THE ROLE OF UPSTREAM ACTIVATING FACTOR IN SUPPRESSING POL II rRNA TRANSCRIPTION IN *SACCHAROMYCES CEREVISIAE*

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DEDICATION

I dedicate this dissertation to Prapti. Thank you for making me stronger and always believing in me.

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ABSTRACT

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Ribosome synthesis is the most resource and energy – intensive process in all eukaryotic cells and is tightly coupled with growth rate. In addition, defects in synthesis and assembly of ribosomal RNA (rRNA) and ribosomal proteins result in G1 arrest and cell death (Bernstein & Baserga, 2004). As the rate limiting step in ribosome synthesis, rRNA transcription is tightly regulated on many levels. RNA polymerase (Pol I) transcribes the ribosomal DNA (rDNA) to generate a 35S ribosomal RNA (rRNA) precursor which is post-transcriptionally modified to mature 18S, 5.8S, 28S rRNAs (Warner, 1999). However, under chronic stress conditions when Pol I transcription is repressed, rRNA can also be synthesized by RNA polymerase II (Pol II) using a cryptic promoter overlapping the Pol I promoter. This phenomenon of rRNA synthesis by Pol II is termed as *polymerase switch* (Conrad-Webb & Butow, 1995). Since this process is conserved throughout eukaryotes including humans and plants, this phenomenon may play a universal role in the regulation of rRNA. Because the Pol I transcription factor, upstream activating factor (UAF), is known to generate rDNA chromatin inhibitory to Pol II during non-stress conditions, we hypothesized that UAF inhibited the polymerase switch during

normal nitrogen conditions and that this inhibition is released during nitrogen deprivation, facilitating the switch. During nitrogen deprivation, UAF steady state levels decreased 2-fold and UAF binding to the rDNA promoter also decreased. Consistent with our hypothesis, UAF subunits H3 and H4 are differentially modified upon nitrogen deprivation with an increase in H3K4 and H3K36 methylation and a decrease in acetylation at H4K5. Contributing to the inhibitory chromatin structure in non-stress conditions, Pol I interacting protein Hmo1 represses polymerase switch as determined by reporter gene assays; whereas, Sir2 does not influence the polymerase switch. Furthermore, transcriptional repressors binding to the Pol II rDNA promoter recruit Ssn6-Tup1 to further repress the Pol II mediated transcription. Thus, during non-stress conditions, UAF triggers the assembly of Pol II inhibitory chromatin and recruitment of HmoI. This inhibitory chromatin is enhanced by the recruitment of the Ssn6-Tup1 repressor. This work has enhanced our understanding of the Pol I regulation during stress conditions and the role Pol II rRNA synthesis plays in overall regulation of ribosome synthesis upon stress.

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ABBREVIATIONS

Amp^r	ampicillin resistance gene
ANOVA	analysis of variance
CF	core factor
ChIP	chromatin immunoprecipitation
CoIP	co-immunoprecipitation
Gln3	glutamine metabolism 3
HDAC	histone deacetylase complex
Hmo1	high mobility group protein 1
HMT	histone methyl transferase
IP	immunoprecipitation
LacZ	beta galactosidase gene
LB	Luria broth
LN	low nitrogen
MAPK	mitogen-activated protein kinase
Mig1	multicopy inhibitor of galactosidase gene expression
Nrg1	negative regulator of glucose repressed genes 1
NTS	non-transcribed spacer
OD	optical density
PEG	polyethylene glycol
PIC	pre-initiation complex
rDNA	ribosomal DNA
RENT	regulator of nucleolar silencing and telophase exit
rRNA	ribosomal RNA
SC	synthetic complete
SEM	standard error of the mean
Sir	sirtuin protein
Sko1	suppressor of kinase overexpression 1
Ssn6	cytochrome C
Sut1	sterol uptake 1
TAP	tandem affinity protein tag
TBP	TATA binding protein
TOR	target of rapamycin
Tup1	dTMP uptake factor 1
UAF	upstream activating factor
UBF	upstream binding factor
UE	upstream element
WCE	whole cell extract
WT	wild type
YPD	yeast peptone dextrose

CHAPTER I

INTRODUCTION

All cells encounter challenges for their survival, one of the most crucial challenges is nutrient exhaustion in the environment. Nutrient availability regulates cell growth and proliferation. Therefore, cells must balance growth with the ability to adapt quickly upon abrupt environmental changes (Gottesman & Maurizi, 2001). *Saccharomyces cerevisiae* (budding yeast) regulates its cell division and growth according to the nutrient availability and environmental conditions. Nutrient depletion leads to an alteration in developmental programs ranging from filamentous development to sporulation during extreme starvation. In yeast, nutrients not only act as substrates, but also serve as signals regulating growth (Broach, 2012).

In yeast, the TOR and PKA signaling pathways signal nitrogen and glucose availability, respectively, activating Pol I and Pol III mediated ribosome synthesis and growth processes. The glucose repression pathway, nitrogen catabolite pathway, and phosphate regulation redirect responses when nutrients become limiting. Together these pathways are necessary in regulating cellular metabolisms during starvation (Conrad et al., 2014). These signaling pathways target rRNA synthesis as cell growth is tightly linked with ribosomes synthesis (Mager & Planta, 1991).

Ribosome Biosynthesis

One of the most crucial cellular processes to regulate is the synthesis of ribosomes. Synthesis of ribosomes is a multistep process that involves transcription and translation of more than 75 different ribosomal proteins, synthesis of four rRNAs and their assembly in the nucleolus (Woolford & Baserga, 2013). This process also requires a host of assembly factors to construct the ribosome. Once assembled, the completed ribosomes are transported to the cytoplasm to perform protein synthesis. Ribosome synthesis expends a large percentage of cellular resources; about 80% of a cell's total transcription apparatus is dedicated to ribosome synthesis. Consequently, ribosome biosynthesis is under strict regulation and linked to environmental challenges, growth rate, and availability of nutrients (Warner, 1999; Woolford & Baserga, 2013).

rDNA Structure

The rate limiting step of ribosome synthesis requiring the highest degree of regulation is the transcription of the 35S rRNA, precursor of the 25S, 18S, and 5.8S rRNAs by RNA polymerase I (Pol I; Warner, 1999). In yeast, rDNA genes are located on the right arm of chromosome XII as tandem repeats (see Figure 1). The number of repeats can vary from less than 100 to 200 or more, and it depends on a cell's age, genetic background, and environmental conditions (Kobayashi, Heck, Nomura, & Horiuchi, 1998; Woolford & Baserga, 2013). The non-transcribed spacer (NTS) between 35S transcription units is split by the 5S rRNA gene into two regions, NTS1 and NTS2. Transcription of the 35S rRNA precursor by Pol I occurs from a rDNA promoter within

the NTS2 region, while the 5S gene is transcribed by Pol III. Within the Pol I rDNA promoter the upstream element (UE) and core element (CE) are essential for regulated initiation of transcription by Pol I *in-vivo* (Choe, Schultz, & Reeder, 1992). rRNA transcription by Pol I is terminated at one of the two terminator sites, T1 or T2, which are present downstream of the 25S rDNA at +93 and + 250 nucleotides respectively in NTS1. The T2 acts also as a fail-safe site for transcripts that fail to terminate at the +93 site.

Along with the promoter sequences, cis elements required for replication are contained within the NTS. In NTS2, there is an autonomous replication site (ARS) required for DNA replication. In addition, NTS 1 contains a replication fork block site (RFB), which arrests the replication fork to prevent collision of Pol I transcription with replication (Brewer & Fangman, 1988).

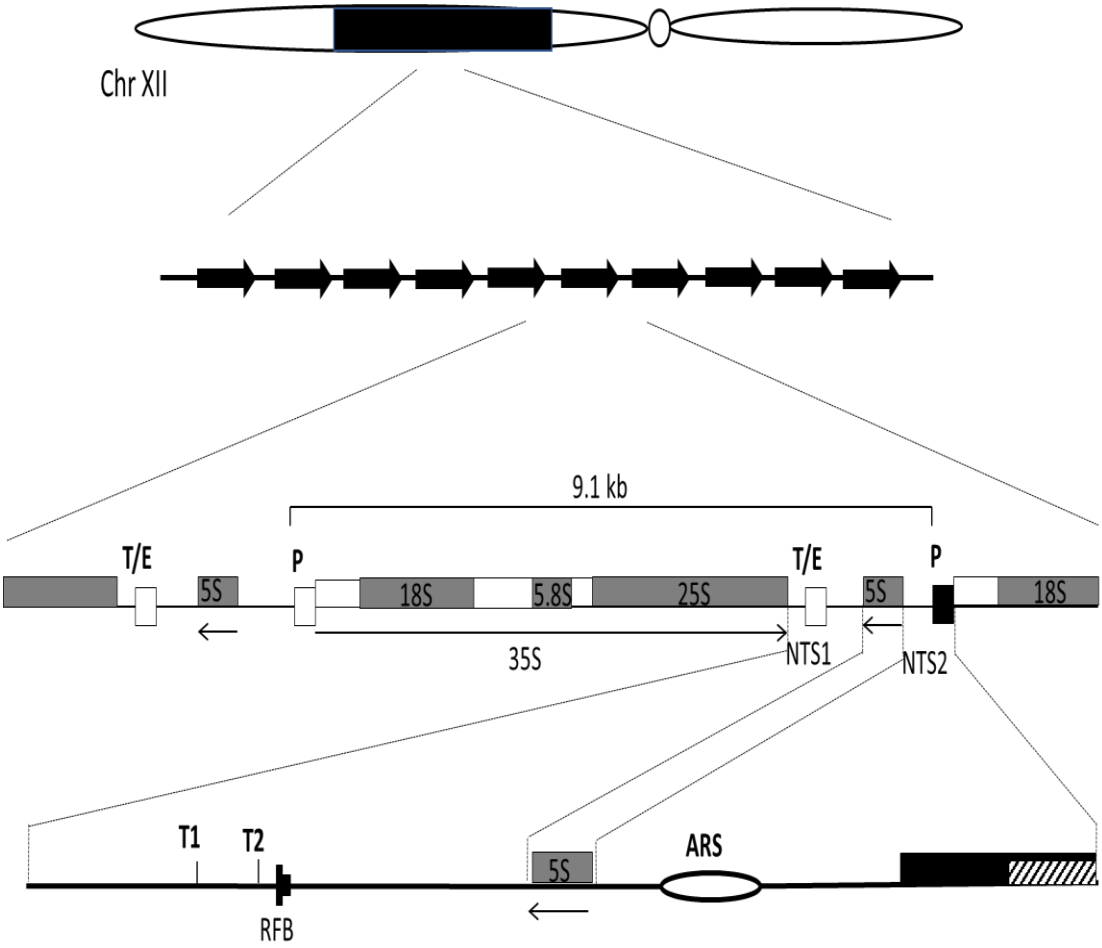

Figure 1. rDNA structure in yeast. The schematic diagram represents location of rDNA repeats on the Q-arm of chromosome XII. There are about 150 – 200 rDNA units arranged in tandem fashion. Each rDNA unit is 9.1 kb and is comprised of a 35S rRNA gene normally transcribed by Pol I which is post-transcriptionally modified to 18S, 5.8S and 25S rRNA, and a 5S rRNA gene that is transcribed by Pol III. The 35S and 5S rRNA genes are separated by two non-transcribed spacer sequences NTS1 and NTS2. Within the NTS2 region the Pol I promoter (cross hatched box) contains the upstream element (UE) and the core element (CE). NTS2 also contains an Autonomous Replicating Sequence (ARS). The NTS1 has two terminators T1 and T2 which are positioned at +93 and + 250 bp downstream of the 35S gene; it also contains a Replication Fork Blocking (RFB) site. Transcription of 35S rRNA and 5S rRNA occurs in opposite directions by Pol I and Pol III respectively.

RNA Polymerase I Transcription of rDNA

Transcription of open rDNA by Pol I is regulated by formation of the pre-initiation complex (PIC). The first step requires binding of upstream activating factor (UAF) a multiprotein complex made up of Rrn5, Rrn9, Rrn10, Uaf30, and histones H3 and H4, to the upstream element (UE; see Figure 2; see Keener, Dodd, Lalo, & Nomura, 1997; Siddiqi et al., 2001). Binding of this UAF complex commits the template for transcription (Lalo, Steffan, Dodd, & Nomura, 1996) and allows the recruitment of core factor (CF), consisting of Rrn6, Rrn7, and Rrn11, by a series of protein-protein interactions (Keys et al., 1996; Keys et al., 1994) to the core element (CE). UAF subunit Rrn9 interacts with TBP allowing Rrn9 to bind CF subunit Rrn7. TBP stabilizes the complex by binding CF's Rrn6 (Lalo et al., 1996; Lin et al., 1996; Steffan, Keys, Dodd, & Nomura, 1996). The binding of TBP, UAF and CF complexes to the rDNA promoter recruits Pol I-Rrn3p resulting in formation of the preinitiation complex (PIC) and leading to stimulated transcription of 35S rDNA. However, in the absence of UAF, basal level of Pol I transcription (~10%) occurs in the presence of CF, Rrn3p and Pol I (Keener, Josaitis, Dodd, & Nomura, 1998; Keys et al., 1996; Steffan et al., 1996) suggesting that UAF is required for maximal and regulated Pol I transcription.

Upon PIC formation (see Figure 2), elongation by Pol I is facilitated by Hmo1 (High Mobility Group protein) binding the coding region since actively transcribed repeats are devoid of nucleosome (Merz et al., 2008; Schneider, 2012). Pol I subunits Rpa49 and Rpa12.2 aid in elongation and act as functional analogs of Pol II elongation

factors (Kuhn et al., 2007). Additionally, the Paf1 complex (Paf1C) has been shown to be associated with Pol I and to aid transcription elongation (Y. Zhang, Smith, Renfrow, & Schneider, 2010).

The termination of Pol I mediated transcription requires the binding of Reb1 and/or Nsi1 to the terminator. *In-vivo* studies have shown that in the absence of Reb1 /Nsi1 binding site at the 3' end of the ribosomal coding region eliminate 3' end formation (Kang, Yokoi, & Holland, 1995). In addition to the Reb1 binding site, termination also requires a poly T rich sequence of several nucleotides to allow release of Pol I (Lang & Reeder, 1995). Following termination, Rrn3-Pol I components are released and recycled to form activated Rrn3-Pol I that can be recruited to rDNA promoter bound UAF – CF complex to initiate another round of rRNA transcription (Russell & Zomerdijk, 2005).

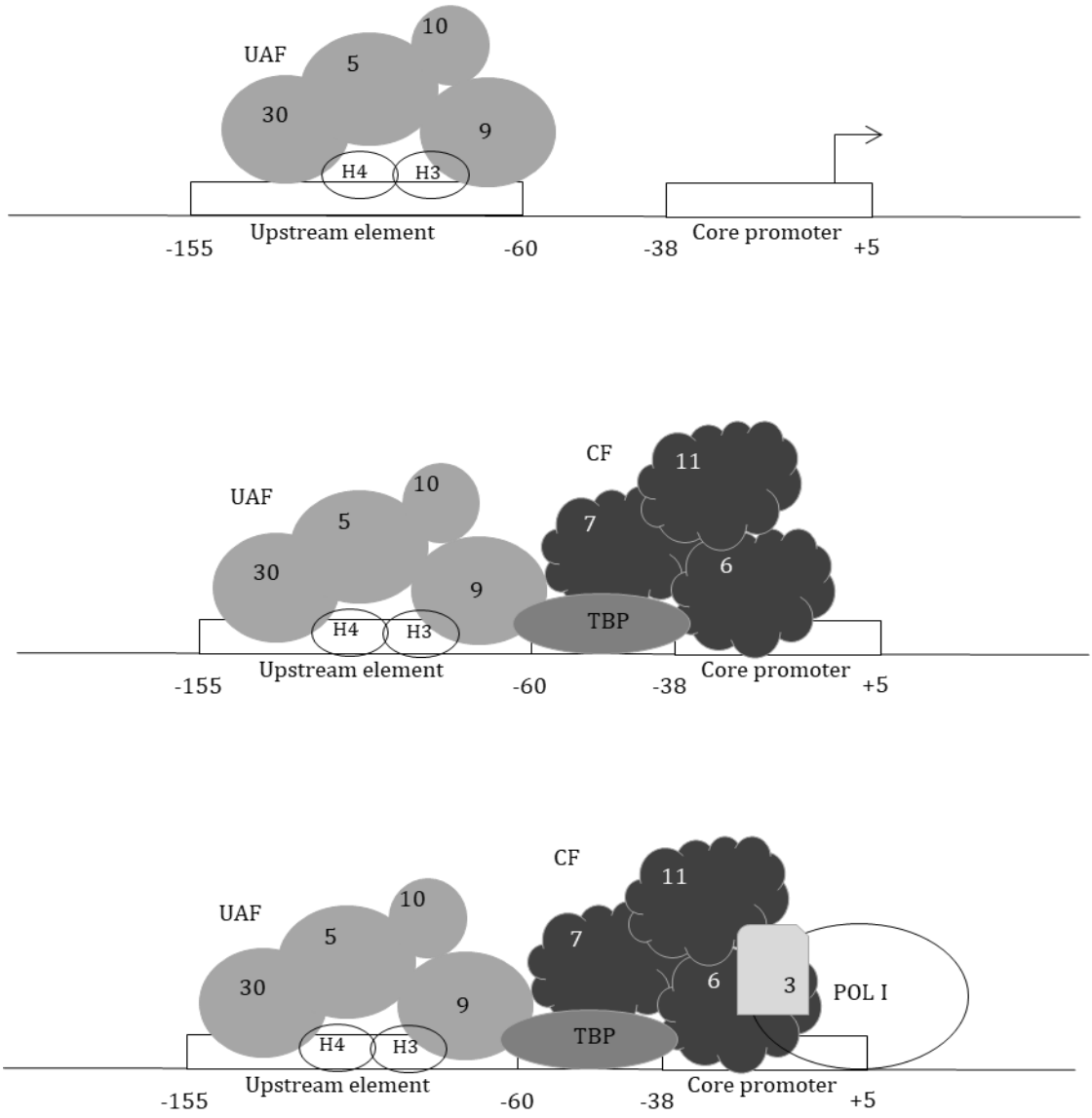

Figure 2. Yeast Pol I transcription initiation. UAF binds to the upstream element to commit the template to the process of transcription. UAF interacts with the core factor (CF) via Rrn9 - Rrn7 respectively. Core factor binds to the core promoter and TBP and recruits the Pol I – Rrn3 complex which interacts with the Rrn6 of core factor. These interactions are necessary for regulated transcription of rDNA. Figure adapted from Nomura, 2000.

Polymerase Switch

In yeast strains lacking mitochondrial DNA, 35S rRNA is synthesized by Pol II as well as Pol I. This Pol II transcribed rRNA is capable of supporting life in the absence of Pol I's second largest subunit, Rpa135 (Conrad-Webb & Butow, 1995). However, strains with deletions of UAF genes *RRN5, RRN9* or *RRN10* survive by Pol II transcription of the 35S rRNA from an overlapping Pol I – Pol II promoter or the Pol II rDNA promoter. This phenomenon has been termed polymerase switch and the strains were termed polymerase switched strains (PSW) (Conrad-Webb & Butow, 1995; Vu, Siddiqi, Lee, Josaitis, & Nomura, 1999).

This polymerase switch has also been observed in cells exposed to environmental stresses such as growth on non-preferred carbon sources, elevated temperature, or chronic nitrogen starvation (Ahmed S., 2001; Vallabhaneni, 2016). Recent studies in *Candida albicans* have shown that nutrient depletion results in an increase of a species of rRNA that maintains a 5'-cap and a poly-A tail, which are hallmarks of Pol II transcription (Fleischmann, Rocha, & Hauser, 2019).

Upstream Activating Factor in Polymerase Switch

UAF is a multiprotein complex consisting of Rrn5, Rrn9, Rrn10, Uaf30, and histones H3 and H4 subunits. The UAF complex has been shown to stimulate transcription of rDNA by Pol I. *In-vitro* assays of Pol I initiation have shown that in the absence of UAF, minimal levels (~ 10% of maximal transcription) of transcription can be performed by Pol I. However, *in-vivo* absence of either Rrn5, 9, or 10 subunit abolishes

Pol I transcription and leads to transcription of chromosomal rDNA using Pol II (Goetze et al., 2010). Interestingly, in the absence of the Uaf30 subunit, the UAF complex retains its stimulatory function for Pol I transcription (Siddiqi et al., 2001), but ~ 10% of rRNA is synthesized by Pol II. Together, this suggests that UAF plays a role in silencing Pol II transcription.

Chromosomal endogenous cleavage (ChEC) DNA foot-printing analysis has revealed that in the absence of UAF complex members, the chromatin architecture of the entire 35S rDNA region is drastically altered. Absence of UAF also caused the loss of Sir2, a histone deacetylase complex (HDAC) and Hmo1 from the rDNA. In contrast, binding of the UAF complex was not altered in the absence of Hmo1 or Sir2. Thus, UAF binding precedes binding of Hmo1 and Sir2 to the 35S rDNA (Vu et al., 1999).

The role played by UAF in the polymerase switch is unique. In cells lacking an essential Pol I subunit such as Rpa135 or Rrn3, or CF, the ability of Pol II mediated rRNA transcription to support cell growth and division was not observed (M. Oakes, Siddiqi, Vu, Aris, & Nomura, 1999). In order for this polymerase switch to occur, two key steps must happen: conversion of rDNA chromatin to a Pol II permissive structure and transcriptional activation.

We hypothesized that under stress conditions, signaling pathways trigger the activation of stress transcription factors and Pol II machinery. These same signaling pathways lead to post-translational modification of UAF subunits. This could ultimately

lead to alteration of rDNA chromatin structure and the release of the UAF complex from the UE allowing Pol II to synthesize rRNA (Keener et al., 1997).

During stress conditions, inhibition of the TOR pathway allows the activation of Rpd3, a HDAC required for Pol II permissive chromatin. Absence of Rpd3 abolishes the switch even in the absence of the UAF complex (M. Oakes et al., 2006). Thus, absence of UAF complex causes a local reorganization of rDNA promoter chromatin making it accessible to Pol II transcription factors and leading to transcription of rRNA genes by Pol II (Vu et al., 1999).

