DISSERTATION

ACTIVATION OF GENE EXPRESSION IN YEAST

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SARAH K. LEE ENTITLED ACTIVATION OF GENE EXPRESSION IN YEAST BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ACTIVATION OF GENE EXPRESSION IN YEAST

Transcription is the generation of RNA from the DNA template, and is the fundamental aspect of gene expression. As such, the initiation of transcription at genes that are transcribed by RNA polymerase II (RNAPII) is a major control point in gene expression. Organisms across the evolutionary spectrum possess genes whose transcription is regulated after recruitment of RNAPII to the promoter, or postrecruitment. This regulatory strategy has been observed in bacteria, yeast, worms, flies, and humans. Therefore, postrecruitment regulation is a conserved strategy for controlling gene expression. Genome-wide studies in *Drosophila* and humans demonstrate that a significant portion of these genomes are postrecruitment regulated. Recent studies in humans indicate two biologically important activators (p53 and c-myc) are involved in releasing paused polymerases from promoter DNA[1,2]. These regulators of cell growth and differentiation are both implicated in carcinogenesis. Thus, further understanding how activators regulate the transition from an inactive to active polymerase will prove crucial in our understanding of transcriptional regulation and human diseases.

Coactivators are conserved, multiprotein complexes involved in regulating the transcription process at most genes. Yet, virtually nothing is known about the role of coactivators at postrecruitment regulated genes in yeast. The work presented in this

dissertation details the identification of postrecruitment functions of two coactivators, the Mediator and SAGA complexes. My studies reveal that coactivators act as intermediaries with activator proteins to stimulate transcription after the recruitment of RNAPII to the promoter. Further, this work demonstrates that this conserved class of factors plays a role in postrecruitment regulation, a previously unappreciated aspect of coactivator function.

Analysis of Mediator function at the postrecruitment regulated *CYC1* gene revealed a functional submodule of the Mediator complex that is required for triggering the preloaded polymerase at the *CYC1* promoter into an active polymerase. This requirement exists even when two different activator proteins control *CYC1* expression, Hap2/3/4/5 and Yap1. Strikingly, this submodule is not required for activation of a recruitment regulated Yap1-dependent gene, *GTT2*.

The Yap1 activator controls the expression of a number of genes during oxidative stress in yeast. Oxidative stress is a damaging condition that haunts all aerobic organisms, and is linked to many human ailments. Yeast respond to this biological assault with a rapid activation of many genes. My investigation of Yap1-dependent transcription demonstrated that postrecruitment regulation is more prevalent in yeast than previously thought. Analysis of SAGA function at Yap1-dependent genes revealed that Yap1 utilizes SAGA during oxidative stress. Despite a common reliance on the SAGA coactivator for expression, each gene has different specific SAGA requirements. This demonstrates an important role for the SAGA coactivator during the important biological response to oxidative stress, and the complexity inherent in transcriptional regulation.

In sum, my findings illustrate the mechanisms of activated transcription yeast utilize in response to important biological stimuli. This work significantly advances our understanding of the regulation of transcription *after* RNAPII arrives at the promoter. It

also reveals the novel role that coactivators play in stimulating transcription at the group of genes that are regulated in this fashion.

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CHAPTER 1. INTRODUCTION

1.1 TRANSCRIPTION: A FUNDAMENTAL AND HIGHLY REGULATED PROCESS

Accurate control of gene expression governs cell growth, differentiation, development, and response to the environment. It is therefore essential for life. Transcription is the generation of RNA from the DNA template, and is the fundamental aspect of gene expression. As such, the initiation of genes transcribed by RNA polymerase II (RNAPII) is a major control point in gene expression, as RNAPII is the enzyme responsible for transcribing genes encoding proteins. Transcription initiation in eukaryotes is a highly regulated and highly conserved process. Initiation requires the presence of the pre-initiation complex (PIC) at promoter DNA. The PIC is composed of the polymerizing enzyme (RNAPII) and the general transcription factors TFIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH[3]. Gene-specific transcriptional activator proteins also play an important role in transcription as they influence the rate of transcript production from target genes. These proteins bind promoter DNA in a sequence-specific manner via a DNA-binding domain (for reviews see[4,5]). Misregulated transcription (from mutation or overexpression of transcription factors or mutations in cis-acting elements) is linked to many human conditions including, but not limited to, β-thalassaemias[6], hemophilia B[7], mental retardation[8], and cancer[9-11].

1.2 ENVIRONMENTAL CONTROL OF RNAPII TRANSCRIPTION

All organisms must effectively utilize transcription to express genes only when required. Therefore, cells must sense their environments and respond with appropriate gene transcription. Completely deregulated transcription is not compatible with life. In the single-celled budding yeast *Saccharomyces cerevisiae*, transcriptional responses to changing environments are essential for survival and are thus finely tuned[12].

1.2.1 Growth in nonfermentable carbon sources:

When yeast grows aerobically in glucose, the majority of the available glucose is fermented. Glucose is the preferred carbon source, indeed, when glucose is present, the levels of enzymes required for metabolizing other carbon sources are absent or greatly repressed[13]. This process ensures glucose is used preferentially, and is referred to as carbon catabolite repression (for reviews see[14,15]).

When glucose is not available, yeast utilizes other carbon sources for energy. Yeast metabolizes nonfermentable sources, such as ethanol, lactate, and acetate, via respiration. This utilizes the TCA cycle, and ATP is produced via oxidative phosphorylation in the mitochondria[16]. Therefore, shifting cells from fermentable to nonfermentable carbon sources requires a change in the metabolic program; this change is facilitated by modifying gene expression patterns. In the laboratory, shifting cultures from glucose-containing media to ethanol-containing media induces this process.

Upon the transition to ethanol as the carbon source, the evolutionarily conserved Hap2/3/4/5 complex of proteins activates an assortment of genes encoding proteins involved in cellular respiration[17]. The Hap2, Hap3 and Hap5 proteins are required for

binding target sequences in gene promoters. The Hap4 protein associates with this complex via a basic region in the N-terminus of the protein. Once bound, Hap4 provides the transcriptional activation function of the complex via two activation domains (**FIGURE 1.1A**)[13]. Transcription of the *HAP4* gene is itself regulated by carbon source (**FIGURE 1.1B**). *HAP4* transcript levels are low during growth in glucose, and are induced upon the transition to ethanol[13].

1.2.2 The response to oxidative stress:

Aerobic organisms are assaulted with the formation of reactive oxygen species (ROS) and the metabolites of ROS generated via respiration[18,19]. ROS also accumulate due to non-metabolic sources such as ultraviolet radiation and chemicals[20]. ROS exposure can lead to a condition of oxidative stress if oxidant levels overwhelm cellular antioxidants[21]. Oxidative stress is a damaging condition as ROS harm all cellular components, including DNA, lipids, and proteins[22,23].

Oxidative stress is implicated in the development of many human ailments. For instance, the process of aging as well as the age-related conditions of atherosclerosis, neurodegenerative diseases, cancer, and inflammatory conditions have all been connected with oxidative stress[24-26]. While it is unclear if this is a causal relationship, there is no debate concerning the correlation between oxidative stress and disease[27,28]. Interestingly, overexpression of some antioxidant enzymes such as superoxide dismutase can extend the lifespan of the model organism *Drosophila melanogaster* by 40%[29].

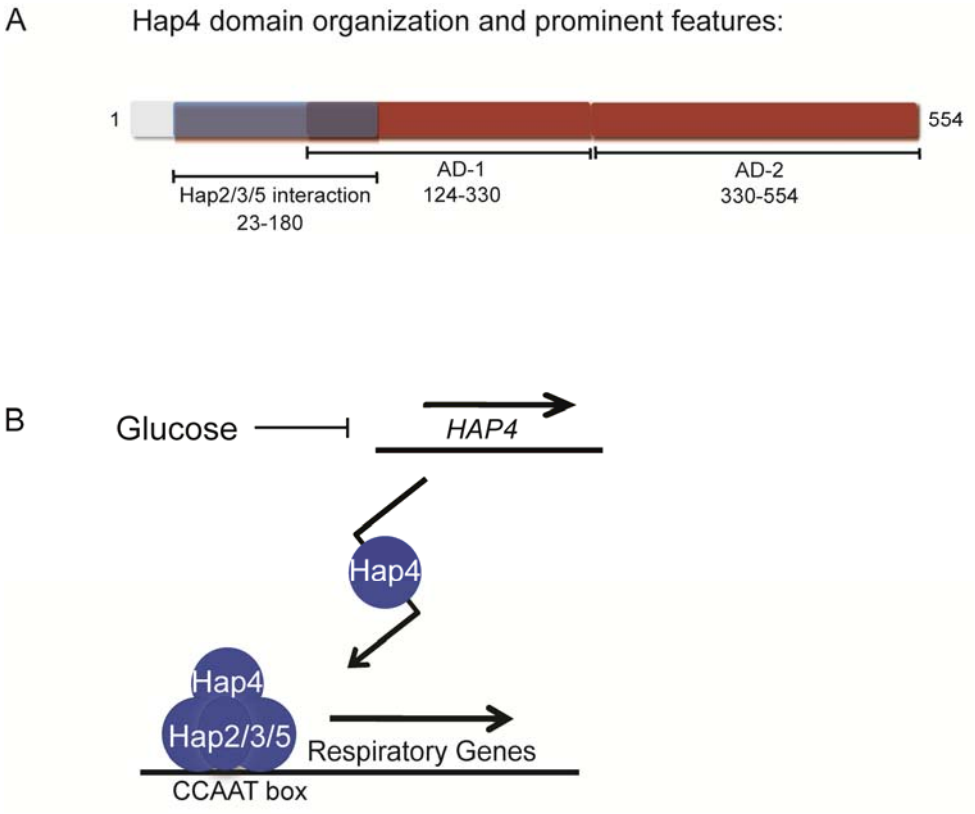

FIGURE 1.1. Features of the Hap4 protein and cellular events leading to transcriptional activation of Hap2/3/4/5 target genes. A) Schematic of the known domain structure of the Hap4 protein. Hap4 contains a basic region at the N-terminus required for interaction with the Hap2/3/5 proteins (blue). Hap4 has two acidic activation domains (AD-1 and AD-2, red)[13,30]. **B)** The presence of glucose represses the expression of *HAP4*. In the absence of glucose, Hap4 can bind with Hap2/3/5 in target promoters and activate transcription.

