ANALYSIS OF THE REGULATION OF THE *S. CEREVISIAE* GENE *PIR3* BY NON-CODING INTERGENIC TRANSCRIPTION

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Robin Monteverde Ceschin, M.S.

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Genome-wide studies have identified pervasive noncoding transcription across prokaryotic and eukaryotic genomes. Although some of these noncoding RNAs (ncRNAs) are likely the result of transcriptional noise, important regulatory functions have been elucidated for a small fraction of these transcripts that have been studied in detail. Previous studies in our lab have elucidated a regulatory mechanism in which transcription of the noncoding RNA, *SRG1*, regulates expression of the adjacent gene *SER3* by directing nucleosome occupancy across the *SER3* promoter, thereby blocking access of activators that induce transcription. A significant portion of noncoding transcription near promoters of protein-coding genes suggests a possible role in the regulation of transcription and initiation of these genes. In this investigation, I describe the identification of a new site of gene regulation by intergenic transcription at *Saccharomyce cerevisiaePIR3* (Proteins with Internal Repeats 3), a gene encoding a cell wall protein identified from RNA pol II genome-wide data. I use a new method of disrupting transcription to assess the effect that loss of intergenic transcription has on *PIR3* expression. This led to the identification of a repressive function for intergenic transcription in *cis* at *PIR3*.

Regulatory functions by ncRNAs have implications in human disease and development, which is often the result of epigenetic changes leading to altered chromatin states. Particularly, investigations of gene expression related to various cancers have provided examples of ncRNAs that can be used as biomarkers in predicting the likelihood of metastasis and silence tumor suppressor genes through epigenetic modifications. From a public health standpoint,

studying the mechanisms by which ncRNAs regulate gene expression or the manner in which epigenetic misregulation leads to disease will allow for more targeted therapies. Additionally, identification of ncRNA is useful in itself as a source of biomarkers in cancer and genetic diseases. This study has additional importance for the development of improved antifungals in the fight against resistant pathogenic strains of yeast, an increasing public health problem, particularly among immunocompromised patients. *S. cerevisiae* is a good model for pathogenic strains of yeast in terms of studying genes and proteins involved in cell wall regulation and biosynthesis which could aid in identifying effective therapies.

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1.0 INTRODUCTION

It has become increasingly apparent that transcription of non-protein-coding DNA (ncDNA) occurs throughout eukaryotic and prokaryotic genomes, often times serving in regulatory roles. This regulation can occur through the act of transcription or by the ncRNA products [50]. Studies of gene regulation by ncRNAs are important based on their roles in human development and disease (reviewed in [59, 23, 78, 50, 28]. *Saccharomyces cerevisae* serves as a model organism for studies on gene regulation and function in eukaryotes.

1.1 TRANSCRIPTION OF PROTEIN CODING GENES

The complex and highly regulated process of mRNA transcription is performed by RNA polymerase II (RNA pol II) a large complex consisting of 12 subunits: Rpb1-12 (reviewed in [70]). There are three major stages of transcription: initiation, elongation, and termination. Each stage is highly regulated by *trans*-acting transcription factors and *cis*-acting regulatory elements. Eukaryotic cells have the added complexity of DNA wrapped around chromatin, creating an obstacle to DNA access that is overcome by various factors. (Figure 1.1).

1.1.1 Transcription Initiation

The transcription cycle begins with the binding of an activator protein to upstream activating sequences (UAS) of gene promoters. The core promoter elements include consensus DNA binding sequences, such as the TATA box, and the transcription initiation site. Bound activators recruit transcription factors in an ordered fashion to assemble the pre-initiation

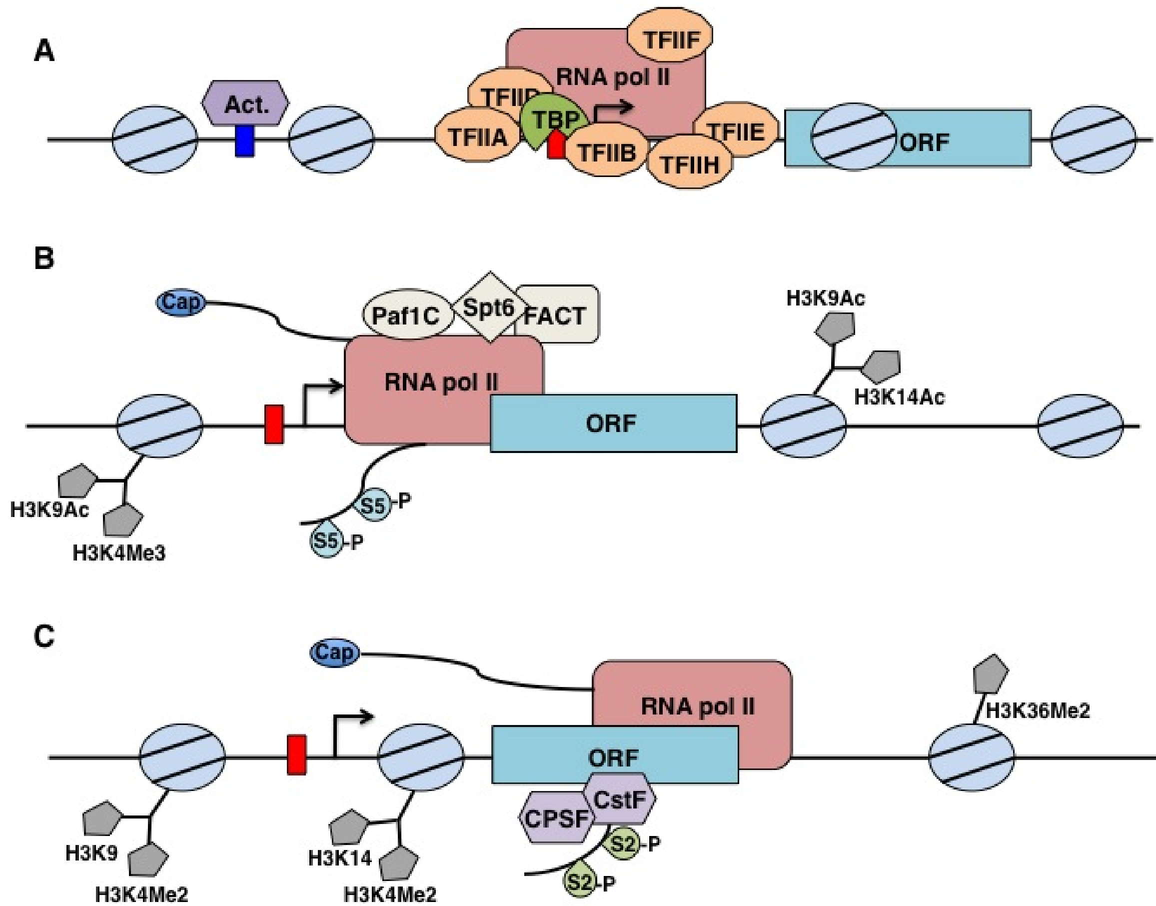

Figure 1.1: **Overview of general transcription of protein coding genes.** Diagrammatic overview of general transcripion of protein coding genes. A) Formation of the PIC with RNA pol II and GTFs at the core promoter. B) Transcription elongation is mediated through phosphorylation of RNA pol II CTD at Ser^5 as well as active transcription marks such as H3 K4 trimethylation and H3 K9 and H3 K14 acetylation. C) Transcription termination is mediated through phosphorylation of RNA pol II CTD at Ser^2 leading to recruitment of poly(A) processing factor CPSF and Ctsf. (see text for details).

complex (PIC) at the core promoter. The PIC is composed of RNA polymerase II (RNA pol II) and general transcription factors (GTFs), including, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. PIC formation is triggered by the binding of TATA-binding protein (TBP), a subunit of TFIID, to the core promoter element through recognition of a consensus sequences (reviewed in [40]). The consensus sequence for TBP is the AT-rich TATA box; however, many promoters lack this element with no consequence to PIC formation. TFIIA and TFIIB are next recruited to stabilize the TFIID-DNA interaction and aid in start site selection. RNA pol II then associates with the forming PIC, an interaction that is stabilized by the simultaneous binding of TFIIF. TFIIH and TFIIE are last to bind to the PIC. The TFIIH helicase activity opens the promoter sequence to begin transcription (reviewed in [67]).

The C-terminal domain (CTD) of Rpb1, the largest subunit of RNA pol II, consists of tandem haptapeptide repeats (YSPTSPS) (27 repeats in yeast and 52 in humans). RNA pol II is hypo-phosphorylated at the time of PIC assembly, but phophorylation states for serines at positions 2, 5, and 7 of the CTD repeats, are necessary depending on the stage of transcription. Following PIC assembly, Kin28, the yeast homologue of human Cdk7 kinase, phophorylates Serine 5 (Ser^5), stimulating RNA pol II clearance of the promoter and productive transcription. After approximately 25 nucleotides (nt) have been produced, the mRNA capping enzyme recognizes Ser^5-P and adds the methylguanosine cap to the 5' end of nascent mRNA, a mark of productive transcription (reviewed in [40]). Di- and tri-methylation of histone H3 at lysine 4 (H3 K4) at the promoter leads to recruitment of histone acetyltransferases (HATs) that acetylate histone resides such as histone H3 K9 and K14 and histone H4 K16 of nucleosomes near the promoter. These histone marks are important for creating a chromatin environment that will promote RNA pol II processivity (reviewed in [67]).

1.1.2 Transcription elongation

Following promoter clearance, RNA pol II moves into the coding sequence to begin the phase of transcription elongation. Elongation is facilitated by a large number of protein coding genes that co-localize with RNA pol II across transcribing genes. These include histone chaperones, histone modifying complexes and other protein complexes that support these actions. Again, modification of the RNA pol II CTD occurs, including a loss of phospho-Ser^5 and addition of phosphate to Serine 2 (Ser^2), mediated by the SSU72 phophatase and Ctk1 (P-TEFb in humans), respectively. Phospho-Ser^2 levels increase toward the 3' end of a gene and stimulate recruitment of cleavage and polyadenylation factors for 3' end processing of the mRNA. An important histone modification associated with elongation is histone H3 K36 methylation, mediated by methlytransferase Set2. Methylated histone H3 K36 recruits Histone deacetylase (HDAC) Rpd3S which removes acetyl-histone modifications returning the chromatin to a non-permissive state, preventing aberrant, or cryptic, transcription from within genes (reviewed in [67].

1.1.3 Transcription termination

Transcription termination is marked by the dissociation of RNA pol II from the 3' end of the transcribing unit. Termination occurs in the context of two separate pathways, the poly(A) signal-dependent pathway, and the Nrd1-Nab3-Sen1-dependent pathway. In the poly(A)-dependent pathway, transcription of the poly(A) signal, a conserved '5-AAUAAA-3' sequence followed by a G/U-rich sequence towards the 3' end of genes, is followed by RNA pol II pausing. The phospho-Ser^2 of the RNA pol II CTD recruits termination complexes, such as the cleavage and polyadenylation specicity factor (CPSF), the cleavage stimulatory factor (CstF) and Poly(A) polymerase, to the 3' end of completed pre-mRNAs. CPSF binds to the transcribed poly(A) track and initiates endoribonucleolytic cleavage of the transcript followed by addition of a poly(A) track by Poly(A) polymerase (reviewed in [67]). The non-poly(A) 3' end formation machinery includes RNA binding proteins Nrd1 (nuclear pre-mRNA down-regulation) and Nab3 (nuclear polyadenylated RNA-binding) and the RNA helicase Sen1 [26]. The Nrd1-Nab3 pathway recruits the TRAMP polyadenylation complex,

of which the Poly(A) polymerase Trf4 is a member, to nascent non-poly(A) transcripts, leading to 3' end processing and/or exosomal degradation [54]. This pathway is utilized in termination of snoRNA, snRNAs, and some long non-coding RNAs, such as cryptic unstable transcripts (CUTs) (reviewed in [48]), [15, 54].

1.1.4 Chromatin and transcription

In eukaryotes, DNA associates with histone proteins to form the compact chromatin structure necessary for securing the genetic code in the nucleus. Chromatin is composed of nucleosomes, which consist of an octamer of histones, around which 147bp of DNA is wrapped 1.65 times. The octamer of histones contains two copies of histones H3 and H4 and two dimers of histones H2A and H2B. Histones are positively charged proteins that fold to form the globular nucleosome structure. The unstructured amino-terminal and C-terminal tails extend from the core nucleosome [43], making contact with DNA or other histone proteins (reviewed in [40]). The chromatin template is formed from the repetition of nucleosomes along the DNA template every 100-200bp [37]. Histone H1 binds to linker DNA between nucleosomes, allowing for the folding of nucleosome into higher order chromatin structure (reviewed in [40]). In yeast, each histone is encoded by two genes and are transcribed as four gene pairs: copies one and two of histone H3 and H4 (*HHT1-HHF1* and *HHT2-HHF2*) and copies one and two of histone H2A and H2B (*HTA1-HTB1* and *HTA2-HTB2*) [43]. The number of histone copies varies among eukaryotes. For example mice and humans have $>$ 50 copies and drosophila have $\sim$100 copies of histone genes. The low copy number in yeast have facilitated studies on the role of histone in various cellular processes (reviewed in [62]).

Chromatin compaction creates an obstacle for DNA access by factors involved in transcription, making the removal or disruption of histones necessary for RNA pol II to move across the transcription unit. The eukaryotic system possesses three main mechanisms for overcoming the chromatin barrier: ATP-dependent chromatin remodeling, addition of histone modifications (ubiquitination, acetylation, methylation, phosphorylation), and histone chaperone activity. Eukaryotic cells have four families of chromatin remodeling complexes, Swi/Snf, Iswi, Chd, and Ino80, that disrupt nucleosome-DNA contacts through the use

of ATP-hydrolysis to laterally slide or remove nucleosomes, aiding in access to gene regulatory elements such as activators. Post-translational histone modifications, particularly histone acetylation, are important for overcoming repressive nucleosome architecture hindering transcription initiation. Paf1 (polymerase-associated factor 1) complex (Paf1C) is a conserved multisubunit complex that has been shown to facilitate transcription elongation in eukaryotes by promoting transcription-dependent post-translational histone modifications (reviewed in [13]). Histone chaperones are important for the removal and replacement of histones from promoters and transcribing sequences in order to both allow passage of RNA pol II and restore repressive chromatin structure in the wake of RNA pol II preventing aberrant transcription from within intragenic or cyrptic promoters. Two well known histone chaperones include Spt6 and the FACT (Facilitates Chromatin Transactions) complex, of which Spt16, Pob3, and auxiliary protein Nhp6 are members (reviewed in [62]).

1.2 YEAST AS A MODEL ORGANISM

In order to better understand regulation of eukaryotic gene expression, we use *Saccharomyces cerevisiae* as a model organism. *S. cerevisiae* is a single cell eukaryote that can exist stably in either a haploid or diploid state. In the haploid state, the genome is composed of 16 chromosomes ranging from 200-2,200 Kb. When the genome was fully sequenced in 1996, approximately 6,183 ORFs were identified and 5,800 were considered to be potential protein coding genes, with only 3.8% containing introns [16, 68]. Genetic analysis in yeast is relatively easy compared to higher eukaryote. In addition to their amenable genetic qualities, *S. cerevisiae* is fast growing (90 minute doubling time), inexpensive, and noninfectious [68].

The ease of genetic manipulation makes this organism ideal for genetic studies. Comparisons between yeast and other eukaryotes lead to the discovery that a considerable degree of homology exists in both protein sequence and function, aiding studies in higher eukaryotes. In addition, mammalian protein function can be studied directly by heterologously expressing human homologues in yeast [8].

An important contribution made by yeast studies toward understanding human disease was recognized in 2001 with the Nobel Prize in Physiology or Medicine, jointly awarded to Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse for their contributions to understanding key regulators of the cell cycle. Dr. Hartwell utilized *S. cerevisiae* in gene mutation experiments that led to the discovery of CDC (cell division cycle) genes and their importance in proper regulation necessary to prevent uncontrolled cell growth or cancer. These findings on cell cycle regulation turned out to be universally applicable to all eukaryotic organisms, again attesting to the high degree of genetic and protein function homology that exists between yeast and higher eukaryotes [58].

1.3 NONCODING RNA (NCRNA)

Important functional noncoding RNAs have long been known. Examples include ncRNAs which are necessary for translation of mRNA into proteins, such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs). Other functional ncRNAs include those involved in RNA processing, such as small nucleolar RNAs (snoRNAs), which function in pre-rRNA cleavage (rRNAs), and small nuclear RNAs (snRNAs) which form the splicesome complex necessary for the removal of introns from pre-mRNAs (reviewed in [18, 34, 48]).

Beyond the well studied examples of functional RNAs, the recent development of new techniques and technology, such as RNA-deep sequencing, high-resolution tiling arrays, and chromatin immunoprecipitation (ChIP) for transcriptome analysis has led to an explosion of ncRNA identification in all organisms (reviewed in [28, 81, 4, 78, 50]). A major question has been whether these ncRNAs serve important biological functions or represent transcriptional noise. While many of these transcripts may represent transcriptional noise, the array of regulatory functions found for ncRNAs has been steadily growing (reviewed in [78]). The discovery of this new class of regulatory RNAs adds a level of complexity to our understanding of gene regulation. Previously, studies on transcription centered around the idea that this process was a means for protein production, not production of RNAs for gene regulation. In gene expression studies in cancer cells, long noncoding RNAs (lncRNAs) have been identified

that have roles in tumor supressor silencing. Additionally, a patient's gene expression profile in cancer cells allows for seemingly accurate predictions in whether the cancer will become metastatic. Therefore, continuing to identify ncRNA and study their regulatory roles can lead to a greater understanding of disease progression and which patients may benefit from more aggressive preemptive treatment ([20] and reviewed in [59, 75]).