Role of High Mobility Group Protein Hmo1 in Polymerase Switch

In addition to transcriptional activation of Pol I, rDNA synthesis is also regulated by template accessibility. Only half of the rDNA repeats are actively transcribed by Pol I, the remaining inactive copies are bound by nucleosomes in a closed chromatin structure (Conconi, Widmer, Koller, & Sogo, 1989). Only actively transcribed rDNA has high mobility group (HMG) protein Hmo1 bound to the 35S rRNA promoter and 35S coding regions. Hmo1 belongs to the HMGB class of proteins and has a single HMG-box (Box B). A second HMG-like box (Box A) acts as a dimerization domain (Albert et al., 2013; Kamau, Bauerle, & Grove, 2004). In yeast, Hmo1 is quite abundant and exhibits a non-sequence specific DNA binding ability (Panday & Grove, 2017). Upon Hmo1 binding to the minor groove, the surrounding DNA is bent stimulating both the binding of Pol I and its transcriptional activity. The bending and remodeling property of Hmo1 could form an

architectural structure specific to Pol I binding (Mitsouras, Wong, Arayata, Johnson, & Carey, 2002; Thomas & Travers, 2001).

Hmo1 in *S. cerevisiae* plays a similar role to the Pol I upstream binding factor (UBF) in vertebrates (Merz et al., 2008; O'Sullivan, Sullivan, & McStay, 2002). Like HmoI, UBF contains HMG domains (Schnapp, Santori, Carles, Riva, & Grummt, 1994). Binding of HMG domains causes DNA to bend resulting in structural changes that could lead to selective recruitment of transcription factors (Hall, Wade, & Struhl, 2006). The binding activity of UBF is regulated by several tumor suppressor genes including p53 and retinoblastoma (RB) in higher eukaryotes (Ruggero & Pandolfi, 2003). In yeast during nutrient exhaustion or acute shift to low nutrient media, there is a rearrangement of Hmo1 to actively transcribed rDNA repeats suggesting that UAF may recruit Hmo1 (Wang, Mansisidor, Prabhakar, & Hochwagen, 2016). We propose that during nitrogen starvation, release of the UAF complex from the promoter region results in chromatin alterations allowing the polymerase switch; one of these changes is the release of HmoI and the loss of rDNA bending. The absence of UAF complex in normal nitrogen may lead to a similar increase in remodeling of chromatin allowing polymerase switch.

Chromatin Remodelers in Polymerase Switch

In yeast the rDNA, telomeres, and mating type loci have Pol II silenced chromatin structures that are established and maintained by the HDAC Sir2 (Bryk et al., 1997; Gottschling, Aparicio, Billington, & Zakian, 1990; J. S. Smith & Boeke, 1997). Sir2 belongs to a group of NAD-dependent enzymes called *sirtuins* found in prokaryotes as

well as in eukaryotes (Dutnall, & Pillus, 2001). In yeast, Sir2 associates with Sir3 and Sir4 to form a silenced complex at mating type loci, and telomeres. Within the rDNA, Sir2 associates with Net1 and Cdc14 to form a RENT complex (regulator of nucleolar silencing and telophase exit) and maintains silencing at rDNA reducing homologous recombination within rDNA repeats (Ghidelli, Donze, Dhillon, & Kamakaka, 2001; Tanny, Kirkpatrick, Gerber, Gygi, & Moazed, 2004). The RENT complex is recruited to the heterochromatin region and once bound, causes deacetylation of nucleosomal H4K16ac, H3K9ac and H3K14ac by Sir2 (Cesarini, D'Alfonso, & Camilloni, 2012; Hoppe et al., 2002; Huang & Moazed, 2003). The RENT complex associates with NTS2 via Net1 and Pol I causing silencing of Pol II transcription of reporter genes inserted into the NTS2 regions of rDNA (Li, Mueller, & Bryk, 2006; Moazed, 2001; Straight et al., 1999). Thus, Sir2 may contribute to the chromatin architecture inhibitory to Pol II rDNA transcription.

In contrast to the silencing mediated by Sir2, Rpd3 is a class I HDAC, which is an essential requirement for the polymerase switch and for transcriptional activation and repression of stress response genes (Sertil, Vemula, Salmon, Morse, & Lowry, 2007; Sharma, Tomar, Dempsey, & Reese, 2007; Vidal & Gaber, 1991). Absence of Rpd3 leads to an increase in silencing at rDNA and silent mating type loci, an antagonistic activity to Sir2 (Sun & Hampsey, 1999; Zhou, Zhou, Lenzmeier, & Zhou, 2009). Rpd3 interacts with several proteins to form two different types of complexes: Rpd3L (large) and Rpd3S (small) (Kasten, Dorland, & Stillman, 1997; Rundlett et al., 1996). During non-stress

conditions, the TOR pathway is activated and represses binding of HDAC Rpd3 to the promoters of genes. During nutrient starvation, deactivation of the TOR signaling pathway results in Rpd3 mediated deacetylation of histones (Goetze et al., 2010). Specifically, Rpd3 inhibits ribosomal DNA silencing by deacetylating histones H4K5 and H4K12. Rpd3 may activate stress induced polymerase switch by deacetylating transcription factors or UAF components at rDNA (Rundlett et al., 1996; Sun & Hampsey, 1999). Starvation studies in *Drosophila melanogaster* have shown an increase in Rpd3 binding at the rDNA promoter correlated with elevation of rRNA synthesis (Nakajima et al., 2016). Thus, binding of Rpd3 to the rDNA promoter may cause deacetylation on H3 and H4 of the UAF complex, which could lead to loss of UAF complex from rDNA promoters.

Histone Modifications at rDNA

There have been many reports of chromatin modifying enzymes affecting silencing at rDNA by altering the state of DNA or histones. One of the crucial modifications involves methylation of histones that regulate rDNA. Lysine on histones can be mono, di, or tri methylated on the same residue. However, not all the consequences of methylation marks at different residues have been clear. Interestingly, different methylation marks could be required for different types of regulation in a cell (Heintzman et al., 2007; Srivastava & Ahn, 2016). Histone methylation is catalyzed by enzymes known as histone methyltransferase (HMTs) and utilizes S-adenosyl-L-methionine (SAM) as a methyl group donor. Two HMTs Set1 and Set2 affect

methylation on histones H3K4 and H3K36, respectively, in yeast and are known to alter rDNA silencing. Methylation at H3K4 and H3K36 regulates silencing at rDNA (Srivastava & Ahn, 2016). Several sites on histones can be methylated, but only H3K4, H3K36, and H3K79 have been associated with transcriptional activation (Martin & Zhang, 2005). The master regulator for nutrient sensing, the TOR pathway, is necessary for Set1 and Set2 function (McDaniel et al., 2017). Set1 is an HMT that tri-methylates histone at H3K4 mostly at the promoters and is associated with accessible chromatin (Black, Van Rechem, & Whetstine, 2012). Set2 methylase negatively affects rDNA silencing by methylating histones at H3K36 in yeast (Ng, Robert, Young, & Struhl, 2003; Strahl et al., 2002). Set2 is required for stress-induced transcription fidelity and its absence leads to a shorter life span in both *S. cerevisiae* and *C. elegans* (McDaniel et al., 2017; Sen et al., 2015). During transcription of genes by Pol II, Set2 is recruited by the elongating phosphorylated Pol II resulting in tri-methylation of H3K36 of coding sequences. This methylation mark signals recruitment of Rpd3S deacetylase complex to suppress cryptic transcription from genes (Venkatesh & Workman, 2013). Therefore, H3K36 methylation at the rDNA promoter may also recruit Rpd3L leading to deacetylation of H3 and H4 within UAF(Joshi & Struhl, 2005; Keogh et al., 2005; Lee et al., 2018; Pokholok et al., 2005; Zhou et al., 2009)

Histone methylation has also been associated with transcriptional memory. When yeast cells are exposed to stressful conditions, they must reprogram their gene expression patterns to adapt with the changing environment. When cells were exposed a second time

to the same stressor, their survival improved and this phenomenon was shown to even pass down to their descendants (Fabrizio, Garvis, & Palladino, 2019). This research shows an increase in di and tri-methylation levels on H3K4 and tri-methylation on H3K36 at rDNA promoter could be important for polymerase switch.

Additional Corepressors in Polymerase Switch

An additional corepressor, Ssn6-Tup1 may be necessary for repression of Pol II transcription of rDNA may be recruited by UAF or independently by transcription repressors (Edmondson, Smith, & Roth, 1996). One of the first corepressor complexes to be identified in *Saccharomyces cerevisiae*, Ssn6-Tup1 is now known to repress more than 3% of the genes during non-stress conditions. This evolutionarily conserved corepressor represses transcription by multiple mechanisms. It can repress the target gene directly, by interacting with under-acetylated histones H3 and H4 (Redd, Arnaud, & Johnson, 1997). Repression can also take place by recruiting the Ssn6-Tup1 corepressor complex, to a transcription repressor bound to the promoter of a specific gene. For example, Ssn6-Tup1 binds at a specific site on the promoter of the *FLO1* gene and recruits HDAC Rpd3 which then deacetylates nucleosomes of the promoter and upstream region causing repression (Fleming, Beggs, Church, Tsukihashi, & Pennings, 2014). Interaction of Ssn6-Tup1 was not only seen with the gene specific repressor proteins, but also with activator proteins suggesting that the repression function of the corepressor complex can be due to masking of the interaction between the activator protein and other transcriptional activation machinery (Wong & Struhl, 2011). The Ssn6-Tup1 corepressor belongs to an

evolutionarily conserved family of proteins that are also present in worms, flies, and mammals (Doelling & Pikaard, 1996; Grbavec, Lo, Liu, Greenfield, & Stifani, 1999; Keleher, Redd, Schultz, Carlson, & Johnson, 1992). In the Pol II rDNA promoter region, many putative binding sites for Ssn6-Tup1 recruiting proteins are present, suggesting that Ssn6-Tup1 might be recruited by a potential repressor protein/s to inhibit Pol II binding during non-stress conditions. In fact, transcriptional proteins that are known to recruit the corepressor complex Ssn6-Tup1 to their respective genes and known to be induced during nutrient depletion have putative binding sites in the Pol II rDNA promoter as shown in (Figure 3). Probable binding sites were deduced using YeTFaSCo software (http://yetfasco.ccbr.utoronto.ca/). For example, the glucose repressible genes are repressed by binding of Mig1 to their promoters and simultaneously recruiting Ssn6-Tup1 corepressor which deacetylates the nucleosomes causing the *SUC2* gene's repression (Rodkaer & Faergeman, 2014). When a surplus amount of glucose is present, Mig1 localizes in the nucleus, binding to the promoter of the *SUC2* gene where it recruits Ssn6-Tup1 resulting in repression. When glucose is depleted, Snf1-mediated phosphorylation of Mig1 at S311 causes Mig1 to dissociate from the Ssn6-Tup1 complex and localize to the cytoplasm releasing repression (Ahuatzi, Riera, Pelaez, Herrero, & Moreno, 2007).

NTS 2 of rDNA Yeast

TTACATTTGGAGGGACGGTTGAAAGTGGACAGAGGAAAAGGTGCGGAAATGGCTGATTTTGAT
TGTTTATGTTTTGTGTGATGATTTTACATTTTTGCATAGTATTAGGTAGTCAGATGAAAGATGAAT
AGACATAGGAGTAAGAAAACATAGAATAGTTACCGTTATTGGTAGGAGTGTGGTGGGGTGGTA
TAGTCCGCATTGGGATGTTACTTTCCTGTTATGGCATGGATTTCCCTTTAGGGTCTCTGAAGCGT
ATTTCCGTCACCGAAAAAGGCAGAAAAAGGGAAACTGAAGGGAGGATAGTAGTAAAGTTTGAA
TGGTGGTAGTGTAATGTATGATATCCGTTGGTTTTGGTTTCGGTTGTGAAAAGTTTTTTGGTATG
ATATTTTGCAAGTAGCATATATTTCTTGTGTGAGAAAGGTATATTTTGTATGTTTTGTATGTTCCC
GCGCGTTTCCGTATTTTCCGCTTCCGCTTCCGCAGTAAAAAATAGTGAGGAACTGGGTTACCCGG
GGCACCTGTCACTTTGGAAAAAAAATATACGCTAAGATTTTTGGAGAATAGCTTAAATTGAAGTT
TTTCTCGGCGAGAAATACGTAGTTAAGGCAGAGCGACAGAGAGGGCAAAAGAAAATAAAAGTA
AGATTTTAGTTTGTAATGGGAGGGGGGGTTTAGTCATGGAGTACAAGTGTGAGGAAAAGTAGT
TGGGAGGTACTTC ATG

Nrg1, Sut1, Mig1, Sko1, UAF binding, Pol I start site

Figure 3. rDNA NTS2 sequence displaying possible binding sites for repressor proteins. Possible binding sites for putative repressor proteins known to recruit Ssn6-Tup1 corepressor complex are shown. It is possible that several proteins could bind at the NTS2 region of rDNA and recruit corepressor complex Ssn6-Tup1resulting in deacetylation of histones altering the chromatin to limit Pol II binding at the rDNA promoter.

A second potential repressor, Sko1 (Suppressor of Kinase), participates in regulating genes during hyperosmotic stress conditions by delocalizing Ssn6-Tup1 (Reinke, Baek, Ashenberg, & Keating, 2013). Sko1 binds to the *SUC2* gene and negatively regulates its expression. During hyperosmotic stress conditions, Hog1 MAP kinase phosphorylates Sko1, converting this repressor into an activator which recruits SWI/SNF complex and SAGA for transcription initiation (Proft & Struhl, 2002).

When non-fermentable carbon sources are unavailable, Snf1 signaling activates Sut1,(Sterol Uptake 1) a nuclear protein that belongs to the Zn[II]2Cys-6 family. Sut1, the regulator of sterol uptake and hypoxic gene expression, represses transcription factor

binding by physically interacting with the Ssn6-Tup1 complex (Regnacq, Alimardani, El Moudni, & Berges, 2001).

Similar to Mig1, Nrg1 (Negative Regulator of Glucose) is another transcriptional repressor involved in repressing STA genes which code for glucoamylase isozymes that break down starch to glucose (Park, Koh, Chun, Hwang, & Kang, 1999). When yeast cells are grown in an adequate level of glucose, Nrg1 binds to the promoter of *STA* genes recruiting Ssn6-Tup1. In the absence of the corepressor, Ssn6-Tup1, the repression by Nrg1 is completely abolished (Hanlon, Rizzo, Tatomer, Lieb, & Buck, 2011).

Unlike other transcriptional proteins described above, Gln3 (Glutamine metabolism) is a transcriptional activator of *NCR* (Nitrogen Catabolite Repressing) genes and is activated to allow utilization of alternative source of nitrogen. When the favored nitrogen source is present, repression of *NCR* genes occurs by phosphorylation of Gln3 protein and its sequestration from the nucleus (Conrad et al., 2014). During nitrogen availability, the TOR signaling pathway represses Gln3 in the NCR pathway.

Significance of Polymerase Switch

The phenomenon of the polymerase switch occurs not only in yeast, but has been observed in higher eukaryotes including humans and plants. When the human rDNA promoter was expressed in monkey cell lines, the reporter is transcribed by Pol II instead of Pol I (Smale & Tjian, 1985). A similar study conducted in plants also showed that Pol I promoter of *Arabidopsis thaliana* transfected into *Brassica oleracea*, led to transcription of *Arabidopsis thaliana* rDNA by Pol II (Doelling & Pikaard, 1996). A recent study in

Candida albicans showed that during nutrient exhaustion the cells had a unique population of rRNAs that had hallmark features of Pol II transcription. These rRNAs had a 5'cap and poly-A tails. These rRNAs were incorporated into functional ribosomes. Most intriguingly, these rRNAs were synthesized later in the growth phase close to nutrient exhaustion. (Fleischmann et al., 2019). Thus, polymerase switch is conserved throughout evolution and may serve as an alternative way to synthesis rRNA during unfavorable conditions.

Synthesis of rRNA is crucial for all cells, as these rRNAs along with ribosomal proteins are assembled into ribosomes in the nucleolus. Ribosomes are important for performing protein synthesis in the cell, the absence of which leads to cell death. In higher eukaryotes, synthesis of ribosomal RNA by Pol I and Pol III is directly regulated by growth signaling pathways including tumor suppressor p53 and retinoblastoma (RB) proteins. Pol I and Pol III dysregulation could ultimately play a role in tumorigenesis (Ruggero & Pandolfi, 2003). Recently, colon cancer patients showed an increased synthesis of pre-45S rRNA in tumor cells compared to normal surrounding tissue. Molecular studies have pinpointed this increase in the synthesis of rRNA by Pol I to the upregulation of the upstream binding factor (UBF) which binds to the promoter of the rDNA in humans and has stimulatory functions in Pol I synthesis similar to its yeast homolog UAF. Downregulating UBF expression resulted in the tumor cells becoming less aggressive in those patients (Tsoi et al., 2017).

The polymerase switch is an evolutionarily conserved mechanism that may be important for stress survival. This study evaluates the role played by chromatin architecture in polymerase switch and how the Pol II transcription of rDNA is inhibited during non-stress conditions. We have used nitrogen deprivation as a stressor. Previous studies demonstrated that nitrogen deprivation triggered polymerase switch and required the mediator, Rpd3 and stress signaling kinase Rim15 (Vallabhaneni, 2016). We were interested in studying the role of UAF in silencing Pol II transcription and chromatin modifications during non-stress and stress conditions. These modifications could ultimately lead to alterations in chromatin structure allowing rRNA synthesis by Pol II. Recruitment of chromatin modifiers at the rDNA promoter regions by UAF could prevent alteration of localized chromatin region preventing synthesis of rRNA by Pol II. Hence, a detailed knowledge of the mechanisms that regulate binding of UAF complex to the UE of promoter will help us understand the regulation of Pol I PIC formation, polymerase switch in yeast, and ultimately provide an insight in its regulation. We propose that in non-stress conditions UAF binding is the key determinant in establishing Pol I accessible rDNA chromatin. Furthermore, Hmo1, Sir2 and Ssn6-Tup1 act to enhance formation of the Pol I PIC. Upon nitrogen deprivation, signaling pathways trigger a reversal of these actions altering rDNA chromatin allowing access by Pol II machinery for rRNA transcription.

Specific Aims:

Aim 1

During non-stress conditions, the UAF complex binds to the RNA polymerase I promoter and commits the template for Pol I stimulated transcription of rDNA. Absence of the UAF complex or chronic stress such as nitrogen deprivation allows transcription of rDNA by RNA polymerase II; therefore, we hypothesize that the interaction between UAF and the promoter must change during low nitrogen to allow RNA polymerase II transcription. To investigate this, we addressed the following questions using chromatin immunoprecipitation (ChIP) and immunoprecipitation (IP).

a) Does UAF binding at the Pol I promoter differ between normal and low nitrogen conditions?
b) Does the Pol I rDNA chromatin show differential histone modifications during nitrogen deprivation?
c) Are UAF's histone H3 and H4 differentially modified during nitrogen deprivation?

Aim 2

The Hmo1 and Sir2 proteins play key roles in establishing the rDNA chromatin structure. To investigate their role in repressing Pol II rRNA synthesis, the following questions were examined in normal and low nitrogen using reporter gene assays.

a) In the absence of Hmo1p, does Pol II rRNA synthesis change as measured by a Pol II – rDNA – LacZ reporter gene?

b) Does absence of Sir2 alter Pol II rRNA synthesis as measured by Pol II reporter plasmid?

Aim 3

The general corepressor complex Ssn6-Tup1 represses transcription of many stress related genes transcribed by RNA polymerase II. In the absence of stress, Ssn6-Tup1 is recruited to the promoters of stress responsive genes by specific transcriptional repressors. Many of these repressors have potential binding sites in the rDNA promoter. To investigate the role of corepressor in the polymerase switch, the following were addressed using reporter gene assays.

a) In the absence of Ssn6-Tup1 complex, is there an increase in Pol II mediated rRNA transcription?
b) Does the absence of Ssn6-Tup1 candidate transcriptional proteins alter the Pol II mediated reporter activity?

CHAPTER II

MATERIALS AND METHODS

Yeast Strains Used in this Study

Strains used in this study are as listed in Tables 1 and 2 with their background and genotype.

Table 1

Yeast Strains Used for Reporter Gene Assays

Phenotype	**Strain Background**	**Genotype**
Wild Type	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*
sko1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 SKO1::KanMAX*
mig1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 MIG1::KanMAX*
nrg1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 NRG1::KanMAX*
sut1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 SUT1::KanMAX*
gln3Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 GLN3::KanMAX*
ssn6Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 SSN6::KanMAX*
tup1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 TUP1::KanMAX*
sir2Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 SIR2::KanMAX*
hmo1Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 HMO1::KanMAX*

uaf30Δ	BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 UAF30::KanMAX*

Table 2

Yeast Strains Used for Chromatin Immunoprecipitation Experiments

Phenotype	**Strain Background**	**Genotype**
Wild type	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
Rrn5-TAP	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RRN5::RRN5-TAP*

Table 3

Primers to calculate plasmid copy number

	Primer sequence	Tm	Ta	Expected Product size (bp)
pFES17	AMP5' - 5'-CTGCATAATTCTCTTACTGTCATGCC-3'	55.8	68.5	124
	AMP3' – 5'-GTATTGACGCCGGGCAAGAGC-3'	61.8		
Genomic DNA	TRP5' – 5'-GTTCAGGCACTCCGAAATACTTGG-3'	57.9	67	138
	TRP3' – 5'-GGAACTCTTGGTATTCTTGCCACG-3'	57.9		

Tm: Melting Temperature (°C), Ta: Annealing Temperature (°C), bp: base pairs

Table 4

Primers to verify TAP and pYW2A4Δ insertion

	Primer sequence	Tm	Ta	Expected Product size (bp)
Rrn5-TAP	Rrn5-TAP5' - 5'-AATGAGCGCTGAGTGGGTTG-3'	58.1	54.5	Non tagged: n/a Tagged: 750
	Rrn5-TAP3' – 5'-AACCCGGGGATCCGTCGACC-3'	64.2		
pYW2A4Δ	25S 5'- GGCGTCCTTGTGGCGTCGCTGAACC-3'	67.9	51.0	Non tagged: n/a Insert: 1500
	λ A 3' – 5'-CGGGAAATACTGATGCGCAG-3'	56.4		

Tm: Melting Temperature (°C), Ta: Annealing Temperature (°C), bp: base pairs

Plasmid DNA Isolation

E.coli containing plasmid DNA was grown on LB agar plates with ampicillin (0.5% yeast extract, 0.5% NaCl, 1% dextrose, 2% agar and 100 µg/ml ampicillin) at 37 °C from freezer stocks. A single isolated colony was inoculated and grown overnight in 100 ml of LB ampicillin broth at 37°C in a shaker. The next day cells were pelleted by centrifugation in a Sorvall RC 5C Plus centrifuge at 5000 *g* for 10 minutes at 4 °C. The pelleted cells were resuspended in 6 ml of freshly prepared lysis buffer (25 mM Tris pH 8.0, 10 mM EDTA, 15% Sucrose and 2 mg/ml lysozyme) and incubated on ice for 10 minutes. Freshly prepared alkaline lysis buffer (0.2M NaOH and 1% SDS) was added, gently mixed by inversion followed by incubation on ice for 10 minutes. To neutralize, 7.5 ml of 3 M sodium acetate pH 4.6 was added and mixed by inversion. The lysate was placed on ice for an additional 20 minutes. The lysate was then centrifuged at 9000 *g* for 15 minutes. The supernatant was transferred to a 30 ml polypropylene tube, 50 µl of RNase A (10 mg/ml) was added, and tubes were incubated at 37 °C for 30 – 45 minutes. The plasmid DNA was extracted twice using an equal volume of phenol: chloroform: isoamyl alcohol (50:40:10). The plasmid DNA was precipitated by adding twice the volume of cold 100% ethanol to the supernatant and incubating the mixture on ice for 60 minutes. The precipitated plasmid DNA was pelleted by centrifuging at 15,000 *g* in a microfuge for 15 minutes. The precipitated plasmid DNA was air dried and dissolved in 1.6 ml of sterile ultrapure water. To the resuspended DNA 0.4 ml of 4 M NaCl was added and mixed followed by addition of 2 ml of 13% PEG (polyethylene glycol, MW 8,000).