During oxidative stress, cells must restore the balance between ROS and antioxidants to return to a normal state. Increased expression of genes that encode proteins involved in cellular protection and detoxification is a primary response to oxidative stress. This reprogramming of gene expression is termed the "oxidative stress response", and its rapidity is critical as this determines the level of cellular damage sustained[31]. In higher eukaryotes, the transcription factors NF-κB and AP-1 are the most prominent in directing the transcriptional response to oxidative stress[32].

Yeast is a model organism for studying the oxidative stress response. Like human AP-1, the yeast AP-1 (referred to hereafter as Yap1) activator is essential for the oxidative stress response in this organism[31]. This yeast protein was identified based on its ability to activate transcription from the AP-1 recognition element[33]. Yap1 contains a basic leucine zipper (b-ZIP) DNA binding domain and is regulated in an oxidation-reduction dependent manner (**FIGURE 1.2A**)[34,35]. It shuttles between the cytoplasm and nucleus, yet under normal conditions, it is predominantly cytoplasmic[34]. The localization of Yap1 is due to an interaction between Yap1 and the karyopherin nuclear exporter protein Crm1, which exports Yap1 from the nucleus to the cytoplasm[36]. Yap1 contains two cysteine-rich domains (CRDs) that form intramolecular disulfide bonds upon oxidation, resulting in a conformational change. This change in conformation masks the nuclear export recognition sequence from Crm1, resulting in Yap1 nuclear localization during oxidative stress[37].

Once in the nucleus, Yap1 binds Yap1 Response Elements (YREs) in the promoter of target genes. Yap1 target genes encode a variety of antioxidants, heat shock proteins, drug transporters, and enzymes involved in carbohydrate metabolism (**FIGURE 1.2B**)[38]. While we know quite a bit about the nuclear localization of Yap1, little is known about its mechanism of transcriptional activation.

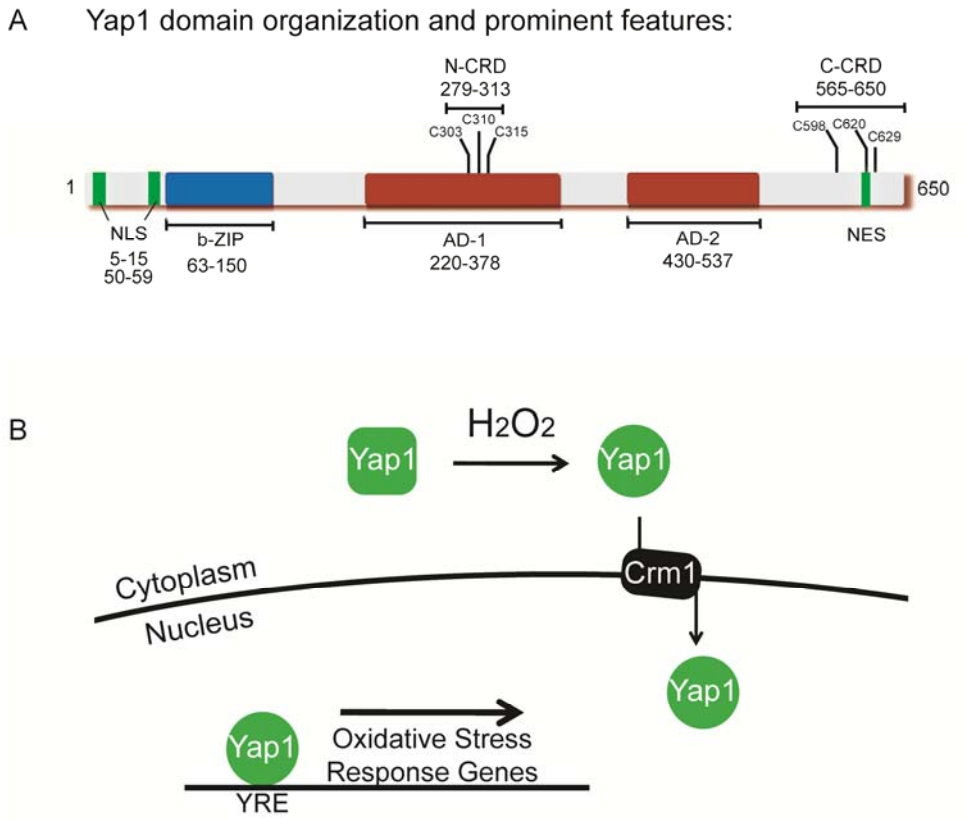

FIGURE 1.2. Important features of the Yap1 protein and cellular events leading to transcriptional activation of Yap1 target genes during oxidative stress. A) Schematic showing the domain structure of the Yap1 protein. Yap1 contains a basic leucine zipper (b-ZIP) DNA binding domain (blue), and two acidic activation domains (AD-1 and AD-2, red). The protein is shuttled into and out of the nucleus via the nuclear localization and export sequences (NLS and NES). Exposure to oxidizing agents such as H_2O_2 results in a conformational change in the Yap1 protein. This occurs via the formation of disulfide bonds between the two cysteine-rich domains (CRDs) of the protein[35,39,40]. **B)** Conformation change allows Yap1 import into the nucleus (via the Crm1 protein), but not export. Once nuclear, Yap1 binds Yap1 Response Elements (YREs) in target genes.

1.3 HOW DO ACTIVATORS STIMULATE TRANSCRIPTION OF TARGET GENES?

How does the presence of activator proteins such as Hap2/3/4/5 and Yap1 at promoter DNA translate into gene activity? One way is through recruitment of the general transcription machinery via direct protein-protein contacts[41-47]. Often recruitment of the GTFs and RNAPII results in transcriptional activity. Genes controlled in this manner are referred to as recruitment-regulated. At a recruitment-regulated gene, an activator protein binds DNA, recruits GTFs and RNAPII to the promoter and transcription ensues. Most well characterized model genes are recruitment-regulated. For instance, the yeast activator Gal4 controls several recruitment-regulated genes (*GAL1, GAL7* and *GAL10*), as does the Gcn4 activator (*ARG1*, *SNZ1* and *ARG4*)[48,49]. At these genes, the rate-limiting step in the transcription process is the formation of the PIC at the promoter. Therefore, the recruitment of RNAPII to the promoter region directly correlates with transcriptional output[50-52]. At recruitment-regulated genes, RNAPII promoter occupancy changes greatly (generally more than 8-fold), along with transcript levels (>10-fold) during induction (**FIGURE 1.3A**). The hallmark of recruitment-regulation is a large change in polymerase occupancy during induction.

Recruitment of the GTFs and RNAPII is not the only way that activators can elicit gene activity. Activators can also function through stimulation of pre-existing complexes at promoters. Genes regulated in this fashion are referred to as postrecruitment regulated. At a postrecruitment regulated gene, GTFs and RNAPII occupy promoter DNA in the absence of transcription. Therefore, RNAPII occupancy is not a marker for transcription. The inactive RNAPII at the promoter regions of postrecruitment regulated genes is referred to as preloaded polymerase. At these genes, steps after the recruitment of polymerase, or postrecruitment, are rate-limiting for the transcription reaction[50-52]. It is unknown how activators trigger postrecruitment regulated genes.

