The Implications of Chromatin Remodelers' Acetylation in INO1 Activation

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The implications of chromatin remodelers’ acetylation in INO1 activation

By

Michelle Marie Esposito
The implications of chromatin remodelers’ acetylation in \textit{INO1} activation

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This manuscript has been read and accepted for the Graduate Faculty in Biology to satisfy the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

It is known that histone acetylases (HATs) regulate gene expression, but only recently have new functional implications about remodelers’ acetylation emerged. For instance, the HAT, Gcn5p, is capable of acetylating the catalytic subunit of the nucleosome remodeling complex SWI/SNF, Snf2p, which results in the dissociation of the complex from chromatin. The implications of this acetylation and subsequent dissociation have yet to be explored with regard to transcriptional regulation and other possible mechanisms. To further understand the implications of remodeler acetylation, I used a yeast model system examining the expression of the inositol-3-phosphate synthase gene INO1. Through chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) assays, I demonstrated that remodeler Snf2p acetylation is required for dissociation of the subunit from the INO1 promoter. Furthermore, employing growth curve and mRNA analyses, along with ChIP targeting of nucleosomal components and polymerase, I showed this acetylation was not required for INO1 transcriptional activation. However, I observed that the chromatin remodeler, Ino80p, was unable to dissociate from the INO1 promoter in the absence of Snf2p acetylation. Even though HAT Gcn5p recruitment remained unaffected, acetylase Esa1p recruitment significantly decreased in the absence of Snf2p acetylation. Although a lack of Snf2p acetylation did not significantly impact the transcription of INO1, it did modify the occupancy/recruitment of remodeler Ino80p and HAT Esa1p at the promoter region, suggesting Snf2p acetylation may promote the recycling of both chromatin remodelers.

In an attempt to understand the biological implications of Snf2p acetylation, I performed sensitivity assays. Our results showed that while DNA damage/replication, osmoregulation, and carbon source utilization were unaffected by acetylation, protection from copper toxicity was
significantly impacted. In conditions where chromatin remodelers were highly concentrated at the *INO1* promoter (i.e., unAcSnf2p cells in the absence or presence of inositol, as well as WT cells in the presence of inositol) copper toxicity defense was hindered, which suggests the possibility a novel link between *INO1* and *CUP1* expression, and remodeler recycling, previously unidentified. Since remodelers SWI/SNF, ISWI, and RSC are capable of being acetylated by HATs, I now demonstrated this acetylation could have significant distinct impact on pathways regulated by SWI/SNF.

I then expanded our study to INO80, which had yet to be shown as an acetylated remodeler. I demonstrated that Ino80p was acetylated, but lacked this acetylation when the DNA-binding HSA domain was deleted, which correlates to studies of other acetylated remodelers in that they tend to be acetylated in regions involved in DNA-binding. This may help explain why Ino80p accumulates at the *INO1* promoter in either HAT mutant strain, *gcn5Δ* or *esa1mt*. To better characterize this acetylation, I then engineered a series of *INO80*-FLAG-tagged HAT deletion mutants and identified that HAT Esa1p was responsible for acetylating Ino80p. Furthermore, I found that the lack of Ino80p acetylation may cause a defect in DNA repair. As such, our findings for Snf2p and Ino80p have revealed insight into the mechanism of chromatin remodeler acetylation and its implications in gene expression regulation.
Dedication and Acknowledgements

This thesis is dedicated to the loved ones I have lost along the way (my dad and my Dr. Elena McCoy), as well as the loved ones who still stand by my side everyday (my mom, my grandparents, and my big brothers).

I would like to acknowledge my family first and foremost; this includes those loved ones I am connected to through blood and those loved ones I have encountered along the way throughout life. Above all else, I would like to thank my parents, as their sacrifices, love, guidance, and protection have made me the person I am today. I will forever be grateful to both of them and consider any success in my life a success of theirs. I miss Dad every minute of every day, but will forever cherish the legacy he has left and appreciate every moment I still get to experience with Mom by my side. In addition to my parents, I would also like to thank my beloved grandparents and my big brothers, as they have been by my side through every obstacle as well as every bright moment of my life. I would not be the person I am today without them either, especially since my brothers have served as an inspiration to me not only with regard to life, but also with regard to the sciences. I am proud to be a copycat of Tony by getting this PhD, as well as a copycat of Mikey by teaching science!

I would also like to thank my mentor, Dr. Chang-Hui Shen who has guided me and supported me for nearly a decade through each of my academic ventures and has helped to mold me into the scientist I am today. I cannot thank him enough for all of the amazing opportunities he has given me and all of the invaluable knowledge he has shared with me. I would also like to extend my gratitude to each of the members of my committee, Dr. Alejandra Alonso, Dr. Yu-Wen Hwang, Dr. Sebastien Poget, and Dr. Jonathan Blaize for their valuable feedback and support throughout my doctoral process. Then of course, I would like to extend my deepest
gratitude and love to Dr. Elena McCoy, who was a valued member of my committee and a valued member of my extended family. She was always one of the first people I would run to in the building in the morning and one of the last people I would say goodnight before leaving. She provided guidance, love, and support that can never be replaced, and she will always serve as my greatest inspiration with regard to the sciences and academic work ethic.

Speaking of extended family…I would also like to thank my other half and partner in crime in 6S for a decade, Paulina Konarzewska, my other partner in crime in 6S, Goldie Sherr, and the former mom of the lab, Roshini Wimalarathna, as well as the biology department, which has served as a second family to me, always looking out for me in every way possible. When you spend as much time in a place as I have spent in 6S, it is so important to be surrounded by people who make it feel more like a home than just a place of work. On that note, I would also like to thank “my children” (you know who you are). There are too many to list them all (just to name a few in alphabetical order… Alicia, Andrew, Brendon, Christine, Dilakshi, Joe, Jwala, Kristi, Kristina, Mays, and Peter), but each has left such a valuable mark on my life that I could write a whole other thesis just raving about all of them. I would also like to thank Dr. William L’Amoreaux for hiding me during the thesis writing process…because well it is tough to be a celebrity! I would also like to further extend my gratitude to Dr. L’Amoreaux for always giving me such valuable feedback and support throughout the years. It has meant so much to me to have been “adopted” and always looked out for every day.

I would like to thank CUNY, especially CSI and the Graduate Center, for giving me the most amazing opportunities to further my education and my career. Lastly, I would like to thank Dr. Xuetong Shen and Dr. Jerry Workman for yeast strains utilized in this research.

Deepest gratitude and love,
Michelle Marie Esposito
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List of Abbreviations

A₂₆₀: 260nm absorbance (nucleic acids)

A₂₈₀: 280nm absorbance (proteins)

A₆₀₀nm: 600nm absorbance (cells)

acH3: acetylated Histone 3

acH4: acetylated Histone 4

ACT1: Actin 1 (housekeeping gene)

Arp8p: Actin-related protein (subunit of Ino80p)

ATP: Adenosine Triphosphate

cDNA: complementary Deoxyribonucleic acid

CHD: Chromodomains Helicase DNA-binding (chromatin remodeler)

ChIP: Chromatin Immunoprecipitation

DEPC: Diethylpyrocarbonate

dNTP: deoxyribose Nucleotide Triphosphates

EDTA: Ethylenediaminetetraacetic acid

Esa1p: Essential Sas-related Acetyltransferase 1 (histone acetyltransferase)

G-6-P: Glucose-6-Phosphate

Gcn5p: General Control Non-derepressible 5 protein (histone acetyltransferase)

H3: Histone 3

H4: Histone 4

HATs: Histone Acetyltransferases

HDACs: Histone Deacetyltransferases

I-3-P: Inositol-3-Phosphate
ino: inositol

INO1: Inositol-3-phosphate synthase gene

INO80: Inositol-requiring 80 (chromatin remodeling family)

Ino80p: Inositol-requiring 80 protein (chromatin remodeler)

IP: Immunoprecipitate

ISWI: Imitation Switch (chromatin remodeling family)

mRNA: messenger Ribonucleic Acid

OD: Optical Density

PCR: Polymerase Chain Reaction

PI: Phosphatidylinositol

PMSF: Phenylmethylsulfonylfluoride

PolIIp: Polymerase II protein

qPCR: quantitative Polymerase Chain Reaction

qRT-PCR: quantitative Reverse Transcriptase Polymerase Chain Reaction

SC: Synthetic Complete

SDS: Sodium Dodecyl Sulfate

Snf2p: Sucrose Non-Fermentable protein (chromatin remodeler)

SWI/SNF: Switch/Sucrose Non-Fermentable (chromatin remodeling family)

TBE: Tris/Borate/EDTA

TE: Tris-EDTA

TES: Tris-Ethanesulfonic Acid

unAcSnf2p: unacetylated Snf2p

WT: wild type
Chapter 1
Introduction

1.1 Packaging of DNA

The entire genome of each eukaryotic organism can result in rather long strands of DNA (approximately 2 meters) that must fit into the nucleus (approximately 2 μm sphere) of each cell (Kornberg, 1974; Peterson & Laniel, 2004). In order to fit this vast amount of macromolecular information into such a small structure, it is necessary for cells to have intricate packaging mechanisms, which require proteinaceous nucleosomes (Cairns, 2009). A nucleosome is made up of highly conserved histone proteins and DNA associated together via electrostatic and hydrogen interactions of the phosphate backbone of DNA with the amino acid residues of the histones (Li et al., 2006). Histone-DNA interactions also include nonpolar interactions between certain amino acid residues and the deoxyribose sugar molecules of the DNA backbone. Note that these histone-DNA interactions do not include the nitrogen bases of DNA, which is why nucleosome formation is not sequence specific and nearly all locations of DNA can be subject to histone-based packaging (Kornberg & Lorch, 1999).

Each nucleosome is composed of two copies of histone proteins H2A, H2B, H3, and H4 assembled into a spool, with approximately 147bp of DNA wrapped around these core histones (Figure 1.1A). To give an estimate of size relations, there are 1.65 turns of left-handed helix per spool (Perez-Martin, 1999; Kornberg & Lorch, 1999; Rando & Wilson, 2012). Extending out of the histone core are unstructured terminal tail chains of amino acids that tend to be rich in positively charged residues such as lysine (Martin & Zhang, 2005). These histone tails are critical to gene regulation as they tend to be targets of post-translational modifications that can significantly impact the interactions between histones and DNA (Perez-Martin, 1999). Multiple nucleosomes are then compacted into a string-like structure called chromatin. The DNA present
between each nucleosome in the chromatin structure represents “linker DNA” (Figure 1.1B).
The varying lengths of this exposed linker DNA is critical in gene expression and allows for
great diversity in gene regulation (Clapier & Cairns, 2009). Outside of the central core octamer
is an additional histone protein, H1, which helps maintain the structural stability of the
nucleosome and aids in proper folding of nucleosomes into higher order supercoiled fibers
(Perez-Martín, 1999). This histone (H1) is called the “linker histone” since it binds to the last 10
bp of linker DNA that enter the nucleosome and the first 10bp of linker DNA that exit the
nucleosome (Happel & Doenecke, 2009). This means that it is the only histone associated with
the linker DNA between nucleosomes (Harshman et al., 2013).
Figure 3.1: Packaging of DNA; A) Nucleosome composed of DNA wrapped around a core octamer of 2 copies of H2A, H2B, H3, and H4, each with amino-terminal tails exposed for post-translational modifications, alongside a linker histone H1 for increased structural stability; B) Chromatin structure in which there is linker DNA of fluctuating lengths between each nucleosome.
1.2 Regulation of Chromatin Structure

The packaging of DNA in nucleosomes is critical to genomic compaction, yet it can leave gene promoters inaccessible to activator proteins or transcriptional machinery and thus prevents transcriptional initiation (Morrison & Shen, 2009). In order for genes to become expressed, a protein complex must find a region, the promoter, which promotes transcription of DNA into RNA. Because of the compaction of the DNA helix around the nucleosomes, transcription is effectively repressed and cannot occur until such time as the barrier is removed. This state characterized by condensed inactive chromosome segments is referred to as heterochromatin, whereas more active, diffusely extended accessible regions of DNA are referred to as euchromatin (Frenster et al., 1963). To overcome these repressive effects of heterochromatin it is necessary for histone tails to interact with regulatory proteins that modify chromatin structure (Li et al., 2007). The two main categories of these regulatory molecules are histone modifying enzymes and chromatin remodelers (Kornberg & Lorch, 1999; Hur et al., 2010).

1.2.1 Histone Acetylases and Histone Deacetylases

HATs (histone acetylases) and HDACs (histone deacetylases) are vital dynamic regulators of gene expression with high sequence specificity (Kouzarides, 2000; Katan-Khaykovich & Struhl, 2002). Transcriptionally active euchromatin tends to be characterized by histone acetylation while inactive heterochromatin tends to be defined by a lack of histone acetylation. HATs can be separated into two categories, Type A and Type B. Type A HATs, such as in the SAGA complex, are nuclear and can be found interacting with chromosomal histones, whereas Type B HATs are cytoplasmic and are mainly observed interacting with newly synthesized histones prior to chromatin formation (Kornberg & Lorch, 1999). Since I am
interested in transcriptional regulation, Type A HATs are the focus of our project; specifically I am interested in Gcn5p and Esa1p. HATs are prevalent targets of research following the discovery that they interact with non-histone proteins, including activator proteins and transcriptional machinery (Glozak et al., 2005).

Recent research demonstrates that some histone acetylases, such as Gcn5p, are capable of acetylating chromatin remodelers, including SWI/SNF, ISWI, and RSC (Ferreira et al., 2007; Kim et al., 2010; Charles et al., 2011). This suggests a level of interactions among coactivators never before considered at gene promoters during transcriptional regulation. Previously, research had focused more on coactivators interactions with the DNA rather than other proteins at the promoter region.

Unlike HATs, HDACs tend to be associated with transcriptional repression (Pazin & Kadonaga, 1997). This repression can be from the direct deacetylation of the histone, or it can be indirect as HDAC complexes contain corepressor proteins that recruit additional repressive proteins to the promoter (Kadosh & Struhl, 1998). Deacetylases, such as Rpd3p, can be physically linked to DNA-binding repressive proteins tethered to promoters (Kadosh & Struhl, 1997; Kornberg & Lorch, 1999; Katan-Khaykovich & Struhl, 2002).

1.2.2 Chromatin Remodelers

Chromatin remodelers are a family of multi-protein complexes that go beyond relieving the condensed nature of nucleosomes; they physically disrupt the nucleosome core structure (Kornberg & Lorch, 1999; Shen et al., 2003). These ATP-dependent remodeling complexes disrupt histone-DNA interactions and trigger movements that include lateral DNA sliding and histone departures (Hur et al., 2010; Rando & Wilson, 2012). Chromatin remodelers are linked
to varying cellular processes including DNA replication, transcriptional initiation, DNA repair, and even transcriptional repression (Clapier & Cairns, 2009; Euskirchen et al., 2012). There are four families of chromatin remodelers: SWI/SNF, ISWI, CHD, and INO80 (Figure 1.2) (Bao & Shen, 2007). These families all share five general properties, including nucleosomal affinity, the ability to detect covalent modifications of histones, a conserved ATPase domain, other proteins or domains that help to regulate the conserved ATPase domain, and other features that allow for chromatin interactions or interactions with transcription factors (Clapier & Cairns, 2009). Despite these shared properties, each family is defined by specific domains (Figure 1.3), which function as follows.

The most critical of all remodeler domains is the highly conserved ATPase domain for catalytic ATP hydrolysis, which can either contain a short insertion or a long insertion. Most remodelers house a short insertion within their ATPase domain, but the INO80 subfamily contains a long insertion (Snf2 ATPase) that contains the Snf2 amino-terminus and Helicase carboxy-terminus (Chen et al., 2013). The ATPase domain recruits Rvb1p and Rvb2p, highly conserved essential members of the AAA+ family of helicases, to bind the actin-related protein Arp5 in an event that is necessary for the remodeling activity of INO80. If this insertion region is deleted, INO80 is inactivated (Shen et al., 2003; Jha & Dutta, 2009).

The HSA (Helicase SANT-Associated) domain, which is a characteristic domain of the SWI/SNF (Switching defective/Sucrose Nonfermenting) and INO80 subfamilies, targets actin and actin-related proteins (Arp7 and Arp9 for SWI/SNF; Arp4 and Arp8 for INO80). The recruitment of these particular actin-related proteins to the INO80 complex is significant as they allow the complex to bind DNA, which led to the identification of the Dbino (DNA-binding domain of INO80) within the HSA domain (Brahmachari et al., 2004; Bartholomew, 2014).
Also involved in DNA binding are the AT hooks located on the SWI/SNF remodelers (Aravind & Landsman, 1998), as well as the HAND/SANT(Swi3/Ada3/N-Cor/TFIIB)/SLIDE(SANT-like ISWI domain) domains of the ISWI and CHD subfamilies (Ryan et al., 2007).

Lastly there are the domains that are critical to recognizing post translational modifications of the exposed histone tails of nucleosomes. This is important as ultimately protein regulators of transcription need to bind both DNA and nucleosomes. The bromodomain of SWI/SNF remodelers recognizes and binds acetylated lysine residues of histones. This domain also aids in DNA binding and helps to maintain the overall stability of the remodeler-nucleosome complex (Hassan et al., 2002). The PHD (Plant Homeodomain) of CHD family remodelers is responsible for recognizing and binding methylated lysine residues. The CHD family also has two tandem chromodomains located on the N-terminus. These tandem domains have been shown to recognize and bind methylated lysine residues, specifically H3K9, and also aid in regulating DNA binding activity as well (Hauk et al., 2010).

With these various domains, there are three ways of regulating the chromatin remodelers. The first is by recruiting the remodeler to the appropriate target site, which can be accomplished by the recognition of various modifications on histones or other proteins. The second means of regulation is by modifying the activities of the domains of the chromatin remodeler, such as the helicase domain. Finally, and most significant to our project, is that chromatin remodelers can be regulated by post-translational modifications to their domains or various subunits (Bartholomew et al., 2014). These modifications can include phosphorylation in the SWI/SNF family or in our case, acetylation in the SWI/SNF, RSC, and SWI families (Muchardt et al., 1996; Sif et al., 1998; Ferreira et al., 2007; Kim et al., 2010; Charles et al., 2011).
Figure 1.4: Four families of yeast chromatin remodelers along with the members of each family.
Figure 1.3: Domains characterizing the four families of chromatin remodelers:
HSA (Helicase SANT-Associated) domain known to target actin and actin-related proteins; ATPase domain for catalytic ATP hydrolysis; AT hooks for binding DNA; Bromodomain for binding acetylated lysine residues; HAND/SANT(Swi3/Ada3/N-Cor/TFIIB)/SLIDE (SANT-like ISWI domain) domains for binding DNA and maintaining complex stability; PHD (Plant homeodomain) for binding methylated lysine residues; and Chromodomains for binding methylated H3K9 and regulating DNA binding activity.
There are various mechanisms proposed for remodeler functions. These mechanisms look to clarify the disruption of histone/DNA interactions and the remodeling outcomes of such disruptions. Three main models for chromatin remodeling are sliding, ejecting, or restructuring nucleosomes (Figure 1.4) (Fazzio et al., 2003; Cairns, 2007; Cairns 2009; Clapier & Cairns, 2009; Morrison & Shen, 2009). In the sliding model of chromatin modification (also called looping or unwrapping; Figure 1.4), which is observed with INO80 and SWI/SNF families (Gangaraju & Bartholomew, 2007; Morrison & Shen, 2009; Cairns, 2009), remodelers are believed to bind the nucleosome with the translocation ATPase domain binding to DNA. The ATPase domain then triggers the translocation of linker DNA toward the core region of the nucleosome, which creates a DNA loop that subsequently proliferates around the nucleosome in a wave-like manner that breaks histone-DNA contacts at the initial end of the loop and replaces them with the lagging end of the loop (Wang et al., 2007; Bao & Shen, 2007; Cairns, 2009; Clapier & Cairns, 2009). This wave-like proliferation allows for the breaking of one or two contacts at a time, so as not to completely lose the integrity of the structure.

The second mechanism, which is observed with the CHD1 family, is ejection (Figure 1.4). In this model, the remodeler breaks the histone-DNA interactions of a nucleosome to make it unstable, so that histone chaperones can remove the octamer from the chromatin. The ejected histone octamer can then be transferred to another area of DNA (Cairns, 2009). The final mechanism, which is described as repositioning or restructuring of nucleosomes is characteristic of the SWR1 remodeler (Figure 1.4). In this model, histone dimers are exchanged for variants in a mechanism that requires the remodeler to bind to a variant dimer, associate with a nucleosomal unit, and finally initiate the physical exchange of the dimers (Morrison & Shen, 2009). The H2A/H2B dimer of nucleosomes is frequently exchanged for the H2A.Z/H2B (HTZ1/H2B in
yeast). This aids in gene regulation, since this variant has an acetylated amino-terminal tail that is associated with active genes. H2A.Z (HTZ1) also buffers against gene silencing and promotes nucleosome ejection, which further modifies the chromatin structure (Mizuguchi, 2004; Cairns, 2009; Morrison & Shen, 2009). ISWI remodelers also reposition nucleosomes, but do not alter the dimer composition. Instead, ISWI remodelers are responsible for nucleosomal spacing and can modify chromatin structure by adjusting the spacing between two nucleosomes (Fazzio et al., 2003; Gangaraju & Bartholomew, 2007). For the studies presented in this dissertation, the remodelers of interest are SWI/SNF and INO80.
Figure 1.4: Three Models of Chromatin Remodeler Activity
1.2.2.1 Chromatin Remodeling Complex SWI/SNF

SWI/SNF is a chromatin remodeler that belongs to the remodeler subfamily of the same name (SWI/SNF) that includes one additional chromatin remodeler, RSC (Bartholomew, 2014). As was already mentioned, SWI/SNF stands for switching defective/sucrose non-fermenting, which is how it was first identified in yeast (Peterson & Workman, 2000). This large 1-2 MDa (~1.2 MDa in yeast and ~2 MDa in mammals), highly conserved complex is composed of approximately 11 tightly associated polypeptide subunits (Peterson et al., 1998; Euskirchen et al., 2012). Snf2p, one of the most widely studied of these subunits, is part of the Swi2/Snf2 catalytic subunit of SWI/SNF, which remodels chromatin via ATP hydrolysis (Smith & Peterson, 2005). This crucial subunit contains a bromodomain identified as necessary for the retention of SWI/SNF on chromatin, as well as for the removal of acetylated histones from the nucleosomal condensed structure (Kim et al., 2010).

SWI/SNF is currently of great interest to researchers as it has been associated with various diseases and ailments. Mutations in SWI/SNF subunits have been linked to malignant progressions as there is a tumor-suppressor role associated with this remodeler. In addition to its tumor-suppressor nature, SWI/SNF interacts with oncogenic viruses, including Epstein-Barr and Kaposi Sarcoma-associated Herpes virus. Both of these viruses recruit SWI/SNF; even HIV is identified as interacting with subunits of SWI/SNF (Euskirchen et al., 2012).

1.2.2.2 Chromatin Remodeling Complex INO80

INO80 is also a major chromatin remodeling complex, which functions via ATPase and helicase activities. It is a large complex with a molecular mass of approximately 1MDa to 1.5MDa and is made up of 15 polypeptides in yeast. The currently established subunits are
Ino80, les1, les2, les3, les4, les5, les6, Nhp10, Anc1/Taf14, Arp4, Arp5, Arp8, actin, Rvb1, and Rvb2 (Bao & Shen, 2007). The INO80 family of chromatin remodelers is defined by a conserved ATPase domain with a long insertion in the middle of it, separating the domain into two regions. This differs from the SWI/SNF family of remodelers, which has the conserved ATPase domain, but only has a short insertion dividing the region (Clapier & Cairns, 2009). The ATPase domain houses a highly conserved GXGKT, which serves as a DNA binding motif within the majority of ATPases. The lysine residues of this conserved motif are critical for ATPase activity since it allows the remodelers to interact with the terminal phosphate group of ATP (Clapier & Cairns, 2009). Another critical domain of Ino80p is the Helicase-SANT-Associated domain (HSA domain), which is the binding domain for various Actin-related proteins (Arps), such as Arp8, Arp4, actin, and Anc1/Taf30 (Shen et al., 2003). These actin-related proteins have recently been demonstrated as crucial components for INO80 ATPase activity, and when they are knocked out, ATPase activity is abolished. Arp8 is necessary to recruit Arp4 and actin (Bao & Shen, 2007). Arp4 is a critical subunit of the nucleosomal acetyltransferase of H4 (NuA4), which modifies histone H4. This is yet another example that chromatin remodelers and acetyltransferases are interconnected.