The mix was incubated on ice for 60 minutes followed by a centrifugation at 15,000 *g* in a microfuge for 5 minutes. The resulting pellet was washed twice with 70% cold ethanol and the pellet air dried to remove traces of ethanol. The pellet was dissolved in TE buffer pH 7.5 and checked for purity using a UV spectrophotometer at 260/280 nm (Spectronic Genesys 5). Restriction mapping was also performed to confirm the plasmids.

Plasmid pFES17 for β-Galactosidase Assay

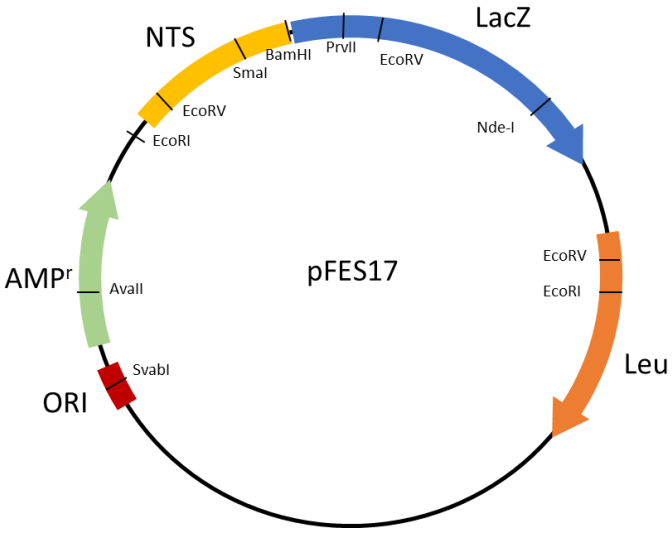

Figure 4. Reporter plasmid pFES17. The plasmid consists of the NTS2 region of rDNA cloned upstream of the *E.coli LacZ* gene. Both RNA Pol I and Pol II can bind to the promoter region on the NTS2 region and transcribe the *LacZ* gene, but only Pol II transcripts will be translated productively. This can be measured using a colorimetric assay. The enzyme activity is indirectly proportional to Pol II activity. Along with this, the plasmid also contains, a yeast selectable marker *Leu2* and *Amp*^{r} for bacterial selection.

The reporter plasmid 35S rDNA-*LacZ*, pFES17 consists of a NTS2 Pol I/ II promoter region upstream of the *E.coli LacZ* coding region. The plasmid also contains a nutrient selectable marker, *LEU2* gene for yeast and an ampicillin resistance gene as a

bacterial selection marker. The transcription of the *lacZ* gene can be performed by both Pol I and Pol II from the 35S rRNA promoter, but only the Pol II transcripts will be polyadenylated and thus translated. The estimation of *lacZ* activity can be performed using *ortho*-Nitrophenyl-β-galactosidase (ONPG) as a substrate and can be assayed spectrophotometrically (*Methods in Yeast Genetics*, 2019)

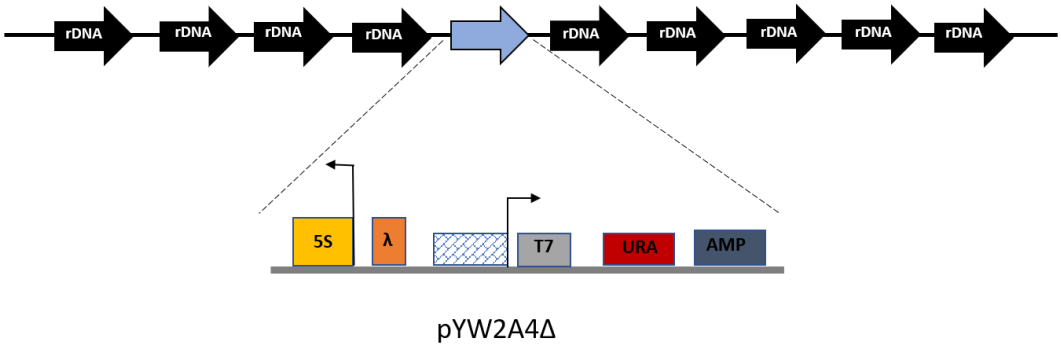

Figure 5. Diagram representing rDNA repeats on chromosome XII with integrated linearized plasmid pYW2A4Δ. This plasmid consists of a 5S gene with NTS1 and NTS2 regions, the native 35S gene is replaced with a T7 bacteriophage gene, a yeast selectable marker *URA3* and an *AMP* gene as a bacterial selection marker. Plasmid pYW2A4Δ was digested with *Hind*III restriction enzyme and transformed into ura3- yeast strains. Transformants were screened for integration into the rDNA repeat region by PCR (see table 4).

The second plasmid used in this study is an integration plasmid, pYW2A4Δ (Conrad-Webb, 1995), that is targeted to the rDNA. When integrated specifically into the rDNA, changes occurring at the chromosomal rDNA promoters can be investigated. The pYW2A4Δ plasmid consists of a bacteriophage T7 gene which, when integrated, replaces the 35S rDNA transcribed unit. This plasmid utilizes *URA3* as a yeast selection marker and an ampicillin resistance gene as a selection marker in bacterial cells. Additionally,

the plasmid also contains a unique λ bacteriophage sequence in the NTS1 region that is used in order to verify the orientation of the insert in rDNA (Conrad-Webb & Butow, 1995).

Transformation of Yeast Cells with Plasmid DNA

The wild type and specific gene knockout strains were inoculated on to YPD (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) plates directly from freezer stocks. Plates were incubated overnight at 30 °C. A loopful of cells were inoculated in 10 ml YPD broth and incubated in a shaker 250 rpm (Innova 4300) overnight. The next morning, cells were harvested by centrifugation at 4000 *g* for 5 minutes in a sterile 15 ml tube. The media was discarded, and the cell pellet was washed by resuspending it in 1 ml sterile distilled water. Cells were transferred to a sterile microfuge tube and pelleted at 13,000*g* for 1 minute in a microfuge. The supernatant was removed and over the cell pellet 240 µl of 50% PEG (polyethylene glycol 4000) was layered without disturbing the cell pellet. Over the PEG layer 36 µl of sterilized 1.0 M lithium acetate was pipetted followed by 50 µl of boiled single-stranded salmon sperm carrier DNA (2 mg/ml) and 34 µl of 0.8 – 1 µg of plasmid DNA. The contents of the tube were resuspended thoroughly using a micropipette and incubated at 42 °C for 45 to 60 minutes. After incubation, the cells were pelleted by centrifugation at 13,000 *g*, the transformation mixture removed using a micropipette followed by washing of cells by resuspending them in 1 ml sterile distilled water. Cells were pelleted again at 13,000 *g* for 1 minute, the supernatant removed, and cells were resuspended in 300 µl of sterile distilled water. The cells

suspension was spread on to synthetic complete minus leucine plates (0.67% yeast nitrogen base, 1% ammonium sulfate, 2% dextrose and 0.2% of SC-leu dropout synthetic mix without leucine and nitrogen base, US Biological) (SC- leu) for transformants and incubated at 30 °C for 3-5 days (Gietz, 2002).

Growth Conditions

The cells transformed with plasmid pFES17 containing the Pol II-rDNA-*LacZ* reporter gene were grown on YPD plates overnight and then transferred to synthetic complete media lacking leucine (SC-leu) plates. Wild type yeast and designated isogenic mutant strains transformed with pFES17 were inoculated into 10 ml of SC-leu broth and incubated overnight at 30 °C in a shaker (Innova) at 250 rpm. The following morning, 100 ml SC-leu media was inoculated from the precultures, incubated by shaking at 30 °C, and harvested once the optical density (OD_{600}) reached 0.8 – 1.0 using spectrophotometer (Spectronic Genesys 5). Cells for low nitrogen were initially grown in SC-leu broth until reaching an OD_{600} of 0.35 using spectrophotometer. These cells were collected by centrifugation at 4000*g* for 10 minutes followed by washing twice with 20 ml of sterile distilled water. The cell pellet was then resuspended in low nitrogen media and transferred to 100 ml of low nitrogen SLAD media in a 250 ml Erlenmeyer flask (0.17% yeast nitrogen base without amino acids, 2% glucose and 0.025% ammonium sulfate and 20 µg/L of uracil and histidine). This was incubated with shaking at 30 °C and harvested after 12 hours (Gasch et al., 2000; Pillai et al., 2003). For protein extracts, 4 aliquots of 7.5 ml of the above cultures were harvested in 15 ml sterile tubes and the cell pellet

resuspended in 300 µl of ice-cold protein breaking buffer (0.1 M Tris-Cl pH 8.0, 1mM dithiothreitol (DTT), and 20% glycerol) and stored at -80 °C until further use. To determine relative plasmid copy number, two aliquots 15 ml each of above cultures were harvested by centrifugation, supernatant discarded, and pellet stored at -80 °C.

Preparation of Crude Extract and β-Galactosidase Assays

RNA polymerase II activity at the plasmid rDNA promoter was confirmed by determining the β-galactosidase activity per plasmid copy number for cell extracts. Whole cell extracts were prepared by thawing cells containing protein breaking buffer on ice and transferring them to 1.7 ml microfuge tubes containing ~0.3 gm of glass beads (0.5 mm Biospec products), 12.5 ul of freshly prepared PMSF (40 mM phenylmethylsulfonylfluoride prepared in 100% isopropanol) was added to the samples. Cells were lysed using a mini beadbeater, (Biospec products) with preset settings for 3 minutes at 4°C. To the cell lysate, additional 250 µl of protein breaking buffer was added followed by another breakage round for 1 minute at 4 °C. The resulting crude extract was transferred to a fresh microfuge tube and centrifuged at 13,000 *g* in a microfuge for 15 minutes to eliminate cell debris and beads. The clarified whole cell extract was transferred to a fresh microfuge tube and stored at -80° C.

β-galactosidase activity from whole cell extracts was assayed by measuring hydrolysis of ONP (o-nitro phenyl) at 420 nm from ONPG (o-nitro phenyl β-D-galactoside). Each extract was assayed twice with each sample in triplicates. Multiple extracts for each sample were assayed to ensure accuracy of results. Enzyme activity for

each sample was normalized to total protein content which was determined by using a Bradford assay (1976) with samples assayed in triplicate (*Methods in Yeast Genetics*, 2019). Enzyme activity was calculated by using the formula:

Enzyme activity (nmoles/minute/mg of protein) =

$$\frac{OD_{420} \times 1.7}{[0.0045 \times \text{protein (mg)} \times \text{extract volume (ml)} \times \text{time (min)}]}$$

The plasmid pFES17 is a multicopy plasmid so normalization of enzyme activity to reporter gene copy number is necessary. This step helped determine whether differences in the β-galactosidase activity were due to regulation by RNA Pol II at the rDNA promoter and not merely due to differences in the plasmid copy number between strains (*Methods in Yeast Genetics*, 2019).

DNA Isolation for Plasmid Copy Number Normalization

The 15 ml cell pellets were transferred to a clean microfuge tube as previously mentioned, resuspended in 300 µl of DNA breakage buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 0.01 M Tris-Cl and 0.001 M EDTA), 300 µl of (1:1) phenol : chloroform and ~0.3 gm of glass beads. The cells were lysed using a bead beater for 5 minutes at 4°C. The crude extract was then centrifuged in a microfuge for 15 minutes. The aqueous phase was transferred to a microfuge tube followed by addition of 1 ml ice-cold 100% ethanol. The DNA was pelleted using a microfuge at 13,000 *g* for 2 minutes at room temperature and the supernatant discarded. The pellet was re-dissolved in 390 µl of Tris

EDTA (TE) (0.1 M Tris-Cl pH 8.0, 0.01 M EDTA pH 8.0) and 10 µl of (10 mg/ml) RNaseA followed by incubation at 37°C for 45 minutes. After incubation, 7 µl of 6 M ammonium acetate and 1 ml of ice-cold 100% ethanol were added to precipitate DNA. The precipitated DNA was collected by centrifugation at 13,000 *g* for 1 minute at room temperature. The DNA was washed twice using 70% ethanol and the pellet air dried for 20 minutes at room temperature. The pellet was then resuspended in 100 µl of TE buffer and quantified using UV spectrophotometer at 260/280 nm ratio. The final concentration of DNA was adjusted to 50 µg/ml with TE buffer for all samples (Hoffman & Winston, 1987).

Strain Confirmation by PCR

After every experiment a strain confirmation was performed to validate the strains. The PCR reaction was performed using a standard PCR machine (Eppendorf Mastercycler gradient) for 30 cycles and the reaction mixture consisting of 12.5 µl of Fail-safe buffer E premix, 1 µl of 10 µM 5' and 3' primers each listed in Tables 5, and 6, 0.4 µl of DNA polymerase enzyme (Fail safe enzyme mix, Cat.No.FSE51100), 6.1 µl of ultra-pure sterile water, 4 µl of the DNA (50 µg/ml). PCR products were subjected to agarose gel electrophoresis and strains were verified by comparing to the wild type gene size. The expected sizes are indicated in Table 5.

Table 5

Primers for strain verification

Strains	Primer sequence	Tm	Ta	Expected Product size (bp)
Ssn6	Ssn6 5' - 5'-CCCTTCCGATTATCAAAGCA-3'	52.5	47.5	WT: 3540
	Ssn6 3' - 5'-GGGAAACCGTTTTAAAGCAA-3'	51.6		Δ*ssn6*: 2250
Tup1	Tup1 5' - 5'-CAAGTTACCTTTCGCACACCA-3'	55.6	48.2	WT: 2376
	Tup1 3' – 5'-TTGTTTTAAAGCGTACCTGGA-3'	52.2		Δ*tup1*: 1866
Hmo1	Hmo1 5' - 5'-AAGTACCAATTAGTCCCAGCG-3'	54.6	49.0	WT: 926
	Hmo1 3' – 5'-CAAGCCCATGCTGCTATATT-3'	53.0		Δ*hmo1*: 1816
Uaf30	Uaf30 5' - 5'-TTGTTGCTTCCTACTTGCGTC-3'	54.0	50.0	WT: 1080
	Uaf30 3' - 5'-ACCCGGGAATGTTAACGTTAC-3'	54.0		Δ*uaf30*: 2016
Sir2	Sir2 5' - 5'-CGCCAGTTGCGGTAAAATAT-3'	53.6	49.6	WT: 2075
	Sir2 3' - 5'-GCCTTGCGTCTTTAGCAGATT-3'	55.8		Δ*sir2*: 2016

Tm: Melting Temperature (°C), Ta: Annealing Temperature (°C), bp: base pairs

Table 5 continued

Primers for strain verification

Strains	Primer sequence	Tm	Ta	Expected Product size (bp)
Mig1	Mig1 5' - 5'-CCCGGTAAAGCATTTCGAAGATAAG-3'	55.9	52.0	WT: 1654
	Mig1 3' - 5'-GTCTTTTGATTTATCTGCACCGCC-3'	56.7		Δ*mig1*: 1755
Sko1	Sko1 5' - 5'-TCATACGGTGATGGTTTTCG-3'	53.0	44.0	WT: 2616
	Sko1 3' – 5'-ATGCTGTAGGAAAATTTACGA-3'	48.0		Δ*sko1*: 2316
Sut1	Sut1 5' - 5'-TTTCTCTCTTTCCGGAACAGG-3'	54.5	50.5	WT: 2078
	Sut1 3' – 5'-CAGGGCCAGTCAGTTTAAGAA-3'	54.8		Δ*sut1*: 2816
Nrg1	Nrg1 5' - 5'-CTCCACCTCGTTTACAGTTT-3'	52.3	47.2	WT: 1090
	Nrg1 3' - 5'-TTGCTTCCGTTTTTTTGACA-3'	51.2		Δ*nrg1*: 2016
Gln3	Gln3 5' - 5'-CAGTAGCCCATCCCACAATAA-3'	54.5	50.0	WT: 2376
	Gln3 3' - 5'-AATGCTCAGGATTGTGGTCA-3'	54.3		Δ*gln3*: 1818

Tm: Melting Temperature (°C), Ta: Annealing Temperature (°C), bp: base pairs

Real Time PCR to Determine Plasmid Copy Number

The plasmid copy number was determined for each strain under normal and low nitrogen growth conditions. Relative copy number was calculated by comparing the average (cycle threshold) Ct value for the ampicillin resistance gene (Amp^r) on the plasmid to the genomic tryptophan *TRP1* gene. The plasmid copy number was normalized to wild type cells grown in normal nitrogen conditions. Real time PCR reaction mix consisted of 12.5 µl of 2X SYBR green premix (iTaq Universal SYBR green supermix 172-5124, BIO-RAD), 1 µl of 5' and 3' primers each (10 nM) (see Table 3, page 25), 5.5 µl ultra-pure sterile water, and 5 µl of the respective DNA (50 µg/ml). Samples were run in quadruplicate and the Ct values were determined using real time PCR (BIO-RAD CFX96 Real time system) for each primer set. Relative plasmid copy number was determined using the following calculations. Copy number was normalized relative to wild type copy number (1.0).

ΔCt Sample = Ct Amp sample – Ct Trp sample

ΔCt calibrator = ΔCt Amp WT normal nitrogen – ΔCt Trp WT normal nitrogen

Δ ΔCt = ΔCt sample – ΔCt Calibrator

Fold difference = $2^{-\Delta\Delta Ct}$

Chromatin Immunoprecipitation (ChIP) assay

To identify the presence of candidate proteins at the rDNA promoter, ChIP experiments were performed using Tandem Affinity Purification (TAP) tagged strains or wild type strains transformed with multicopy pFES17 or linearized integrated YW2 A4Δ

plasmids. Non-TAP tagged strains were used as negative controls for the experiment. The strains were subjected to growth in normal and low nitrogen media (200 ml) as described in growth conditions. The cells were harvested by centrifugation at 4000 *g* at 4 °C and washed twice with ice-cold phosphate buffered saline (1X PBS) (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 8.0). To perform protein-protein crosslinking, cells were resuspended and incubated with gentle shaking in 40 ml freshly prepared 10 mM dimethyl adipimate (DMA) in ice-cold PBS with 0.25% DMSO (Kurdistani & Grunstein, 2003). The cells are harvested by centrifuging at 4000 *g* at 4 °C and supernatant discarded.

Cells are washed in ice-cold PBS followed by resuspension in 50 ml of cold PBS. To the resuspended cells 1.35 ml of 37% formaldehyde was added and incubated along with gentle shaking at room temperature for 60 minutes to perform protein-DNA crosslinking. The crosslinking was terminated by addition of 2.5 ml of 2.5 M glycine to the flask and incubated for 5 minutes with gently mixing on a shaker at low speed setting. Crosslinked cells were collected by centrifugation at 4000 *g* for 5 minutes at 4 °C. Cells were then washed once with ice-cold 1.25 mM PBS-glycine followed by a wash with 20 ml ice-cold PBS. Cell pellets were stored at -80 °C until further use. The cell pellet was resuspended in 1 ml of freshly prepared ChIP lysis buffer with protease inhibitor cocktail (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate) with a protease inhibitor tablet (AEBSF 2 mM, Bestatin 130 µM, E-64 14 µM, Leupeptin 1µM, Aprotinin 0.3 µM)

(Sigma Cat# S8830). One mM PMSF (Sigma # 78830-5G), freshly prepared, was added to the individual tubes. Cells were lysed using 0.5 mm glass beads in a bead beater (Biospec) for 5 cycles of 1 minute followed by incubation on ice for 5 minutes. The whole cell extract was collected in fresh polystyrene tubes (Evergreen scientific #214-3721-010) for sonication. Sonication of chromatin was performed using a Q-sonica sonicator for 3 minutes at 100% amplitude with 20 seconds ON and 40 seconds OFF cycles to obtain DNA fragments ranging from 0.4 kb to 1 kb. An aliquot of 200 µl was removed for DNA isolation; following isolation, 30% of each sample was separated by electrophoresis on a 0.7% on agarose gel to confirm shearing efficiency.