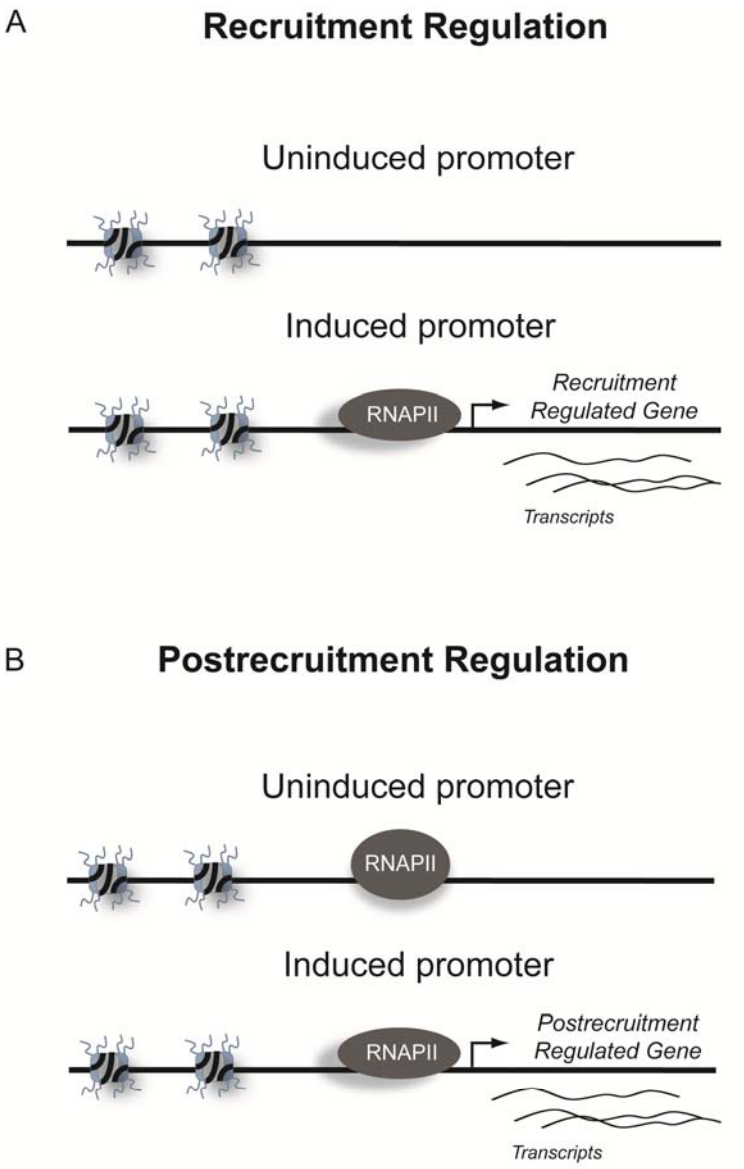

FIGURE 1.3. Key features of recruitment-regulated and postrecruitment regulated promoters. A) Recruitment regulation. In the uninduced state, RNAPII does not occupy promoter DNA, and there is little/no transcripts detected. Upon induction, RNAPII occupies promoter DNA, with corresponding high levels of transcript. It is important to note that recruitment-regulated genes may contain some RNAPII occupancy prior to induction (if they are transcribed), this schematic is meant to represent the large change in occupancy that occurs upon induction. **B)** Postrecruitment regulation. In the uninduced state, RNAPII occupies promoter DNA, but there is little/no transcript detected. Upon induction, high levels of transcript are detected with little change in RNAPII promoter occupancy.

Preloaded, yet transcriptionally inactive, promoters are poised for subsequent activation. Upon induction, the amount of RNAPII present at the promoter changes slightly (generally less than 3-fold), despite large changes in transcript levels (>10-fold) (**FIGURE 1.3B**). The best-characterized postrecruitment regulated gene in yeast is the *CYC1* gene[48,52,53].

1.3.1 Conservation of postrecruitment regulation:

The proteins and mechanisms driving transcription are conserved from yeast to humans[54]. Poised promoters are found across the evolutionary spectrum, indicating that postrecruitment regulation is a conserved regulatory strategy. This regulatory scheme has been observed in bacteria, yeast, *C. elegans*, *Drosophila*, and humans[50-53,55-58]. Recent genome-wide studies in *Drosophila* demonstrate that a large number of developmental and stress-inducible genes have RNAPII preloaded at promoter-proximal regions[59-61]. In humans, similar studies suggest that transcription of a significant part of the genome may be regulated at rate-limiting steps after recruitment of the PIC[51,59]. Interestingly, this includes viral genes (HIV)[62] and proto-oncogenes such as c-myc[63], c-myb[64], and c-fos[65,66]. Clearly, this type of regulation plays a significant role in human biology. Elucidating the mechanisms driving expression of this group of genes in yeast can potentially transform our understanding of transcriptional regulation and human diseases.

1.4 RECRUITMENT-REGULATED GENES REQUIRE COACTIVATORS FOR PROPER EXPRESSION:

Transcription by RNAPII is a complex process that depends upon the coordinate activities of a large number of factors. In addition to RNAPII and the general transcription factors, coactivators are an important and highly conserved class of factors that are

required for transcription of recruitment-regulated genes. At these genes, coactivators function as intermediaries between transcriptional activator and repressor proteins and RNAPII. Therefore, coactivators mediate and integrate signals from the cell to the transcription machinery at this group of genes[67]. This dynamic process allows for the appropriate level of gene expression of individual genes during a particular condition. It is currently unknown if coactivators are required for transcription of postrecruitment genes in yeast.

1.4.1 SAGA: a multi-functional coactivator

SAGA is a conserved, multi-functional coactivator that regulates transcription at a subset of RNAPII-dependent genes[68]. The complex contains distinct activities involved in transcription regulation, and is named for its protein components (Spt-Ada-Gcn5 acetyltransferase). Structural analysis of SAGA demonstrates the functional activities are spatially separated; therefore, SAGA has a modular composition[69] (**FIGURE 1.4A**). SAGA is required for recruitment of the transcription machinery (TBP and/or RNAPII) at several recruitment-regulated genes[45,48,70]. For instance, at the galactose-inducible *GAL10* gene, SAGA is absolutely required for gene expression. Without this coactivator, the Gal4 activator protein cannot stimulate transcription, and RNAPII is not recruited to the promoter DNA[70].

SAGA is also involved in modifying chromatin structure. It contains two enzymatic activities with this function, the Gcn5 histone acetyltransferase (HAT) enzyme and the Ubp8 deubiquitinating enzyme. Both of these activities generate chromatin marks classically associated with active chromatin (**FIGURE 1.4B**). The Gcn5 protein can acetylate the tails of histones H3 and H2B[71-74]. Histone acetylation strongly correlates

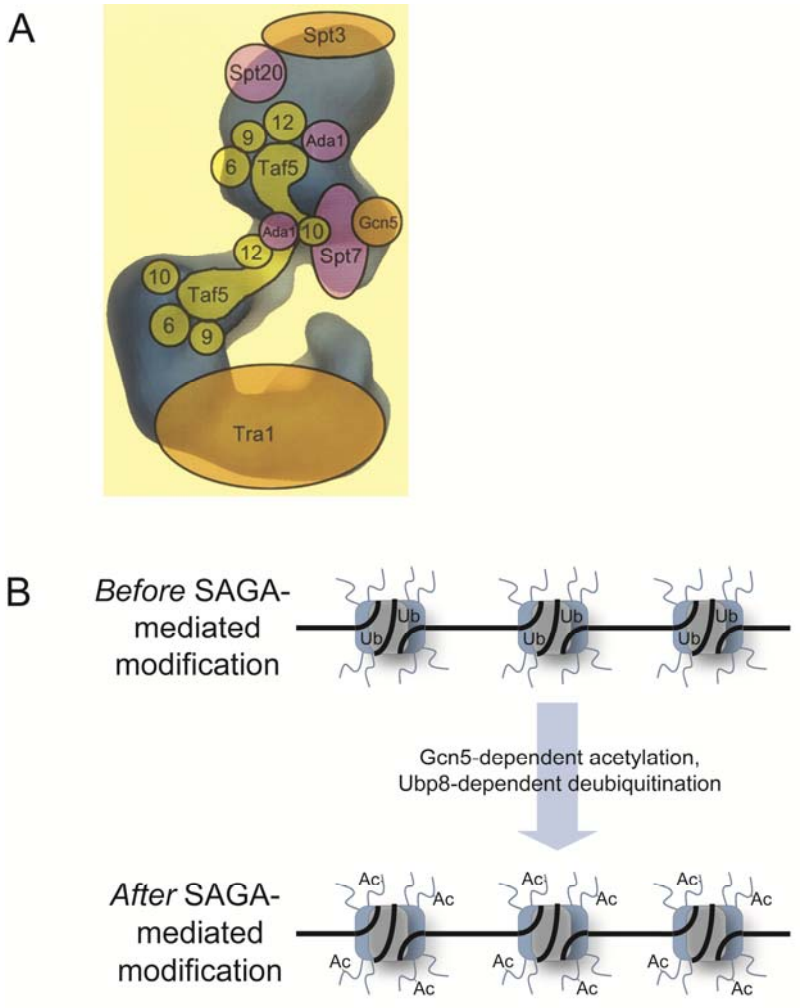

FIGURE 1.4. SAGA structure and function. A) Model of the EM structure of the SAGA complex with the mapped location of several subunits indicated. Figure modified from[69]. **B)** Schematic of the nucleosome modifications SAGA catalyzes. The N-terminal tails of histones H2B and H3 are acetylated (Ac) via the Gcn5 HAT protein. H2B is deubiquitinated (ubiquitin=Ub) via the Ubp8 enzyme.

with transcriptional activation[75-77]. The mechanism behind this correlation likely involves increased recruitment of bromodomain-containing proteins[78], and a reduction in inter-nucleosomal interactions resulting in fiber unfolding[79-81]. SAGA also cleaves monoubiquitin from lysine 123 of histone H2B via the Ubp8 subunit[82-84]. In vivo, Ubp8 relies on the Sgf11, Sgf73 and Sus1 accessory proteins for activity[85,86]. The role of histone ubiquitination and deubiquitination in transcription is still evolving, but sequential ubiquitination and deubiquitination has been shown to play a positive role in transcriptional activation of the recruitment-regulated *GAL1* and *GAL10* genes[87,88].