Although chromatin remodelers are mainly studied for their roles in transcription, INO80 is also involved in DNA recombination and repair (Shen et al., 2004). INO80 is involved with DNA replication, which may be linked to its DNA damage repair activities as INO80 is required for replication fork continuation after replication has been stalled with methylmethanesulfonate (MMS) or hydroxyurea (Shen et al., 2009). Ultimately, due to its versatility and implications in recombination-based repair, INO80 is implicated in an array of diseases, including cancer, similar to SWI/SNF (Shi et al., 2007).
1.3 INO1 transcriptional model

In order to better characterize yeast transcriptional regulation, I have chosen INO1 as our model gene. INO1 is responsible for the rate-limiting step of de novo phospholipid synthesis (Ford et al., 2007, 2008; Esposito et al., 2009; Konarzewska et al., 2012). Phospholipid biosynthesis is a crucial process in cells that is conserved throughout higher eukaryotes (Carman & Han, 2009). Phospholipids are the predominant molecule in cellular membranes and thus are key contributors to the structural integrity of cells. Phospholipids also serve a variety of functions within cells. These amphipathic molecules can be molecular chaperones, precursors to macromolecules and secondary messengers, and are key members of complex signaling pathways, such as the phosphatidylinositol bisphosphate pathway (Iwanyshyn et al., 2004).

Phosphatidylinositol (PI) is one of the main phospholipids in yeast. PI can either be synthesized from inositol found in the surrounding environment or it can be synthesized de novo through INO1 and PIS1 (Greenberg & Lopes, 1996). In Saccharomyces cerevisiae, INO1 encodes for inositol-3-phosphate-synthase (I-3-P synthase), which converts glucose-6-phosphate (G-6-P) to inositol-3-phosphate (I-3-P). I-3-P is then dephosphorylated to form inositol by inositol monophosphatases, encoded by INM1 and INM2. This inositol then leads to the synthesis of PI through the actions of PIS1, which codes for PI synthase (Figure 1.5) (Shaldubina et al., 2002; Gardocki et al., 2005).

In order to better understand the transcriptional activation process involved in the expression of INO1, it is of interest to explore the various coactivators that are recruited to the INO1 promoter during activation. The precise mechanism and functional roles of these coactivators still remains elusive, but recent studies have given great insight into potential roles for them beyond just acetylating histones and remodeling chromatin to make DNA more
accessible to transcriptional machinery. Previous studies have demonstrated that the expression of *INO1* is an Ino2p activator-dependent event (Schwank *et al*., 1995; Shetty & Lopes, 2010). Ino2p binds as an Ino2p/Ino4p heterodimer to the *INO1* upstream activating sequence (UAS) (Figure 1.6), and subsequently recruits coactivators, such as chromatin remodelers, Snf2p and Ino80p, as well as, histone acetylases, Esa1p and Gcn5p (Ford *et al*., 2007, 2008; Esposito *et al*., 2009; Konarzewska *et al*., 2012). The activator Ino2p is necessary for the recruitment of coactivators, but then some coactivators depend on other coactivators for recruitment as well. For instance, chromatin remodeler Ino80p is required at the *INO1* promoter; otherwise the remodeler Snf2p will not be recruited. The precise interaction between these two remodelers remains elusive (Ford *et al*., 2008).

In addition to being heavily dependent upon the activator and coactivators, *INO1* expression is also prominently influenced by environmental conditions, principally the presence or absence of the carbohydrate inositol (Kelley *et al*., 1988), as well as the nutrient, choline (Hirsch & Henry, 1986). In the absence of inositol, phosphatidic acid (PA) levels increase. The PA and anchor protein Scs2p sequester Opi1p, a known repressor of *INO1*, in the endoplasmic reticulum (ER) (Figure 1.6). This prevents Opi1p from having access to the nucleus and, more specifically, the *INO1* promoter. If, however, inositol is added to the environment, a rapid reduction of PA occurs since inositol allows for the activation of PA phosphatase and for the production of PI from the PI precursor, PA (Carman & Henry, 2003; Loewen *et al*., 2004). As PA is rapidly consumed in this process, Opi1p is released from ER confinement, which allows the Opi1p to freely diffuse into the nucleus (Figure 1.6). Once Opi1p enters the nucleus, it binds to the Ino2p/Ino4p activator complex and blocks the recruitment of coactivators or initiation machinery to the *INO1* promoter (Young *et al*., 2010). Another repressive protein recruited in
the presence of inositol, is repressor, Ume6p, which binds to the *URS1* element of the *INO1* promoter region and subsequently recruits corepressor, Sin3p, and HDAC, Rpd3 (Shetty and Lopes, 2010). In other words, *INO1* is induced in the absence of inositol and repressed in the presence of inositol (Figure 1.6). The presence of choline in addition to inositol further enhances this repression of *INO1* (Jesch *et al*., 2005). Based on ChIP and real-time PCR experiments in which coactivator presence at the upstream regulatory region was normalized to a control gene, *Poll*, certain patterns of coactivator recruitment have been characterized. Chromatin remodelers appear to be highly recruited during *INO1* repression, and then dissociate from the promoter once *INO1* induction begins (Ford *et al*., 2008). Histone acetylases, on the other hand, seem to have the opposite pattern of expression. They are present in low numbers during repression, but greatly increase upon induction of *INO1* (Konarzewska *et al*., 2012). This interesting inverse recruitment pattern led to further exploration of potential interactions between the remodelers and acetylases. In order to better understand potential dependencies among these coactivators, knockout strains of yeast have proven valuable. When the chromatin remodelers are knocked out, histone acetylase Esa1p recruitment is unaffected, but histone acetylase Gcn5p recruitment decreases nearly fifty percent (Konarzewska *et al*., 2012). The results are even more dramatic if histone acetylases are knocked out instead. When the acetylases are knocked out, the recruitment or presence of the remodelers at the *INO1* promoter no longer declines upon *INO1* induction. Instead of dissociating, it appears that both remodelers accumulate at the promoter in the absence of either of the acetylases, Gcn5p or Esa1p (Konarzewska *et al*., 2012).
Figure 1.5: De novo synthesis pathway for inositol includes *INO1* as a rate-limiting step: Glucose-6-Phosphate (G-6-P) is converted into Inositol-3-Phosphate (I-3-P) by I-3-P synthase, which is coded for by the *INO1* gene. I-3-P is then dephosphorylated into inositol, which is converted to Phosphatidylinositol (PI) by PI synthase.
Figure 1.6: The original model of INO1 transcriptional induction; Under repressing conditions, PA levels are depleted and thus Opi1p is freed from the ER and translocates to the nucleus where it binds to the Ino2p/Ino4p heterodimer, subsequently blocking the interaction between Ino2p and RNA polymerase. HDACs are bound to the URS1 further maintaining a repressed state. Under inducing conditions, a lack of inositol elevates PA levels, which allows for a complex with ER membrane protein, Scs2p, which binds and sequesters repressor Opi1p in the ER. Ino2p/Ino4p is now accessible to transcriptional machinery.
1.4 Our hypothesized model of *INO1* regulation

Although HATs are crucial dynamic regulators of gene expression (Kouzarides, 2000; Katan-Khaykovich, 2002), little is known with regard to their ability to target non-histone proteins (Glozak, 2005). Recent research has shown that some histone acetylases, such as Gcn5p, are capable of acetylating chromatin remodelers, such as Snf2p, which can lead to the dissociation of the SWI/SNF complex off of chromatin (Kim *et al.*, 2010). The INO80 complex, however, has not been examined yet with regard to acetylation. Based upon the coactivator recruitment patterns observed, along with the effects of knocking out particular coactivators, and the recent discoveries of Gcn5p acetylation capabilities, I hypothesize that histone acetylases recruited to the *INO1* promoter by Ino2p may play a role in the acetylation of chromatin remodelers, perhaps to promote the removal of chromatin remodelers from the *INO1* promoter once induction has begun (Figure 1.7). In other words, a recycling role may be occurring here to maximize the availability of chromatin remodelers for various gene activations. Such a recycling effect would be quite valuable to cells since certain remodelers, such as Snf2p, are present in rather low numbers (100-500 copies) in yeast cells, despite being responsible for transcriptional regulation of roughly 5% of all yeast genes (Kim *et al.*, 2010). This dissociation may also have a wide range of implications with respect to transcriptional initiation, elongation, or even repression, which will be discussed in a later section.

The ability of histone acetylases to modify non-histone proteins is an emerging area of research in the field of chromatin. p53 was the first reported non-histone target of HATs (Gu & Roeder, 1997), but recent discoveries show that multiple transcription factors make up the largest class of newly identified non-histone targets of HATs. Acetylation of these proteins can have varying effects, which can be drastically different depending on precisely which lysine residue
undergoes acetylation. For instance, with HMG proteins (high mobility group proteins), acetylation of lysine residue 71 (K71) is associated with positive regulation of transcription, whereas acetylation of K65 is tied to negative regulation. In addition to the acetylation of non-histone proteins by HATs, little is known with respect to the deacetylation of such proteins by HDACs (Glozak et al., 2005). Ultimately, there is great possibility that such previously unidentified modifications may be occurring within the INO1 model.

Thus, I hypothesize that in repressing conditions (100μM inositol), Opi1p remains bound to the Ino2p/Ino4p activator complex, blocking transcription, while repressor proteins, Rpd3p, Sin3p, and Ume6p, are bound to the URS further repressing transcription. In this model, the chromatin remodelers, Ino80p and Snf2p, are unacetylated, and are highly present at the promoter region. Upon induction of INO1 (in the absence of inositol), repressor proteins, Rpd3p, Sin3p, and Ume6p vacate the region, and Hac1p inactivates Opi1p, to promote the repressor’s dissociation from the Ino2p/Ino4p complex (Cox et al., 1997; Nikawa & Kimura, 2012). The lack of inositol, then triggers an increase in PA levels (Carman & Henry, 2003). The PA and anchor protein, Scs2p, sequester Opi1p in the endoplasmic reticulum (ER), which prevents Opi1p from inhibiting the activator complex, Ino2p/Ino4p. The HATs, Gcn5p and Esa1p, are then recruited to the promoter region, where they acetylate Ino80p and Snf2p in the DNA-binding domain regions of the chromatin remodelers. I hypothesize this acetylation triggers the dissociation of these remodelers away from the INO1 promoter (Figure 1.7), which makes the region more accessible for the transcriptional proteins, and allows for the remodelers to mobilize elsewhere as they are required to activate many other genes in yeast.
Figure 1.7: Our hypothesized model of INO1 transcriptional activation in which HATs are suggested to acetylate the chromatin remodelers to promote remodeler dissociation from the INO1 promoter
1.5 Significance of chromatin remodeler acetylation in INO1 gene regulation

Some regulatory proteins, such as the Ino2p/Ino4p activator dimer, are constitutively bound to promoters (Brickner & Walter, 2004). Other regulators, such as remodelers and HATs, do not remain constitutively bound. Just as their recruitment is of significance, the dissociation of these regulators must also have significance. ATPase chromatin remodelers, Ino80p and Snf2p, are recruited to gene promoters in order to break and reestablish the interactions between histones and DNA. When the remodelers break these interactions, they make the DNA sites more accessible for transcription factors and various other regulatory proteins (Tyler & Kadonaga, 1999). I am proposing that the acetylation of these remodelers may lead to their dissociation from the promoter region, which in turn would prevent them from blocking the DNA sites that they had just loosened away from the histones. If this acetylation did not occur, the remodelers would accumulate at the promoter region and may prevent transcriptional initiation by physically blocking the region where Transcription Factor IIA (TFIIA) binds upstream of the TATA. This would hinder transcription since TFIIA is necessary for the binding of RNA polymerase II (Li et al., 2007; Clark & Pazdernik, 2012). Decreased levels of TFIIA impair INO1 transcriptional activation (Graves & Henry, 2000). Other transcription factors, which form the upper layers of the transcriptional machinery, would also have trouble accessing their normal complex locations if the large remodeler complexes remained at the promoter. Two such factors may include TFIID and TFIIH. TFIID, consisting of TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs), is associated with the recognition of a polymerase II specific promoter. TFIIH is responsible for the phosphorylation of polymerase II (Li et al., 2007; Clark & Pazdernik, 2012). Mutants for TFIIH are unable to properly express INO1 (Feaver et al., 1999).
Another possibility is that the acetylation of the remodelers and their subsequent dissociation from the promoter may play a role in transcriptional elongation rather than initiation, since TFIJJ, which is involved in promoter clearance and elongation, and binds TFIIA (Li et al., 2007; Clark & Pazdernik, 2012), may be blocked if bulky remodelers remained constitutively present at the promoter.

Finally, there is the alternative possibility that the acetylation role may not be related to transcriptional initiation or elongation, but may instead play a repressive role on transcription to prevent overexpression of INO1. Just as I suggested that the removal of the bulky remodelers from the promoter region makes the DNA more accessible to positive regulators such as transcription factors, it should also be taken into account that this removal of remodelers may also be making the DNA more accessible to negative regulators, such as HDACs, including Sin3p and Rpd3p. Ultimately, the transcriptional activation and regulation of INO1 is quite complex with a plethora of factors that need to be taken into account, and have yet to be fully elucidated.

Even if in the end, the acetylation does not directly affect INO1 transcription, it still may have a significant impact on coactivators’ recruitment or occupancy at the INO1 promoter, which may in turn affect other genes that are regulated by these coactivators. As previously stated, this form of a recycling role may be occurring here to maximize the availability of chromatin remodelers and other coactivators for various gene activations. Such a recycling effect would be quite valuable to cells since certain remodelers, such as Snf2p, are present in rather low numbers (100-500 copies) in yeast cells, despite being responsible for transcriptional regulation of roughly 5% of all yeast genes (Kim et al., 2010). Among these 5% of genes, SWI/SNF is involved in regulating genes that control DNA damage repair and osmoregulation (Holstege et
al., 1998; Young et al., 1998), as well as genes involved in the utilization of various carbon sources (Schöler et al., 1994), and protection from copper toxicity (Wimalarathna et al., 2013).

This project aims to provide a more in-depth gene model for INO1, which is a crucial gene in yeast phospholipid biosynthesis. In this study I have two specific aims to better characterize that model. The first is to determine the roles of Snf2p acetylation in INO1 activation and its biological implications. The second aim is to demonstrate that Ino80p is also capable of being acetylated and to identify the HAT(s) responsible for this acetylation. Much is still unknown with regard to the roles of the coactivators recruited in this model, including the potential interplay among remodelers and acetylases. Chromatin remodelers and histone acetylases are key coactivators in a vast variety of gene pathways, so by delving into their roles in the INO1 induction pathway, valuable information may be gained that can be explored in the pathways of other genes as well. Also, by better elucidating the roles of various coactivators in the gene regulation process, I hope to better understand the factors that affect gene activation and gene repression. This will not only be valuable with respect to better understanding phospholipid synthesis, but will also shed some light on the key regulatory molecules, histone acetylases and histone deacetylases, which have valuable roles in crucial processes in development. Deregulation of these versatile proteins has been connected to various cancers and disorders, such as breast cancer, leukemia, and fragile X syndrome (Eberharter & Becker, 2002), just as deregulation of gene expression in general has been linked to cancer, obesity, and diabetes (Dubé and Tremblay, 2005).
Chapter 2
Materials and Methods

2.1 Media

**SC (Synthetic complete media):** 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 114027522), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022); 10 μM myo-inositol

*For Plates 1.5% Difco Agar (BD, Cat: 214530) added

**SC + inositol:** 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 114027522), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022), 100 μM myo-inositol (Sigma, Cat: I5125).

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added

**SC – inositol:** 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB without inositol (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 4027 412), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022).

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added

**SC-His:** 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 114027522), 0.079% CSM-His (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4510 322)

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added
**SC-Trp**: 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedical, Cat: 114027522), 0.079% CSM-Trp (complete supplement mixture) (w/v) (MP Biomedical, Cat: 4511 022)

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added*

**SC-Trp-Leu**: 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedical, Cat: 114027522), 0.079% CSM-Trp-Leu (complete supplement mixture) (w/v) (MP Biomedical, Cat: 4520 012)

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added*

**SC-Ura**: 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedical, Cat: 114027522), 0.079% CSM-Ura (complete supplement mixture) (w/v) (MP Biomedical, Cat: 4510 022)

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added*

**LB (Luria-Bertani)**: 10g Bacto-Tryptone (Difco, Cat: 211705), 5g Bacto Yeast Extract (Difco, Cat: 212750), 10g NaCl per 1L volume

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added per 500 ml volume*

**LB/AMP (Luria-Bertani with Ampicillin)**: 5g Bacto-Tryptone (Difco, Cat: 211705), 2.5g Bacto Yeast Extract (Difco, Cat: 212750), 5g NaCl per 500 ml volume, 0.1mg/ml Ampicillin added upon cooling

*For Plates 7.5g Difco Agar (BD, Cat: 214530) added*
2.2 Reagents and Solutions

**10x Buffer**: 1M Tris HCl pH 8; 1M MgCl₂; DEPC water. Filter Sterilized

**Acid Phenol (pH 4.5)**: Shelton Scientific Cat# IB05184

**Chloroform**: 99.8% Acros Cat# 61003-0040

**DEPC treated water**: 0.1% of diethyl pyrocarbonate (Sigma Cat# D5758) was added to distilled water and autoclaved.

**DNase 1**: RNase free DNase (Qiagen Cat# 79254)

**EDTA**: 0.5M (DNase RNase and proteases free (Quality Biological Cat# 351-027-100))

**Formaldehyde**: 37.5% Sigma Cat# 252549

**Glycerol**: 99.5% UltraPure™ Glycerol (Invitrogen, Cat# 15514-029)

**Glycine**: 2.5 M Fisher Scientific Cat# G46-1

**High Salt Buffer**: 0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl, pH8; 500mM NaCl

**LiCl Buffer**: 0.25M LiCl; 1mM EDTA; 10mM Tris-HCl, pH8; 1% Triton X-100; 0.1%

**Deoxycholic Acid**: Sigma Cat# D2510

**Low Salt Buffer**: 0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl, pH8; 150mM NaCl

**Lysis Buffer**: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100

**Phenol (pH 6.6)**: Thermo-Fisher Scientific Cat# 108-95-2

**Phenylmethylsulfonylfluoride**: Sigma Cat# 78830

**Protease Inhibitor Cocktail Set III**: CalBioChem Cat# 539134 (added as 100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64 Protease Inhibitor, 2 mM leupeptin and 1 mM pepstatin A)
**Proteinase K**: 20mg/ml stock (Sigma-Aldrich Cat# P2038-100MG)

**RNA sample buffer**: 2mM EDTA; 10mM Tris HCl (pH 8); 1% β-mercaptoethanol; 1% SDS; 10% glycerol.

**SDS PAGE gel**: (8%) Resolving Gel Layer (1.56ml 40% acrylamide; 2.45ml 1M Tris pH 8.8; 62.5ul 10% SDS; 1.45ml MiliQ water; 1ml 50% sucrose; 1.56µl TEMED; 156µl 10% ammonium persulfate) and Stacking Gel Layer (310µl 40% acrylamide; 1.05ml 0.375M Tris pH 6.6; 1.575ml MiliQ water; 31.25µl 10% SDS; 1.25µl TEMED; 125µl 10% ammonium persulfate)

**Sodium acetate**: (3M) 408.1g sodium acetate final volume 1L with distilled water.

**Sorbitol**: (1M) 9.10g D-sorbitol (Acros organics Cat# 132730010) final volume 50 ml distilled water. Filter Sterilized.

**SYBER® GreenER™ Two-Step qRT-PCR Kit Universal**: (Invitrogen Cat#: 11765-100)

- SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (RT enzyme mix: RNaseOUT™ recombinant ribonuclease inhibitor and SuperScript™ III reverse transcriptase, 2X RT reaction mix: 2.5µM oligo (dT)20, 2.5ng/µl random hexamers, 10mM MgCl2 and dNTPs, and E.coli RNase H). Stored at -20°C.
- SYBR® GreenER™ qPCR SuperMix Universal kit (2X SYBR® GreenER™ qPCR SuperMix Universal: Taq DNA polymerase, SYBR® GreenER™ fluorescent dye, MgCl2 dNTPs with dUTP instead of dTTP, UDG and ROX reference dye). Stored at 4°C.

**Taq Man Gene Expression Master Mix**: (Applied Biosystems Cat# 4369016) 2X concentration. AmpliTaq Gold® DNA Polymerase; UP (UltraPure); Uracil-DNA Glycosylase (UDG); dTNPs with dUTP.
**TE Buffer**: 1X: 10mM Tris-HCl; 1mM EDTA

**TES (Tris-EDTA-SDS) buffer**: 10mM Tris-HCl pH 7.5; 10mM EDTA; 0.5% SDS. Autoclave sterilized

**Trichostatin A**: (10mM) 1mg trichostatin A in 330ul 100% ethanol (Wako Cat# 203-17561)

**Tris-HCl**: (1M) 78.82g Tris HCl; distilled autoclaved water to 500ml final volume; pH adjusted using NaOH

**Triton X-100**: Sigma Cat# T8532-500ML
### 2.3 Yeast Strains

**Table 2.1 Genotypes of yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT BY4741 (Acetylateable Snf2p Wild Type)</td>
<td>MATa his3Δ200 leu2Δ0 met1Δ50 ura3Δ0 SNF2-FLAG::LEU</td>
</tr>
<tr>
<td><strong>unAcSnf2p (Unacetylateable Snf2p K1493R, K1497R)</strong></td>
<td>MATa his3Δ200 leu2Δ0 met1Δ50 ura3Δ0 snf2-K1493R-K1497R-FLAG::LEU</td>
</tr>
<tr>
<td>snf2Δ</td>
<td>MATa his3Δ200 leu2Δ0 met1Δ50 ura3Δ0 snf2Δ</td>
</tr>
<tr>
<td>WT BY4733</td>
<td>MATa his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG</strong></td>
<td>MATa INO80-FLAG his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
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<td>MATa ino80Δ::TRP1 his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>ΔN</strong></td>
<td>MATa INO80-FLAG-ΔN his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>K737A</strong></td>
<td>MATa INO80-FLAG-K737A his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>sas2Δ</strong></td>
<td>MATa sas2Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>sas3Δ</strong></td>
<td>MATa sas3Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>hat1Δ</strong></td>
<td>MATa hat1Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
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<tr>
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<td><strong>esa1mt</strong></td>
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</tr>
<tr>
<td><strong>INO80-FLAG/WT (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/ino80Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG ino80Δ::TRP1 his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/sas2Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 sas2Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/sas3Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 sas3Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/hat1Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 hat1Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/hat2Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 hat2Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/gcn5Δ (created in this study)</strong></td>
<td>MATa INO80-FLAG-URA3 gcn5Δ his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>INO80-FLAG/esa1mt (created in this study)</strong></td>
<td>MATa INO80-FLAG-HIS3 esa1mt his3Δ200 leu2Δ0 met1Δ50 trp1Δ63 ura3Δ0</td>
</tr>
</tbody>
</table>
2.4 Chromatin Immunoprecipitation (ChIP)

2.4.1 Crosslinking Cells:

Cells were grown at 30°C from a single colony in 6ml of appropriate media, depending upon the yeast strain. Cells were then transferred to 394ml of the same media. Once cells reached an optical density of approximately $A_{600nm}$ 0.8 (mid-logarithmic phase), they were divided in half and pelleted. One pellet was washed twice with inducing media (0μM inositol) and the second pellet was washed twice with repressing media (100μM inositol). Each pellet was then resuspended in 200mL of the appropriate media, either inducing or repressing, and incubated for 2 hours at 30°C, 300 rpm. Each flask of cells was then crosslinked with 5.4ml of Formaldehyde for 35 minutes at 125 rpm. Each flask of cells was then subsequently quenched with 32.7ml of 2.5 M Glycine for 5 minutes at 90 rpm. Cells were then pelleted and washed twice with Cell Wash Buffer.