If the fragmented DNA ranged from 0.4 kb to 1 kb, the remainder of the whole cell extract was centrifuged at 4500 *g* for 15 minutes at 4 °C and the supernatant transferred to a fresh tube. Protein estimation for the clarified lysate was performed using a BCA assay kit (Thermo scientific Cat# 23250). Immunoprecipitation reactions were performed overnight using lysate containing 400 µg of protein along with 1 % input controls. Each sample received 2 µg of rabbit anti-TAP antibody (Thermoscientific # CAB1001), or 5 µg of rabbit anti-H3K14 acetylation antibody (abcam # ab52946), or 5 µl of rabbit anti-H4K5 acetylation antibody (Millipore # ABE535), or 5 µg of rabbit anti-H3K4 di, tri-methylation antibody (abcam # ab6000), or 5 µg of rabbit anti-H3K36 tri-methylation antibody (abcam # ab9050) and incubated on a rocker overnight at 4°C. Normal rabbit IgG 2 µg (Cell Signaling Technologies # 2729S) was used as a negative control and 18 µg of rabbit anti-histone H3 (Cell Signaling Technologies # 4499) and 18

µg of rabbit anti-histone H2A (abcam # ab188312) were used as positive controls. Strains without the TAP tag were processed in parallel to control for non-specific binding. The following day, 30 µl of protein G magnetic beads, freshly washed twice in ChIP lysis buffer (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) were added to the lysates and incubated on a rocker at 4°C for 2 hours. The protein G beads, and bound complexes were collected using a magnet (BIO-RAD) and the unbound sample was removed using a micropipette, transferred to a fresh tube, and stored at -20 °C for further analysis. The beads were then washed thrice with ChIP washing buffer (50 mM HEPES-KOH pH 7.5, 350 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) with protease inhibitor tablet (AEBSF 2 mM, Bestatin 130 µM, E-64 14 µM, Leupeptin 1µM, Aprotinin 0.3 µM) and once with ChIP lysis buffer. Any protein-DNA complex was eluted by incubating at 65 °C with elution buffer (50 mM tris pH 8.0, 1% SDS and 10 mM EDTA). The eluted material was transferred to a fresh microfuge tube, proteinase K (100 µg) was added and allowed to digest for 2 hours at 65°C with gentle vortexing continuously (Mukhopadhyay, Deplancke, Walhout, & Tissenbaum, 2008). DNA was isolated using a kit and provided manufacturer's protocol (Qiagen QIAquick PCR purification kit # 28104).

Quantification of DNA was performed using real time PCR with appropriate primer sets (see Table 6). Real time PCR set up contained 12.5 µl of 2X SYBR green mix (BIO-RAD # 1725274), 1 µl of 5' and 3' primers each, 5.5 µl of sterile ultrapure water

and 5 µl of DNA. Samples were assayed twice in duplicates with each primer set to determine the C_t values for each sample and calculated using the percent input method. The C_t for 1% input was adjusted as shown below.

The percentage input method used for ChIP experiments calculations is as follows:

i. Adjusted Input to 100% = [Raw Ct input – (Log2 of 100)]

   Dilution factor 100 for 1% input is Log2 of 100 = 6.644

ii. Percent Input = 100*2^ (Adjusted input – Ct IP)

Table 6

Primers for Chromatin Immunoprecipitation experiment

	Primer sequence	Tm	Ta	Expected Product size (bp)
Genomic rDNA promoter	Critical 2 5' - 5'-GTAGCATATATTTCTTGTGTGAGAAAGG-3'	54	52.0	320
	Critical 2 3' - 5'-ACCTCCCAACTACTTTTCCTCACACTTGT-3'	61.1		
Integrated plasmid pYW2A4Δ	Critical 2 5' - 5'- GTAGCATATATTTCTTGTGTGAGAAAGG -3'	54	57.2	554
	T7 3' – 5'-TACAACGTGTGGAACTTCGG-3'	60		
Multicopy reporter pFES17	Critical 2 5' - 5'- GTAGCATATATTTCTTGTGTGAGAAAGG -3'	54	57.8	492
	LacZ 3' – 5'-CAGTCACGACGTTGTAAACGAC-3'	56.1		

Tm: Melting Temperature (°C), Ta: Annealing Temperature (°C), bp: base pairs

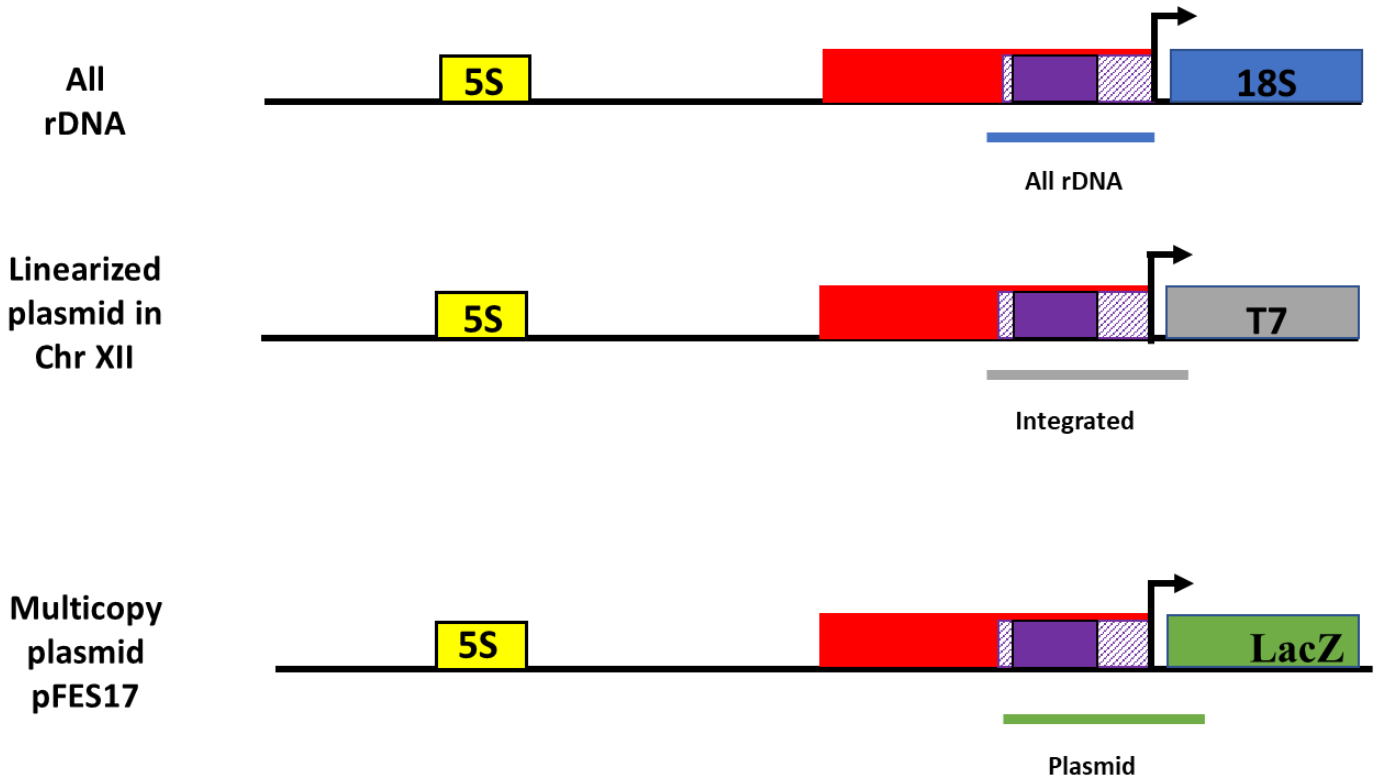

Figure 6 : Diagram representing rDNA regions analyzed for ChIP assay using specific primers

Yeast cells contain about 150 – 200 copies of rDNA tandemly and only 50% of them are active at a given time point. In order to study changes happening at a single active loci we introduced a linearized plasmid consisting of a unique T7 fragment. pFES17 multicopy plasmid represents events occurring at the rDNA promoter of the plasmid. To delineate between different regions different 3' primers were used with 5' Critical 2 primer which anneals upstream of UAF binding site. Using 5' & 3' critical 2 primers would amplify all the rDNA promoters. 5' critical 2 & 3' T7 primers would only amplify integrated linearized plasmid in the chromosome. 5' critical & 3' LacZ would amplify only the plasmid pFES17.

Co-Immunoprecipitation to study the UAF complex

Protein complexes were isolated using a TAP purification technique to assess any post translational modifications that may lead to UAF's dissociation from the promoter region. Wild type and TAP-tagged strains (1 liter) were grown under non-stress and stress conditions and cells were collected by centrifugation at 4000 *g* at 4 °C for 5 minutes once they reached an OD_{600} of 0.8 – 1.0. Cell pellet was washed with 20 ml ice cold lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 10% glycerol, 0.2% v/v Nonident P-40 (NP-40)) the pellet collected by centrifugation at 4000 *g* for 5 minutes and stored at -80 °C. Ice cold lysis buffer (1 ml) containing protease inhibitor cocktail and 1 mM PMSF was added to the pelleted cells along with 0.5 mm glass beads. Cells were ruptured using 5 cycles of a bead beater for 1 minute followed by incubation in an ice bath for 5 minutes. Lysate was sonicated using the QSONICA (Q800R) sonicator for 3 minutes with 20 seconds on and 40 seconds off at 100% amplitude at 4°C yielding fragments up to 1 kb in size. To collect the cell extract, the bottom of Eppendorf tubes were punctured with a needle (18G), placed in a 15 ml tube and centrifuged for 1 minute at 300 *g*.

Tandem Affinity Purification: IgG sepharose beads (GE healthcare, # 17-0969-01) were prewashed twice with 5 ml of ice-cold lysis buffer. The cell lysates were incubated with prewashed IgG sepharose beads for 2 hours at 4°C with continuous rotation (Labnet mini-labroller). After incubation, beads were collected by centrifugation at 100 *g* for 1 minute, the supernatant was transferred to fresh tubes and stored at -20 °C as the unbound

fraction. The beads were washed three times with 1 ml ice-cold lysis buffer, followed by two more washes in 1 ml of ice-cold (Tobacco Etch Virus) TEV cleavage buffer. After each wash the beads were pelleted by centrifugation at 100*g* for 1 minute.

TEV (Tobacco Etch Virus) cleavage: To the washed beads 1 ml of TEV cleavage buffer (150 mM NaCl, 10 mM Tris-Cl pH 8.0, 0.5 mM EDTA, 0.1% NP-40, (1 mM DTT just before use)) was added followed by addition of acTEV protease (10 units/ µl). The mix was incubated at room temperature for 2 hours followed by overnight incubation at 4 °C with continuous rotation. The beads were centrifuged at 100 *g* for 1 minute and the supernatant transferred to fresh microfuge tubes.

Calmodulin binding: The eluate after TEV treatment was added to calmodulin beads, which had been previously pre-washed with calmodulin binding buffer. These samples were incubated for 4 hours at 4 °C on a rocker. The beads were collected by centrifugation at 100 *g* for 1 minute, and the supernatant stored as the calmodulin unbound fraction. The beads were washed three times with calmodulin binding buffer (150 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM $MgCl_2$, 1 mM Imidazole, 0.1% NP-40, 10 mM β-mercaptoethanol and 2 mM $CaCl_2$). For elution, 100 µl of 1X Laemmli sample buffer was added to the beads and incubated at 100°C for 10 minutes in a water bath. The supernatant was collected by centrifugation at 15,000 *g* for 1 minute and stored as the purified sample at -80°C (Kaiser, Meierhofer, Wang, & Huang, 2008).

Western blotting: SDS-PAGE gels 8-20% pre-cast SDS acrylamide gels (BIO-RAD Cat # 456-8094) were run using 1X Tris-Glycine SDS buffer (25 mM Tris base, 192 mM

Glycine, 0.1% SDS) at 150 volts using electrophoresis unit (BIO-RAD Cat # 1658004) until the loading dye exits the gel. Wet transfer was performed using 1X Tris-glycine buffer (25 mM Tris base, 192mM Glycine, 20% methanol) on nitrocellulose membrane (*LI-COR*, # 926-31092) using electrophoretic transfer cell (BIO-RAD Cat # 1703930), overnight in 4 °C cold room at 20 volts. The efficiency of pull-down for each step was checked using western blotting by specific antibodies using conditions provided below.

Antibodies used for CoIP western blots

Antibodies for western blot analysis were rabbit anti-TAP (2.5 µg) in 5% non-fat milk tri-buffered saline (50 mM Tris-Cl pH 7.5, 150 mM NaCl) (0.05%) tween-20 (TBST), rabbit anti-H3 (9 µg) in 5% BSA TBST, rabbit anti-H3K4 di, tri-methylation (14.5 µg) in 5% BSA TBST, rabbit anti-H3K36 tri-methylation (4 µg) in 5% BSA TBST, mouse anti-H3 (1 µg) in 5% BSA TBST. Primary antibodies were incubated overnight at 4 °C. Next day blots were washed three times with 15 ml TBST buffer followed by incubation for 1 hour at room temperature with goat anti-rabbit 700 antibody (*LI-COR* IRDye® 800CW # 926-32211). Following incubation blots were washed two times with 15 ml of TBST and detected using ODYSSEY Clx imaging system (*LI-COR*, Inc).

Statistical Analysis

To establish if there was statistically significant difference in β-galactosidase activity between wild type and knockout strain, one-way ANOVA with Tukey's post-hoc test was performed. To reject a null hypothesis of no difference in enzyme activity between wild type and knockout strain and to accept alternate hypothesis that there is a

difference between wild type and knockout, significance of $p \leq 0.05$ was considered. To test for differences between normal (SC) and low nitrogen conditions (LN) in the same primer set in ChIP experiments, two-tailed independent student's *t*-test was performed. To reject null hypothesis and accept the alternate hypothesis, significance of $p \leq 0.05$ in the enrichment of the protein between non-stress and stress conditions was used.

CHAPTER III

RESULTS

Upstream Activating Factor (UAF) Commits the rDNA Template for Pol I Transcription

UAF is a multiprotein complex composed of six protein subunits Rrn5, Rrn9, Rrn10, Uaf30, histones H3 and H4. UAF along with core factor (CF), Rrn3 and RNA polymerase I, are required for stimulated rDNA transcription *in-vivo*. UAF is required for silencing transcription of rDNA by RNA polymerase II. UAF-defective mutants can survive by invoking the polymerase switch (Nogi, Vu, & Nomura, 1991). In order for this switch to occur, a mutation in UAF followed by chromosomal rDNA repeat expansion must occur. A null mutation in any protein of UAF complex can exclusively reduce RNA polymerase I transcription; however null mutations of RNA polymerase I, core factor or Rrn3 cannot lead to RNA polymerase II transcription of rDNA and are lethal. Therefore, UAF seems to play a key role in silencing RNA polymerase II transcription of rDNA (M. Oakes et al., 1999). The Uaf30 subunit of the UAF complex is important in recruiting the complex to the promoter of rDNA, and in its absence transcription of rDNA by RNA polymerase I goes down by 70% leaving only a few copies of rDNA open for transcription which are overloaded with RNA polymerase I and RNA polymerase II (Hontz et al., 2008). Thus, we hypothesized that during stress

conditions, signaling pathways could trigger a decrease in binding of UAF complex to the rDNA promoter region and hence allowing chromatin remodeling and transcription of rDNA by RNA polymerase II. Our results (see Table 7) indicate that there was a significant increase in β-galactosidase activity of *uaf30Δ* strains in regular nitrogen conditions compared to wild type strains reflecting an increase in Pol II rDNA transcription (see Figure 7). Also, there was equivalent transcription by RNA polymerase II in low nitrogen conditions.

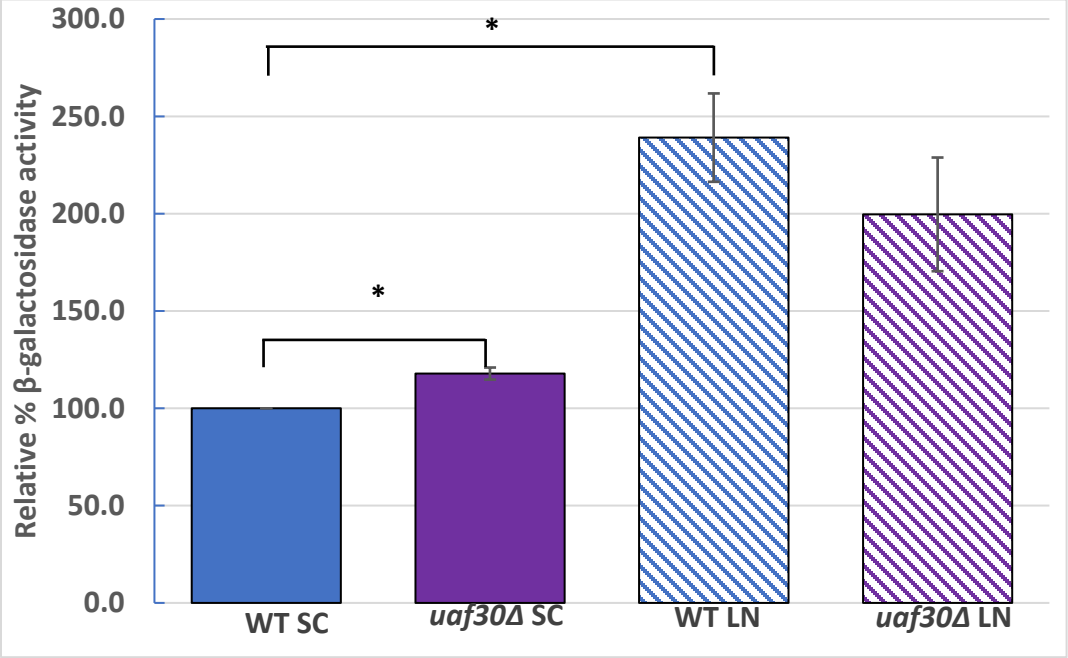

Figure 7. Increase in polymerase switch due to absence of Uaf30: Relative β-galactosidase activity was measured in wild type BY4743, *uaf30Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. Absence of Uaf30 resulted in a significant increase in Pol II activity in regular nitrogen (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was similar in low nitrogen conditions for *uaf30Δ* compared to wild type cells (striped bars) indicating that the Uaf30 could play a role in keeping the polymerase switch off during non-stress conditions. (one-way ANOVA at $p \leq 0.05$, $n = 3$).

Table 7

Relative β-galactosidase activity/ plasmid copy number for wild type and uaf30Δ grown in regular and low nitrogen media.

Strains	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0
WT LN	291.1	196.2	229.9	**239.1***	22.7
***uaf30Δ* SC**	114.6	113.6	125.4	**117.8***	3.1
***uaf30Δ* LN**	258.9	135.2	204.8	**199.6**	29.2

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. Average enzyme activity represents 3 independent experiments **#** compared to wild type cells in low nitrogen. (one-way ANOVA at $p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean

Saccharomyces cerevisiae cells undergoing logarithmic growth have between 100 – 150 copies of rDNA and approximately 50% of them are active (Warner, 1999). The inactive copies differ from the active ones in their chromatin organization. These differences in the chromatin structure can determine which 35S rRNA genes are transcribed and by which polymerase Pol I or Pol II (Merz et al., 2008). We were interested in analyzing the differences in the chromatin architecture and protein complexes bound to the promoter region of rDNA during stress versus non-stress conditions. To study this, we employed ChIP by utilizing strains in which individual genes are tagged with TAP. However, having only half of the chromosomal rDNA copies in open confirmation complicates the assay. Also, rRNA is transcribed by both Pol I and Pol II from the ectopic circular copies of rDNA (ERC) during mitochondrial dysfunction

(Conrad-Webb & Butow, 1995). To reduce the noise from inactive rDNA and to differentiate between the chromosomal and ectopic rDNA, strains were transformed with pFES17 circular episomal plasmid and a single open repeat was tagged by integrating pYW2A4Δ.

Mutation or absence of any UAF subunit abolishes all the transcription of rDNA by Pol I in turn allowing Pol II rDNA transcription. To investigate the difference in enrichment of the UAF complex at rDNA promoters between the non-stress and stress conditions, we grew a Rrn5-TAP tagged strain transformed with pFES17 and pYW2A4Δ both under normal nitrogen and low nitrogen conditions. We observed that there was a significant decrease in the occupancy of the UAF complex at the chromosomal rDNA locations. However, there was no significant difference in UAF binding at the rDNA-*LacZ* promoter region between the non-stress and stress conditions (see Table 8 and Figure 8).

Table 8

Normalized percent input values of non-tagged and Rrn5-TAP tagged strains using primers specific to the rDNA promoter region(s) as indicated in figure 1.

	WT SC (±SEM)	**WT LN (±SEM)**	**Rrn5-TAP SC (±SEM)**	**Rrn5-TAP LN (±SEM)**
All rDNA	0.05 (0.01)	0.01 (0.00)	1.40 (0.17)	0.55* (0.09)
T7	0.05 (0.01)	0.02 (0.01)	3.28 (0.59)	1.73* (0.25)
LacZ	0.07 (0.00)	0.02 (0.01)	0.19 (0.02)	0.22 (0.04)

All samples were significantly different from nontagged strains. * Independent student *t*-test was used to compare Rrn5-TAP binding in non-stress conditions to Rrn5-TAP binding in low nitrogen conditions of the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

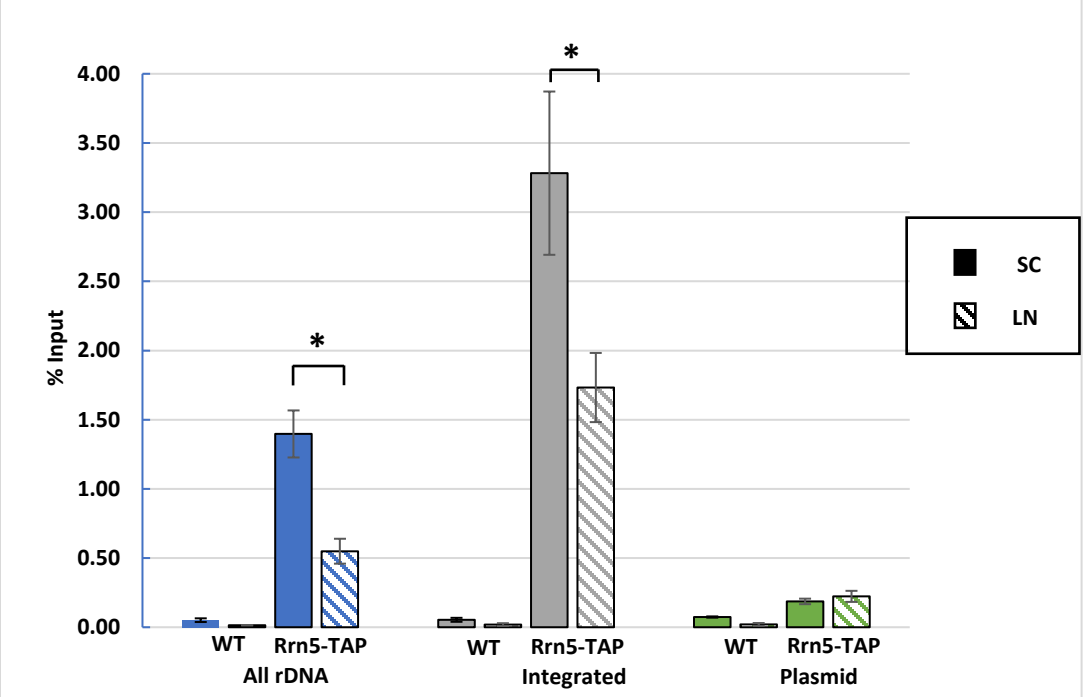

Figure 8. Decrease in UAF occupancy at the rDNA promoter(s) during nitrogen deprivation. Wild type non-tagged strains and Rrn5-TAP tagged strains were grown under normal nitrogen and low nitrogen conditions. Chromatin IP was performed using anti-TAP antibody for all untagged and tagged samples and subjected to qPCR. Occupancy of UAF complex was normalized to 1% input. * Independent student *t*-test was used to compare Rrn5-TAP binding in non-stress conditions to Rrn5-TAP binding in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Table 9

Percent input values of non-stress and stress conditions calculated for histone H3 using primers specific to the rDNA promoter region(s).