SAGA also contains several Spt (Suppressor of Ty) proteins, including Spt3, Spt7, Spt8 and Spt20. The *SPT* family of genes encodes proteins intimately involved in various transcription-related processes. In fact, TBP itself is encoded by the essential *SPT15* gene. Products of the yeast *SPT* gene family are implicated in various processes such as transcription initiation, elongation and RNA processing, and maintaining chromatin structure[89-92]. Spt3 and Spt8 are involved in regulating TBP-TATA interaction[93-98]. The Spt20 protein is required for the structural integrity of the complex, along with the Spt7 and Ada1 proteins. The complex fails to form in strains containing deletions in any of these three subunits[72,96,99].

While we know that SAGA plays a role at recruitment-regulated genes, it is unknown if this coactivator functions at postrecruitment regulated genes. If it is important for expression of postrecruitment regulated genes, this could rely on its previously characterized functions described in terms of recruitment regulation above. On the other hand, perhaps a previously unknown function of the complex is required at postrecruitment regulated genes. This is certainly possible given that SAGA function has not been studied at this distinct class of promoters.

1.4.2 Mediator: a moderator of transcriptional activation

Mediator is a large co-regulatory complex that plays essential roles in the transcription of most RNAPII-dependent genes[100]. This integral member of the transcription machinery is conserved from yeast to humans[101]. The complex contains 25 subunits in yeast, and is over 1 MDa in mass. The core complex has been described as consisting of three modules termed the head, middle and tail. A fourth module transiently interacts with the rest of Mediator. This module consists of four proteins, two of which are a cyclin-dependent kinase/cyclin pair, and is referred to as the CDK8 module. These proteins are thought to primarily contribute to the negative functions of the complex[102-104].

The classical function of Mediator, defined from in vitro experiments, is threefold. Mediator can stimulate basal transcription in vitro, it can stimulate TFIIH activity, and it can stimulate activated transcription[105]. Understanding the activity of Mediator in vivo has been more elusive. Current knowledge is limited to mutational analysis (both phenotypic and molecular analysis of transcription), and occupancy and order of recruitment studies at a variety of recruitment-regulated promoters. At the majority of these genes, an interaction with DNA binding activator proteins results in Mediator recruitment to promoter DNA[44,106-108]. Once recruited to the proper location, in vivo investigation of recruitment-regulated promoters point to three primary functions of the Mediator complex including stability/assembly of the PIC[45,109,110], phosphorylation of a component of the transcription machinery[111,112], and stimulation of TFIIH activity[110].

Mediator also interacts extensively with RNAPII. Electron microscopy analysis of Mediator particles with RNAPII reveals a broad interface between Mediator and polymerase[113,114]. This interface localizes to the head and middle modules of Mediator[115,116] (**FIGURE 1.5**). The in vivo significance of the large interface is currently not

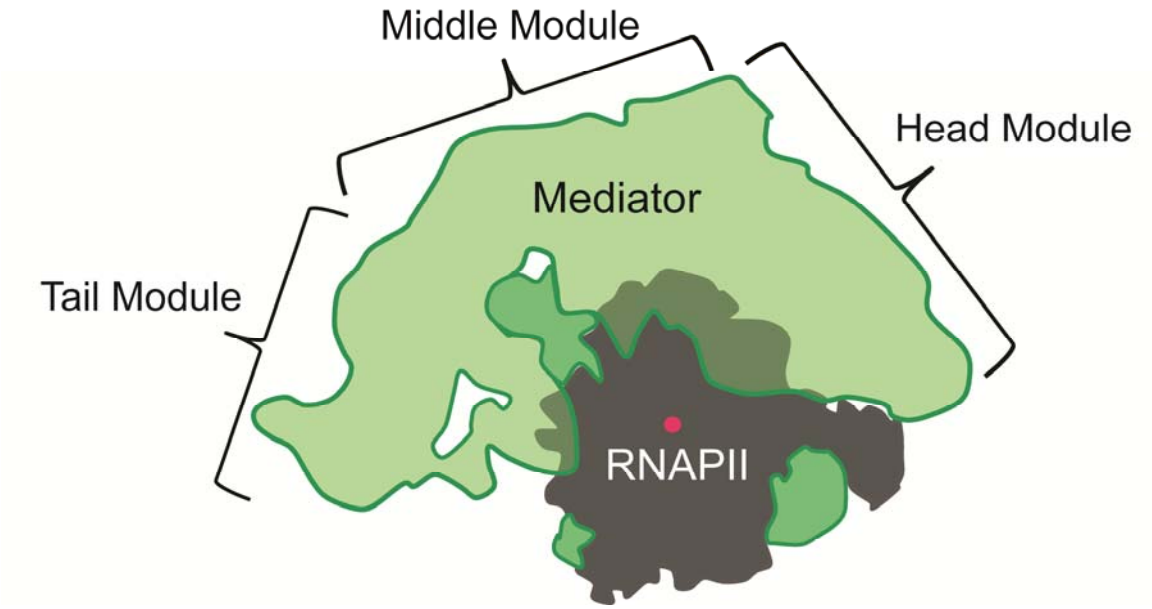

FIGURE 1.5. Mediator structure and interaction with RNAPII. Model of Mediator/RNAPII complex modified from[115]. The crystal structure of RNAPII was modeled into the EM structure of the Mediator complex. Location of the RNAPII active site is indicated by the magenta dot. The relative locations of each Mediator module are indicated[117].

understood. However, there have been numerous speculations that Mediator binding RNAPII results in a conformational change in the polymerase[1,118-120]. It is unknown if Mediator plays a role at postrecruitment regulated genes. Is it possible that the intruiging Mediator-polymerase interface could be important for stimulation of preloaded polymerases?

1.5 GAPS IN THE FIELD:

Despite the prevalence of postrecruitment regulated genes across evolution, it is unknown what regulatory factors are involved in the transition from a transcriptionally incompetent to a transcriptionally active polymerase. Consequently, we have an incomplete mechanistic understanding of these poised, yet inactive promoters. For instance, it is unknown if coactivators are required for transcription of this class of genes. We know that coactivators have roles in recruitment regulation, but are they also required for governing postrecruitment regulated genes? It is also unclear if the mechanisms operating at an individual postrecruitment regulated gene are conserved between genes. Can we glean rules governing this type of regulation from studying multiple preloaded genes, and will studying these genes illuminate the purpose of sequestering RNAPII in an inactive form at some genomic locations?

To define the factors involved in postrecruitment regulation, we set out to identify gene products with important roles in transcription after RNAPII occupies promoter DNA. Using a genetic screen aimed at identifying proteins involved in postrecruitment regulation, we discovered two coactivator complexes are indeed involved in this type of regulation, the SAGA complex and Mediator. This study is detailed in **CHAPTER 3**.

SAGA function at postrecruitment regulated genes is completely unknown. A molecular analysis of SAGA dependencies has only been performed at a few recruitment-regulated model genes. When the genetic screen revealed that SAGA plays a role in postrecruitment regulation, we next wondered what functions of the complex are required for activation of preloaded polymerases. In **CHAPTER 3**, I set out to determine the SAGA dependency at the preloaded *CYC1* gene during activation by the Hap2/3/4/5 complex. Intriguingly, we found SAGA does not use its previously characterized functions to stimulate *CYC1*, yet it is still essential for activity of this gene. We next wondered if SAGA-dependency is a common feature of other postrecruitment regulated genes. To address this question, I expanded my analysis to four oxidative stress response genes, three of which are postrecruitment regulated (**CHAPTER 5**). Studying the role of this important and conserved coactivator at postrecruitment regulated genes provides a new perspective of SAGA's role in the transcription process. We show that SAGA is not limited to activation of recruitment-regulated promoters, but also plays an essential role in the transition from an inactive to active complex at postrecruitment regulated genes.

Analysis of Mediator function in the transcription process is limited to the investigation of recruitment-regulated promoters. Therefore, many questions remain regarding its role at poised promoters. For instance, a portion of Mediator termed the head module interacts extensively with the RNAPII enzyme. Does this interaction play a role at genes with preloaded inactive polymerase? This question is answered in **CHAPTER 4**. In **CHAPTER 5**, I analyze the timing of Mediator recruitment to a group of four genes induced by oxidative stress, and investigated the relationship between SAGA and Mediator by determining occupancy of Mediator in SAGA-deficient cells.

In the course of this study, I discovered that coactivators are important in the regulation of poised promoters. This is an important finding, as yeast coactivators have only been implicated in recruitment regulation until this point. Functional analysis reveals that particular roles of the SAGA complex are not uniformly required at a group of postrecruitment regulated promoters. Instead, this coactivator has gene-specific functions within the postrecruitment regulated class of genes. Mediator is also important in postrecruitment regulation. I found this coactivator is required for stimulation of the preloaded *CYC1* gene. This requirement is not activator-specific; two activators involved in responding to the environment, Hap2/3/4/5 and Yap1, utilize Mediator to stimulate the *CYC1* gene. This highlights the important function of Mediator in regulating the *CYC1* gene.

The postrecruitment regulatory strategy as well as SAGA and Mediator are conserved from yeast to higher eukaryotes. Therefore, the findings outlined in this study not only change our view of transcriptional regulation in budding yeast, but also have the potential to illuminate mechanisms of regulation at poised promoters in higher eukaryotes.

CHAPTER 2. MATERIALS AND METHODS

2.1 YEAST STRAINS:

Strains used in this study are in **TABLE 2.1**. The parent BY4741 (*MAT***a** *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*) strain was purchased from Research Genetics. The *med2Δ* strain was generated using common protocols[48]. All other deletion strains were purchased from Research Genetics.