2.4.2 Cell Lysate Preparation and Chromatin Isolation

Cell pellets were resuspended in 400μl of +PMSF Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1.5 μM trichostatin A (WAKO)(added as 1.5 mM solution in ethanol), 0.2mM phenylmethylsulfonylfluoride (Sigma Cat#78830) and 0.4 μl of Protease Inhibitor Cocktail Set III (CalBioChem Cat#539134)(added as 100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64 Protease Inhibitor, 2 mM leupeptin and 1 mM pepstatin A). This cell suspension was then slowly dripped onto 0.45g of acid washed glass beads (0.5mm diameter Sigma, Cat: G8772-500G) in microcentrifuge tube. Each sample was then vortexed in a 4°C cold room for 20 minutes and spun down at 2K for 10 seconds. The supernatant was then transferred to a new tube and was centrifuged for 15 minutes
at 13K in the 4°C cold room. The supernatant was now discarded and the pellet was resuspended in 1ml ice cold –PMSF Lysis Buffer (same as above but without PMSF). Each sample was then sonicated in 10 second intervals for 1 minute and spun down at 2K for 1 minute at 4°C. The supernatant was collected and stored at -80°C.

2.4.3 Quantification (Input DNA Preparation)

10μl of cell lysate was combined with 390μl elution buffer (1% SDS; 0.1M NaHCO₃) and 16μl 5M NaCl. Samples were incubated in a 65°C water bath for 6 hours, then 8μl 0.5M EDTA, 16μl 1M Tris-HCl, pH6.5, and 2μl Proteinase K were added and samples were moved to 45°C for 2 hours. Phenol/chloroform extraction was then performed and the A₂₆₀/A₂₈₀ ratio was determined by UV spectrophotometer analysis with 2μl of sample and 68μl water. DNA concentration was calculated as A₂₆₀*50μg/ml*35 dilution factor.

2.4.4 Immunoprecipitation

11μg of cell lysate was diluted 10 fold in ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2mM EDTA; 16.7mM Tris-HCl, pH8; 167mM NaCl) with 1μl TSA and 1μl protease inhibitor cocktail. 35μl of Protein A agarose slurry was then added and samples were precleared at 4°C for 1 hour with gentle rotations.

Samples were then pelleted at 2K for 10 seconds. Supernatant was collected into a new tube, the appropriate antibody was added, and samples were left at 4°C for overnight rotation. The next morning, 60μl of Protein A agarose slurry was added to each tube and all samples remained rotating at 4°C for an additional hour. Samples were then pelleted at 2K for 10 seconds and each pellet was then washed with Low Salt Buffer (0.1% SDS; 1% Triton X-100; 2mM
EDTA; 20mM Tris-HCl, pH8; 150mM NaCl), High Salt Buffer (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl, pH8; 500mM NaCl), LiCl Buffer (0.25M LiCl; 1mM EDTA; 10mM Tris-HCl, pH8; 1% Triton X-100; 0.1% Deoxycholic Acid), and two rounds of 1X TE Buffer (10mM Tris-HCl; 1mM EDTA). After washes, protein-DNA complexes were then eluted from the resin with two 15 minute incubations gently rotating at room temperature with 250μl of elution buffer each time. The supernatant from each elution was collected, 16μl of NaCl was added, and samples were reverse cross-linked at 65°C for 6 hours. 8μl 0.5M EDTA, 16μl 1M Tris-HCl, pH6.5, and 2μl Proteinase K were added and samples were moved to 45°C for 2 hours. Phenol/chloroform extraction was then performed and samples were stored for qPCR analysis.

Table 2.2: Antibodies used for ChIP experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-acH3</td>
<td>Millipore</td>
<td>06-599</td>
</tr>
<tr>
<td>α-acH4</td>
<td>Millipore</td>
<td>06-866</td>
</tr>
<tr>
<td>α-arp8</td>
<td>Abcam</td>
<td>12098-100</td>
</tr>
<tr>
<td>α-esa1</td>
<td>Abcam</td>
<td>4466-100</td>
</tr>
<tr>
<td>α-FLAG</td>
<td>Sigma</td>
<td>7425</td>
</tr>
<tr>
<td>α-gcn5</td>
<td>Santa Cruz</td>
<td>y-300</td>
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<tr>
<td>α-H3</td>
<td>Abcam</td>
<td>1791</td>
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<tr>
<td>α-H4</td>
<td>Abcam</td>
<td>10158</td>
</tr>
<tr>
<td>α-pol II</td>
<td>Millipore</td>
<td>05-623</td>
</tr>
</tbody>
</table>

2.4.5 Real-Time qPCR

Real-time qPCR (Applied Biosystems 7500 Real-Time PCR system) using Taqman probes was applied to analyze IP samples, along with mock and input samples. Input was prepared as all genomic DNA sequences from the cell lysate without any selection or immunoprecipitation. Mock samples were prepared as a no-antibody signal background in
which all ChIP steps were performed on cell lysate, except for the addition of the selective antibody.

Each reaction was set up using 12.5μl Taqman Gene Expression Master Mix (Applied Biosystems, Cat #4369016), 2.5μl forward primer, 2.5μl reverse primer, 2.5μl probe, 1μl DNA, 4μl autoclaved water.

All experiments were performed with three repeat colonies and all PCR reactions were completed in at least duplicate. The IP for each sample was calculated as:

\[
[2^{-((IP Ct - mock Ct) - (input Ct - mock Ct))}]*\frac{100}{1100}*(A_{260}*50\mu g/ml*35 \text{ dilution factor})
\]

The IP for the INO1 promoter was then graphed as mean ± standard deviation normalized to input and mock samples. Primer and Probe sequences utilized are listed below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INO1 Forward</td>
<td>GAAATATGCGGAGGCCAAGTAT</td>
</tr>
<tr>
<td>INO1 Reverse</td>
<td>ACGCAGAGGTGCCTTCT</td>
</tr>
<tr>
<td>INO1 Probe</td>
<td>[6FAM]CGCTTCCGGCGGCT[BHQ1a-Q]</td>
</tr>
</tbody>
</table>

### 2.5 Growth Curve Studies

Each yeast strain was grown initially in 10 μM inositol SC media (30°C; 300rpm) and subsequently washed and diluted to an A_{600nm} of 0.2 in repressing or inducing conditions (100μM inositol and 0μM inositol, respectively) or in the absence or presence of copper (0mM, 1.5mM, or 3mM). Cell growth was monitored spectrophotometrically using the A_{600nm} density every 3 hours (except overnight) until stationary phase was reached. Repeat colonies were averaged and standard deviation was calculated.

---

35
Average 24th hour $A_{600}$ values (with an $A_{600}$ of 1.0 correlating to approximately $0.8 \times 10^8$ cells per ml) for WT, unacetylated Snf2p, and snf2Δ cells were used to calculate the average number of generations for that cell line as:

$$\left( \frac{\log \text{ # of cells at the end of 24hrs} - \log \text{ # of cells at the start of 24hrs}}{\log(2)} \right)$$

The number of generations for 24 hours was then used to determine the generation time in minutes/generation as:

$$\left( \frac{60 \text{min/hr} \times 24 \text{hr}}{\text{number of generations in 24hr}} \right)$$

### 2.6 Sensitivity Assays

Cells were grown overnight in SC media at 30°C with slow shaking to saturation and subsequently diluted with autoclaved water to an $A_{600}$ of 2. Five 10-fold serial dilutions of all strains were performed using autoclaved water. Each dilution was plated as a 4μl droplet onto the appropriate plates: SC plates, SC plates containing 50 mM hydroxurea, SC plates containing 0.8M KCl, 2% Glucose, 2% Sucrose, 2% Galactose, 2% Maltose, 3% Ethanol, or SC plates containing 1.5mM copper, and 3mM copper. Plates were incubated for 48 hours at 30°C. All experiments were repeated in duplicate.

### 2.7 Copper Sensitivity Growth Analyses

Growth analyses were performed using the yeast strains (Table 3.1); all were grown initially in 10 μM inositol SC media and subsequently washed and diluted to an $A_{600}$ of 0.2 in repressing or inducing conditions as before, that also contained either 0mM Cu, 1.5mM Cu, or 3mM Cu. Cell growth was monitored spectrophotometrically using the $A_{600}$ density every 3
hours (except overnight) until stationary phase was reached. Repeat colonies were averaged and standard deviation was calculated.

Average 24th hour $A_{600nm}$ values for WT, unAcSnf2p, and $snf2\Delta$ cells were used to calculate the average number of generations for that cell line as:

$$\left( \log \text{ # of cells at the end of 24hrs} - \log \text{ # of cells at the start of 24hrs} \right) / \log(2)$$

The number of generations for 24 hours was then used to determine the generation time in minutes/generation as:

$$\left( 60 \text{min/hr} \times 24 \text{hr} \right) / \text{number of generations in 24hr}$$

2.8 RNA Analyses

2.8.1 Total RNA Preparation

Yeast cells were grown to mid-log phase (0.8 $A_{600nm}$) in 10 μM inositol SC media, washed, subjected to repressing or inducing conditions (100μM inositol and 0μM inositol, respectively) for 2 hours, and pelleted. Pellets were resuspended with 400μl ice cold autoclaved water, then centrifuged (13K, 15 seconds) at 4°C. The supernatant was discarded, the pellet was resuspended in 400μl cold TES (10mM Tris-HCl, pH 7.5; 10mM EDTA; 0.5% SDS), and 400μl of acid phenol (pH 4.5) was added. Samples were vortexed and incubated for 1 hour at 65°C. Samples were placed on ice for 5 minutes, centrifuged (13K, 5 minutes, 4°C), and the top layer was collected into a new tube where an additional 400μl of acid phenol (pH 4.5) was added. Samples were again placed on ice for 5 minutes, centrifuged (13K, 5 minutes, 4°C), and the top layer was collected into a new tube where 400μl of chloroform was added. Samples were again placed on ice for 5 minutes, centrifuged (13K, 5 minutes, 4°C), and 360μl of the top layer was collected into a new tube. 40μl of 3M NaOAc and 1ml of ice cold 100% ethanol were added.
then samples were placed in dry ice for 20 minutes, and centrifuged (13K, 5 minutes, 4°C). The
supernatant was removed and the pellet was washed with 1ml of 70% ethanol. Samples were
centrifuged (13K, 5 minutes, 4°C), the supernatant was removed, and the pellet was air dried for
10 minutes. The pellet was then resuspended in 200μl of RNA sample buffer (2mM EDTA;
10mM Tris-HCl, pH 8; 1% β-mercaptoethanol; 1% SDS; 10% glycerol), and stored at -80°C for
further analysis.

2.8.2 RNA Quantification

260nm and 280nm absorbance was read with a UV spectrophotometer (2μl sample + 98μl
water). Concentration of RNA in μg/ml was calculated as A_{260}*40μg/ml*50. To determine the
quantity necessary for 10μg of RNA for DNase treatment, 10 was divided by the RNA
concentration.

2.8.3 DNase Treatment

10μg of RNA was combined with 5μl 10x buffer (0.1M Tris-HCl, pH8; 25mM MgCl₂;
5mM CaCl₂), 3.5μl DNase, and DEPC water to total volume of 50μl. Samples were incubated
for 1 hour at 37°C, then 150μl of DEPC water was added. 200μl of phenol:chloroform in a 3:1
ratio was added, samples were vortexed and placed on ice for 5 minutes. Samples were then
centrifuged (13K, 10 minutes) and the supernatant was transferred to a new tube with 600μl
100% ethanol and 20μl of 3M NaOAc (pH 5.2). Samples were gently mixed and kept on ice for
20 minutes, then centrifuged (13K, 14 minutes), and the supernatant was discarded. Pellets were
washed with 600μl of 70% ethanol, centrifuged (13K, 3 minutes), and dried by speed-vacuum.
Pellets were resuspended in 20μl DEPC treated TE buffer. RNA concentration was once again determined by \( A_{260} \) and \( A_{280} \) absorbance readings.

2.8.4 First Strand Synthesis

All reactions were performed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR and were carried out in a PCR thermocycler. Each reaction contained 10μl 2x RT Reaction Mix, 2μl RT enzyme mix, 1μg of mRNA, and DEPC water to a final volume of 20μl. Samples were gently mixed, incubated at 25˚C for 10 minutes, and then incubated at 50˚C for 30 minutes. Reactions were terminated at 85˚C for 5 minutes and then chilled on ice. 1μl of E.coli RNase H was added and samples were incubated at 37˚C for 20 minutes. Samples were then stored at -20˚C.

2.8.5 Reverse-Transcriptase qPCR

The cDNA synthesized from the purified RNA by reverse transcriptase PCR, was then amplified and quantified via qPCR using the SYBR GreenER qPCR SuperMix Universal kit with forward and reverse primers targeting the promoter sequence of \( INO1 \) and reference gene \( ACT1 \). Each reaction was set up with 7.8μl SYBR® GreenERTM qPCR SuperMix Universal, 0.4μl forward primer, 0.4μl reverse primer, 0.4μl ROX reference dye, 2μl cDNA template, and autoclaved water to a final volume of 25μl. Each reaction was gently stirred when pipetted, and the 96 well PCR plate was sealed. The reactions were placed in the Applied Biosystems 7500 real time PCR system and the following thermocycler program was utilized: 50˚C for 2 minutes hold, 95˚C for 10 minutes hold, and 45 cycles of: 95˚C for 15 seconds, 60˚C for 60 seconds.
All experiments were performed with three repeat colonies and all PCR reactions were performed in duplicate. RNA expression levels were normalized to the constitutive ACT1 housekeeping gene using the formula $2^{-\Delta Ct}$ in which $\Delta Ct$ represents the difference between the Ct value of INO1 and the Ct value of ACT1. Final data was graphed as mean ± standard deviation.

Table 2.4 Primer sequences for Reverse Transcriptase PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INO1 Forward</td>
<td>CCATGGTTAGCCCAAAACGA</td>
</tr>
<tr>
<td>INO1 Reverse</td>
<td>GCCTTCAAGCGTGTGGCA</td>
</tr>
<tr>
<td>ACT1 Forward</td>
<td>CCAAGGCGTTTGTACCTTGT</td>
</tr>
<tr>
<td>ACT1 Reverse</td>
<td>ACCGGCCAAATCGATTTC</td>
</tr>
</tbody>
</table>

2.9 Protein Analyses

2.9.1 Cell Lysate Preparation

Yeast cells were grown at 30°C in SC media (synthetic complete media) containing 2% glucose (wt/vol) to mid-log. Cells were then pelleted and resuspended in 400 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1.5 μM trichostatin A (WAKO)(added as 1.5 mM solution in ethanol), 0.2mM PMSF (Sigma Cat#78830) and 0.4 μl of Protease Inhibitor Cocktail Set III (CalBioChem Cat#539134)(added as 100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64 Protease Inhibitor, 2 mM leupeptin and 1 mM pepstatin A). Equal volume glass beads (Sigma Cat#G8772, 0.5mm diameter, acid washed,) were added and the cells were vortexed for 1 hour at 4°C. The supernatant was transferred to a new tube and briefly centrifuged at 4K in 4°C. The supernatant containing proteins was collected and stored for further analysis.
2.9.2 Immunoprecipitation Pull-Down Procedure

200 μl lysate was then precleared for 1 hour with 20 μl of Protein A Agarose Slurry (Millipore Cat#16-157) and was transferred to 50 μl of equilibrated M2 Anti-Flag Affinity Agarose (EzView Red Anti-FLAG M2 Affinity Gel, Sigma Cat#F2426). This mixture was then gently rotated for 1 hour and was subsequently washed three times with TBS (50mM Tris HCl, 150mM NaCl, pH 7.4). Samples were eluted with 50 μl 2X Laemmli Sample Buffer (Sigma Cat#S3401), boiled for 5 min, and vortexed. These samples were then briefly centrifuged (5sec) and the supernatants were transferred to fresh tubes.

2.9.3 SDS PAGE and Western Blot

20μl of boiled cell lysate was then electrophoresed on 8% SDS-PAGE with standards at 60V and was subsequently silver stained (Pierce SilverSNAP Stain Kit II Cat#24612). For Western blot, the same SDS-PAGE procedure was utilized. Subsequently, gels were transferred to nitrocellulose membranes at 22V for 2 hours. Transfer of protein was confirmed with Ponceau staining. Blots were blocked in 5% BSA for 1hr followed by incubation overnight at 4°C with 1:1000 dilution of Anti-FLAG (Sigma Anti-FLAG, antibody produced in rabbit, Cat#F7425-.2MG) or Anti-acK primary antibody (Cell Signalling Technology Acetylated Lysine Rb Antibody, Cat#9441S). Excess primary antibody was washed away with three 10min washes of 1X TTBS, pH 7.6 (0.05% Tween20, 20mM Tris, 150mM NaCl) and a single wash of 1X TBS, pH 7.6 (20mM Tris, 150mM NaCl). Secondary antibody (Abcam Cat#ab6721) was then applied for 1 hour. Blots were washed again as stated above and lastly detection was performed via the BioRad Immunstar HRP substrate kit (BioRad Cat#170-5040).
2.9.4 Silver Staining

Silver staining was performed with the Thermo Scientific Pierce Silver Stain Kit (Cat#24612). The SDS PAGE was run as described above, then the gel was washed in 5 minute intervals twice in sterile water, with gentle rocking. The gel was then fixed with two 15 minute incubations in a 30% ethanol: 10% acetic acid solution, and was subsequently washed twice (5 minute intervals) in 10% ethanol, and then in sterile water again. The gel was then sensitized for 1 minute with Sensitizer Working Solution (50μl Sensitizer with 25mL water), and washed twice (1 minute intervals) with sterile water. The gel was then stained for 45 minutes with Stain Working Solution (0.5mL Enhancer with 25mL Stain), briefly washed twice with sterile water (20 second intervals), and developed for 30 seconds with Developer Working Solution (0.5mL Enhancer with 25mL Developer). Developing was then stopped with 5% acetic acid for 10 minutes.

2.10 Mutagenesis Experiments

2.10.1 Restriction Digest and DNA Gel Extraction

In order to engineer an INO80-FLAG-HIS3 vector (Figure 2.4), restriction digest, gel purification, and ligation were performed as shown in Figure 2.1. For vectors, restriction was set up with 9μl of autoclaved water, 2μl Tango buffer, 8μl plasmid DNA (pRS416-INO80-2FLAG; Figure 2.2), and 2μl NsiI. Samples were incubated at 37°C for 1 hour, and then 1μl PfoI was added. Samples were then incubated at 37°C for an additional 3 hours. 1μl of CIP was added with 30min of incubation, followed by another 1μl of CIP with 30 min of incubation.
For inserts, restriction was set up with 11μl of autoclaved water, 2μl Tango buffer, 8μl plasmid DNA (pRS413; Figure 2.3), and 2μl NsiI. Samples were incubated at 37°C for 1 hour, and then 1μl PfoI was added. Samples were then incubated at 37°C for an additional 3 hours.

Loading dye was added to each sample and they were run on a 0.8% agarose gel at 85V. The desired bands were cut out and weighed in a 1.5ml tube. The Qiagen gel extraction kit (cat#28704) was then utilized. 3 volumes of QG buffer (5.5M guanidine thiocyanate and 20mM Tris-HCl pH 6.6) was added to 1 volume of gel and samples were incubated at 50°C for a minimum of 10 min to dissolve the gel fragment. 1 volume of isopropanol was added and the sample was centrifuged in a spin column 600μl at a time in 1min 13K intervals. The spin column was then washed with 750μl of PE buffer (10mM Tris-HCl pH 7.5 and 80% ethanol) and final DNA was eluted using 22μl of TE (10mM Tris-HCl pH8; 1mM EDTA).
Figure 2.1: Genetic engineering of INO80-FLAG-HIS3 plasmid DNA
pRS413 and pRS416 were digested with NsiI and PfoI. The gel purified HIS3 insert fragment of pRS413 was then ligated to the gel purified INO80-FLAG pRS416 vector fragment, and the recombinant vector was transformed into E.coli and purified via miniprep and phenol chloroform extraction.
2.10.2 Ligation and Transformation

Optimal vector and insert quantities for a minimum 3:1 (insert:vector) molar ratio were calculated as follows for a 50ng vector ligation:

**Vector (pRS416-INO80-2FLAG)**

10339bp (plasmid) - 1193bp (URA3) = 9146bp

50ng/20μl = 0.0025ng/μl

\[
(0.0025\text{ng}/\mu l)/(9146\text{bp} \times 650 \text{ Daltons}) \times 2\text{ends} = 0.84\text{nM}
\]

0.84nM * 3 = 2.52nM of insert necessary

**Insert (pRS413 HIS3 segment)**

\[
\frac{x}{(1238\text{bp} \times 650 \text{ Daltons})} \times 2 = 2.52\text{nM}
\]

\[
x = 0.001\mu g/\mu l \times 20\mu l = 0.02\mu g = 20\text{ng of insert}
\]

The calculated vector and insert quantities were added to 10μl of NEB 2x quick ligation buffer and 1μl of quick T4 ligase (NEB quick ligation kit cat#M2200S) for a 45 minute room temperature incubation. They were then placed on ice for transformation.

Transformation was performed by adding E.coli to 250μl of ice cold Calcium Chloride (50mM; Fisher cat#C79-500). 10μl of the ligation was added (or 1μl of control plasmid DNA), and the samples were left on ice for approximately 50min. Heat shock was performed at 42°C for 45sec, and samples were returned to ice for 2min. 250μl of LB was added and samples were incubated shaking at 37°C for 30min before each was plated on the appropriate selective plates for overnight 37°C incubation.
2.10.3 Agarose Gel Electrophoresis

0.8% gels were made by heating 0.4g of agarose (Fisher cat#BP160-100) with 50ml of 1X TBE (89mM TrisBase, 89mM Boric Acid, and 2mM EDTA). The dissolved solution was then poured into a mold with 1.5μl Ethidium Bromide (Fisher cat#15585011) and solidified at room temperature. Gels were then run at 85V in 1X TBE with NEB 6x loading dye (cat#B7021S) in each sample. Markers that were run alongside samples were composed of NEB 6x loading dye and NEB 1kb DNA ladder (cat#N3232S) or NEB 100bp DNA ladder (cat#N3231S).

2.10.4 Plasmid Miniprep

A single bacterial colony was inoculated into 6ml of LB/AMP (final concentration 100μg/ml) and grown overnight to saturation at 37°C 300rpm. The culture was then pelleted and resuspended in 300μl of ice cold Qiagen P1 (50mM Tris-HCl; 10mM EDTA, and 100μg/ml RNase A) and was left on ice for 5 min. 300μl of room temperature Qiagen P2 (200mM NaOH and 1% SDS) was then added and gently mixed prior to a 5min room temperature incubation. 300μl of ice-cold P3 (3M potassium acetate, pH 5.5) was then added, gently mixed, and incubated for 5 min on ice. The sample was then centrifuged at max speed for 10 min and the supernatant was transferred to a new tube.

2.10.5 Phenol Chloroform Extraction

400μl of phenol was added, and the sample was vortexed and centrifuged for 3 min at 13K. The top layer was transferred to a new tube and 400μl of chloroform was added prior to vortexing and centrifugation for 3 min at 13K. Again the top layer was transferred to a new tube
to which 1ml of 100% Ethanol and 40μl of Sodium Acetate was added. The sample was then placed in dry ice for 20min and centrifuged for 15 min at 13K. All supernatant was removed and replaced with 1ml of 70% Ethanol prior to a 5 min 13K spin. Lastly, the sample was air dried and the pellet of DNA was resuspended with 20μl TE (10mM Tris-HCl pH8; 1mM EDTA).

Plasmid maps of plasmids purified for use in our experiments are represented in Figures 2.2-2.4.