1.3.1 Classification of ncRNAs

ncRNAs represent a diverse class of molecules that vary in size, stability, and function. In yeast, there are three classifications of ncRNAs based on stability: SUT, CUTs, and XUTs (reviewed in [3]). These transcripts can be found either sense or anti-sense to protein-coding genes. Stable unannotated transcripts (SUTs) are a group of stable non-coding transcripts, that are capped and polyadenylated, exhibiting a median length of 761 nucleotides. XUTs are Xrn1-sensitive unstable transcripts that are degraded by the cytoplasmic 5' to 3' exonuclease Xrn1, and are only detected in *xrn1* deletion strains [74]. Cryptic unstable transcripts (CUTs) are a recently described class of ncRNAs in yeast that vary in size (200-800bp) but share the common feature of being rapidly degraded by the nuclear exosome such that these transcripts are only detected in strains with deleted exosome components. Termination of CUTs is dependent on the Nrd1-Nab3 pathway which recruits the TRAMP polyadenylation complex, of which the poly(A) polymerase Trf4 is a member, marking them for degradation by the exosome, accounting for the fast turnover of CUTs in cells [54, 80]. Rrp6 is a 3' exonuclease associated with the exosome that rapidly degrades cryptic or unstable transcripts, making deletions of Rrp6 and/or Trf4 necessary for visualization of CUTs [54, 15].

In other eukaryotes, ncRNAs are described primarily based on size, and, in some cases origin of transcription. Long noncoding RNAs (lncRNAs) are described as being greater than 200 nucleotides in length with small noncoding RNAs (sRNAs) being less than 200 nucleotides long, both tending to be associated with gene boundaries. For example, promoter-associated sRNAs (PASRs) are transcribed near gene promoters, whereas those associated with the 3' ends of genes are termed terminator-associated sRNAs (TASRs)
[33]. microRNAs (miRNAs) are another well characterized group of small ncRNAs that are

transcribed and target mRNA with complementary sequences leading to mRNA degradation or translational repression (reviewed in [11]). These long noncoding RNAs can be transcribed in the sense or anti-sense direction of protein coding genes, as well as from intronic sites (reviewed in [50]). In humans, short, polyadenylated and highly unstable transcripts known as PROMPTs (for promoter upstream transcripts) have been identified that, like CUTs, are only detectable in mutants of the human exosome (reviewed in [3]).

1.3.2 Multiple roles for ncRNAs in gene regulation

Long ncRNAs (lncRNAs) have been implicated in gene regulation in multiple ways including affecting chromatin modifications, post-transcriptional processing, and preventing access of transcription factors (reviewed in [50]). These regulatory functions can be performed in *cis* or in *trans*.

1.3.3 Regulation of noncoding RNAs in *trans*.

Long noncoding RNAs can act in *trans* to mediate epigenetic regulation through altered chromatin states with implications in human disease and development. In these cases, the RNA product is coordinated with the regulation of gene expression, often times by associating with proteins that perform histone modifications. For example, X-chromosome inactivation, important for maintaining proper gene dosage in mammalian females, is regulated by the concerted action of ncRNAs *Tsix* and *Xist*. *Tsix* and *Xist* block or recruit, respectively, the polycomb repressive complex (PRC2), which silences genes by promoting repressive H3 lysine 27 trimethylation (H3 K27me3) mark. Also, the human *HOTAIR* (Hox transcript antisense RNA) ncRNA, transcribed from *HOXC* locus of the homeobox domain, recruits PRC2 to the *HOXD* locus, inducing a repressive chromatin state, which is important for regulating proper timing of gene expression (reviewed in [50]). Recently, misregulation of recruitment of PRC2 by *HOTAIR* has been implicated in breast cancer metastasis with the finding that *HOTAIR* can recruit PRC2 to tumor suppressor genes, resulting in their epigenetic silencing [20, 75] (Figure 1.3.3).

A cancer-associated virus, Kaposi's sarcoma-associated herpesvirus (KSHV), produces a polyadenylated nuclear RNA (PAN RNA) that interacts with host proteins in the nucleus and is necessary for expression of late viral genes [7]. Recent data suggests that PAN RNA activates KSHV gene expression through interaction with demethylases and methyltransferases resulting in the removal of repressive H3 K27me3 marks with the simultaneous addition of the activating H3 K4me3 marks [66]. *trans* interactions of ncRNAs with transcription factors can also act in repression, for example a ncRNA that interacts with TFIIB prevents stable PIC formation, specifically at the human *DHFR* (Dihydrofolate reductase) locus (reviewed in [78]). Antisense noncoding transcription can also affect gene regulation. For example, the *p15* human tumor supressor gene has been shown to be silenced by the *p15AS* ncRNA transcribed from the 3' end and across the promoter of *p15*, altering histone modification that result in heterochromatin formation (reviewed in [23]).

1.3.4 Regulation by noncoding RNA is *cis*.

Transcription of ncRNAs in *cis* performs various regulatory functions, including nucleosome remodeling, promoting histone modifications, and blocking promoter access. The *Schizosaccharomyces pombe fbp1*$^{+}$ gene is activated in response to glucose starvation through nucleosome remodeling by ncRNA transcription. At this locus, ncRNA transcription across the promoter yields a chromatin structure that is open, making access by activators and transcription factors possible (reviewed in [78]). Repressive histone marks can also be regulated in *cis*. At the divergently transcribed *GAL1* and *GAL10* genes, transcription originating from the 3' end of *GAL10* induces repressive histone modifications, such as H3 K36 methylation (reviewed in [23]). Transcription interference is another *cis*-acting regulatory mechanism that results in blocking promoter access by activators or transcription factors as a result of elongating noncoding transcription. Examples of this include activator eviction at *ADH1* and *ADH3* as a result of *ZZR1* transcription across the promoter [5].

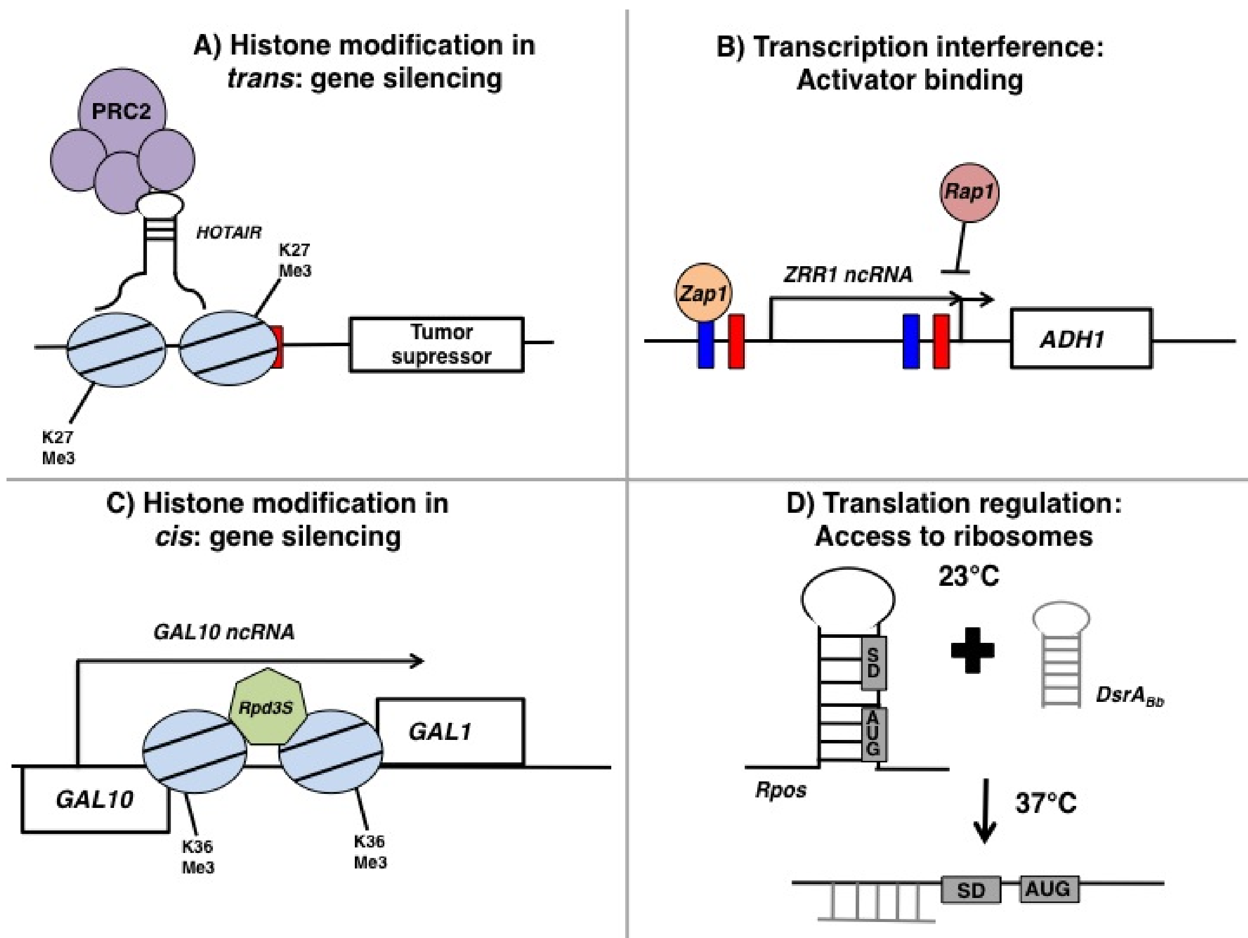

Figure 1.2: **Diagram of gene regulation by ncRNA.** **(A)** Diagram of *trans* acting *HOTAIR* recruiting PCR2 to silence tumor supressor genes [20]. **(B)** Transcription interference model at *ADH1* where intergenic transcription of *ZRR1* evicts the Rap1 activator protein at the *ADH1* promoter [5]. **(C)** Transcription in *cis* across the *GAL10/GAL1* promoter mediates recruitment of the Rpd3S HDAC resulting in repressive histone H3 K36 methylation [27, 57]. **(D)** Translation regulation by ncRNA by the *B. burgderfori DsrA$_{Bb}$* sRNA. At 23°C the *rpoS* mRNA is in a hairpin formation which sequesters the Shine-Delgaro (SD) sequence. At 37°C, DsrA$_{Bb}$ binds to complementary sequence on *rpoS* mRNA, relaxing the hairpin for ribosome access [44].

1.3.5 Noncoding RNAs can regulate mRNA translation.

Beyond transcription regulation of gene expression, translation of mRNAs can be regulated by ncRNA association. For example, translation of a virulence-associated gene in *Borrelia burgdorferi*, the causative agent of Lyme disease, is indirectly dependent on a ncRNA. A proposed mode of regulation for this small ncRNA (sRNA), DsrABb, is that it can rapidly induce translation of the RpoS sigma factor required for transcription of the outer membrane protein OspC, necessary for transmission of *B.burgdorferi* from tick to mammalian host. Translation of the long *rpoS* mRNA is thought to be rapidly induced by the binding of DsrABb which relaxes a proposed 5' hairpin structure in the mRNA, which obscures the Shine-Delgarno sequence necessary for translation [44] (reviewed in Samuels2011.

Another interesting example of ncRNA-mediated translation has been implicated in Alzheimer's disease. This ncRNA, β-site amyloid precursor protein (APP)-cleaving enzyme-Antisense (BACE1-AS) is transcribed in the opposite direction of BACE1. The ncRNA interacts with the mRNA at BACE1 for stabilization during translation. If BACE1-AS transcription is increased, it may lead to increasingly stable BACE1 mRNA and thus higher translation of this protein. Cleavage of the BACE1 target, APP, leads to the production of amyloid β-peptide (Aβ), of which high levels have been implicated in neurological disorders, such as Alzheimer's disease (reviewed in[75]).

These studies make it is apparent that ncRNAs have a variety of distinct roles in gene regulation. Some of these examples have implications in human disease and cancer. Studying ncRNAs will lead to a greater understanding of how they regulate gene expression leading to possible insights in cancer progression and prognosis. We can use our knowledge of ncRNA in disease as biomarkers to aid in treatment decisions. In the future, ncRNAs may even become therapeutic targets for RNAi to prevent ncRNAs from silencing tumor supressors or operating to exacerbate disease states (reviewed in [59]).

1.4 *SRG1/SER3* AS A MODEL FOR REGULATION BY NCRNA TRANSCRIPTION

Previous studies have discovered a noncoding DNA (ncDNA) sequence, *SRG1* (SER3 Regulatory Gene 1), that is transcribed across the promoter of the adjacent downstream gene, *SER3*, resulting in *SER3* repression [45]. Based on the prevalence of transcription that occurs in proximity to promoters of protein-coding genes, this mechanism serves as a general model for gene regulation by intergenic transcription. *SER3* encodes an enzyme that catalyzes a step in the serine biosynthesis pathway [1] and its expression is tightly regulated by the availability of serine [46]. Transcription of *SRG1* serves as the regulatory switch that responds to serine availability [46]. This is a complex regulatory system involving many factors including the Spt6 and Spt16 histone chaperones, Paf1 elongation complex, and their control of chromatin [22, 60, 21].

In high serine conditions, the Cha4 activator protein recruits the SAGA HAT and the Swi/Snf chromatin remodeling complex to initiate RNA pol II transcription of *SRG1* (Figure 1.3). As RNA pol II transcribes *SRG1* through the *SER3* promoter sequence, nucleosomes are removed in front of the elongating RNA pol II and redeposited behind it in a manner dependent on histone chaperones Stp6 and Spt16. The presence of these nucleosomes block transcription factors from binding to the *SER3* promoter [24]. When serine levels become low in the cell, Cha4 no longer associates with SAGA and Swi/Snf, resulting in the loss of *SRG1* transcription-coupled nucleosome remodeling across the *SER3* activating sequence. This provides transcription factor access to the *SER3* promoter [46, 24]. Two positioned nucleosomes remain at the 5' end of *SRG1* during times of high serine when *SER3* is expressed, which may be the source of histones that regulate *SER3* expression through promoter occlusion in a transcription dependent manner [24]. From these and other studies, nucleosome occlusion of protein coding promoters in a manner dependent on transcription of the ncDNA represents a novel mechanism of gene regulation. These studies provide a framework for investigating new cases of gene regulation by ncDNA transcription.

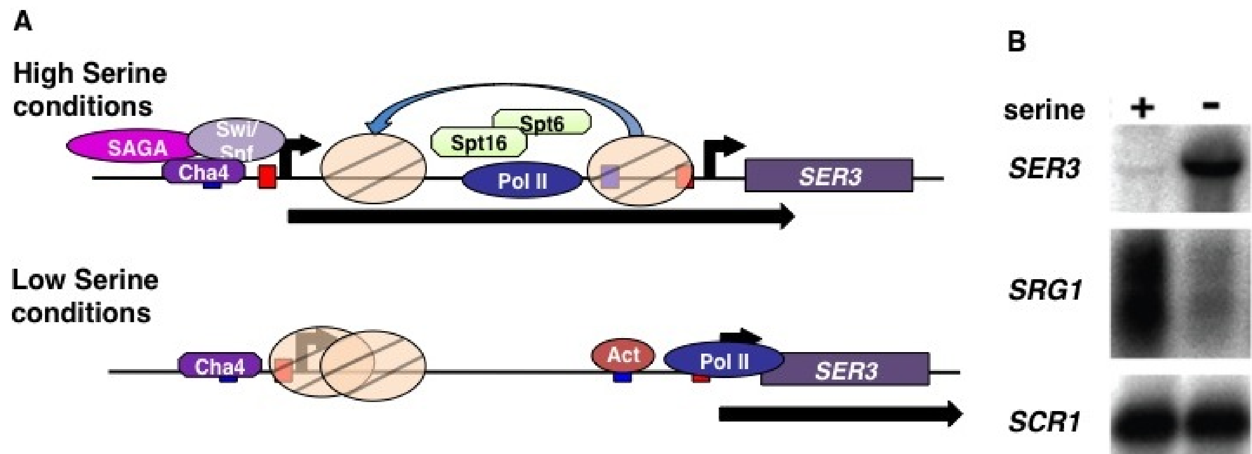

Figure 1.3: **Model for regulation of *SER3* by transcription of the ncDNA *SRG1*.** (A) A model for repression of *SER3* by *SRG1* transcription. In high serine conditions, nucleosomes are remodeled and maintained over the *SER3* promoter by *SRG1* transcription. These transiently positioned nucleosomes prevent the binding of an activator, leaving *SER3* in a repressed state. The cell responds to serine starvation by turning off *SRG1* transcription, leading to a loss of the nucleosomes across the promoter, allowing activator binding and *SER3* expression. (B) Northern blot analysis examining the effects of serine on the expression of *SRG1* and *SER3*. Cells were grown to a density of 1-2 X 10^7 cells/ml at 30°C in SC+serine media and shifted to SC-serine media for 25 min. *SCR1* serves as a loading control.

