 121 genes with an Opi$^-$ phenotype. These genes identified pathways previously unknown to regulate the phospholipid genes like the Ubiquitin/Proteasome system. It also identified the essential subunits of NuA4 HAT. Genes involved in the Ubiquitin/Proteasome system and NuA4 HAT were tested for a repression defect in

the most highly regulated phospholipid biosynthetic genes, *INO1*. Neither mutant identified from these pathways showed a repression defect under repressing conditions. Phospholipid biosynthetic genes are also growth phase regulated that is under activating conditions (no inositol) *INO1* is active, but when cells reach the stationary phase *INO1* is repressed. Both NuA4 HAT and Ubiquitin/Proteasome genes showed a repression defect at the stationary phase of the cellular growth suggesting that these biological processes are responsible for the regulation of *INO1* at the stationary phase of the cellular growth.

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# I. OVERVIEW

*Saccharomyces cerevisiae* has proven to be an excellent model for the study of eukaryotic gene expression. Seminal studies on genes such as *PHO5*, *GAL1-10*, *HIS3* and *CYC1* have yielded a wealth of information about regulatory mechanisms that involve orchestrated interactions between specific regulatory proteins the general transcription machinery, chromatin remodeling and histone modifications[1]. The regulation of the *INO1* phospholipid biosynthetic gene has been studied for over three decades by labs all over the world and therefore contributed significantly to our understanding of gene expression. Most of these studies have focused on how the Ino2p:Ino4p:Opi1p regulatory network control *INO1* expression in response to inositol. Given the number of studies on *INO1* expression, it is surprising that new mechanisms that regulate its expression continue to be discovered (*eg*, transcriptional memory and Cbf1-mediated regulation). This underscores the results reported here which show that protein degradation and the NuA4 histone acetyltransferase (HAT) also contribute to regulating to *INO1* expression. These discoveries were made possible by the ability to easily screen specific mutant collections that cover >90% of the yeast genome.

As a backdrop to the results reported here, it is necessary to briefly summarize our understanding of the regulation of *INO1* expression, the usefulness

of genetic and genomic screens for the understanding of *INO1* regulation, the process of protein degradation, the role of the NuA4 HAT complex.

## Membrane synthesis and *INO1* regulation

In yeast, inositol is a critical phospholipid precursor and its synthesis is highly regulated. We know that *INO1* encodes for inositol-3-phosphate synthase, which uses glucose-6-P and converts it into inositol[2]. Early on, researchers successfully purified this enzyme and characterized its activity. They also identified mutants like *ino2* and *ino4* which prevent expression of inositol-3-phosphate synthase, in contrast to *opi1* mutations that render the enzyme constitutive. Using genetic approaches, the *INO1* gene was isolated and cloned into a plasmid[3]. This was very important for the field since the cloning of *INO1* allowed researchers to identify the regulatory system. This lead to the identification of the *INO2* and *INO4* genes as positive regulators of *INO1*[4,5].

*INO1* is a structural gene that encodes inositol-3-phosphate synthase which converts glucose-6-phosphate into inositol-3-phosphate in the *de novo* synthesis of phosphatidylinositol (PI)[2,3]. Transcriptional regulation of *INO1* is responsive to inositol and choline. *INO1* is highly expressed when inositol and choline are absent, partially repressed when inositol alone is present, and fully repressed when both inositol and choline are present[6]. The activation mechanism for *INO1* expression requires a *cis*-acting regulatory element called UAS$_{INO}$[5,7,8] and the Ino2p and Ino4p activator proteins.

The UAS*INO* element consists of a 10bp sequence (5'-CATGTGAAAT-3') that serves as a binding site for the Ino2p/Ino4p heterodimer activator complex[4,9,10]. Ino2p and Ino4p belong to the basic Helix-Loop-Helix (bHLH) family of regulatory proteins which form dimers in order to bind DNA. While the *INO1* promoter has nine UAS*INO* elements, only two have been shown to be functional[8].

Activation requires a sequence of events initiated by the binding of the Ino2/Ino4p complex to target promoters. This mechanism includes the Snf1p histone kinase and the SAGA histone acetyltransferase complex (HAT)[11–15]. When bound to the *INO1* promoter the transcriptional activation domain of Ino2p recruits Snf1p and phosphorylates Serine 10 of histone H3[15]. This phosphorylated H3 then recruits the SAGA complex which acetylates Lysine 14 of histone H3 resulting in a relaxing of the chromatin. The phosphorylated H3 is also responsible for recruiting the TATA-binding protein (TBP)[15].

The mechanism for repression has also been worked out and involves a repressor protein called Opi1p. The *OPI1* locus was originally identified based on the phenotype of ***o***ver***p***roduction of ***i***nositol (Opi$^-$)[16]. Opi1p is a leucine zipper protein with two poly-glutamine rich domains. In general, leucine zipper proteins are known to dimerize and bind DNA[17], however there is no evidence in support of Opi1p interacting directly with DNA. In the absence of inositol, Opi1p binds to phosphatidic acid (PA), stabilizing an interaction with the endoplasmic reticulum (ER) integral membrane protein, Scs2p [18–21]. The signal that actually dictates inositol repression is PA levels, rather than inositol itself. PA is an early precursor in the synthesis of phospholipids that accumulates in the absence of inositol.

However, when inositol is present, PA is consumed leading to Opi1p release from the ER, translocation into the nucleus and inhibiting transcription of *INO1* by directly interacting with Ino2p bound to *INO1* promoter[20]. Predictably, *scs2* mutant strains are inositol auxotrophs because Opi1p constitutively translocates to the nucleus repressing *INO1* expression[19]. The mechanism for Opi1p repression in response to inositol involves the Sin3p-Rpd3p Histone Deacetylase Complex (HDAC)[22–33]. Op1p bound to Ino2p recruits the HDAC which compresses chromatin structure by deacetylating the Lys5 and Lys 12 residues in histone H4[22].

The HDAC complex also regulates *INO1* expression via a second independent mechanism involving Ume6p. Ume6p is a global repressor known to repress meiosis genes and was shown to repress *INO1* by binding to a URS1 (upstream repressing sequence) element (5'-AGCCGCCA-3')[22,26–28,30,31] in the *INO1* promoter and recruiting the Sin3p-Rpd3p HDAC complex.

Opi1p activity is also regulated by phosphorylation via protein kinase C (PKC), protein kinase A (PKA) and casein kinase II[34–36]. PKC phosphorylates Opi1p, at Ser26 and mutating this residue results in decreased expression of *INO1-lacZ* under derepressing conditions[35]. PKA phosphorylates Ser31 and Ser 251 and mutating these residues yielded increased expression of *INO1-lacZ* under both repressing and derepressing conditions[36]. Lastly, casein kinase II phosphorylates Ser10 and mutating this residue resulted in increased expression of *INO1* under derepressing conditions[34]. These experiments suggest that phosphorylation of Opi1p plays a role in both repressing and derepressing conditions, regulating Opi1p activity positively and/or negatively.


Figure 1: **Summary of the regulatory proteins that control *INO1* transcription.** Generally, positive regulators are highlighted in green and negative regulators in red. The summary shows ~100 bp of the *INO1* promoter which is required for all of its regulation. The interactors that take place at the $UAS_{INO}$ elements are shown for only one of the two elements to simplify the figure. Arrows indicate positive roles and lines ending in bars indicate negative roles. **Cheng et al. BBA, 2007, Vol 1771; 310-321**

Transcriptional memory regulation of *INO1*

In many organisms including yeast, prior experiences alter the regulation and transcriptional rate of many genes. This phenomenon is sustained through various cell division cycles and is called epigenetic transcriptional memory[37]. The *INO1* gene is a model system for this type of regulation. The process works by allowing pre-binding of RNA polymerase to the *INO1* promoter, bypassing the recruiting step of the RNAPII to the promoter during reactivation.

The nuclear pore complex (NPC) is an essential player in the transit of RNA and protein between the nucleus and the cytoplasm[38–42]. Early studies suggested that the NPC physically interacts with silenced genes, but other data has shown that the NPC also interacts with many active genes creating a memory of prior transcription events[43]. Transcriptional memory of *INO1* requires physical interaction of the *INO1* promoter with the NPC which will remain associated for several generations after switching to repressing conditions. This NPC association requires an altered chromatin structure and binding of a poised RNAPII to the recently repressed promoter[37,44,45]. This is possible because *INO1* moves to the nuclear periphery and physically interacts with the NPC upon activation[18,43,46]. The interaction of *INO1* promoter with the NPC requires small *cis*-acting DNA elements[46]. Two elements called GRS I and GRS II in the *INO1* promoter are necessary for targeting it to the NPC[46]. These elements work as DNA zip codes which are essential for *INO1* targeting to the NPC and activation of gene expression. They are also required for subsequent repositioning to the nuclear periphery (that is, memory)[46–48]. GRS I binds to a transcription factor Put3p, which will dictate GRS

I-mediated positioning[47], this DNA zip code encode subnuclear positioning through transcription factor binding sites. In addition, transcriptional memory leads to interchromosomal clustering[48], clustering during memory requires clustering of active *INO1* and the MRS zip code, but not GRS I. This suggests that the *INO1* gene has the ability to cluster with different gene partners under activating and memory conditions which leads to the conclusion that interchromosomal clusters can be remodeled. In yeast, several stress-induced genes and the *GAL* genes show a very similar memory behavior, however, the mechanism by which their transcriptional memory works is similar but not identical to that of *INO1*[49,50].

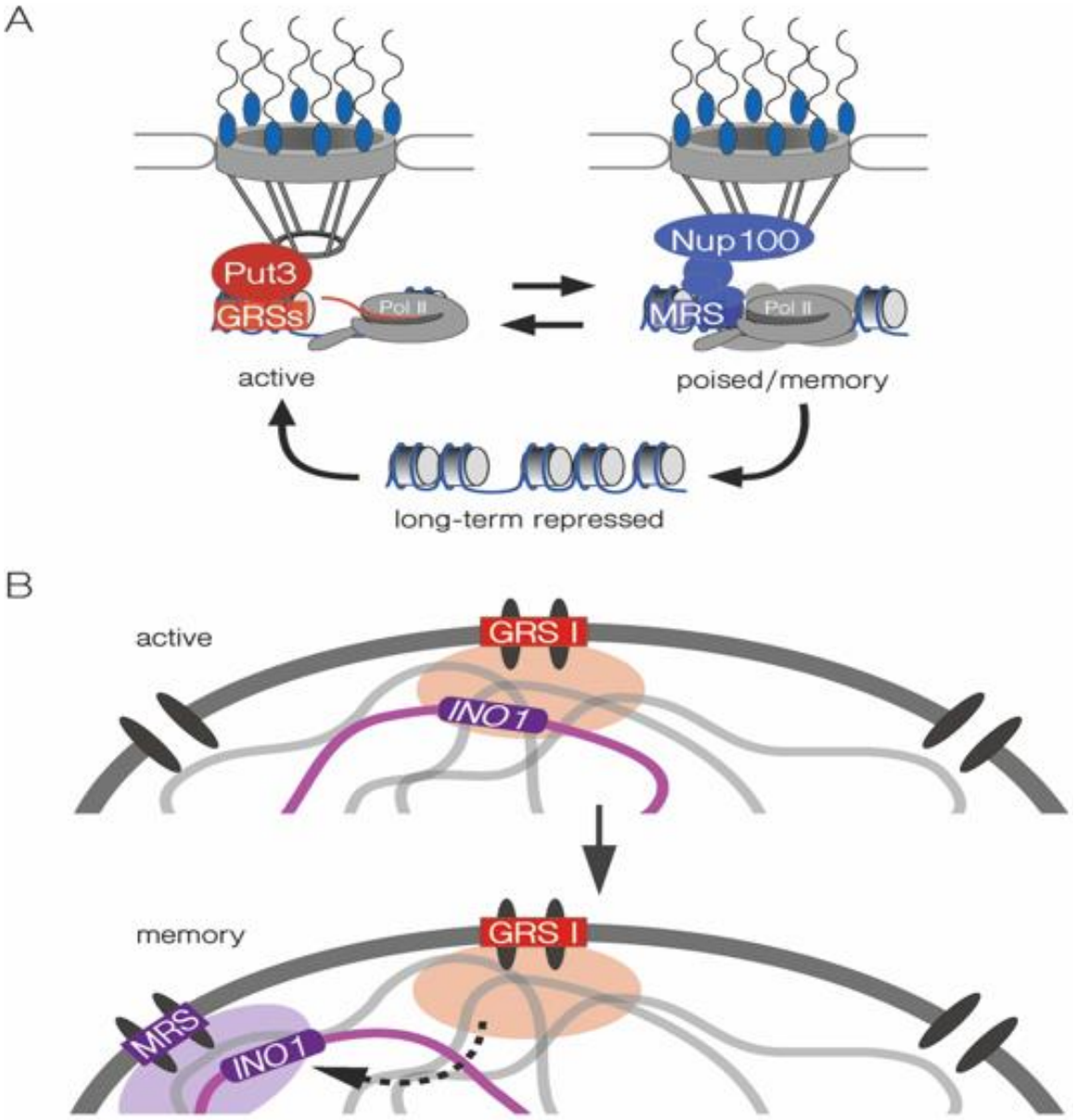


Figure 2: **Model for zip code-dependent *INO1* targeting to the NPC and interchromosomal clustering.**
**(A)** The long-term repressed gene is positioned in the nucleoplasm and both the active and recently repressed memory state of the gene are positioned at the nuclear periphery through interaction with the NPC. The GRS elements control targeting to the NPC under activating conditions. The Put3 transcription factor binds the GRS I zip code and is required for GRS I-mediated peripheral targeting and interchromosomal clustering [7]. The MRS element controls targeting to the NPC under memory conditions and requires Nup100 [10].
**(B)** The *INO1* gene clusters with other GRS I-containing loci under activating conditions (top) and this is a prerequisite for clustering with itself (and potentially other loci) in an MRS-dependent cluster for several generations after repression, during transcriptional memory. **Brickner et al Microbial Cell, Vol. 2, No. 12, pp. 481 - 490; doi: 10.15698/mic2015.12.242**

Cooperative derepression of *INO1* via centromere-binding factor 1 (Cbf1p)

In previous sections we have discussed in detail *INO1* regulation by the Ino2p:Ino4p:Opi1p circuit. Even though, *INO1* regulation has been studied for over three decades, novel players in *INO1* regulation have recently being discovered as is the case with Cbf1p. Cbf1p is a well-known regulator of *MET* gene expression and a centromere DNA element I (CDEI) binding protein that belongs to the bHLH protein family (just like Ino2p and Ino4p)[51,52].

Previous studies of yeast bHLH proteins were restricted to understanding the regulation of single genes or pathways[53,54]. With that in mind our lab studied cross regulation of biological process by different bHLH proteins. One such study sought to know if *INO1* was regulated by other bHLH proteins. When testing *INO1* transcription on *cbf1Δ* mutant, its transcription was found to be reduced to 21% under derepressing conditions when compared to WT. Cbf1p was subsequently shown to bind to sites upstream and distal to the *INO1* gene (up to 1.6 kb upstream). Indeed two Cbf1p binding sites were included in the promoter of the upstream *SNA3* gene[55]. It was also discovered that Cbf1p is required for maximal binding of Ino2p/Ino4p to the *INO1* promoter in a cooperative matter[55] and that likewise Ino2p and Ino4p are required for the recruitment of Cbf1p upstream of *INO1*.

Cbf1p is known to regulate transcription by recruiting chromatin remodelers of the imitation switch (ISWI) class family (including Isw2p), a family known to be involved in *INO1* repression[56–58]. In the published study, Cbf1p was established to be an important player in Isw2p binding to the *INO1* promoter[55].

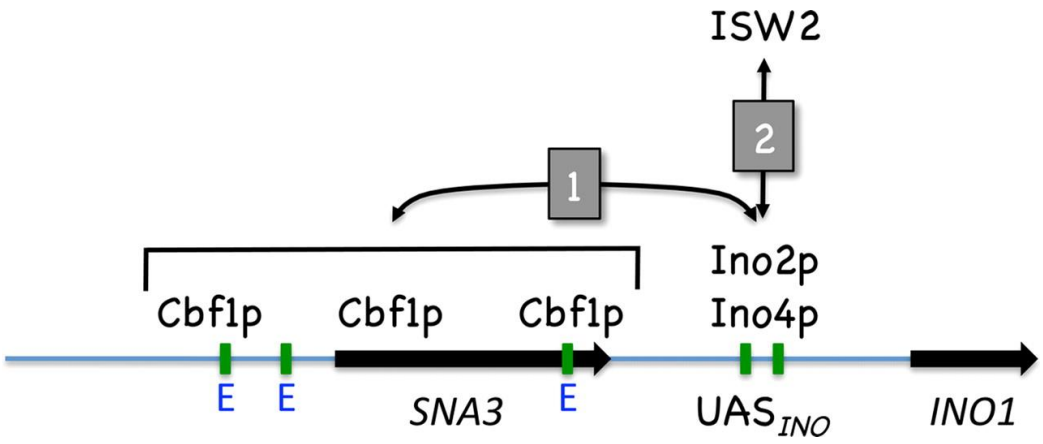


Figure 3: **Model of regulation of *INO1* transcription by Ino2p-Ino4p, Cbf1p, and ISW2.**
Black arrows indicate the positions of genes, and green bars indicate the positions of UAS$_{INO}$ elements and other potential E boxes. Numbered arrows indicate the sequence of events. **Ameet Shetty, and John M. Lopes Eukaryotic Cell 2010; doi:10.1128/EC.00144-10**

The power of inositol auxotrophy for the study of biological processes

A powerful aspect of *Saccharomyces cerevisiae* is the myriad phenotypes that can be screened/selected for in order to understand a particular biological process. One report described 80 easily assessable phenotypes that are grouped into different categories. These categories include, conditional phenotypes such as temperature, ethanol, and growth sensitivity. Cell cycle defect phenotypes include $G_1$ arrest, failure to arrest in $G_1$, and $G_2$/M arrest. Mating and sporulation defects can help identify genes important for mating, sporulation and meiosis among other processes. Other categories include defects in cell morphology, cell wall synthesis, responses to environmental stresses, nucleic acid metabolism, and sensitivity to drugs. Auxotrophies represents the biggest group, and include auxotrophy for certain amino acids, phosphate, ability to grow on different carbon sources like galactose, maltose, and sucrose, nitrogen utilization, and the most important for the purpose of this thesis inositol auxotrophy[59].

Auxotrophies typically are failures in gene expression that are required for the synthesis of a specific nutrient or biochemical intermediate. Many auxotrophies occur when mutants have a defect in a specific transcriptional regulatory mechanism, although other auxotrophies can be associated with general transcription defects[59].

Surprisingly, inositol auxotrophy has proven to be indicative of defects in the general transcriptional apparatus, as it appears that the *INO1* gene has an extreme sensitivity to general transcription machinery perturbations[60]. For example, altering proteins in RNA polymerase II (RNAPII), TBP, Spt7p, SWI/SNF complex, all

yield an Ino$^-$ phenotype[61–64]. RNA polymerase II (RNAPII) is the enzyme responsible for transcription of all genes that code for class II genes. It is a multi-subunit enzyme and its structure is conserved throughout eukaryotes[65]. In the past many researchers aiming to identify mutations that could affect RNAPII activity, classified them into two different categories: assembly and/or stability of the enzyme. Inositol auxotrophy has been useful for studying RNAPII, because inositol auxotrophs are often associated with mutations that affect RNAPII, regardless of the type of defect[66]. The power of this phenotype is that *INO1* expression is not derepressed in mutant RNAPII cells in contrast with many other genes that are transcribed sufficiently to avoid yielding an auxotrophy. Inositol auxotrophy in RNAPII mutants, can happen due of the reduced assembly of RNAPII. This has been observed when a mutation is present in RPO21 (RNAPII largest subunit)[66,65].

## Whole genome Opi$^-$ mutant screens to further understand transcription regulation

Many studies have been done demonstrating that screening the yeast genome is useful in generating valuable information about well-studied processes[67–70]. Our lab has focused on genome-wide screens to identify mutants with an Opi$^-$ phenotype to further understand repression of phospholipid biosynthesis[71,72]. Our screen using the Viable Yeast Deletion Set (VYDS), which includes ~4,800 mutants, identified 91 Opi$^-$ mutants. Several of the mutants identified here were previously known, but a number of genes were identified that were previously unknown to play a role in *INO1* regulation. Over-represented biological functions include components of the Rpd3p HDAC complex and six of the non-essential subunits of

NuA4 HAT complex[71]. The screen also identified the *REG1* gene which is involved in regulating gene expression in response to changes in glucose. Initially this was thought to suggest a coordination between glucose usage and phospholipid synthesis, but now it is known that the Opi$^-$ phenotype is due to an altered protonation status of PA, as a function of cellular pH (altered in a *reg1* mutant). The altered protonation status affects Opi1p translocation into the nucleus[73].

Many genes involved in the unfolded protein response (UPR) system were also identified, which was expected based on previous studies showing that there is a coordination between UPR and phospholipid synthesis[37,74–76]. The VYDS screen identified an ubiquitin E2 enzyme-encoding gene, *UBC13*. This was the first time a gene involved in the ubiquitination pathway was associated with an Opi$^-$ phenotype and this will become an important piece of the puzzle later in this thesis. The subject of this thesis is an Opi$^-$ screen using an essential gene library and it yielded further information about the regulation of phospholipid genes. On one hand it further complemented many processes identified in the VYDS screen like the essential components of NuA4 HAT, and known Opi$^-$ phenotype genes like *CDS1*, but also identified novel functions like components of the Nuclear pore complex (*NIC96*, *NUP1*, *NUP145*, *NUP49*, *NUP82*, and *NUP85*), gene looping (*PTA1* and *SSU72*), protein modifications like SUMOylation (*AOS1* and *UBC9*), and it further confirmed that the protein degradation genes play a role in the regulation of the phospholipid biosynthetic pathway. Identifying different subunits from the proteasome and also identifying missing components of the ubiquitination pathway, including the only E1 enzyme in yeast (*UBA1*) and the only essential E3 enzyme (*RSP5*), combined with

the previously identified E2 enzyme, UBC13, yields all relevant components of the E1-E2-E3 enzyme cascade from the ubiquitination pathway, strongly suggesting that this pathway might be responsible for the regulation of *INO1*[72].