Strains containing proteins tagged with either the HA or myc epitope were generated according to the literature[121], except for strains which also contain deletions. These strains were generated by first amplifying a portion of the open reading frame (ORF) and the tag from the BY4741 tagged strain. PCR amplified DNA was ethanol precipitated, and the DNA resuspended in TE. The DNA was then run on a 1% agarose gel. The PCR amplified band was cut out of the gel and the DNA was extracted using a spin column. DNA was precipitated and resuspended in 40-50 µL of TE. The wild-type strain and given deletion strain were transformed with 2.5-5 µg of the purified DNA. Transformations were performed using the standard LiAC/TE procedure.

The Med15 (Gal11)-myc, Med15-myc *spt20Δ*, Med15-myc *gcn5Δ*, and Med15-myc *med20Δ* strains were a gift from Alan Hinnebusch[44,96]. The *gcn5*^{E173Q} ORF was a gift from Shelley Berger[122]. The ORF was subcloned into the pRS313 plasmid, which was transformed into the BY4741 background.

TABLE 2.1. ***S. cerevisiae*** **strains used in this work.**

STRAIN	GENOTYPE	SOURCE
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
med2Δ	BY4741 *med2Δ::URA3*	Chapter 3[48]
gcn5Δ[a]	BY4741 *gcn5Δ::kanMX4*	Research Genetics
gcn5^{E173Q}	BY4741 *gcn5Δ::kanMX4*/pRS313-*GCN5-*^{E173Q} *(HIS3)*	Chapter 3[48]
SPT20-HA	BY4741 *SPT20-HA*$_3$*::HIS3*	Chapter 3[48]
GCN5-myc	BY4741 *GCN5-myc*$_{13}$*::HIS3*	Chapter 3[48]
SPT8-HA	BY4741 *SPT8-HA*$_3$*::HIS3*	Chapter 3[48]
TAF1-HA	BY4741 *TAF1-HA*$_3$*::HIS3*	Chapter 3[48]
MED12-HA	BY4741 *MED12-HA*$_3$*::HIS3*	Chapter 3[48]
MED12-HA, spt20Δ	BY4741 *MED12-HA*$_3$*::HIS3, spt20Δ::kanMX4*	Chapter 3[48]
MED15-myc	BY4741 *MED15-myc*$_{13}$*::HIS3*	[96]
MED15-myc, spt20Δ	BY4741 *MED15-myc*$_{13}$*::HIS3, spt20Δ::kanMX4*	[96]
MED15-myc, gcn5Δ	BY4741 *MED15-myc*$_{13}$*::HIS3, gcn5Δ::kanMX4*	[96]
MED15-myc, med20Δ	BY4741 *MED15-myc*$_{13}$*::HIS3, med20Δ::kanMX*	[44]
YAP1-myc	BY4741 *YAP1-myc*$_{13}$*::HIS3*	This work
MED14-HA	BY4741 *MED14-HA*$_3$*::HIS3*	This work

[a] All other deletion strains were purchased from Research Genetics. The marker is the *kanMX* gene.

2.2 YEAST MEDIA:

Media used for routine culture of yeast is as described[123]. YPD plates have 2% final concentration of glucose. YP-galactose plates have 2% galactose as the carbon source. YP-raffinose plates have 2% raffinose. YP-Glycerol plates were made by supplementing YP with 2% glycerol. YPEG plates were made by supplementing YP with ethanol (3%) and glycerol (3%). YPD plates with hydrogen peroxide were made by supplementing cooled YPD with hydrogen peroxide (Sigma) to a final concentration between 2.5 and 4.5 mM.

2.3 CELL CULTURING CONDITIONS:

For ethanol induction, yeast cultures were grown overnight in YPD, then diluted and allowed to undergo 2 doublings in YPD. Cells were washed with YP three times and diluted into YP containing 3% ethanol as the sole carbon source and were cultured at

30° with shaking for various times (30 minutes to 6 hours, as indicated). For uninduced samples, cells were grown in YPD for the indicated time at 30°C.

For galactose induction, cells were grown in YP containing 2% glucose, then washed and transferred to YP galactose (2%). For uninduced samples, cells were grown in 2% glucose at 30° C to an optical density of 0.8-1.0.

For oxidative stress induction, cultures were grown overnight in YP and allowed to undergo 2 cell doublings the next day. When cultures reached an OD_{600} of 0.7-0.8 cells were treated with hydrogen peroxide (Sigma) to a final concentration of 0.3 mM. Cultures were incubated at 30°C with shaking. Samples were taken at various time points after the addition of hydrogen peroxide to the culture, as indicated.

2.4 WESTERN BLOT ANALYSIS:

Yeast cells (10 mL) were grown to an OD_{600} of ~0.8-1.0. Cells were harvested, washed with sterile water, and resuspended in 200 µL lysis buffer (25 mM Tris Phosphate, pH 6.7, 2 mM PMSF). Whole cell extracts were prepared by vigorous bead beating. Cellular debris was removed by spinning the extracts at 3000 rpm at 4°C for 15 minutes. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equal amount of whole cell extracts were separated on 7.5-10% SDS-PAGE and transferred to a nitrocellulose membrane (80 V, 1-1.5 hours). The following antibodies were used at the given dilutions: anti-HA (12CA5, from Covance Inc; 1:1000), anti-myc (Upstate Inc., 1:500), polyclonal anti-Toa1 or anti-TBP (1:10,000). Horseradish peroxidase (HRP)-conjugated secondary antibodies were used at a 1:20,000 dilution and protein bands detected using ECL Plus reagents from Amersham Biosciences.

2.5 PHENOTYPIC ASSAYS:

For phenotypic analysis, yeast cultures were grown overnight in YPD. The next morning, cultures were diluted and allowed to undergo two cell doublings to an OD_{600} of 0.7-0.9. Cells were collected and diluted in water to an OD_{600} of 0.1. 10-fold serial dilutions were plated to the indicated condition and plates were incubated at 30^{o}C for 2-5 days before photographing.

2.6 PLASMID-BASED TBP TETHERING SCREEN:

Yeast cells were transformed with plasmids using standard procedures[124]. Cells were first transformed with the LexAop*HIS3* plasmid. *LEU2*$^{+}$ cells were then transformed with the LexA and LexA-TBP fusion constructs. Strains were streaked or spotted in serial dilutions onto SC-UL and SC-ULH plates containing 20-40 mM AT, based on cell growth. Cell growth was scored as ranging from "+/-" to "+++", with "+/-" indicating little or no growth and "+++" indicating robust growth.

To assay reporter gene expression, SC-based plates lacking uracil, leucine, and histidine were supplemented with 3-aminotriazol (AT). The reporter plasmid was created by first amplifying the *HIS3* gene from the SK1 strain[125], which has the Gcn4 binding sites replaced by the LexA operator. The amplified product was subcloned into the YCp111 plasmid (*LEU2*, CEN). LexA and LexA fused TBP derivatives cloned into pRS316 (*URA3*, CEN) were obtained from previous studies[126]. Both LexA plasmids have an HA epitope in front of the LexA-protein fusion sequence.

2.7 RNA ABUNDANCE:

S1 nuclease assays were conducted as described[127]. Briefly, yeast cells were harvested and total RNA was extracted by the hot-phenol extraction method. 30 µg of total RNA was hybridized with excess ^{32}P labeled probe in a 55^{o}C water bath overnight. S1 nuclease (Promega) digestion was performed on hybridized samples for 25-30 minutes at 37^{o}C. Reactions were stopped and precipitated with EDTA, ssDNA, and linear polyacrylamide. Digested samples were separated on a 10% sequencing gel (run with 1X TBE, 18 mAmps for 1-2 hours). Gels were dried at 80^{o}C for 1 hour. The probe was visualized by PhosphorImager, and band intensity normalized to the intensity of the tRNA^{w} band.

TABLE 2.2. Sequences of oligonucleotides used for S1 RNA analysis in this work.