1.5 IDENTIFICATION OF *PIR3* FOR REGULATION BY NCRNA TRANSCRIPTION

The pervasiveness of non-coding transcription throughout eukaryotic genomes and the increasing number of regulatory roles being found for ncRNAs suggest the possibility that the act of transcription and/or RNA products may regulate a large number of protein coding genes. To discover candidate ncRNAs that may have regulatory roles, we filtered genome-wide RNA pol II microarray data (J. Martens, unpublished genome-wide data) to find intergenic regions displaying high RNA pol II density across promoters of annotated protein-coding genes. *PIR3* was selected based on the presence of significant RNA pol II occupancy over its promoter, indicating that intergenic ncRNA transcription could have a regulatory role at the promoter (Figure 1.4). Previous studies have identified *PIR3* as a gene that encodes the cell wall protein (CWP) Pir3, part of the Pir (proteins with internal repeats) family. These proteins were first identified based upon their N-terminal amino acid repeats (18-19 residues, tandemly repeated 7-10 time in the case of Pir1, Pir2, and Pir3), and were found to be non-essential [73]. A subsequent study aimed at systemically identifying cell wall components, discovered the Pir family of proteins to be covalently linked, non-soluble, major cell wall stability proteins. The alias given to these proteins is Ccw (covalently linked cell wall proteins), with *PIR3* designated as *Ccw8* [51]. Clues as to how *PIR3* expression may be physiologically regulated by intergenic transcription could come from understanding the role that Pir3 plays in cell wall construction and stability.

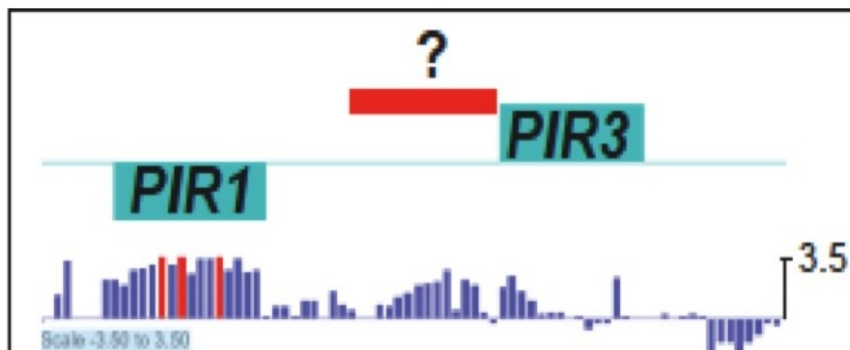

Figure 1.4: **Identification of intergenic transcription at *PIR3*.** Genome-wide RNA pol II ChIP reveals high levels of Pol II binding upstream of *PIR3*, indicative of potential intergenic transcription (Joe Martens, unpublished).

1.6 ROLE OF *PIR3* PROTEIN IN CELL WALL

The yeast cell wall, of which the Pir3 protein is a part, is a complex and adaptable structure with important functions including maintaining turgor pressure and cell shape, acting as a protein scaffold, and protecting the cell against physical stress (reviewed in [42]) (Figure 1.5). Load-bearing β-1,3 and 1,6-glucans make up the inner layer of the cell wall and serve as a scaffold for an outer layer of mannoproteins. Some chitin chains, accounting for a small percentage of the total cell wall, are found glycosidically attached to the β-1,3-glucans with attachment of chitin to β-1,3 and 1,6-glucans increasing in response to cell wall stress. The flexible and helical shape of the β-1,3-glucans allow for cell wall flexibility, necessary for remodeling during growth, mating, and response to cell wall stress. The β-1,6-glucans are highly branched and are found external to the β-1,3-glucans, linked through an unknown mechanism (reviewed in [35, 42]).

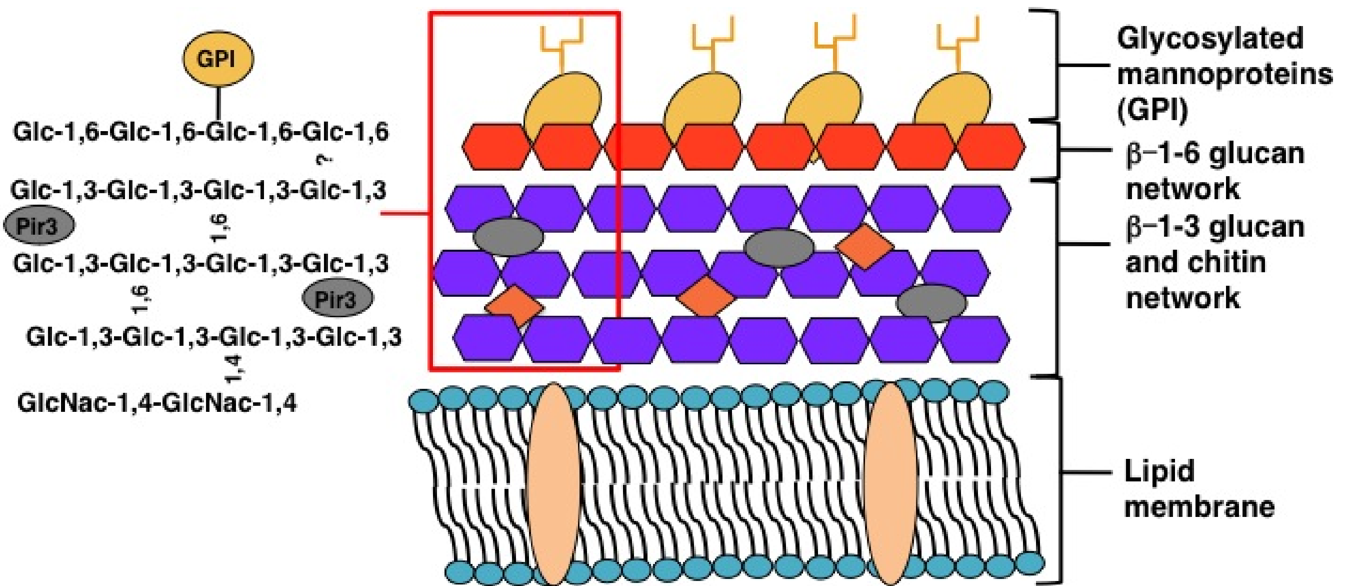

Figure 1.5: **Representation of the yeast cell wall.** External to the lipid cell membrane, β-1,3 and 1,6-glucans make up the inner layer of the cell wall. External to the glucan network, glycproteins, such as mannoproteins, are linked to the β-1,6-glucans through modified GPIr anchors, protecting the cell wall by reducing permeability. Cell wall protein (CWPs), like Pir3, are covalently attached to β-1,3-glucans. Chitin (yellow diamonds) chains are found predominately attached to β-1,3-glucans during normal cell growth.

1.6.1 Protein components of the cell wall

Outer layer CWPs, decrease cell wall permeability, leading to protection against cell wall perturbing agents (reviewed in[35]). Other CWPs are found directly attached to the the β-1,3-glucan network through an alkali soluble linkage. This protein group is composed of the Pir-CWPs with specific linkage occurring between the repetitive and conserved amino acid motif and the β-1,3-glucans. No enzyme has been found to catalyze covalent linkage, but an autocatalytic reaction has been suggested [17]. Evidence to support the involvement of these CWPs in increasing cell wall strength comes from the fact that their expression is increased in response to cell wall stress [6, 19]. Additionally, loss of multiple Pir proteins leads to increased susceptibility to cell wall stress agents (reviewed in [42]).

1.6.2 Cell wall stress

Cell wall stress arising from environmental pressures or structural changes during normal cell growth is addressed through the cell wall integrity (CWI) pathway (reviewed in [42]) (Figure 1.6). Two sub-families of plasma-membrane spanning sensors are responsible for initiating CWI signaling. One group consists of the Wsc-type sensors encompassing Wsc1, Wsc2 and Wsc3, and the other group includes Mid2 (Mating Induced Death) and Mtl1 (Mid-two-like protein). It is believed the cell wall contacts of these transmembrane proteins are altered as a result of cell wall perturbations, implicating them in the role of mechanosensors, rather than molecule sensing proteins (reviewed in [29]).

Wsc1 and Mid2 interact with the guanosine nucleotide exchange factors (GEFs), Rom1 and Rom2. These GEFs can simultaneously interact with Rho1 to catalyze nucleotide exchange. Rho1 is an essential protein and a member of the (Ras-homologous) family of GTPases that are integral to mediating polarized cell growth in mammalian and fungal cells. The GTP-bound Rho1 can interact with and activate Pkc1 (protein kinase C), initiating the linear Pkc1-activating MAPK (Mitogen Activated Protein Kinase) cascade. Pkc1 has been shown to phosphorylate Bck1 MEKK, which in turn phosphorylates the Mkk1/2 MEKs. Mkk1 and -2 phosphorylate the Mpk1/Slt2 MAPK [42]. Mpk1 activates the Rlm1 (resistant to the lethality of constitutive Mkk1) transcription factor through phosphorylation

of its transcriptional activation domain (reviewed in [42]) [31]. It has been demonstrated that Rlm1 is an important activator of at least 25 genes involved in cell wall biogenesis [31]. Rlm1 contains an N-terminal binding domain with specificity for [CTA(T/A)4TAG] conserved sequence. In particular, *PIR3* possesses a canonical binding site for the Rlm1 transcription factor which may be important for *PIR3* expression in cell wall stress-inducing conditions [19, 30].

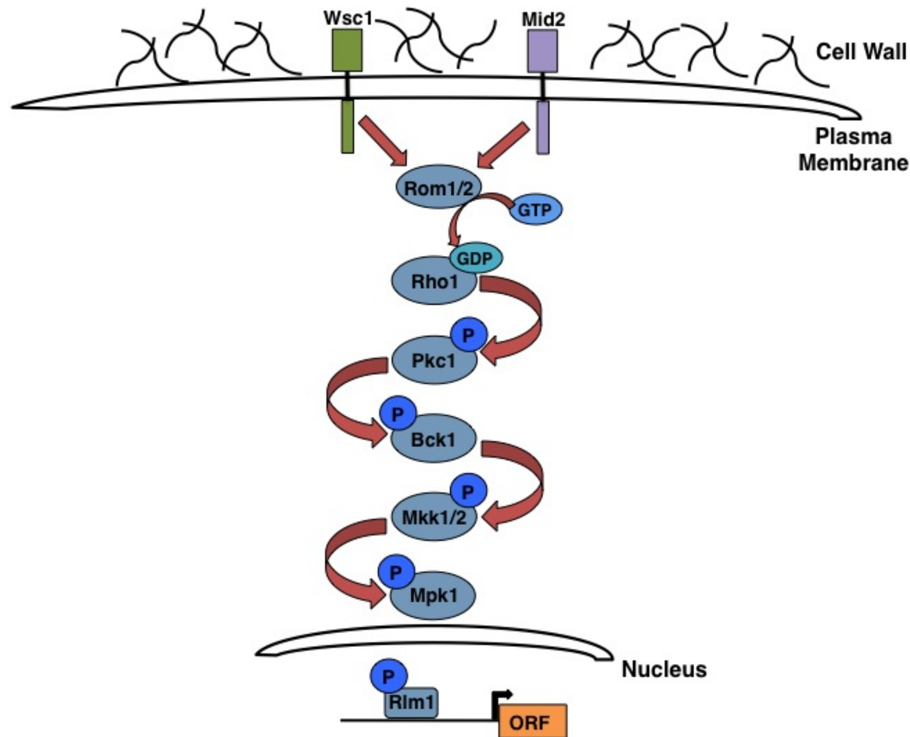

Figure 1.6: **Schematic representation of the CWI MAPK pathway for Rlm1 activation.** Signaling is initiated by surface receptors, Mid2 or Wsc1, that interact with GEFs Rom1 or Rom2, stimulating nucleotide exchange on Rho1. Activated Rho1 phosphorylates Pkc1, initiating the Pkc1-activated MAPK cascade. The Rlm1 transcription factor is phophorylated at the completion of cascade and activates expression of genes important in cell wall biogenesis.

1.7 *PIR3* REGULATION IN RESPONSE TO CELL WALL STRESS

As previously mentioned, the CWI pathway is upregulated in response to cell wall stress from a variety of sources. Through genome-wide analysis assessing the transcriptional response to cell wall stress agents and cell wall mutations, *PIR3* has demonstrated increased mRNA expression, presumably through Slt2 mediated activation of the Rlm1 transcription factor [6, 39, 19]. *PIR3* has been shown to be induced upon over-expression of Mkk1, also a part of the MAPK kinase pathway that activates Rlm1, in response to prolonged elevations in temperature (39°C) [30]. Additionally, *pir3* deletions, particularly when combined with other deletion of the Pir family proteins, leads to increased sensitivity to osmotin (plant antifungal protein), congo red (β-1,3-glucan-binding) calcoflour white (chitin antagonist), and caffeine (activator of Pkc1-MAPK kinase cascade) [83, 52, 71, 49]. Rlm1 has been demonstrated to be an important factor in activating genes associated with caffeine-induced stress [76]. Evidence suggests that this protective effect may be the result of multiple β-1,3-glucans linking to the internal amino acid repeats of the Pir proteins, decreasing the permeability of the cell wall to these agents [35]. Although much remains to be discovered about the role of *PIR3* in inducing cell wall strength and stability, we can use these studies to assist in our analysis of regulation by ncRNA. Particularly, growing cells in conditions that induce cell wall stress with subsequent analysis of expression of the ncRNA and *PIR3* ORF may lead to identification of natural conditions in which intergenic transcription plays a regulatory role. From this investigation I am able to demonstrate a new source for repression by intergenic transciption at *PIR3*.

2.0 THESIS AIMS

Accumulating evidence is implicating ncRNAs in gene regulation, rather than as transcriptional by-products. However, many sources of ncRNA transcription have been identified without implication in defined regulatory roles. I began this investigation with the hypothesis that intergenic noncoding RNA across the *PIR3* promoter enacts a regulatory function. This hypothesis is supported by studies at *SRG1/SER3* where nucleosome deposition across the *SER3* promoter occurs in a manner dependent on transcription of the intergenic ncRNA *SRG1*. My goal in this study was to provide evidence supporting regulation of *PIR3* expression by intergenic transcription. Here, I provide evidence that *PIR3* expression is in fact affected by intergenic transcription. This regulation appears to be occurring in a manner independent of the Rlm1-mediated cell wall stress response. Although my studies into the mechanism of regulation at *PIR3* are not complete, my data suggest that *PIR3* repression by intergenic transcription occurs by a mechanism that is distinct from what we have previously described for *SER3*.

Specific Aim #1: Analyze ncRNA transcription upstream of *PIR3*.
The goal of this Aim is to determine if intergenic transcription upstream of *PIR3* has a regulatory function. Following verification of ncRNA, a method for inserting a strong transcription termination sequence within the region of intergenic transcription was employed to stop intergenic transcription across the *PIR3* promoter. The affect that loss of the ncRNA has on *PIR3* expression was investigated.

Specific Aim #2: Determine the start site *PIR3* ncRNA.

The goal of this Aim is to map start sites for the intergenic and *PIR3* transcripts. This will help to characterize the ncRNA and *PIR3* in terms of start site of transcription initiation.

Specific Aim #3: Determine a model for regulation at *PIR3*.

Regulation of gene expression by intergenic transcription could occur by a number of mechanisms, including the ncRNA product in *trans* or promoter occlusion in *cis*. Promoter occlusion in *cis* could occur through nucleosome remodeling at the promoter or through repressive post-translational histone modifications. The transcription interference mechanism regulates gene expression by blocking access of activators or transcription factors to the gene promoter through the act of transcription. There are three goals of this Aim:

1) Determine if the intergenic ncRNA at *PIR3* regulates in *cis* or in *trans*.

2) Test the role of chromatin in *PIR3* regulation.

3) Determine if transcription interference is working at *PIR3* by assessing TBP occupancy at the *PIR3* promoter in the absence or presence of intergenic transcription.

Specific Aim #4: Determine if regulation by intergenic transcription has a physiological basis in cell wall stress.

Previous studies have implicated *PIR3* expression to increase in response to cell wall stress in an manner dependent on the Rlm1 transcription activator. Some of these inducing conditions include caffeine and prolonged temperature increases, which can activate the CWI pathway. The goal of this Aim is to determine if these conditions may activate *PIR3* through loss of intergenic transcription, implicating the ncRNA transcription in interfering with Rlm1 binding in none-stress conditions. This hypothesis will be tested by growing cells and measuring the levels of ncRNA and *PIR3* mRNA at elevated temperatures as well as in caffeine in order to determine if regulation by the *PIR3* ncRNA has a basis in CWI signaling [76, 30, 32].

3.0 MATERIALS AND METHODS

3.1 YEAST STRAINS AND MEDIA

Saccharomyces cerevisae strains are derived from a *GAL2*$^{+}$ strain of S288C and are listed in Table 3.1 [79]. All strains were constructed using standard transformation techniques and genetic crosses [2]. Strains were grown in the following media as indicated in the figure legends: YPD (1% yeast extract, 2% peptone, 2% glucose), YPD + caffeine (1% yeast extract, 2% peptone, 2% glucose, 12mM caffeine) [65].