### Role of protein degradation in *INO1* regulation

Biological processes which includes cell cycle, DNA repair, transcription, tumor repression, and neurogenesis have been reported to be regulated by protein degradation[77–80]. One pathway for protein degradation requires the proteasome which is a protein complex present in all eukaryotes, archea, and some bacteria. Its specific function is to destroy damaged or unnecessary proteins[81]. Its structure consists of two main subassemblies; the 19S regulatory particle (RP), which includes the lid and base, and the 20S core particle (CP). The RP recognizes peptides to be degraded, while the CP contains the proteolytic active sites to degrade targeted proteins[78]. A regulatory process that is necessary to recognize and target proteins to the proteasome for destruction is modification involving a covalent attachment of ubiquitin. This process dynamically sculpts the proteasome with hundreds of yeast proteins being rapidly and selectively degraded[82]. Ubiquitination is governed by an E1-E2-E3 cascade of enzymes. An ubiquitin-activating enzyme (E1) utilizes ATP to transfer the ubiquitin peptide (Ub) to an Ub-conjugating enzyme (E2), which will transfer the Ub to an Ub-ligase (E3) holding the target substrate to be ubiquitinated[83,84]. After several rounds of conjugation, polyubiquitination is achieved. In higher eukaryotes the most common site residue to be modified by Ub is Lysine[85], however, serine, threonine, and cysteine

ubiquitination have been observed in both yeast and mammals[86–89]. The conjugation machinery in yeast consists of a single E1 enzyme, eleven E2 enzymes, and a large family of E3 enzymes (60-100). E3 enzymes mediate the selection and specificity of ubiquitination throughout direct interactions with the substrate[78].

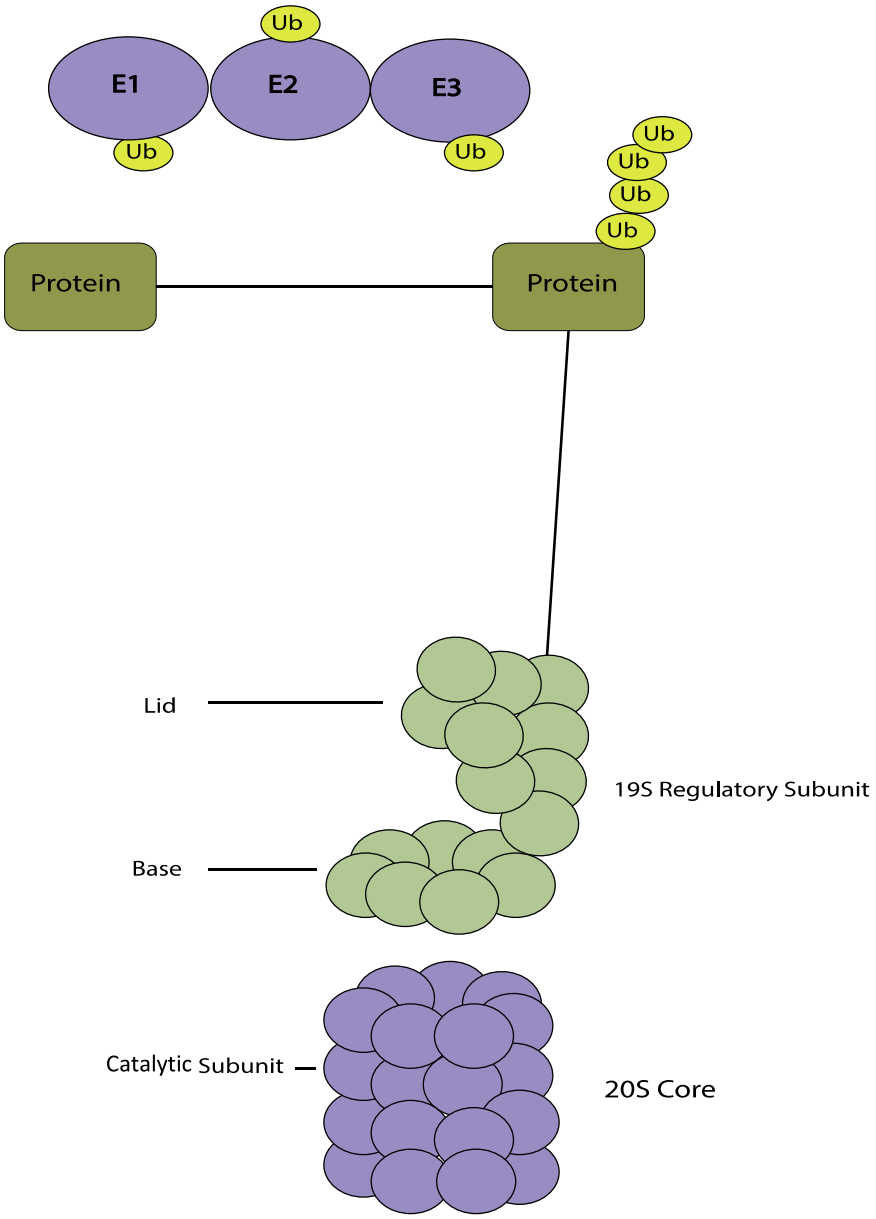


Figure 4: **Ubiquitin-Proteasome System in *Saccharomyces cerevisiae***
In eukaryotes proteins can be degraded through the Ubiquitin (Ub)-Proteasome system. It consists of a E1-E2-E3 cascade of enzymes which will transfer a Ub onto the targeted protein. Several rounds of ubiquitination result in polyubiquitination which is the main recognition path for the proteasome. The proteasome is a barrel-like complex consisting with a Regulatory Subunit (RS) which includes the lid for target recognition and base with ATPase activity, and the 20S Core subunit which is the area where proteins are degraded.

## NuA4 HAT and chromatin regulation

In eukaryotes, gene expression has a higher degree of difficulty in that DNA is tangled in a complex of proteins collectively called chromatin. Chromatin is very dynamic and active in processes that requires regulation of genes[90]. Histones are a family of basic proteins that are connected with DNA in the nucleus and help to wind DNA into what we call the chromatin. Histones tails can have a number of modifications that include methylation, phosphorylation, ubiquitination, and acetylation. Acetylation of histones is known to play a double role in the cell. Acetylation of Lysine residues is known to neutralize the positive charge of histone tails which leads to weaker interaction with DNA, this causes the chromatin to relax and decondense making genes available for transcription by unwinding it from the chromatin. Alternatively, acetylation can provide an epigenetic marker for gene expression by blocking the heterochromatin-stabilizing association complexes[91–94]. This type of modification is driven by Histone Acetyl Transferases (HAT), which exist in large complexes such as NuA4 (Nucleosome acetyltransferase of H4), one of the most conserved HAT complexes in eukaryotes[95,96]. The NuA4 complex is important because of its role in different essential processes like DNA repair and transcription regulation[97–101].

The NuA4 HAT consists of a 12-subunit complex with Esa1p as the catalytic subunit that acetylates proteins. It is a primary regulator of gene expression and cell cycle progression. Acetylation of Lysine is dictated by HAT complexes such as NuA4, which are associated with transcriptional activation[102]. HATs are directed to promoters throughout interaction with histone tails or chromatin binding

proteins[103]. In addition to histones, transcription factors are also modified by acetylation. In addition, it is known that acetylation is important for targeting some proteins for degradation[104]. Nine of the thirteen subunits that compile the NuA4 HAT have been identified with an Opi$^-$ phenotype, this includes the Lysine catalytic subunit Esa1p[71,72]. With such overrepresentation of NuA4 HAT in previous studies, it is suspected that NuA4 HAT plays a novel type of regulation of the phospholipid biosynthetic genes.

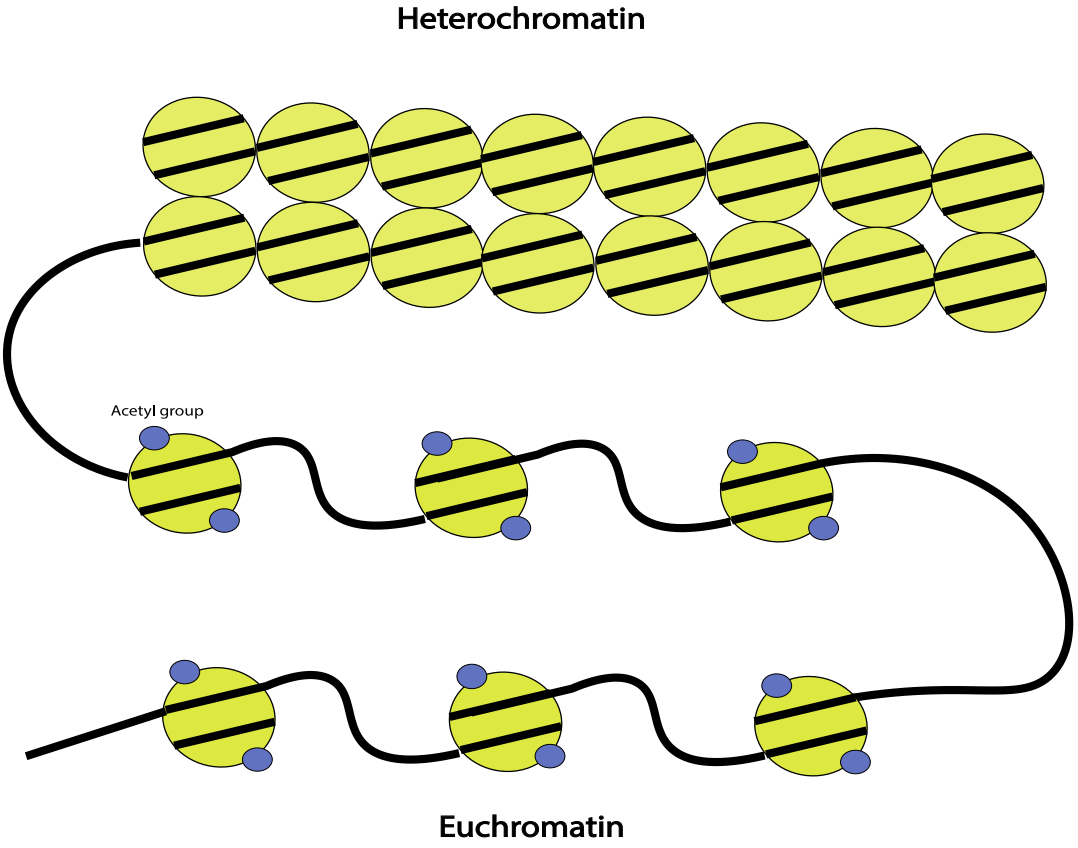


Figure 5: **Heterochromatin vs Euchromatin**
Heterochromatin is when DNA is tightly bound to the histone complex forming a higher level structure. Usually gene expression is reduced in a heterochromatin structure because of the poor accessibility to DNA. Euchromatin is referred to areas of DNA where gene expression is active. DNA chromatin is relaxed and DNA is available for the transcription machinery to access. Typically acetylation of histones is one mechanism whereby DNA can be relaxed from its interaction with histones. Acetylation of histones is an indication of gene expression.

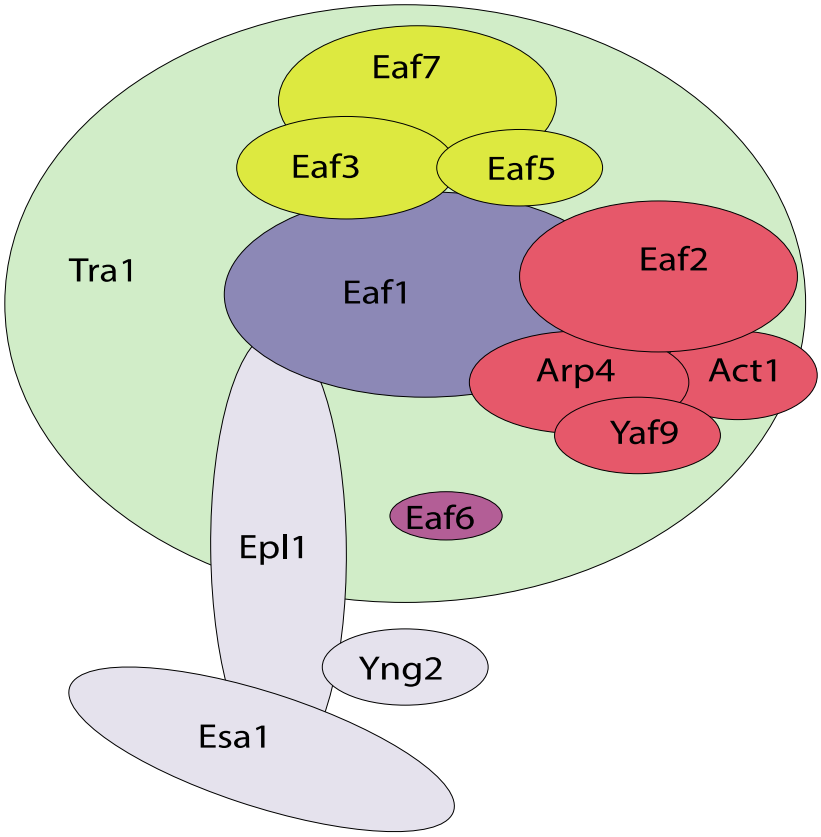


Figure 6: **NuA4 HAT Complex**
Image of the NuA4 HAT complex.

CHAPTER

# II. *SACCHAROMYCES CEREVISIAE* ESSENTIAL GENES WITH AN OPI$^-$ PHENOTYPE

## Introduction

Phospholipid biosynthetic genes in yeast are regulated by inositol and choline[76,105–108]. These genes are fully repressed in the presence of inositol and choline and derepressed when these are limiting. This regulation requires several transcription factors that when mutated display one of two phenotypes: inositol auxotrophy (Ino$^-$) or overproduction and secretion of inositol (Opi$^-$)[106,107]. Some of these mutants were identified over the last three decades through traditional genetic screens. However, we previously reported a genomic screen of the viable yeast deletion set (VYDS) for Opi$^-$ mutants that identified 91 mutants[71]. Here, we report a screen of the essential yeast gene set using a conditional-expression library[69].

Well studied regulators of the phospholipid biosynthetic genes include Ino2p:Ino4p activators, the Opi1 repressor, and the Ume6p-Sin3p-Rpd3p histone deacetylase complex (HDAC), the SAGA histone acetyltransferase complex, the ISW2, INO80, SWI/SNF chromatin remodeling complexes, and Mot1p[4,10,27,28,31,109]. Ino2p and Ino4p belong to the family of basic-helix-loop-helix regulatory proteins (bHLH). These proteins form a heterodimer that binds to a UAS$_{INO}$ sequence to activate transcription of most of the phospholipid biosynthetic genes (eg. *INO1*, *CHO2*, and *OPI3* in Figure 7)[76,108]. The Ume6p-Sin3p-Rpd3p HDAC, the ISW2, and

INO80 chromatin remodeling complexes , and Mot1p are global regulators that play a negative role in phospholipid biosynthetic gene expression[22,25,27–30,110–112]. Opi1p was the first, and to date the only repressor found that specifically regulates the phospholipid biosynthetic pathway.

The *OPI1* locus was first identified in a screen for mutants that overproduce and excrete inositol (Opi$^-$ phenotype) into the medium of growth in the absence of inositol and choline[16]. The original *opi1* mutant and a small set of similar mutants identified over the next two decades showed that the Opi$^-$ phenotype correlated with a defect in repression of the *INO1* gene[6,22,23], which is required for inositol synthesis *de novo* (Figure 7)[113]. However, most of the ninety-one Opi$^-$ mutants identified in a more recent screen of the VYDS did not affect inositol-mediated repression of an *INO1-lacZ* reporter[71].

Our current understanding of the mechanism for inositol-mediated repression of phospholipid biosynthetic gene expression is that it requires translocation of Opi1p from the endoplasmic reticulum (ER) to the nucleus. Repression in response to inositol and choline is mediated by phosphatidic acid (PA). In the absence of inositol, PA levels are elevated and Opi1p binds to PA[20] and is tethered in the ER by Scs2p, an integral membrane protein[19–21,114,115]. When inositol is added, phosphatidylinositol (PI) synthesis increases, causing a decrease in PA levels and Opi1p is released from the ER. Opi1p rapidly translocates into the nucleus where it represses transcription by directly interacting with the Ino2p transcriptional activator and recruiting several HDACs to repress transcription[25,116–118]. The addition of choline by itself has little effect on PA levels, however in

combination with inositol, choline further reduces PA levels resulting in additional repression. Not surprisingly, blocks in the *de novo* phosphatidylcholine (PC) biosynthesis that elevate PA levels also yield an Opi$^-$ phenotype[119–122]. Thus *cds1*, *cho2,* and *opi3* mutants all have an Opi$^-$ phenotype (Figure 7). The Opi$^-$ phenotype of these mutants is conditional and it can be suppressed by adding choline (C) to the medium. Choline restores PC synthesis through the Kennedy pathway thereby alleviating the accumulation of PA caused by the block in the *de novo* PC pathway (Figure 7).

Consistent with the role of PA as the signal, we reported that reduced expression of the *PIS1* gene (Figure 7) yields an Opi$^-$ phenotype[123]. Because PI is an essential gene, we created a strain harboring a *GAL1-PIS1* gene that allowed us to reduce *PIS1* gene expression by growth in glucose or low galactose concentrations[123]. These results are consistent with another study showing that GFP-Opi1p translocation into the nucleus is slow and impaired in a *pis1* partial function mutant[20].

Many studies have shown that screening the VYDS[67,68] and an essential yeast mutant gene set[69] can yield valuable insight into well-studied processes such as regulation in response to phosphate concentration[70]. We previously reported the results of a VYDS screen for the Opi$^-$ phenotype to further understand repression of phospholipid biosynthesis[71]. That screen identified all seven of the Opi$^-$ mutants that had been identified by several labs over the previous thirty years, but also identified 84 new Opi$^-$ mutants. Highly represented in this mutant set were components of the Rpd3p HDAC complex and five of the six nonessential

components of NuA4 HAT complex (*EAF1*, *EAF3*, *EAF5*, *EAF7*, and *YAF9*)[71]. The screen also identified the *reg1* mutants[71], which was known to regulate gene expression in response to changes in glucose. Early hypotheses suggested a coordination of glucose utilization and phospholipid synthesis, however the mechanism for this coordination was unknown. More recently, it was found that the Opi$^-$ phenotype of a *reg1* mutant is actually due to the altered protonation status of PA, as function of cellular pH, which affects Opi1 translocation to the nucleus[73].

It is well established that phospholipid biosynthesis is coordinated with the Unfolded Protein Response (UPR) and that Opi1p plays a role in this coordination[74–76]. The UPR is initiated in the ER in response to accumulation of unfolded proteins[124] and is also induced by depleting inositol[74,75]. Upon UPR induction, Ire1p is activated initiating splicing of *HAC1* mRNA[125]. The spiced *HAC1* transcript produces the Hac1p basic leucine zipper transcription factor that binds to the UPR element (UPRE) of genes such as *KAR2* but also regulates UAS$_{INO}$ containing promoters by counteracting the function of Opi1p[126]. Thus, it was predictable that the VYDS Opi$^-$ screen identified genes that are known to affect the UPR. Screening the VYDS for the Opi$^-$ phenotype provided a wealth of information about other functions that affect regulation of phospholipid synthesis.

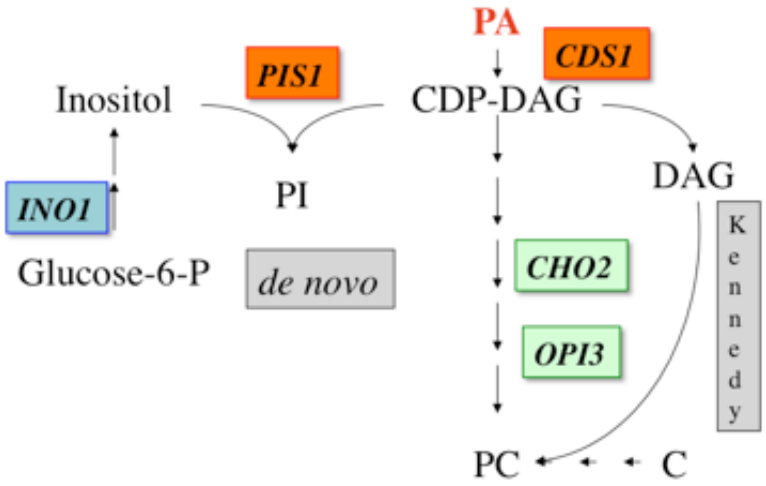


Figure 7**: Abridged yeast phospholipid biosynthetic *de novo* and Kennedy pathways**.
Genes encoding biosynthetic enzymes are italicized and boxed. Those genes noted in green and orange are non-essential and essential (respectively) and yield an $Opi^-$ phenotype when mutated. PA, phosphatidic acid; CDP-DAG, CDP-diacylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; and C, choline

## Materials and Methods

### Strains and Growth Conditions

This study used the BY4742 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ1*) wild-type and doxycycline (Dox) titratable strains[69]. The BRS1005 diploid tester strain is a homozygous for the *ino1-13* and *ade1* alleles[71]. Yeast cultures were grown at 30°C in complete synthetic medium[127] containing 2% glucose (w/v) but lacking inositol and choline (I-C-). For the Opi$^-$ screen, agarose was reduced at 1.2% and Dox was added at 0, 5, and 10 μg/ml.