2.10.6 Yeast Electroporation

Yeast cells were inoculated into 6ml of appropriate media and grown overnight at 30˚C, 300rpm. Cells were then diluted to a starting $A_{600nm}$ of 0.4 in 50ml of appropriate media and grown until they reached an $A_{600nm}$ of approximately 0.8-1.0, at which point they were pelleted and washed three times with ice cold 1M sorbitol. Final pellets were then resuspended in 200μl of 1M sorbitol and were divided into 40μl aliquots. 2-4μl of purified plasmid DNA was then added to a 40μl aliquot and kept on ice for 5min. The cell/DNA mixture was then transferred to a cold electroporation cuvette (Bio Rad 0.2cm electrode gap) and electroporated at 1.5kV using the Biorad E. coli Pulser (one pulse) with a time constant between 5.0 and 6.0. 450μl of ice cold 1M sorbitol was then added to the cuvette and cells were plated on selective agar as 100μl and remaining volume quantities. All plates were incubated at 30˚C overnight and were flipped the following day for additional 1-2 days of incubation at 30˚C.

*All statistical analyses were performed using the Multifactorial ANOVA with Tukey Posthoc in Statistica.
Figure 2.2: Plasmid map of pRS416-IN080-2FLAG
Vector engineered by Xuetong Shen by cloning a PCR fragment including a BamHI restriction site followed by the IN080 native promoter (-500) to the terminator HindIII restriction site. A double FLAG sequence was inserted before the stop codon. ΔN mutants from Xuetong Shen were made by introducing mutations into this vector, which was then inserted into the ino80Δ strain. Histone acetyltransferase mutants containing IN080-FLAG were engineered by Michelle Esposito using this vector. The selectable marker used to screen for mutants containing this vector is URA3.
Figure 2.3: Plasmid map of pRS413
Vector containing HIS3 selectable marker within its PfoI to NsiI restricted region. Utilized as a HIS3 source to engineer a new version of the pRS416-INO80-2FLAG vector that would have a unique selectable marker for use in the esa1mt strain.
Figure 2.4: Plasmid map of engineered pRS416-INO80-2FLAG with HIS3 selectable marker. Vector engineered in which pRS416-INO80-FLAG selectable marker URA3 was replaced by the HIS3 selectable marker of pRS413 using the restriction sites PfoI and NsiI. As with the original pRS416-INO80-2FLAG vector, this engineered vector includes a BamHI restriction site followed by the INO80 native promoter (-500) to the terminator HindIII restriction site, with a double FLAG sequence inserted before the stop codon.
Chapter 3
The role of Snf2p acetylation in INO1 activation and its biological implications

I have previously demonstrated that chromatin remodelers, Snf2p and Ino80p, exhibit high occupancy levels during INO1 repression, and then dissociate from the promoter as INO1 induction commences (Ford et al., 2008). Histone acetylases, Gcn5p and Esa1p, on the other hand, exhibit an opposite pattern of recruitment in which they are present in low numbers at the INO1 promoter during repression, but greatly increase upon INO1 induction (Konarzewska et al., 2012). Although Snf2p, along with Ino80p, is a necessary regulator of INO1 expression in budding yeast (Ford et al., 2007; Esposito et al., 2009), the interplay of this critical remodeler with other transcriptional regulators present at the INO1 promoter is not fully elucidated.

It has long been known that histone acetylases regulate gene expression, but only recently have new functional implications about remodelers’ acetylation emerged. Recent data show that some histone acetylases, such as Gcn5p, are capable of acetylating chromatin remodelers, such as Snf2p, which can lead to the dissociation of the SWI/SNF complex from chromatin (Kim et al., 2010). I also observed when the acetylases are absent in knockout strains, the recruitment or presence of the remodelers at the INO1 promoter no longer demonstrates a decreased level upon INO1 induction. Instead of dissociating, both remodelers may accumulate at the promoter in the absence of acetylases (Konarzewska et al., 2012). This leads us to the hypothesis that the histone acetylases recruited to the INO1 promoter may acetylate the Snf2p remodeler and promote its dissociation from the INO1 promoter (Figure 3.1), which may then have significant implications on the activation of INO1, as well as other genes regulated by Snf2p. The full implications of this acetylation and subsequent dissociation have yet to be explored with regard to transcriptional regulation and other possible implications.
To test this hypothesis and examine the implications of Snf2p acetylation on \textit{INO1} activation, ChIP analysis, coupled with qPCR, followed by growth analysis, mRNA analyses, and further ChIP analyses targeting coactivators and transcriptional proteins, were performed on WT cells in which Snf2p can become acetylated, unAcSnf2p cells in which Snf2p cannot become acetylated (target lysine residues were converted to arginine residues), and \textit{snf2\Delta} control cells, under \textit{INO1} inducing and repressing conditions. Further studies were then performed to examine whether or not the Snf2p acetylation and occupancy changes influenced other gene activities, as many coactivators are involved in regulating large numbers of genes within cells and may need highly regulated recycling patterns. Since SWI/SNF is involved in regulating genes that control the cell’s ability to overcome DNA damage, osmotic stress, sugar or carbon-source metabolism, and copper toxicity (Wimalarathna \textit{et al.}, 2013; Holstege \textit{et al.}, 1998; Young \textit{et al.}, 1998; Schöler \textit{et al.}, 1994), I performed a series of sensitivity assays and growth analyses on various media sources using serial dilutions of cells on each plate to determine which pathways would be affected by the absence of Snf2p acetylation.
Figure 3.1: Our hypothesized model of \textit{INO1} transcriptional activation in which HATs are suggested to acetylate the chromatin remodelers to promote remodeler dissociation from the \textit{INO1} promoter
3.1 Materials and Methods

3.1.1 Chromatin Immunoprecipitation (ChIP)

ChIP was performed on each of the strains (Table 3.1) grown to mid-logarithmic phase (0.8 A$_{600nm}$) in 10 μM inositol synthetic complete (SC) media, washed and subjected to conditions to repress (100 μM inositol (+ino)) or induce (0 μM inositol (-ino)) INO1 transcription for 2 hours prior to collection. Cells were cross-linked with 3.7% formaldehyde and crosslinking was subsequently quenched with 2.5 M glycine prior to pelleting and treatment with cell wash buffer (0.2 M NaCl, 20 mM TrisHCl pH8).

Cells were lysed via chemical and mechanical disruption, in which cell pellets were resuspended in 400μl of +PMSF Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1.5 μM trichostatin A (WAKO)(added as 1.5 mM solution in ethanol), 0.2mM PMSF (Sigma Cat#78830) and 0.4 µl of Protease Inhibitor Cocktail Set III (CalBioChem Cat#539134)(added as 100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64 Protease Inhibitor, 2 mM leupeptin and 1 mM pepstatin A). This cell suspension was then slowly dripped onto 0.45g of acid washed glass beads (0.5mm diameter Sigma, Cat: G8772-500G) in a microcentrifuge tube. Each sample was then vortexed in a 4°C cold room for 20 minutes and spun down at 2K for 10 seconds. The supernatant was then transferred to a new tube and was centrifuged for 15 minutes at 13K in the 4°C cold room. The supernatant was now discarded and the pellet was resuspended in 1ml ice cold –PMSF Lysis Buffer (same as above but without PMSF). Each sample was then sonicated (Misonix Incorporated Sonicator 3000) in 10 second intervals for 1 minute and spun down at 2K for 1 minute at 4°C. The supernatant was collected and stored at -80°C.
Quantification was then performed in which 10μl of cell lysate was combined with 390μl elution buffer (1% SDS; 0.1M NaHCO₃) and 16μl 5M NaCl. Samples were incubated in a 65°C water bath for 6 hours, then 8μl 0.5M EDTA, 16μl 1M Tris-HCl, pH6.5, and 2μl Proteinase K were added and samples were incubated at 45°C for 2 hours. Phenol/chloroform extraction was then performed and the A₂₆₀/A₂₈₀ ratio was determined by UV spectrophotometer analysis with 2μl of sample and 68μl water. DNA concentration was calculated as A₂₆₀*50μg/ml*35 dilution factor. Samples were then saved as input DNA.

Immunoprecipitation (IP) was then performed as follows. 11μg of cell lysate was diluted 10 fold in ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2mM EDTA; 16.7mM Tris-HCl, pH8; 167mM NaCl) with 1μl TSA and 1μl protease inhibitor cocktail. 35μl of Protein A agarose slurry was then added and samples were precleared at 4°C for 1 hour with gentle rotations. Each pre-cleared sample was gently rotated overnight at 4°C with the appropriate antibody (α-FLAG (Sigma Cat: 7425), α-Arp8 (Abcam Cat: 12098-100), α-Gcn5 (Santa Cruz Cat: y-300), α-Esa1 (Abcam Cat: 4466-100), α-Pol II (Millipore Cat: 05-623), α-H3 (Abcam Cat: 1791), α-acH3 (Millipore Cat: 06-599), α-H4 (Abcam Cat:10158), α-acH4 (Millipore Cat: 06-866). 60μl of protein A-agarose slurry was added; each sample was rotated for 1 hour at 4°C then pelleted with a 30 second 1K spin. The pellet was washed with 1ml of low salt buffer rotating for 3 minutes and was spun down at 1K for 1 minute. The wash was then repeated with high salt buffer and then LiCl buffer. The rotation was extended to 5 minutes for a 1x TBE wash. Lastly, 1ml of 1x TBE was added, samples were shaken 3 times, spun down, and all supernatant was removed. Bound Snf2-FLAG protein-DNA complexes were eluted from resin at room temperature employing an SDS buffer. Reverse cross-linking at 65°C was then
performed on eluted complexes and all samples were further purified with phenol-chloroform extraction prior to analysis with qPCR.

**Table 3.1: Yeast strain genotypes utilized in ChIP experiments**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Acetylateable Snf2p Wild Type; BY4741)</td>
<td>MATa his3Δ200 leu2Δ0 met15Δ0 ura3Δ0 SNF2-FLAG::LEU</td>
</tr>
<tr>
<td>unAcSnf2p (Unacetylateable Snf2p K1493R, K1497R)</td>
<td>MATa his3Δ200 leu2Δ0 met15Δ0 ura3Δ0 snf2-K1493R-K1497R-FLAG::LEU</td>
</tr>
<tr>
<td>snf2Δ</td>
<td>MATa his3Δ200 leu2Δ0 met15Δ0 ura3Δ0 snf2Δ</td>
</tr>
</tbody>
</table>

*Parental strain BY4741 for each mutant in Table 3.1

### 3.1.2 Real-Time qPCR

Real-time qPCR using Taqman probes (Applied Biosystems) was employed to analyze IP samples, along with mock and input samples. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate.

The IP for each sample was calculated as:

\[ 2^{-(Ct_{IP} - Ct_{mock}) - (Ct_{input} - Ct_{mock})}} \times (100/1100) \times (A_{260} \times 50\text{µg/ml} \times 35 \text{ dilution factor}) \]

The IP for the INO1 promoter was then graphed as mean standard ± deviation normalized to input and mock. Input was prepared as all genomic DNA sequences from the cell lysate without any selection or immunoprecipitation. Mock, on the other hand, was prepared as a no-antibody signal background in which all ChIP steps were performed on cell lysate, except for the addition of the selective antibody. Primer and Probe sequences utilized are listed in Table 3.2 and mapped in Figure 3.2A.

**Table 3.2: Primer and Probe sequences for Real-Time qPCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INO1 Forward</td>
<td>GAAATATGCGGAGGCCAAGTAT</td>
</tr>
<tr>
<td>INO1 Reverse</td>
<td>ACGCAGAGGTGCGCTTTTCT</td>
</tr>
<tr>
<td>INO1 Probe</td>
<td>[6FAM]CGCTTCCGGCGGCT[BHQ1a-Q]</td>
</tr>
</tbody>
</table>
3.1.3 Growth Analysis

Growth analyses were performed using the yeast strains as shown in Table 3.1; all were grown initially in 10 μM inositol SC media until mid-log phase and subsequently washed and diluted to an A$_{600nm}$ of 0.2 in repressing or inducing conditions as before. Cell growth was monitored spectrophotometrically using the A$_{600}$ density every 3 hours (except overnight) until stationary phase was reached. Repeat colonies were averaged and standard deviation was calculated.

Average 24$^{th}$ hour OD values for WT, unAcSnf2p, and snf2Δ cells were used to calculate the average number of generations for that cell line as:

$$\text{[(log # of cells at the end of 24hrs - log # of cells at the start of 24hrs)/log(2)]}$$

The number of generations for 24 hours was then used to determine the generation time in minutes/generation as:

$$\text{[(60min/hr x 24hr)/number of generations in 24hr]}$$

3.1.4 mRNA Preparation and Quantification

WT, unAcSnf2p, and snf2Δ cells (Table 3.1) were grown to mid-log phase (0.8 A$_{600nm}$) in 10 μM inositol SC media, washed and subjected to repressing or inducing conditions as before for 2 hours prior to analyses. RNA was isolated and purified via acid phenol-chloroform extraction, in which the cell pellet was resuspended in TES (10mM Tris-HCl pH 7.5; 10mM EDTA; 0.5% SDS), incubated with equal volume acid phenol for 1 hour at 65°C, placed on ice for 5 minutes, and centrifuged. The top layer was then transferred to a new tube and additional equal volume acid phenol was added. Samples were then placed on ice for 5 minutes, centrifuged, and the top layer was transferred to a new tube where equal volume chloroform was added. Samples were again placed on ice, centrifuged and the top layer was transferred to a new
tube in which 3M NaOAc (final 0.3M concentration) and 100% ethanol were added. Samples were placed in dry ice for 20 min, centrifuged, and the supernatant was removed. The pellet was washed with 70% ethanol, centrifuged, and the supernatant was discarded. The pellet was then air dried, resuspended in 200μl of RNA sample buffer (2mM EDTA; 10mM Tris-HCl, pH 8; 1% β-mercaptoethanol; 1% SDS; 10% glycerol), and the concentration of isolated RNA was determined as A$_{260}$*40μg/ml*50. Samples were then purified by DNase treatment to remove any contaminating DNA.

### 3.1.5 DNase Treatment

10μg of RNA was combined with 5μl 10x buffer (0.1M Tris-HCl, pH8; 25mM MgCl$_2$; 5mM CaCl$_2$), 3.5μl DNase, and DEPC water to total volume of 50μl. Samples were incubated for 1 hour at 37°C, then 150μl of DEPC water was added. 200μl of phenol:chloroform in a 3:1 ratio was added, samples were vortexed and placed on ice for 5 minutes. Samples were then centrifuged (13K, 10 minutes) and the supernatant was transferred to a new tube with 600μl 100% ethanol and 20μl of 3M NaOAc (pH 5.2). Samples were gently mixed and kept on ice for 20 minutes, then centrifuged (13K, 14 minutes), and the supernatant was discarded. Pellets were washed with 600μl of 70% ethanol, centrifuged (13K, 3 minutes), and dried by speed-vacuum. Pellets were resuspended in 20μl DEPC treated TE buffer. RNA concentration was once again determined by A$_{260}$ and A$_{280}$ absorbance readings.

### 3.1.6 First Strand Synthesis

All reactions were performed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Cat: 11752-050) and were carried out in a PCR thermocycler. Each
reaction contained 10μl 2x RT Reaction Mix, 2μl RT enzyme mix, 1μg of mRNA, and DEPC water to a final volume of 20μl. Samples were gently mixed, incubated at 25˚C for 10 minutes, and then incubated at 50˚C for 30 minutes. Reactions were terminated at 85˚C for 5 minutes and then chilled on ice. 1μl of *E.coli* RNase H was added and samples were incubated at 37˚C for 20 minutes. Samples were then stored at -20˚C.

3.1.7 Reverse-Transcriptase qPCR

The cDNA synthesized from the purified RNA by reverse transcriptase PCR, was then amplified and quantified via qPCR using the SYBR GreenER qPCR SuperMix Universal kit (Invitrogen, Cat: 11762-500) with forward and reverse primers targeting the ORF of *INO1* and reference gene *ACT1* (Table 3.3; Figure 3.2B). Each reaction was set up with 7.8μl SYBR® GreenER™ qPCR SuperMix Universal, 0.4μl forward primer, 0.4μl reverse primer, 0.4μl ROX reference dye, 2μl cDNA template, and autoclaved water to a final volume of 25μl. Each reaction was gently stirred when pipetted, and the 96 well PCR plate was sealed. The reactions were placed in the Applied Biosystems 7500 real time PCR system and the following thermocycler program was utilized: 50˚C for 2 minutes hold, 95˚C for 10 minutes hold, and 45 cycles of: 95˚C for 15 seconds, 60˚C for 60 seconds.

All experiments were performed with three repeat colonies and all PCR reactions were performed in duplicate. RNA expression levels were normalized to the constitutive *ACT1* housekeeping gene using the formula 2^-∆Ct in which ∆Ct represents the difference between the Ct value of *INO1* and the Ct value of *ACT1*. Final data was graphed as mean ± standard deviation.
Table 3.3: Primer sequences for Reverse Transcriptase PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INO1 Forward</td>
<td>CCATGGTTAGCCCAAACGA</td>
</tr>
<tr>
<td>INO1 Reverse</td>
<td>GCCTTCAAGCGTTGTGCA</td>
</tr>
<tr>
<td>ACT1 Forward</td>
<td>CCAAGCCGTTTTGTCCTTGT</td>
</tr>
<tr>
<td>ACT1 Reverse</td>
<td>ACCGGCCAAATCGATTTC</td>
</tr>
</tbody>
</table>

3.1.8 Sensitivity Assays

Strains (Table 3.1) were grown overnight in SC media at 300rpm, 30°C to saturation and subsequently diluted with autoclaved water to an A₆₀₀nm of 2.0 (approximately 1.6 x 1₀⁹ cells/ml). Five 10-fold serial dilutions of all strains were performed using autoclaved water. Each dilution was plated as a 2μl droplet onto SC plates as a control to demonstrate cell viability. Dilutions were then plated on SC plates containing 50 mM hydroxyurea, which inhibits DNA damage repair and DNA replication. SC plates containing 0.8M KCl were then utilized as a high osmotic stress source. Cells were also plated on SC plates containing 1.5mM copper (activates CUP1) or 3mM copper (copper toxicity condition) (Wimalarathna et al., 2013). Lastly each serial dilution was plated on fermentable 2% Glucose plates, 2% Sucrose plates, 2% Galactose plates, 2% Maltose plates, and non-fermentable 3% Ethanol plates to demonstrate cellular utilization of carbon sources (Schöler et al., 1994; Neigeborn & Carlson, 1984). Plates were incubated for 48 hours at 30°C. All experiments were repeated in duplicate.

3.1.9 Copper Sensitivity Growth Analyses

Growth analyses were performed using the yeast strains as listed in Table 3.1; all were grown initially in 10 μM inositol SC media and subsequently washed and diluted to an A₆₀₀nm of 0.2 in repressing or inducing conditions as before, that also contained either 0mM Cu, 1.5mM Cu, or 3mM Cu. Cell growth was monitored spectrophotometrically using the A₆₀₀nm density.
every 3 hours (except overnight) until stationary phase was reached. All growth experiments were performed in duplicate; repeats were averaged and standard deviation was calculated.

Average 24th hour $A_{600nm}$ values (with an $A_{600nm}$ of 1.0 correlating to approximately $0.8 \times 10^8$ cells per ml) for WT, unAcSnf2p, and snf2Δ cells were used to calculate the average number of generations for that cell line as:

$$\frac{\log \text{# of cells at the end of 24hrs} - \log \text{# of cells at the start of 24hrs}}{\log 2}$$

The number of generations for 24 hours was then used to determine the generation time in minutes/generation as:

$$\frac{(60\text{min/hr x 24hr})}{\text{number of generations in 24hr}}$$
Figure 3.2: Primers used in qPCR experiments
A) Forward and reverse primers used in ChIP experiments to target the URS of INO1
B) Forward and reverse primers used in mRNA experiments to target the ORF of INO1 and ACT1
3.2 Results

3.2.1 Unacetylated Snf2p accumulates at the INO1 promoter

Since transcriptional activation is a highly regulated process that requires the recruitment of an assortment of activators and coactivators to a gene’s upstream regulatory region, it is important to better characterize the post-translational modifications of these coactivators as they occupy a promoter region, especially when that modification is necessary for the coactivators to vacate the region. Previous studies have demonstrated that the post-translational modification of acetylation can lead to the dissociation of coactivators from gene promoters (Kim et al., 2010); however, this has yet to be studied for its biological implication on gene expression. Before the implications of Snf2p acetylation at the INO1 promoter could be explored, it was first necessary to confirm that the acetylation was indeed necessary for Snf2p dissociation in our model (Figure 3.1). To confirm this, I have conducted chromatin immunoprecipitation (ChIP) to pull down Snf2p bound to DNA in WT cells and in an unAcSnf2p mutant in which the acetylatable lysine residues of the remodeler were replaced with unacetylatable arginine residues (Kim et al., 2010). In both strains, Snf2p contained a C-terminal double-FLAG tag that could be targeted with an antibody against the octapeptide FLAG. A snf2Δ strain was utilized as a negative control. Quantitative real-time PCR (qPCR) was then utilized to examine the presence of Snf2p in these strains under repressing and inducing conditions, to determine if Snf2p accumulated at the INO1 promoter when Snf2p was unable to become acetylated.

For repressing and inducing conditions, the IP signals of Snf2p-FLAG were examined at the upstream regulatory sequence (URS) of INO1 and were subsequently normalized to the INO1 input and mock. In the WT strain, the relative IP values demonstrated a significant difference ($p<0.01$), as the Snf2p-FLAG-IP were $1.26 \pm 0.19$ under repressing conditions and $0.10 \pm 0.01$
under inducing conditions, which demonstrated the dissociation of Snf2p from the promoter region. This result is in agreement with the previous findings (Ford et al., 2008). On the other hand, relative IP values for the Snf2p-FLAG-IP in the unAcSnf2p mutant resulted in an insignificant change ($p=0.996$) in IP under repressing conditions (1.40±0.29) compared to the IP under inducing conditions (1.35±0.47; Figure 3.3). The negative control, snf2Δ, demonstrated minimal IP values with no significant difference between conditions, with 0.01±0.001 and 0.01±0.002 under repressing and inducing conditions, respectively. These data demonstrate the accumulation of unacetylated Snf2p at the INO1 promoter upon INO1 induction in the absence of Snf2p acetylation.
Unacetylatable Snf2p accumulates at the INO1 promoter instead of dissociating. Real-time PCR analysis of DNA immunoprecipitated through Chromatin Immunoprecipitation (ChIP) with an antibody against FLAG-tagged Snf2p in WT cells, unAcSnf2p cells, and snf2Δ cells that were grown to mid-log phase (0.8 A600nm) in 10 μM inositol Synthetic Complete (SC) media and were subsequently subjected to repressing or inducing conditions, 100 μM inositol (+ino) and 0 μM inositol (-ino) synthetic complete media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the INO1 promoter is graphed as mean ± standard deviation normalized to input and mock. Input represents all genomic DNA sequences from the cell lysate without any selection or immunoprecipitation. Mock, on the other hand, represents a no-antibody signal background in which all ChIP steps were performed on cell lysate, except for the addition of the selective antibody, so no DNA should theoretically have been immunoprecipitated in these samples.
3.2.2 The acetylation of Snf2p is not required for cell growth in the absence of inositol

Since I observed that Snf2p acetylation is required for Snf2p dissociation from the INO1 promoter, it was subsequently instructed to determine if this acetylation is required for cell growth in the absence of inositol. Growth analysis was utilized as a preliminary means to examine the implications on INO1 expression. Since INO1 plays a rate-limiting role in the de novo synthesis of inositol in cells (Greenberg & Lopes, 1996), cells that have impeded INO1 expression are expected to demonstrate a growth deficiency in media lacking inositol, whereas cells with fully functioning INO1 gene activity can successfully synthesize inositol that is absent from the environment and can thus still thrive in inositol depleted media.