The *pir3*$_{TTS}$ allele was created to terminate transcription of the *PIR3* ncRNA by insertion of a 171bp DNA fragment containing a minimal *HIS3* transcription termination sequences, *HIS3*-TTS (Figure 4.3). The *pir3*$_{TTS}$ allele was constructed by PCR-mediated two-step gene integration as has been previously described [65]. First, a 1276bp sequence containing *URA3* flanked by 171bp of the *HIS3* termination sequence was amplified from pDW1 (unpublished Danielle Wagner) using a pair of primers, each having 60bp of sequence on their 5 ends derived from the sense and antisense strands on either side of the site of integration into the genome. Ura^{+} transformants were passaged on non-selective media (YPD). Following overnight growth at 30°C, they were replica-plated onto media containing 5-Fluoroorotic acid (5-FOA) (1mg/μl, US Biological), a drug that is toxic to Ura^{+} yeast cells, to select for cells that have lost the *URA3* gene by homologous recombination leaving behind a single copy of the *HIS3*-TTS. Correct insertion of the *HIS3*-TTS at -486bp relative to the translation start of *PIR3* was verified by PCR. The *srg1*$_{TTS}$ was created in the same manner with placement of the *HIS3*-TTS at -450bp, relative to *SER3* ATG. The *ura3*$_{TTS}$ allele was generated from the *pir3*$_{TTS}$ allele with replacement of the *PIR3* ORF (from +1 to +977; *PIR3* ATG=+1) with a PCR-generated *URA3* ORF (from +1 to +804bp; *URA3* ATG=+1).

Strains containing the histone H3 K122A double mutation were created by a one-step integration of plasmids expressing a synthetic histone gene targeting the *HHT1/HHF1* locus and tagged with a selectable hygromycin resistant cassette (kind gift from J. Dai, Tsinghua University). The strains used in this transformation were JDY86 strains expressing another H3 K122A synthetic histone gene sequence replacing *HHT2/HHF2* marked by a selectable *URA3* marker. Both histone mutations, at *HHT2/HHF2* and *HHT1/HHF1*, were created as part of a plasmid library of histone H3 and H4 mutants containing a number of systematic amino acid substitutions (previously described [14]). The plasmids containing these mutations can be integrated into the yeast genome through homologous recombination between sequences flanking the selectable marker and mutation on the plasmid and the genomic site of the H3/H4 genes. Plasmids were linearized with *Bci*VI and transformed into the JDY86 strain with the *(hht2-hhf2)*Δ*::hhts-K122A/HHTS-URA3* mutation by homologous recombination (previously described [14]). Transformants were selected on YPD media containing 200μg/mL of hygromycin, and confirmed through PCR and sequencing (unpublished, S. Hainer).

pir3-nctata1, pir3-nctata2, pir3-nctata3 (pir3 noncoding tata) alleles were generated by two-step gene replacement as previously described [65]. Putative *PIR3* ncRNA TATA elements (Figure 4.1) were mutated to a *Avr*II restriction site by tranformation with integrating *URA3*-marked plasmids pRM13, pRM14, pRM15 (see description below) into *ura3*Δ strains, that had been linearized with *Eco*RI. Transformants were selected for on plates containing SC-URA. Ura$^+$ transformants were passaged on non-selective media (YPD) and then replicaplated onto media containing 5-FOA to select for strains that have lost the integrating sequence by homologous recombination. Replacement of the TATA elements with *Avr*II was verified by PCR and subsequent restriction digest.

3.2 PLASMID CONSTRUCTION AND ANALYSIS

Plasmids were constructed and isolated from *Escherichia coli* by standard methods [2].

pDW1 was created by three successive cloning steps (D. Wagner). First, one copy of the *HIS3*-TTS sequence with flanking *Not*I restrcition sites was PCR-generated from genomic DNA. The *Not*I restriction sites were used to ligate *HIS3*-TTS into the polylinker of pBluescript II SK- (Stragene). Correct orientation of inserts was tested by restriction digest. *URA3* was excised from pRS406 and ligated into the same pBluescript II SK- using the *Eco*RI and *Xba*I restriction sites. A second copy of the *HIS3*-TTS sequence with flanking *Eco*RI and *Xho*I restriction sites was PCR-generated from genomic DNA and ligated into a separate pBluescript II SK-. This *Eco*RI-*HIS3*TTS-*Xho*I fragment was then cut and ligated into the *Not*I-*HIS3*TTS-*Not*I containing plasmid. The resulting pDW1 contains *URA3* flanked by copies of *HIS3*-TTS sequences (Figure 4.3)

An *Eco*RI to *Bam*HI DNA fragment containing the *PIR3* sequence from -1319 to -225 (*PIR3* ATG=+1) that was generated by PCR and cloned into pBluescript II SK- to generate pRM04. This sequences contains putative ncRNA TATA elements, sites 1, 2, and 3 (Figure 4.1) that may promote *PIR3* ncRNA transcription. Each site was mutated to an *Avr*II site (CCTAGG) by Quick-Change Mutagenesis on three separate plasmids [84]. pRM06 contains the site 1 *Avr*II mutation (from -682 to -677; *PIR3* ATG=+1); pRM09 contains the site 2 *Avr*II mutation (from -765 to -760; *PIR3* ATG=+1); pRM10 contains the site 3 *Avr*II mutation (-870 to -865; *PIR3* ATG=+1). Adaptations to the standard site-directed mutagenesis protocol included use of phusion polymerase with the 5X high fidelity (HF) mix (Fermentas), 0.15μl of each 100μM primer, 10-50ng plasmid template, and 1.0μl of a mix containing 10mM of each dNTP, for a total reaction volume of 50μl. The PCR conditions were 98°C for 10 sec; 20 cycles of 98°C for 30 sec, 55°C for 30 sec and 72°C for 7 min, for a $\sim$4Kb plasmid, with annealing temperatures adjusted based on primer melting temperature. Following *Dpn*I digestion, 5μl of each reaction was transformed into DH5α cells using standard methods and selected for on LuriaBertani (LB) plate containing 100μg/ml ampicillin. Following confirmation by sequencing, the mutated fragments were sublconed by

excision from pBluescript II SK- and ligation into individual integrating plasmids pRS406 [12] using *Eco*RI and *Bam*HI restriction enzymes resulting in pRM13, pRM14, and pRM15, containing *pir3-nctata1, pir3-nctata2, pir3-nctata3*, respectively. p*SPT16-URA*, p*SPT16-LEU2*, and p*spt16-E857K-LEU2* have been previously described [21].

Table 3.1: Yeast Strains

Strains	**Genotype**	**Source**
FY4	*MAT**a***	F. Winston
FY2431	*MAT**a** his3*Δ*200 lys1-128*δ *leu2*Δ*1 spt2*Δ*0*	F. Winston
KY719	*MAT**a** ura3*Δ*0*	K. Arndt
KY912	*MAT**a** hhis3*Δ*200 leu2*Δ*1 lys2-128*δ *ura3-52 set2*Δ*::HIS3*	K. Arndt
KY934	*MAT*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 dot1*Δ*::HIS3*	K. Arndt
KY938	*MAT*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 set1*Δ*::HIS3*	K. Arndt
KY1235	*MAT**a** his3*Δ*200 lys2-128*δ *ura3-52 rco1*Δ*::HIS3*	K. Arndt
KY1703	*MAT**a** rtf1::KanMX rtf1*Δ*::kanMX4*	K. Arndt
JD86	*MAT**a** his3*Δ*200 leu2*Δ*0 lys2*Δ*0 trp1*Δ*63 ura3*Δ*0 met15*Δ*0 (hht1-hhf)*Δ*::NatMX4 (hht2-hhf2)*Δ*::HHTS/HHFS (or containing substitution)-URA3 can1*Δ*::MFApr-HIS3*	[14]
YJ785	*MAT**a** paf1*Δ*::kanMX4*	K. Petrov
YJ787	*MAT*α *rtf1*Δ*::kanMX4*	K. Petrov
YJ788	*MAT*α *his3*Δ*200 rtf11*Δ*::kanMX4*	K. Petrov
YJ807	*MAT*α *his3*Δ*200 paf1*Δ*::kanMX4*	K. Petrov
YJ809	*MAT*α *ura3*Δ*0 paf1*Δ*::kanMX4*	K. Petrov
YJ822	*MAT**a** srg1::*$srg1_{TTS}$	D. Wagner
YJ1091	*MAT*α *ura3*Δ*0 leu2*Δ*0 his4-912*δ *lys2-128*δ *trp1*Δ*63 spt16*Δ*::KanMX pSTP16-URA3*	[21]

Table 3.1: Yeast Strains (continued)

Strains	**Genotype**	**Source**
YJ1092	*MAT**a** ura3Δ0 leu2Δ0 his3Δ200 spt16Δ::KanMX KanMX-Gal1-prFLO8-HIS3 pSPT16-URA3*	[21]
YJ1100	*MAT**a** pir3::pir3*$_{TTS}$	This study
YJ1101	*MAT**a** pir3::pir3*$_{TTS}$	This study
YJ1102	*MAT**a** pir3::pir3*$_{TTS}$	This study
YJ1103	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::HHTS-HHFS*-Hygro *(hht2-hhf2)Δ::HHTS-HHFS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer
YJ1104	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::HHTS-HHFS*-Hygro *(hht2-hhf2)Δ::HHTS-HHFS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer
YJ1105	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::HHTS-HHFS*-Hygro *(hht2-hhf2)Δ::HHTS-HHFS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer
YJ1106	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::hhts-K122A/HHFS*-Hygro *(hht2-hhf2)Δ::hhts-K122A/HHTS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer
YJ1107	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::hhts-K122A/HHFS*-Hygro *(hht2-hhf2)Δ::hhts-K122A/HHTS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer
YJ1108	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::hhts-K122A/HHFS*-Hygro *(hht2-hhf2)Δ::hhts-K122A/HHTS-URA3 can1Δ::MFA*pr-*HIS3*	S. Hainer

Table 3.1: Yeast Strains (continued)

Strains	Genotype	Source
YJ1109	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::ura3*$_{TTS}$*/PIR3*	This study
YJ1110	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::ura3*$_{TTS}$*/PIR3*	This study
YJ1111	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::ura3*$_{TTS}$*/PIR3*	This study
YJ1112	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::URA3/pir3::pir3*$_{TTS}$	This study
YJ1113	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::URA3/pir3::pir3*$_{TTS}$	This study
YJ1114	*MAT**a**/MATα ura3Δ0/ura3Δ0 pir3Δ::URA3/pir3::pir3*$_{TTS}$	This study
YJ1115	*MAT**a** ura3Δ0*	This study
YJ1116	*MAT**a** ura3Δ0*	This study
YJ1117	*MAT**a**/MATα*	This study
YJ1118	*MAT**a**/MATα*	This study
YJ1119	*MAT**a**/MATα*	This study

3.3 NORTHERN ANALYSIS

Cells were grown in the media as indicated in figure legend to mid-log (1-2 X 10^7 cells/ml) at 30°C. Total RNA isolation and Northern blot analysis was performed as previously described [2]. Double-stranded DNA probes were radiolabled with [α-^{32}P] dATP by random-primed labeling of purified PCR fragments. Location of probes are relative to the ATG=+1 of each gene and are as follows: *SER3* (+111 to +1342; OJ63 & OJ64), *SRG1* (-454 to -123,

relative to *SER3* ATG), *SCR1* (+163 to +284, relative to *SCR1* ATG), *PIR3* (-479 to -101; OJ1334 & OJ1335 and +501 to +709; OJ1465 & OJ1467) *URA3* (+261 to +690; OJ1388 & OJ1389). Phosphorimages were obtained on a GE Typhoon FLA 7000 and analyzed using the ImageJ software package.

3.4 CHROMATIN IMMUNOPRECIPITATION (CHIP)

Cells were grown in YPD at 30°C to a density of 1-2x10^7 cells/ml and then treated with 1% formaldehyde for 20 min. Chromatin was isolated and sonicated as previously described [69] and then incubated with antibodies overnight at 4°C, while rotating. 2μl anti-TBP was used to immunoprecipitate TBP. Primary antibody-protein conjugates were isolated by incubating with 30μl Protein A-coupled Sepharose beads (GE Healthcare) at 4°C for 2 to 3 hr. After purifying DNA through PCR purification columns (Qiagen), the amount of immunoprecipitated (IP) DNA relative to input DNA was determined by quantitative PCR (qPCR) by normalizing to a template located within a region of chromosome V that lacks ORFs (No ORF) [36].

3.5 REAL-TIME QPCR

ChIP assays were analyzed by qPRC using an ABI StepOnePlus real-time PCR machine and SYBR green (Fermentas). Quantitation of real-time PCR results were calculated based on comparative ΔCT using the Pfaffl method which takes into account variations in primer efficiency while normalizing to a standard control (No ORF) [55]. Primer set locations are relative to the translation start of *PIR3* ATG=+1 and include the following: Internal to the *PIR3* ORF (+548 to +644; OJ1652 & OJ1653), targeting the putative *PIR3* TATA box (-214 to -117; OJ1654 & OJ1655), targeting the putative *PIR3* ncRNA TATA site 1 (-713 to -634; OJ1656 & OJ1657), targeting the putative *PIR3* ncRNA TATA site 3 (-893 to -791; OJ1658 & OJ1659), No ORF (OJ477 & OJ478) (previously described in [36]).

3.6 PRIMER EXTENSION

Primer extension reactions were performed on 20μg of total RNA. Protocols followed were based on previously described methods [64]. The oligo labeling reaction was performed with 4μl of 50 ng/μl purified oligo and 6000μci/μl γ ^{32}P. The primer extension reaction was carried out with 5 X 10^6 CPMs of radioactively labeled oligo and the sequencing reaction was performed with 5 X 10^5 CPMs of radioactively labeled oligo using the USB sequencing kit, Sequenase version 2.0. Oligo positions are relative to the translation start of *PIR3* and are as follows: *PIR3*-o2 (+12 to +35; OJ1367), *PIR3*-i7 (-620 to -600; OJ1486). The *ADH1* control primer (OJ1371) corresponds to bases +32 to +10 of the *ADH1*-coding sequence as previously described [45]. Phosphorimages were obtained on a GE Typhoon FLA 7000.

4.0 RESULTS

4.1 MAP OF THE *PIR3* LOCUS

PIR3 was chosen as a candidate gene for regulation by ncRNA due to the presence of significant RNA polymerase II occupancy over its promoter based on previous genome-wide RNA pol II ChIP data (Figure 1.4). Throughout this study, I have gathered information on expression data and regulatory sequences at the *PIR3* locus. In figure 4.1, I have summarized the details of this information. All sites indicated by base pair position are relative to the *PIR3* ATG at +1bp of the 978bp coding sequence. Regulatory elements are based on consensus sequence alignment or published genome-wide analysis. For example, a potential TATA box for the *PIR3* ORF was identified at -145bp by consensus sequence alignment that I performed. Approximately 100bp upstream of this TATA site at -251bp, a sequence was identified as a consensus recognition site for the Rlm1 transcription activator based on a genome-wide study assessing the effects on gene expression in response to activation of the MAP kinase, Mpk1/Slt2 [30]. Transcriptome analysis surveying transcript structure and expression levels across the yeast genome as well as a study mapping transcription boundaries by RNA-Seq, identified the same 5' end for the *PIR3* mRNA at -250bp, indicated by the arrow directly downstream of the Rlm1 binding site [80, 53]. Additional elements identified from genome-wide studies include putative TATA sites for the intergenic ncRNA discovered from Chip-exo data aimed at identifying sites of PIC assembly (red rectangles site 1 and site 3) [63]. The light red rectangle at site 2 also represents a putative intergenic TATA element, which was determined by consensus sequence alignment. In our lab, transcription start sites (TSS) for the intergenic transcript were mapped by 5' RACE and are indicated by the green pentagons (Rapid Amplification of cDNA Ends) (unpublished, Danielle Wagner).

Two sites were identified in repeat RACE experiments at -610bp and -810bp. Transcriptome data was also a source for identifying annotated noncoding RNAs that may be important at *PIR3* [80]. This search led to the identification of *SUT228*, which is estimated to be 1.1Kb long. It appears to initiate at -1.218Kb and extend to -82bp upstream of the *PIR3* ATG. Given that *SUT228* is transcribed within the intergenic region of *PIR3*, it would seem to be a likely candidate for regulation by ncRNA. However, when we attempted to disrupt intergenic transcription (details below and in Materials and Methods) near the mapped TSS at -810bp, a transcript signal was still visible by Northern analysis (data not shown). If *SUT228* were transcribing across this region, we would have expected to see a loss of transcription. In addition, the predicted start site for *SUT228* is much farther upstream of either TSS mapped by 5' RACE, and the 600bp ncRNA that I have predicted from my Northerns is about half the size predicted for *SUT228*. Consistent with these observations, insertion of the TTS at -486bp (purple diamond) was successful in terminating intergenic transcription, which will be expanded upon later in the results section.

4.2 IDENTIFICATION OF INTERGENIC TRANSCRIPTION AT *PIR3*

The genome-wide RNA pol II data provided strong evidence for intergenic transcription overlapping the *PIR3* promoter (Figure 1.4). In order to confirm this data, Nothern analysis was performed. Using a probe 5' of *PIR3*, we detected an intergenic transcript of approximately 600bp in length. Reprobing this Northern using a probe contained within the *PIR3* coding sequence, detected the *PIR3* mRNA with an estimated size of 1.2Kb (Figure 4.1). This result supports the RNA poI II ChIP data that intergenic transcription is occurring upstream of *PIR3*.