MESSAGE DETECTED	PRIMER NAME	SEQUENCE
CYC1	*STA 297*	5’ GTA GCA CCT TTC TTA GCA GAA CCG GCC TTG AAT TCA GTC ATT ATT AAT TTA GRG TGT GTA TTT GTA CCG TA 3’
FLR1	*STA 497*	5’ GGG GCC AGT TTT GTG GGT TCT CAG GAT CAC TGG GGC CGT TCC AAT CCA CCC TGA AAG GAT CTA AAA A 3’
TRX2	*STA 520*	5' GGC ACC GAC GAC TCT GGT AAC CTC CTT ACC GCC CTT GTA GAA GAT TAG GGT AGG CAT GGA AGA AAC AAG TCG 3'
GTT2	*STA 528*	5' CCT CAC AAA TTG CAC ACT TGA TAG CAT GTT CTT CTC AGC CAA GGC AAT GCG GAC TCG GGC CGG ATA TCC GGG 3'
GLR1	*STA 557*	5'- GCG GAA GCA ACA CCC CCT GAG CCA CCC CCG ATG ACG AGG TAA TCG TAA TGC AAC CAC-3'
AIM13	*STA 560*	5'- GCG GAG TAT AGA CTT GTT GTT TTT CTG CAC CAG CAC CCA CTT TGG AAG TGT AAC TTG -3
GAL1	*STA 531*	5’ CGG CCA ATG GTC TTG GTA ATT CCT TTG CGC TAG AAT TGA ACT CAG GTA CAA TCT GAA GA 3’
GAL10	*STA 535*	5’ CAG CAA AGT GAA TTA CCG AAT CAA TTT TAT ATT CTT TGA AAA CCT TTT CCA GAC CTT TTC GGT CAC ACA AAT CAA CCA GTA TC 3’
tRNA	*STA 303*	5’ GGA ATT TCC AAG ATT TAA TTG GAG TCG AAA GCT CGC CTT A 3’

2.8 CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS:

Cultures were induced as described above (section **2.3**). When cells reached an OD_{600} of 0.8-1.0 cultures were cross-linked with 1% formaldehyde for 15 minutes. Glycine was added to a concentration of 125 mM to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were then resuspended in FA-lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 1x Protease Inhibitor Cocktail: PMSF, benzamidine, pepstatin, leupeptin, and chymostatin). Chromatin was sheared by sonication using a Branson W-350 model sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). Ten percent of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. Chromatin material (500 µL) was incubated with 10 µL of anti-TBP, anti-RNAPII (8WG16, Covance Inc.), anti-HA (Santa Cruz) or anti-Myc (Upstate) antibodies, rotating overnight at 4^{o}C. 50 µL protein-A sepharose beads (Pharmacia-prepared as slurry) were incubated with the chromatin material for 3 hours at room temperature, spinning on a rotator. The beads were collected by centrifugation and the antigen-antibody complexes recovered and treated with elution buffer (50 mM Tris, 10 mM EDTA, 1% SDS) for 15 minutes at 65^{o}C to elute the complexes. Protein-DNA cross-links were reversed by incubation overnight at 65^{o}C and the DNA was purified by phenol-chloroform extraction. DNA was resuspended in 50-750 µL of ddH_2O and used for linear PCR analysis or quantitative PCR analysis. DNA was stored at -80^{o}C.

2.9 LINEAR PCR ANALYSIS:

Linear PCR reactions were carried out in a volume of 25 µL. Each reaction contained 1 µL of 1/100 dilution of ^{32}P labeled-ATP. Different dilutions of each input and

immunoprecipitated material were used to determine the linear range of the PCR reaction. Samples were analyzed on a 5% native polyacrylamide gel in 0.5X TBE buffer. The gels were dried and exposed to a PhosphorImager screen. The image was scanned on a STORM and quantified using ImageQuant software to detect the signal intensities. Samples with no antibody were used as controls. The ratio between the precipitated sample and the input, minus background of no antibody control, was used as an indication of the protein occupancy.

For linear PCR analysis of the occupancy of the LexA derivative, primers were designed to encompass the engineered *HIS3* reporter promoter region and amplified a product of 646 bp. Primers were designed to the promoter region of the *CYC1* gene (-230 to +80).

2.10 QUANTITATIVE PCR ANALYSIS:

Quantitative PCR reactions were carried out in a volume of 25 µL using a BioRad iCycler and ABsolute SYBR fluorescein mix (ThermoScientific). Standard curves were generated using 10-fold serial dilutions of input DNA and were run with each PCR reaction. PCR efficiencies ranged from 85-100%, with a correlation coefficient of 0.95 or greater. Threshold cycle data were quantified relative to the input, as described[128].

Occupancy at a negative control region was subtracted from the occupancy of the region of interest in each case. A region proximal to the telomere on the right arm of Chromosome VI or the *GAL10* promoter was used as the negative control, as indicated in the figure legends.

The PCR reaction consists of a 10 second hold at 95^{o}C (to melt the DNA), followed by 30 seconds at a lower temperature to anneal the DNA, then 30 seconds at

73°C for polymerase extension. This cycle is repeated 50 times. The annealing temperature varies based on the primer set and is listed in **TABLE 2.3**. The optimal temperature for each primer set was empirically determined. The final primer concentration in each reaction was empirically determined and ranges from 70-200 nM (**TABLE 2.3**).

TABLE 2.3. Oligonucleotide sequences for linear and quantitative PCR. Sequence for the reverse complement is shown for the reverse primer.

AMPLICON NAME	PRIMER NAME	SEQUENCE	REACTION CONDITIONS
CYC1 core promoter	*STA 487 (F)*	5' CAT ATG GCA TGC ATG TGC TCT GT 3'	70 nM[a], 53°C[b]
	STA 434 (R)	5' ACC TTT CTT AGC AGA ACC GGC C 3'	
CYC1 promoter	*STA 446 (F)*	5' AGG CGT GTA TAT ATA GCG TGG AT 3'	70 nM, 52°C
	STA 445 (R)	5' CCA CGG TGT GGC ATT GTA GAC AT 3'	
CYC1 UAS	*STA 568 (F)*	5' ATC TAA AAT TCC CGG GAG CA 3'	200 nM, 56°C
	STA 569 (R)	5' CTT GAT CCA CCA ACC AAC G 3'	
FLR1 promoter	*STA 362 (F)*	5' CAG TGC GAA AAG GGA CAT GAT AG 3'	100 nM, 61.4°C
	STA 363 (R)	5' CTT CAC GGG CAC TCT GTA AAG 3'	
TRX2 promoter	*STA 540 (F)*	5'- CAC ACA TAC ACG AGA GTC TAC GA -3'	100 nM, 61.4°C
	STA 494 (R)	5'- CAA CAA CGA CTA ACT TGT CGC C -3'	
GTT2 promoter	*STA 561 (F)*	5'- CTT CTA CTA CCG TGT GCA AAA CAG GG -3'	70 nM, 58°C
	STA 358 (R)	5'- AAG GCA ATG CGG ACT CGG GC -3'	
GLR1 promoter	*STA 533 (F)*	5'- CTC ATG CGC TTC TCA CTC TCA G -3'	70 nM, 50.5°C
	STA 534 (R)	5'- GAC GAG GTA ATC GTA ATG CTT G -3'	
RPL11a promoter	*STA 529 (F)*	5' TCA CAT CCA CGT GAC CAG TT 3'	200 nM, 51°C
	STA 530 (R)	5' AAC TTT CGC ATA GCT GAG TGG 3'	
AIM13 promoter	*STA 562 (F)*	5'- CTA CGA ATA TTC GTG GTA TGT CGC -3'	100 nM, 49.7°C
	STA 563 (R)	5'- GAC TCT GTA TTA GTC GAT ATA CCA CC -3'	
GAL10 promoter	*STA 373 (F)*	5' GGG GCT CTT TAC ATT TCC ACA 3'	200 nM, 52°C
	STA 372 (R)	5' CGG AAT TCG ACA GGT TAT CAG CAA CA 3'	
Region proximal to telomere, Chr. VI	*STA 555 (F)*	5' CGT AAC AAA GCC ATA ATG CC 3'	100 nM, 55°C
	STA 556 (R)	5' CAG AAA GTA GTC CAG CCG 3'	

[a]Concentration of each primer used in the PCR reactions

[b]Annealing temperature used in PCR reaction

CHAPTER 3. ACTIVATION OF A POISED RNAPII-DEPENDENT PROMOTER REQUIRES BOTH SAGA AND MEDIATOR

This chapter is published in the **March 2010** issue of ***GENETICS***. It is listed as reference number 48 in this dissertation. The literature citation for this work is as follows:

Lee, S.K., Fletcher, A.G.L., Zhang, L., Chen, X., Fischbeck, J.A., and Stargell, L.A. Genetics. March 2010. 184(3):659-72.

This chapter is the result of collaboration with several members of the Stargell laboratory. I wrote the manuscript based on my findings (shown in **FIGURES 3.5**, **3.6B**, **3.10**, and **3.11**), and the work of Aaron Fletcher, Lei Zhang, Xu Chen, and Julie Fischbeck. Aaron collected the majority of the data found in **TABLE 3.1**, Lei, Julie and I participated in this aspect of the project as well. Lei Zhang also contributed to **FIGURES 3.2**, **3.3**, **3.6A**, and **3.8**. Aaron Fletcher contributed to **FIGURES 3.7** and **3.8**. Xu Chen collected the data in **FIGURES 3.4** and **3.9**. I formatted the text and all figures. We would like to thank Shelley Berger for providing the *GCN5* histone acetyltransferase mutant derivative, $gcn5^{E173Q}$, Alan Hinnebusch for providing the Med15-myc tagged strain in the *spt20*Δ and *gcn5*Δ backgrounds, and Carlos Herrera for his participation in the tethering screen.

3.1 ABSTRACT

A growing number of promoters have key components of the transcription machinery, like TATA-Binding Protein (TBP) and RNA polymerase II (RNAPII), present at the promoter prior to activation of transcription. Thus, while transcriptional output undergoes a dramatic increase between uninduced and induced conditions, occupancy of a large portion of the transcription machinery does not. As such, activation of these poised promoters depends on rate-limiting steps after recruitment of TBP and RNAPII for regulated expression. Little is known about the transcription components required in these latter steps of transcription in vivo. To identify components with critical roles in transcription after recruitment of TBP in *Saccharomyces cerevisiae*, we screened for loss of gene expression activity from promoter-tethered TBP in over 100 mutant strains deleted for a transcription-related gene. The assay revealed a dramatic enrichment for strains containing deletions in genes encoding subunits of the SAGA complex and Mediator. Analysis of an authentic postrecruitment regulated gene (*CYC1*) reveals that SAGA occupies the promoter under both uninduced and induced conditions. In contrast, Mediator is recruited only after transfer to inducing conditions, and correlates with activation of the preloaded polymerase at *CYC1*. These studies indicate critical functions of SAGA and Mediator in the mechanism of activation of genes with rate-limiting steps after recruitment of TBP.