### The Opi$^-$ genetic screen

The essential gene library contains 838 essential genes driven by a Tet-regulated promoter that are shut off by the addition of Dox[69]. The screen was done using a laborious but sensitive screening assay[128] that consisted in streaking the Tet-driven strain at the top of the plates containing different Dox concentrations (0, 5, and 10 μg/ml). These were allowed to grow for 1-2 days. The tester strain was streaked perpendicular to the Tet-driven strain. This process was done in duplicate.

## Results and Discussion

### Screen of an essential yeast gene library driven by titratable promoter identifies 122 Opi$^-$ mutants

To date there had been no screen of the essential genes for defects in phospholipid synthesis and it is clear that the essential gene set and VYDS are not identical with respect to the biological processes they affect[68]. Motivated by this and the success of the VYDS Opi$^-$ screen[71], our lab conducted an Opi$^-$ screen using the essential gene library driven by titratable promoter[69]. The collection we used contains 838 essential yeast genes driven by a Tet-regulated promoter that is shut off by the addition of Doxycycline (Dox). We used a range of Dox concentrations because strains can have different growth sensitivity[69]. Our screen of the VYDS for the Opi$^-$ phenotype used a pining strategy[71], but this strategy was unsuccessful for the essential gene screen. We used a more laborious but also more sensitive screening assay (Figure 8)[128]. The technique works by streaking a Tet-driven strain at the top of the plates containing different concentrations of Dox (0, 5, and 10 μg/ml), lacking inositol and allowed to grow for 1-2 days. A tester strain was then streaked perpendicular to the Tet-driven strain. The tester strain is a diploid homozygous for *ino1* and *ade1* mutants[129]. This strain does not grow on media lacking inositol because of the *ino1* mutation. If the Tet-driven strain has an Opi$^-$ phenotype, it will excrete inositol into the medium, feeding the tester strain and allowing it to grow. As inositol levels increase in the media, the tester grows more robustly as a red streak (*ade1* phenotype). The tester strain was streaked 3 times on each plate and each Tet-driven strain was analyzed in duplicate. The growth of

the tester was scored as 0 (no growth), 1, 2, or 3 for progressively varying growth phenotypes. Three researchers independently scored each plate. The screen identified 122 mutants that all three researchers agreed had positive tests on the two independent assays (Figure 8B and Supporting Information, Table S1). As a control we used a wild type strain (BY4742) and an *opi1* mutant, which had an Opi$^-$ phenotype under all Dox concentrations. Sometimes the tester strain will show a papillar pattern rather than a uniform growth pattern (Figure 8B). These are not revertants or a result of rare mating since the tester is homozygous diploid. We have observed this pattern previously and shown that it correlates with a defect in transcription regulation[22,71]

Most mutant strains did not show an Opi$^-$ phenotype in the absence of Dox but did have the phenotype when increasing Dox (Figure 8B). In few cases the Opi$^-$ phenotype was observed at lower Dox concentrations but not higher (top Figure 8B). The reason of this was because higher concentrations of Dox were lethal for the strain and did not grow. In a few cases, the mutant strain yielded an Opi$^-$ phenotype in the absence of Dox and did not grow in the presence of Dox (bottom Figure 8B). These results may be possible from a reduced expression from the Tet promoter (no Dox) when compared with the native promoter and lethality when expression is more reduced by adding Dox. As expected, the screen identified thr *cds1* mutant which is the only essential gene previously known to have an Opi$^-$ phenotype (*pis1* allele was not present in the collection)[119,120]. In addition, the screen also identified five mutants that are duplicated in the collection (*use1*, *cks1*, *rpn11*, *sec4*, and *vrg4*). These results suggest that the screen was successful in

identifying legitimate Opi$^-$ mutants. We should also note that four mutants with an Opi$^-$ phenotype (*YNG2*, *HSC82*, *KIC1*, and *SMB1*) are not classified as essential genes in the *Saccharomyces* Genome Data-base (http://www.yeastgenome.org/). Regardless of this fact, down-regulation did yield an Opi$^-$ phenotype so these mutants are retained in our database.

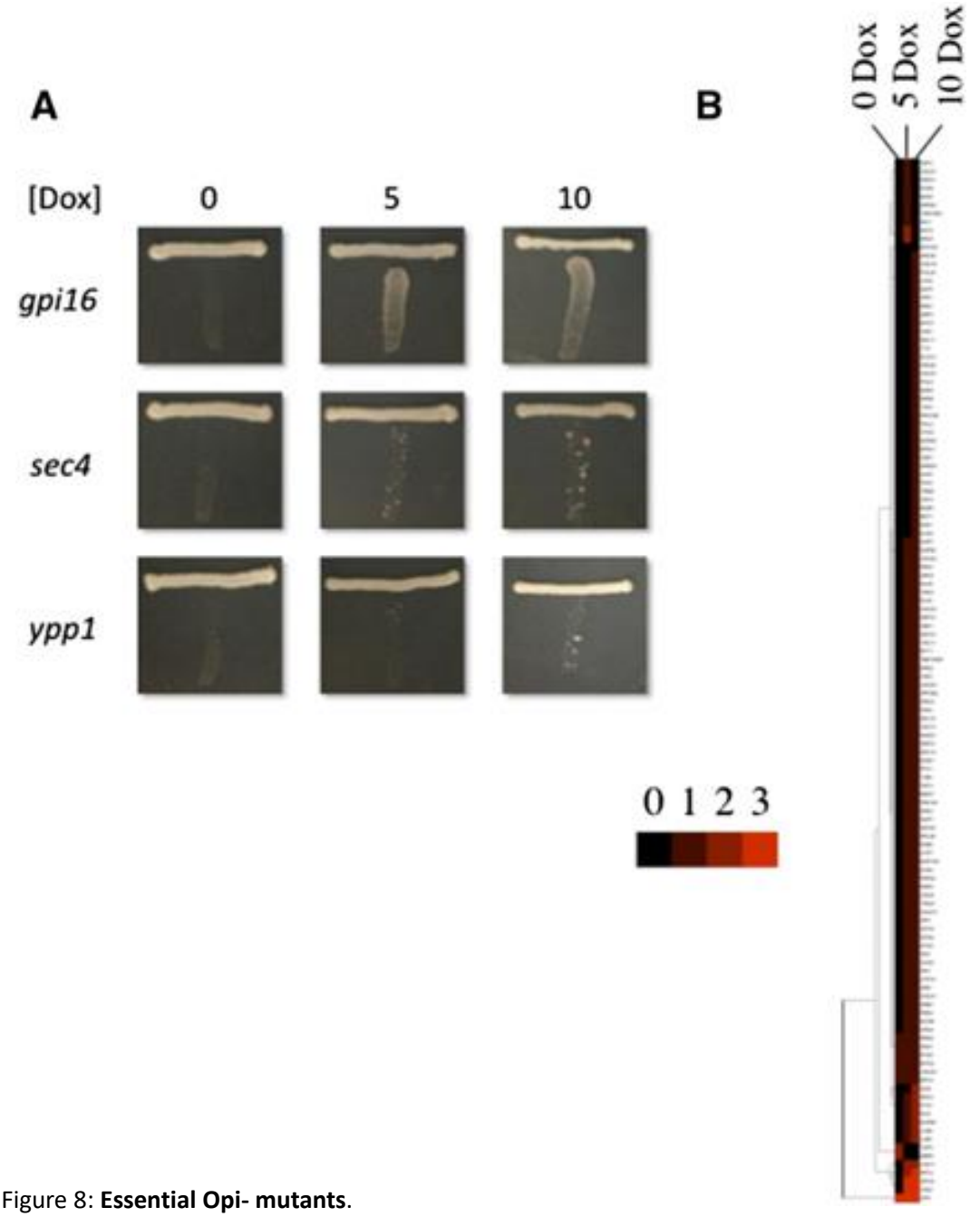


Figure 8: **Essential Opi- mutants**.
(A) Representative Opi- phenotype for the gpi16 (0,3,3), sec4 (0,1,2), and ypp1 (0,0,1) mutants grown under three Dox concentrations. (B) Mutants were clustered with respect to phenotype severity using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and displayed using Java Treeview (Saldanha 2004).

## The essential gene and VYDS screens identify mutants in different sets of biological processes

We predicted that the screen might reveal novel process when compared to the VYDS screen. To test this, the mutants were clustered based on biological processes using the SGD Yeast Go Slim Mapper software (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The results clearly showed that the two screens yielded different information with respect to biological processes (Figure 9). The essential mutant collection showed significantly more mutants affecting RNA metabolic processes, cell cycle, and cell division whereas the VYDS screen identified more mutants in transport, cellular localization, transcription, and response to stimulus.

Consistent with the results from the VYDS screen and the coordination of phospholipid biosynthesis with the UPR, this screen identified multiple mutants that affect protein modification (Figure 10 and Table S1). These include several genes that glycosylate proteins in the ER (*ALG2*, *ALG13*, *OST2*, *PIM40*, *RFT1*, and *SEC53*). The screen also identified several genes required for synthesis of glycosylphosphatidylinositol anchors (*GPI12*, *GPI16*, and *PGA1*) and sphingolipid synthesis (*LCB1*, *LCB2*, and *TSC11*). This is the first report linking these two processes to phospholipid synthesis.

*INO1* gene expression is affected by a mechanism that involves both gene looping and association of *INO1* promoter with the nuclear pore complex[130,131]. In our screen, genes involved in both gene looping and nuclear pore complex were identified with the Opi$^-$ phenotype (Figure 10). Both *pta1* and *ssu72* mutants were

identified in the essential gene screen. These proteins are known to be required for gene looping of the *INO1* gene[132]. It is not immediately obvious why they should also have an Opi$^-$ phenotype but this does provide the first phenotype for gene looping. A significant number of nuclear pore complex (NPC) mutants were identified in the two screens[133]. The VYDS screen identified *NUP84*, and the essential gene screen identified *NIC96*, *NUP1*, *NUP49*, *NUP82*, *NUP85*, and *NUP145*. On activation, the *INO1* promoter is recruited to the nuclear pore complex via *cis* sequences called DNA Zip codes (GRS1 and II) within the *INO1* promoter and the adjoining *SNA3* ORF[44,46] Upon transfer to repressing conditions, the *INO1* promoter remains associated with the nuclear periphery for up to three to four generations[45]. This association is a mechanism for transcriptional memory of recently repressed *INO1* transcription[45,46]. This memory requires an eleven bp sequence, the memory recruitment sequence (MRS), within the *INO1* promoter[46]. Thus, identification of NPC mutants in the Opi$^-$ screens is consistent with its role in recruiting and regulating the *INO1* promoter.

A group of interesting mutants was identified in the essential gene screen that was not present in the VYDS screen. There was an overrepresentation of mutants in the ubiquitin/proteasome degradation pathway (Figure 10 and Table S1). This includes *UBA1* and *RSP5* that encode E1 and E3 ubiquitinating enzymes[134]. Interestingly, an *rsp5* mutant has been shown to affect expression of an *INO1-lacZ* reporter under derepressing conditions[135]. The screen also identified several genes required for proteasome function[136,137] including: the *PRE4* gene that is required for assembly of the 20S proteolytic core particle; the *RPN11* gene that encodes a

deubiquitylase present in the lid of the 19S regulatory particle[138]; and the *RPT2* and *RPT4* genes that are required for unfolding and translocating the protein substrates as well as opening of the proteasome gate (*RPT2*)[136,137]. Another protein modification pathway that was identified by the screen was a ubiquitin -like modification, SUMO. The screen identified both E1 (*AOS1*) and E2 (*UBC9*) encoding genes[134,139]. This finding is consistent with published work showing that a mutation in a deubiquitylation enzyme (*ULP2*) affects *INO1* expression under derepressing conditions by altering the sumoylation status of Scs2p, which normally retains Opi1p in the ER under derepressing conditions[140].

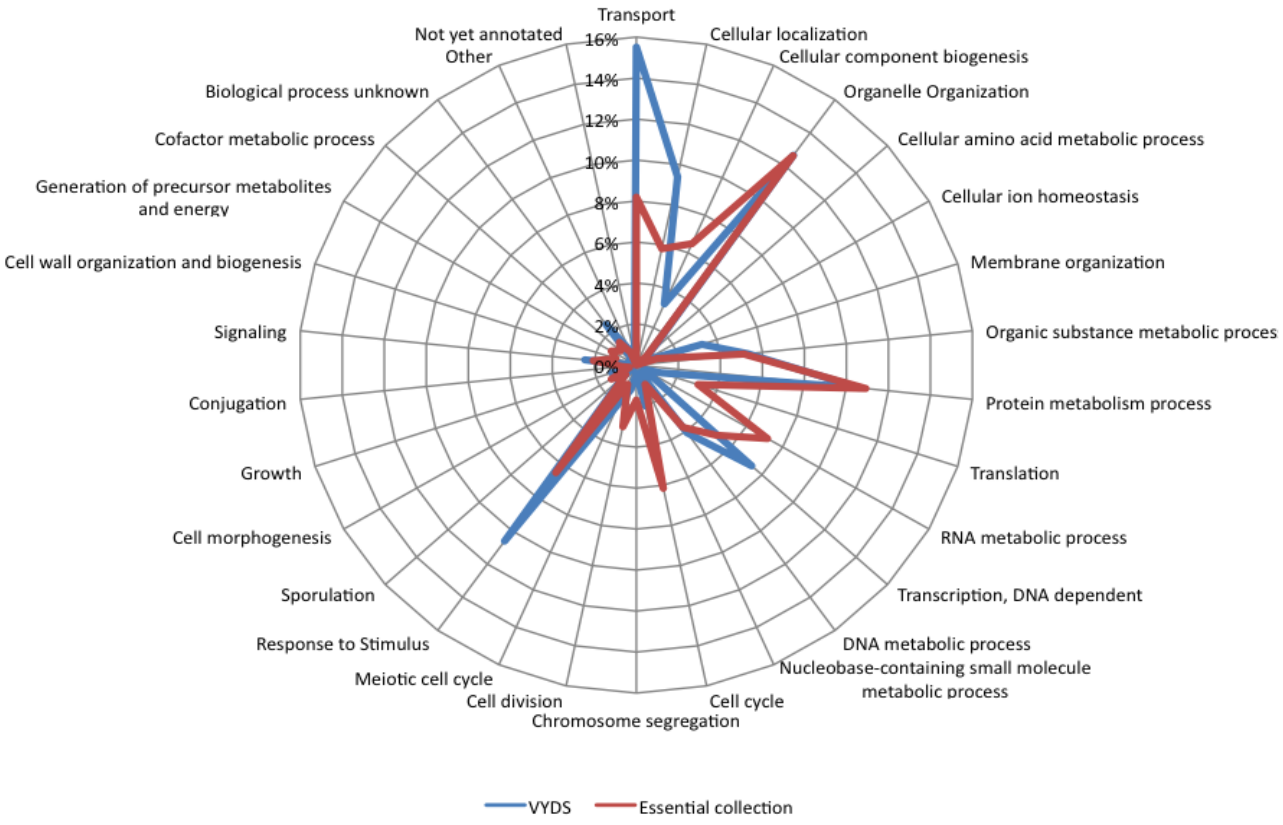


Figure 9: **Radar Chart**

Radar chart comparing percentage of Opi$^-$ mutants in different biological processes for the VYDS (blue) and essential (red) mutant collections. Each point on the graph represents the percentage of mutants within each of the Opi$^-$ mutant sets in each functional category.

Both Opi$^-$ screens identified subunits of NuA4 HAT complex

We previously reported that the VYDS screen identified five of the six nonessential subunits of the NuA4 HAT complex[71]. The essential collection screen also identified three of the six essential subunits (*ARP4*, *ESA1*, and *SWC4*) (Note: *YNG2* is included in the screen but it is not essential) (Figure 10). One of the essential subunits (*ACT1*) was not present in the collection. Our screen identified *ESA1*, which is the catalytic subunit of the complex and contains a chromodomain that interacts with methylated histones as well as *YNG2*, which contains a PHD domain that also interacts with methylated histones[141]. Thus, both screens collectively identified nine of the 12 NuA4 subunits.

It is possible that the proteasome and NuA4 complexes may regulate *INO1* gene expression via a direct role since it has been shown that a 19S proteasome subcomplex works with NuA4 to regulate expression of ribosomal protein genes[142]. However, the finding that mutations in the 20S complex and the ubiquitin modification pathway yield an Opi$^-$ phenotype suggest that protein degradation is more likely explanation for the phenotype. With respect to the NuA4 complex it is interesting that it functions in activation of gene expression while mutants in other transcription factors that also yield the Opi$^-$ phenotype (e.g. *opi1, ume6, sin3, and rpd3*) function in repression[71,90,141]. In the case of the non-essential Opi$^-$ mutants, the mutants yielded elevated expression of the *INO1* target gene under both repressing and derepressing growth conditions, that is, they had a defect in repression[71]. A trivial explanation for this would be that NuA4 affects repression of *INO1* indirectly by controlling the activation of the *OPI1* repressor gene. However,

we found that these mutants did not affect activation of the *OPI1* gene[71]. Moreover there is evidence that NuA4 binds the *INO1* promoter[143]. It is also important to note that some of the subunits of the NuA4 complex are shared with the SWR-C complex that is responsible for loading the modified H2A.Z into nucleosomes and H2A.Z is involved in the regulation of *INO1*[144]. However, none of the SWR-C-specific components were identified in our screens suggesting that the Opi$^-$ phenotype is specific to the NuA4 complex. A more likely explanation is that NuA4 may be acetylating a non-histone regulatory protein that controls *INO1* expression. Consistent with this, an *in vitro* protein acetylation microarray identified many non-histone targets of NuA4[145]. Along this line it is important that another HAT, Gcn5p, acetylates the Ume6p regulatory protein, which targets it for degradation via the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase[104,146]. This occurs as cells are initiating the meiotic program. Consistent with this model the essential gene screen did identify *CDC27*, which is a component of the APC/C (Figure 10 and Table S1). While *INO1* is not a meiotic gene, it is regulated by Ume6p and its associated Sin3/Rpd3 complex[22,23,27–29,147,148]. Thus, NuA4 could be regulating *INO1* either through Opi1p or Ume6p via a mechanism that includes protein degradation. Future experiments will address this possibility.

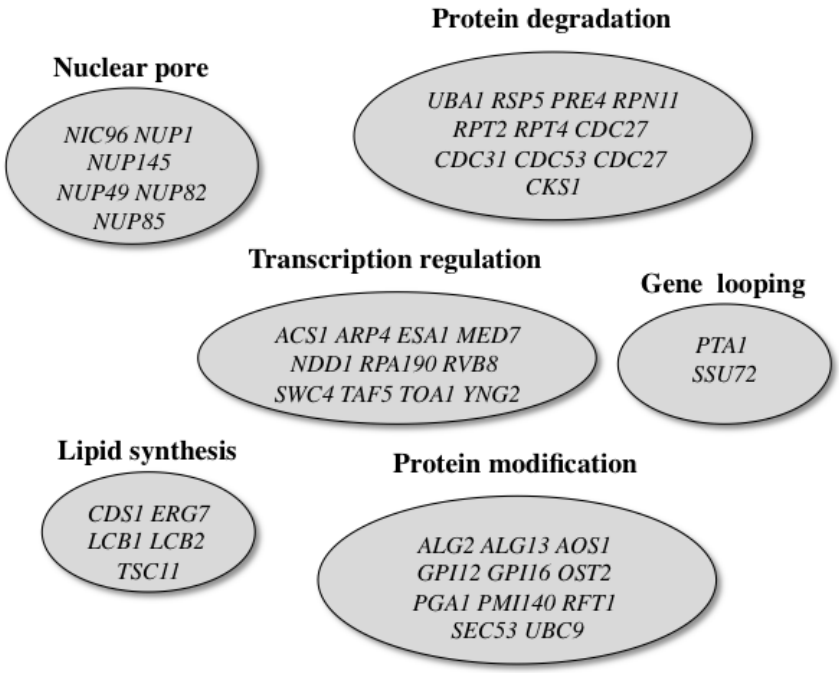


Figure 10: **Opi- mutants cluster by functional categories**.
Shown are those cases where a significant set of mutants affected a biological function.