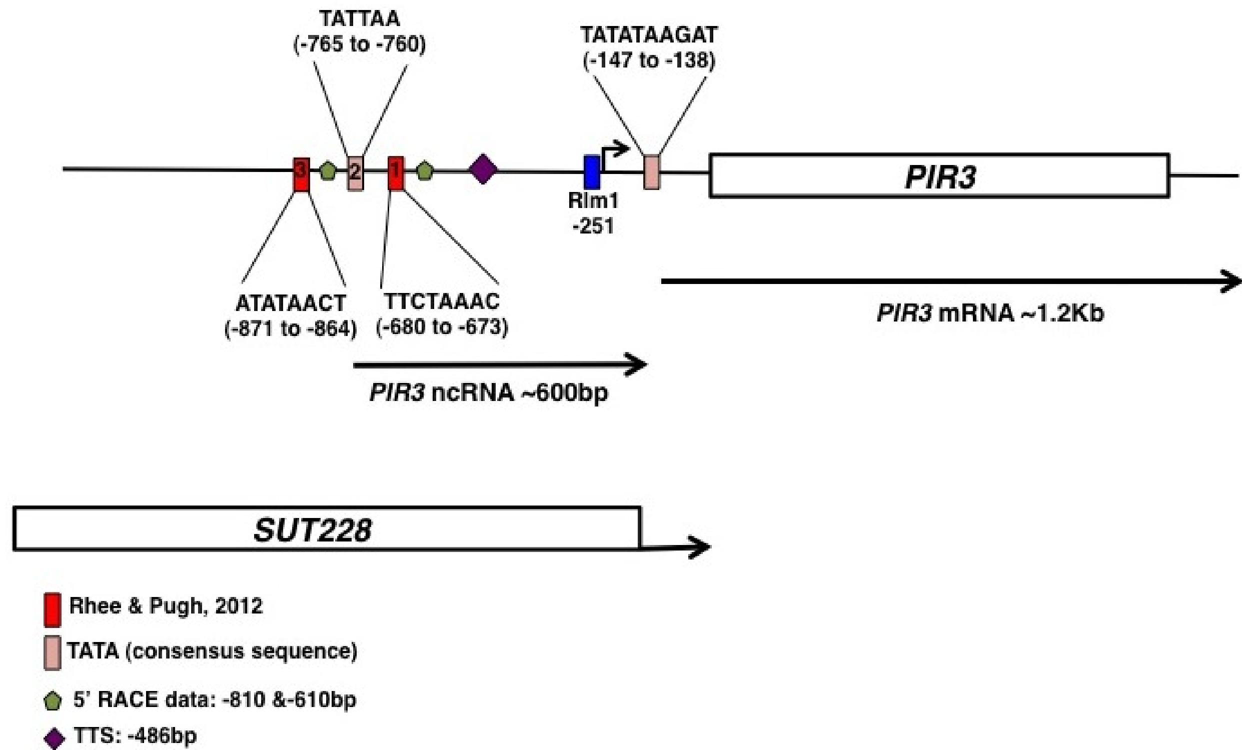

Figure 4.1: **Map of the *PIR3* locus.** The *PIR3* locus is mapped with estimated sizes and locations of the ncRNA and ORF transcripts. Sizes were estimated from Northern analysis by comparison to a standard RNA ladder. Important elements are noted such as the Rlm1 conserved DNA binding sequence [30]. 5' RACE mapped transcription start sites (green pentagons) and putative regulatory elements determined from genome-wide Chip-exo data [63] (red rectangles) and sequence alignment (light red rectangles). A putative transcription start site for *PIR3* mRNA is indicated by the arrow in between the Rlm1 binding site and the TATA box. The purple diamond indicated the location of the TTS that disrupts ncRNA transcription.

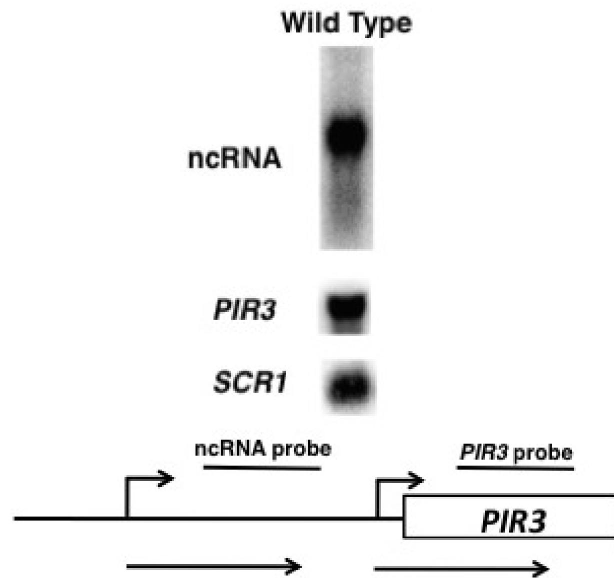

Figure 4.2: **Northern analysis validating *PIR3* ncRNA and *PIR3* transcript**. Representative Northern analysis of three independent experiments to confirm the presence of intergenic transcription based on the genome-wide RNA pol II ChIP data. Below the Northern blot, a diagram of a *PIR3* locus indicates the approximate locations of Northern probes. Total RNA was isolated from the wild type KY719 strain grown to a density of 1-2 X 10^7 cells/ml in YPD at 30°C. *SCR1* is used as a loading control.

4.3 DEVELOPMENT OF TRANSCRIPTION TERMINATION STRATEGY TO PREVENT INTERGENIC TRANSCRIPTION

In order to determine if intergenic transcription affects *PIR3* mRNA levels, I needed a method to stop intergenic transcription. When I began this study, potential regulatory sequences for the *PIR3* ncRNA were not defined, making disruption of transcription by mutation difficult. Therefore, a strategy was developed to utilize a strong transcription termination sequence from the *HIS3* gene (*HIS3*-TTS) to prematurely terminate the *PIR3* ncRNA. Briefly, a plasmid, pDW1, containing a construct of tandem copies of the *HIS3*-TTS, consisting of 171bp from the 3' end of *HIS3*, flanking the *URA3* gene as a selectable marker was generated (D. Wagner, unpublished). In order to target a particular genomic site for disruption of transcription, this construct can be PCR-amplified using primers that bear homologous sequence to the genomic integration site for transformation into yeast. After selection, the *URA3* gene can be lost by homologous recombination between the two copies of the TTS, yielding a strain with one copy of the TTS at the desired location (Figure 4.3). The desired result will be premature termination of the intergenic transcription without any alteration of regulatory elements. Using this method I constructed the $pir3_{TTS}$ in addition to the already constructed $srg1_{TTS}$ allele (D. Wagner) (see Materials and Methods for more details).

In order to test the efficacy of this strategy, I first assessed the effects of TTS insertion on repression of *SER3* by *SRG1*. A transformation was performed for insertion of the *HIS3*-TTS at -450bp, relative to the *SER3* ATG. The $srg1_{TTS}$ and wild type control strains were grown in serine-rich media (rich media, YPD). Normally in high serine conditions, *SRG1* is transcribed, resulting in strong repression of *SER3*. This result is in fact replicated in the wild type strain (Figure 4.3, panel **C**, first lane). In contrast, *SRG1* transcript signal is lost in $srg1_{TTS}$ strain, and *SER3* mRNA levels are strongly increased, reproducing the results observed when the *SRG1* TATA box is mutated and nonfunctional (Figure 4.3, panel **C**, second lane). These results indicate that insertion of the *HIS3*-TTS is a practical method for disrupting transcription in lieu of promoter mutations.

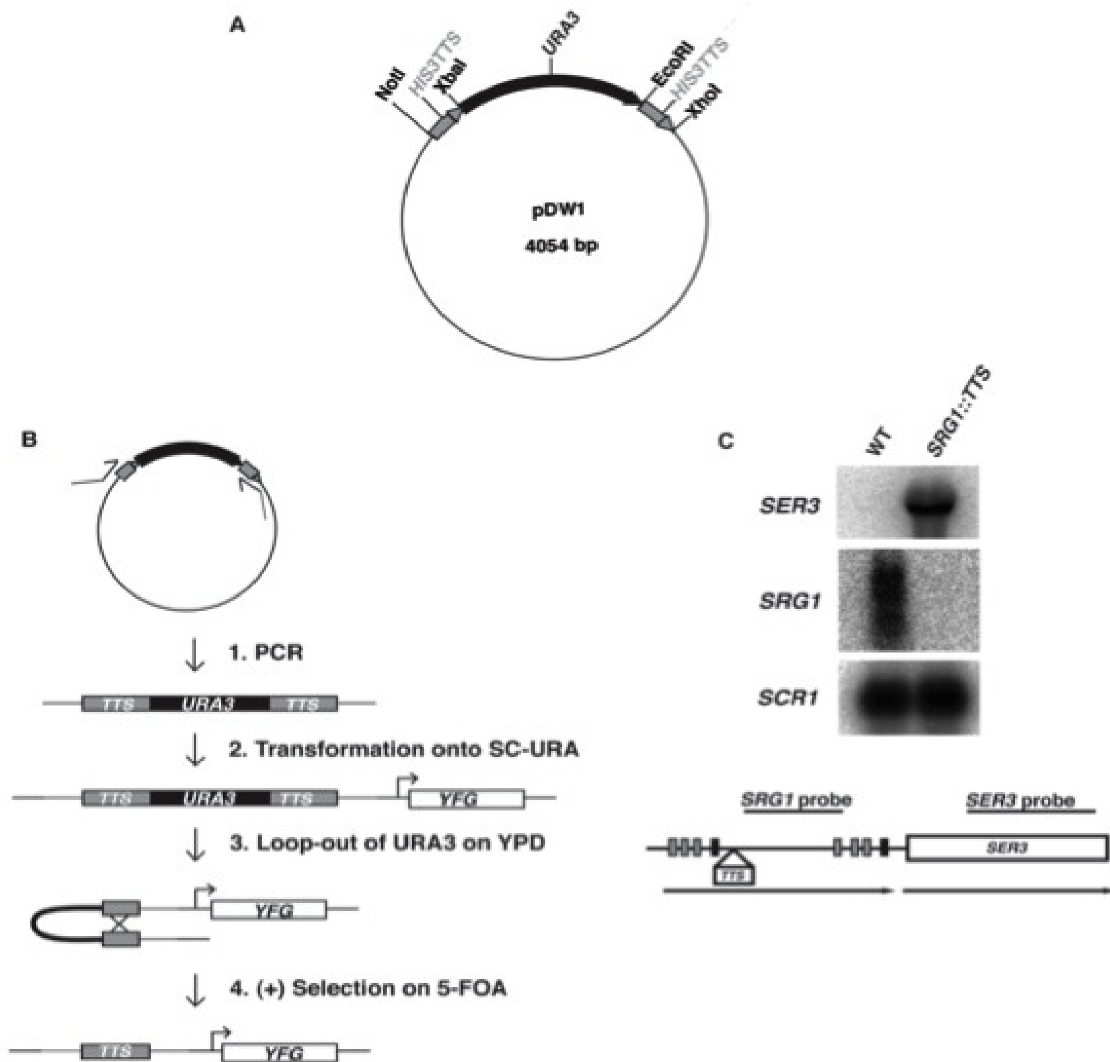

Figure 4.3: **A strategy for disrupting intergenic transcription.**

The TTS sequence is derived from the terminal 171bp of the *HIS3* gene. **(A)** Diagram of the pDW1 plasmid used as the PCR template for the selectable *URA3* gene flanked by the *HIS3*-TTS. **(B)** Outline for integration of the *HIS3*-TTS into the yeast genome. **(C)** Northern analysis for control experiment using *SRG1/SER3*. Yeast strain YJ822 ($srg1_{TTS}$) and KY719 (wild type) were grown in rich media (YPD) at 30°C to a density of 1-2 X 10^7 cells/ml. *SCR1* serves as a loading control.

4.4 LOSS OF NCRNA LEADS TO AN INCREASE IN *PIR3* EXPRESSION

Having successfully developed a method for disruption of intergenic transcription, I assessed the effect of *PIR3* ncRNA on transcription of *PIR3*. In order to determine a target site for insertion of the *HIS3*-TTS within the sequence of the *PIR3* ncRNA, the transcription start sites for the *PIR3* ncRNA were determined by 5' RACE, as previously described (Figure 4.1). The start site farthest upstream (-810bp) of the *PIR3* ATG was initially targeted for disruption; however, insertion of the TTS at -800bp did not result in loss of intergenic transcript (data not shown). Therefore, I inserted the TTS at -486bp, 3' of a second transcription start site, mapped at -610bp, generating the $pir3_{TTS}$ allele. Wild type and $pir3_{TTS}$ strains were grown in rich media and transcript expression was tested by Northern analysis. For the wild type strains, the Northern results displayed the expected signal for the *PIR3* ncRNA and ORF transcripts (Figure 4.4, panel A, lane 1). Interestingly, insertion of the TTS successfully terminated the intergenic transcript, and resulted in a 2-fold increase in *PIR3* expression (Figure 4.4, panel A, lane 2). These results support a potential regulatory mechanism whereby the loss of ncRNA leads to an increase in *PIR3* expression, indicative of a repressive function for intergenic transcription.

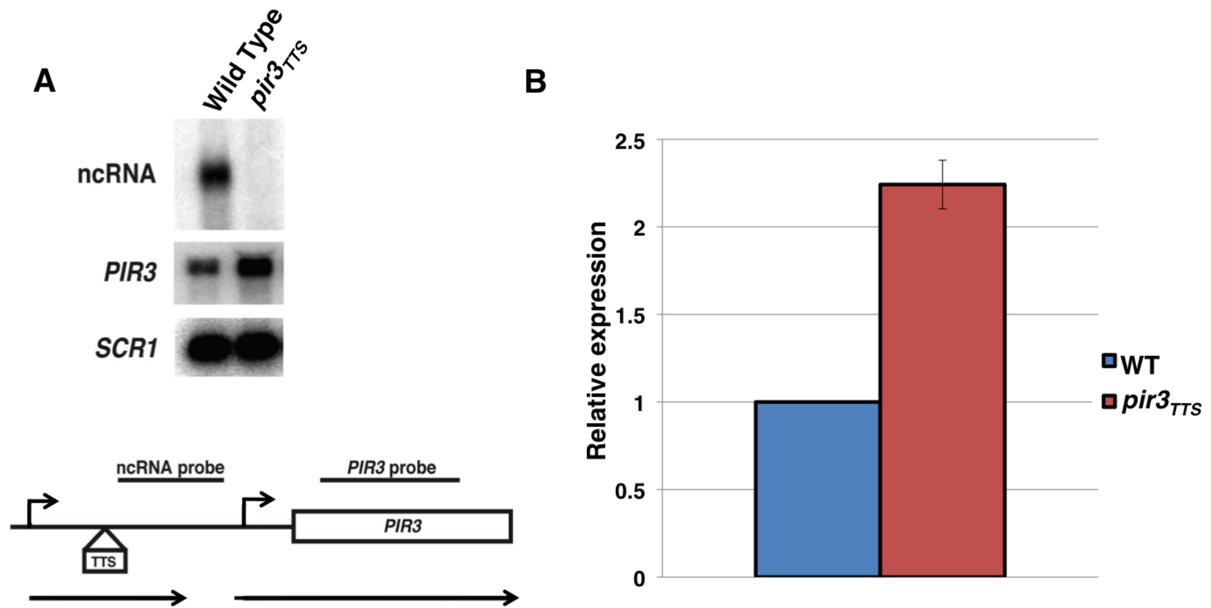

Figure 4.4: **Termination of intergenic transcription at *PIR3* results in increased *PIR3* expression.** **(A)** Representative Northern analysis of three independent experiments assessing the effect that loss of the ncRNA has on *PIR3* expression. Start sites of ncRNA at *PIR3* were determined by 5' RACE, directing placement of the *HIS3*-TTS. Insertion of *HIS3*-TTS, results in loss of the ncRNA with a subsequent increase in expression of the *PIR3* ORF. **(B)** Quantitation of **(A)**. *PIR3* levels in the $pir3_{TTS}$ strains are normalized to the *SCR1* loading control and made relative to *PIR3* levels of the wild type strain (arbitrarily set to 1). Strains KY719 (wild type or WT), YJ1100, YJ1101, and YJ1102 ($pir3_{TTS}$) were grown in rich media (YPD) at 30°C to a density of 1-2 X 10^7 cells/ml. *SCR1* serves as a loading control.

4.5 DETERMINATION OF TRANSCRIPTION START SITES (TSS) FOR *PIR3* NCRNA AND *PIR3*

To better characterize the ncRNA, I performed primer extension to map the 5' ends of the *PIR3* ncRNA and *PIR3* mRNA. Separate oligos were designed to target the 5' ends of the *PIR3* ncRNA and *PIR3* mRNA, using the 5' RACE and genome-wide data to direct the position of oligos most likely to map a TSS [80]. Oligo *PIR3*-o2 was designed to target the 5' end of the *PIR3* mRNA and anneals from +35bp to +12bp of the *PIR3* coding sequence. Oligo *PIR3*-i7 was designed to target the 5' end of the *PIR3* ncRNA and anneals from -600 to -620 upstream of the *PIR3* ATG. In each primer extension analysis, I compared a wild type to a *pir3*$_{TTS}$ strain. Primer extension at *ADH1* results in two strong TSS signals. This reaction was used as a positive control for quality of RNA samples and amount of RNA loaded for each sample.