In media containing 100 μM inositol, which serves as a repressor to the INO1 gene and an external source of inositol for the cells, the WT type strain thrived and reached an average peak optical density $A_{600\text{nm}}$ of approximately 7.9±0.07. In inositol depleted media, the WT cells were still able to survive, but only reached an average peak $A_{600\text{nm}}$ of approximately 6.0±0.07 (Figure 3.4; Table 3.4). The unAcSnf2p strain demonstrated a growth pattern similar to the WT in both the INO1 repressing and inducing conditions ($p$=0.3 and $p$=0.14, respectively), with an average peak $A_{600\text{nm}}$ around 7.5±0.14 in the presence of inositol and around 5.6±0.00 in the absence of inositol. The snf2Δ strain, however, demonstrated a significantly different growth pattern compared to the unAcSnf2p strain ($p$<0.01 for repressing and inducing conditions), as the average peak $A_{600\text{nm}}$ in 100μM inositol was around 5.0±0.07, but in 0μM inositol it was only around 1.3±0.07.

When the number of generations per 24 hour period, as well as generation times, were calculated from the average $A_{600\text{nm}}$ values, the unAcSnf2p strain continued to demonstrate values more comparable to the WT strain as opposed to the snf2Δ strain. The WT number of
generations was 5.25 under repressing conditions and 4.92 under inducing conditions, as the unacetylateable strain number of generations was 5.16 and 4.78 for repressing and inducing conditions respectively (Table 3.4). These values correlated to generation times of 274.29 min/generation and 292.68 min/generation for the WT, and 279.07 and 301.36 min/generation for the unAcSnf2p strain under repressing and inducing conditions, respectively (Table 3.4). The snf2∆ strain, on the other hand, exhibited 4.25 and 2.52 generations for repressing and inducing respectively in a 24 hour period timespan, which correlated to generation times of 338.82 min/generation and 571.43 min/generation (Table 3.4). Taken together, these results demonstrated that the unAcSnf2p strain exhibited growth patterns more characteristic of the WT strain rather than the snf2∆ strain, as only snf2∆ in 0μM inositol demonstrated a 2 fold difference of generation times when compared to other strains, whereas the ratios of WT to unAcSnf2p generation times in each condition yielded 1, representative of no difference. Our results demonstrated that Snf2p acetylation is not required for cell growth in the absence of inositol.
Figure 3.4: UnAcSnf2p mutant exhibits growth pattern similar to Wild Type cells. WT, unAcSnf2p, and snf2Δ cells were grown in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and diluted to an A_{600nm} of 0.2 in repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete media, respectively. The 600nm optical density was then noted every 3 hours (except overnight) until stationary phase was reached. Repeat colonies were averaged prior to being graphed.

Table 3.4: unAcSnf2p strain Growth Experiment 24th hour analyses

<table>
<thead>
<tr>
<th>Cell Strain</th>
<th>Average O.D. at 100μM inositol (24th hour)</th>
<th>Average O.D. at 0μM inositol (24th hour)</th>
<th># of generations in 24 hours at 100μM inositol</th>
<th># of generations in 24 hours at 0μM inositol</th>
<th>Generation Time (min/generation) at 100μM inositol</th>
<th>Generation Time (min/generation) at 0μM inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>7.6±0.14</td>
<td>6.05±0.07</td>
<td>5.25</td>
<td>4.92</td>
<td>274.29</td>
<td>292.68</td>
</tr>
<tr>
<td>unAcSnf2p</td>
<td>7.15±0.35</td>
<td>5.5±0.14</td>
<td>5.16</td>
<td>4.78</td>
<td>279.07</td>
<td>301.26</td>
</tr>
<tr>
<td>snf2Δ</td>
<td>3.8±0.14</td>
<td>1.15±0.07</td>
<td>4.25</td>
<td>2.52</td>
<td>338.82</td>
<td>571.43</td>
</tr>
</tbody>
</table>
3.2.3 The acetylation of Snf2p is not required for INO1 activity

Growth analyses suggested that the acetylation of Snf2p was not required for cell growth in the absence of inositol. To further identify that these results are due to the fact that Snf2p acetylation is not required for INO1 expression, mRNA analysis was employed. The mRNA from the WT, unAcSnf2p, and snf2Δ strains cultured in repressing and inducing conditions were used as a template to synthesize cDNA, which was then in turn analyzed via qPCR using primers targeting the INO1 open reading frame region, as well as the housekeeping gene ACT1 as a normalizing control (Table 3.3). A significant difference in mRNA levels between repressing and inducing conditions (0.005±0.001 and 0.266±0.076, under repressing and inducing conditions, respectively; \( p<0.01 \)) was observed in the WT strain (\( p<0.01 \)), as well as in the unAcSnf2p strain (0.004±0.003 and 0.355±0.101, under repressing and inducing conditions, respectively; \( p<0.01 \)); whereas no significant differences were observed in the snf2Δ strain (0.002±0.001 and 0.048±0.002, under repressing and inducing conditions, respectively; \( p=0.9 \)) (Figure 3.5B). This demonstrated that the INO1 expression patterns in the unacetylateable strain were comparable to those observed in the WT strain rather than the deletion strain, as both strains demonstrated significant upregulation of INO1 upon induction. Ultimately, there was no significant difference in INO1 expression between the WT strain and the unAcSnf2p strain for repressing conditions (\( p=1.0 \)) and inducing conditions (\( p=0.3 \)) during this upregulation. Taken together with the growth analyses, the mRNA analyses confirmed that the acetylation of Snf2p was not required for INO1 activity.
Figure 3.5: UnAcSnf2p mutant exhibits *INO1* expression comparable to WT cells. 

(A) Amplification plot showing variation of log (∆Rn) against cycle number with threshold denoted as the horizontal green line (B) WT, unAcSnf2p, and *snf2Δ* cells were grown to mid-log phase (0.8 A_{600nm}) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete media, respectively, for 2 hours prior to collection. RNA was isolated and purified via Acid Phenol-Chloroform extraction, followed by DNase treatment. Gene expression was then analyzed via a two-step process in which cDNA was first synthesized from the purified RNA in a reverse transcriptase polymerase chain reaction, and then was amplified and quantified via qPCR using SYBR Green and ROX reference dye, with forward and reverse primers targeting the ORF sequence of *INO1*. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. RNA expression levels were normalized to the constitutive *ACT1* housekeeping gene and graphed as mean ± standard deviation.
3.2.4 Chromatin remodeler, Ino80p, accumulates at the \textit{INO1} promoter in the absence of Snf2p acetylation, but \textit{INO1} nucleosome density is unaltered

Although Snf2p acetylation is independent of the \textit{INO1} expression, I was interested to see whether Snf2p acetylation influences the recruitment of transcription co-activators. In this study, I focused on the various known coactivators involved in the \textit{INO1} model of gene regulation. These transcriptional coactivators include the chromatin remodeler Ino80p, and the histone acetyltransferases, Gcn5p and Esa1p. Based on previous data, in the WT, Ino80p should demonstrate a promoter occupancy pattern similar to Snf2p, in that both remodelers are present in high concentrations during \textit{INO1} repression in the presence of inositol, but then this abundance significantly diminishes upon induction in the absence of inositol (Ford \textit{et al.}, 2008). The histone acetyltransferases, however, are known to demonstrate the inverse pattern, as they exhibit lower occupancy during repression, but then significantly increase upon induction. Ultimately, these two acetyltransferases are dispensable in comparison to either of the chromatin remodelers, as \textit{INO1} expression is not influenced by the absence of either one, and the remodeler activities of Ino80p and Snf2p are sufficient to activate \textit{INO1} transcription (Konarzewska \textit{et al.}, 2012). Previous data also demonstrated that Ino80p and Snf2p are both required at the \textit{INO1} promoter for transcription to occur (Ford \textit{et al.}, 2008), and that both remodelers accumulate in the absence of the histone acetyltransferases, Gcn5p and Esa1p (Konarzewska \textit{et al.}, 2012). Taken together this demonstrates that there is likely strong cooperation among the chromatin remodelers and histone acetylases at the promoter region, so it is of interest to determine if the absence of Snf2p acetylation at the \textit{INO1} promoter results in modified transcriptional complexes. These coactivators are required for the expression of other genes within yeast cells, so even if \textit{INO1} transcription does not significantly change in the absence of Snf2p acetylation, it is still
important to further examine the interactions occurring at the *INO1* promoter region. Changes in the activation complex at *INO1* could impact other genes if certain coactivators are accumulating at this *INO1* location and are thus not available in their necessary quantity elsewhere.

To determine if the acetylation of Snf2p impacts Ino80p during gene activation, ChIP coupled with qPCR was performed on WT, unAcSnf2p, and *snf2Δ* strains. IP signals using an antibody against Arp8p of INO80 were scrutinized at the upstream regulatory sequence (*URS*) of *INO1* under repressing and inducing conditions, 100μM and 0μM inositol respectively, and were subsequently normalized to *INO1* input and *INO1* mock DNA. In the WT strain, the relative ChIP values for the Arp8p-IP were 0.78±0.07 under repressing conditions and 0.17±0.03 upon induction of *INO1*. Statistically, I show that induction resulted in a significant dissociation of the remodeler from the promoter upon *INO1* induction (*p*<0.01), which was also observed in the *snf2Δ* strain (0.74±0.13 with repressing conditions and 0.15±0.04 under inducing conditions; *p*<0.01) as Ino80p arrives at the *INO1* prior to and independent of Snf2p (Ford *et al.*, 2008). In the unAcSnf2p mutant strain, however, the relative IP values for the Arp8p-IP in the repressed condition were statistically similar to the induced condition (0.67±0.11 with repressing conditions and 0.61±0.12 under inducing conditions; *p*=0.97) (Figure 3.6), which demonstrates the absence of Ino80p dissociation from the *INO1* promoter.

Since remodelers Snf2p and Ino80p accumulated at the *INO1* promoter, it is interesting to see if remodeler activity is affected in the absence of Snf2p acetylation. One way to monitor the remodeling activity is to examine nucleosome density. Previously, it has been shown that nucleosome density decreased at the *INO1* promoter upon induction (Esposito *et al.*, 2009). If nucleosome density remains similar to wild type nucleosome patterns, it demonstrates that the
remodeler is still capable of loosening the condensed DNA around histones upon the induction of a gene. In order to examine nucleosome density, ChIP coupled with qPCR was performed in which antibodies targeted histones H3 and H4.

In the WT strain, the relative H3-IP values demonstrated a significant difference in nucleosome density upon induction of INO1 in the absence of inositol ($p<0.01$), which is expected as chromatin remodelers are modifying the nucleosomal structure. Under these same conditions, the unAcSnf2p mutant demonstrated a similar nucleosome density pattern as the wild type strain, with a significant difference in H3 nucleosome density upon INO1 induction ($p<0.01$), which suggested that the chromatin remodeler activities at the INO1 promoter were not affected by Snf2p acetylation (Figure 3.7A; Note that the IP range for H3 is on a slightly higher scale than my other antibody IP values. When using polyclonal antibodies, as opposed to monoclonal antibodies, lot-to-lot variability is characteristic since polyclonal antibodies recognize multiple epitopes. Although this can result in a higher or lower range of IP values compared to a different antibody, the trend of all samples tested with that particular antibody holds true. In other words, each IP signal with that antibody may reach a higher peak than other antibodies, but this does not alter the comparison of IP values among different strains or conditions tested with the single antibody. Overall, the trend of samples tested with the antibody against H3 matches the trend observed with the same samples tested against H4, although the exact values are on a slightly higher scale.).

Similar results were also observed with regard to histone H4. In the WT strain, the relative H4-IP values were 1.10±0.24 and 0.35±0.01 under repressing and inducing conditions, respectively. This demonstrated the expected significant difference in H4 density upon INO1 induction ($p=0.01$) characteristic of chromatin remodeling, while the snf2Δ strain demonstrated
the expected lack of remodeling (1.11±0.21 under repressing conditions and 1.37±0.05 under inducing conditions; p=0.47). The unAcSnf2p mutant also demonstrated a significant difference between repressing and inducing conditions (p=0.04), with relative H4-IP values of 0.91±0.01 and 0.34±0.07 (Figure 3.7B). As with H3, the results for H4 in the absence of Snf2p acetylation ultimately demonstrated a loss of nucleosomes upon INO1 induction characteristic of nucleosome repositioning by chromatin remodelers.
Figure 3.6: Ino80p accumulates at the *INO1* promoter in unAcSnf2p cells. Real-time PCR analysis of DNA immunoprecipitated through ChIP with an antibody against Arp8p of Ino80p in WT cells, unAcSnf2p, and *snf2Δ* cells that were grown to mid-log phase (0.8 A₆₀₀nm) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the *INO1* promoter is graphed as mean ± standard deviation normalized to input and mock. Input represents all genomic DNA sequences from the cell lysate without any selection or immunoprecipitation. Mock, on the other hand, represents a no-antibody signal background in which all ChIP steps were performed on cell lysate, except for the addition of the selective antibody, so no DNA should theoretically have been immunoprecipitated in these samples.
H3 occupancy at the \textit{INO1} promoter

\begin{figure}
\centering
\includegraphics[width=\textwidth]{h3_occupancy}
\end{figure}

WT Strain | unAcSnf2p Strain | snf2δ Strain
---|---|---
+ino | -ino | +ino
\text{IP (Normalized to input & mock)}

p=0.0003

\[\text{p-value} = 0.0003\]
Figure 3.7: Nucleosome density at the INO1 promoter does not vary from wild type in unacetylatable Snf2p cells. Real-time PCR analysis of DNA immunoprecipitated through ChIP with antibodies against (A) Histone 3, (B) Histone 4 in WT cells, unAcSnf2p cells, and snf2Δ cells that were grown to mid-log phase (0.8 A600nm) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the INO1 promoter is graphed as mean ± standard deviation normalized to input and mock.
3.2.5 HAT, Gcn5p, is unaffected, but Esa1p recruitment decreases in unAcSnf2p cells.

INO1 histone acetylation remains unchanged.

Our previous studies characterized HAT recruitment in the standard INO1 model in which acetylase occupancy was low at the INO1 promoter under repressing conditions but then significantly increased upon induction (Konarzewska et al., 2012). Since both acetylases are regulators of gene expression in yeast cells, it is of interest to explore whether the acetylation of Snf2p impacts the recruitment of either of these acetyltransferases to the INO1 promoter. To determine if any fluctuations in histone acetyltransferase patterns occurred, chromatin immunoprecipitation coupled with real-time qPCR was performed on WT, unAcSnf2p, and snf2Δ strains.

Relative Gcn5p-IP values were examined at the URS of INO1 in cells prepared in repressing and inducing conditions. All IP values were subsequently normalized to INO1 input and INO1 mock DNA. Gcn5p-IP in WT cells had IP values of 0.22±0.02 under repressing conditions and 1.46±0.34 under inducing conditions. This pattern of significant increase under inducing conditions \( (p<0.01) \) demonstrated the expected WT histone acetylase recruitment in which the Gcn5p is recruited to the INO1 promoter once induction commences. In snf2Δ cells, a significant difference is still observed between repressing and inducing conditions (0.04±0.04 with repressing conditions and 0.86±0.01 under inducing conditions; \( p=0.01 \)), as expected from our previous data in which Gcn5p decreases in the absence of Snf2p or Ino80p remodelers (Konarzewska et al., 2012). Gcn5p-IP values in the unAcSnf2p mutant strain resulted in occupancy levels under repressing and inducing conditions, 0.10±0.07 and 1.08±0.19 respectively. While statistically different from each other \( (p<0.01) \), this pattern did not significantly differ from the WT strain \( (p=0.93) \) (Figure 3.8). This suggested that the acetylation
of Snf2p, and any modifications it caused at the INO1 promoter, did not significantly influence Gcn5p occupancy.

Next, I explored any potential impact of Snf2p acetylation on histone acetyltransferase Esa1p. As with Gcn5p, relative Esa1p-IP values were determined at the URS of INO1 under repressing and inducing conditions, and were subsequently normalized to INO1 input and INO1 mock DNA. Under repressing conditions, the Esa1p-IP of the WT strain exhibited a relative value of 0.24±0.008 under repressing conditions and 1.97±0.09 under inducing conditions, which was a similar pattern to the snf2∆ strain (0.07±0.02 under repressing conditions; 1.88±0.06 under inducing conditions). The Esa1p-IP of the unAcSnf2p mutant, however, exhibited a relative value of 0.17±0.02, but then only exhibited a relative value of 0.67±0.18 upon induction. This induction value was still significantly different from the repressing condition (p=0.03), but not to the degree usually observed in the WT strain (p<0.01) or snf2∆ strain (p<0.01). We have previously shown that the deletion of Ino80p or Snf2p does not interfere with the recruitment of Esa1p, even though it results in an approximately 35% reduction of Gcn5p recruitment (Konarzewska et al., 2012). I now show that there was a significant difference between the relative Esa1p-IP value under inducing conditions in the unAcSnf2p strain versus the WT strain (p<0.01) (Figure 3.9). This suggested that Esa1p was still recruited to the INO1 promoter, but in the absence of Snf2p acetylation, this recruitment was significantly diminished, as less Esa1p was able to occupy the promoter region.

Next, I wanted to evaluate histone acetylation, which is one of the characteristics of actively transcribed genes. Chromatin immunoprecipitation coupled with qPCR was performed using antibodies against acetylated H3 (acH3) and acetylated H4 (acH4). In the WT strain, the relative acH3-IP values were 0.11±0.005 and 1.22±0.26 under repressing and inducing
conditions, respectively. This demonstrated a significant increase in acetylated H3 upon induction \((p<0.01)\). A similar pattern was then observed with regard to the unAcSnf2p mutant in which the relative acH3-IP values were 0.07±0.01 and 0.90±0.04 under repressing and inducing conditions, with a significant increase upon induction \((p<0.01)\), whereas the \(snf2\Delta\) strain demonstrated no significant difference between repressing and inducing conditions \((p=0.62)\) (Figure 3.10A).

Acetylated H4 also demonstrated a similar pattern in both the WT strain and the unAcSnf2p strain. Under WT repressing and inducing conditions, the relative acH4-IP values were 0.06±0.01 and 1.22±0.28, respectively, which demonstrated a significant difference during induction conditions \((p<0.01)\). In the unAcSnf2p mutant strain, a significant increase upon induction \((p<0.01)\) was also observed with relative acH4-IP values of 0.03±0.004 under repressing conditions and 1.39±0.10, whereas the \(snf2\Delta\) demonstrated no significant difference \((p=0.99)\) (Figure 3.10B). These results suggest that even though Esa1p recruitment is altered at the \(INO1\) promoter in unAcSnf2p cells, the acetylation of histones in this region is still maintained.
Figure 3.8: Histone acetyltransferase, Gcn5p, maintains a WT recruitment pattern at the *INO1* promoter in *unAcSnf2p* cells.

Real-time PCR analysis of DNA immunoprecipitated through ChIP with an antibody against Gcn5p in WT cells, *unAcSnf2p* cells, and *snf2Δ* cells that were grown to mid-log phase (0.8 A₆₀₀nm) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the *INO1* promoter is graphed as mean ± standard deviation normalized to input and mock.
Figure 3.9: Histone acetyltransferase, Esa1p, recruitment to the INO1 promoter decreases in the absence of Snf2p acetylation. Real-time PCR analysis of DNA immunoprecipitated through ChIP with an antibody against Esa1p in WT cells, unAcSnf2p cells, and snf2Δ cells that were grown to mid-log phase (0.8 A_{\text{600nm}}) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the INO1 promoter is graphed as mean ± standard deviation normalized to input and mock.
A) ach3 occupancy at the INO1 promoter

WT Strain  unAcSnf2p Strain  snf2Δ Strain

p=0.0003  p=0.0008  p=0.0008
Figure 3.10: Acetylation of histones at the INO1 promoter does not vary from wild type in unAcSnf2p cells.

Real-time PCR analysis of DNA immunoprecipitated through ChIP with antibodies against (A) acetylated Histone 3 and (B) acetylated Histone 4 in WT cells, unAcSnf2p cells, and snf2Δ cells that were grown to mid-log phase (0.8 A600nm) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the INO1 promoter is graphed as mean ± standard deviation normalized to input and mock.
3.2.6 The recruitment of RNA polymerase II to \textit{INO1} is independent of Snf2p acetylation

Although the transcriptional activation of \textit{INO1} is independent of Snf2p acetylation, I want to examine whether the Snf2p acetylation influences transcription machinery. To examine RNA polymerase recruitment to the \textit{INO1} promoter, immunoprecipitation was performed using an antibody directed against RNA Pol II p (Pol IIp). In the WT strain, PolIIp-IP had relative IP values of 0.39±0.16 and 1.35±0.33 under repressing and inducing conditions, respectively. This demonstrated a significant difference of RNA pol II recruitment during induction ($p<0.01$). A similar pattern was observed in the unAcSnf2p mutant strain, with relative IP values of 0.30±0.18 and 1.54±0.03 under repressing and inducing conditions, respectively, whereas the \textit{snf2Δ} mutant exhibited no significant difference between repressing and inducing conditions ($p=0.99$). In the absence of Snf2p acetylation, a significant difference in PolIIp-IP upon induction ($p<0.01$) resulted (Figure 3.11). Taken together, these results confirmed that the recruitment of the RNA polymerase II to the \textit{INO1} promoter was not dependent upon the acetylation of Snf2p and the polymerase was still able to be recruited in the absence of Snf2p acetylation. This validates the \textit{INO1} expression results, as there was no significant difference between \textit{INO1} mRNA in WT cells and in unAcSnf2p mutant cells (Figure 3.5; $p=0.3$), and thus polymerase function is maintained.
Figure 3.11: PolII recruitment to the INO1 promoter in unAcSnf2p cells does not significantly differ from WT. Real-time PCR analysis of DNA immunoprecipitated through ChIP with an antibody against RNA Polymerase II in WT cells, unAcSnf2p cells, and snf2Δ cells that were grown to mid-log phase (0.8 A600nm) in 10μM inositol Synthetic Complete (SC) media and were subsequently washed and subjected to repressing or inducing conditions, 100μM inositol (+ino) and 0μM inositol (-ino) synthetic complete (SC) media, respectively, for 2 hours prior to collection. All experiments were performed with three repeat colonies and all PCR reactions were done in at least duplicate. The IP for the INO1 promoter is graphed as mean ± standard deviation normalized to input and mock.
3.2.7 Biological implications of Snf2p acetylation

Even though I demonstrated that Snf2p acetylation was not required for INO1 expression, its absence did result in a modified regulatory complex at the INO1 promoter as INO1 was induced, where Ino80p and Snf2p failed to dissociate while Esa1p recruitment decreased. To demonstrate whether this modified occupancy of INO1 coactivators in unAcSnf2p cells was influencing pathways Snf2p regulates, I performed plate sensitivity assays targeting osmoregulation, DNA damage, carbon source usage, and copper toxicity.

WT, unAcSnf2p, and snf2Δ strains were first plated on SC media to demonstrate cell viability. All strains demonstrated viable growth at the five dilutions used (Figure 3.12A; stock cell concentration 1.6x10⁹ cells per ml diluted 10⁻¹ through 10⁻⁵). These same strains were then analyzed under high osmolarity conditions. Osmoregulation is a common stress pathway that is highly regulated in yeast and can easily be tested for disregulation via plate sensitivity assays, in which yeast growth is screened in the presence of high salt concentrations (De Nadal et al., 2011). Microarray analyses demonstrated that SWI/SNF is associated with various genes involved in osmoregulation (Holstege et al., 1998), so synthetic complete plates containing 0.8M KCl were utilized to demonstrate whether there were any effects on osmoregulation when Snf2p lacks acetylation.

In the presence of high salt, the WT strain was still visible at the greatest dilution and maximum growth time. The unAcSnf2p strain demonstrated growth comparable to the WT at most dilutions, but was not observed at the greatest dilution of 10⁻⁵. Lastly, the snf2Δ exhibited significantly diminished growth compared to the other strains, as it had very weak growth at the 10⁻³ dilution and no growth at the 10⁻⁴ or 10⁻⁵ dilutions (Figure 3.12B). As the unAcSnf2p strain demonstrated growth more comparable to the WT versus the deletion strain, the sensitivity of the
cells in the presence of osmotic stress did not appear to be significantly altered in the absence of Snf2p acetylation.