**Table S1 List of essential genes with an Opi$^-$ phenotype.**

| Gene | Aliases | Function |
|---|---|---|
| *AOS1* | *RHC31* | Subunit of a heterodimeric nuclear SUMO activating enzyme (E1) with Uba2p; activates Smt3p (SUMO) before its conjugation to proteins (sumoylation) |
| *ACS2* | | Acetyl-coA synthetase isoform which, along with Acs1p, is the nuclear source of acetyl-coA for histone acetylation; mutants affect global transcription |
| *AFG2* | *DRG1* | ATPase of the CDC48/PAS1/SEC18 (AAA) family, forms a hexameric complex; is essential for pre-60S maturation and release of several preribosome maturation factors |
| *ALG13* | | Catalytic component of UDP-GlcNAc transferase, required for the second step of dolichyl-linked oligosaccharide synthesis; anchored to the ER membrane via interaction with Alg14p |
| *ALG2* | | Mannosyltransferase that catalyzes two consecutive steps in the N-linked glycosylation pathway |
| *ARC40* | | Subunit of the ARP2/3 complex, which is required for the motility and integrity of cortical actin patches |
| *ARP4* | *ACT3* | Nuclear actin-related protein involved in chromatin remodeling, component of chromatin-remodeling enzyme complexes including NuA4 complex |
| *CDC11* | *PSL9* | Component of the septin ring of the mother-bud neck that is required for cytokinesis |
| *CDC19* | *PYK1* | Pyruvate kinase, functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate |
| *CDC25* | *CTN1* | Membrane bound guanine nucleotide exchange factor (GEF or GDP-release factor); indirectly regulates adenylate cyclase through activation of Ras1p and Ras2p by stimulating the exchange of GDP for GTP; required for progression through G1 |
| *CDC27* | *APC3, SNB1* | Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C), which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors |
| *CDC31* | *DSK1* | Calcium-binding component of the spindle pole body (SPB) half-bridge, required for SPB duplication in mitosis and meiosis II; binds multiubiquitinated proteins and is involved in proteasomal protein degradation |
| *CDC33* | *TIF45* | Cytoplasmic mRNA cap binding protein and translation initiation factor eIF4E |
| *CDC37* | *SMO1* | Essential Hsp90p co-chaperone; necessary for passage through the START phase of the cell cycle; stabilizes protein kinase nascent chains and participates along with Hsp90p in their folding |
| *CDC42* | | Small rho-like GTPase, essential for establishment and maintenance of cell polarity; mutants have defects in the organization of actin and septins |
| *CDC53* | | Cullin, structural protein of SCF complexes (which also contain Skp1p, Cdc34p, Hrt1p and an F-box protein) involved in ubiquitination; SCF promotes the G1-S transition by targeting G1 cyclins and the Cln-CDK inhibitor Sic1p for degradation |
| *CDC8* | *MDP1, MUT2, NPI1, UBY1, SMM1* | Thymidylate and uridylate kinase, functions in de novo biosynthesis of pyrimidine deoxyribonucleotides; converts dTMP to dTDP and dUMP to dUTP; essential for mitotic and meiotic DNA replication |
| *CDS1* | *CDG1* | Phosphatidate cytidylyltransferase (CDP-diglyceride synthetase); an enzyme that catalyzes that conversion of CTP + phosphate into diphosphate + CDP-diacylglyerol, a critical step in the synthesis of all major yeast phospholipids |
| *CKS1* | | Cyclin-dependent protein kinase regulatory subunit and adaptor; modulates proteolysis of M-phase targets through interactions with the proteasome; role in transcriptional regulation, recruiting proteasomal subunits to target gene promoters |
| *DBP6* | | Essential protein involved in ribosome biogenesis; putative ATP-dependent RNA helicase of the DEAD-box protein family |
| *DIM1* | *CDH1* | Essential 18S rRNA dimethylase (dimethyladenosine transferase), responsible for conserved m6(2)Am6(2)A dimethylation in 3'-terminal loop of 18S rRNA, part of 90S and 40S pre-particles in nucleolus, involved in pre-ribosomal RNA processing |
| *DOP1* | | Golgi-localized, leucine-zipper domain containing protein; involved in endosome to Golgi transport, organization of the ER, establishing cell polarity, and morphogenesis |
| *ERD2* | | HDEL receptor, an integral membrane protein that binds to the HDEL motif in proteins destined for retention in the endoplasmic reticulum; has a role in maintenance of normal levels of ER-resident proteins |
| *ERG7* | | Lanosterol synthase, an essential enzyme that catalyzes the cyclization of squalene 2,3-epoxide, |

| | | |
|---|---|---|
| | | a step in ergosterol biosynthesis |
| *ESA1* | *TAS1* | Catalytic subunit of the histone acetyltransferase complex (NuA4) that acetylates four conserved internal lysines of histone H4 N-terminal tail |
| *GCD14* | *TRM61* | Subunit of tRNA (1-methyladenosine) methyltransferase, with Gcd10p, required for the modification of the adenine at position 58 in tRNAs, especially tRNAi-Met |
| *GCD2* | *GCD12* | Delta subunit of the translation initiation factor eIF2B, the guanine-nucleotide exchange factor for eIF2; activity subsequently regulated by phosphorylated eIF2 |
| *GPI12* | *GCR4* | Protein involved in the synthesis of N-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI), the first intermediate in the synthesis of glycosylphosphatidylinositol (GPI) anchors |
| *GPI16* | | Transmembrane protein subunit of the glycosylphosphatidylinositol transamidase complex that adds GPIs to newly synthesized proteins |
| *HIP1* | | High-affinity histidine permease, also involved in the transport of manganese ions |
| *HRR25* | *KTI14* | Protein kinase involved in regulating diverse events including vesicular trafficking, DNA repair, and chromosome segregation; binds the CTD of RNA pol II |
| *HSC82* | *HSP90* | Cytoplasmic chaperone of the Hsp90 family, redundant in function and nearly identical with Hsp82p, and together they are essential |
| *KIC1* | *NRK1* | Protein kinase of the PAK/Ste20 kinase family, required for cell integrity possibly through regulating 1,6-beta-glucan levels in the wall; physically interacts with Cdc31p (centrin), which is a component of the spindle pole body |
| *LAS17* | *BEE1* | Actin assembly factor, activates the Arp2/3 protein complex that nucleates branched actin filaments; localizes with the Arp2/3 complex to actin patches |
| *LCB1* | *END8, TSC2* | Component of serine palmitoyltransferase, responsible along with Lcb2p for the first committed step in sphingolipid synthesis, which is the condensation of serine with palmitoyl-CoA to form 3-ketosphinganine |
| *LCB2* | *SCS1, TSC1* | Component of serine palmitoyltransferase, responsible along with Lcb1p for the first committed step in sphingolipid synthesis, which is the condensation of serine with palmitoyl-CoA to form 3-ketosphinganine |
| *LST8* | | Protein required for the transport of amino acid permease Gap1p from the Golgi to the cell surface; component of the TOR signaling pathway; associates with both Tor1p and Tor2p |
| *MAK21* | *NOC1* | Constituent of 66S pre-ribosomal particles, required for large (60S) ribosomal subunit biogenesis; involved in nuclear export of pre-ribosomes |
| *MAS1* | *MIF1* | Smaller subunit of the mitochondrial processing protease (MPP), essential processing enzyme that cleaves the N-terminal targeting sequences from mitochondrially imported proteins |
| *MDN1* | *REA1* | Huge dynein-related AAA-type ATPase (midasin), forms extended pre-60S particle with the Rix1 complex (Rix1p-Ipi1p-Ipi3p); acts in removal of ribosomal biogenesis factors at successive steps of pre-60S assembly and export from nucleus |
| *MED7* | | Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation |
| *MGE1* | *YGE1* | Mitochondrial matrix cochaperone, acts as a nucleotide release factor for Ssc1p in protein translocation and folding; also acts as cochaperone for Ssq1p in folding of Fe-S cluster proteins |
| *MOB1* | | Component of the mitotic exit network; associates with and is required for the activation and Cdc15p-dependent phosphorylation of the Dbf2p kinase; required for cytokinesis and cell separation; component of the CCR4 transcriptional complex |
| *MYO1* | | Type II myosin heavy chain, required for wild-type cytokinesis and cell separation; localizes to the actomyosin ring; binds to myosin light chains Mlc1p and Mlc2p through its IQ1 and IQ2 motifs respectively |
| *MYO2* | *CDC66* | One of two type V myosin motors (along with MYO4) involved in actin-based transport of cargos; required for the polarized delivery of secretory vesicles, the vacuole, late Golgi elements, peroxisomes, and the mitotic spindle |
| *NAT2* | | Protein with an apparent role in acetylation of N-terminal methionine residues |
| *NDD1* | | Transcriptional activator essential for nuclear division; localized to the nucleus; essential component of the mechanism that activates the expression of a set of late-S-phase-specific genes |
| *NET1* | *ESC5, CFI1, SRM8* | Core subunit of the RENT complex, which is a complex involved in nucleolar silencing and telophase exit; stimulates transcription by RNA polymerase I |
| *NIC96* | | Component of the nuclear pore complex, required for nuclear pore formation; forms a subcomplex with Nsp1p, Nup57p, and Nup49p |

| | | |
|---|---|---|
| *NOP7* | *YPH1* | Component of several different pre-ribosomal particles; forms a complex with Ytm1p and Erb1p that is required for maturation of the large ribosomal subunit; required for exit from G0 and the initiation of cell proliferation |
| *NUP1* | | Nuclear pore complex (NPC) subunit, involved in protein import/export and in export of RNAs, possible karyopherin release factor that accelerates release of karyopherin-cargo complexes after transport across NPC; potential Cdc28p substrate |
| *NUP49* | *NSP49* | Subunit of the Nsp1p-Nup57p-Nup49p-Nic96p subcomplex of the nuclear pore complex (NPC), required for nuclear export of ribosomes |
| *NUP82* | *HRB187* | Nucleoporin, subunit of the nuclear pore complex (NPC); forms a subcomplex with Gle2p, Nup159p, Nsp1p, and Nup116p and is required for proper localization of Nup116p in the NPC |
| *NUP85* | *RAT9* | Subunit of the Nup84p subcomplex of the nuclear pore complex (NPC), required for assembly of the subcomplex and also for formation of the nucleocytoplasmic Gsp1p concentration gradient that plays a role in nuclear trafficking |
| *NUP145* | *RAT10* | Essential nucleoporin, catalyzes its own cleavage in vivo to generate a C-terminal fragment that assembles into the Nup84p subcomplex of the nuclear pore complex, and an N-terminal fragment of unknown function that is homologous to Nup100p |
| *OST2* | | Epsilon subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins |
| *PAN1* | *MDP3, MIP3, DIM2* | Part of actin cytoskeleton-regulatory complex Pan1p-Sla1p-End3p, associates with actin patches on the cell cortex; promotes protein-protein interactions essential for endocytosis |
| *PFY1* | *PRF1* | Profilin, binds actin, phosphatidylinositol 4,5-bisphosphate, and polyproline regions; involved in cytoskeleton organization; required for normal timing of actin polymerization in response to thermal stress |
| *PGA1* | | Essential component of GPI-mannosyltransferase II, responsible for second mannose addition to GPI precursors as a partner of Gpi18p; required for maturation of Gas1p and Pho8p |
| *PGI1* | *CDC30* | Glycolytic enzyme phosphoglucose isomerase, catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate; required for cell cycle progression and completion of the gluconeogenic events of sporulation |
| *PIK1* | *PIK41, PIK120* | Phosphatidylinositol 4-kinase; catalyzes first step in the biosynthesis of phosphatidylinositol-4,5-biphosphate |
| *PLP2* | | Essential protein that interacts with the CCT (chaperonin containing TCP-1) complex to stimulate actin folding |
| *PMI40* | | Mannose-6-phosphate isomerase, catalyzes the interconversion of fructose-6-P and mannose-6-P; required for early steps in protein mannosylation |
| *POL2* | *DUN2* | Catalytic subunit of DNA polymerase (II) epsilon, a chromosomal DNA replication polymerase that exhibits processivity and proofreading exonuclease activity; also involved in DNA synthesis during DNA repair |
| *POL30* | | Proliferating cell nuclear antigen (PCNA), functions as the sliding clamp for DNA polymerase delta; may function as a docking site for other proteins required for mitotic and meiotic chromosomal DNA replication and for DNA repair |
| *POL5* | | DNA Polymerase phi; has sequence similarity to the human MybBP1A and weak sequence similarity to B-type DNA polymerases, not required for chromosomal DNA replication; required for the synthesis of rRNA |
| *POP6* | | Subunit of both RNase MRP and nuclear RNase P; RNase MRP cleaves pre-rRNA, while nuclear RNase P cleaves tRNA precursors to generate mature 5' ends and facilitates turnover of nuclear RNAs |
| *PRE4* | | Beta 7 subunit of the 20S proteasome |
| *PRP21* | *SPP91* | Subunit of the SF3a splicing factor complex, required for spliceosome assembly |
| *PRP22* | | DEAH-box RNA-dependent ATPase/ATP-dependent RNA helicase, associates with lariat intermediates before the second catalytic step of splicing; mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes |
| *PSF2* | *CDC102* | Subunit of the GINS complex (Sld5p, Psf1p, Psf2p, Psf3p), which is localized to DNA replication origins and implicated in assembly of the DNA replication machinery |
| *PTA1* | *FUN39* | Subunit of holo-CPF, a multiprotein complex and functional homolog of mammalian CPSF, required for the cleavage and polyadenylation of mRNA and snoRNA 3' ends; involved in pre-tRNA processing; binds to the phosphorylated CTD of RNAPII |
| *RFT1* | | Essential integral membrane protein that is required for translocation of Man5GlcNac2-PP-Dol from the cytoplasmic side to the lumenal side of the ER membrane |

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|---|---|---|
| *RNA1* | | GTPase activating protein (GAP) for Gsp1p, involved in nuclear transport |
| *RPA190* | *RRN1* | RNA polymerase I largest subunit A190 |
| *RPL28* | *CYH2* | Ribosomal protein of the large (60S) ribosomal subunit; may have peptidyl transferase activity |
| *RPN11* | *MPR1* | alloprotease subunit of the 19S regulatory particle of the 26S proteasome lid; couples the deubiquitination and degradation of proteasome substrates |
| *RPT2* | *YHS4, YTA5* | One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for normal peptide hydrolysis by the core 20S particle |
| *RPT4* | *CRL13, PCS1, SUG2* | One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in degradation of ubiquitinated substrates |
| *RRN3* | | Protein required for transcription of rDNA by RNA polymerase I; transcription factor independent of DNA template; involved in recruitment of RNA polymerase I to rDNA |
| *RRN5* | | Protein involved in transcription of rDNA by RNA polymerase I; transcription factor, member of UAF (upstream activation factor) family along with Rrn9p and Rrn10p |
| *RRP45* | | Exosome non-catalytic core component; involved in 3'-5' RNA processing and degradation in both the nucleus and the cytoplasm |
| *RRS1* | | Essential protein that binds ribosomal protein L11 and is required for nuclear export of the 60S pre-ribosomal subunit during ribosome biogenesis |
| *RSP5* | | E3 ubiquitin ligase of the NEDD4 family; involved in regulating many cellular processes including MVB sorting, heat shock response, transcription, endocytosis, and ribosome stability |
| *RTS2* | | Basic zinc-finger protein |
| *RVB2* | *TIP49B, TIH2, TIP48* | Essential protein involved in transcription regulation; component of chromatin remodeling complexes; required for assembly and function of the INO80 complex |
| *SDA1* | | Highly conserved nuclear protein required for actin cytoskeleton organization and passage through Start, plays a critical role in G1 events, binds Nap1p, also involved in 60S ribosome biogenesis |
| *SEC10* | | Essential 100kDa subunit of the exocyst complex (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p), which has the essential function of mediating polarized targeting of secretory vesicles to active sites of exocytosis |
| *SEC11* | | 18kDa catalytic subunit of the Signal Peptidase Complex (SPC; Spc1p, Spc2p, Spc3p, and Sec11p) which cleaves the signal sequence of proteins targeted to the endoplasmic reticulum |
| *SEC14* | *PIT1* | Phosphatidylinositol/phosphatidylcholine transfer protein; involved in regulating PtdIns, PtdCho, and ceramide metabolism, products of which regulate intracellular transport and UPR |
| *SEC4* | *SRO6* | Rab family GTPase essential for vesicle-mediated exocytic secretion and autophagy; associates with the exocyst component Sec15p and may regulate polarized delivery of transport vesicles to the exocyst at the plasma membrane |
| *SEC53* | *ALG4* | Phosphomannomutase, involved in synthesis of GDP-mannose and dolichol-phosphate-mannose; required for folding and glycosylation of secretory proteins in the ER lumen |
| *SIS1* | | Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p; not functionally redundant with Ydj1p due to due to substrate specificity |
| *SLD5* | *CDC105* | Subunit of the GINS complex (Sld5p, Psf1p, Psf2p, Psf3p), which is localized to DNA replication origins and implicated in assembly of the DNA replication machinery |
| *SMB1* | | Core Sm protein Sm B; part of heteroheptameric complex (with Smd1p, Smd2p, Smd3p, Sme1p, Smx3p, and Smx2p) that is part of the spliceosomal U1, U2, U4, and U5 snRNPs |
| *SOG2* | | Key component of the RAM signaling network, required for proper cell morphogenesis and cell separation after mitosis |
| *SPP382* | *CCF8, NTR1* | Essential protein that forms a dimer with Ntr2p; also forms a trimer, with Ntr2p and Prp43p, that is involved in spliceosome disassembly |
| *SRP21* | | Subunit of the signal recognition particle (SRP), which functions in protein targeting to the endoplasmic reticulum membrane |
| *SRP72* | | Core component of the signal recognition particle (SRP) ribonucleoprotein (RNP) complex that functions in targeting nascent secretory proteins to the endoplasmic reticulum (ER) membrane |
| *SSU72* | | Transcription/RNA-processing factor that associates with TFIIB and cleavage/polyadenylation factor Pta1p; exhibits phosphatase activity on serine-5 of the RNA polymerase II C-terminal domain; affects start site selection in vivo |
| *SUI2* | | Alpha subunit of the translation initiation factor eIF2, involved in the identification of the start codon; phosphorylation of Ser51 is required for regulation of translation by inhibiting the |

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|---|---|---|
| | | exchange of GDP for GTP |
| *SWC4* | *EAF2, GOD1* | Component of the Swr1p complex that incorporates Htz1p into chromatin; component of the NuA4 histone acetyltransferase complex |
| *TAF5* | *TAF90* | Subunit (90 kDa) of TFIID and SAGA complexes, involved in RNA polymerase II transcription initiation and in chromatin modification |
| *TIF35* | | eIF3g subunit of the core complex of translation initiation factor 3 (eIF3), which is essential for translation; stimulates resumption of ribosomal scanning during translation reinitiation |
| *TOA1* | | TFIIA large subunit; involved in transcriptional activation, acts as antirepressor or as coactivator |
| *TRM5* | | tRNA(m(1)G37)methyltransferase, methylates a tRNA base adjacent to the anticodon that has a role in prevention of frameshifting |
| *TRS31* | | One of 10 subunits of the transport protein particle (TRAPP) complex of the cis-Golgi which mediates vesicle docking and fusion; involved in endoplasmic reticulum (ER) to Golgi membrane traffic |
| *TSC11* | *AVO3* | Subunit of TORC2 (Tor2p-Lst8p-Avo1-Avo2-Tsc11p-Bit61p), a membrane-associated complex that regulates actin cytoskeletal dynamics during polarized growth and cell wall integrity; involved in sphingolipid metabolism |
| *TTI1* | | Putative protein of unknown function; subunit of the ASTRA complex which is part of the chromatin remodeling machinery |
| *TUB1* | | Alpha-tubulin; associates with beta-tubulin (Tub2p) to form tubulin dimer, which polymerizes to form microtubules |
| *UBA1* | | Ubiquitin activating enzyme (E1), involved in ubiquitin-mediated protein degradation |
| *UBC9* | | SUMO-conjugating enzyme involved in the Smt3p conjugation pathway; nuclear protein required for S- and M-phase cyclin degradation and mitotic control; involved in proteolysis mediated by the anaphase-promoting complex cyclosome (APCC) |
| *USE1* | *SLT1* | Essential SNARE protein localized to the ER, involved in retrograde traffic from the Golgi to the ER; forms a complex with the SNAREs Sec22p, Sec20p and Ufe1p |
| *UTP14* | | Subunit of U3-containing Small Subunit (SSU) processome complex involved in production of 18S rRNA and assembly of small ribosomal subunit |
| *UTP7* | *KRE31* | Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA |
| *VAS1* | | Mitochondrial and cytoplasmic valyl-tRNA synthetase |
| *VRG4* | *VAN2, GOG5, LDB3, VIG4* | Golgi GDP-mannose transporter; regulates Golgi function and glycosylation in Golgi |
| *YEF3* | *TEF3* | Gamma subunit of translational elongation factor eEF1B, stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing eEF1A (Tef1p/Tef2p) from the ribosomal complex; contains two ABC cassettes; binds and hydrolyzes ATP |
| *YNG2* | *NBN1, EAF4* | Subunit of the NuA4 histone acetyltransferase complex that acetylates histone H4 and H2A |
| *YPP1* | | Cargo-transport protein involved in endocytosis; interacts with phosphatidylinositol-4-kinase Stt4 |
| *YBR190w* | | Dubious open reading frame |
| *YGR190c* | | Dubious open reading frame |

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CHAPTER

# III. GROWTH PHASE REGULATION OF PHOSPHOLIPID BIOSYNTHETIC GENES IN YEAST

## Introduction

*Saccharomyces cerevisiae* has been a fantastic model for the understanding of biological processes such as phospholipid synthesis. Phospholipid synthesis is a key step in the formation of membranes and requires precise metabolic coordination by the cell to maintain cellular stability. In budding yeast, the phospholipid biosynthetic genes are repressed in response to inositol and choline[105,106] (I+C+). These genes are maximally derepressed when inositol and choline are both limited (I-C-). However, many phospholipid biosynthetic genes have been reported to also be growth phase regulated. This regulation is characterized by an increase in gene expression that correlates with exponential growth of a culture and a severe decrease in expression as cells enter stationary phase[149,150]. Little is known about the mechanism for growth phase regulation. The current study shows that repression in stationary phase requires the proteasome protein degradation process.

*INO1* is a phospholipid biosynthetic gene that has served as a model for understanding coordinated regulation of this system. *INO1* gene regulation requires several transcription factors including Ino2p, Ino4p, and Opi1p, the Ume6p-Sin3p-Rpd3p histone deacetylase (HDAC) complex, ISW2, and INO80 chromatin-remodeling complexes[4,10,27,28,31,109]. Ino2p and Ino4p form a heterodimer that binds

to a $UAS_{INO}$ promoter sequence to activate transcription of most of the phospholipid biosynthetic genes[76,108]. Opi1p is a repressor that specifically regulates the phospholipid biosynthetic pathway[16]. Under repressing conditions (I+C+) Opi1p can be found in the endoplasmic reticulum (ER) physically interacting with ER membrane protein Scs2p and phosphatidic acid (PA)[19,114]. Under derepressing conditions (I-C-), PA levels drop and when this happens Opi1p gets released from the ER and translocates to the nucleus where it represses transcription by directly interacting with the Ino2p transcriptional activator[116–118]. This is the current understanding of *INO1* gene regulation during the exponential growth phase.