H3	rDNA (±SEM)	T7 (±SEM)	LacZ (±SEM)
WT SC	35.69 (2.30)	54.75 (3.25)	45.53 (2.42)
WT LN	34.03 (2.07)	44.48* (3.51)	36.78* (2.64)

Independent student *t*-test was used to compare H3 enrichment in non-stress conditions to H3 enrichment in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

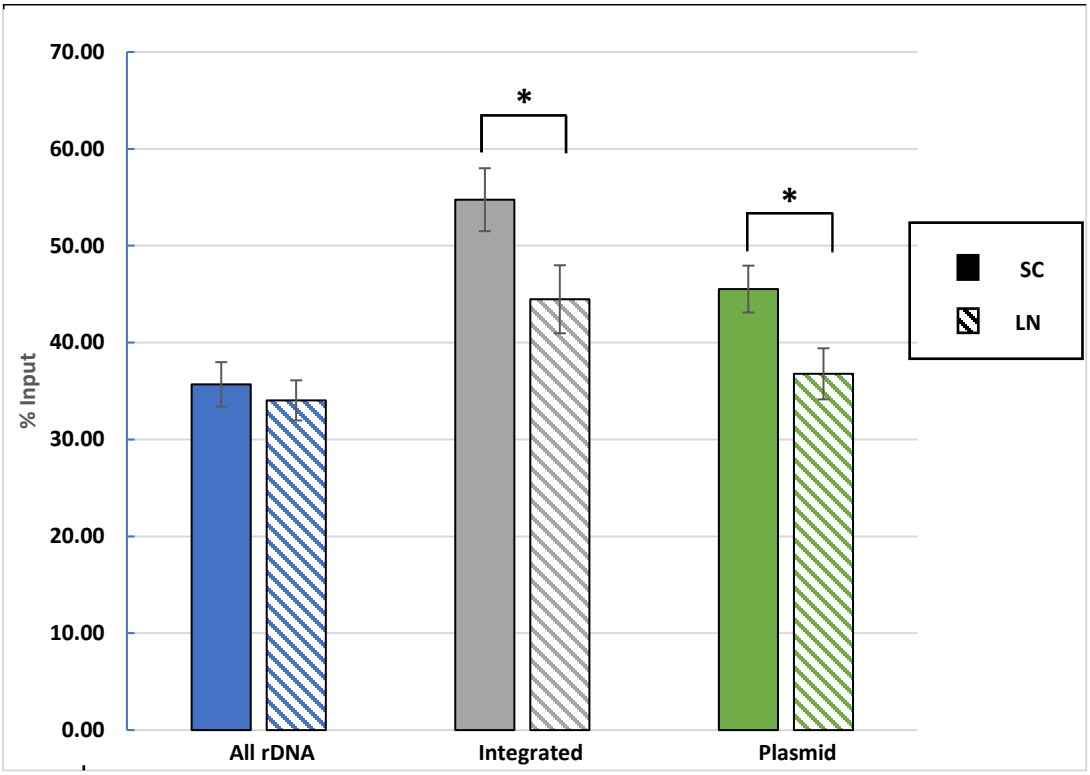

Figure 9. Histone H3 occupancy at the rDNA promoter(s) during nitrogen deprivation: Wild type non-tagged strains were grown under normal nitrogen and low nitrogen conditions. Chromatin IP was performed using anti-H3 antibody and subjected to qPCR. Occupancy of histone H3 is normalized to 1% input. * Independent student *t*-test was used to compare H3 occupancy in non-stress conditions to H3 occupancy in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

We further wanted to investigate if there were any changes in UAF components; histone H3 and H4 at the rDNA promoter. The ChIP data revealed that there was a significant decrease in histone H3 at the promoter of chromosomal integrated plasmid. To confirm this decrease was not due to an overall reduction of nucleosomes, we performed ChIP assay on H2A which is not part of the UAF complex. No changes in H2A levels were observed at the integrated plasmid. Also, no significant changes in histone H3 and H2A occupancy was observed at the overall chromosomal rDNA promoters. However, there

was a significant decrease in the levels of both histone H3 (see Table 9 and Figure 9) and H2A on the promoter of rDNA-*LacZ* plasmid (see Table 10 and Figure 10). This probably reflects the unique chromatin environment of the episomal plasmid as well as its potentially non-nucleolar location.

Table 10.

Percent input values of non-stress and stress conditions calculated for histone H2A using primers specific to the rDNA promoter region(s)

H2A	rDNA (±SEM)	T7 (±SEM)	LacZ (±SEM)
WT SC	2.51 (0.2)	3.91 (0.3)	4.74 (1.0)
WT LN	2.91 (0.2)	3.94 (0.4)	3.22* (0.8)

* Independent student *t*-test was used to compare H2A occupancy in non-stress conditions to H2A occupancy in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

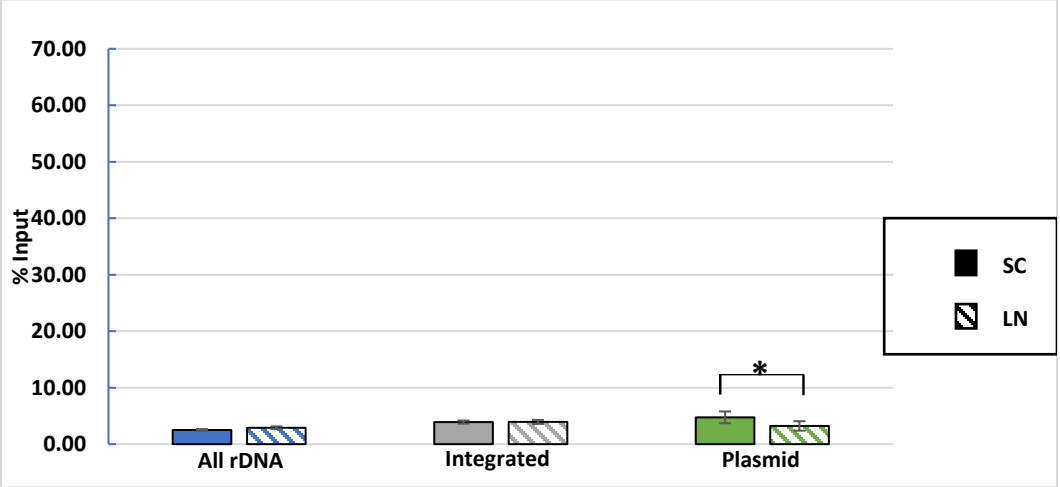

Figure 10. Histone H2A occupancy at the rDNA promoter(s) during nitrogen deprivation: Wild type non-tagged strains were grown under normal nitrogen and low nitrogen conditions. Chromatin IP was performed using anti-H2A antibody and subjected to qPCR. Occupancy of histone H2A is normalized to 1% input. * Independent student *t*-test was used to compare H2A enrichment in non-stress conditions to H2A enrichment in low

nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Histone Modifications at the rDNA Promoter

External environmental conditions trigger a cascade of signaling pathways that ultimately leads to changes in gene regulation which are crucial for cell survival. These changes necessitate alteration in the chromatin structure including several modifications of histone residues. These post-translational modifications (PTMs) are caused by several enzymes, ultimately leading to changes in rDNA promoter's architecture. Histone modifying enzymes can lead to PTMs like methylation, acetylation, and ubiquitination. We were interested in studying modifications brought to the rDNA promoter chromatin by complexes known to alter rDNA chromatin such as Rpd3 or Sir2 histone deacetylase complex and Set1 and Set2 histone methylases. These post-translational modifiers may alter the UAF complex binding to the rDNA promoter during stress conditions permitting transcription of rRNA genes by Pol II. To investigate this hypothesis, we performed ChIP assay using histone PTM specific antibodies on yeast cells grown in normal nitrogen and low nitrogen conditions.

Increased methylation on H3K4 at rDNA promoter(s) during stress conditions

We evaluated histone H3 lysine 4 residues for di and tri-methylation modifications. Methylation at this site is a result of Set1 activity on promoters of actively transcribed genes. We detected a significant increase in the levels of di and tri-methylation on the H3 lysine 4 site at the genomic rDNA promoters and on the integrated plasmid (see Table 11). However, there were no significant changes in the levels of

methylation on histone H3 lysine 4 sites at the *LacZ*-rDNA promoter (see Table 11 and Figure 11)

Table 11

Percent input values of non-stress and stress conditions calculated for H3K4 di me tri me antibodies and histone H3 using primers specific to the rDNA promoter region.

H3K4 di me tri me/H3	WT SC	WT LN (±SEM)
All rDNA	1.00	1.35* (0.07)
T7	1.00	2.10* (0.50)
LacZ	1.00	1.71 (0.33)

Ratios of histone modification to histone H3 was calculated and represented as relative to non-stress conditions as indicated in figure 11. * Independent student *t*-test was used to compare H3K4me2, 3 in non-stress conditions to H3K4me2, 3 in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

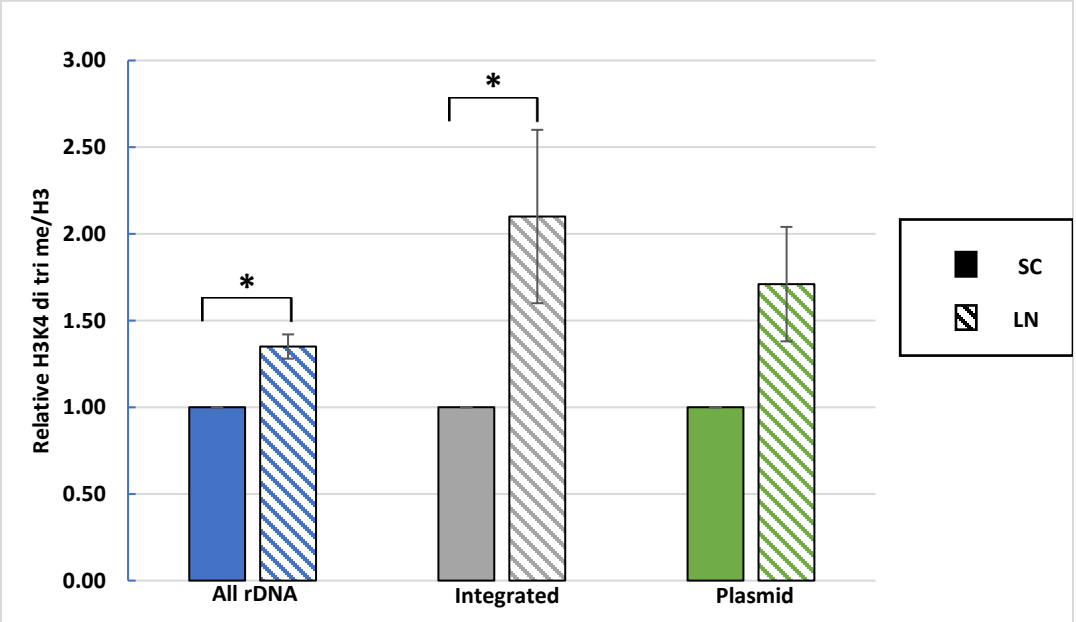

Figure 11. Increase in histone di & tri-methylation on H3K4 at promoters of genomic rDNA and integrated plasmid during nitrogen deprivation. Wild type strains were grown under normal nitrogen and low nitrogen conditions and subjected to IP using anti-H3K4

di me tri me antibody. Percent input calculated for each primer pair for histone modifications and total H3 and the ratios calculated to show relative enrichment of H3K4di me tri me at rDNA promoter(s). Independent student *t*-test was used to compare H3K4me2, 3 in non-stress conditions to H3K4me2, 3 in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Increased methylation on H3K36 at rDNA promoter(s) during stress conditions

Histone methylation was also investigated at rDNA promoters for the H3K36 site using anti-H3K36tri methylation antibodies. Methylation at this site is mediated by Set2 HMT and is associated with active Pol II transcription of a gene. We detected a significant increase in the tri-methylation levels on all the rDNA promoters, rDNA promoter of integrated plasmid as well as on the promoter of rDNA-*LacZ* reporter plasmid during low nitrogen conditions (see Table 12 and Figure 12).

Table 12

Percent input values of non-stress and stress conditions calculated for H3K36 tri me antibodies and histone H3 using primers specific to rDNA promoter(s).

H3K36 tri me/ H3	WT SC	WT LN (±SEM)
All rDNA	1.00	1.57* (0.27)
T7	1.00	1.41* (0.10)
LacZ	1.00	1.50* (0.06)

Ratios of histone modification to histone H3 calculated and represented as relative to non-stress conditions as indicated in figure 12. * Independent student *t*-test was used to compare H3K36me3 in non-stress conditions to H3K36me3 in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

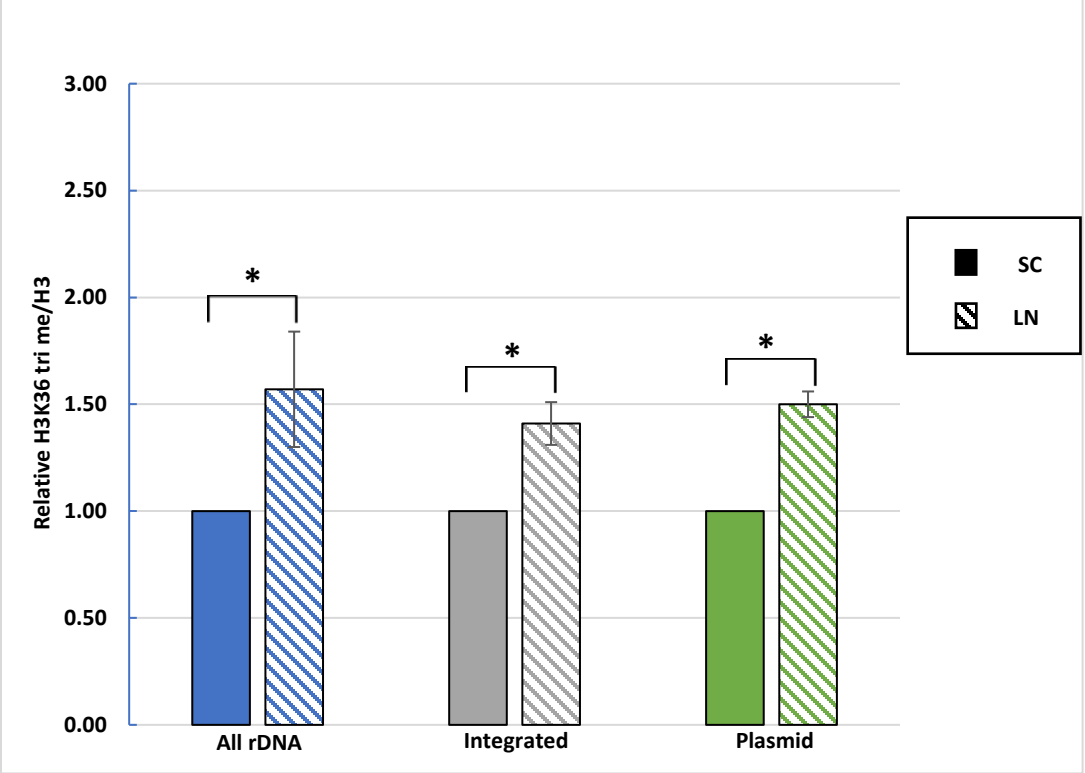

Figure 12. Increase in histone tri-methylation on H3K36 at rDNA promoter during nitrogen deprivation. Wild type strains were grown under normal nitrogen and low nitrogen conditions and subjected to IP using anti-H3K4 tri me antibody. Percent input calculated for each primer pair for histone modifications and H3 IPs and the ratios calculated to show relative enrichment of H3K4 tri me at rDNA promoter. * Independent student *t*-test was used to compare H3K36me3 in non-stress conditions to H3K36me3 in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Histone Acetylation at rDNA Promoter

Decrease in acetylation on H4K5 at rDNA promoter(s) during stress conditions

Histones H3 and H4 of the UAF complex are likely targets for modifications. Rpd3 is a known HDAC required for Pol II mediated rRNA synthesis. Here, we investigated levels of acetylations on known Rpd3 targets H3K4 and H3K14 by

performing ChIP assays. We detected a significant decrease in the levels of acetylation at H4K5 site for all three rDNA promoter regions (see Table 13 and Figure 13).

Table 13

Percent input values of non-stress and stress conditions calculated for H4K5ac antibodies and histone H3 using primers specific to the rDNA promoter region.

H4K5ac/ H3	WT SC	WT LN (±SEM)
All rDNA	1.00	0.66* (0.16)
T7	1.00	0.63* (0.15)
LacZ	1.00	0.57* (0.18)

Ratios of histone modification to histone H3 calculated and represented as relative to non-stress conditions as indicated in figure 13. * Independent student *t*-test was used to compare H4K5ac in non-stress conditions to H4K5ac in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

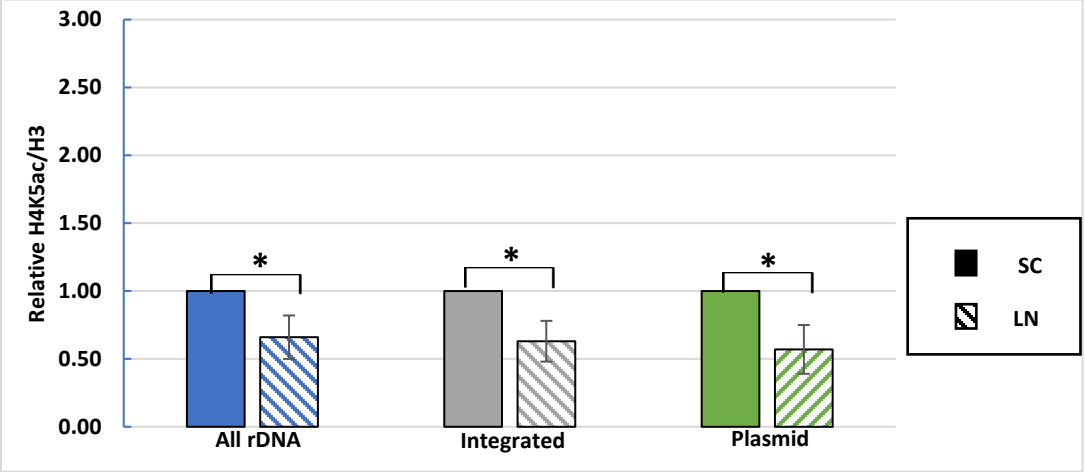

Figure 13. Decrease in histone acetylation on H4K5 at rDNA promoters during nitrogen deprivation. Wild type strains were grown under normal nitrogen and low nitrogen conditions and subjected to IP using anti-H4K5ac antibody. Percent input calculated for each primer pair for histone modifications and H3 IPs and the ratios calculated to show relative enrichment of H4K5ac at rDNA promoter. * Independent student *t*-test was used to compare H4K5ac in non-stress conditions to H4K5ac in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Decreased acetylation on H3K14 at genomic rDNA promoters during stress conditions

The analysis of acetylation on H3K14 loci indicated a slight decrease in the acetylation levels at the location overall for genomic rDNA promoter. However, there was no difference in the levels of acetylation at this site for the promotor of integrated or *LacZ* reporter plasmids (see Table 14 and Figure 14).

Table 14

Percent input values of non-stress and stress conditions calculated for H3K14ac antibodies and histone H3 using specific primers which amplify the rDNA promoter region.

H3K14ac/ H3	WT SC	WT LN (±SEM)
All rDNA	1.00	0.87* (0.03)
T7	1.00	0.96 (0.07)
LacZ	1.00	0.92 (0.07)

Ratios of histone modification to histone H3 were calculated and represented as relative to non-stress conditions as indicated in figure 14. * Independent student *t*-test was used to compare H3K14ac in non-stress conditions to H3K14ac in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

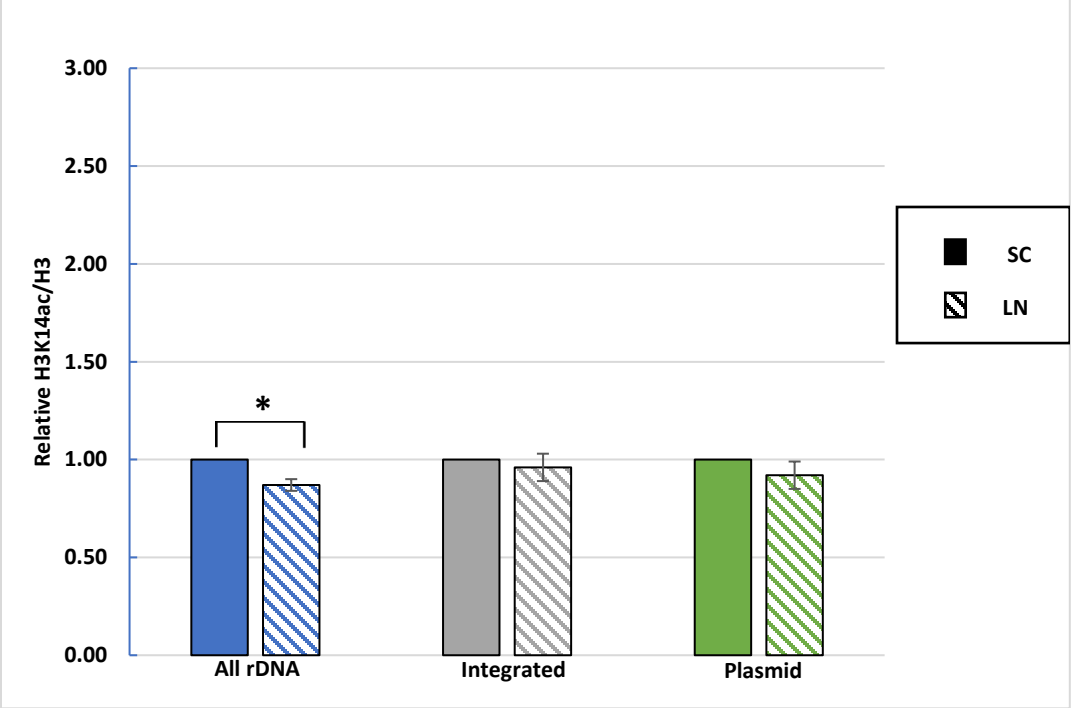

Figure 14. Changes in histone acetylation on H3K14 at the genomic rDNA promoters during nitrogen deprivation. Wild type strains were grown under normal nitrogen and low nitrogen conditions and subjected to IP using anti-H3K14ac antibody. Percent input calculated for each primer pair for histone modifications and H3 IPs and the ratios calculated to show relative enrichment of H3K14ac at rDNA promoter. * Independent student *t*-test was used to compare H3K14ac in non-stress conditions to H3K14ac in low nitrogen conditions for the same primer set. ($p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Co-Immunoprecipitation of Upstream Activating Factor

UAF plays a stimulatory role in transcription of rDNA by Pol I. *In-vivo* and *in-vitro* studies have shown that binding of this multiprotein complex comprised of Rrn5, Rrn9, Rrn10, Uaf30, and histones H3 and H4 commits rDNA for Pol I transcription. The absence of Pol I itself does not lead to a polymerase switch (Vu et al., 1999); however, the absence of Rrn5, Rrn 9, or Rrn10 subunits will lead to a switch to predominantly Pol II transcription while the absence of Uaf30 leads to transcription of rDNA by both Pol I

and Pol II (Siddiqi et al., 2001). Histones H3 and H4 are part of the UAF multiprotein complex and a preferred site for modifications by several acetyl and methyl transferases. In open chromosomal repeats, the UAF binding region is non-nucleosomal and presumably all the modification are occurring on H3 and H4 histones of UAF complex. ChIP experiments showed a significant increase in histone methylation at H3K4 and H3K36 as well as a decrease in histone acetylation levels at H4K5 and H3K14. To investigate post-translational modification differences of H3 and H4 components of UAF in non-stress and stress conditions, we performed a co-immunoprecipitation study on cells with Rrn5-TAP tagged strains grown in regular and low nitrogen conditions along with non-tagged strains as controls.