Similar results were observed with regard to DNA damage sensitivity in which all strains were plated in the presence of 50mM hydroxyurea. Hydroxyurea is a known inhibitor of the DNA replication enzyme ribonucleotide reductase responsible for dNTP pool expansion during G1/S phase of the cell cycle. In other words, hydroxyurea reduces the purine pools that polymerase requires in order to synthesize new DNA strands at the replication fork. While mammalian cells have compensatory mechanisms to overcome this effect, yeast are fully dependent upon ribonucleotide reductase during replication (Merrill et al., 2004). In the presence of hydroxyurea, the WT strain and the unAcSnf2p strain both demonstrated similar growth with each being observed through the $10^{-4}$ dilution, whereas the $snf2\Delta$ strain was unable to viably grow beyond the $10^{-2}$ dilution (Figure 3.12C). As with the osmotic results, the DNA damage sensitivity plates demonstrated that the lack of Snf2p acetylation did not hinder DNA damage repair.

Another highly regulated process in yeast cells, which is known to be associated with SWI/SNF family remodelers, is the utilization of various carbon sources in the form of fermentable substrates, such as glucose, sucrose, galactose, and maltose, as well as non-fermentable carbon sources, such as ethanol (Schöler et al., 1994). In yeast, SUC2 is gene coding for an invertase involved in the glucose repression system that allows cells to utilize sources other than glucose for carbon (Neigeborn & Carlson, 1984; Abrams et al., 1986). In $snf2\Delta$ strains, this regulatory mechanism is known to be disrupted so cells grow slower in sucrose media, and fail to grow in galactose (Neigeborn & Carlson, 1984). To determine if the acetylation of Snf2p affects the utilization of alternate carbon sources, synthetic complete plates
with 2% glucose, 2% sucrose, 2% galactose, 2% maltose, or 3% ethanol were utilized. On each plate, the unAcSnf2p mutant grew comparable to the WT. Ultimately the unAcSnf2p strain survived all conditions, including the fermentable carbon sources, such as 2% glucose, 2% sucrose, 2% galactose, and 2% maltose, as well as the non-fermentable carbon source of 3% ethanol (Figure 3.13).

Lastly, yeast copper metabolism was examined. Recent research has demonstrated that the chromatin remodelers, Snf2p and Ino80p, which I study at the INO1 promoter, are also required at the CUP1 promoter (Wimalarathna et al., 2013). The regulation of CUP1 is essential in yeast cells for defending the cells from copper toxicity, as CUP1 codes for a yeast metallothionein, which is a cysteine-rich protein that binds metals, such as copper (Thiele, 1988). When examining CUP1 expression and copper sensitivity in yeast cells, it should be noted that 3 mM copper in media is necessary for toxicity, whereas 1.5 mM copper is necessary for CUP1 induction, the gene responsible for protecting yeast cells from copper toxicity (Wimalarathna et al., 2013). Thus, to determine if Snf2p acetylation is significant in copper resistance, all strains were plated on synthetic complete plates containing 1.5 mM or 3 mM copper. Chromatin remodelers Snf2p and Ino80p are required for CUP1 activation, which then leads to the production of the metallothionein protein that binds free-copper in the cytoplasm of cells (Wimalarathna et al., 2013). As was expected, no strain was able to survive in the presence of 3 mM copper (Figure 3.14A). In the presence of 1.5 mM, WT cells demonstrated strong viability, whereas the unAcSnf2p strain demonstrated significant sensitivity comparable to the snf2Δ strain (Figure 3.14B). This suggests that Snf2p acetylation is necessary for proper copper protection in yeast cells, perhaps through its regulation of CUP1. Further studies, such as growth analyses, were then required to better characterize this mechanism.
WT, unAcSnf2p, and snf2Δ cellular growth in (A) Synthetic Complete media (SC), (B) SC with 0.8M KCl, (C) SC with 50mM Hydroxyurea. All cells were grown overnight to saturation, and then diluted to a starting optical density of 2.0 at 600nm absorbance as a stock solution (approximately 1.6x10⁹ cells per ml). Five serial dilutions were plated for each strain (from left to right 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵). Experiments were repeated in duplicate.
The lack of Snf2p acetylation does not significantly affect cell growth on alternate sugar sources or nonfermentable carbon sources. WT, unAcSnf2p, and snf2Δ cellular growth in (A) 2% Glucose, (B) 2% Sucrose, (C) 2% Galactose, (D) 2% Maltose, (E) 3% Ethanol. All cells were grown overnight to saturation, and then diluted to a starting optical density of 2.0 at 600nm absorbance as a stock solution (approximately 1.6x10⁹ cells per ml). Five serial dilutions were plated for each strain (from left to right 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵). Experiments were repeated in duplicate.
Figure 3.14: Snf2p acetylation is required for copper toxicity survival. WT, unAcSnf2p, and snf2Δ cellular growth in (A) SC with 3 mM copper and (B) SC with 1.5 mM copper. All cells were grown overnight to saturation, and then diluted to a starting optical density of 2.0 at 600nm absorbance as a stock solution (approximately 1.6x10^9 cells per ml). Five serial dilutions were plated for each strain (from left to right 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). Experiments were repeated in duplicate.
3.2.8 Increased sensitivity to copper toxicity when remodelers accumulate at the INO1 promoter

As mentioned above, copper resistance in yeast cells is regulated by the CUP1 gene, which encodes for a cysteine-rich metallothionein protein that binds copper and thus promotes detoxification (Jensen et al., 1996). This gene is highly regulated by chromatin remodelers, Ino80p and Snf2p, which are absent from the CUP1 promoter under un-induced conditions, but then become highly recruited upon induction (Wimalarathna, 2013). This remodeler pattern is the opposite of that observed at the INO1 gene, in which chromatin remodelers, Ino80p and Snf2p, are highly present at the promoter under repressed conditions, then dissociate and leave the region upon INO1 induction (Ford et al., 2008). To determine if this inverse pattern of chromatin remodeler occupancy requires that Snf2p dissociate from the INO1 promoter in order to arrive at sufficient levels to the CUP1 promoter, WT cells, unAcSnf2p, and snf2Δ cells were grown under un-induced and induced conditions for both genes.

In standard SC media, which contains 10μM inositol, all three strains were able to grow well in the absence of copper (Figure 3.15A), with no significant difference observed between WT and unAcSnf2p (p=0.99). When 1.5 mM copper was added to the media, however, only the WT cells were still able to grow (Figure 3.15B). This WT growth demonstrated a generation time two-folds slower than observed in the absence of copper (Table 3.5). UnAcSnf2p cells, on the other hand, demonstrated a sensitivity to copper significantly different from the WT (p=0.04) and more comparable to the snf2Δ strain (p=0.98), as they only had an A600nm of 0.5 after 24 hours (Table 3.5). This suggests that in the absence of Snf2p acetylation, yeast cells are more sensitive to copper toxicity.
Next, copper resistance growth was examined under INO1 repressing and inducing conditions, 0 μM and 100 μM inositol, respectively. In 0 μM inositol, all three strains were able to grow in the absence of copper, although snf2Δ cells exhibited a significantly reduced growth pattern \((p<0.01)\) compared to when inositol was present (Figure 3.16A). In the presence of 1.5 mM copper, the WT cells were once again able to survive; whereas the unAcSnf2p cells and snf2Δ cells were sensitive to the copper (Figure 3.16B). The unAcSnf2p cells only reached an \(A_{600nm}\) of 0.59 after 24 hours, with a generation time two-folds less than the WT cells (Table 3.6). Ultimately, in the absence of inositol, when no copper was present, there was no significant difference between WT and unAcSnf2p growth \((p=0.9)\), yet when copper was present, a significant difference was observed \((p<0.01)\) between WT and unAcSnf2p cells, with no significant difference observed \((p=0.13)\) between unAcSnf2p and snf2Δ cells. This suggests that copper toxicity defense is hindered in unAcSnf2p cells under INO1 inducing conditions.

Lastly, cells were examined in 100μM inositol, where cells are known to have Snf2p highly recruited at the INO1 promoter. In the absence of copper, all three strains, including the snf2Δ cells, demonstrated strong growth in 100μM inositol with no significant difference \((p=0.98)\) observed between the WT and unAcSnf2p cells (Figure 3.17A). Unlike the 0μM and 10μM inositol media, however, WT cells in 100μM inositol were no longer able to defend against copper toxicity in the presence of 1.5mM copper and demonstrated a lack of growth comparable to the unAcSnf2p cells and the snf2Δ cells \((p=0.81 \text{ and } p=0.99, \text{ respectively})\) (Figure 3.17B; Table 3.7). It should also be noted that when looking at copper resistance during INO1 repression and induction, not only did WT cells fail to grow during INO1 repression when Snf2p is collected at the INO1 promoter, but they also exhibited the strongest growth during INO1 induction (0μM inositol condition) when Snf2p is known to dissociate from the INO1 promoter.
(Figure 3.17B). When comparing the WT strain’s growth in the presence of 1.5mM copper, there was a significant difference between no inositol (when remodelers have dissociated away from the INO1 promoter) and 10μM inositol ($p=0.02$). An even more significant difference was then observed between the 0μM inositol WT growth and 100μM inositol WT growth (when remodelers are most heavily present at the INO1 promoter) in the presence of 1.5mM copper ($p<0.01$). In other words, the 100μM inositol WT growth was significantly different from the 10μM inositol WT growth ($p=0.004$) and the 0μM inositol WT growth ($p=0.0004$), but was not significantly different from the unAcSnf2p growth in each condition (0μM ino: $p=0.26$; 10μM ino: $p=0.08$; 100μM ino: $p=0.11$). This suggests that copper toxicity defense is hindered when remodelers are accumulated at the INO1 promoter.

Ultimately, in all three inositol conditions, unAcSnf2p mutants failed to recover from copper toxicity, which thus suggests that the acetylation of Snf2p is necessary for copper detoxification. It also became apparent that WT cells demonstrate a similar copper sensitivity to the unacetylatable mutant and deletion mutant when grown under INO1 repressing conditions, as remodelers are occupying the INO1 promoter.

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Table 3.5: 10μM Growth Experiment 24th hour analyses

<table>
<thead>
<tr>
<th>Cell Strain</th>
<th>Average O.D. at 0mM Cu (24th hour)</th>
<th>Average O.D. at 1.5mM Cu (24th hour)</th>
<th># of generations in 24 hr at 0mM Cu</th>
<th># of generations in 24hr at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 0mM Cu</th>
<th>Generation Time (min/gen) at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 3mM Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>6.55 ± 0.21</td>
<td>1.25 ± 0.21</td>
<td>4.80</td>
<td>2.54</td>
<td>299.92</td>
<td>566.98</td>
<td>1587.68</td>
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<tr>
<td>unAcSnf2</td>
<td>6.45 ± 0.07</td>
<td>0.5 ± 0.00</td>
<td>4.91</td>
<td>0.92</td>
<td>293.44</td>
<td>1572.01</td>
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<td>snf2Δ</td>
<td>5.1 ± 0.42</td>
<td>0.35 ± 0.21</td>
<td>4.79</td>
<td>0.77</td>
<td>300.92</td>
<td>1865.75</td>
<td>N/A</td>
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</table>

Table 3.6: 0μM Growth Experiment 24th hour analyses

<table>
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<tr>
<th>Cell Strain</th>
<th>Average O.D. at 0mM Cu (24th hour)</th>
<th>Average O.D. at 1.5mM Cu (24th hour)</th>
<th># of generations in 24 hr at 0mM Cu</th>
<th># of generations in 24hr at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 0mM Cu</th>
<th>Generation Time (min/gen) at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 3mM Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>6.55 ± 0.07</td>
<td>1.85 ± 0.21</td>
<td>4.86</td>
<td>3.07</td>
<td>296.05</td>
<td>468.71</td>
<td>3117.22</td>
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<td>unAcSnf2</td>
<td>6.4 ± 0.14</td>
<td>0.59 ± 0.03</td>
<td>4.90</td>
<td>1.33</td>
<td>294.11</td>
<td>1084.19</td>
<td>3827.89</td>
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<td>snf2Δ</td>
<td>2.65 ± 0.21</td>
<td>0.185 ± 0.04</td>
<td>4.53</td>
<td>0.35</td>
<td>318.11</td>
<td>4096.64</td>
<td>9472.55</td>
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Table 3.7: 100μM Growth Experiment 24th hour analyses

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<th>Cell Strain</th>
<th>Average O.D. at 0mM Cu (24th hour)</th>
<th>Average O.D. at 1.5mM Cu (24th hour)</th>
<th># of generations in 24 hr at 0mM Cu</th>
<th># of generations in 24hr at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 0mM Cu</th>
<th>Generation Time (min/gen) at 1.5mM Cu</th>
<th>Generation Time (min/gen) at 3mM Cu</th>
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</thead>
<tbody>
<tr>
<td>Wild Type</td>
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<td>0.81 ± 0.01</td>
<td>5.38</td>
<td>2.17</td>
<td>267.59</td>
<td>663.55</td>
<td>1835.92</td>
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<tr>
<td>unAcSnf2</td>
<td>7.35 ± 0.07</td>
<td>0.525 ± 0.02</td>
<td>5.57</td>
<td>1.81</td>
<td>258.62</td>
<td>796.67</td>
<td>29439.14</td>
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<td>snf2Δ</td>
<td>6.8 ± 0.57</td>
<td>0.46 ± 0.04</td>
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<td>1.67</td>
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<td>864.48</td>
<td>7724.01</td>
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Table 3.8: 1.5mM Cu 24th hour OD comparison

<table>
<thead>
<tr>
<th>Cell Strain</th>
<th>Average O.D. at 0μM ino (24th hour)</th>
<th>Average O.D. at 10μM ino (24th hour)</th>
<th>Average O.D. at 100μM ino (24th hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>1.85 ± 0.21</td>
<td>1.25 ± 0.21</td>
<td>0.81 ± 0.01</td>
</tr>
<tr>
<td>unAcSnf2</td>
<td>0.59 ± 0.03</td>
<td>0.5 ± 0.00</td>
<td>0.525 ± 0.02</td>
</tr>
<tr>
<td>snf2Δ</td>
<td>0.185 ± 0.04</td>
<td>0.35 ± 0.21</td>
<td>0.46 ± 0.04</td>
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</tbody>
</table>
Figure 3.15: Unacetylated Snf2p results in increased sensitivity to copper in SC media. WT, unAcSnf2p, and snf2Δ cells were grown to saturation then diluted to a starting A\textsubscript{600nm} of 0.2 (approximately 1.6x10\textsuperscript{8} cells per ml) in (A) 10μM inositol; 0mM, 1.5mM, and 3mM Cu, (B) Panel only observing copper toxicity conditions: 10μM inositol; 1.5mM and 3mM Cu. Repeat colonies were averaged prior to graphing.
Figure 3.16: Unacylated Snf2p results in increased sensitivity to copper in 0μM media. WT, unAcSnf2p, and snf2Δ cells were grown to saturation then diluted to a starting $A_{600\text{nm}}$ of 0.2 (approximately $1.6 \times 10^8$ cells per ml) in (A) 0μM inositol; 0mM, 1.5mM, and 3mM Cu, (B) Panel only observing copper toxicity conditions: 0μM inositol; 1.5mM and 3mM Cu. Repeat colonies were averaged prior to graphing.
Figure 3.17: Accumulated chromatin remodelers at the INO1 promoter result in increased sensitivity to copper. WT, unAcSnf2p, and snf2∆ cells were grown to saturation then diluted to a starting $A_{600\text{nm}}$ of 0.2 (approximately $1.6 \times 10^8$ cells per ml) in (A) 100μM inositol; 0μM, 1.5μM, and 3μM Cu, and (B) Panel only observing copper toxicity conditions during INO1 repression and induction: 1.5μM Cu; 0μM, 10μM, and 100μM inositol. Repeat colonies were averaged prior to graphing.
3.2.9 Discussion

In yeast *Saccharomyces cerevisiae*, transcriptional activation involves a series of activator and coactivators interactions with the upstream regulatory region of the gene, as well as interactions with each other during the process. Although recent research shows that some histone acetylases (e.g., Gcn5p) are capable of acetylating chromatin remodelers (e.g., SWI/SNF, RSC, or ISWI) (Ferreira *et al*., 2007; Van Demarck *et al*., 2007; Kim *et al*., 2010), the implications of this post-translational modification has yet to be thoroughly examined.

Through chromatin immunoprecipitation and qPCR, I demonstrated that Snf2p acetylation is required for Snf2p dissociation from the INO1 promoter upon induction in inositol depleted media. When acetylation was prevented by residue replacement of lysine to arginine, Snf2p accumulated at the promoter (Figure 3.3). This helps explain the mechanism behind our previous findings in which the chromatin remodeler, Snf2p, accumulated at the INO1 promoter in the absence of Gcn5p (Konarzewska *et al*., 2012). With the use of an unacetylateable Snf2p mutant, I am able to provide evidence that the accumulation was due to the acetylation of Snf2p (Figure 3.3), which is known to be a post-translational modification performed by HAT, Gcn5p (Kim *et al*., 2010). Since our previous work demonstrated that Snf2p accumulated in the absence of Esa1p, and since Ino80p accumulated in the absence of either Gcn5p or Esa1p (Konarzewska *et al*., 2012), further studies are necessary to better characterize any additional interactions occurring among these transcriptional regulators.

Based on the finding that Snf2p acetylation was required for dissociation from the INO1 promoter, it was necessary to next explore the possible implications of this dissociation with regard to transcriptional activation and coactivator activities. Regarding transcriptional activation, growth survival and mRNA analyses confirmed that although the acetylation was
necessary for dissociation from the promoter, it was not necessary for *INO1* gene expression, as
the unAcSnf2p strain was able to survive in the absence of inositol and expressed normal mRNA
levels under this inducing condition (Figures 3.4 and 3.5). This growth in the absence of inositol
demonstrated that the cells were capable of adapting to the lack of inositol in the environment,
most likely by activating the *INO1* biosynthetic pathway in which *INO1* encodes for Inositol-3-
Phosphate-Synthase (I-3-P synthase), which converts Glucose-6-Phosphate (G-6-P) into Inositol-
3-Phosphate (I-3-P). I-3-P is then dephosphorylated to form inositol by inositol
monophosphatases, encoded by *INM1* and *INM2*. This inositol then leads to the synthesis of PI
through the actions of *PIS1*, which codes for PI synthase (Figure 1.5) (Shaldubina *et al.*, 2002;
Gardocki *et al.*, 2005). This pathway requires the activation of a series of genes, which can
explain the decreased absorbance observed in the inductive conditions compared to the
repressive conditions in WT and unAcSnf2p strains. I previously demonstrated that Snf2p is
required for *INO1* activation (Ford *et al.*, 2008), so snf2Δ cells are not capable of activating the
*INO1* biosynthetic pathway required for survival in inositol depleted conditions, and thus
demonstrated a lack of growth in both conditions.

PolIIp continued to demonstrate wild type patterns of *INO1* promoter occupancy, even in
the absence of Snf2p acetylation (Figure 3.11), which further confirmed that *INO1*
transcriptional activation was not hindered without Snf2p acetylation. This suggested that
acetylation plays a role beyond transcriptional activation and may instead have additional
significances, either in coactivators’ activities at the promoter or in the recycling of remodelers
to mobilize elsewhere.

*INO1* activation is a highly regulated process that is heavily dependent upon the
recruitment and dissociation of various coactivators, such as Snf2p, Ino80p, Gcn5p, and Esa1p,
in order to modify the nucleosome structure at the promoter region and promote appropriate polymerase activity (Ford et al., 2008; Esposito et al., 2009; Konarzewska et al., 2012). Once I demonstrated that Snf2p acetylation was responsible for the dissociation of Snf2p from the INO1 promoter, it was then necessary to determine if the lack of this acetylation affected the other coactivators and nucleosomal components in our INO1 model, as any modifications in coactivators at one gene promoter could potentially influence activities at other gene promoters requiring the same coactivators.

Through chromatin immunoprecipitation coupled with qPCR, I demonstrated that although the chromatin structure and histone acetylation levels remained the same as WT, a lack of Snf2p acetylation resulted in an accumulation of Ino80p at the INO1 promoter (Figure 3.6). Our previous findings had demonstrated that not only does Ino80p arrive at the INO1 promoter before Snf2p, but that this binding of Ino80p is necessary in order to subsequently recruit Snf2p to the promoter (Ford et al., 2008). Our most recent findings now suggest that since the INO80 complex binds to the promoter first and then SWI/SNF binds, it is likely that the removal of the bulky 2 mDa SWI/SNF complex (Winston et al., 1999) from the region is necessary for INO80 to be free to dissociate. Without Snf2p acetylation to trigger Snf2p dissociation, the majority of Ino80p basically remains trapped at the INO1 promoter region. In the unAcSnf2p mutant strain, the relative IP values for the Arp8p-IP in the repressed condition were statistically similar to the induced condition (p > 0.05) (Figure 3.6), which demonstrated that in the absence of Snf2p acetylation, Ino80p failed to dissociate from the INO1 promoter upon induction, and instead remained occupying the region, perhaps trapped by the accumulated unacetylated Snf2p, which is part of a much bulkier SWI/SNF complex. Despite the accumulation of Snf2p and Ino80p remodelers, the chromatin remodeler activities at the INO1 promoter were not affected by Snf2p
acetylation (Figure 3.7). Since at this point, INO1 has already been induced, this modified regulatory complex may not directly affect INO1 expression, hence the standard PolIIp levels observed in Figure 3.11. It may instead, however, affect the various actions that Ino80p would require to perform upon dissociation from the promoter, as Ino80p is a critical yeast chromatin remodeler responsible for regulating more than just the INO1 gene.

In addition to the altered Ino80p occupancy pattern observed, the Esa1p recruitment pattern also varied from WT expectations in the absence of Snf2p acetylation (Figure 3.9). Our previous data demonstrated that both Ino80p and Snf2p not only fail to dissociate from the INO1 promoter in the absence of Gcn5p, but also in the absence of Esa1p (Konarzewska et al., 2012). Taken together with our new finding that Esa1p had significantly diminished recruitment in the absence of Snf2p acetylation (Figure 3.9) where Snf2p and Ino80p failed to vacate the promoter, this suggests that Esa1p may have a more direct connection to the remodelers than previously believed. Since Gcn5p interacts with and acetylate chromatin remodelers, such as Snf2p, then it is plausible that perhaps Esa1p is also involved in remodeler acetylation and dissociation at promoter regions. Or perhaps the diminished recruitment is simply a byproduct of the steric hindrance created when bulky remodeling complexes, such as SWI/SNF and INO80 fail to dissociate from the promoter. Although a lack of Snf2p acetylation did not significantly impact the transcription of INO1, it did result in the accumulation of remodelers, Snf2p and Ino80p, as well as a diminished presence of Esa1p. These modified recruitment/dissociation patterns may have a significant impact on other genes that depend upon the availability of these regulatory proteins for activation.