As noted above, the current study revealed that *INO1* repression in stationary phase requires protein degradation. Many biological processes including the cell cycle, tumor suppression, DNA repair, and transcription of genes are known to be regulated by protein degradation[77,78]. In eukaryotes, archaea, and some bacteria, a main process for protein degradation involves a protein complex called the proteasome and a protein ubiquitination pathway. The proteasome's function is specifically to destroy proteins that are damaged or unnecessary at the moment[81]. The proteasome is organized into two main subassemblies: the 19S regulatory particle (RP), which includes the lid and base, and the 20S core particle (CP). The RP function is to recognize peptides to be degraded, while the CP contains the proteolytic active sites to degrade targeted proteins[78]. One function of ubiquitination is to target proteins for degradation by the proteasome[82]. It involves an E1-E2-E3 cascade of enzymes. Ubiquitin-activating enzymes (E1), utilize ATP to transfer ubiquitin (Ub) peptides to an Ub-conjugating enzyme (E2), which will

transfer Ub to a Ub-ligase (E3) that holds the target protein to be ubiquitinated[83]. Polyubiquitination is achieved through several rounds of conjugation. In eukaryotes, the most common residue to be modified by Ub is Lysine[85]. In yeast, the conjugation machinery consists of a single E1 enzyme, eleven E2 enzymes, and a large family of E3 enzymes (60-100). E3 enzymes mediate the selectivity and specificity of ubiquitination throughout direct interactions with the substrate[78]. Many E3 enzymes were categorized into two major classes: RING domain E3s and HECT domain E3s. Most belong to the RING domain E3s with only five HECT domain E3s are encoded in the yeast genome[78].

Our lab previously reported an essential gene screen looking for mutants with an Opi$^-$ phenotype to further understand phospholipid regulation in yeast[72]. One of the most overrepresented groups identified in the screen were genes in the Ubiquitin/Proteasome system[72], suggesting for the first time that this system is important for the transcriptional regulation of the phospholipid biosynthetic genes. Our screen successfully identified the single yeast E1 enzyme (*UBA1*), an E2 enzyme (*UBC13*), and an E3 enzyme (*RSP5*). In addition, subunits from the proteasome were identified including *RPN11* from the lid, *RPT2* and *RPT4* from the base, and *PRE4* from the core particle[72].

The Opi$^-$ phenotype has historically been correlated with a repression defect in *INO1*. However, when testing different proteasome subunits identified from our essential gene screen, they did not show a repression defect similar to what happens in an *opi1Δ* mutant. However, as we noted previously, in addition to the inositol-mediated repression, the phospholipid biosynthetic genes are also growth phase

regulated[150]. Our results here suggest that the protein degradation pathway plays an important role in the growth phase regulation of *INO1*. In this chapter, we attempt to decipher a possible mechanism responsible for *INO1* growth phase regulation via protein degradation by the proteasome.

## Materials and Methods

### Plasmid construction

Plasmid pCR-Blunt II- TOPO from Invitrogen Zero Blunt® TOPO® PCR Cloning Kit was used to insert a fragment containing from 500 bp upstream to 500 bp downstream of *INO2* ORF to generate pBS101 (Table 2). Likewise, plasmid pCR-Blunt II- TOPO was used to insert a fragment containing from 500 bp upstream to 500 bp downstream of *INO4* ORF to generate pBS104 (Table 2).

### Site-directed mutagenesis

Site directed mutagenesis was performed on *INO2* ORF using Agilent Technologies QuikChange XL Site-Directed Mutagenesis Kit on codons K110R (a329g) and K158R (a473g) (Table 1). Site directed mutagenesis was performed on *INO4* ORF codons K19R (a56g), K115R (a344g), and K138R (a413g) (Table 1). Primers were designed based on recommendations in the Agilent Technologies QuikChange XL Site-Directed Mutagenesis Kit. Mutagenized plasmids were transformed into XL10-Gold Ultracompetent Cells (Agilent), collected using a Zyppy Plasmid Miniprep Kit (Genesee) and sequenced to confirm the presence of the mutations (Eurofins).

Yeast strains, media, and growth conditions

The *S. cerevisiae* strains used in this study were BY4741 (*MATa*, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*); isogenic strains containing *ino2Δ*, *ino4Δ*, *pdr5Δ*, *opi1Δ* (VYDS), and doxycycline (Dox) titratable strains *rpn11 Dox and rpt2 Dox* [67–69]. Strains with Tandem Affinity Purification (TAP) tagged *INO2* and *INO4* were purchased from Open Biosystems [151] (Table 3). Gene-specific cassettes containing a C-terminally positioned TAP tag were synthesized by PCR using pFA6a-TAP-His3MX (CBP-TEV-ZZ-His3MX6) as template and transformed into relevant strains to generate *pdr5Δ INO2-TAP* and *pdr5Δ INO4-TAP*, *rpn11 Dox INO2-TAP* and *rpn11 Dox INO4-TAP*, and *rpt2 Dox INO2-TAP* and *rpt2 Dox INO4-TAP* strains. BY4741 *opi1Δ* was transformed with pMK139 containing HA-OPI1[152] to determine Opi1p stability throughout growth phase. Yeast strain BRS2011 contains a *GAL1-INO2* gene inserted at the *GAL4* site[153]. The INO2 gene was TAP-tagged using the strategy described above.

Alleles generated by mutagenesis were digested with *Eco*RI releasing the entire gene. These DNA fragments were transformed into *ino2Δ*, *ino4Δ* strains using a Yeast Maker Transformation Kit (Clontech) which resulted in insertion of the mutant alleles at endogenous loci. Subsequently, the mutant alleles were tagged using the same gene-specific cassette described above.

Yeast cultures were grown at 30˚C in complete synthetic medium containing 2% glucose (w/v) but lacking inositol and choline (I-C)[127]. When indicated 75 μM of inositol and 1 mM of choline where added (I+C+). Media used for titratable strains

included 10 μg/ml of Doxycycline [69]. BRS2011 (*GAL1-INO2*) was grown in 2% Raffinose (w/v) and 0.25% Galactose in media with and without inositol and choline.

### Growth phase assays

Cells were pre-cultured in YEPD until saturation. Cells were pelleted and washed with $dH_20$ and transferred to complete I-C- synthetic medium at a 1:10 dilution. Samples were taken at different $OD_{600}$ measurements (0.4, 0.6, 0.8, 1.0, 1.2) until cultures reached the stationary phase of cell growth. When assaying titratable strains, Doxycycline (10 μg/ml) was added when cells reached 0.4 units at $OD_{600}$. When assaying *pdr5Δ* mutants, 10 μM of protease inhibitor MG132 was added after cultures reached 0.8 units at $OD_{600}$.

### RNA extraction and quantitative real-time PCR (QRT-PCR) analysis

RNA was extracted by a hot-acid phenol method[55], followed by DNase digestion using RQ1 DNase (Promega), and purified using an RNA Clean & Concentrator™ (ZYMO) kit. RNA was used to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen). For quantification, cDNA was diluted 1:10, and quantitative PCR (QPCR) was performed as described previously[123]. *INO1, INO2,* and *TCM1* transcripts were quantified using the INO1-ORF, INO2-ORF, and TCM1-ORF primer pairs (Table 1)

### Protein extraction, SDS-PAGE, Western blotting

Whole cell extracts from *S. cerevisiae* were prepared by sonicating cells in Extraction Buffer (40 mM HEPES pH7.4, 350 mM Sodium chloride, 0.1% NP40, 10% Glycerol, 100 μM PMSF, 2 μg/ml Pepstatin A). Protein concentration was determined by the Bradford Method. Proteins were denatured in Laemlli Buffer, DMSO and 95°C and fractionated in 8% polyacrylamide gels, in Tris/Glycine/SDS Buffer (Bio-Rad), transferred in 1X Tris/Glycine, 20% methanol onto PVDF membranes at 4°C overnight. Membranes were washed with 1X PBS Tween 0.05% (Genesee) blocked with 1X PBST 5% dry milk. Peroxidase Anti-Peroxidase Antibody (Sigma) was incubated for 3 hrs for detecting TAP-tags. TBP served as an internal standard and was detected using a Monoclonal Primary Antibody (Mouse Anti-TBP (Sigma)) for 2 hrs. HA was detected using a Monoclonal Primary Antibody produced in mouse (Sigma) for 2hrs, followed by a secondary antibody used, Donkey Anti-mouse HRP (Thermo), for 1 hr. Proteins were detected using an ImageQuant LAS4000 mini Luminescent Image Analyzer (GE) with ProSignal™ Dura ECL reagents (Genesee)

**Table 1: List of oligonucleotides**

| Primer | Sequence |
|---|---|
| INO4-TAP Fwd | GGAGTTAATTTGGGAGCTGGGTGATGGACAGAGTGGTCAGGGTCGACGGATCCCCGGGTT |
| INO4-TAP Rev | TTTTCTTTGTGGGATATGCACCACTCCCCTCTGCAAAGTGTCGATGAATTCGAGCTCGTT |
| INO2-TAP Fwd | AAGCGCAAATGAAGCACTACAGCACATACTGGATGATTCCGGTCGACGGATCCCCGGGTT |
| INO2-TAP Rev | CTATAGATCAGAGGTTACATGGCCAAGATTGAAACTTAGAGTCGCTGAATTCGAGCTCGT |
| INO4-TAP Conf Fwd | GAAGGAGTTAATTTGGGAGCTGGGTGATGGACAGAGTGGT |
| INO2-TAP Conf Fwd | GAAGGTACGGAAATGGAAACACGTTCAAATGGAGAAGATAC |
| TAP Conf Rev | CCTACCCATGGTTGTTTATGTTCGGATGTGATGTGAGAAC |
| GAL4-TAP Rev | AATTAAAACGAATATTCAAGAAATGCAATGATGTGCCTCTTCGATGAATTCGAGCTCGTT |
| INO1-ORF F | GTATTAAACCGGTCTCCATTGC |
| INO1-ORF R | CCGACGGGCTTCATATATTTG |
| INO2-ORF F | CCTGAAACACATCCACACAG |
| INO2-ORF R | CATTTGAACGTGTTTCCATTTC |
| TCM1-ORF F | CCAGAGCTGGTCAAAGAGGT |
| TCM1-ORF R | ACCGTAGTGGACGAAACCAC |
| INO2 K110R (a329g)F | CAAGCGCTTGGTCTCAGGTTGTCTCCTTCCAGT |
| INO2 K110R (a329g)R | ACTGGAAGGAGACAACCTGAGACCAAGCGCTTG |
| INO2 K158R (a473g)F | CATCTACATATAAGATCACCAAGAAAGCAGCATAGGTATACCGAA |
| INO2 K158R (a473g)R | TTCGGTATACCTATGCTGCTTTCTTGGTGATCTTATATGTAGATG |
| INO4 K19R (a56g)F | CCGGGATTGTCCGAGATTAGGGAGATAAAGGGTGA |
| INO4 K19R (a56g)R | TCACCCTTTATCTCCCTAATCTCGGACAATCCCGG |
| INO4 K115R (a344g)F | ATCATAGCTAAGCATGAGGCAAGAACCGGCAGCAG |
| INO4 K115R (a344g)R | CTGCTGCCGGTTCTTGCCTCATGCTTAGCTATGAT |
| INO4 K138R (a413g)F | GAAACATTCGGGATTTAGTACCGAGGGAGTTAATTTGGGAG |
| INO4 K138R (a413g) R | CTCCCAAATTAACTCCCTCGGTACTAAATCCCGAATGTTTC |
| INO2 +1to20 seq | ATGCAACAAGCAACTGGGAA |
| INO2 +200to220 seq | ACCCTCTCACGTAGAAACTA |
| INO4 +1to20 seq | ATGACGAACGATATTAAGGA |
| INO4 -500to454 F | GCACCTTCCAAGCTTTAGTGTCGATGAAGTTGGAT |
| INO4 +901to928 R | AAGAAAGTAAAAATATGAGCGGTACGAGAATCGA |
| INO2 -500to454 F | GAGTTACTTTGAATCCTTGATAATATGGATCGAG |
| INO2 1366to1409 R | CGTCCCTTAATTGGTATTATTTATAAATAAATGGCACGTACTTT |

## Table 2: Vector list

| Plasmid | Description | Source |
|---|---|---|
| pCR-Blunt II TOPO | Empty vector | Agilent Technologies |
| pBS101 | -500bp INO2 to +500 pCR-Blunt II TOPO | This study |
| pBS102 | INO2 K110R on pCR-Blunt II TOPO | This study |
| pBS103 | INO2 K110R K158R on pCR-Blunt II TOPO | This study |
| pBS104 | INO2 K158R on pCR-Blunt II TOPO | This study |
| pBS105 | -500bp INO4 to +500 pCR-Blunt II TOPO | This study |
| pBS106 | INO4 K19R on pCR-Blunt II TOPO | This study |
| pMK139 | HA-Opi1p on pRS200 | Kaadige and Lopes 2006 |
| pJH330 | INO1-LacZ | Elkhaimi et al. 2000 |

## Table 3: Yeast strains and genotype

| Yeast strains | Description | Source |
|---|---|---|
| BY4741 | Mat a *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* | VYDS |
| *Δopi1* | BY4741 *Δopi1* | VYDS |
| *Δino2* | BY4741 *Δino2* | VYDS |
| *Δino4* | BY4741 *Δino4* | VYDS |
| Rpn11 Doxy | BY4741 *Dox trititable rpn11* | Essential gene collection |
| Rpt2 Doxy | BY4741 *Dox trititable rpt2* | Essential gene collection |
| *Δpdr5* | BY4741 *Δpdr5* | VYDS |
| INO2-TAP | BY4741 with INO2-HIS5-TAP | Open Biosystems |
| INO2-TAP *rpn11 Doxy* | INO2-TAP in Rpn11 Doxy strain | This study |
| INO2-TAP *rpt2 Doxy* | INO2-TAP in Rpt2 Doxy strain | This study |
| INO2-TAP *Δpdr5* (VYDS) | INO2-TAP in *Δpdr5* strain | This study |
| INO2-TAP K110R | INO2 K110R allele inserted in *Δino2* then tagged with TAP-tag | This study |
| INO2-TAP K110R K158R | INO2 K110R K158R allele inserted in *Δino2* then tagged with TAP-tag | This study |
| INO2-TAP K158R | INO2 K158R allele inserted in *Δino2* then tagged with TAP-tag | This study |
| INO4-TAP | BY4741 Open Biosystems | Open Biosystems |
| INO4-TAP *rpn11 Doxy* | INO4-TAP in Rpn11 Doxy strain | This study |
| INO4-TAP *rpt2 Doxy* | INO4-TAP in Rpt2 Doxy strain | This study |
| INO4-TAP *Δpdr5* (VYDS) | INO4-TAP in *Δpdr5* strain | This study |
| INO4-TAP K19R | INO4 K19R allele inserted in *Δino4* then tagged with TAP-tag | This study |
| BRS2011 | GAL1-INO2 | Ashburner and Lopes 1995 |
| *Δeaf7* | BY4741 *Δeaf7* | VYDS |
| *Δeaf7* INO2-TAP | *Δeaf7* strain with INO2-TAP | This study |

## Results

### A novel *INO1* transcriptional defect happens at the stationary phase of growth

Historically when a mutant was identified with an Opi$^-$ phenotype, it was associated with a repression defect under repressing growth conditions (I+C+)[6]. For example, an *opi1* mutant shows elevated levels of *INO1* mRNA under repressing conditions, (Figure 11A). Having identified several mutants from the proteasome with an Opi$^-$ phenotype, we hypothesized that these are playing a role in *INO1* repression in repressing media. However, when we tested *INO1* mRNA levels under activating and repressing conditions, strains containing mutations in two proteasome subunits tested (*rpn11* and *rpt2*) failed to show the repression defect typically associated with the Opi$^-$ phenotype, showing full repression of *INO1* under repressing conditions (Fig 11A).

In addition to *INO1* regulation in response to inositol and choline, there is an additional regulation of *INO1* which depends on the growth phase[149,150]. This growth regulation shows that *INO1* is active at the exponential phase of growth but repressed when cells reach stationary phase. Thus, we decided to test the effect of *rpn11* and *rpt2* mutant alleles on *INO1* expression at the stationary phase (Fig 11B). Surprisingly, when *RPN11* and/or *RPT2* were conditionally shutdown by the addition of Doxycycline, *INO1* expression was now elevated in stationary phase. This result suggests that *RPN11* and *RPT2* play a role in repressing *INO1* at the stationary phase in I-C- conditions. Because eukaryotes may require both ubiquitination and the proteasome to degrade a protein, we tested the only E1 enzyme yeast has from the ubiquitination pathway to determine if this part of the

protein degradation pathway plays an important role in regulating *INO1* at stationary phase. When looking at an *UBA1* conditional shutdown strain, we identified a repression defect at stationary phase that was similar to what we observed with the proteasome shutdown mutants, further confirming that the Ub pathway is important for the growth phase regulation of *INO1* (Figure 12).

As a control for cell growth, we quantified *INO1* transcription in WT cells under activating conditions (I-C-) (Figure 13). Samples were taken at different $OD_{600}$ levels (Figure 12B). As previously reported, *INO1* mRNA levels increase throughout exponential growth and drop dramatically until almost undetectable levels on entry into stationary phase (1.2 $OD_{600}$).

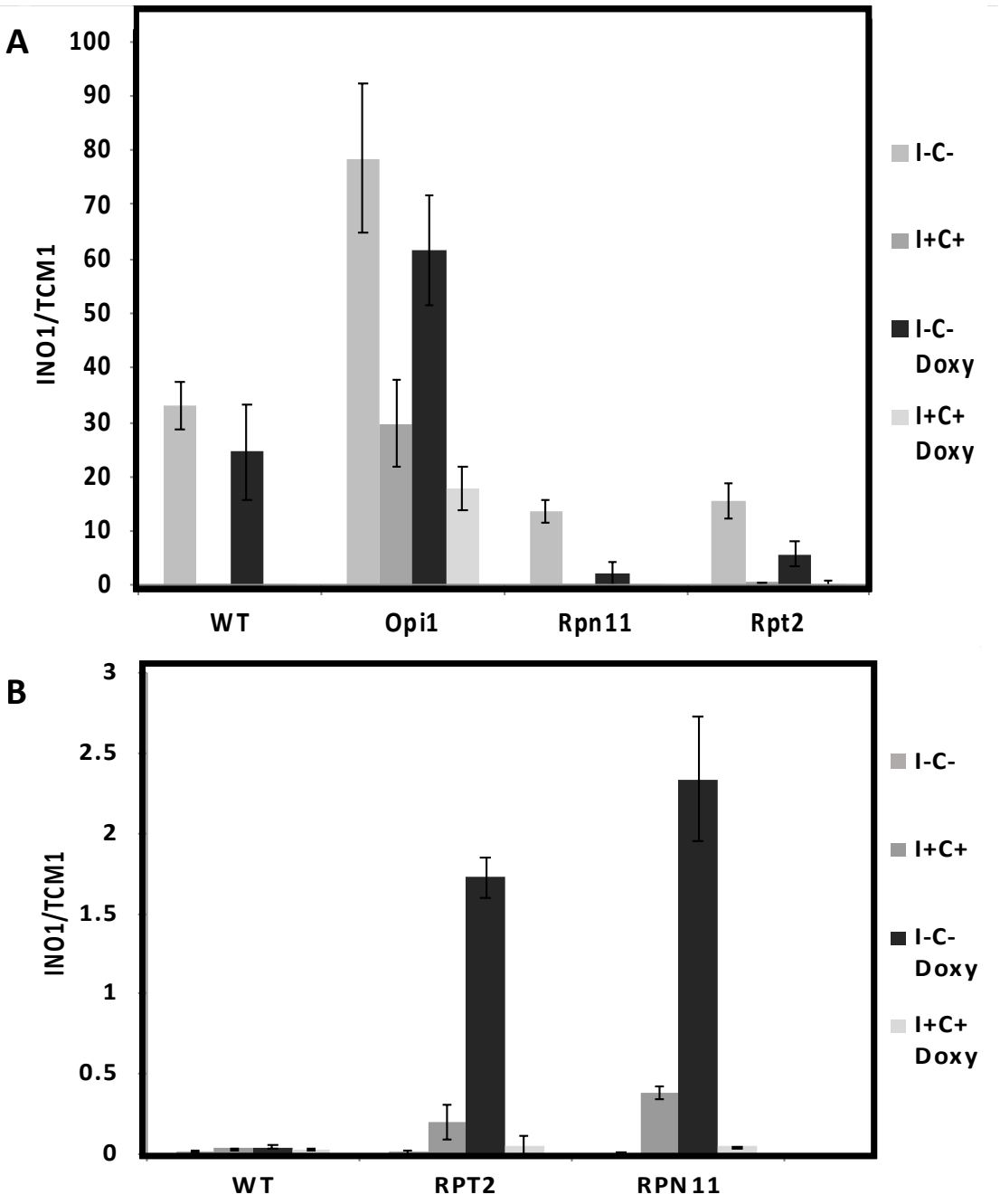


Figure 11: ***INO1* mRNA levels at exponential and stationary phase in response to inositol**

*INO1* mRNA levels in repressing (I+C+) and derepressing conditions (I-C-). 10 ∝g/ml of Doxycycline was added to induce conditional shutdown of essential genes *RPN11* and *RPT2*. (A) Samples were taken at exponential phase ($OD_{600}$= > 0.6 < 0.9) or (B) at late stationary phase ($OD_{600}$= 1.1-1.2).