Table 15

Quantification of Western blots show a significant decrease in the UAF complex in low nitrogen condition in whole cell extract and in CoIP's.

	CoIP-1	**CoIP-2**	**AVG**
Rrn5-TAP SC WCE	1.00	1.00	1.00
Rrn5-TAP LN WCE	0.41	0.17	0.29
Rrn5-TAP SC CoIP	1.00	1.00	1.00
Rrn5-TAP LN CoIP	0.33	0.40	0.36

Data represents average of 2 experiments.

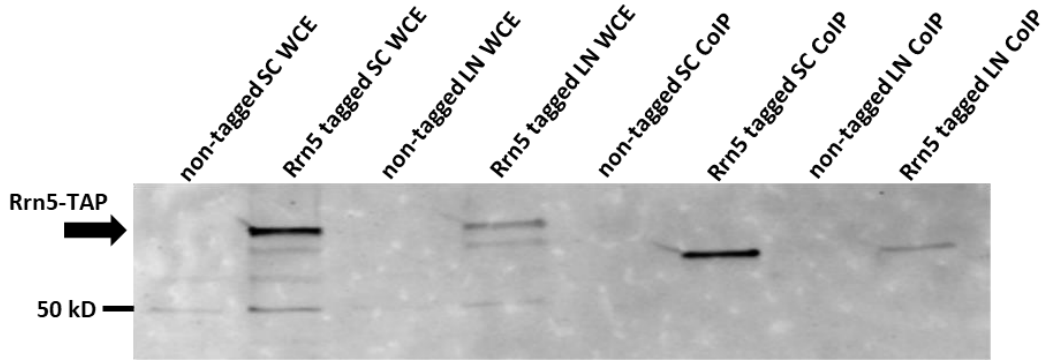

Figure 15. Immunoprecipitation of Rrn5-TAP tagged strain. Representative Western blot showing pull down of Rrn5-TAP using human IgG Sepharose beads and probed with rabbit anti-TAP antibody. Arrows represent Rrn5-TAP tagged before and after TEV cleavage. WCE: Whole cell extract, SC: Synthetic complete, LN: Low Nitrogen. Representative blot for 2 independent experiments shown.

Immunoprecipitation (IP) of Rrn5-TAP showed no detectable signal for non-tagged strain and an overall decrease in the a UAF complex occurs during stress conditions (see Table 15 and Figure 15). Similar to the Rrn5-TAP IP western blot, histone H3 Western blot from the same gel show a decrease in histone H3's enrichment (see Table 16 and Figure 16A). To confirm that histone H3 detected by co-IP belongs to the UAF complex, IP was performed on non-chromatin samples of Rrn5-TAP tagged strains and western blot showed no detection of histone H3

Table 16.

Quantification of Western blot showing decrease in histone H3 associated with UAF complex in low nitrogen conditions

H3	CoIP-1	CoIP-2	AVG
Rrn5-TAP SC CoIP	1.00	1.00	1.00
Rrn5-TAP LN CoIP	0.64	0.43	0.53

Data represents average of 2 independent experiments.

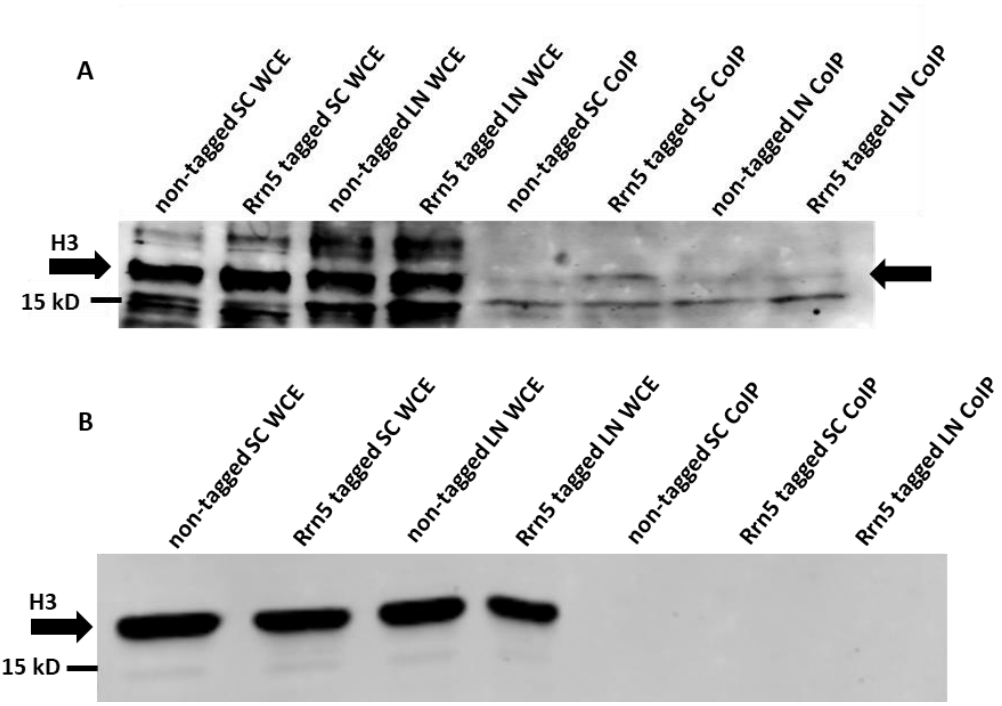

Figure 16. Co-immunoprecipitation of Rrn5-TAP tagged strain. A) Representative Western blot showing CoIP of histone H3 associated with UAF complex. Arrows indicate histone H3 bands. WCE: Whole cell extract, SC: Synthetic complete, LN: Low Nitrogen. Representative blot for 2 independent experiments shown. B) Western blot representing absence of H3 association with UAF complex when immunoprecipitation performed using non-chromatin fraction.

High Mobility Group Protein Hmo1 Bound to Actively Transcribed rDNA Promotes Pol I rDNA Transcription.

The HMG proteins when bound to DNA result in the modification of rDNA chromatin architecture. One of the members of the HMG family is Hmo1, which is known to be associated with the entire 35S rDNA region and interacts with multiprotein complexes on DNA to stabilize them (Hall et al., 2006). Hmo1 is enriched on actively transcribed rDNA and is colocalized with RNA polymerase I during transcription (Merz et al., 2008). We proposed that in the absence of Hmo1 there would be an increase in polymerase switch compared to wild type strain. The polymerase switch requires a reduction in Pol I activity as well as modification of chromatin. We investigated the Pol II reporter gene activity in *hmo1Δ* cells (see Table 17 and Figure 17). The absence of Hmo1 resulted in a significant increase in Pol II activity during normal nitrogen conditions compared to wild type cells. In low nitrogen conditions, Pol II reporter gene activity of *hmo1Δ* was equivalent to that of wild type cells. Thus, Hmo1 appears to repress Pol II mediated transcription of rRNA in non-stress conditions.

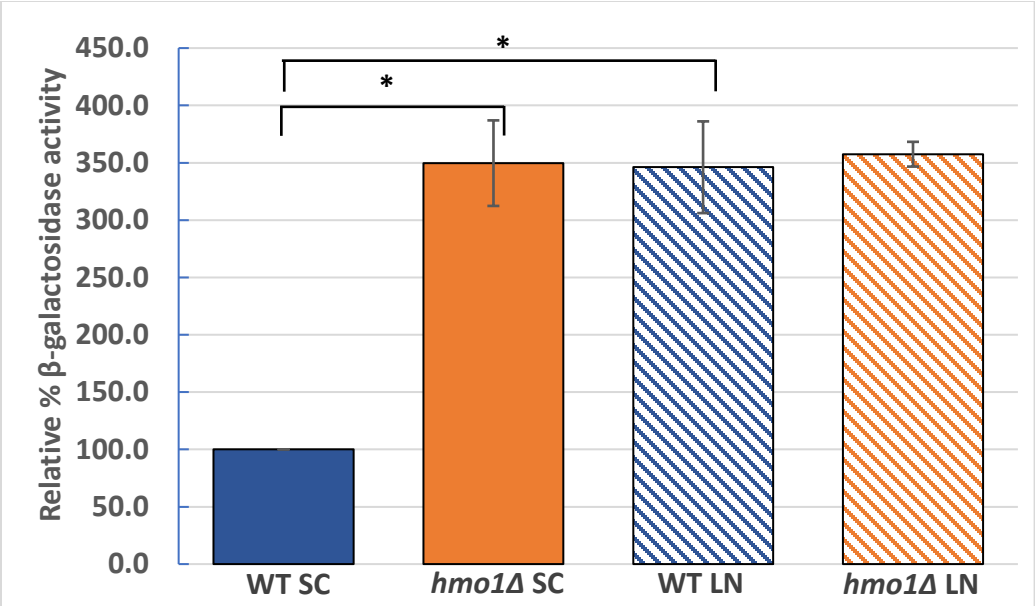

Figure 17. Increase in polymerase switch in the absence of Hmo1. Relative β-galactosidase activity was measured in wild type BY4743, *hmo1Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. In the absence of Hmo1 we saw a significant increase in Pol II activity in regular nitrogen (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was significantly higher in low nitrogen conditions for *hmo1Δ* compared to wild type cells in low nitrogen (striped bars) indicating that the Hmo1 could play a role in keeping the polymerase switch off during non-stress and stress conditions. (one-way ANOVA at $p \leq 0.05$, $n = 3$).

Table 17.

Relative β-galactosidase activity/ plasmid copy number for wild type and hmo1Δ grown in regular and low nitrogen media.

Strains	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0.0
***hmo1Δ* SC**	430.2	272.2	346.6	**349.7***	37.3
WT LN	416.4	252.0	370.0	**346.1***	40.0
***hmo1Δ* LN**	383.9	346.1	342.3	**357.5**	10.8

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. **#** compared to wild type cells in low nitrogen. (one-way ANOVA at $p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

Histone Deacetylase Sir2 is not Involved in Polymerase Switch

Sirtuin family proteins are involved in silencing of various regions of the yeast genome including telomeres, mating type loci and rDNA. Sir2 is a NAD-dependent protein deacetylase (Imai, Armstrong, Kaeberlein, & Guarente, 2000; Landry et al., 2000) that silences Pol II reporter genes integrated in rDNA by formation of the RENT complex. The RENT complex consisting of proteins like Net1, Sir2 and Cdc14 is recruited to the NTS1 by associating with Fob1 and to NTS2 by Pol I (Shou et al., 1999; Straight et al., 1999) (Buck et al., 2016). In silencing of rDNA, proteins like Sir2 and Net1 have been shown to be bound to most of the 9.1 kb of rDNA along with Sir2's presence mostly in the NTS1 region. Therefore, Sir2 could play a role in silencing of Pol II rRNA transcription by forming a chromatin state favorable for Pol I

transcription and not Pol II. Hence, we hypothesized that during non-stress conditions, the presence of Sir2 at the rDNA promoter would cause the polymerase switch to remain off. Our results indicate that in the absence of Sir2 β-galactosidase activity was like that of wild type cells in both normal and low nitrogen (see Table 18 and Figure 18). This suggested that Sir2 does not inhibit the polymerase switch.

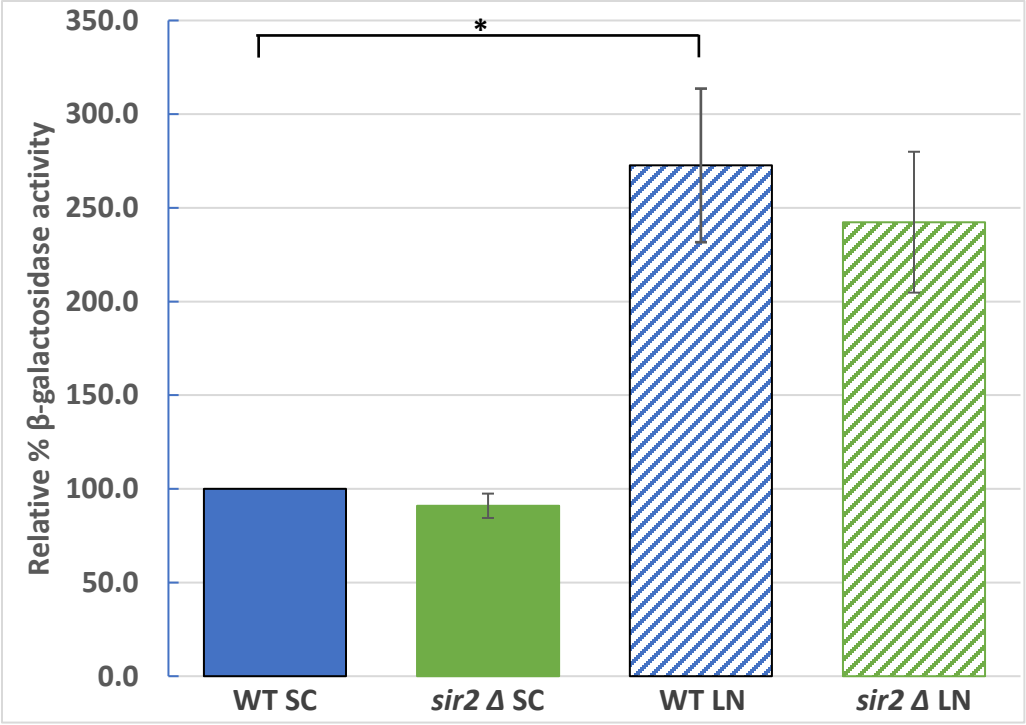

Figure 18. Polymerase switch in absence of Sir2. Relative β-galactosidase activity was measured in wild type BY4743, *sir2Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. Absence of Sir2 resulted in a similar Pol II activity in regular nitrogen (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was similar in low nitrogen conditions for *sir2Δ* compared to wild type cells (striped bars) indicating that the Sir2 might not play a role in keeping the polymerase switch off during non-stress conditions. (one-way ANOVA at $p \leq 0.05$, $n = 3$).

Table 18.

Relative β-galactosidase activity/ plasmid copy number for wild type and sir2Δ grown in regular and low nitrogen media.

Strain	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0.0
***sir2Δ* SC**	108.5	60.3	104.1	**91.0**	6.5
WT LN	455.2	131.2	231.6	**272.6***	41.0
***sir2Δ* LN**	422.0	128.8	176.2	**242.3**	37.6

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. **#** compared to wild type cells in low nitrogen. (one-way ANOVA at $p \leq 0.05$, $n = 3$). SEM: Standard Error of Mean.

General Corepressor Complex Ssn6-Tup1 Plays a Role in Silencing Pol II mediated rRNA transcription

Stress adaptions requires an orchestrated response of several genes, to activate stress response genes while repressing genes required for active growth. One of the mechanisms involves the Ssn6-Tup1 corepressor complex, which is known to repress osmotic stress, glucose repression, and nitrogen stress induced Pol II transcribed genes in non-stress conditions. This corepressor complex is recruited to stress response promoters of candidate genes bound by gene specific transcriptional repressor proteins. The transcriptional repressor protein interact with the Ssn6 subunit of the complex while the Tup1 subunit has the repression function. (R. L. Smith & Johnson, 2000). Tup1 also binds preferentially to the under-acetylated histones tails of H3 and H4

(Edmondson et al., 1996) presumably following deacetylation by HDAC's such as Rpd3 and Hda1that are activated during stress (Davie, Edmondson, Coco, & Dent, 2003; Fleming et al., 2014). *Rrn9Δ* cells are unable to synthesize rRNA by Pol II in absence of Rpd3 (M. L. Oakes et al., 2006).

The presence of multiple putative binding sites on rDNA promoter for repressor proteins reported to recruit the Ssn6-Tup1 corepressor complex. This led us to hypothesize that a similar Ssn6-Tup1 repression mechanism could play a role in suppressing Pol II rRNA synthesis in coordination with HDAC like Sir2. To test this hypothesis, we performed β-galactosidase assays on individual knockout strains of ssn6Δ and tup1Δ, grown in normal and low nitrogen conditions. The absence of either Tup1 or Ssn6 resulted in 1.5-fold increase in Pol II activity during normal nitrogen conditions indicating that the corepressor complex could play a role in repressing RNA polymerase II transcription of rRNA during non-stress conditions (see Table 19 and Figure 19). During nitrogen deprivation, Pol II reporter gene activity of *tup1Δ* was not significantly different than WT, but the absence of Ssn6 resulted in lower β-galactosidase activity suggesting role played by Ssn6 and Tup1 in enhancing Pol II inaccessible chromatin.

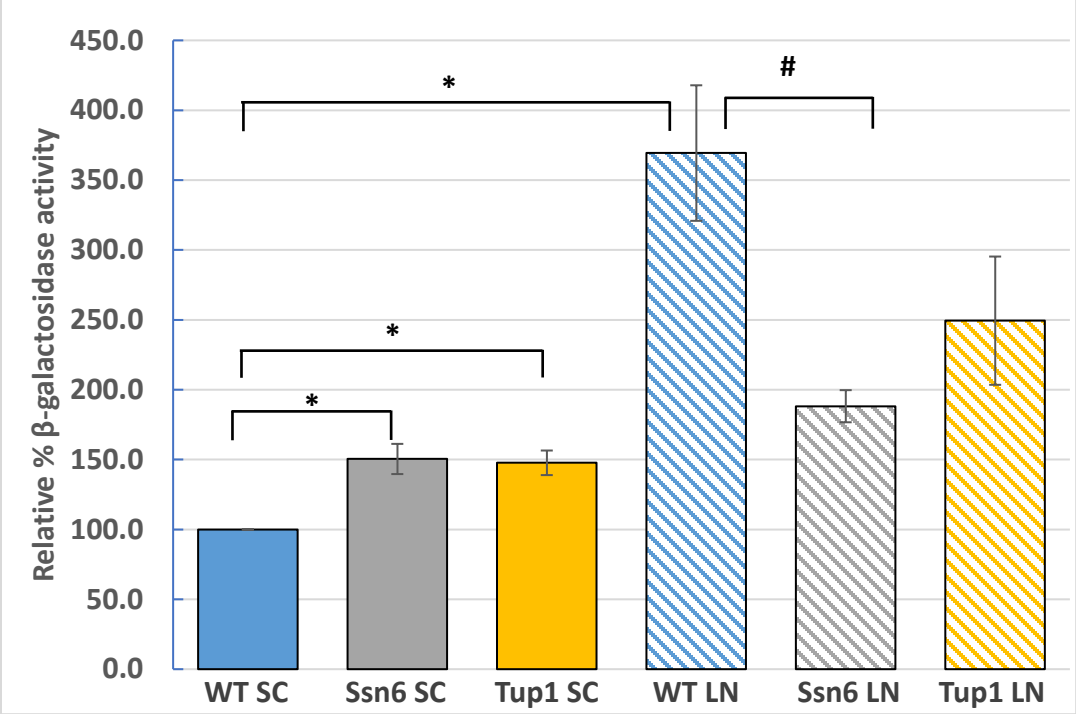

Figure 19. Increase in polymerase switch due to absence of corepressor complex Ssn6-Tup1. Relative β-galactosidase activity was measured in wild type BY4743, *ssn6Δ* and *tup1Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. In the absence of Ssn6 and Tup1 of the corepressor complex we saw a significant increase in Pol II activity in regular (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was significantly reduced in low nitrogen conditions for ssn6Δ strain compared to low nitrogen condition of wild type cells (striped bars). (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$).

Table 19.

Relative β-galactosidase activity/ plasmid copy number for wild type and ssn6Δ and tup1Δ grown in regular and low nitrogen media.

Strains	Exp-1	Exp-2	Exp-3	Exp-4	AVG	±SEM
WT SC	100.0	100.0	100.0	100.0	**100.0**	0
***ssn6Δ* SC**	161.8	179.8	125.0	135.1	**150.4***	10.8
***tup1Δ* SC**	141.7	177.4	140.2	131.5	**147.7***	8.8
WT LN	366.4	442.8	416.4	251.9	**369.4***	48.5
***ssn6Δ* LN**	209.1	201.3	149.6	192.7	**188.2#**	11.5
***tup1Δ* LN**	133.3	341.0	223.9	299.4	**249.4**	45.9

SC- Normal Nitrogen, LN- Low Nitrogen, SEM Standard Error of Mean. * compared to wild type cells in regular nitrogen. # compared to wild type cells in low nitrogen. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$). SEM: Standard Error of Mean.

Transcription Repressor Proteins Binding at the rDNA Promoter and Potentially Recruiting Ssn6-Tup1 Complex.