While, hydroxyurea-induced DNA damage repair, osmoregulation, and carbon source utilization were not significantly affected by Snf2p acetylation, protection from copper toxicity
was noticeably impeded, as demonstrated by sensitivity plate assays and growth analyses. In each situation where Snf2p is known to be accumulated at the \textit{INO1} promoter, for instance in unAcSnf2p cells in any condition or WT cells in 100μM inositol, a severe lack of copper detoxification was observed (Figure 3.17). The growth of unAcSnf2p was significantly reduced in the presence of copper in the absence or presence of inositol. In the presence of copper, the growth of WT cells in 100μM inositol was also significantly diminished. In other words, these results suggested that Sfn2p must vacate the \textit{INO1} promoter in order to be readily available at the necessary levels to aid in the induction of \textit{CUP1}. As mentioned earlier, \textit{INO1} and \textit{CUP1} demonstrate inverse patterns of remodeler recruitment (Ford \textit{et al.}, 2008; Wimalarathna \textit{et al.}, 2013), which further support this hypothesis. So even though Sfn2p acetylation was not necessary for \textit{INO1} transcriptional activation, it is still a significant post-translational modification in yeast cells, as our results suggest a recycling role may be in effect for chromatin remodeler acetylation. As Sfn2p and Ino80p, known regulators of \textit{CUP1}, accumulate at the \textit{INO1} promoter in the absence of Sfn2p acetylation, other genes requiring Sfn2p and Ino80p, such as \textit{CUP1}, may be affected, as Sfn2p is required for the activation of nearly 5% of all yeast genes, yet is fairly rare within the cells at an estimated 100-500 copies per nucleus (Peterson & Workman, 2000). Further studies will be necessary to directly confirm this relationship and to identify other genes that may be impeded by a lack of Sfn2p acetylation.
Chapter 4
The acetylation of chromatin remodeler, Ino80p

It has already been demonstrated that certain chromatin remodelers, including SWI/SNF, ISWI and RSC, are targeted for acetylation by histone acetyltransferases, such as Gcn5p (Ferreira et al., 2007; Kim et al., 2010; Charles et al., 2011). The INO80 complex, however, despite being a versatile and highly conserved remodeler, had not been examined yet with regard to acetylation. Within this complex, the fundamental subunit is Ino80p, which has been characterized as having ATPase, helicase, and DNA-binding activities comparable to the SWI/SNF complex that includes Snf2p (Shen et al., 2000; Bao et al., 2007). We have previously shown that under repressing conditions, Ino80p is highly recruited, but then drastically decreases or dissociates from the INO1 promoter once induction commences (Ford et al., 2008). We then demonstrated that this pattern becomes altered when histone acetyltransferases are no longer present in the yeast strain. Instead of dissociating, Ino80p accumulates at the INO1 promoter upon induction in Gcn5p or Esa1p knockout strains (Konarzewska et al., 2012). Based upon these coactivator recruitment patterns and similarities to the Snf2p patterns previously discussed in this thesis, I hypothesize that Ino80p may also be subjected to acetylation in a similar manner to Snf2p. I further propose that the histone acetylases recruited to the INO1 promoter by the activator, Ino2p, may play a role in the acetylation of Ino80p, perhaps to promote the removal of Ino80p from the INO1 promoter once induction has begun.

In this chapter, I aim to demonstrate that Ino80p can be acetylated. WT yeast cells carrying Ino80p engineered with a double FLAG tag were used in IP and Western blot analysis. Our results demonstrated that Ino80p can be acetylated in vivo. Subsequently, histone acetylase knockout mutants were created and were subjected to Western blot analysis to identify which
histone acetylase is responsible for Ino80p acetylation. I also evaluated the possible implication of Ino80p acetylation in DNA damage repair.
4.1 Materials and Methods:

4.1.1 Yeast Growth Conditions and Lysis

Yeast strains utilized in these experiments are listed in Table 4.1 and key differences in Ino80p in select strains can be viewed in Figure 4.5. Yeast cells were grown to mid-log phase at 30°C in SC media (synthetic complete media) containing 2% glucose (wt/vol) except ino80Δ cells which were grown in SC-trp (SC medium lacking tryptophan). Cells were then pelleted and resuspended in 400 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1.5 μM trichostatin A (WAKO)(added as 1.5 mM solution in ethanol), 0.2mM PMSF (Sigma Cat#78830) and 0.4 μl of Protease Inhibitor Cocktail Set III (CalBioChem Cat#539134)(added as 100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64 Protease Inhibitor, 2 mM leupeptin and 1 mM pepstatin A). Equal volume glass beads (Sigma Cat#G8772, 0.5mm diameter, acid washed,) were added and the cells were vortexed for 1 hour at 4°C. After centrifugation, the supernatant was collected and stored for further analysis.
**Table 4.1: Yeast strain genotypes utilized**

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<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td><strong>Wild Type (BY4733)</strong></td>
<td>MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
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<tr>
<td><strong>INO80-FLAG</strong></td>
<td>MATa INO80-FLAG his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>ino80Δ</strong></td>
<td>MATa ino80Δ::TRP1 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
</tr>
<tr>
<td><strong>ΔN</strong></td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td><strong>INO80-FLAG/hat2Δ</strong></td>
<td>MATa INO80-FLAG-URA3 hat2Δ his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
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<td><strong>INO80-FLAG/gcn5Δ</strong></td>
<td>MATa INO80-FLAG-URA3 gcn5Δ his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
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<td><strong>INO80-FLAG/esa1mt</strong></td>
<td>MATa INO80-FLAG-HIS3 esa1mt his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
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*Parental strain for all strains listed above is BY4733*
4.1.2 Immunoprecipitation Pull-down Procedure

200 μl lysate was then precleared for 1 hour with 20 μl of Protein A Agarose Slurry (Millipore Cat#16-157) and was transferred to 50 μl of equilibrated M2 Anti-Flag Affinity Agarose (EzView Red Anti-FLAG M2 Affinity Gel, Sigma Cat#F2426). This mixture was then gently rotated for 1 hour at 4°C and was subsequently washed three times with TBS (50mM Tris Hcl, 150mM NaCl, pH 7.4). Samples were eluted with 50 μl 2X Laemmli Sample Buffer (Sigma Cat#S3401), boiled for 5min, and vortexed. These samples were then briefly centrifuged (5 sec) and the supernatants were transferred to fresh tubes.

4.1.3 Protein Gel Analysis and Western Blot

20μl of boiled cell lysate was then electrophoresed on 8% SDS-PAGE with standards at 60V and was subsequently silver stained (Pierce SilverSNAP Stain Kit II Cat#24612). For Western blot, the same SDS-PAGE procedure was utilized without Silver staining. Subsequently, gels were transferred to nitrocellulose membranes at 22V for 2 hours (Invitrogen Xcell II Blot Module). Transfer of protein was confirmed by gently shaking the blot with 0.1% Ponceau in 5% acetic acid. Blots were blocked in 5% BSA for 1hr followed by incubation overnight at 4°C with 1:1000 dilution of Anti-FLAG (Sigma Anti-FLAG, antibody produced in rabbit, Cat#F7425-.2MG) or Anti-acK primary antibody (Cell Signalling Technology Acetylated Lysine Rb Antibody, Cat#9441S). Excess primary antibody was washed away with three times 10min washes of 1X TTBS, pH 7.6 (0.05% Tween20, 20mM Tris, 150mM NaCl) and a single wash of 1X TBS, pH 7.6 (20mM Tris, 150mM NaCl). Secondary antibody (Goat Anti-Rabbit HRP, Abcam Cat#ab6721) was then applied for 1 hour. Blots were washed again as stated above.
and lastly detection was performed via the BioRad Immunstar HRP substrate kit (BioRad Cat#170-5040).

4.1.4 Restriction Digest and DNA Gel Extraction

In order to engineer an \textit{INO80-FLAG-HIS3} vector (Figure 4.4), restriction digest, gel purification, and ligation were performed as shown in Figure 4.1. For vectors, restriction was set up with 9μl of autoclaved water, 2μl Tango buffer, 8μl plasmid DNA (pRS416-\textit{INO80-2FLAG}; Figure 4.2), and 2μl NsiI. Samples were incubated at 37˚C for 1hour, and then 1μl PfoI was added. Samples were then incubated at 37˚C for an additional 3 hours. 1μl of CIP was added with 30min of incubation, followed by another 1μl of CIP with 30min of incubation.

For inserts, restriction was set up with 11μl of autoclaved water, 2μl Tango buffer, 8μl plasmid DNA (pRS413; Figure 4.3), and 2μl NsiI. Samples were incubated at 37˚C for 1hour, and then 1μl PfoI was added. Samples were then incubated at 37˚C for an additional 3 hours.

Loading dye was added to each sample and they were run on a 0.8% agarose gel at 85V. The desired bands were cut out and weighed in a 1.5ml tube. The Qiagen gel extraction kit (cat#28704) was then utilized. 3 volumes of QG buffer (5.5M guanidine thiocyanate and 20mM Tris-HCl pH 6.6) was added to 1 volume of gel and samples were incubated at 50˚C for a minimum of 10min to dissolve the gel fragment. 1 volume of isopropanol was added and the sample was centrifuged in a spin column 600μl at a time in 1min 13K intervals. The spin column was then washed with 750μl of PE buffer (10mM Tris-HCl pH 7.5 and 80% ethanol) and final DNA was eluted using 22μl of TE (10mM Tris-HCl pH8; 1mM EDTA). DNA elution was confirmed by running 2μl of sample on a 0.8% agarose gel at 85V.
4.1.5 Ligation and Transformation

Optimal vector and insert quantities for a minimum 3:1 (insert:vector) molar ratio were calculated as follows for a 50ng vector ligation:

**Vector (pRS416-INO80-2FLAG)**

10339bp (plasmid) - 1193bp (URA3) = 9146bp

50ng/20μl = 0.0025ng/μl

\[
\frac{(0.0025\text{ng/μl})}{(9146\text{bp} \times 650\ \text{Daltons})} \times 2\text{ends} = 0.84\text{nM}
\]

0.84nM*3 = 2.52nM of insert necessary

**Insert (pRS413 HIS3 segment)**

\[
\left(\frac{x}{1238\text{bp} \times 650\ \text{Daltons}}\right) \times 2 = 2.52\text{nM}
\]

\[x = \frac{0.001\ μg/μl \times 20μl}{0.02μg} = \text{20ng of insert}\]

The calculated vector and insert quantities were added to 10μl of NEB 2x quick ligation buffer and 1μl of quick T4 ligase (NEB quick ligation kit cat#M2200S) for a 45 minute room temperature incubation. They were then placed on ice for transformation.

Transformation was performed by adding *E.coli* to 250μl of ice cold Calcium Chloride (50mM; Fisher cat#C79-500). 10μl of the ligation was added (or 1μl of control plasmid DNA), and the samples were left on ice for approximately 50min. Heat shock was performed at 42°C for 45sec, and samples were returned to ice for 2min. 250μl of LB was added and samples were incubated shaking at 37°C for 30min before each was plated on the appropriate selective plates for overnight 37°C incubation.
Figure 4.1: Genetic engineering of *INO80-FLAG-HIS3* plasmid DNA

pRS413 and pRS416 were digested with NsiI and PfoI. The gel purified *HIS3* insert fragment of pRS413 was then ligated to the gel purified *INO80-FLAG* pRS416 vector fragment, and the recombinant vector was transformed into *E.coli* and purified via miniprep and phenol chloroform extraction.
Figure 4.2: Plasmid map of pRS416-INO80-2FLAG
Vector engineered by Xuetong Shen by cloning a PCR fragment including a BamHI restriction site followed by the INO80 native promoter (-500) to the terminator HindIII restriction site. A double FLAG sequence was inserted before the stop codon. ΔN mutants from Xuetong Shen were made by introducing mutations into this vector, which was then inserted into the ino80Δ strain. Histone acetyltransferase mutants containing INO80-FLAG were engineered by Michelle Esposito using this vector. The selectable marker used to screen for mutants containing this vector is URA3.
Figure 4.3: Plasmid map of pRS413
Vector containing HIS3 selectable marker within its PfoI to NsiI restricted region. Utilized as a HIS3 source to engineer a new version of the pRS416-INO80-2FLAG vector that would have a unique selectable marker for use in the esa1mt strain.
Vector engineered in which pRS416-INO80-2FLAG selectable marker URA3 was replaced by the HIS3 selectable marker of pRS413 using the restriction sites PfoI and NsiI. As with the original pRS416-INO80-2FLAG vector, this engineered vector includes a BamHI restriction site followed by the INO80 native promoter (-500) to the terminator HindIII restriction site, with a double FLAG sequence inserted before the stop codon.
4.1.6 Agarose Gel Electrophoresis

0.8% gels were made by heating 0.4g of agarose (Fisher cat#BP160-100) with 50ml of 1X TBE (89mM TrisBase, 89mM Boric Acid, and 2mM EDTA). The dissolved solution was then poured into a mold with 1.5μl Ethidium Bromide (Fisher cat#15585011) and solidified at room temperature. Gels were then run at 85V in 1X TBE with NEB 6x loading dye (cat#B7021S) in each sample. Markers that were run alongside samples were composed of NEB 6x loading dye and NEB 1kb DNA ladder (cat#N3232S) or NEB 100bp DNA ladder (cat#N3231S).

4.1.7 Plasmid Miniprep and Phenol Chloroform Extraction

A single bacterial colony was inoculated into 6ml of LB/AMP (final concentration 100μg/ml) and grown overnight to saturation at 37°C 300rpm. The culture was then pelleted and resuspended in 300μl of ice cold Qiagen P1 (50mM Tris-HCl, 10mM EDTA, and 100μg/ml RNase A) and was left on ice for 5min. 300μl of room temperature Qiagen P2 (200mM NaOH and 1% SDS) was then added and gently mixed prior to a 5min room temperature incubation. 300μl of ice-cold P3 (3M potassium acetate, pH 5.5) was then added, gently mixed, and incubated for 5min on ice. The sample was then centrifuged at max speed for 10min and the supernatant was transferred to a new tube. 400μl of phenol was added, and the sample was vortexed and centrifuged for 3min at 13K. The top layer was transferred to a new tube and 400μl of chloroform was added prior to vortexing and centrifugation for 3min at 13K. Again the top layer was transferred to a new tube to which 1ml of 100% Ethanol and 40μl of Sodium Acetate was added. The sample was then placed in dry ice for 20min and centrifuged for 15min at 13K. All supernatant was removed and replaced with 1ml of 70% Ethanol prior to a 5min 13K spin.
Lastly, the sample was air dried and the pellet of DNA was resuspended with 20μl TE (10mM Tris-HCl pH8; 1mM EDTA).

4.1.8 Yeast Electroporation

Yeast cells were inoculated into 6ml of appropriate media and grown overnight at 30°C, 300rpm. Cells were then diluted to a starting A_600nm of 0.4 in 50ml of appropriate media and grown until they reached an A_600nm of approximately 0.8-1.0, at which point they were pelleted and washed three times with ice cold 1M sorbitol. Final pellets were then resuspended in 200μl of 1M sorbitol and were divided into 40μl aliquots. 2-4μl of purified plasmid DNA was then added to a 40μl aliquot and kept on ice for 5min. The cell/DNA mixture was then transferred to a cold electroporation cuvette (Bio Rad 0.2cm electrode gap) and electroporated at 1.5kV using the Biorad E. coli Pulser (one pulse) with a time constant between 5.0 and 6.0. 450μl of ice cold 1M sorbitol was then added to the cuvette and cells were plated on selective agar as 100μl and remaining volume quantities. All plates were incubated at 30°C overnight and were flipped the following day for additional 1-2 days of incubation at 30°C.
4.2 Results

4.2.1 Ino80p is acetylated and this acetylation is lost when the HSA/DNA-binding domain is removed

In order to confirm that Ino80p becomes acetylated, *Saccharomyces cerevisiae* cells carrying Ino80p engineered with a double FLAG tag were used (INO80-FLAG; Figure 4.5), along with non-tagged WT and *ino80Δ* cells as controls. Immunoprecipitation was used to pull down Ino80p and Western blot was performed to examine whether or not acetylation was detectable.

*INO80-FLAG* cells were grown to mid log phase, as were WT and *ino80Δ* cells as controls. Cells were harvested and the cell lysate analyzed by SDS-PAGE and subsequent silver staining (Figure 4.6A). This was followed by Western blot probing with antibodies against FLAG and acetyl lysine, respectively. For the blot probed with FLAG, our result showed that a band representing the correct molecular weight of Ino80p, which is approximately 174kD was detected in the *INO80-FLAG* lysate but not in the negative control non-tagged WT or *ino80Δ* strain lysate (Figure 4.6B). This confirmed the specificity of the α-FLAG antibody. For the blot probed with the α-acetyl lysine antibody, I observed that multiple upper molecular weight proteins with acetylated residues were present in all cell lysates (Figure 4.6B). This suggests the presence of acetylated proteins in all lysates.

To examine whether Ino80p can be acetylated, the lysates were subjected to immunoprecipitation (IP) with α-FLAG resin to isolate Ino80p. These eluted IP samples were again subject to SDS-PAGE and Western blot analysis. Subsequently, the blots were probed with the α-FLAG and α-acetyl lysine antibodies, respectively. For the blot probed with α-FLAG, one prominent band was shown in the *INO80-FLAG* cells, but no band was detected in the IP
samples from the negative controls, WT and \textit{ino80Δ} cells (Figure 4.6C). This confirmed the effectiveness and purity of the pull-down materials. For the blot probed with the $\alpha$-acetyl lysine antibody, I detected acetylated lysine residues of Ino80p in \textit{INO80-FLAG} strain while no bands were observed in the control strains (Figure 4.6C). This suggested that Ino80p is thus capable of being acetylated.

Although Ino80p can be acetylated, the full characterization of this acetylation, as well as its functional implications in transcriptional activation, will require further analysis. Not only will a better understanding of Ino80p acetylation provide insight into transcriptional activation, but it also gives insight into the interactions occurring between coactivators at gene promoters during gene regulation. One of the first steps of this characterization would be to narrow down and ultimately identify the exact residues acetylated and determine which acetyltransferase is responsible for this modification. Once it was determined that Ino80p was able to be acetylated, the exact location of these acetylated residues became the focus of attention. Since the known acetylated chromatin remodelers, such as Snf2p, are acetylated on lysine residues located within the DNA-binding domain of the remodeler (Kim \textit{et al}., 2010), I utilized an \textit{INO80-FLAG} strain lacking a DNA-binding domain, to attempt to narrow down the location of acetylated residues. The mutant was the \textit{INO80-FLAG-ΔN} strain in which the segment of amino acids 356-682 was removed (Shen \textit{et al}., 2003). This removed region was part of the HSA domain of Ino80p, which is adjacent to the ATPase domain (Figure 4.5). Intriguingly, the pull-down and subsequent Western blot of this particular Ino80p mutant resulted in the absence of acetylated residues of Ino80p (Figure 4.6C). This suggests that the acetylated residues of Ino80p fall within this region that includes the HSA domain and Ino80p DNA-binding domain. As stated, this correlates to the other remodelers that have been shown to be acetylated, as they too were
acetylated on residues within their DNA-binding domains, which explains why this acetylation triggers remodeler dissociation from promoter regions during transcriptional activation models. Further mutagenesis experiments and Western blot analyses would be necessary to identify the exact lysine residues in this region that are acetylated.
Figure 4.5: Modifications of Ino80-FLAG protein in yeast strains utilized in these experiments. A double FLAG coding sequence separated by a KpnI endonuclease recognition site (5′-GACTACAAGGACGACGATGACAAGGGTACCGATTACAAGGATGATGACGACAAG-3′) was added directly in front of the INO80 stop codon to yield a translated protein with a C-terminal FLAG tag.

In the ∆N mutant, the sequence coding for amino acids 356-682 were removed, which included the DNA-binding region of Ino80p as well as the binding sites for Arp4 and Arp8, which are also involved in DNA-binding. This mutant demonstrated a phenotype characteristic of ino80Δ.
Figure 4.6: Ino80p can be acetylated and this acetylation is lost in ∆N mutants. (A) An 8% SDS-PAGE analysis of whole cell lysates of the WT, INO80-FLAG, and ino80Δ visualized with silver stain. (B) Western blot analysis of whole cell lysates of the WT, INO80-FLAG, ∆N and ino80Δ cells on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against FLAG (α-FLAG) and an antibody against acetylated lysine residues (α-AcK), respectively. (C) Immunoprecipitated FLAG from either WT, INO80-FLAG, ∆N or ino80Δ cells was analyzed by an 8% SDS PAGE immunoblotted with α-FLAG and α-AcK, respectively.
4.2.2 Creation and confirmation of *INO80* FLAG-tagged non-essential histone acetylase knockout mutants

Upon confirmation of acetylation, it was then necessary to better characterize the acetylation and confirm that it was indeed due to a histone acetyltransferase. To identify which acetylase was responsible for this acetylation, it was necessary to engineer a series of mutants in which I could study Ino80p in the absence of each histone acetylase. Since there was no commercially available antibody to pull down Ino80p in yeast, I needed to engineer a series of mutant strains in which Ino80p was FLAG-tagged and a target histone acetylase was knocked out or mutated. To genetically engineer these strains, a yeast centromere vector, pRS416, containing a *URA3* selectable marker, as well as *INO80*, from the native *INO80* promoter to the terminal region, with a double FLAG coding sequence directly in front of the *INO80* stop codon, was purified and introduced into a series of histone acetyltransferase knockout strains, such as *gcn5Δ*, *hat1Δ*, *hat2Δ*, *sas2Δ*, and *sas3Δ*, as well as into a wild type strain and an *ino80Δ* strain.

To engineer these mutants, it was first necessary to isolate and purify plasmid pRS416-*INO80*-FLAG (Figure 4.2) DNA via plasmid miniprep. Purified plasmid DNA was confirmed on a 0.8% agarose gel, which demonstrated a strong concentration of supercoiled pRS416-*INO80*-FLAG without any RNA contamination (Figure 4.7).

The appropriate yeast cells, *WT, ino80Δ, sas2Δ, sas3Δ, hat1Δ, hat2Δ*, and *gcn5Δ*, were grown to an optical density of 0.9 at *A*$_{600nm}$ and were made competent with ice cold 1M sorbitol treatments. The purified pRS416-*INO80*-FLAG plasmid DNA was then electroporated into the competent cells and colonies were selected for on synthetic complete agar plates lacking uracil. The wild type controls for the experiments were prepared by electroporating the plasmid DNA into WT and *ino80Δ* cells (Figure 4.8). Plasmid DNA was also electroporated into *sas2Δ, sas3Δ,*
hat1Δ, hat2Δ, and gcn5Δ. Colonies were observed for each strain (Figure 4.9). Since the esa1mt strain contained the same selectable marker (URA3) as the vector, further experimentation would be required to engineer that strain, which will be discussed at a later time.

True selectivity of the plates utilized was demonstrated by streaking the mutant colony alongside the non-resistant parental strain on the same uracil deficient plate. Only the newly electroporated mutant strain was capable of growing on the plate (Figure 4.10A).

Engineered strains were then confirmed via Western blot in which each mutant strain was grown to mid-log phase in media lacking uracil. Cells were then harvested and cell lysates were run on an 8% SDS-PAGE gel. This was followed by Western blot with an antibody against FLAG to confirm that Ino80p with a FLAG tag was now present in these cells. The same INO80-FLAG strain used in our previously described experiments was run in the first lane as a control. Ino80-FLAG was detected in all samples, which confirmed that the engineered mutants successfully produced FLAG-tagged Ino80p (Figure 4.10B).
Figure 4.7: pRS416-INO80-FLAG plasmid DNA is isolated and purified without RNA contamination. A strong concentration of RNA-free pRS416-INO80-FLAG plasmid DNA (~10.3kB) was isolated and purified with miniprep and phenol chloroform extraction. DNA was confirmed on a 0.8% agarose-TBE gel visualized with Ethidium Bromide. More than one band is present in each lane as plasmid DNA is isolated in the nicked, linear, and supercoiled forms.
Figure 4.8: Colonies grew on selective uracil deficient media after pRS416-INO80-FLAG was electroporated into competent wild type and ino80Δ cells. Wild type and ino80Δ cells were made competent with ice cold 1M sorbitol treatment and pRS416-INO80-FLAG was electroporated into them. Colonies were selected for based on survival in the absence of uracil, as pRS416 contains URA3. An example of a recombinant colony is denoted by the black arrow.
Colonies grow on selective uracil deficient media after pRS416-INO80-FLAG was electroporated into competent sas2Δ, sas3Δ, hat1Δ, hat2Δ, and gcn5Δ cells. Wildtype and ino80Δ cells were made competent with ice cold 1M sorbitol treatment and pRS416-INO80-FLAG was electroporated into them. Colonies were selected for based on survival in the absence of uracil, as pRS416 contains URA3.