3.2 INTRODUCTION

The regulation of gene expression by RNA polymerase II (RNAPII) is a fundamental and highly complex process. Transcription by RNAPII involves a number of steps including the recruitment of a pre-initiation complex to the promoter, promoter melting, initiation of transcription, promoter clearance, elongation, and termination (for

review see[129]). An assortment of factors is required for these events to take place efficiently and accurately. Initiation of transcription is dependent upon RNAPII, and the general transcription factors (GTFs), TFIID (comprised of the TATA-binding protein (TBP) and TBP-associated factors or TAFs), TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH[3], which together form the pre-initiation complex (PIC). For a large number of well-characterized promoters the rate-limiting step in the transcription process is the formation of the PIC at the promoter. For these genes, the recruitment and occupancy of TBP and RNAPII to the promoter correlates strongly with transcriptional output[50-52]. Indeed, artificially tethering TBP or RNAPII to a promoter is sufficient for gene activation in many contexts[52,130,131]. Despite this, an increasing number of promoters are regulated after recruitment of the PIC (for reviews see[132-134]). These preloaded, yet transcriptionally inactive, promoters can be defined as poised for subsequent activation. Poised promoters are found across the evolutionary spectrum, including bacteria, yeast, *Drosophila*, and humans[50-53,55-57]. Indeed, whole genome studies suggest that transcription of a significant part of the human genome may be regulated at rate-limiting steps after recruitment of the PIC[51,59]. Importantly, the transcription factors involved in this mechanism of regulation in vivo are currently poorly defined.

To discover transcription factors with roles in rate-limiting steps after formation of the PIC, we took advantage of the fact that tethering TBP to a reporter promoter in a wild-type strain results in robust gene expression[126,135,136]. We used this plasmid-based system to screen mutant strains in search of those that are unable to activate the reporter gene, which would suggest involvement of the gene product in essential steps in transcription after TBP recruitment. We initially analyzed 10 *SPT* (Suppressor of Ty) yeast deletion strains in the screen since this family of genes encodes proteins intimately involved in various transcription-related processes. In fact, TBP itself is encoded by the

essential *SPT15* gene. Products of the yeast *SPT* gene family are implicated in various processes such as transcription initiation, elongation and RNA processing, and maintaining chromatin structure[89-92]. The Spt1, Spt10 and Spt21 proteins are the regulatory factors that control the expression levels of histone genes[137-140]. *SPT23* encodes an activator protein involved in transcription of genes involved in lipid biosynthesis[141]. *SPT2* and *SPT4* encode transcription elongation factors[142,143]. Finally, several *SPT* genes are subunits of the SAGA (Spt-Ada-Gcn5-acetyltransferase) coactivator complex including Spt3, Spt7, Spt8, and Spt20[72,144].

Using the TBP-tethering approach, we identified several subunits of SAGA and Mediator with potential postrecruitment functions. These results were corroborated with studies of the authentic poised promoter at the *CYC1* gene. Timing of SAGA and Mediator occupancy at *CYC1*, and the lack of interdependency of the two coactivator complexes, indicates distinct functional roles for each complex in activating the poised promoter. Our results underscore the versatility of SAGA and Mediator in mechanisms of gene regulation, since both complexes also have well-established roles in the regulation of recruitment-regulated genes.

3.3 RESULTS

3.3.1 Classification of mutant strains in a TBP tethering assay suggests roles in postrecruitment functions: We used a TBP tethering assay to identify non-essential *SPT* gene family members with potential functions in rate-limiting steps after TBP recruitment. The assay consists of two plasmids: a *HIS3* reporter plasmid with the *HIS3* promoter replaced by a LexA operator, and a plasmid expressing either the LexA DNA binding domain or LexA fused to TBP (**FIGURE 3.1**). This fusion results in binding of LexA-TBP to the promoter, which drives *HIS3* expression[135]. Reporter gene expression

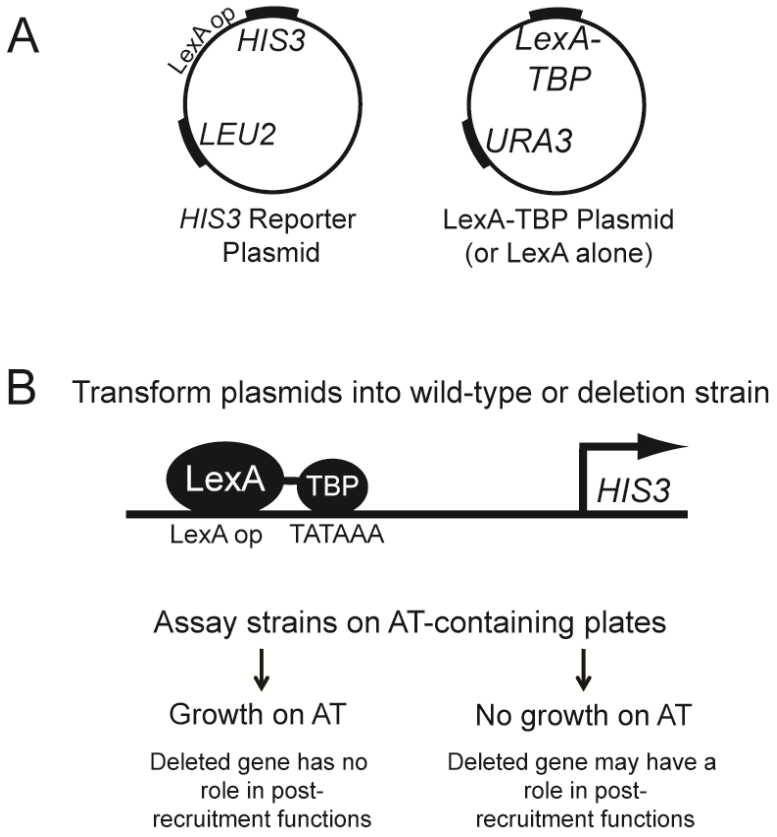

FIGURE 3.1. Schematic of the tethering assay. A) The two plasmid system for the tethering assay. The *LEU2* marked plasmid contains the LexAoperator-*HIS3* reporter. The *URA3* marked plasmid contains either LexA-TBP, or LexA alone. **B)** The wild-type strain or a strain with a deletion in one non-essential gene is transformed with the *HIS3* reporter plasmid and the LexA-TBP or LexA alone expressing plasmid. LexA-TBP binds to the LexA operator in the *HIS3* reporter plasmid, and results in TBP tethering. *HIS3* gene expression is assayed by monitoring cell growth on media containing a competitive inhibitor of the *HIS3* gene product, 3-aminotriazole (AT).

assayed by cell growth on plates containing 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. Growth properties on AT correlate very well with quantitative measurements of *HIS3* RNA[145]. In wild-type cells expressing LexA-TBP, growth on plates containing AT is robust, whereas LexA alone shows little growth (**FIGURE 3.2**). To assay the postrecruitment functions of the *SPT* gene family members, the reporter system was transformed into a variety of strains, each with a deletion of one non-essential *SPT* gene. If the *SPT* deletion strains are defective for TBP recruitment, artificially recruiting TBP in the tethering assay will correct these defects and growth on AT will be similar to the wild-type strain. However, if the *SPT* deletion strains are defective for functions after TBP recruitment, these defects will not be corrected and growth on AT will be poor. Therefore, the behavior of the deletion strain reflects the involvement of the wild-type protein in regulation of transcription after recruitment of TBP.

A majority of strains (*spt1*Δ, *spt2*Δ, *spt3*Δ, *spt4*Δ, *spt8*Δ, *spt10*Δ, *spt21*Δ, and *spt23*Δ) transformed with the two plasmids grew similar to the wild-type strain on plates containing 20 to 40 mM AT (**TABLE 3.1 and FIGURE 3.2**). Thus, the proteins expressed by these *SPT* genes are unlikely to play critical functions after TBP associates with the promoter. In contrast, strains containing deletions of *SPT7* and *SPT20* grew poorly on plates containing AT (**TABLE 3.1 and FIGURE 3.2**). Loss of reporter gene expression in strains lacking *SPT7* and *SPT20* suggests these genes have a post-TBP recruitment role in transcription, but could also be due to less interesting indirect effects. For example, poor reporter expression could be due to low expression of the LexA-TBP fusion protein, since low levels of LexA-TBP would prevent the formation of the PIC on the reporter gene and result in no growth on AT. To test this, levels of LexA-TBP protein were assayed via immunoblot analysis. Expression levels of LexA-TBP were