When using oligo *PIR3*-o2, I would expect to see a TSS signal that is stronger in the *pir3*$_{TTS}$ strain as compared to the wild type strain. This would support the Northern result demonstrating the increase in *PIR3* expression in the *pir3*$_{TTS}$ strain. Oligo *PIR3*-o2 mapped two starts sites at -38bp and -80bp upstream of the *PIR3* ORF (Figure 4.5, panel **B**). In comparing the wild type to the *pir3*$_{TTS}$ strain, I do see increased signal in the *pir3*$_{TTS}$ strain at both locations. In mapping the *PIR3* ncRNA, I would expect to see signal from both the wild type and the *pir3*$_{TTS}$ strain. The start sites mapped by oligo *PIR3*-i7 in the wild type strains were at -718bp and -663bp (Figure 4.5 **C**). Both sites map 5' of the putative TATA elements and they are also near the -610bp TSS mapped by 5' RACE, making them attractive candidates for a TSS (Figure 4.1). However, I did not see the same signal intensity in the *pir3*$_{TTS}$ strain. RNA degradation was likely the cause of this negative result, as the *ADH1* control reaction for the *pir3*$_{TTS}$ strain displayed no signal. Overall, I identified putative transcription start sites for both *PIR3* ncRNAs and *PIR3* mRNA; however, additional analysis will be required to confirm these sites. Additional experiments will be required to confirm these mapped sites.

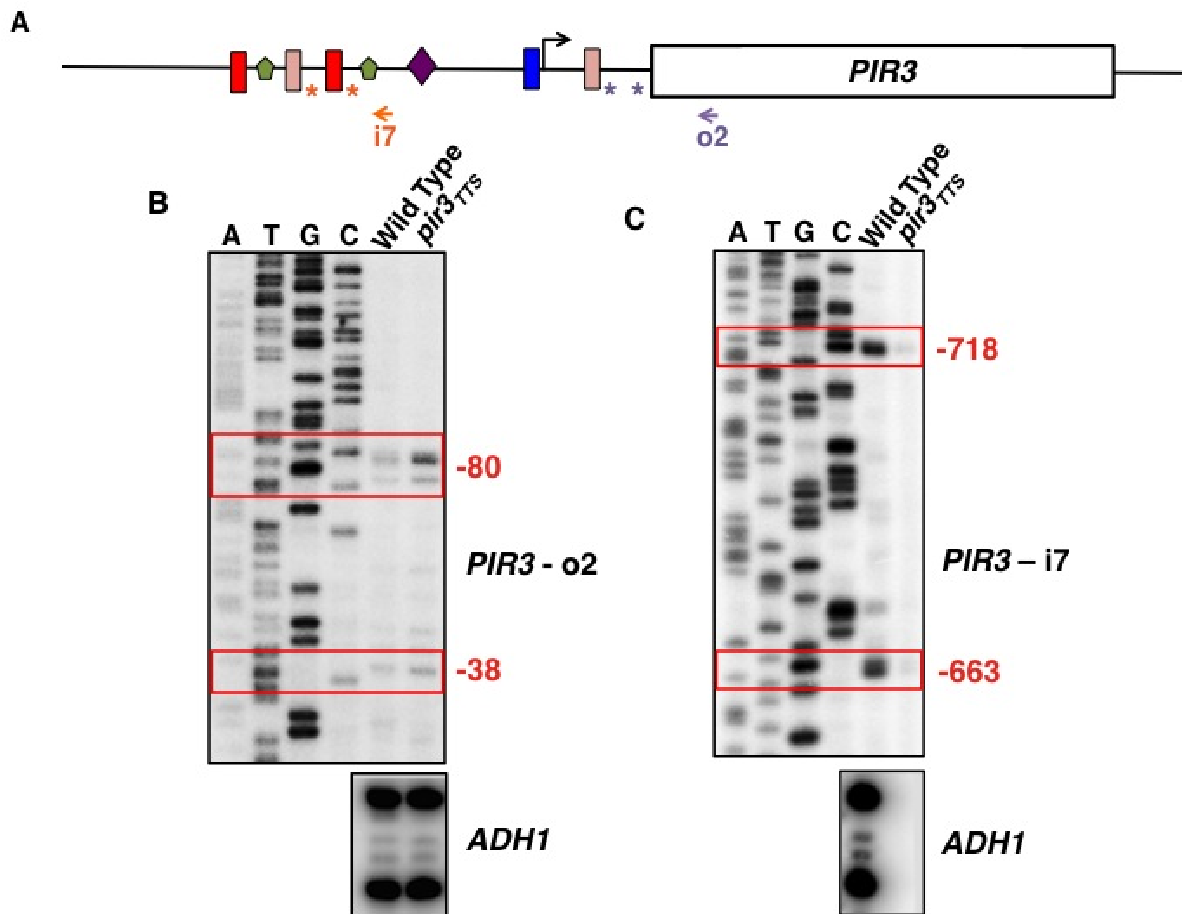

Figure 4.5: **Determination of transcription start sites (TSS) for *PIR3* ncRNA and *PIR3*. (A)** A diagram of the *PIR3* locus highlighting the oligos used in primer extension. The color of the oligo corresponds to the mapped start site marked by * of matching color. **(B and C)** Primer extension analyses. Wild type strains were compared to $pir3_{TTS}$ strains. Oligos are listed next to each gel. Oligo *PIR3*-o2 mapped two start sites at -38bp and -80bp *PIR3*. Oligo *PIR3*-i7 mapped two start site at -718bp and -663bp for the *PIR3* ncRNA. The mapped start sites are highlighted in red. Primer extension at *ADH1* displayed below each image was used as a control to measure amount of RNA loaded and to assess RNA quality. Strains KY719 (Wild type), YJ1100, YJ1101, YJ1102 ($pir3_{TTS}$) were grown in rich media at 30°C to a density of 1-2 X 10^7 cells/ml.

4.6 *PIR3* IS REPRESSED IN *CIS* BY INTERGENIC TRANSCRIPTION

After determining that loss of the *PIR3* ncRNA leads to a 2.0-fold increase in *PIR3* expression, I next wanted to determine a model for the mechanism of regulation. My hypothesis was that intergenic transcription across the *PIR3* promoter will produce a regulatory effect in *cis*, as observed at the *SRG1/SER3* locus, supporting the concept that the act of transcription and not the ncRNA product represses *PIR3*. To test this, I used a previously described *cis/trans* experiment (Martens et al., 2004). Briefly, I constructed diploid strains in which one copy of the *PIR3*-coding sequence was replaced by the sequence for *URA3*, allowing expression from each *PIR3* promoter to be assayed independently. Diploid 1 expressed the $ura3_{TTS}$ allele where the *PIR3* intergenic transcription will be terminated upstream of the allele containing *URA3* in place of *PIR3*, and Diploid 2 expressed the $pir3_{TTS}$ allele where *PIR3* intergenic transcription will be terminated upstream of the allele containing *PIR3* (see Materials and Methods for strain construction details).

Each diploid was grown in YPD and subjected to Northern analysis (Figure 4.6). In the first two lanes of the Northern blot, the haploid wild type strain and the $pir3_{TTS}$ strain (lanes 1 and 2, respectively) again demonstrate the 2-fold increase in *PIR3* ORF expression when the intergenic transcript is disrupted. When comparing signals from diploid 1 and diploid 2, wild type *PIR3* ncRNA transcription represses the *PIR3* promoter only when in *cis* ((Figure 4.6, lane 4, Diploid 2)). Additionally, repression of the *URA3* was also observed, but only when adjacent to the wild type *PIR3* ncRNA (Figure 4.6, lane 3, Diploid 1). If the ncRNA were working in *trans* we would have expected to see an equal level of expression in both protein coding genes regardless of location of the TTS. These results show that the repressive effect of intergenic trasncription on the *PIR3* ORF occurs in *cis*. Although I cannot exclude a possible role for the ncRNA in repression of *PIR3*, our data suggest that *PIR3* is most likely repressed by the act of intergenic transcription.

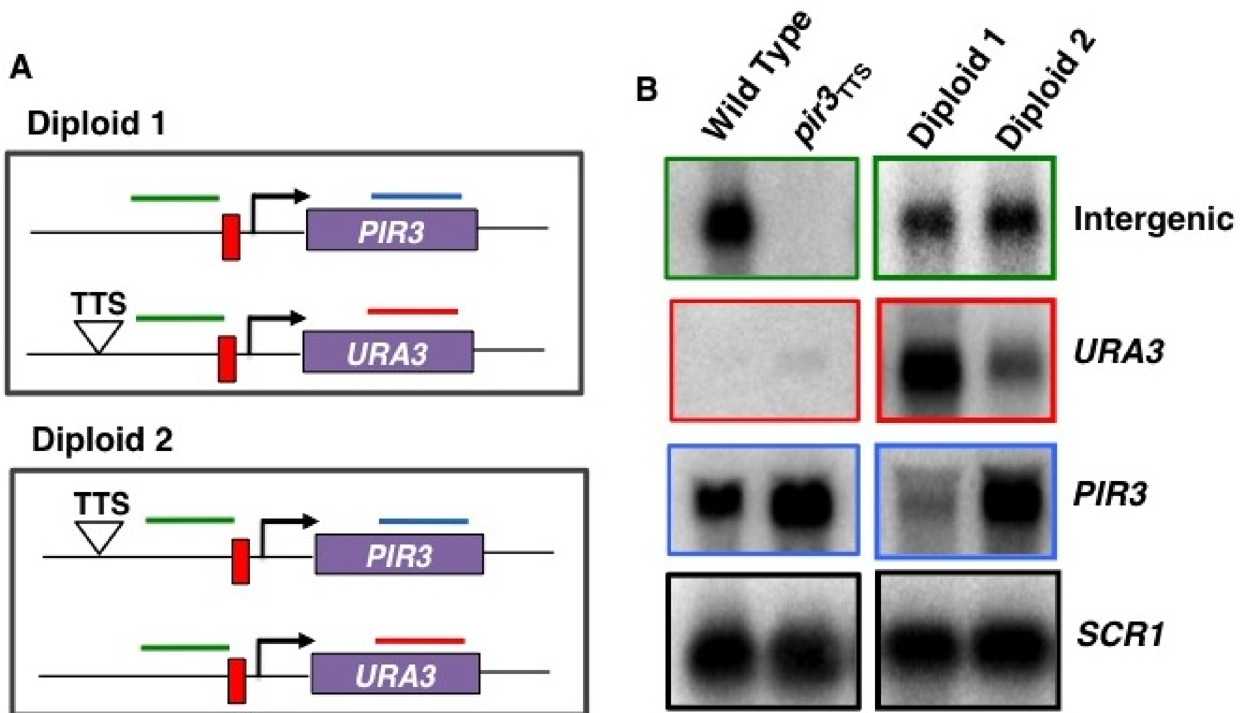

Figure 4.6: **Intergenic transcription represses*PIR3* in *cis*.** **(A)** Diagram of the diploid strains used in an assay to determine whether the intergenic transcript has a *cis* or *trans* effect on the *PIR3* ORF. Diploids were constructed in which a second copy of the *PIR3* ORF was replaced, in frame, by the *URA3* ORF. Intergenic transcription is disrupted by insertion of the TTS either upstream of *URA3* (Diploid 1; *ura3*$_{TTS}$ allele) or upstream of *PIR3* (Diploid 2; *pir3*$_{TTS}$ allele). **(B)** Representative Northern analysis of three independent experiments depicting the results of the *cis/trans* test, which indicate repression in *cis*. Diploid 1 strains YJ1109, YJ1110, YJ1111 and Diploid 2 strains YJ1112, YJ1113, and YJ1114 were grown in rich media (YPD) at 30°C to a density of 1-2 X 10^7 cells/ml. *SCR1* serves as a loading control

4.7 EFFECT OF INTERGENIC TRANSCRIPTION ON TBP BINDING AT THE *PIR3* PROMOTER

Repression by intergenic transcription in *cis* could occur through a number of regulatory mechanisms including promoter occlusion through transcription interference or altered chromatin structure. In the transcription interference model, transcription of *PIR3* ncRNA across the *PIR3* promoter would prevent access of transcription factors or disrupt activator binding. Occlusion of the yeast *FLO11* promoter by upstream ncRNA results in repression through blocked access of general transcription factors [9], whereas *ADH1/ADH3* repression occurs through ncRNA transcritption-mediated removal of bound activators [5]. In order to test whether *PIR3* ncRNA may be repressing *PIR3* by denying access of transcription factors, I performed ChIP and quantitative real-time PCR to assess occupancy of TBP, an integral factor in promoter initiation complex (PIC) formation for transcription initiation.

Primers flanking a putative TATA sequence for *PIR3* expression were used to assess TBP binding at *PIR3*. A primer sets was designed to target the *PIR3* consensus TATA sequence ("ORF TATA"). (Figure 4.7). Low levels of TBP occupancy at the *PIR3* TATA were detected in the wild type strain. TBP occupancy was observed in the *pir3* $_{TTS}$-where *PIR3* mRNA levels are increased 2-fold-with no significant increase as compared to wild type occupancy. It is possible that this small increase in TBP could account for the two-fold increase in *PIR3* expression, but that increase cannot be detected by ChIP. From this result, it does not appear that intergenic transcription significantly interferes with TBP occupancy at the *PIR3* promoter.

I also examined TBP occupancy at the *PIR3* ncRNA TATA sites using primers sets designed to target the *PIR3* ncRNA sites 1 and 3 ("ncRNA TATA1" and "ncRNA TATA3," respectively), previously identified by genome-wide ChIP-exo data [63]. In both cases, the signal for TBP occupancy is above background levels, particularly for TATA site 3. This indicates a likely TATA box upstream of the *PIR3* ncRNA. However, because of the resolution of ChIP I cannot distinguish between one or both TATA sequences contributing to TBP binding. These two sites are candidates for TATA sequences that promote *PIR3* ncRNA synthesis.

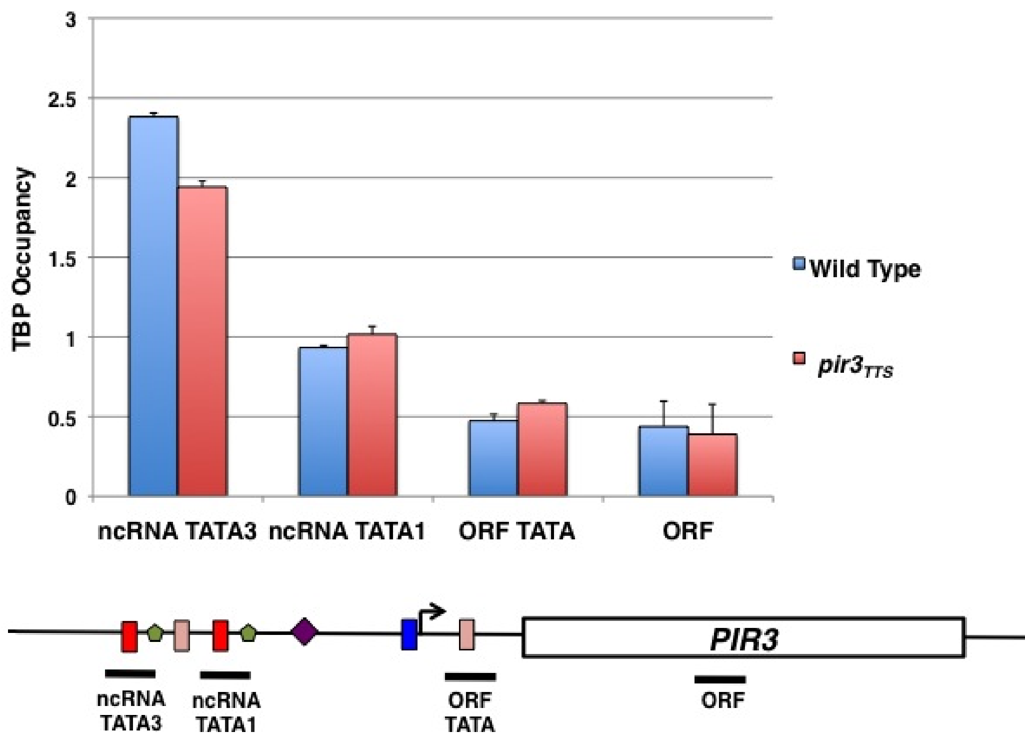

Figure 4.7: **Effects of intergenic transcription on TBP binding at the *PIR3* promoter.** ChIP analaysis was performed using anti-TBP to characterize binding sites for *PIR3* ORF and ncRNA. This quantitation is the result of three independent real-time PCR experiments. Relative locations of primer sets are indicated on the diagram below the graph. The control primer set, to which all values are normalized, amplifies a region of chromosome V that lacks open reading frames and represent the mean of three biological replicates +/- SEM. Strains KY719, YJ1115 and YJ1116 (Wild type) and YJ1100, YJ1101, YJ1102 ($pir3_{TTS}$) were grown at 30°C to 1-2 X 10^7 cells/ml in YPD.