Since corepressors Ssn6 and Tup1 inhibit Pol II rRNA transcription, we investigated transcription repressor p roteins that have potential binding sites at the rDNA promoter and have been reported to recruit the Ssn6-Tup1 complex at other stress inducible genes. Examination of the Pol II rDNA promoter revealed several DNA binding proteins with putative binding sites: Sko1, Sut1, Nrg1, Gln3, and Mig1. Four repressor proteins have putative binding sites at the rDNA Pol II promoter (see Figure 3). Mig1 is a transcriptional factor involved in glucose repression and has been reported to recruit Ssn6-Tup1 corepressor to glucose-repressed promoters (Treitel & Carlson, 1995). Knockout strains for Mig1 showed no significant difference in Pol II

activity compared to wild type cells in non-stress and stress conditions (see Table 20, Figure 20). Sko1 is a basic leucine zipper transcription factor that forms a complex with Ssn6-Tup1 to activate transcription of osmotic and oxidative stress genes (Proft & Struhl, 2002; Rep et al., 2001). In the absence of Sko1, we detected a small but significant increase in reporter gene activity in non-stress conditions (see Table 21 and Figure 21). Similarly, for the Sut1 repressor protein, also reported to interact with Ssn6-Tup1 and represses genes involved in expression during non-hypoxic conditions (Rizzo, Mieczkowski, & Buck, 2011) showed a significant increase in Pol II activity during non-stress conditions compared to wild type in non-stress conditions (see Table 21 and Figure 21). Nrg1 is a transcriptional repressor protein that recruits the corepressor complex and causes repression of genes during glucose repression(Park et al., 1999). In absence of Nrg1, we observed a significant increase of Pol II activity during stress conditions (see Table 22 and Figure 22) compared to wild type cells in non-stress and stress conditions.

We also investigated an activator protein, Gln3 which is a transcriptional activator of genes regulated by nitrogen catabolite repression and its activity regulated by quality of nitrogen sources (Minehart & Magasanik, 1991). In the absence of Gln3, we saw a significant increase in Pol II activity compared to wild type cells in non-stress and stress conditions (see Table 22 and Figure 22).

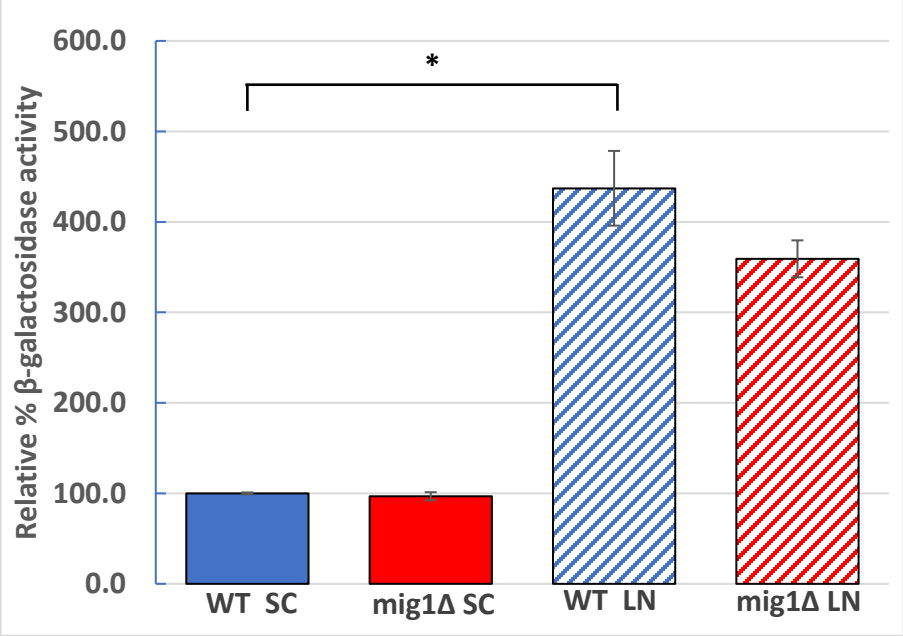

Figure 20. Polymerase switch is not inhibited by Mig1. Relative β-galactosidase activity was measured in wild type BY4743, *mig1Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. Absence of Mig1 resulted in a similar Pol II activity in regular nitrogen (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was no different in low nitrogen conditions for *mig1Δ* strain compared to wild type cells (striped bars) indicating that the Mig1 might not play a role in keeping the polymerase switch off during non-stress conditions. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$).

Table 20.

Relative β-galactosidase activity/ plasmid copy number for wild type and mig1Δ grown in regular and low nitrogen media.

Strain	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0.0
***mig1Δ* SC**	85.6	93.9	110.8	**96.8**	4.5
WT LN	620.3	293.0	398.1	**437.2***	41.3
***mig1Δ* LN**	389.3	300.9	387.4	**359.2**	20.4

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. **#** compared to wild type cells in low nitrogen. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

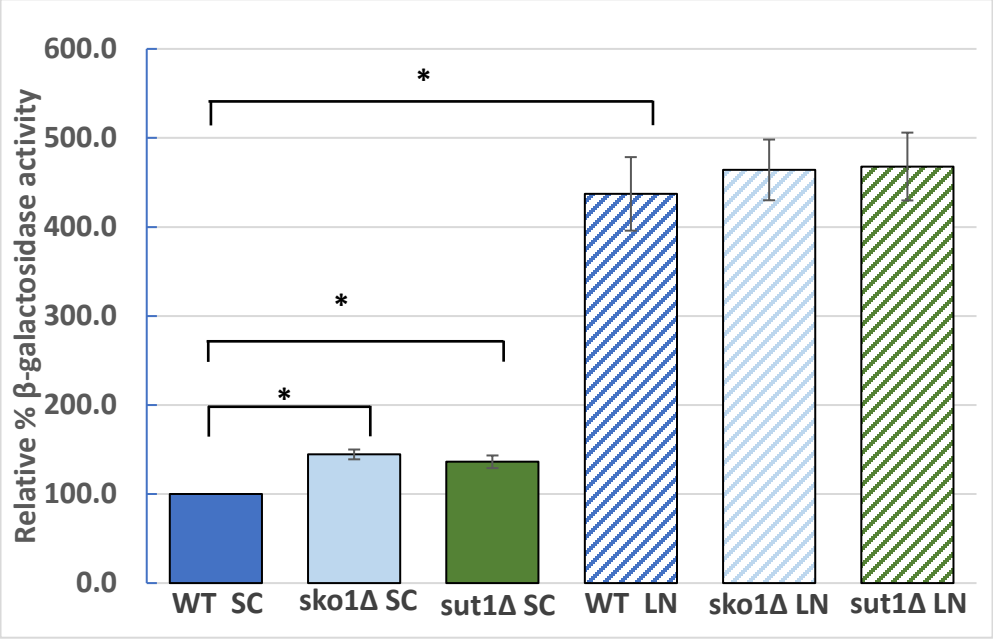

Figure 21. Increase in polymerase switch due to absence of Sko1, and Sut1. Relative β-galactosidase activity was measured in wild type BY4743, *sko1Δ* and *sut1Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. Absence of Sko1, and Sut1 resulted in an increase of Pol II activity in regular nitrogen (solid bars) compared to wild type cells in regular nitrogen. The Pol II activity was similar in low nitrogen conditions for *sko1Δ,* and *sut1Δ* compared to wild type cells (striped bars) This

indicates that the Sko1, and Sut1 might play a role in keeping the polymerase switch off during non-stress conditions. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, n=3).

Table 21.

Relative β-galactosidase activity/ plasmid copy number for wild type and sko1Δ and sut1Δ grown in regular and low nitrogen media.

Strain	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0.0
***sko1Δ* SC**	146.4	133.1	154.0	**144.5***	5.5
***sut1Δ* SC**	157.3	112.2	139.3	**136.3***	7.1
WT LN	620.3	293.0	398.1	**437.2***	41.3
***sko1Δ* LN**	545.2	338.8	508.2	**464.1**	34.1
***sut1Δ* LN**	631.2	349.2	423.3	**467.9**	38.1

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. **#** compared to wild type cells in low nitrogen. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean.

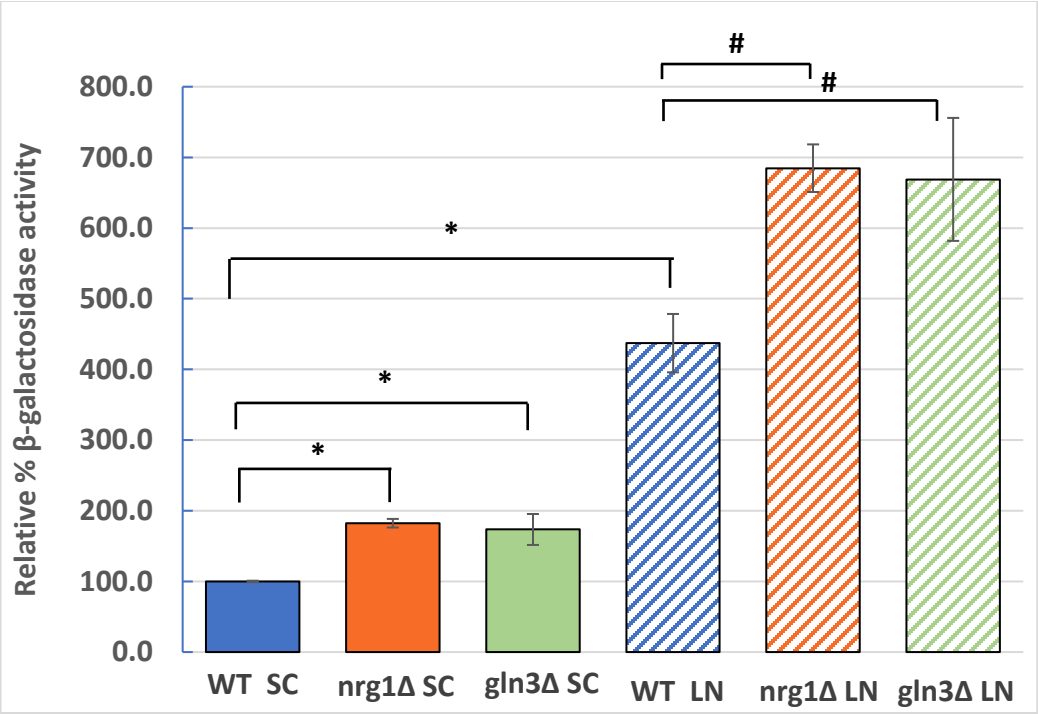

Figure 22. Increase in polymerase switch in non-stress and stress conditions due to absence of Nrg1, and Gln3. Relative β-galactosidase activity was measured in wild type BY4743, *nrg1Δ* and *gln3Δ* strains which were transformed with 35S rDNA-*LacZ* reporter construct, pFES17. Absence of Nrg1, and Gln3 resulted in a significantly higher Pol II activity compared to wild type cells in regular nitrogen (solid bars). The Pol II activity was significantly higher in low nitrogen conditions for *nrg1Δ* and *gln3Δ* compared to wild type cells (striped bars) indicating that the Nrg1 and Gln3 might play a role in keeping the polymerase switch off during non-stress conditions and the combined effect of repressor protein absence with stress conditions leading to a significantly higher Pol II switch. (one-way ANOVA with Tukey's posthoc test at $p \leq 0.05$, $n = 3$).

Table 22

Relative β-galactosidase activity/ plasmid copy number for wild type and nrg1Δ and gln3Δ grown in regular and low nitrogen media.

Strain	Exp-1	Exp-2	Exp-3	AVG	±SEM
WT SC	100.0	100.0	100.0	**100.0**	0.0
***nrg1Δ* SC**	166.1	-	198.5	**182.3***	6.1
***gln3Δ* SC**	-	120.4	226.6	**173.5***	21.9
WT LN	620.3	293.0	398.1	**437.2***	41.3
***nrg1Δ* LN**	659.2	-	710.1	**684.7#**	33.7
***gln3Δ* LN**	-	445.3	892.4	**668.9#**	87.0

SC- Normal Nitrogen, LN- Low Nitrogen. ***** compared to wild type cells in regular nitrogen. **#** compared to wild type cells in low nitrogen. (one-way ANOVA with Tukey posthoc test at $p \leq 0.05$, $n = 3$) SEM: Standard Error of Mean. "-" indicates β-galactosidase activity could not be determined for those experiments.

CHAPTER IV

DISCUSSION

This research investigated possible mechanisms for suppression of 35S rRNA synthesis by Pol II during non-stress condition and role of UAF in invoking an inaccessible chromatin structure for the polymerase switch. Since the absence of UAF components Rrn5, Rrn9, or Rrn10 triggers sufficient Pol II rRNA synthesis to support growth in the absence of Pol I (Nogi et al., 1991), we hypothesized that UAF interaction with the Pol I promoter during nitrogen deprivation must change to permit the switch to RNA polymerase II. Indeed, absence of UAF30 resulted in an increase in Pol II rRNA synthesis as measured by reporter gene activity. Furthermore, during nitrogen starvation UAF binding to the rDNA promoter decreases. This decrease in UAF binding parallels an overall decrease in steady-state Rrn5 levels. Since the open Pol I rDNA promoter is devoid of nucleosomes, acetylation and methylation of H3 and H4 at the rDNA promoter, it most likely reflect changes in UAF components H3 and H4. During nitrogen deprivation, UAF subunits H3 and H4 are differentially modified with an increase in H3K9 and H3K36 methylation and a decrease in H4K5 acetylation supporting the hypothesis that UAF is a target of stress response signaling and suggesting its importance in altering the chromatin structure and polymerase switch. Studies of deletion strains

reveal that DNA binding protein Hmo1 contributes to Pol II inhibitory chromatin during non-stress conditions, while Sir2 does not appear to alter the polymerase switch. In concert with HmoI and UAF, the repressor proteins Sut1, Nrg1, and Sko1 may recruit Ssn6-Tup1 to supplement Pol II inaccessible chromatin structure suppressing polymerase switch during non-stress conditions. Thus, UAF triggers the assembly of Pol II suppressive chromatin at the rDNA promoter with the aid of Hmo1 and Ssn6-Tup1.

Upstream Activating Factor Prevents Polymerase Switch

UAF binding to the rDNA promoter has been reported to inhibit Pol II transcription of 35S rRNA (M. Oakes et al., 1999). Interestingly, the absence of CF, Rrn3, or Pol I does not trigger the switch. Furthermore, absence of UAF components Rrn9 result in major chromatin architecture alterations along with the inability to recruit Sir2, HmoI, CF, and Pol I (Vu et al., 1999). It is only in the absence of UAF, and coupled with the dramatic changes in rDNA chromatin, that sufficient Pol II rRNA synthesis occurs to support life in the absence of Pol I (Goetze et al., 2010). Together, these results suggested that UAF is the linchpin in suppressing the polymerase switch. In fact, *rrn5Δ, rrn9Δ,* or *rrn10Δ* cells, even in presence of Pol I, synthesize majority of their rRNA using Pol II; however, *uaf30Δ* cells show ~ 90% Pol I and ~ 10% Pol II rRNA transcription (Siddiqi et al., 2001). As expected, we observed a modest increase in β-galactosidase activity for *uaf30Δ* cells grown in regular nitrogen media supporting UAF's role in polymerase switch suppression. We hypothesized that UAF binding would decrease during nitrogen deprivation to accompany the increase in Pol II rRNA synthesis.

Wild type Rrn5-TAP tagged strains showed a 2-fold reduction in UAF binding to all rDNA promoters as well as a two-fold reduction in binding at a single open tagged promoter under low nitrogen conditions. Supporting the reduction of UAF binding to the rDNA promoter in low nitrogen, ChIP assays revealed a similar reduction in UAF component H3 binding to the single open tagged repeat. Although binding to all rDNA promoters (chromosomal, integrated and plasmid) did not show a reduction in H3 binding, this may be due to the fact that at least 50% of the signal was derived from closed nucleosomal repeats. In fact, the number of closed repeats may have increased in low nitrogen. Supporting the conclusion that the decrease in H3 binding is due to a decrease in UAF binding versus nucleosomal H3, H2A binding to the rDNA promoter was not changed in low nitrogen. Therefore, the decrease in H3 binding is mostly likely due to a decrease in UAF binding.

In agreement with a reduction in UAF binding to the rDNA promoter during stress conditions, nitrogen deprived cells showed a three times reduction in steady-state Rrn5 in whole cell extract, as well as in immunoprecipitation (IP) samples. Immunoprecipitation of Rrn5-TAP with anti-TAP antibody from chromatin revealed a similar three-fold reduction in H3 in nitrogen deprived cells. Interestingly, immunoprecipitation from the non-chromatin fraction (see Figure 16 B) did not reveal association of UAF to H3 suggesting that the UAF complex may contain H3 and H4 only in the chromatin bound state. Altogether, binding of UAF to the rDNA promoter decreases during nitrogen deprivation.

UAF Component H3 and H4 Modifications at rDNA Chromatin during Nitrogen Deprivation

A decrease in UAF binding triggered by nitrogen deprivation suggests an alteration in rDNA chromatin such as is seen in strains lacking UAF components (Goetze et al., 2010). As the UAF complex consists of histones H3 and H4, these components are likely targets for modifications by signaling pathways. Modification of H3 and H4 within the UAF complex may lead to reduction in UAF binding and/or the recruitment of other chromatin modifiers. We observed a 1.5-fold increase in the levels of tri-methylation on histone H3K36 at the rDNA promoter during low nitrogen conditions. This modification is also associated with non-ribosomal genes that are actively transcribed by Pol II and Pol II transcription elongation (Hampsey & Reinberg, 2003). Set2, the HMT responsible for H3K36 methylation, is associated with Pol II during transcription and methylates H3K36 within the nucleosome inhibiting spurious Pol II transcription. Accumulation of these Pol II spurious transcripts are known to decrease longevity in yeast (Sen et al., 2015). In addition to increased aging, absence of Set2, resulted in decreased resistance to heat and nitrogen utilization (McDaniel et al., 2017) suggesting that stress conditions could trigger increased H3K36 tri-methylation by Set2. Since Set2 methylation of H3K36 inhibits silencing of reporter genes with in the NTS2 region of rDNA (Briggs et al., 2001; Strahl,

Ohba, Cook, & Allis, 1999) Set 2 must be present within the nucleoli and potentially available to methylate H3K36 within the UAF complex.

During nutrient deprivation conditions, Set1 di and tri-methylation of H3K36 increases globally due to the inhibition of TOR signaling (Cohen et al., 2018). Similarly, during nitrogen deprivation we observed a two-fold increase in levels of di and tri-methylation on histone H3K4 at the rDNA at the promoter of single tagged repeat and a 1.5-fold increase at all the rDNA promoters during low nitrogen conditions. A similar increase in H3K4 methylation at rDNA promoters has been shown in ageing yeast cells where it has been linked with suppression of aberrant Pol II transcription within the gene body and the subsequent reduction of aging (Cruz et al., 2018). These results suggest a link between increased H3K4 di- and tri-methylation and stress response. Since H3K4 methylation is known to recruit Rpd3 (Kim & Buratowski, 2009), methylation of UAF H3 subunit may facilitate the recruitment of Rpd3.

In mammalian cells, signaling pathways trigger the acetylation and phosphorylation of Pol I transcription factors, UBF, SL1, TIF-IA (Rrn3) and Pol I to regulate Pol I preinitiation complex formation (Russell & Zomerdijk, 2005). Although the Pol I/TIFIA (Rrn3) complex appears to be the primary target for modification, both SL1 and UBF have been reported to be acetylated in response to abundant nutrients (Shen et al., 2013). Similarly, our data from yeast show an ~60% decrease in H4K5 acetylation levels at the rDNA promoters under low nitrogen conditions. Since Rpd3 can deacetylate H4K5, this decrease likely reflects Rpd3 activity (Sun & Hampsey, 1999). Furthermore,

during nutrient starvation and TOR inactivation, Rpd3 binding at rDNA promoters increases resulting in a decrease of H4K5 acetylation. Since Rpd3 activity is essential for the polymerase switch (M. L. Oakes et al., 2006), it can be proposed that methylation by Set1 recruits Rpd3 to UAF resulting in the deacetylation of H4K5 subunit.

Role of Additional Chromatin Modifiers in Inhibition of the Polymerase Switch

Reduction in UAF binding results in decreased recruitment of CF and Pol I; however, this is not sufficient to trigger the polymerase switch which requires additional transcriptional activation. Although Sir2 suppresses Pol II transcription in NTS1 of rDNA, deletion strains of Sir2 did not alter the levels of polymerase switch, consistent with previous studies which failed to detect any Pol II-derived 35S rRNA transcripts in *sir2*Δ strains. (M. Oakes et al., 1999). Thus, Sir2 is not required for the polymerase switch

In contrast, the absence of Hmo1, a normally bound component of actively transcribed rDNA, results in a polymerase switch in non-stress conditions comparable to that of wild type cells undergoing stress. Since *hmo1Δ* does not invoke sufficient Pol II rRNA to support growth in the absence of Pol I, Hmo1 must contribute to Pol II inaccessible environment established by UAF binding at the rDNA promoter. Hmo1 also stimulates Pol I transcription of rDNA and remains associated with the 35S transcription unit even in absence of Pol I (Merz et al., 2008). Since the absence of Hmo1 does not influence binding of UAF or CF (Merz et al., 2008), this stimulatory role could be due to its DNA-bending properties which might generate architectural structures that would

favor binding of Pol I (Mitsouras et al., 2002; Thomas & Travers, 2001). One mechanism that stabilizes the non-nucleosomal DNA involves formation of bridges and loops by dimerization of HmoI box A domains. This looping of rDNA by Hmo1 can be easily disrupted by the transcribing Pol I (Bennink et al., 2001; Brower-Toland et al., 2002). Furthermore, Atomic Force microscopy shows that looping caused by Hmo1 allows Pol I to easily transcribe through them and requires much less energy than to shift nucleosomes (Murugesapillai et al., 2014). Inactivation of TOR signaling pathways during stress conditions represses Hmo1 expression, absence of Hmo1 results in altered chromatin (Berger et al., 2007; Xiao, Kamau, Donze, & Grove, 2011). Hence, Hmo1 could facilitate repression of the polymerase switch by forming specialized Pol I favored structures and indirectly inhibiting Rpd3 recruitment to the rDNA promoter (Rohde & Cardenas, 2003).