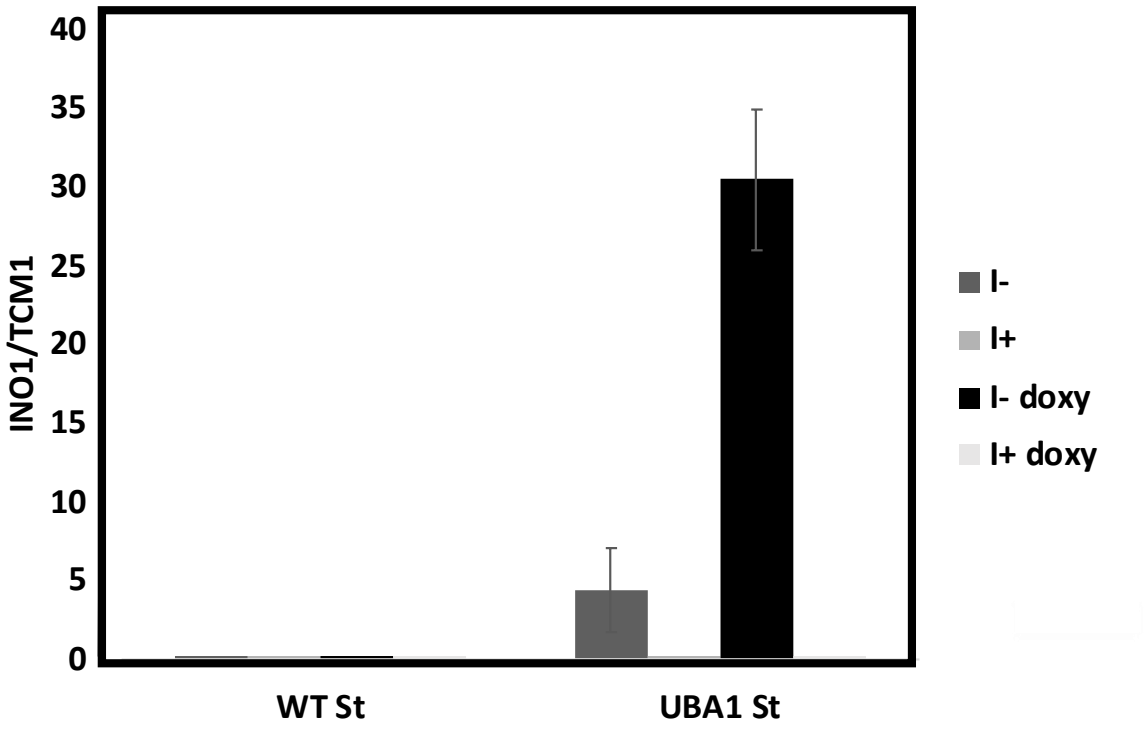


Figure 12: ***INO1* expression in an *UBA1* conditional shutdown strain**
*UBA1* is yeasts only Ubiquitination pathway E1 enzyme. *INO1* mRNA levels in repressing (I+C+) and derepressing conditions (I-C-). 10 ug/ml of Doxycycline was added to induce conditional shutdown of essential genes *RPN11* and *RPT2*. Samples were taken at late stationary phase ($OD_{600}$= 1.1-1.2).

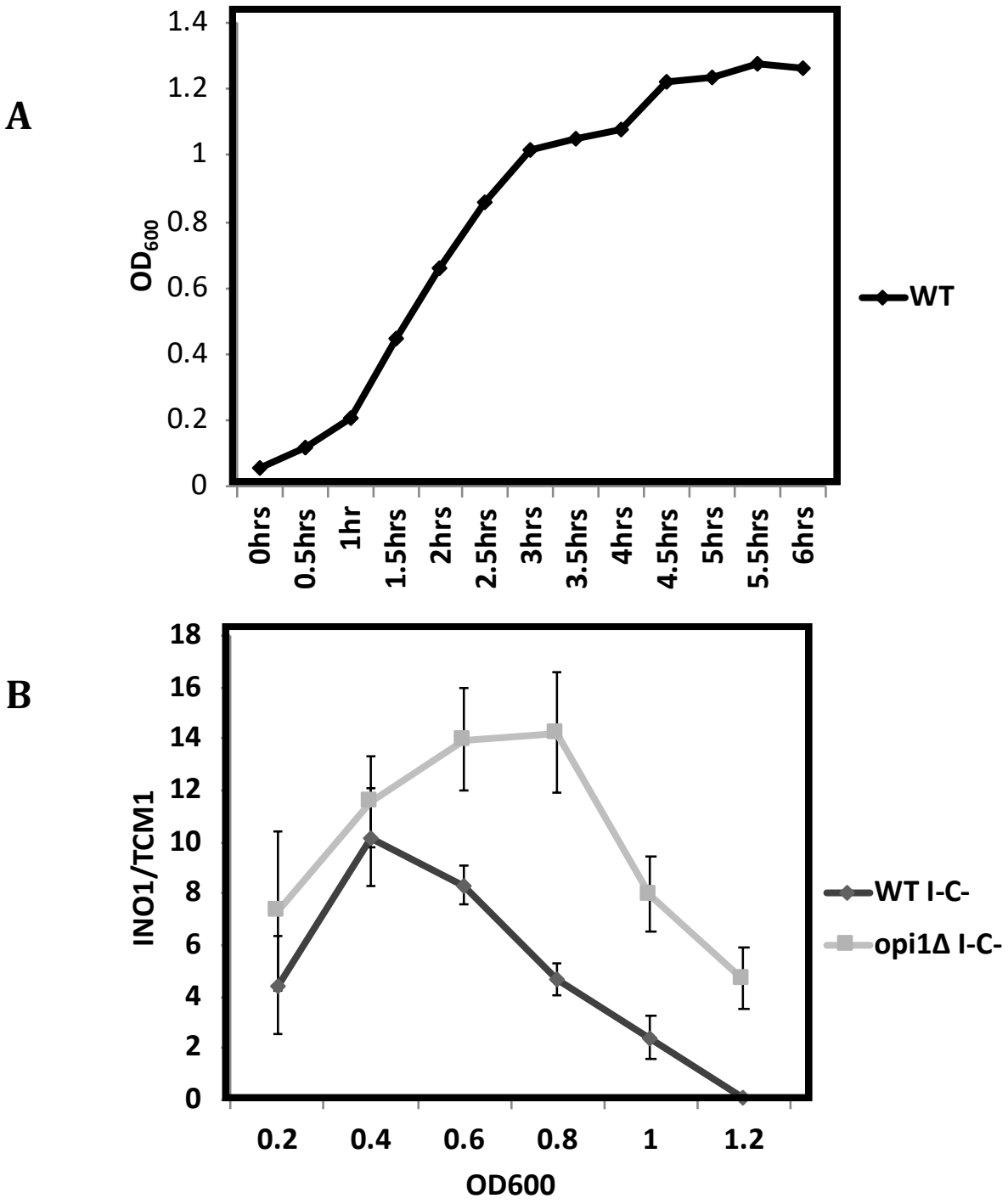


Figure 13: ***INO1* mRNA transcription levels throughout growth phase**
(A) Growth of yeast in I-C- media. Maximum $OD_{600}$ that yeast reach in this media is little over 1.2.
(B) Samples were taken at different stages of the cell growth and *INO1* mRNA levels were quantitifed.

*INO1* transcriptional activators are degraded during stationary phase

Given our limited understanding of *INO1* regulation at stationary phase and our results with the proteasome subunits (Figure 11), we reasoned that under the conditional shutdown of *RPN11* and *RPT2,* some transcription factor might be stabilized causing *INO1* transcription to remain high. First, we decided to test Ino2p and Ino4p activator stability using TAP-tagged strains (tag is inserted at the native location) (Figure 14). The data show that both Ino2p-TAP and Ino4p-TAP levels decrease dramatically when cells approach stationary phase. In fact, Ino2p-TAP decreased throughout exponential phase and into stationary phase (Figure 14A).

We wanted to create an Opi1p-TAP to determine if its stability is affected as a function of growth. For reasons that are not clear, we and others have been unable to insert a TAP-tag at the endogenous location. Instead, we transformed an *opiΔ* strain with pMK139, which contains an HA-tagged *OPI1* gene. The results show that HA-Opi1p levels did not show any regulation throughout growth. Opi1p levels remained stable through different growth phases suggesting that Opi1p does not play a role in the growth phase regulation of *INO1* (Figure 15).

These results suggest that Ino2p and Ino4p levels are the main targets that could explain *INO1* growth phase regulation which suggests that genes involved in protein degradation play a direct role in the regulation of *INO1*. This is the first evidence we have showing that Ino2p and Ino4p are getting degraded at stationary phase. With this understanding, we hypothesized that during the conditional shutdown of the proteasome subunits, Ino2p and Ino4p are stabilized during stationary phase. To test this, we generated strains combining *RPN11* and *RPT2*

conditional shutdown with Ino2p-TAP and Ino4p-TAP. Consistent with our previous results (Figure 14), both Ino2p-TAP and Ino4p-TAP levels decreased during stationary phase, but when proteasome subunit genes where shutdown by adding Doxycycline, both Ino2p-TAP and Ino4p-TAP were stabilized during stationary phase (Figure 16). This experiment suggests that the protein degradation pathway plays a direct role in the regulation of *INO1* at stationary growth phase, showing for the first time that the protein degradation pathway is an important regulator of the phospholipid biosynthetic genes.

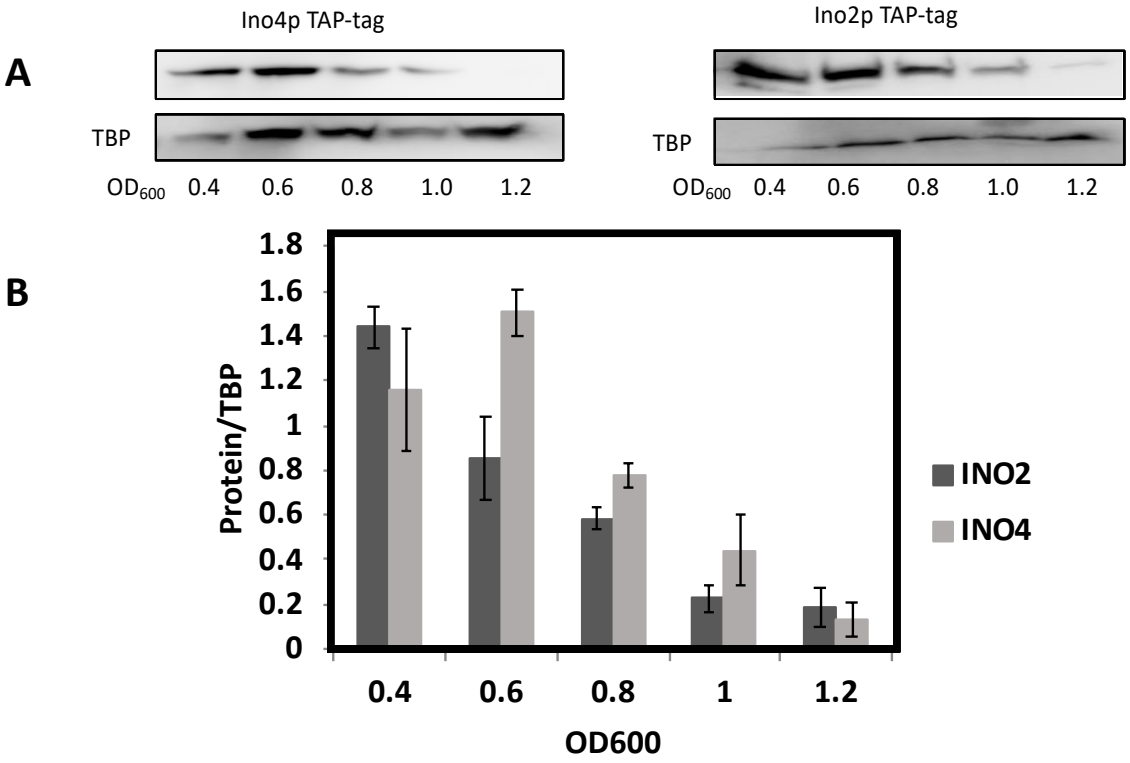


Figure 14: ***INO1* activators' stability throughout growth phase**
(A) Western blot showing Ino4p-TAP and Ino2p-TAP levels at different stages in the cell growth. (B) Quantification of Ino4p-TAP and Ino2p-TAP protein levels (triplicate) throughout cell growth.

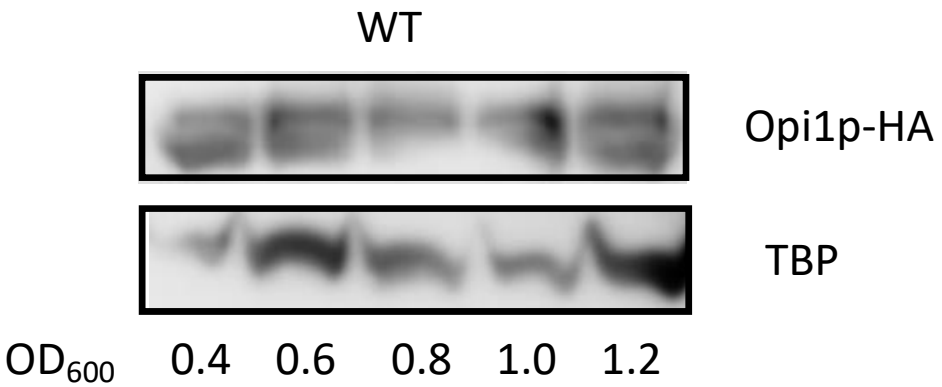


Figure 15: **HA-Opi1p stability throughout growth phase**
Western blot showing HA-Opi1p (from pMK139) in an *opi1Δ strain* grown in I-C- media at different stages of growth.

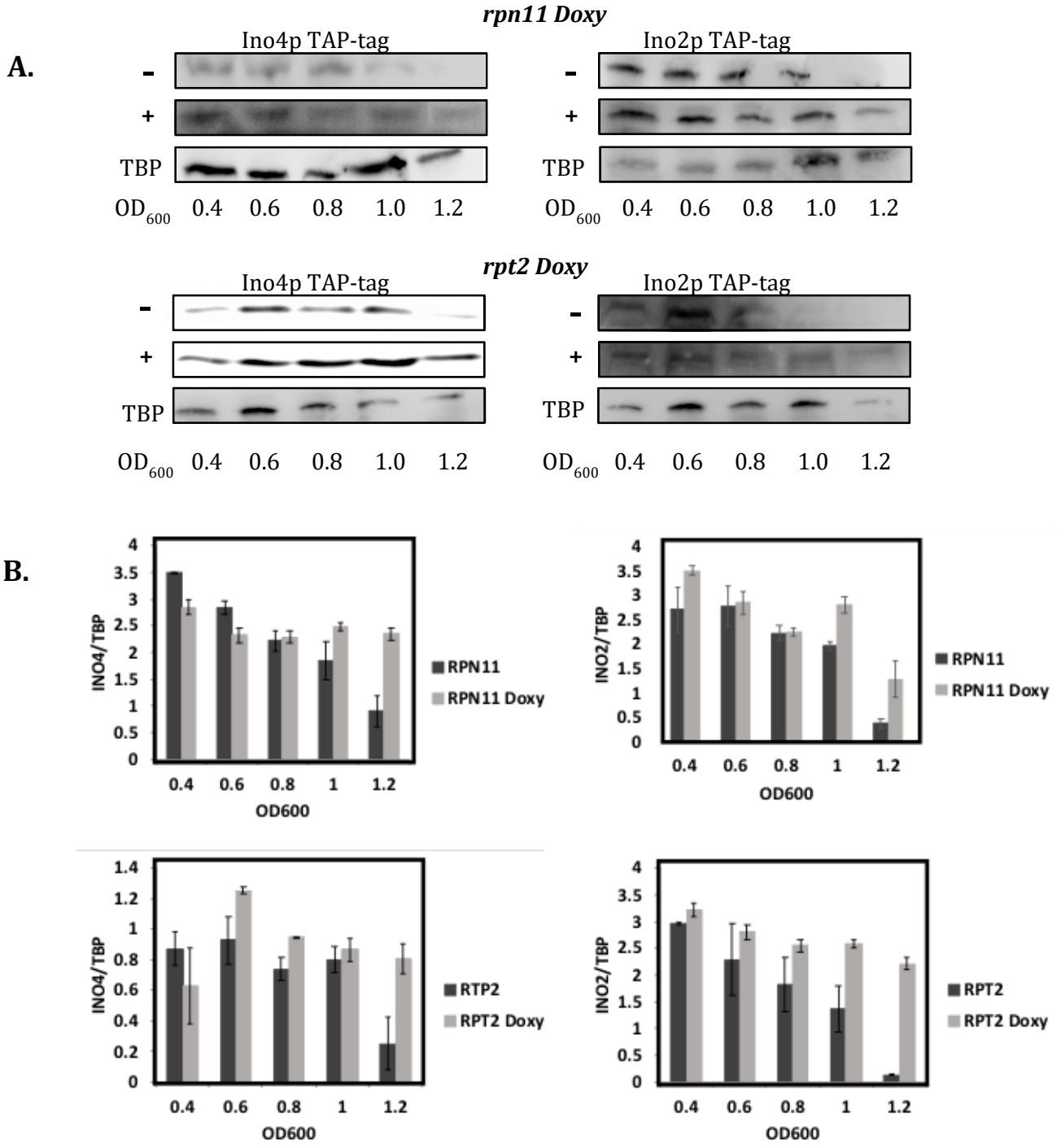


Figure 16: **Ino2p and Ino4p stability in proteasome subunits throughout strains growth phase**
(A) INO1 activators stability throughout growth phase under proteasome subunits (RPT2 and RPN11) conditional shutdown by doxycycline (+). (B) Quantification of INO1 activators throughout growth phase as function of proteasome subunit (RPT2 and RPN11) conditional shutdown.

Chemical inhibition of the proteasome stabilizes *INO1* activators

The proteasome is a primary pathway responsible for degrading proteins in eukaryotes. Our previous results suggest that the proteasome might degrade Ino2p and Ino4p at stationary phase. To confirm that the proteasome is involved in the degradation of Ino2p and Ino4p we tested their stability following treatment with the proteasome chemical inhibitor MG132. MG132 is a potent, reversible, cell-permeable proteasome inhibitor. In yeast, it is capable of reducing degradation of Ub-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities. To use MG132 in yeast, it is necessary to use a *pdr5Δ* mutant strain. *PDR5* encodes a multidrug transporter that is important for cellular detoxification[154]. Thus, we generated Ino2p-TAP and Ino4p-TAP strains containing a *pdr5Δ* allele and tested Ino2p and Ino4p stability following MG132 treatment (Figure 17). Ino2p and Ino4p levels decrease dramatically when adding DMSO, but with the addition of MG132, both Ino2p and Ino4p levels were stabilized at stationary phase. This further confirms our model that Ino2p and Ino4p are degraded at the stationary phase.

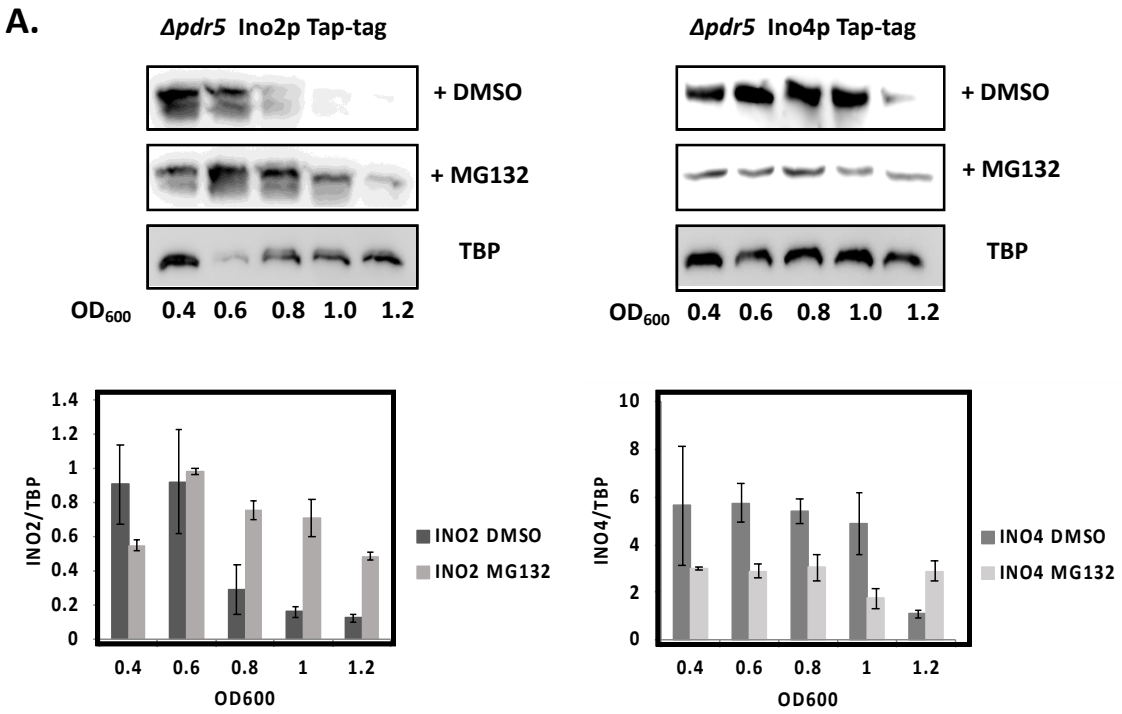


Figure 17**: *INO1* activator stability throughout growth phase in the presence of the proteasome chemical inhibitor MG132**

(A) Western blot of Ino2p-TAP and Ino4p-TAP in a *pdr5*D deletion mutant. Cells were exposed to either DMSO, or the proteasome chemical inhibitor MG132. (B) Quantification of Ino2p-TAP and Ino4p-TAP throughout growth phase.

Mutagenesis of predicted Ub sites yields stabilization of Ino2p and Ino4p

Proteins that are targeted to the proteasome often must be modified by poly-Ub. Typically, ubiquitination occurs on Lysine residues[92]. Using a bioinformatics tool (www.ubpred.com) that predicts Ub sites, we identified several potential Ub sites for both Ino2p and Ino4p. Ino2p has two potential sites, K110 and K158. In order to determine if these sites are required for Ino2p degradation we conducted site-directed mutagenesis on these sites. DNA fragments containing mutant versions of *INO2* were introduced into an *ino2Δ* strain thus placing the mutation at the native location and the ORF was subsequently tagged with TAP. These yeast strains were used to determine Ino2p stability throughout the growth phase (Figure 18). The K110R single *INO2* mutant showed no significant stabilization when compared to WT (Figure 18). However, when both K110, K158 sites or the K158 site alone were mutated, Ino2p-TAP was significantly stabilized in stationary phase. This suggests that the K158 site might by modified by Ub and is important for Ino2p stability in stationary phase.