4.8 INTERGENIC TRANSCRIPTION DOES NOT REGULATE *PIR3* EXPRESSION IN RESPONSE TO CELL WALL STRESS

The role for *PIR3* in maintaining cell wall integrity in times of cell wall stress may provide a physiological mechanism for *PIR3* regulation by intergenic transcription. Specifically, *PIR3* expression has been shown to increase in response to elevated temperatures or in response to cell wall perturbing agents such as congo red, zymolyase, or calcoflour white, in what is speculated to be an Rlm1-dependent manner [30, 6, 19, 32].

To determine if *PIR3* regulation by cell wall stress involves intergenic transcription, we first assayed *PIR3* ncRNA and *PIR3* mRNA levels in response to heat shock. Wild type cells were grown to mid-log at room temperature and then shifted to 39°C, with time points taken every 30 min for three hours. *PIR3* ncRNA and*PIR3* mRNA expression was assessed by Northern analysis, with each time point compared to time 0 min (Figure 4.8). In response to elevated temperatures, I observed increased *PIR3* mRNA levels, as has been previously described [30]. Interestingly *PIR3* ncRNA levels also increased under these conditions. From this result I can conclude that the increased expression of *PIR3* in response to induction of cell wall stress through elevated temperatures is independent of intergenic transcription.

Growth of yeast cells in the presence of caffeine has also been shown to activate cell wall stress [76, 77, 47, 82, 38]. Therefore, I assayed changes in *PIR3* ncRNA and *PIR3* mRNA after expression in caffeine by Northern analysis. Cells were grown to mid-log before adding 12mM caffeine. Time points were taken prior to caffeine addition and at 30 min intervals after caffeine addition for three hours. Northern analysis shows increased *PIR3* mRNA by 30 min after addition of 12mM caffeine (Figure 4.9). Consistent with our temperature experiment, expression of the ncRNA also increased in cell wall stress conditions. From this result I can conclude that the increased expression of *PIR3* in response to caffeine-induced cell wall stress is independent of intergenic transcription.

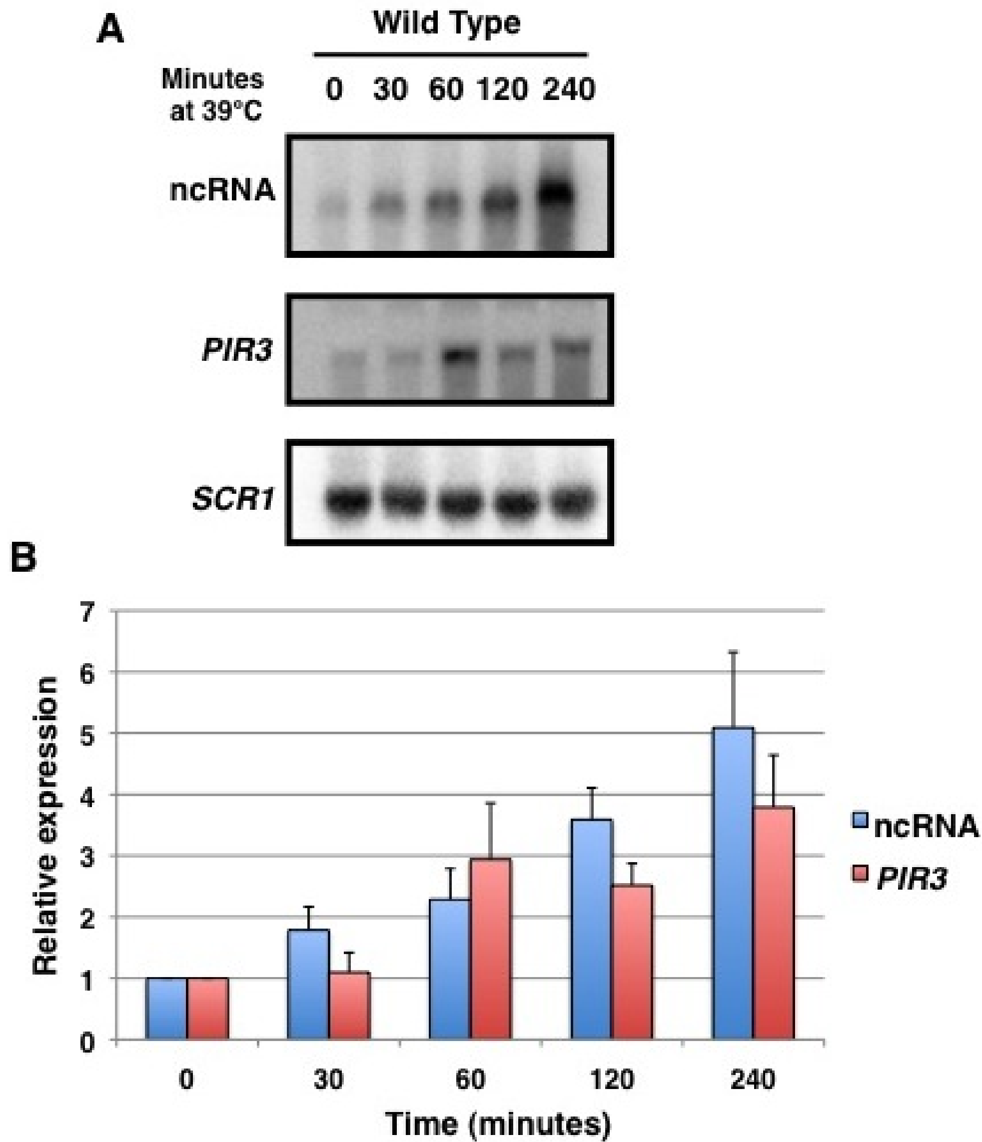

Figure 4.8: **Increased expression of *PIR3* in response to elevated temperatures is independent of intergenic transcription.** Northern analyses. **(A)** Strains KY710, RMY104, RMY109 were grown at 25°C in YPD to a density of 1 X 10^7 cells/ml before being shifted to 39°C by spinning cells down, removing media and re-suspending in pre-warmed media. Samples were collected for RNA isolation at the indicated times. **(B)** Quantitation of Northern in **(A)** from three biological replicates. *PIR3* mRNA and ncRNA levels are normalized to the *SCR1* loading control and are relative to the *PIR3* mRNA and ncRNA levels measured at time 0 (arbitrarily set to 1).

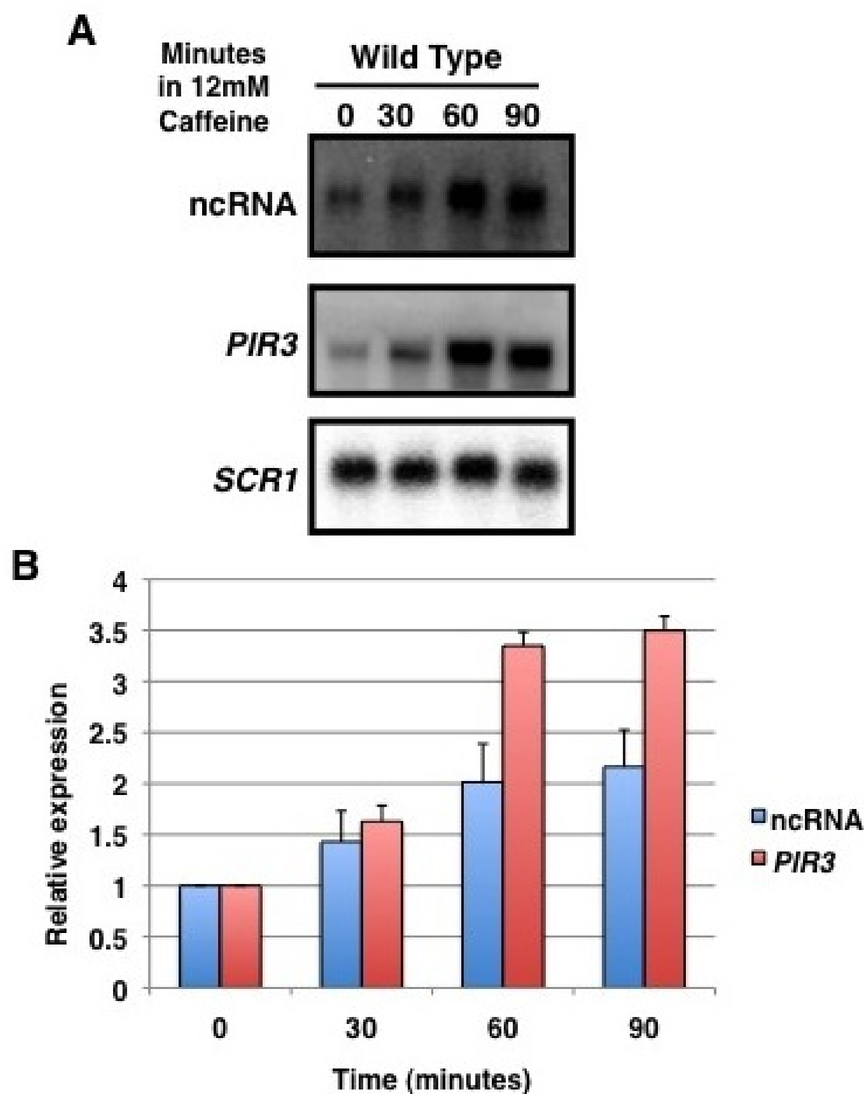

Figure 4.9: **Increased expression of *PIR3* in response to caffeine is independent of intergenic transcription.** Northern analyses. **(A)** Strains KY710, RMY104, RMY109 were grown at 30°C in YPD to a density of 1 X 10^7 cells/ml prior to addition of 12mM caffeine. Samples were collected for RNA isolation at the indicated times. **(B)** Quantitation of Northern in **(A)** from three biological replicates. *PIR3* mRNA and ncRNA levels are normalized to the *SCR1* loading control and are relative to the *PIR3* mRNA and ncRNA levels measured at time 0 (arbitrarily set to 1).

4.9 CHROMATIN EFFECTS IN *PIR3* NCRNA FUNCTION

Thus far, my data indicates that *PIR3* is repressed by intergenic transcription. Several studies have indicated that ncRNA transcription regulates gene expression by altering chromatin. *SER3* repression has been shown to be regulated by intergenic transcription-dependent nucleosome remodeling across the *SER3* promoter [24]. Transcription of ncRNA can also lead to repressive histone marks to control gene expression which has been described for the divergently transcribed yeast *GAL10* and *GAL1* genes [27, 57].

To determine if chromatin structure could be causing repression of *PIR3*, I compared *PIR3* expression levels in cells expressing mutant forms of Spt16 (*spt16-E857K*) or histone H3 (H3 K122A) which are known to derepress *SER3* by impaired *SRG1* transcription-dependent nucleosome assembly over the *SER3* promoter. If chromatin structure is important for *PIR3* repression, I would expect to see an increase in *PIR3* expression in the presence of these mutations. By Northern analysis, I observed a 2-fold ($p < 0.08$) increase of the *PIR3* mRNA in *spt16-E857K* mutant as compared to wild type *SPT16* strain (Figure 4.10, lanes 1 and 2). However, no significant increase in expression of *PIR3* mRNA was observed for the histone H3 K122A mutants as compared to the wild type strain (Figure 4.10, lanes 3 and 4). Analysis of *SER3* was performed as a control, with results similar to previously described results [22, 21]. This may indicate that functional Spt16 is more important for *PIR3* regulation than the histone H3 K122A mutation. The conflicting results of these two mutants suggests one of two possibilities. First, *PIR3* regulation by intergenic transcription mediated by transcription-dependent changes in chromatin is more sensitive to the Spt16 mutant than the histone H3 mutant. Alternatively, Spt16 may repress *PIR3* by a mechanism that is not related to its role in transcription-coupled nucleosome assembly.

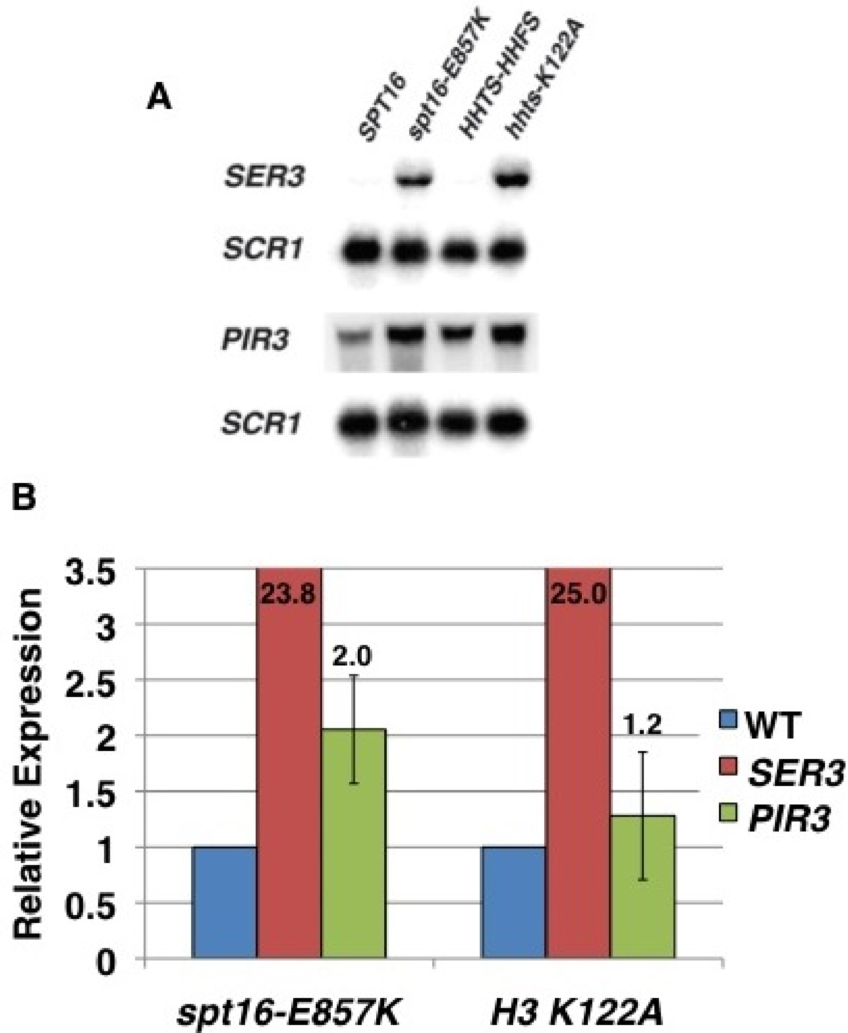

Figure 4.10: **Analysis of the effects on gene expression in the presence of histone H3 K122A and p*spt16-E857K*. (A)** Representative Northern analysis of three independent experiments assessing expression of the *PIR3* and *SER3* ORFs in strains harboring mutations known to affect nucleosome occupancy at *SER3*. The mutant alleles are listed above the blots. **(B)** Quantitation of Northern analyses. *PIR3* and *SER3* RNA levels for the mutant strains are normalized to the *SCR1* loading control and are relative to the *PIR3* and *SER3* RNA levels measured in wild type strains (arbitrarily set to 1). Strains YJ1091, YJ1092, YS417, YS418, YS419, YS420, YS421, YS404 were grown at 30°C to 1-2 X 10^7 cells/ml in YPD. *SCR1* is used as the loading control.

4.10 DELETIONS IN OTHER CHROMATIN-RELATED FACTORS HAVE NO EFFECT ON *PIR3* REPRESSION

Post-translation histone modifications prevent cyrptic intragenic transcription from occurring in the wake of elongating RNA pol II across protein coding genes. Specifically, Set2 methylation of histone H3 on lysine 36 (H3 K36), a mark that occurs in the wake of elongating RNA pol II, recruits the Rpd3S histone deacetylase for the removal of acetylation marks which returns chromatin to a transcriptionally inactive state. This series of events occurs in the context of active transcription and is dependent on the Paf1C elongation complex. Specifically, H2B K123 monoubiquitylation by the Rad6 ubiquitin conjugating enzyme and the Bre1 ubiquitin ligase are necessary for downstream methyl marks by Set1 and Dot1 methyltransferases on H3 K4 and H3 K79, respectively. These marks are promoted by Paf1 and Rtf1 and are associated with active transcription. Additionally, H3 K4 di-methylation occurs at 5' ends of genes in the wake of elongation RNA pol II and leads to the recruitment of the Set3 HDAC, which is also responsible for removal of histone acetyl marks (reviewed in [13]). From this data, I hypothesized that regulation of *PIR3* repression could be dependent on histone modifications, whereby the *PIR3* ncRNA is actively transcribed across the *PIR3* promoter and the recruitment of histone deacteylases prevents transcription activation from the *PIR3* promoter. Evidence to support this model comes from the *GAL1/GAL10* locus, where a similar mechanisms of regulation by ncRNA occurs (reviewed in [23]).

To test this possibility, I performed Northern analysis to determine the effect that loss of histone methyltransferases Set1, Set2, Dot1, and Rco1, an essential subunit of the Rpd3S HDAC [10], have on *PIR3* expression. I would expect to see increased *PIR3* expression when one or more of these genes are deleted if histone methylation is important for maintenance of repression. Surprisingly, I found that both the *PIR3* ncRNA and *PIR3* mRNA levels were reduced in these mutants. These results indicate that histone methylation is not involved in *PIR3* repression by intergenic transcription. Rather these marks are required for normal transcription of both the *PIR3* ncRNA and *PIR3* mRNA.