Pol II Repression during Non-Stress Conditions by a General Co-repressor

The Ssn6-Tup1 corepressor complex has been reported to repress more than 150 genes including many stress-induced genes (DeRisi, Iyer, & Brown, 1997). We observed a similar increase in the polymerase switch in both *ssn6*Δ and *tup1*Δ cells in regular nitrogen conditions indicating that the corepressors contribute to the repression of Pol II transcription of rDNA during non-stress conditions. However, in low nitrogen conditions Pol II reporter gene activity was induced but to a lesser extent compared to wild type cells. The Ssn6-Tup1 corepressor complex represses Pol II transcription by recruiting HDACs, interacting with histone tails or interfering with the Pol II transcriptional machinery (Watson et al., 2000). In addition, Ssn6-Tup1 plays a role in activating

osmotic stress inducible genes (Proft & Struhl, 2002). Potentially, the absence of Ssn6 or Tup1 may indirectly impede maximal Pol II transcription of rRNA. Thus, corepressor complex Ssn6-Tup1 could contribute to repressing Pol II rRNA transcription either by altering chromatin structure for Pol II transcription factors or by inhibition of transcription activation (Z. Zhang, Varanasi, & Trumbly, 2002).

The Ssn6-Tup1 corepressors are recruited to Pol II transcribed genes during normal conditions by gene specific repressor proteins. Of the several potential repressors with binding sites in the Pol II rDNA promoter (Figure 3), the absence of several repressors increased the Pol II-mediated rRNA transcription. Mig1 is a glucose gene repressor which is known to induce repression by recruiting Ssn6-Tup1 (Alipourfard et al., 2019; Nehlin, Carlberg, & Ronne, 1991). The *mig1*Δ strains showed no changes in β-galactosidase activity for either conditions indicating that Mig1 is not playing a role in recruiting Ssn6-Tup1 to the rDNA promoter. Since putative Mig1 binding sites overlap the UAF and Reb1 binding site, we predict that during non-stress conditions binding of UAF could restrict Mig1 binding at that site.

The absence of either Sko1 or Sut1 showed elevated β-galactosidase activity in non-stress conditions, while having no effect during nutrient deprivation. In fact, these mutants behaved similarly to the absence of either repressor component under normal nitrogen conditions. However, the absence of Nrg1 that targets the corepressor complex to glucose-repressed genes (Park et al., 1999) resulted in elevated Pol II polymerase activity in both stress and non-stress conditions suggesting it induces a change in

chromatin via Ssn6-Tup1 release, as well as, transcriptional activation of the polymerase switch during low nitrogen. Supporting this conclusion, the absence of Nrg1 leads to myo-inositol auxotropy (Villa-Garcia et al., 2011) and sensitivity to acids and cations suggesting these stresses, as well as, the reduction in carbon source may transcriptionally trigger the switch under low nitrogen conditions.

Consistent with the role of these repressors triggering the polymerase switch during nutrient deprivation or stress conditions. Sko1 is necessary for repression of genes responsible for countering oxidative stress and osmotic stress by recruiting corepressor complex Ssn6-Tup1 (Proft & Struhl, 2002). Sut1 directly interacts with Ssn6-Tup1 in low nitrogen conditions (Holland, Bergenholm, Borlin, Liu, & Nielsen, 2019; Regnacq et al., 2001). Thus after 12 hours of nitrogen deprivation as nutrients are depleted and oxidative stress increases (Gasch et al., 2000), stress signaling pathways would inactivate repressor binding and releasing Ssn6-Tup1 and transcriptionally activate the polymerase switch.

We investigated several stress responsive repressors with potential binding sites in the rDNA promoter that are known to recruit Ssn6-Tup1 corepressor complex. In general, during non-stress conditions repressor proteins recruit Ssn6-Tup1 to form Pol II suppressive chromatin, which is relaxed upon stress. The absence Sko1, Sut1 and Nrg1 all show an increase in reporter gene activity by ~50%; however, the absence of no one repressor released the inhibition. Ssn6-Tup1 can also interact with multiple repressors at the same time (R. L. Smith, Redd, & Johnson, 1995) suggesting that more than one

repressor protein may recruit Ssn6-Tup1to the rDNA promoter during normal conditions. Furthermore, these repressor proteins may be redundant as deletion of one repressor protein can be rescued by another one (Hanlon et al., 2011).

Contrary to the transcriptional repressor proteins, the Gln3 transcription factor activates genes responsible for utilizing alternative sources of nitrogen (Minehart & Magasanik, 1991). We observed a significant increase in polymerase switch in *gln3*Δ strains compared to wild type in regular and low nitrogen conditions. As Gln3 is responsible for utilizing alternative source of nitrogen, its absence results in glutamate auxotropy inducing a level of nitrogen stress even in rich media (Rai et al., 2015). Thus, absence of Gln3 in non-stress conditions mimics nitrogen starvation as the cells are unable to utilize alternate form of nitrogen source.

Proposed Model

During non-stress conditions (see Figure 23), activation of TOR and PKA signaling pathways phosphorylate Rrn3 resulting in the formation of the transcriptionally competent and activated Rrn3-Pol I. In parallel, TOR and PKA signaling recruits histone acetyl transferase Esa1 to acetylate UAF component H3 facilitating the binding of the UAF complex at the UE of open rDNA promoters. Binding of UAF primes the template for transcription, recruiting CF, TBP and Pol I/Rrn3 for PIC formation. During PIC formation, HmoI is recruited via TBP and Pol 1 interactions creating a Pol I favorable chromatin environment. In parallel, TOR/PKA signaling facilitates the binding of Sko1,

Sut1 or Nrg1 transcriptional repressors and the Ssn6-Tup1 corepressor to the promoter further repressing Pol II mediated rDNA transcription.

In response to nutrient starvation, TOR and PKA pathways are inhibited resulting in the activation of Set1 and Set2 HMT (see Figure 24). Set1 and Set2 methylates H3K4 and H3K36 of UAF. Rpd3 may be recruited to the PIC via interactions with HmoI, Set1, or Set2 resulting in binding of Rpd3 to methylated H3K4. Once bound to UAF, Rpd3 deacetylates H3 and potentially other UAF components resulting in reduced UAF binding at rDNA. With the loss of UAF binding, CF, Pol I, HmoI would be released from the rDNA promoter. Additionally, TOR/PKA inactivation triggers the release of Ssn6-Tup1 and its repressors. All of these changes in binding of Pol I transcription factors lead to decreased Pol I transcription and chromatin alterations, ultimately allowing rDNA promoter access to Pol II (see Figure 25). In parallel, inactivation of TOR pathway inhibits phosphorylation of the downstream Rim15 stress kinase. Unphosphorylated Rim15 is translocated to the nucleus where it activates stress transcription factors Gis1, Hsf1 and Yap. Binding of these transcription factors to the altered rDNA promoter promotes binding of mediator complex facilitating Pol II binding at rDNA promoter and transcription of the 35S rRNA (Vallabhaneni, 2016).

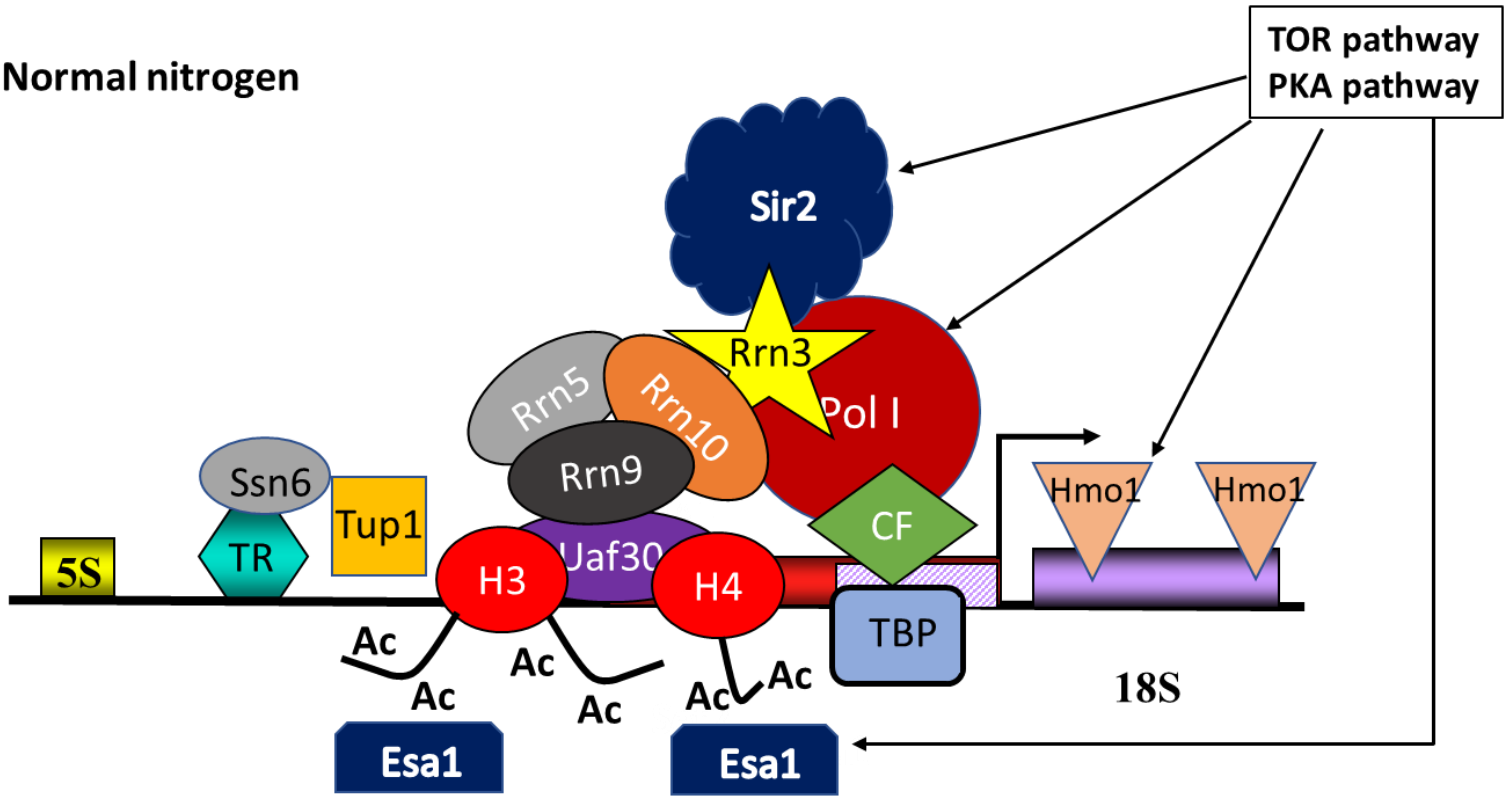

Figure 23: The proposed model represents Pol I mediated rDNA transcription during non-stress conditions: When nutrient supply is adequate, activated TOR signaling pathway recruits histone acetyl transferases (HAT) like Esa1 to acetylate H3 and H4 histones of UAF complex to facilitate its binding to the promoter region. Binding of UAF complex recruits CF (Core Factor), TBP (TATA Binding Protein) and Hmo1 to rDNA. General corepressor Ssn6-Tup1 interacting with one or more transcriptional repressor proteins bound to rDNA contributing to the Pol II repressive chromatin. Together these mechanisms create chromatin structure that is favorable to Pol I. Activated Pol I-Rrn3 is recruited to the rDNA promoter to initiate transcription of rDNA.

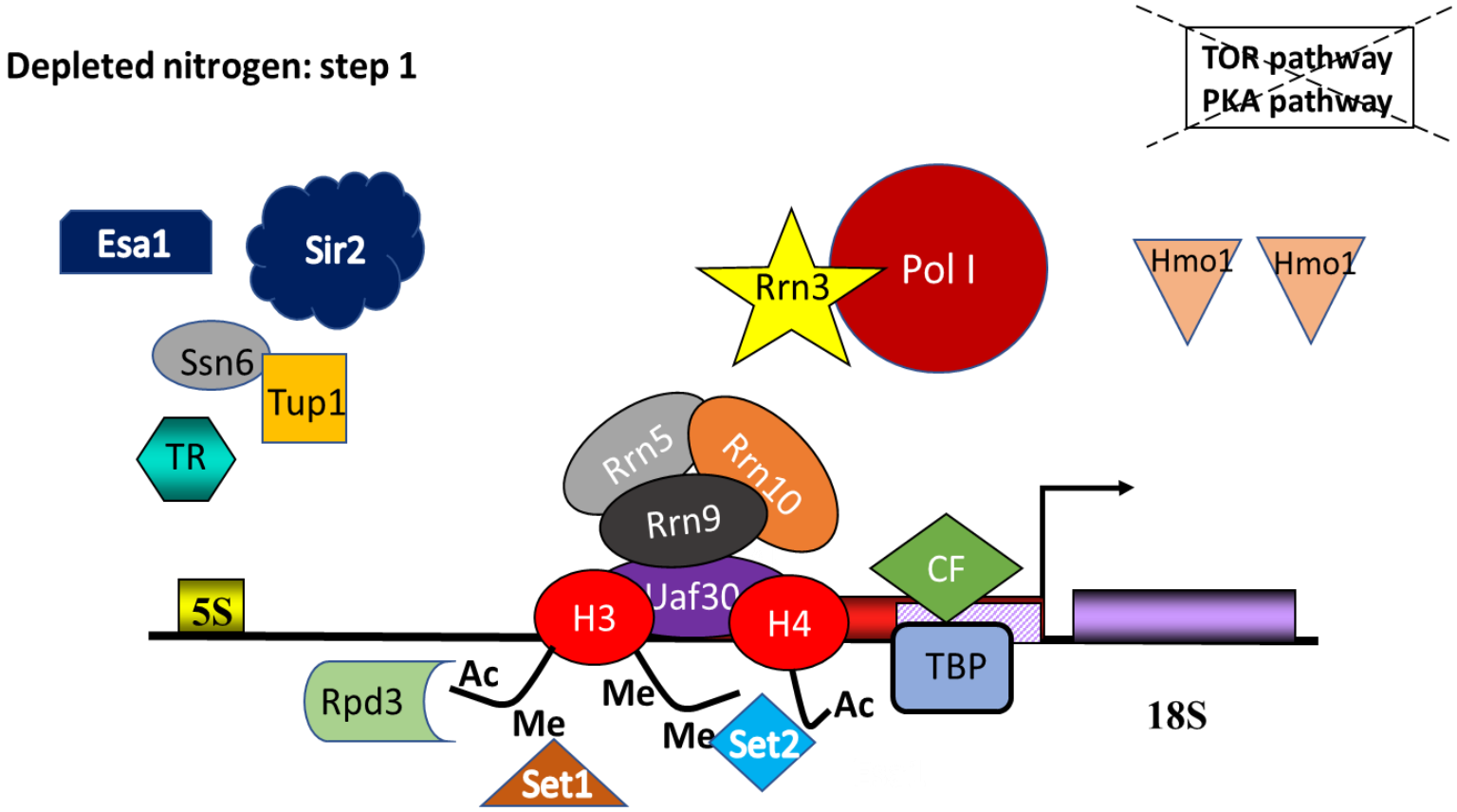

Figure 24: The proposed model represents changes occurring at rDNA promoter during stress conditions: Upon stress, inactivation of TOR pathway results in recruitment of histone methyltransferases SET1 and SET2 increasing the methylation of H3 and H4 of UAF complex. TOR inactivation releases Esa1 from the promoter site and the methylation of histones of the UAF complex in-turn recruits Rpd3 HDAC to deacetylate histones on UAF complex. In parallel TOR pathway inactivation also reduces transcription repressor (TR) protein's affinity to rDNA promoter releasing Ssn6-Tup1 mediated Pol II repression. This orchestration leads to removal of UAF complex from the promoter along with the subsequent release of Hmo1 creating chromatin favorable for Pol II transcription factors to interact.

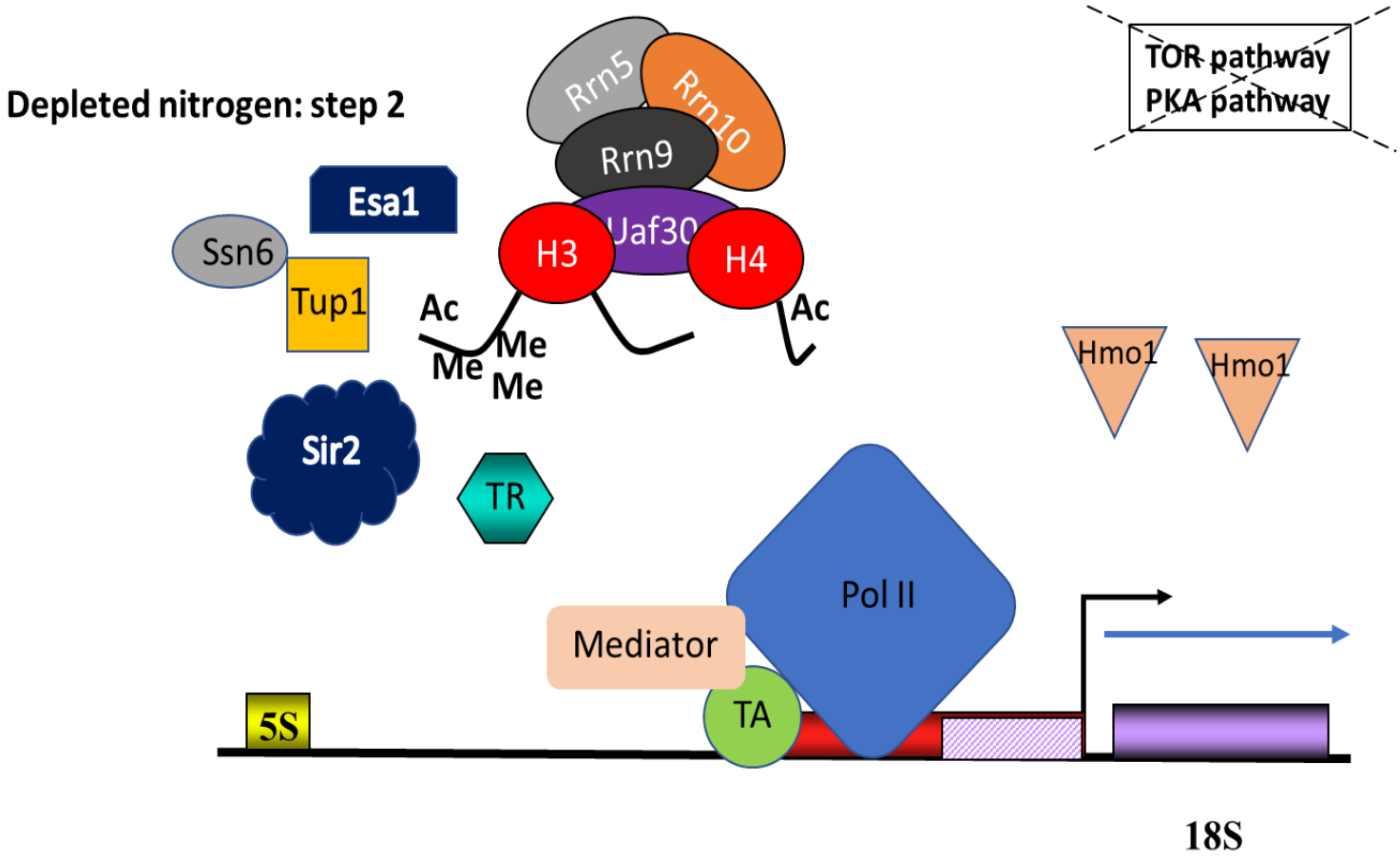

Figure 25: The proposed model represents Pol II transcription occurring at rDNA promoter during stress conditions: Release of UAF complex by Rpd3 deacetylation of UAF components histones H3 and H4 leads to changes in chromatin confirmation that would allow Pol II transcriptional activator proteins (TA) like Hsf1, Yap1 to bind to rDNA promoter and recruit the mediator complex required for Pol II binding to the rDNA promoter and transcription.

Significance of the Study

This research advanced our understanding of the regulation of rRNA synthesis during chronic nitrogen deprivation. The regulation of ribosome synthesis is essential since 80% of total cellular transcriptional machinary is dedicated to ribosome production and defects in ribosome synthesis result in G1 arrest and cell death. During a single cell cycle, a yeast cell can synthesize up to 200,000 ribosomes (Warner, 1999). The rate limiting step in ribosome biosynthesis is rRNA production. Synthesis of ribosomal RNA is under regulation by growth signaling pathways as well as stress pathways allowing a cell to modulate its production of ribosomes dependent on environmental conditions.

Dysregulation of rRNA and ribosome synthesis in humans leads to ribosomopathies- pathologies that are a consequence of aberrant or insufficient ribosome synthesis. The usually autosomal dominant disorder, Treacher Collins Syndrome is caused by defects in the *TCOF1* gene and is characterized by midface hypoplasia, under-developed external ears and abnormal brain development (Chang & Steinbacher, 2012). Mutations in *TCOF1* gene leads to an abnormal nucleolar phosphoprotein Treacle. Treacle normally associates with UBF1 and Pol I to regulate rDNA transcription and rRNA processing (Valdez, Henning, So, Dixon, & Dixon, 2004). Cockayne syndrome A (CSA) is a genetic disorder causing premature aging and pre-mature death. In addition to acting in transcription coupled nucleotide excision repair system, CSA also acts as an important transcription factor for Pol I and stimulates ribosome biogenesis in a cell (Koch et al., 2014). Many other diseases are also associated with ribosomal RNA abnormalities.

Malignant cells show a dramatic increase in rRNA and ribosome synthesis. Recent studies in colon cancer cells showed an increase in pre-45S rRNA expression compared to surrounding non-cancerous cells. This increased pre-45S rRNA expression was due to activated UBF. RNAi or pharmacological disruption of UBF in colon cancer cells led to reduction in pre-45S rRNA synthesis resulting in lowered cancer cell proliferation in tumors (Tsoi et al., 2017). Hence, an understanding of the regulation of rRNA synthesis is crucial and may lead to novel cancer therapies.

The ability to use Pol II to synthesize rRNA is conserved in eukaryotes from yeast to humans (Warner, 1999), suggesting it is an additional mechanism to regulate rRNA synthesis and it may be an important mechanism for cell survival during chronic stress. Since absence of ribosomes or aberrant assembly can lead to cell death, Pol II rRNA synthesis may serve as an alternate mechanism to synthesize the ribosomes necessary to produce protein necessary for survival. Furthermore, ribosomes containing Pol II synthesized rRNA may differ in translation efficiency, fidelity, or stability, differences that evolved to facilitate adaptation and survival. This study of polymerase switch during nitrogen starvation would further our understanding of stress-induced changes in rRNA synthesis that are indispensable for cell survival.

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