Ino4p also has two potential sites, K19 and K115. Similar to the situation with Ino2p, mutating the Ino4p K19 significantly stabilized Ino4p-TAP in stationary phase (Figure 19). This suggests that the Ino4p K19 site might be modified by Ub and is important for Ino4p stability in stationary phase.

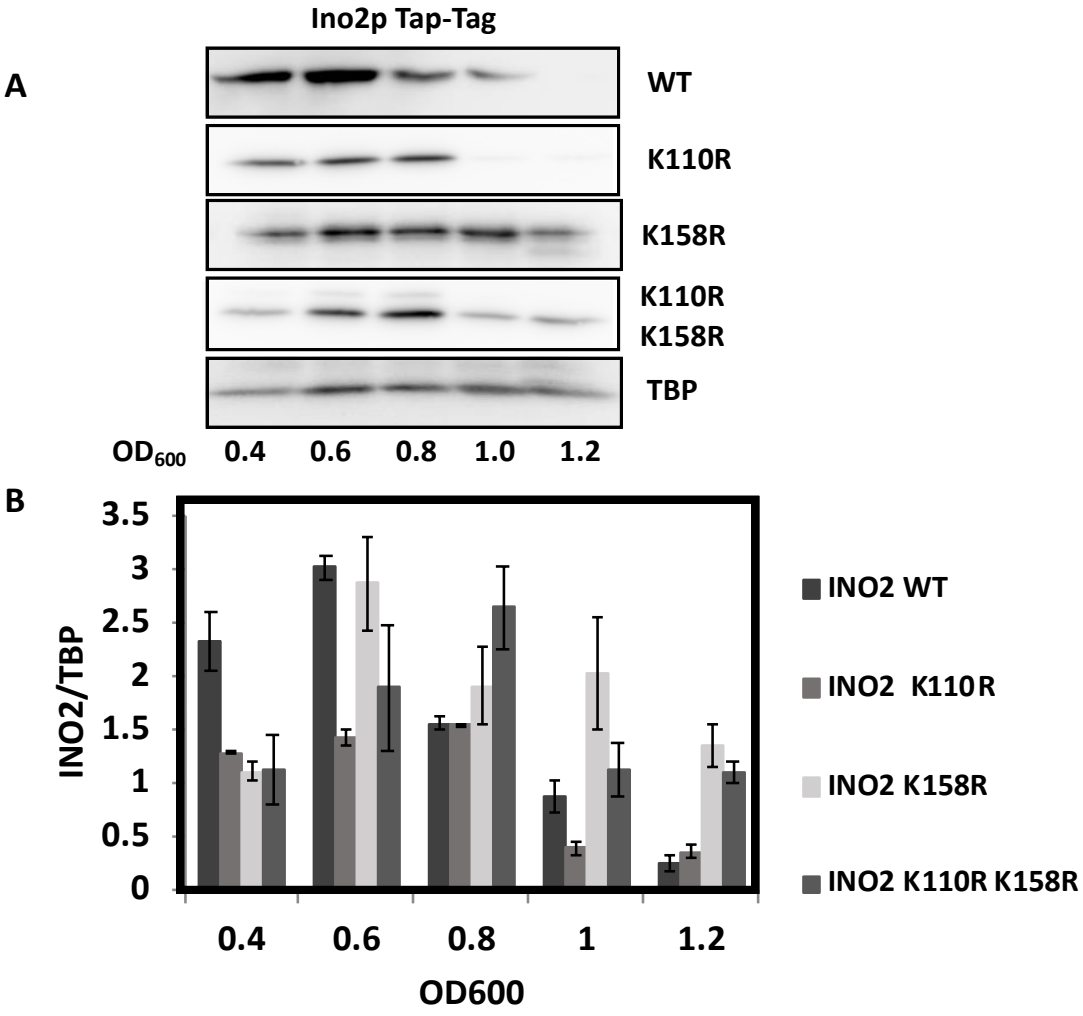


Figure 18: **Site-directed mutagenesis of K158 residue stabilizes Ino2p in stationary phase**
(A) Western blots showing wild type Ino2p and K110R and K148R Ino2p mutants throughout the growth phase. (B) Quantification of Ino2p throughout growth phase.

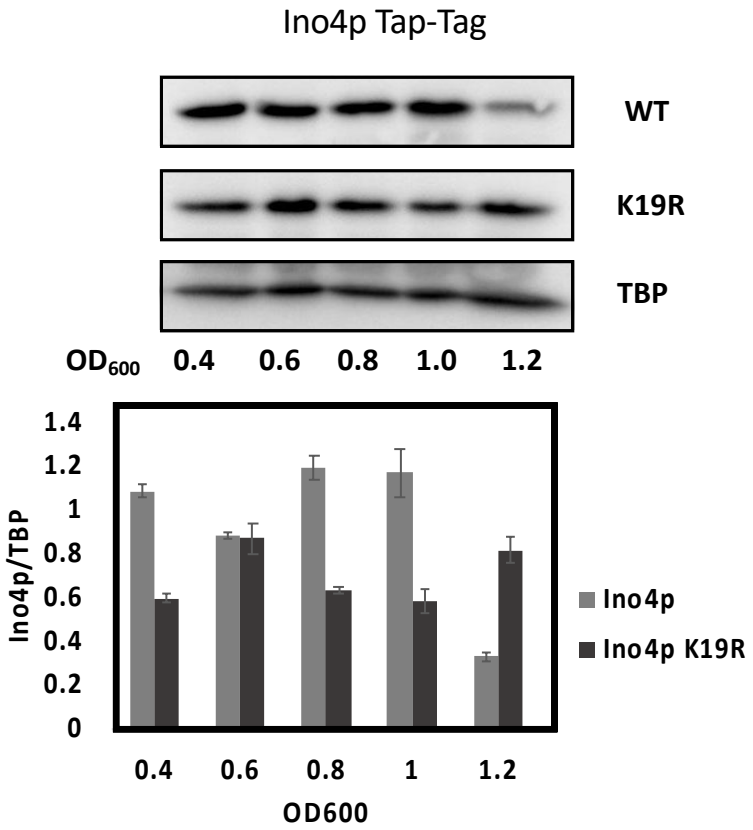


Figure 19: **Site-directed mutagenesis of K19 residue stabilizes Ino4p in stationary phase**(A) Western blots showing wild type Ino4p and a K19R Ino2p mutants throughout the growth phase. (B) Quantification of Ino4p throughout growth phase.

## Regulation of *INO1* in stationary phase occurs at the activator protein level not at the transcription level

The decrease in protein levels of *INO1* transcriptional activators during stationary phase can be explained if these are being degraded by the proteasome, but there is a possibility that this decrease is caused by a decrease in the transcription levels of *INO2* or *INO4*. This possibility exists because *INO2* is auto regulated[147]. The *INO2* promoter contains an $UAS_{INO}$, and is autoregulated in response to inositol, in a pattern that is similar to *INO1* regulation[107]. To determine if *INO1* repression at the stationary phase is indeed due to altered activator protein levels we investigated the effect on *INO1* transcription if we control transcript levels of the *INO2* activator gene.

For this purpose, we used a strain (BRS2011) that contains a single copy of a *GAL1-INO2* gene inserted in the genome[153]. By controlling *INO2* levels with galactose, we can determine if the drop in Ino2p levels and *INO1* transcription is based on either *INO2* genetic repression or degradation of Ino2p. We grew the *GAL1-INO2* strain (BRS2011) on 0.25% galactose, since *INO1* expression has been shown to be maximal at this concentration of galactose[153]. Under these conditions, *INO1* regulation remains high during exponential phase and dropped in stationary phase mimicking *INO1* expression in a WT strain (Figure 20). These data suggest that *INO1* repression in stationary phase is in fact due to decreased levels of Ino2p.

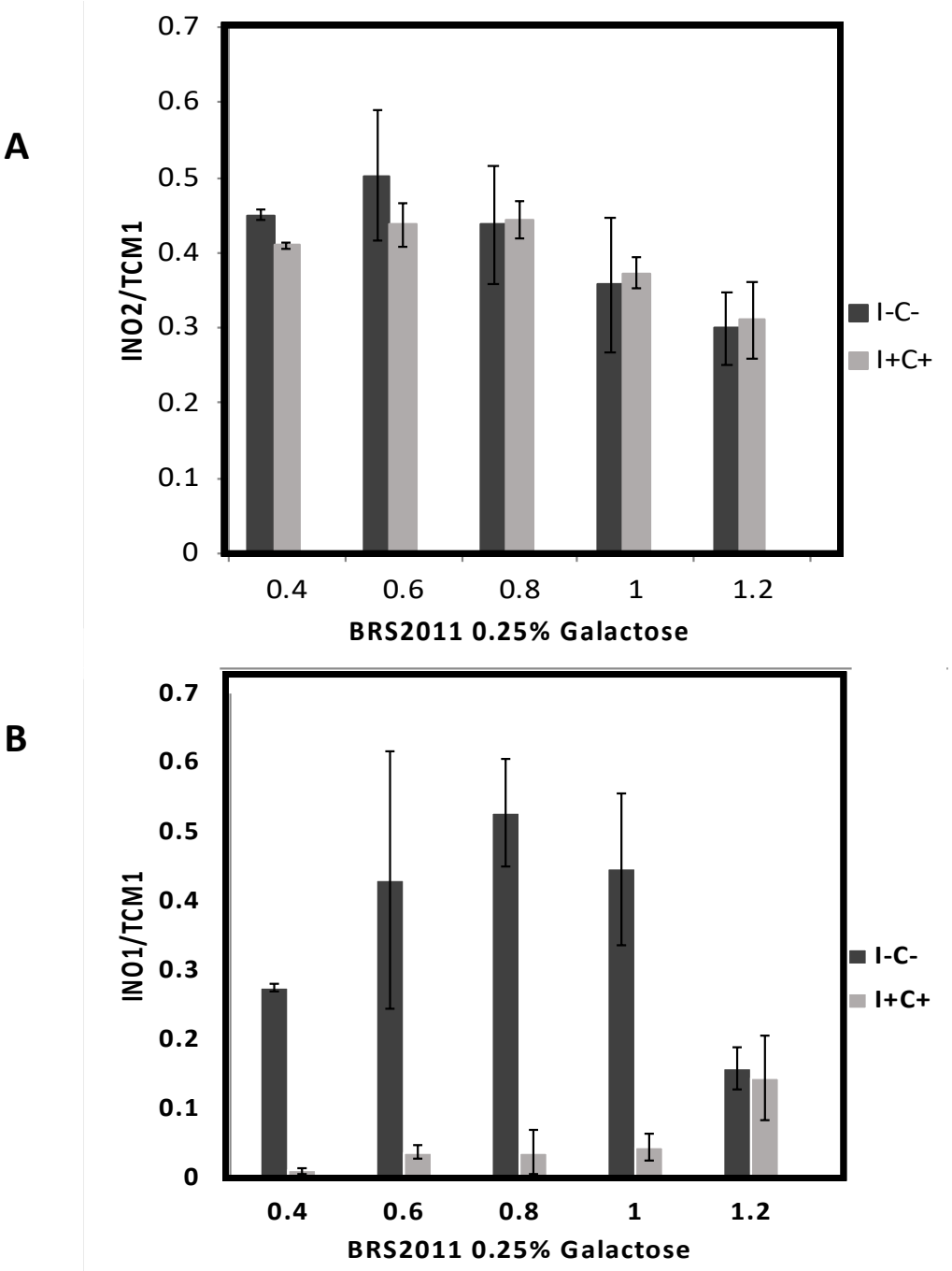


Figure 20: ***INO1* growth phase regulation in a *GAL1-INO2* strain**
(A) Transcript levels of *INO2* (A) and *INO1* (B) in a *GAL1-INO2* strain (BRS2011) in 0.25% galactose throughout the growth phase.

## Discussion

In this chapter we sought to decipher how the phospholipid biosynthetic genes are regulated as cells enter stationary phase. All of the research done in trying to understand how the phospholipid biosynthetic genes are regulated has predominantly focused on the response to inositol and choline. While, growth phase regulation has been known for around thirty years, little to nothing was known about the mechanism for this regulation[23,150]. This is very important to understand as our studies will open a new area focused on understanding how phospholipid biosynthetic genes are regulated, how they are coordinated with other process through protein degradation and stationary phase and it identified new genes involved in the transition to stationary phase.

In eukaryotes, proteins are often degraded by the UB-proteasome system[78], making this a very important process for the cell. In our previous screens looking for the Opi$^-$ phenotype[71,72], different genes involved in the ubiquitination pathway and proteasome complex were identified with an Opi$^-$ phenotype suggesting that this process is involved in the regulation of the phospholipid biosynthetic genes in yeast. This constituted the first formal evidence that the protein degradation pathway plays an important role in the regulation of the phospholipid biosynthetic genes.

Prior to this study, the Opi$^-$ phenotype had been strongly correlated with a repression defect in *INO1* transcription wherein cells become unresponsive to inositol and choline. Our results here showed that downregulation of proteasome subunit genes did not show this same defect under repression growth conditions

(I+C+). Knowing *INO1* can be regulated in response to growth, we tested for *INO1* transcriptional regulation throughout the growth phase. Our results showed that the protein degradation genes play a role in repressing *INO1* at stationary phase under activating conditions (I-C-).

Further research in the role of the protein degradation genes in the regulation of *INO1* showed how its activators, Ino2p and Ino4p, are present during the exponential phase but decrease dramatically at stationary phase to undetectable levels. This correlates with *INO1* expression throughout the growth phase, since *INO1* and other phospholipid biosynthetic genes are active at exponential phase but repressed in stationary phase[23,150]. The proteasome subunit genes (*RPN11* and *RPT2*) tested for effects on *INO1* transcription at stationary phase, were also tested to determine the effect on Ino2p and Ino4p throughout the growth phase. A conditional shutdown of the proteasome subunit genes, yielded stabilization of the activators at stationary phase, correlating with what we see with *INO1* mRNA levels under these same conditions. This stabilization of *INO1* activators was further confirmed when using the chemical inhibitor MG132. This is the first compelling evidence that supports that Ino2p and Ino4p are being degraded at the stationary phase, which links the protein degradation pathway as an important growth phase regulator of *INO1* transcription.

Using a bioinformatic tool (www.ubpred.com), we identified potential sites for ubiquitination in Ino2p and Ino4p. When these sites were mutagenized, we were able to determine that Lysine 158 in Ino2p and Lysine 19 in Ino4p are important amino acid residues for the stability of these proteins. These results are

consistent with, but do not prove that, Ino2p and Ino4p are being targeted for degradation by ubiquitination. Notably, it is known that protein degradation is possible in a Ub-independent matter in eukaryotes[155,156]. In this project, we tried identifying if ubiquitination does take place on either Ino2p or Ino4p by using an anti-Ub antibody following immunoprecipitation, using mass spectrometry. We took samples at different $OD_{600}$ in the presence of de-ubiquitinase inhibitor N-Ethylmaleimide (NEM) in order to determine if Ino2p or Ino4p had a different band size that could lead us to suspect that they are being modified by Ub. All experiments failed at trying to identify if ubiquitination is taking place. it is worth noting that there have been recent reports that the proteasome is responsible for regulating phospholipid synthesis in a Ub-independent matter via the Kennedy pathway[157].

Based on our data, we built a model that explains how *INO1* repression behaves when the reach stationary phase (Figure 21). Under activating conditions, it is known that *INO1* is active because the transcription activators, Ino2p and Ino4p, heterodimerize to form a complex that binds the *INO1* promoter. When approaching stationary phase, we propose that an unknown signal triggers Ino2p and Ino4p to be targeted to the proteasome for destruction. By the time the cells have fully entered stationary phase, both Ino2p and Ino4p levels have decreased dramatically leading to reduced *INO1* expression.

Both protein degradation and phospholipid synthesis are essential processes that the cell needs for proliferation. In addition to both processes being important for its proliferation, recently it was reported that in mammals genes involved in

protein degradation, specifically the ubiquitination pathway, play an essential role in controlling neurogenesis[79,80]. There is a direct connection with autism, as it has been reported that a common phenotype between individuals with autism is that there may be too many synapses in the brain[158]. The study shows how the Ub-ligase (E3) RNF8 in mammals (*DMA1* and *DMA2* in yeast) and Ub-conjugase (E2) UBC13 play an important role in suppressing synapse formation in the mammalian brain *in vivo*. This result is interesting, especially considering that UBC13 was identified to have an Opi$^-$ phenotype in yeast[71]. Taking in consideration all we have learned about the role of protein degradation in regulating the phospholipid biosynthetic genes, its role in controlling neurogenesis, and both processes having UBC13 as a common player, this may lead to using the behavior of *INO1* transcription for early detection in autism. This is not the first instance of yeast research being used to understand the human condition. A significant body of study has shown a role for *INO1* transcription in bipolar disorder[159]. Since so much is still unknown about the role of the protein degradation genes in regulating the phospholipid biosynthetic genes, it is still too early to suggest that a connection can be found in the phospholipid genes behavior and autism. More research is needed to further confirm a connection between both pathways, but if this indicates a connection, it could lead to the use of *INO1* transcriptional behavior in early detection in autistic children.

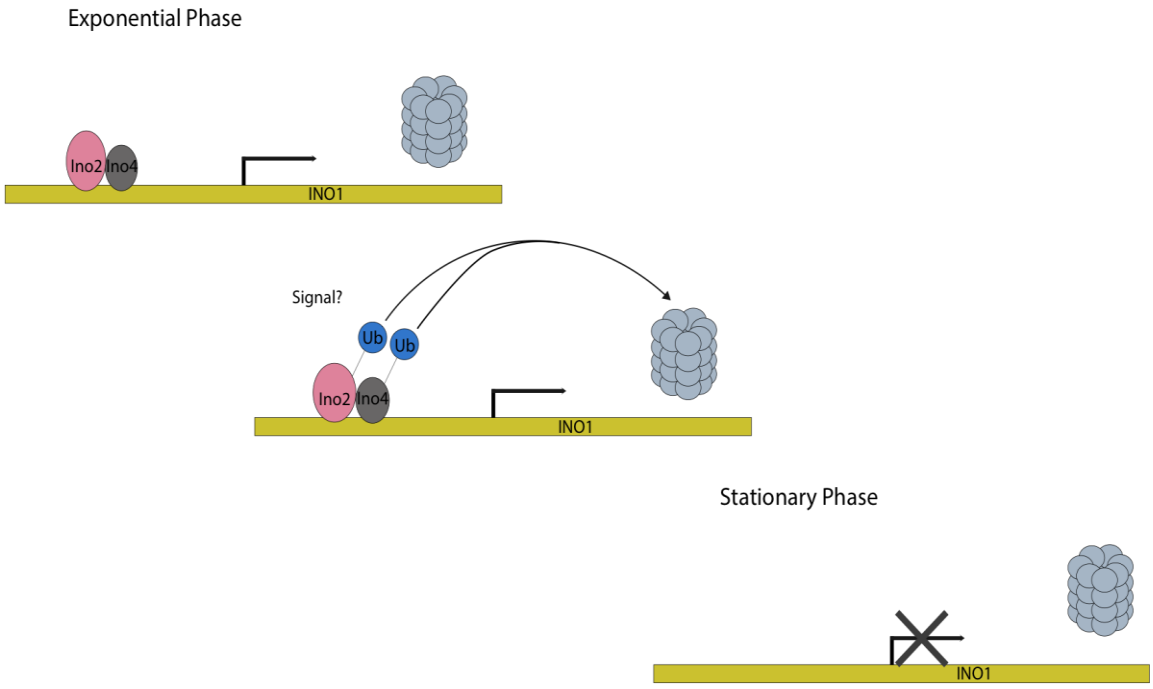


Figure 21: **INO1 Growth Phase regulation model**
Proposed model which shows possible mechanism of *INO1* regulation at stationary phase. Refer to text for complete description.

CHAPTER

# IV. NUA4 HAT IS A REGULATOR OF PHOSPHOLIPID BIOSYNTHETIC GENE EXPRESSION

## Introduction

Eukaryotes show a higher degree of complexity in terms of regulation of gene expression. Eukaryotic DNA is organized into a complex structure called chromatin[91]. Chromatin in turn is a very active and dynamic player in many processes that involve gene regulation[90]. One type of modification that plays an important role for regulating the function of chromatin is a post-translational modification of histones, acetylation[160]. Histones are a family of basic proteins that are associated with DNA in the nucleus and help condense it into chromatin. Histones can have several types of modifications including methylation, phosphorylation, ubiquitination, and acetylation[161]. Acetylation of histones is proposed to play a double role in the cell. It is known that acetylation of lysines in histones neutralizes the positive charge of histone tails which leads to weaker interaction with DNA, this leads the chromatin to decondense and make promoters more accessible for transcription. Second, acetylation can provide an epigenetic marker for gene expression by blocking the heterochromatin-stabilizing association complexes[91–94]

Acetylation is driven by Histone Acetyl Transferases (HAT), which exist in large complexes, such as NuA4 (Nucleosome acetyltransferase of H4). NuA4 is one of the most conserved HAT complexes in eukaryotes[95,96] (Figure 6). The NuA4

complex is important because of its role in different essential processes such as DNA repair and transcription regulation[97–101]. It is composed by 13 subunits encoded in both essential and non-essential genes. The essential genes include *ESA1* (encodes the catalytic subunit), *EPL1*, *TRA1*, *ARP4*, *ACT1*, *EAF2* (*SWC4*), and *YNG2*, while *EAF1*, *EAF3*, *EAF5*, *EAF6*, *EAF7*, and *YAF9* are non-essential in *Saccharomyces cerevisiae*. However, all of these are very well conserved throughout eukaryotes[90]. HAT complexes are well known for their ability to acetylate histones tails, but in addition to these well-established substrates NuA4 and other HAT complexes are known to acetylate non-histone substrates, which can control different processes such as metabolism, autophagy, and homeostasis[104,145,162–164]. Some subunits of NuA4 have also been associated with tumorigenesis in colon, breast and prostate cancers[111,165].