I also tested the deletion mutants of Spt2 and Paf1, two factors that have also been shown to be important for maintenance of repressive nucleosome occupancy at *SER3*

[60, 72]. Spt2 binds DNA through an HMG (High Mobility Group)-like domain at transcribing regions, including *SRG1/SER3*, and has been shown to mediate chromatin dynamics [72]. I performed Northern analysis, which revealed decreased levels of both the *PIR3* ncRNA as well as *PIR3* mRNA. These results indicate that chromatin associated activities of Spt2 or Paf1 are not involved in *PIR3* repression.

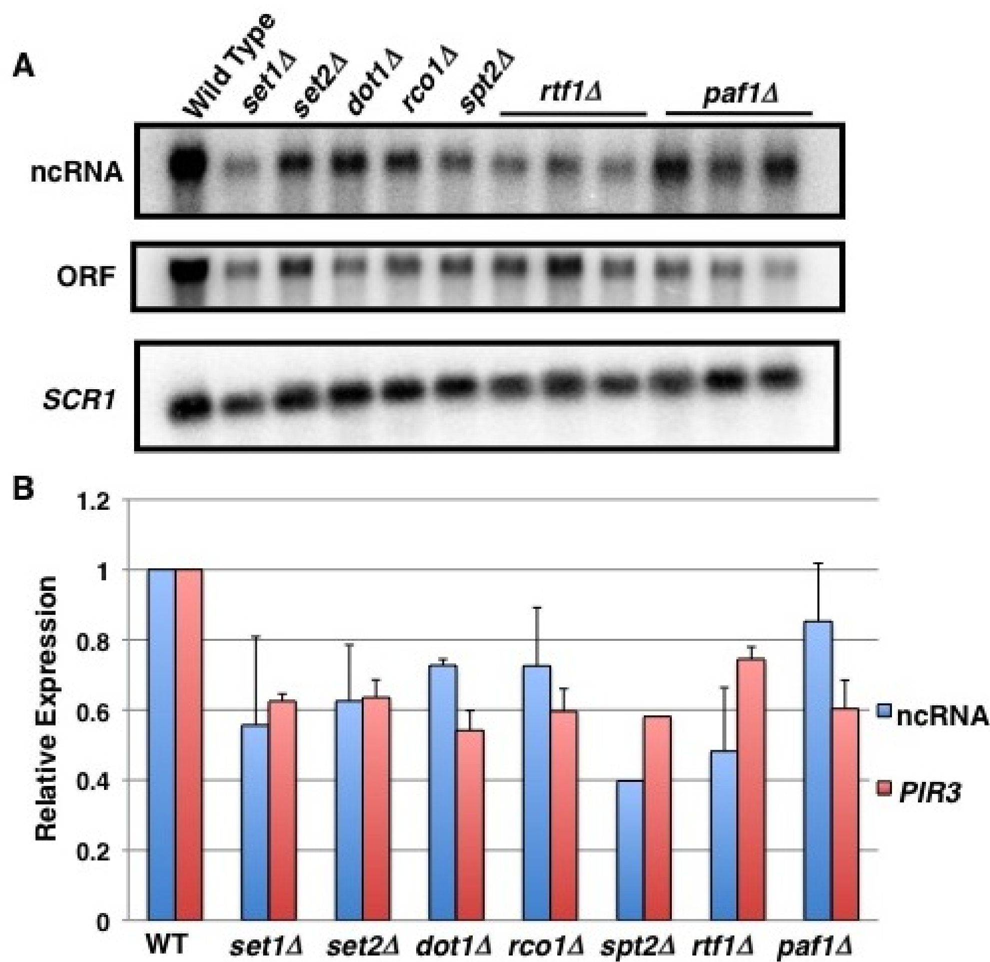

Figure 4.11: **Deletions in other chromatin-related factors have no effect on *PIR3* repression. (A)** Representative Northern analysis of three independent experiments assessing *PIR3* and ncRNA expression in wild type *set1*Δ, *set2*Δ, *dot1*Δ, *paf1*Δ, *rco1*Δ, *rtf1*Δ, *and spt2*Δ *strains*. **(B)** Quantitation of results from a minimum of three biological replicates, where error bars are given. Expression values are normalized to the *SCR1* loading control and made relative to the wild-type strains, arbitrarily set to 1. All strains were grown at 30°C to 1-2 X 10^7 cells/ml in YPD.

5.0 DISCUSSION

In this study, I provide evidence to support a new case of gene regulation by intergenic transcription at the *Saccharomyces cerevisiae PIR3* gene. Similar to our previous studies of *SER3*, *PIR3* repression by intergenic transcription occurs in *cis*. However, my results suggest that intergenic transcription is repressing *PIR3* through a different mechanism than what is observed in *SER3* repression [22, 24, 21, 60].

SER3 regulation has been shown to be dependent on nucleosome occupancy across its promoter. Factors and histone residues found to be important for maintaining this repressive architecture, include Paf1, Spt16, Spt2, and specific residues of histone H3
[22] [24, 21, 60, 72]. When these factors have been deleted or mutated, *SER3* is no longer repressed, even when *SRG1* is being transcribed. This is due to loss of repressive nucleosome occupancy at the *SER3* promoter. If *PIR3* expression were derepressed in strains with deletion or mutations in these factors, it would have provided evidence for repression by nucleosome occupancy at the *PIR3* promoter. Deletions in *paf1*Δ and *spt2*Δ showed a decrease in *PIR3* expression. Additionally, no significant change in *PIR3* mRNA levels in the histone H3 K122A mutant was detected (Figures 4.10 and 4.11). This would suggest that nucleosome occupancy at the *PIR3* promoter is not mediating repression, which is different from *SER3* repression. However, the same 2-fold increase in *PIR3* expression seen when intergenic transcription is abolished by TTS insertion, was seen in the *spt16-E857K* mutant. This would seem to support a role for nucleosome occupancy in *PIR3* expression (Figure 4.10). Both the *spt16-E857K* and the histone H3 K122A mutations have been associated with loss of histone H3 occupancy across the *SER3* promoter. Furthermore, the histone H3 K122A and the *spt16-E857K* have been shown to reduce histone H3 occupancy at highly transcribed genes, but not at lowly transcribed genes [24, 21]. Therefore, one possibility

to account for the different effect of these mutants on *PIR3* expression is that the level of *PIR3* ncRNA or *PIR3* expression occur at some intermediate level, where there is a greater sensitivity to defects in *spt16-E857K* than for the other factors. Alternatively, Spt16 has a role at *PIR3* independent of its role in transcription-coupled nucleosome occupancy. To resolve these models, I need to examine the chromatin architecture at *PIR3* by performing histone ChIP experiments. In order to test the effects of *spt16-E857K* and histone H3 K122 have on nucleosome architecture at *PIR3*, I would need to perform ChIP analysis of histone H3. If histone H3 occupancy is significantly decreased in the *spt16-E857K* mutant but not in the histone H3 K122A strain, this would provide evidence for nucleosome-mediated repression at *PIR3* that is regulated by Spt16 activity. If I do not see any significant loss in histone H3 occupancy in these experiments, it would provide support for some other role of Spt16 in maintaining *PIR3* repression.

It is possible that *PIR3* repression is regulated by histone modifications coordinated by transcription-dependent recruitment of histone modifying proteins. This mode of repression by ncRNA is seen at the yeast *GAL10/ GAL1* locus, where the Rpd3S HDAC is recruited by histone H3 K4 di- and H3 K36 tri-methylation for removal of activating acetylation modification at the *GAL10/ GAL1* promoter (reviewed in [23]). Therefore, I investigated the effects that loss of histone methyltransferases Set1, Set2, Dot1, and the Rpds3 subunit, Rco1, have on *PIR3* repression. I found that *PIR3* repression by intergenic transcription appears to be independent of histone methylation and the removal of histone acetylation by the Rpd3S HDAC. In general, both a loss of *PIR3* ncRNA and *PIR3* mRNA expresion is seen in this mutants. This indicates that histone modifications may be important for maintaining normal levels of transcription, but not for maintaining repression by intergenic transcription at *PIR3*.

Two other possible mechanisms of *PIR3* repression include transcription interference and promoter competition. If promoter competition were occurring, the promoter for the *PIR3* ncRNA would outcompete the *PIR3* promoter for binding of transcription factors, resulting in repressed *PIR3* transcription [25]. However, promoter competition is likely not the model for *PIR3* repression, based on the increased *PIR3* expression observed in the *pir3*$_{TTS}$ strain. Transcription termination has no effect on PIC formation, as the promoter

and regulatory elements are still in tact. Therefore, if promoter competition had been acting in *PIR3* repression, I would not have expected to see an increase in *PIR3* expression upon TTS insertion within the intergenic sequence because the intact intergenic promoter would still outcompete the *PIR3* promoter.

With promoter competition an unlikely model for *PIR3* repression, I tested the hypothesis that transcription interference by intergenic transcription may result in repression of *PIR3*. To test this, I assessed the levels of TBP occupancy at the putative *PIR3* consensus TATA site. Since no significant increase in TBP occupancy at the *PIR3* consensus TATA site was seen in the absence of the ncRNA, I do not have strong evidence to support transcription interference in blocking access of TBP at *PIR3* (Figure 4.7). It is still possible that intergenic transcription blocks access of other transcription factors. It is also possible that TBP presence at *PIR3* is in general low, making it difficult to see changes in TBP occupancy using ChIP.

Interestingly, I found significant TBP occupancy at two TATA sequences 5' of *PIR3* ncRNA transcription start sites, indicating that these sequences may be the site of PIC formation for intergenic transcription. The resolution of this ChIP is 200bp, and the distance between *PIR3*-ncTATA2 and *PIR3*-ncTATA3 is only 99bp, making it difficult to say with certainty which site TBP may be occupying. In the future, the TATA sites will need to be mutated in order to make any conclusive arguments about PIC assembly sites. Once the the ncTATA sites 1, 2, and 3 (Figure 4.1) have been mutated, I can assess whether loss of TBP binding is associated with one of these TATA mutants to more accurately designate the true regulatory elements. (See Materials and Methods for details on TATA mutations.)

It has been determined that *PIR3* is upregulated in an Rlm1-dependent manner in response to the cell wall integrity pathway [30]. This led me to hypothesize that ncRNA at *PIR3* may regulate *PIR3* expression in response to cell wall stress through transcription interference of this activator. If this were the case, I would have expected to see a decrease in *PIR3* ncRNA expression in concert with an increase in *PIR3* expression when strains were grown in cell wall stress-inducing conditions. This would support a model where loss of intergenic transcription allows for binding of the Rlm1 activator protein to allow for increased *PIR3* expression to aid in stabilizing the cell wall. After growing cells in the stress inducing

conditions of elevated temperatures and 12mM caffeine, both *PIR3* ncRNA and *PIR3* mRNA expression were increased. This result does not support transcription interference of activator binding as a model for regulation of *PIR3* expression in response to cell wall stress. Although this data does not exclude the possibility that intergenic transcription interferes with the binding of some other activator, it does, however, refute Rlm1 involvement in inducing *PIR3* expression as a result of decreased intergenic transcription. In future experiments, I will confirm that repression by intergenic transcription is not dependent on blocking Rlm1 binding by performing Northern analysis in *rlm1*Δ strains in elevated temperatures and 12mM caffeine. If Rlm1 is only involved in cell wall stress, I would likely see no change in *PIR3* expression, regardless of levels of intergenic transcription. In addition, I will compare wild type to $pir3_{TTS}$ strains to prove if derepression of *PIR3* by loss of intergenic transcription is independent on Rlm1.

In order to further characterize regulation by intergenic transcription at *PIR3*, I attempted to map the ncRNA and ORF transcription start sites. Using primer extension, I found two potential start sites for both the *PIR3* ncRNA and the *PIR3* mRNA transcripts (Figure 4.5). This data is preliminary and needs to be repeated.

To summarize, I have discovered a new source of repression in *cis* by intergenic transcription at *PIR3*. A model for regulation in terms of how the ncRNA or the act of intergenic transcription may be repressing *PIR3* can only be speculated. Repression does not appear to be mediated through chromatin structure, although comparing histone H3 ChIPs in the presence and absence of *spt16-E857K* mutation will provide further evidence to support or refute this model. Furthermore, histone modifications do not appear to be repressing *PIR3*. The ncRNA TATA mutations at the *PIR3* ncRNA should lead to a better understanding of repression at *PIR3*. First, I will be able to confirm that the increased *PIR3* expression I see in the $pir3_{TTS}$ strains is due to loss of intergenic transcription and not some secondary effect resulting from insertion of 171bp of the TTS sequence upstream of*PIR3*. Secondly, I will be able to confirm the correct TATA site for the intergenic transcript by repeating the TBP ChIP analysis comparing wild type strain and each TATA mutant to determine which site results in loss of TBP occupancy. Third, if promoter competition is operating at *PIR3*, I would expect to see an even larger signal for *PIR3* derepression. We cannot rule out

promoter competition without preventing PIC formation at the ncRNA through mutation of the regulatory elements.

During the course of this study, I have aided in the development of a method for disrupting intergenic transcription through insertion of a minimal transcription termination sequence (Figure 4.3). The utility of this method was confirmed at *SER3*. Briefly, I inserted 171bp of the *HIS3*-TTS at -450bp upstream of the *SER3* ATG, within the *SRG1* sequence. I demonstrated a loss of *SRG1* with concurrent derepression of *SER3* despite growth in high serine conditions. This shows that by abolishing *SRG1* transcirption with the TTS, I also disrupted the repressive nucleosome occupancy at the *SER3* promoter. Having confirmed the efficacy of this method, I employed it at *PIR3*. With insertion of the TTS at -486bp, relative to the *PIR3* ATG, I was able to establish that repression by intergenic transcription was occurring. This method could prove useful for studying the effects of disrupted transcription at any nonessential gene. The advantage to using the *HIS3*-TTS to disrupt transcription is that mutational analysis of regulatory sequences is not needed, faciliting studies where these sites are unknown, as was the case for *PIR3*. A caveat, however, is the fact that 171bp of DNA sequence is added to the site under investigation. If sequence structure is important in regulating expression, this method may not lead to identification of true mechanisms of gene expression.

Studying mechanisms of gene regulation by ncRNA will lead to a greater understanding of their role in disease and development. ncRNAs have been shown to have importance in neural function, cellular development, and cancer (Reviewed in [61, 50, 3, 75][59]). The discovery of these ncRNAs was made possible by improved technologies for transcriptome analysis [50][3]. As technologies advance that can identify RNAs expressed within particular cells at particular times, the number of genes identified to be regulated by ncRNA will likely increase. Ideally, current studies of gene regulation by ncRNA will provide guidelines for identifying and studying these new ncRNAs. Additionally, these ncRNA can be used as targets in treating disease or as biomarkers, as is the case with certain cancers or neurological function (Reviewed in [61, 59]). For example, a ncRNA that is aberrantly expressed, resulting in disease development, could be targeted by RNA silencing technologies. On the other hand,

if a ncRNA has been shown to be present in high levels in aggressive cancers, this biomarker could be useful in deciding to treat a patient more aggressively despite adverse side effects. Thus, studying gene regulation by ncRNAs will lead to improved understanding and treatment of disease.

PIR3 does not appear to be regulated by ncRNA in response to cell wall stress; however, the fact that this study involved gene regulation important in cell wall synthesis may have implications beyond noncoding RNA. Certain yeast species, such as *Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans*, act as human pathogens. *S. cerevisae* serves as a model for yeast pathogens, due to conservation in cell wall construction and stress signaling, especially in *C. albicans* [41]. With increases in the number of fungal infections as well as increases in antifungal resistance since the 1990's [56], improving our knowledge of cell wall biogenesis and regulation could aid in the development of more effective treatments for pathogenic yeast, especially in immune compromised people.

APPENDIX

NON-SPECIFIC BAND FOR *PIR3* NORTHERN PROBE

In my initial studies, I thought that, in addition to the increase in *PIR3* expression in the *pir3*$_{TTS}$ strain, a second larger transcript was expressed at *PIR3* by Northern analysis. However, I began to see this band in the wild type strains as well as inconsistencies in the bands appearance in *pir3*$_{TTS}$ strain. This prompted me to assess transcript signals from the *PIR3* probe in a *pir3*Δ strain. In the Northern analysis, I saw the larger band even when *PIR3* was deleted, indicating that this signal is non-specific (Figure A1). I continued to use this probe despite this result since I wanted a probe within the *PIR3* ORF that had no overlap with untranslated regions, and attempts to design other probes had homology to *PIR1*.

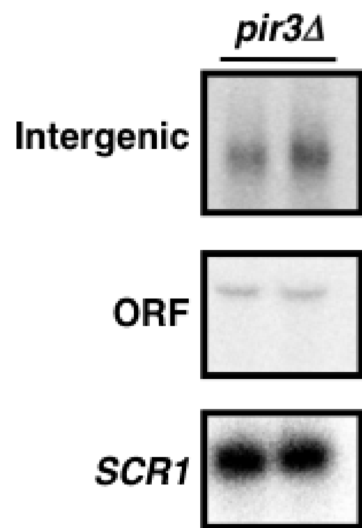

Figure A1: **Northern analysis assessing the specificity of *PIR3* probe** used *pir3*Δ from ko collection.

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