Figure 4.9: Colonies grow on selective uracil deficient media after pRS416-INO80-FLAG was electroporated into competent sas2Δ, sas3Δ, hat1Δ, hat2Δ, and gcn5Δ cells. Wildtype and ino80Δ cells were made competent with ice cold 1M sorbitol treatment and pRS416-INO80-FLAG was electroporated into them. Colonies were selected for based on survival in the absence of uracil, as pRS416 contains URA3.
Figure 4.10: Ino80-FLAG mutants are confirmed with selective media and Western blot.
A) Electroporated strain with URA3 selectivity and parental strain streaked on uracil deficient selective plates.
B) Western blot analysis of whole cell lysates of the INO80-FLAG control alongside newly engineered INO80-FLAG/WT, INO80-FLAG/ino80Δ, INO80-FLAG/gcn5Δ, INO80-FLAG/sas2Δ, INO80-FLAG/sas3Δ, INO80-FLAG/hat1Δ, and INO80-FLAG/hat2Δ cells on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against FLAG (α-FLAG)
4.2.3 Creation of an *esa1* viable mutant carrying *INO80-FLAG* plasmid

All other mutant strains that were used as parental strains in the mutagenesis experiments lacked the *URA3* gene and were thus compatible with the pRS416-*INO80-FLAG* vector. The *esa1mt* strain, however, already contained the *URA3* gene and thus was unable to undergo electroporation selectivity with the pRS416-*INO80-FLAG* vector. To modify the vector so that it would no longer rely on *URA3* as a selectable marker, but would instead have a *HIS3* marker, pRS416-*INO80-FLAG* (Figure 4.2) plasmid DNA was isolated and purified with miniprep and phenol chloroform extraction to serve as a vector, while pRS413 (Figure 4.3) was prepared so that its *HIS3* gene could be isolated as an insert for our new vector (Figure 4.4). Collected DNA of both plasmids was confirmed on a 0.8% agarose gel with 1kB and 100bp DNA ladders, with pRS416-*INO80-FLAG* exhibiting a size around 10kB and pRS413 exhibiting a size around 5kb as expected (Figure 4.11A, B, respectively).

The pRS416-*INO80-FLAG* and pRS413 plasmid DNA was then digested with PfoI and NsiI and each sample was visualized on a 0.8% gel to identify the desired fragment for engineering pRS416-*INO80-FLAG-HIS3*. The non-*URA3* fragment of prs416-*INO80-FLAG* (expected to be 9,146bp as opposed to the *URA3* fragment of 1,193bp), would serve as the vector, and the *HIS3* fragment of pRS413 (expected to be 1,238bp as opposed to the non-*HIS3* fragment of 3,732bp), would serve as the insert for ligation to engineer our modified vector (Figure 4.12A). These two fragments were then excised from the gel and purified with a Qiagen gel extraction kit (cat#28704). To confirm the purification and estimate the concentration of our starting material for ligation, the products, which should be 9,146bp (vector) and 1,238bp (insert), were run on a 0.8% gel after gel extraction (Figure 4.12B).
Once the desired vector fragment and insert fragment were isolated and purified, the final step of engineering the modified vector was to ligate the fragments together and transform the new vector into bacterial cells to amplify it for collection. In order to perform the ligation with a 3:1 insert to vector molar ratio, 20ng of our 1,238bp insert was necessary with 50ng of our 10,339bp vector. The DNA mixture was then treated with the NEB quick ligation kit at room temperature and 10μl was aliquoted to transformation with calcium chloride competent E.coli.

Transformation was performed with the original pRS416-INO80-FLAG as a control, as well as the ligation mixture. Successfully transformed bacteria containing the ligated pRS416-INO80-FLAG-HIS3 plasmid were selected on Luria-Bertani agar plates containing 100μg/ml ampicillin (Figure 4.13). Note that ligation decreases the efficiency of transformation, so fewer colonies are observed than the control. Isolated colonies were then transferred to 6ml of LB media containing 100μg/ml ampicillin and grown to saturation for further analysis. Miniprep and phenol chloroform extraction were performed to isolate and purify the newly engineered vector, which was expected to have a size of 10,378bp. Confirmation of isolated plasmid DNA was determined by visualizing the samples on a 0.8% agarose gel (Figure 4.14A).

To confirm that the colonies had the true pRS416-INO80-FLAG-HIS3 plasmid, a series of restriction digests were performed on the purified pRS416-INO80-FLAG-HIS3 plasmid DNA using the BstBI enzyme and the NheI enzyme. Since pRS416-INO80-FLAG only has one BstBI recognition site and only one NheI recognition site, either of these enzymes would only result in a linearized plasmid fragment on an agarose gel. If, however, the ligation was successful and the isolated plasmid was indeed pRS416-INO80-FLAG-HIS3, then the plasmid would have two recognition sites for BstBI, which would result in a 9,928bp fragment and a 450bp fragment. The pRS416-INO80-FLAG-HIS3 plasmid would also have two recognition sites for NheI, which
would result in a 6,489bp fragment and a 3,889bp fragment. Since one of the expected fragments was far smaller than the rest, the gel was first stopped earlier to capture the small 450bp fragment, but was then run longer to better visualize the larger 6,489bp versus 3,889bp fragments for the NheI digest. Ultimately, all four expected bands (two for BstB1 and two for NheI) indicative of pRS416-INO80-FLAG-HIS3 were observed to confirm the modified plasmid (Figure 4.14B).

Once the desired plasmid was confirmed, it was then necessary to introduce it into the appropriate mutant strain to yield our target INO80-FLAG-esa1mt strain. This was accomplished by electroporating the purified pRS416-INO80-FLAG-HIS3 DNA into the esa1mt cells, which were made competent through ice cold 1M sorbitol treatments. Successful mutant colonies were selected for on synthetic complete agar plates lacking histidine (Figure 4.15A). Western blot analysis of INO80-FLAG-esa1mt with an antibody against FLAG (α-FLAG) confirmed this strain contained FLAG-tagged Ino80p (Figure 4.15B).
Figure 4.11: pRS416-INO80-FLAG (A) and pRS413 (B) plasmid DNA isolated. Plasmid DNA was isolated and purified by miniprep and phenol chloroform extraction. pRS416-INO80-FLAG (A) and pRS413 (B) plasmid DNA was then confirmed on a 0.8% agarose gel visualized with Ethidium Bromide. More than one band is visible in each lane due to plasmid DNA being collected in the nicked, linear, and supercoiled forms.
Figure 4.12: Vector and insert for engineering pRS416-IN080-FLAG-HIS3 isolated and purified.  
A) Restriction digest with PfoI and NsiI yielded the desired 9,146bp vector from pRS416-IN080-FLAG and the desired 1,238bp insert from pRS413 on a 0.8% agarose gel.  B) The desired vector and insert fragments were excised and purified with a Qiagen gel extraction kit, then run on a 0.8% agarose gel to estimate the concentration and confirm purification.
Control with original pRS416-INO80-FLAG DNA

Figure 4.13: pRS416-IN080-FLAG-HIS3 is engineered through ligation and introduced into E.coli. A control for transformation was set up using the original pRS416-INO80-FLAG plasmid in calcium chloride treated competent E.coli with samples plated on LB plates containing 100μg/ml ampicillin. The ligated pRS416-IN080-FLAG-HIS3 underwent the same transformation protocol. Successfully ligated and transformed cells were then transferred from the plates to liquid media for further confirmation of the plasmid.
Figure 4.14: pRS416-INO80-FLAG-HIS3 plasmid is confirmed with restriction digest.
A) Plasmid DNA from transformation plates expected to be pRS416-INO80-FLAG-HIS3 was isolated with miniprep and purified with phenol chloroform extraction. Size and purity was confirmed by running the final products on a 0.8% agarose gel visualized with Ethidium Bromide.
B) Restriction digests of pRS416-INO80-FLAG-HIS3 treated with BstBI and NheI enzymes run for a short duration of time to visualize the 9,928bp and 450bp fragments of pRS416-INO80-FLAG-HIS3 when cut by BstBI and the same gel run for a longer duration of time to visualize the 6,489bp and 3,889bp fragments of pRS416-INO80-FLAG-HIS3 when cut by NheI.
Figure 4.15: INO80-FLAG/esa1mt colonies confirmed with selective media and Western blot
A) INO80-FLAG-esa1mt colonies grow on synthetic complete plates lacking histidine after electroporation. esa1mt cells were made competent with ice cold 1M sorbitol treatment and pRS416-INO80-FLAG-HIS3 was electroporated into them. Colonies were selected on plates lacking histidine, as pRS416-INO80-FLAG-HIS3 was engineered to contain the HIS3 selectable marker.
B) Western blot analysis of INO80-FLAG/esa1mt and control ino80Δ on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against FLAG (α-FLAG).
4.2.4 Esa1p is responsible for Ino80p acetylation in *Saccharomyces cerevisiae*

In order to determine which histone acetyltransferase is responsible for acetylating the chromatin remodeler, Ino80p, all of our engineered mutants (*INO80-FLAG/WT, INO80-FLAG/ino80Δ, INO80-FLAG/sas2Δ, INO80-FLAG/sas3Δ, INO80-FLAG/hat1Δ, INO80-FLAG/hat2Δ, INO80-FLAG/gcn5Δ, and INO80-FLAG/esa1mt*) were grown to mid-log phase, as was the *ino80Δ* strain lacking any FLAG-tag as a control. Cells were harvested and the cell lysate was subjected to IP in which α-FLAG resin was used to pull down the FLAG-tagged Ino80p in each strain. The IP samples were then subjected to SDS-PAGE and Western blot probing with antibodies against FLAG and acetyl lysine, respectively. For the blot probed with FLAG, our result showed that a band representing the correct molecular weight of Ino80p, which is approximately 174kD, was detected in the pull-down from each of our engineered *INO80-FLAG* strains, but was absent from the *ino80Δ* strain (Figure 4.16A). This result confirmed the effectiveness and purity of the pull-down materials, and identified the blot location of Ino80p.

The eluted IP samples were then subjected to analysis in which the blot was probed with the α-acetyl lysine antibody. I detected acetylated lysine residues of Ino80p in *INO80-FLAG/WT, INO80-FLAG/ino80Δ, INO80-FLAG/sas2Δ, INO80-FLAG/sas3Δ, INO80-FLAG/hat1Δ, INO80-FLAG/hat2Δ, INO80-FLAG/gcn5Δ* but not in the *INO80-FLAG/esa1mt* strain (Figure 4.16B). Repeat samples demonstrated the same result in which the *INO80-FLAG/esa1mt* and *ino80Δ* IP samples probed with α-acetyl lysine antibody lacked any band, but the *INO80-FLAG* wild type control demonstrated the expected acetylated Ino80p band (Figure 4.16C).
Figure 4.16: Histone acetyltransferase Esa1p is responsible for acetylating Ino80p
(A) Western blot analysis of IP of INO80-FLAG/ino80Δ, INO80-FLAG/WT, INO80-FLAG/sas2Δ, INO80-FLAG/sas3Δ, INO80-FLAG/hat1Δ, INO80-FLAG/hat2Δ, INO80-FLAG/gcn5Δ, INO80-FLAG/esa1mt and ino80Δ cells on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against FLAG (α-FLAG) to detect pulled down Ino80p.
(B) Western blot analysis of IP of INO80-FLAG/ino80Δ, INO80-FLAG/WT, INO80-FLAG/sas2Δ, INO80-FLAG/sas3Δ, INO80-FLAG/hat1Δ, INO80-FLAG/hat2Δ, INO80-FLAG/gcn5Δ, and INO80-FLAG/esa1mt cells on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against acetylated lysine residues (α-AcK).
(C) Western blot analysis of repeat samples of INO80-FLAG wild type, INO80-FLAG/esa1mt and ino80Δ cells on an 8% SDS-PAGE and analyzed by immunoblotting with an antibody against acetylated lysine residues (α-AcK).
4.2.5 Strains demonstrating a loss of Ino80p acetylation, also demonstrate preliminary sensitivity to DNA damage and osmotic stress

Once Ino80p was shown to be acetylated, I needed to delve into characterizing this acetylation and its various implications within cells. In order to gain some preliminary insights into the implications of this post-translational modification, I examined how strains lacking Ino80p acetylation were affected by DNA damage and osmotic stress, two important pathways in cells that tend to involve the recruitment of chromatin remodelers. Any strains demonstrating a loss of Ino80p acetylation were then subjected to the DNA damage and osmotic stress sensitivity assays utilized with the Snf2p experiments, in which each strain was plated on SC media, SC media containing 50mM Hydroxyurea, and SC plates containing 0.8M KCl, to determine if the loss of Ino80p acetylation resulted in an increased sensitivity to DNA damage or osmotic stress.

To explore the impact of DNA damage and osmotic stress on our mutant strains that lacked Ino80p acetylation, each strain was first plated as a serial dilution on synthetic complete media to confirm the viability of all strains involved. All four strains (wild type, ΔN, and INO80-FLAG/esa1mt, and ino80∆) demonstrated viable growth, with wild type, ΔN, and INO80-FLAG/esa1mt all showing strongly visible growth at all five dilutions of the assay, while ino80∆ grew strongly at the lower dilutions, but failed to be visible at the final two dilutions (Figure 4.17A). When introduced to media containing hyperosmotic conditions (0.8M KCl), all four strains again demonstrated growth, although the two strains lacking Ino80p acetylation, which are the ΔN and INO80-FLAG/esa1mt, as well as ino80∆, demonstrated weaker growth than the wild type strain, suggesting minor sensitivity to the osmotic stress (Figure 4.17B).
The INO80 chromatin remodeling complex is known to be directly involved in double-stranded DNA break repair through its interaction with the DNA damage-induced phosphorylated histone H2A, which recruits it to damaged sites for its chromatin remodeling activity (Morrison et al., 2004). As such, I was interested in examining the effect of Ino80p acetylation in DNA repair. In the presence of DNA damage-inducing hydroxyurea, a known inhibitor of the DNA replication enzyme responsible for dNTP pool expansion during G1/S phase of the cell cycle (Merrill et al., 2004), the wild type cells were able to recover from the DNA damage and were observed growing even at the fifth dilution. The strains lacking Ino80p acetylation, ΔN and INO80-FLAG/esa1mt, however, more closely mimicked the sensitivity of ino80Δ, as they were unable to survive in the presence of hydroxyurea (Figure 4.17C). This preliminary result suggests an increased sensitivity to double-stranded DNA break damage in the absence of Ino80p acetylation.
Figure 4.17: Mutants lacking Ino80p acetylation demonstrate preliminary strong sensitivity to DNA damage. Wild type, ΔN, INO80-FLAG-esa1mt, and ino80Δ cells were grown to saturation, then diluted to a stock optical density of 2.0 at 600nm absorbance. A serial dilution of 10^{1}, 10^{2}, 10^{3}, 10^{4}, and 10^{5} was performed and each was plated on (A) Synthetic Complete media (SC), (B) SC with 0.8M KCl, and (C) SC with 50mM Hydroxyurea. Experiments were repeated in duplicate.
4.2.6 Discussion

The acetylation of non-histone proteins by histone acetyltransferases (HATs) is an emerging field of study in epigenetics, particularly with regard to coactivators, such as chromatin remodelers. Tumor suppressor p53 was the first reported non-histone target of HATs (Gu et al., 1997), but since then, it has been discovered that multiple transcription factors make up the largest class of newly identified non-histone targets of HATs (Glozak et al., 2005). Acetylation of these proteins can have varying effects, which can be drastically different depending on precisely which lysine residue undergoes acetylation. For instance, with HMG proteins (High Mobility Group proteins), acetylation of lysine residue 71 has been associated with positive regulation of transcription, whereas acetylation of lysine residue 65 has been tied to negative regulation (Munshi et al., 1998). In addition to the acetylation of non-histone proteins by HATs, little is known with respect to the deacetylation of such proteins by HDACs (Glozak et al., 2005). Ultimately, these modifications are biologically significant since the addition or removal of acetyl groups from lysine residues of proteins alters the electrostatic interactions of the protein’s functional groups, which in turn modifies the protein’s abilities and actions.

With respect to chromatin remodelers, it has already recently been shown that SWI/SNF, ISWI, and RSC are capable of being acetylated by HATs (Ferreira et al., 2007; Kim et al., 2010; Charles et al., 2011). It has been suggested that the acetylation of these remodelers is involved in their dissociation from promoters, as well as the regulation of DNA damage resistance (Cairns, 2007; Kim et al., 2010; Charles et al., 2011). Even though remodelers SWI/SNF, ISWI, and RSC (Ferreira et al., 2007; Kim et al., 2010; Charles et al., 2011) had been examined with regard to acetylation, until now, Ino80p had yet to be shown as an acetylated remodeler. Here, I demonstrated through immunoprecipitation and Western analysis that Ino80p can be acetylated
This acetylation can explain the co-activator’s recruitment patterns observed in current gene activation models. Previously, in the yeast INO1 model, it has been shown that Ino80p is highly recruited during INO1 repression, and then dissociates from the promoter once de-repression begins. Histone acetylases, on the other hand, have demonstrated the opposite pattern of recruitment in this model, as they have an increased presence at the promoter as INO1 de-repression commences. The Ino80p recruitment pattern described by this model, significantly changes when HAT mutant strains are studied. It has been shown that instead of dissociating, Ino80p accumulates at the promoter in the absence of functional HATs, such as Gcn5p or Esa1p, under de-repressing processes (Konarzewska et al., 2012). As such, this post-translational modification of Ino80p may be required for its proper dissociation from the INO1 promoter, as I have demonstrated with regard to Snf2p (Figure 3.3).

The remodelers’ dissociation mechanism may also have a wide range of implications with respect to remodeler recycling, as I have demonstrated with regard to Snf2p acetylation, as it allows for increased spatial access to the promoter for the various transcription factors and regulators that need to bind in that region. Our findings here suggest a previously uncharacterized interaction between Ino80p and other co-activators recruited to promoters (Figure 3.6 and Figure 4.16). Although I have now demonstrated that Ino80p can be acetylated, the full characterization of this acetylation, as well as its functional implications in transcriptional activation, will require further analysis. Ultimately, not only will a better understanding of Ino80p acetylation provide insight into the role of epigenetic modifications in transcriptional activation, but it also gives insight into the interactions occurring between co-activators at gene promoters during gene regulation.
To better characterize this acetylation, I then narrowed down the location of acetylation on Ino80p with immunoprecipitation and Western blot analysis using the ΔN strain in which Ino80-FLAG lacks the HSA/DNA-binding domain. Western blot analysis demonstrated that this mutant lacked Ino80p acetylation (Figure 4.6C). This correlates to studies with other acetylated remodelers in that the chromatin remodelers tend to be acetylated in regions involved in DNA-binding, such as the AT-hook domain of Snf2p (Kim et al., 2010). Further mutagenesis experiments will be required to identify the exact lysine residues in this region that are targeted for acetylation. Once these residues are identified, mutants that cannot be acetylated can be engineered by replacing the target lysines with arginines. A non-acetylatable mutant containing the arginine residues would then be useful in studying the implications of Ino80p acetylation.

In order to better characterize the acetylation of Ino80p, I also identified which histone acetyltransferase was responsible for acetylating Ino80p. In order to identify the correct acetylase, I engineered a series of mutant strains in which INO80 was FLAG-tagged and a particular histone acetylase was knocked out. Once I had all of the necessary histone acetyltransferase mutant strains, I used immunoprecipitation and Western blot analysis to demonstrate that Esa1p is responsible for the acetylation of Ino80p (Figure 4.16). Taken together with our results from studying Sfn2p acetylation in which Ino80p accumulates at the INO1 promoter in the absence of Sfn2p acetylation and Esa1p recruitment significantly decreases, I hypothesize that one of two events may be occurring. Either, Ino80p accumulates as the lack SWI/SNF dissociation physically traps the Ino80p at the promoter since Ino80p was recruited prior to Sfn2p or Ino80p accumulates because the accumulation of the SWI/SNF complex hinders Esa1p recruitment to the region, and as I have now shown, Esa1p is required to acetylate Ino80p to subsequently dissociate from the promoter. In either mechanism, there are
previously unidentified interactions among transcriptional coactivators occurring at the INO1 promoter during transcriptional activation.

Once the acetylation mechanism of Ino80p was better characterized, I then wanted to commence preliminary exploration into the implication of this post-translational modification. As Ino80p is a known regulator in DNA-damage repair pathways, I performed initial sensitivity assays with the strains that demonstrated a loss of Ino80p acetylation (ΔN and INO80-FLAG-esa1mt). Both strains demonstrated significant sensitivity to hydroxyurea-induced DNA damage, and only minimal sensitivity to osmotic stress (Figure 4.17). In order to demonstrate a direct connection between Ino80p acetylation and DNA-damage regulation, it would be necessary to engineer the unacetylateable Ino80p mutant strain, once the exact acetylated lysine residues in the HSA/DNA-binding domain are identified. Future studies can then use the unacetylateable Ino80p mutant to demonstrate if Ino80p acetylation is necessary for a recycling role to promote the dissociation of Ino80p away from INO1 to activate other genes, which include genes involved in DNA-damage repair.

Ultimately I have provided insight into the mechanism of chromatin remodeler acetylation and its implications in gene expression regulation. I have also further characterized the evolving model of INO1 transcriptional regulation. Further studies, however, will be necessary to better characterize the potential recycling roles of this acetylation of Sfn2p and Ino80p, and to identify the exact residues involved in Ino80p acetylation. Ultimately, it would be of interest to determine the implication when neither Sfn2p nor Ino80p can be acetylated in a cell, as this would prevent any compensatory mechanisms from masking results.
Chapter 5
Concluding Remarks

Transcriptional activation is a highly regulated process in eukaryotes that requires the interplay of various co-activator proteins, such as histone acetylases and chromatin remodelers, at the promoter sequence of genes. The intricacies and implications of these interactions during the activation process have not yet been fully elucidated. In this study, I focused on the acetylation of chromatin remodelers, Snf2p and Ino80p, in the INO1 model of budding yeast.

Snf2p has been shown to be acetylated, but the implications of this post-translational modification had yet to be examined. Here I determined that this acetylation is required for Snf2p dissociation from the INO1 promoter (Figure 3.3). Through growth analyses and mRNA analyses I then showed that this acetylation is not required for INO1 transcriptional activation or polymerase recruitment (Figures 3.4, 3.5, and 3.11). In its absence, however, I observed a significant difference in the occupancy of co-activators at the promoter, as chromatin remodelers, Snf2p and Ino80p, accumulated, while histone acetylase, Esa1p, decreased (Figures 3.3, 3.6, and 3.9). Based on our previous work in which Ino80p was shown to be recruited prior to Snf2p (Ford et al., 2008), this modified occupancy may be due to the Ino80p being trapped by the accumulated Snf2p that arrived after Ino80p. With both bulky chromatin remodeler complexes accumulated at the promoter, Esa1p may not have as much access to the region and thus is unable to occupy the promoter as much as it usually would. The reduced Esa1p and accumulation of Ino80p may also be connected to the data discussed in chapter 4, where I not only demonstrated that Ino80p becomes acetylated, but also narrowed down the location of targeted residues and determined this acetylation is due to the actions of Esa1p (Figures 4.6 and 4.16). Since I demonstrated that Esa1p acetylates Ino80p, the decreased presence of Esa1p when unAcSnf2p accumulates, means reduced ability for the Ino80p to become acetylated and thus
reduced ability to dissociate from the promoter. The identification of Esa1p as the HAT responsible for acetylating Ino80p, along with our results that Snf2p must be acetylated to dissociate from the *INO1* promoter otherwise both remodelers accumulate, help to explain our previous data in which both chromatin remodelers (Snf2p and Ino80p) accumulated at the *INO1* promoter in either HAT mutant strain, *gcn5Δ* or *esa1mt* (Konarzewska et al., 2012).

Our findings also suggest that this acetylation-driven mechanism of dissociation is critical to the regulation of genes elsewhere as remodelers are necessary to help activate a plethora of genes, but are rather rare with in the cell, and thus appear to need a highly regulated recycling process. In this study, I revealed that when chromatin remodelers are highly occupying the *INO1* promoter, the copper toxicity defense pathway, which is dependent upon those same remodelers for activation through *CUP1* regulation, is unable to properly function and protect the cells. In other words, our results suggest that Gcn5p must acetylate Snf2p, and Esa1p must acetylate Ino80p, to allow the remodelers to dissociate from the *INO1* promoter. Once freed from the *INO1* promoter region, it is possible that the remodelers can mobilize at the *CUP1* promoter (Figure 5.1), which is the gene coding for the production of a metallothionein that binds free copper to protect yeast cells from toxicity. Ultimately, our findings for Snf2p and Ino80p have revealed insight into the mechanism of chromatin remodeler acetylation and its implications in gene expression regulation.
Figure 5.1: Our model of chromatin remodeler recycling

Ino80p is acetylated by Esa1p, while Snf2p is acetylated by Gcn5p, which promotes the dissociation of the remodelers from the INO1 promoter so they can mobilize and activate other genes, including CUP1.
REFERENCES