Our lab previously conducted genome-wide screens that discovered interactions between the NuA4 HAT and phospholipid biosynthetic gene regulation[71,72]. Nine of the thirteen NuA4 HAT subunits displayed an Opi$^-$ phenotype, suggesting a role in repressing expression of the phospholipid biosynthetic genes. While HAT complexes are known to play a role in gene activation, the Opi$^-$ phenotype that we see with NuA4 suggests that it may play a role in repression. Interestingly, published experiments suggest NuA4 HAT binds the *INO1* promoter but is not required for transcriptional activation[143], which suggests that the role of NuA4 HAT in the regulation of the phospholipid genes is due to an uncharacterized role. In this project, we explore the effect of mutating NuA4 on *INO1* transcriptional regulation.

## Materials and Methods

### Yeast strains, media, and growth conditions

The *S. cerevisiae* strains used in this study were BY4741 (*MATa*, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and an isogenic *eaf7Δ* strain (VYDS)[67,68]. Strains with Tandem Affinity Purification (TAP)-tagged *INO2* and *INO4* were purchased from Open Biosystems [151]. Gene-specific cassettes containing a C-terminally positioned TAP tag were synthesized by PCR using pFA6a-TAP-His3MX (CBP-TEV-ZZ-His3MX6) as template to generate *eaf7Δ*, *INO2-TAP* strain.

### Growth phase assays*:*

Precultures of cells were grown on YEPD until saturation. Cells were pelleted and washed with $dH_20$ and transferred to complete I-C- synthetic medium at a 1:10 dilution. Samples were taken at different $OD_{600}$ measurements (0.4, 0.6, 0.8, 1.0, 1.2) until cultures reached the stationary phase of cell growth.

### RNA extraction and quantitative real-time PCR (QRT-PCR) analysis*:*

RNA was extracted by a hot-acid phenol method[55], followed by DNase digestion using RQ1 DNase (Promega), and purification using an RNA Clean & Concentrator™ kit (ZYMO). RNA was used to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen). For quantification, cDNA was diluted 1:10, and quantitative PCR (QPCR) was performed as described previously[123]. *INO1* and *TCM1* transcripts were quantified by using the INO1-ORF and TCM1-ORF primer pairs (Table 1).

β-galactosidase assay:

β-galactosidase assays were performed as previously described[9]. Assays were performed in β-Gal Assay Buffer (20% glycerol, 0.1 M Tris/HCl pH8.0 1 mM DTT, 10 μM PMSF, 1 μg/ml Pepstatin A) following the addition of 160 μg/μl of ONPG. The plasmid pJH330 contains an *INO1-lacZ* construct which was transformed into the relevant strains. Kinetic activity was measured in a microtiter plate reader at $OD_{420}$ measured every 12 seconds for 30 minutes.

Protein extraction, SDS-PAGE, Western blotting*:*

Whole cell extracts from *S. cerevisiae* were prepared by sonicating cells in Extraction Buffer (40 mM HEPES pH7.4, 350 mM Sodium chloride, 0.1% NP40, 10% Glycerol, 100 μM PMSF, and 2 μg/ml Pepstatin A). Protein extract concentration was quantified by the Bradford Method and denatured on Laemlli Buffer, DMSO at 95˚C and fractionated on 8% polyacrylamide gels in Tris/Glycine/SDS Buffer (Bio-Rad). Proteins were transferred in 1X Tris/Glycine, 20% methanol onto PVDF membranes at 4°C overnight. Membranes were washed with 1X PBS Tween 0.05% (Genesee) blocked with 1X PBST 5% dry milk. Membranes were incubated 3 hrs with Peroxidase Anti-Peroxidase Antibody (Sigma) for detecting the TAP-tag. TBP was detected using a Monoclonal Primary Antibody, Mouse Anti-TBP (Sigma) for 2 hrs followed by a secondary antibody, Donkey Anti-mouse HRP (Thermo) for 1 hr. Proteins were detected using an ImageQuant LAS4000 mini Luminescent Image Analyzer (GE).

## Results

### *INO1* regulation is affected in NuA4 HAT mutants in stationary phase

Since the Nua4 HAT complex has been identified in both screens our lab has conducted looking for mutants with an Opi$^-$ phenotype[71,72], we tested to determine if NuA4 HAT plays a role in the regulation of *INO1* in response to inositol. To do this we assayed β-gal activity in strains harboring and *INO1-lacZ* reporter (pJH330) grown in exponential phase (Figure 22). The *eaf7* NuA4 HAT subunit mutant strain did not show any sign of a repression defect in I+C+. However, *INO1-lacZ* expression was higher under activating conditions. This suggests that if NuA4 HAT plays a role in the regulation of *INO1*, it is not in the response to inositol.

With the understanding that *INO1* is growth phase regulated, we tested to determine if *INO1* regulation in the NuA4 HAT mutant is affected as cells enter stationary phase (Figure 23). As was the case with the proteasome mutants, *INO1* mRNA was significantly elevated in I-C- conditions when compared to WT at stationary phase. This suggest that NuA4 HAT is important for *INO1* regulation at the stationary phase of growth.

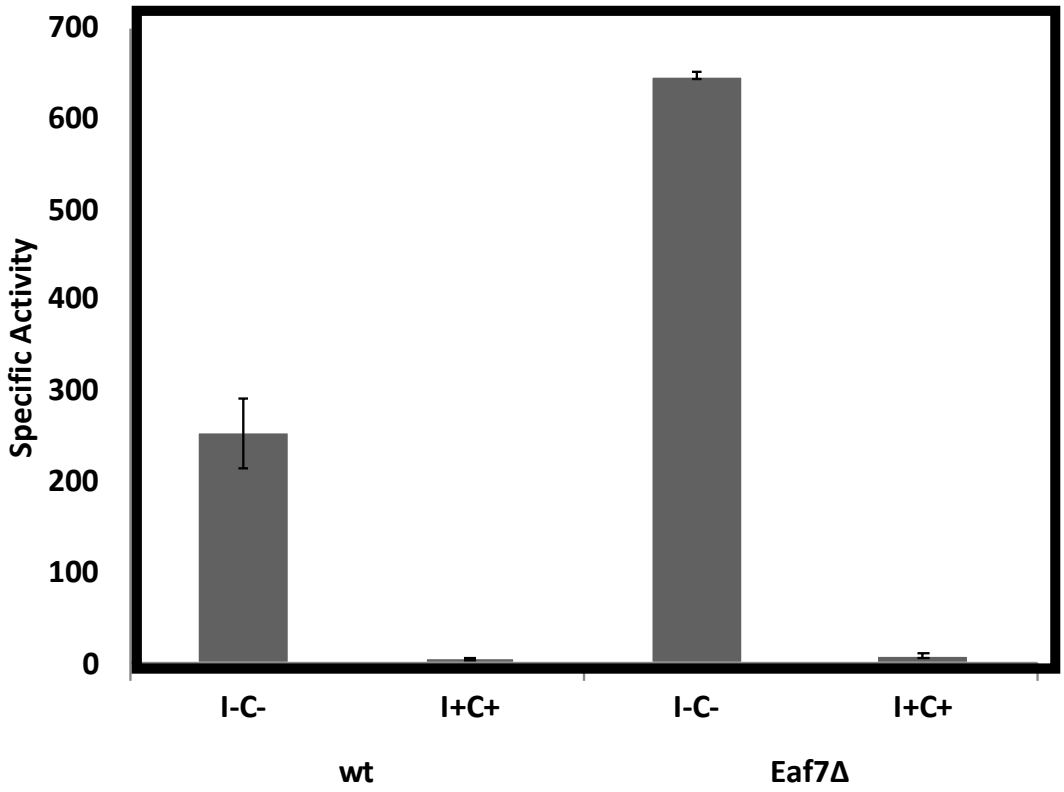


Figure 22: ***INO1* expression in a NuA4 HAT mutant at exponential phase**
b-galactosidase activity from an *INO1-LacZ* reporter in a NuA4 mutant, *eaf7*, grown in I-C- and I+C+ media to exponential phase.

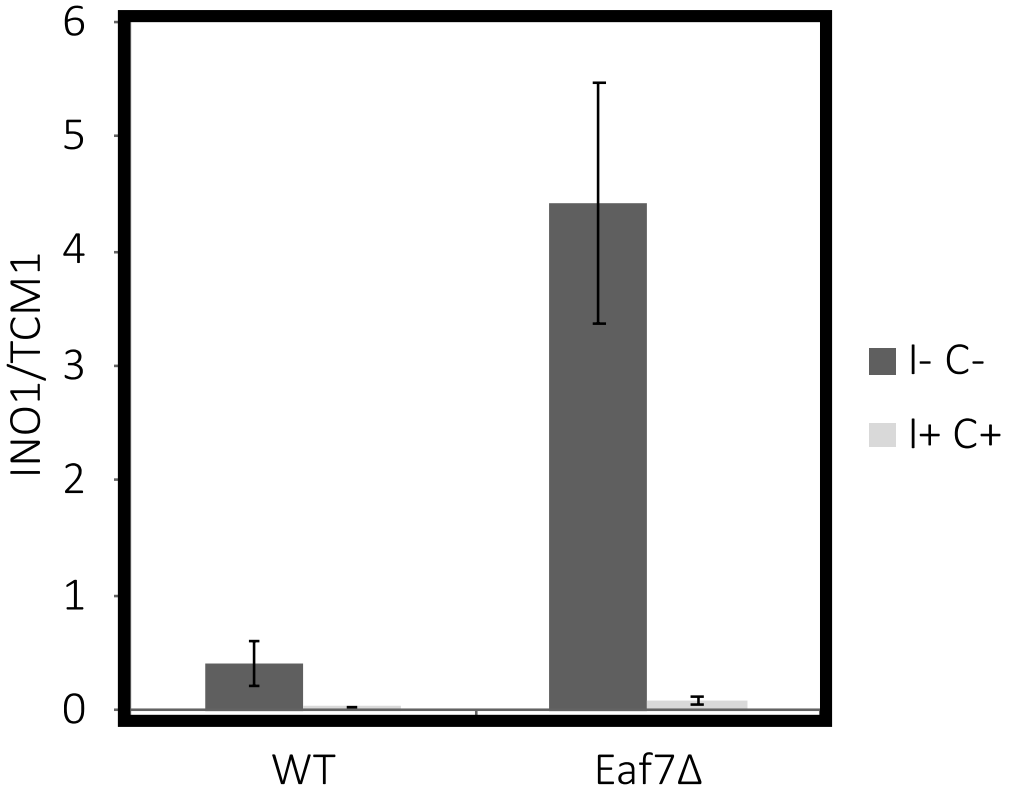


Figure 23: ***INO1* expression in a NuA4 HAT mutant at stationary phase**
β-galactosidase activity from an *INO1-LacZ* reporter in a NuA4 mutant, *eaf7*, grown in I-C- and I+C+ media to stationary phase.

## Ino2p activator protein levels are regulated by the Eaf7p NuA4 subunit in stationary phase

Our current understanding of *INO1* regulation proposes that the protein degradation pathway is involved in regulation at stationary phase. Interestingly, in yeast it has been shown previously that in order for cells to enter meiosis, a transcriptional repressor Ume6p needs to be degraded in order to activate the meiosis activating genes. The first step for degradation of Ume6p has been shown to require acetylation by the SAGA HAT complex[104,164]. Knowing that the protein degradation pathway and NuA4 HAT are important regulators of *INO1* during stationary phase, we tested if NuA4 HAT plays a role in the protein stability of the *INO1* regulator, Ino2p.

Ino2p levels throughout growth phase were similar to the pattern of expression of WT cells (Figure 24). This led us to conclude that NuA4 HAT does not necessarily play a role in *INO1* regulation via transcription factor degradation.

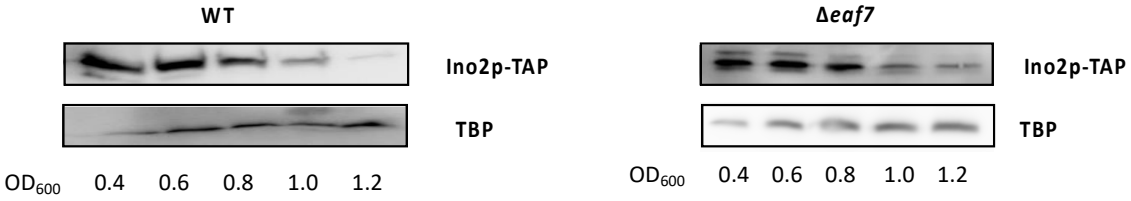


Figure 24**: Ino2p stability in a NuA4 HAT mutant throughout growth phase.** Western blots showing stability of *INO1* activator (Ino2p) stability in a strain harboring an *eaf7Δ* mutant (NuA4 HAT subunit).

Discussion

Currently our understanding of how NuA4 affects *INO1* expression is not completely formalized. With respect to NuA4 playing a role in *INO1* regulation in response to inositol it appears to have a slight effect under activating conditions, but no evidence of a role in inositol-mediated repression in exponential phase (Figure 22). Knowing *INO1* can be growth phase regulated, we tested *INO1* transcription at stationary phase in a NuA4 mutant and found that *INO1* mRNA levels remained high in stationary phase (Figure 23). We learned in the earlier project, that *INO1* growth phase regulation depends on the protein degradation pathway. Also, there is evidence in yeast that acetylation of the Ume6p transcription factor by a HAT complex is a first step towards protein degradation[104,164]. However, Ino2p levels were not affected in a NuA4 mutant (Figure 24). This suggested that the role NuA4 might be playing in order to regulate *INO1* is not through protein degradation.

It has been shown that lysine acetylation contributes to lipid metabolism by regulating gene expression and metabolic enzymes[161]. It is also known that NuA4 has the ability to bind the *INO1* promoter, despite not being required for transcriptional activation[143,166]. NuA4 is also an important negative interactor with Sec14p[167], a phospholipid-remodeling protein. This could can provide a way to decipher a possible role of NuA4 with respect to regulating phospholipid synthesis. Sec14p is an essential phospholipid-binding protein important for the metabolism of phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylcholine (PC) at the Golgi apparatus. This protein functions to create a favorable environment for lipid trafficking[168]. *SEC14* mutants display an increase in intracellular PC, a decrease in

PI-4-P, and a growth deficiency in media lacking inositol[169–174]. Recently a study attempted to decipher the role NuA4 plays in phospholipid synthesis. It was hypothesized that since NuA4 mutants over-produce inositol (Opi$^-$ phenotype), combining both NuA4 mutants with *SEC14* mutant, would suppress the growth defects that Sec14 displays in inositol-depleted conditions. Surprisingly, NuA4 mutants increased the growth defects present in a *sec14-1$^{ts}$* under inositol-depleted conditions[175]**.** Consistent with our previous data, *INO1* was upregulated in NuA4 mutants and in combination with *sec14-1$^{ts}$,* but other genes that have UAS$_{INO}$ in their promoter were not affected by these mutants. This suggests that the role NuA4 plays may be through other aspects of homeostasis. In fact, through genetic and chemical approaches, it was suggested that the role NuA4 plays might lie in phospholipid homeostasis through regulation of fatty acid synthesis and lipid droplets[175].

CHAPTER

# V. DISCUSSION

## Summary

This study adds considerable insight to our understanding of phospholipid biosynthetic gene regulation and which biological processes are important for regulation. Our research focused on learning how *INO1* is regulated in response to growth, which has been known to be occur for over twenty years, but little to nothing was known about the mechanism.

Our lab's genome-wide screens looking for potential repressors for *INO1* transcription revealed that our understanding of the regulation of the phospholipid biosynthetic pathway is incomplete[71,72]. During the course of studying this pathway, there were only eight genes identified (by many labs) to have an Opi$^-$ phenotype, a phenotype that is correlated with a repression defect. In our first project (Chapter II) we identified 122 essential genes with an Opi$^-$ phenotype which adds to the 91 Opi$^-$ mutants found screening the VYDS[71,72], for a total of over 200 genes responsible for potentially repressing *INO1* transcription. Processes identified in our screen included, gene looping, protein degradation, protein post-translational modification, the nuclear pore complex, transcriptional regulation, and lipid synthesis. With these results it was reasonable to conclude that more research is needed in order to fully understand transcriptional regulation of the phospholipid biosynthetic genes.

In our second project (Chapter III), we focused on trying to understand how one of the novel biological processes identified in the essential gene screen affects

phospholipid transcriptional regulation. The protein degradation machinery is a biological process that was overrepresented in our screen leading us to suspect that it plays an important role in gene regulation. We learned that the protein degradation genes are responsible for the transcriptional regulation of *INO1* in response to growth. We successfully identified that at stationary phase *INO1* shows a repression defect in the presence of proteasome mutants. In addition, we determined that *INO1* transcription activators (Ino2p and Ino4p) levels decrease as cells enter stationary phase, leading to lack of activation of *INO1*. When inducing a conditional shutdown of the proteasome or using a chemical inhibitor of the proteasome (MG132) we determined that both Ino2p and Ino4p levels stabilize in stationary phase, suggesting that the proteasome degrades both activators. We attempted to identify the role of ubiquitination, although we were not successful in identifying Ub-modified activators, we were able to mutate potential sites for ubiquitination in both Ino2p and Ino4p and found that these were stabilized in stationary phase. This suggests that ubiquitination might be happening in the high confident sites we mutated. With this new knowledge about *INO1* growth phase regulation we were able to build a preliminary model that explains the growth phase regulation with respect to entry in stationary phase.

In our third project (Chapter IV), we focused on trying to understand the role of the NuA4 HAT complex in the regulation of *INO1*. Nine of the thirteen NuA4 subunits have been identified in screens looking for the Opi$^-$ phenotype, which includes Esa1p the catalytic subunit of the complex. Our initial thought was that NuA4 could be acetylating a non-histone protein and that could be the initial signal

that could target the *INO1* regulators for protein degradation, something that has been shown to be possible in yeast[104,164]. In addition to determining that NuA4 mutants show higher levels of *INO1* during activating conditions, we also determined that NuA4 is playing a role in the growth phase regulation of *INO1*. When we tested for the effect of NuA4 mutants on Ino2p stability throughout growth phase, we did not see a clear effect in Ino2p stability. Recent reports have suggested that NuA4 HAT could be contributing to phospholipid homeostasis in yeast[175]. With this limited knowledge about the effect of NuA4 in *INO1* transcription we concluded that further investigation is needed for a better understanding of its role in regulating transcription of phospholipid biosynthetic genes.

## Future Directions

### Growth phase regulation via protein degradation

In our work we discovered a possible explanation for how genes involved in protein degradation are involved in *INO1* growth phase regulation. Still, many questions remain that could be answered in the future. Although, we found sites in both Ino2p and Ino4p that are suspicious for ubiquitination, physical evidence of ubiquitination is still non-existent. Determining if these activators are being modified by Ub will be another piece of the puzzle. In addition, there is an alternative view that could explain why we didn't identify ubiquitination, namely that there are Ub-independent pathways that lead to protein degradation[155]. This pathway is still not well understood in terms of how it works, but it has been already suggested that it plays a role in regulating Pah1p, an important player in the

Kennedy pathway (the recycling pathway for phospholipids)[157]. With this knowledge it is necessary to determine if *INO1* activators are being degraded by an Ub-dependent mechanism or an Ub-independent mechanism. This will also help decipher if the Ub-independent regulation that was reported in the past in a different part of the phospholipid genes is a phenomena specific for the phospholipid genes or just for the particular case of Pah1p.

Knowing that protein degradation is important for synapse formation in mammals[158], it will be worth investigating if *INO1* regulation can give us a clue about synapse formation. This is driven by the observation that *INO1* is regulated by protein degradation and both synapse formation and *INO1* regulation via protein degradation require the ubiquitin conjugation enzyme encoded by *UBC13*. In order to determine this, first we will need to test if *UBC13* is required for *INO1* regulation (similar to *UBA1* in the current study). If *INO1* shows signs that *UBC13* is required for its regulation we will need a different model system to study this in depth, in order to test directly synapse formation and *INO1* regulation.

### Role of NuA4 HAT in the phospholipid biosynthetic pathway

Thanks to de development of innovative computational technologies, the field has been able to advance and increase our understanding and capacity in studying complex systems. Recently a workflow called Octopus-toolkit was developed to automate mining of public epigenomic and transcriptomic next-generation sequencing (NSG) data[176]. It retrieves and processes large sets of NGS data from the most popular model organisms like humans, mouse, dog, plant,

zebrafish, worm, and yeast. Researchers used this tool to try to identify DNA-binding proteins that recruit histone-modifying complexes. They analyzed a ChIP-seq data set (GSE52339) and their results show that the majority of Esa1p-binding sites contained a significant number of Opi1p-binding motifs. Their results suggested that a spatiotemporal recruitment of Esa1p-containing NuA4 HAT might be mediated by Opi1p[176]. This suggests new mechanistic insight between the NuA4 complex and phospholipid homeostasis[72,175].

In addition, if NuA4 plays a role in phospholipid homeostasis it has been suggested that the reason NuA4 mutants show an Opi$^-$ phenotype is due to a downregulation of *CDS1* expression[175]. Downregulation of *CDS1* leads to accumulation of PA which reflects an Opi$^-$ phenotype[72,120]. Under those circumstances, it will be worth studying the role NuA4 HAT plays in the regulation of *CDS1*. If NuA4 positively regulates *CDS1*, it could give an explanation on why NuA4 mutants show an Opi$^-$ phenotype.

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