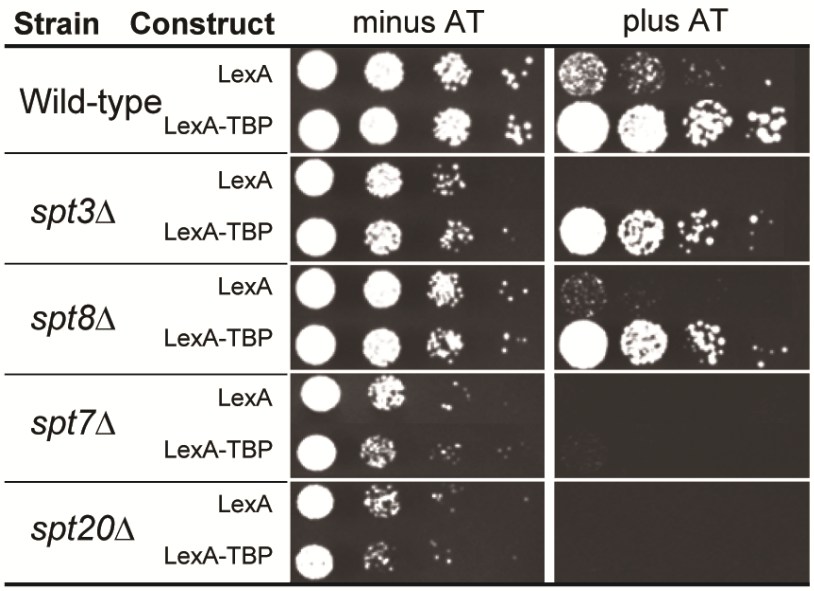

FIGURE 3.2. The *spt7*Δ and *spt20*Δ strains are compromised for function in the tethering system. The wild-type strain and representative *SPT* gene deletion strains (as indicated) were transformed with the tethering plasmids and monitored for growth. Serial dilutions of each strain were spotted on media with or without AT, and incubated for 3 days.

comparable in all strains tested (**FIGURE 3.3A**). Another indirect explanation for failure to grow on AT is that *SPT7* or *SPT20* are required for LexA-TBP protein occupancy at the reporter promoter. To test this, we used a chromatin immunoprecipitation (ChIP) assay to measure the occupancy of LexA-TBP at the *HIS3* reporter promoter. We found LexA-TBP was recruited to the *HIS3* reporter gene promoter to comparable levels in the wild-type strain and the *spt7*Δ and the *spt20*Δ strains (**FIGURE 3.3B**). These results indicate LexA-TBP is expressed and recruited to the promoter, but this is not sufficient for reporter gene expression in the absence of the gene products encoded by *SPT7* and *SPT20.* This suggests that these two gene products are involved in regulatory steps after the recruitment of TBP.

3.3.2 Proper regulation of the poised *CYC1* promoter requires the function of *SPT7* and *SPT20*: We next compared the results from the tethering assay to transcription of an authentic postrecruitment regulated promoter. *CYC1* is regulated after the recruitment of TBP and RNAPII[52,53,146-148]. Therefore, RNAPII occupies the promoter to a similar degree under both uninduced and induced conditions (**FIGURE 3.4A**). This is despite a dramatic change in transcript levels during induction (**FIGURE 3.4B**). This preloading of key members of the transcription machinery at the promoter of *CYC1* is fundamentally different from recruitment-regulated genes such as *GAL1*. Occupancy of RNAPII at the *GAL1* promoter undergoes a large change (10-fold) upon transcriptional activation (**FIGURE 3.4A and B, respectively**). *CYC1* is therefore regulated in a postrecruitment fashion. We refer to *CYC1* as having a poised promoter, as preloaded TBP and RNAPII mark the promoter for future activation.

To examine the correlation between the tethering assay and regulation of the poised *CYC1* promoter, we tested whether *SPT* genes were required for *CYC1* expression. Transcript levels of *CYC1* were measured using RNA harvested from wild-

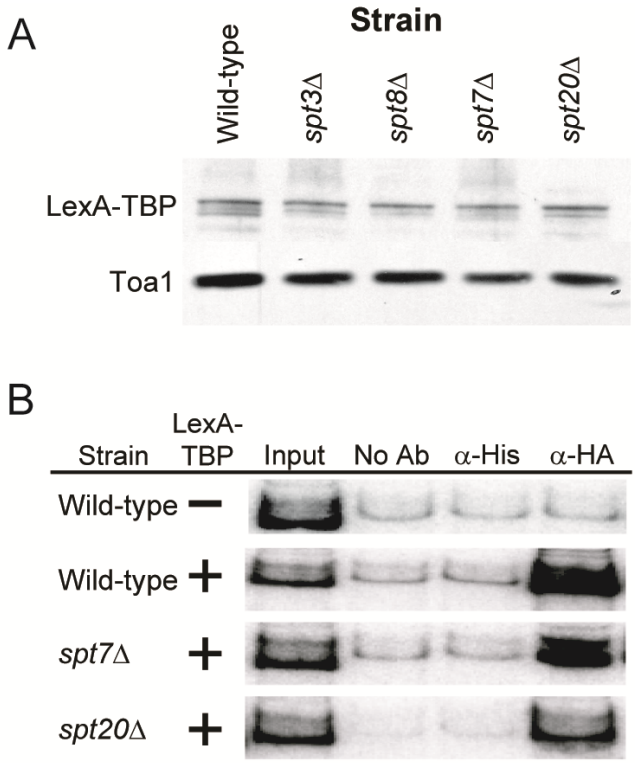

FIGURE 3.3. LexA-TBP is expressed in various *SPT* gene deletion strains and is recruited to the promoter of the *HIS3* reporter plasmid. A) Expression levels of LexA-TBP protein are similar in *SPT* deletion strains to the wild-type parent strain. Protein extracts from the indicated strains expressing LexA-TBP were separated on an SDS-PAGE gel and subjected to western blot analysis. Levels of LexA-TBP were detected via anti-HA antibody against the HA tag on the N-terminus of the fusion protein. Anti-Toa1 antibody was used to detect Toa1 levels for a loading control. **B)** LexA-TBP is recruited to the reporter *HIS3* gene in the deletion strains. Chromatin immunoprecipitation (ChIP) assays using anti-HA antibody from strains expressing LexA-TBP were performed to determine the occupancy of LexA-TBP on the *HIS3* promoter. Antibody to an irrelevant His-tag was used as a control. ChIP assays were repeated a minimum of three times using independent cultures of cells.

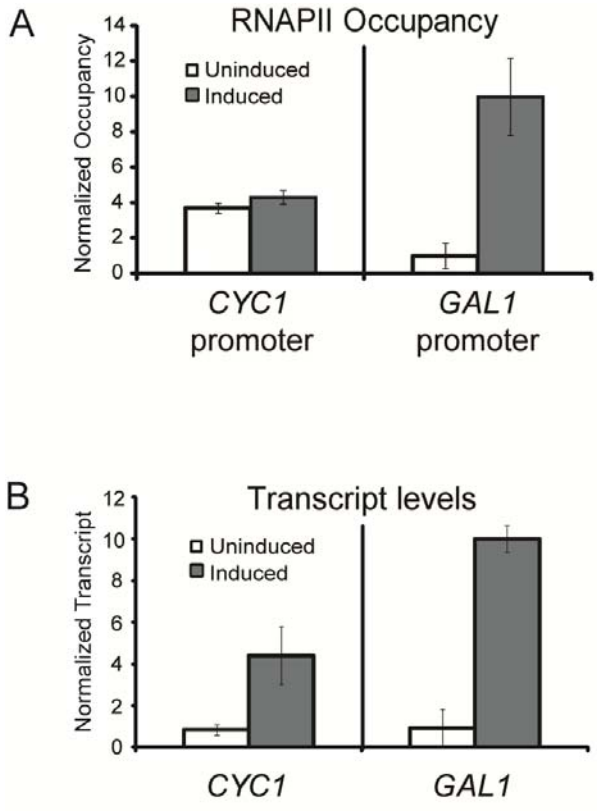

FIGURE 3.4. *CYC1* and *GAL1* represent two different classes of gene regulation. A) Chromatin immunoprecipitation for RNAPII during uninduced (glucose; unfilled bars) and induced (galactose; filled bars) conditions at the *CYC1* and *GAL1* promoter regions. RNAPII occupies the *CYC1* promoter in both conditions, but is recruited to the *GAL1* promoter during the same conditions. **B)** *CYC1* and *GAL1* transcript levels are induced during growth in medium containing galactose as a carbon source. Total RNA from the wild-type strain grown in glucose (uninduced; unfilled bars) or in galactose (induced; filled bars) was analyzed via S1 nuclease assay using ^{32}P labeled *CYC1, GAL1* and tryptophan tRNA probes. The $tRNA^{w}$ signal was used as a loading control and to normalize transcript levels. In both panels, the mean ± SD of three separate biological samples is shown.

type and *SPT* deletion strains and S1 nuclease protection assays. *CYC1* transcript levels in the uninduced condition were not significantly changed upon deletion of any of the *SPT* genes (**FIGURE 3.5**). However, during induction activated transcription from *CYC1* was dramatically abolished in strains deleted for *SPT7* and *SPT20*. Thus, *SPT7* and *SPT20* are specifically required for activation of the poised *CYC1* promoter. Significantly, these are the two *SPT* strains that were also identified in the tethering assay. *SPT7* and *SPT20* both encode subunits of the yeast SAGA (Spt-Ada-Gcn5-acetyltransferase) complex[72,144]. As these subunits are both required for the structural integrity of the complex[72,99], we next focused on SAGA.

3.3.3 *ADA1* and *GCN5* are also critical for postrecruitment regulation: SAGA is a highly conserved, multiple subunit coactivator complex comprised of Spt proteins, TAFs, Ada proteins, and the histone acetyltransferase enzyme Gcn5 (for reviews see[149,150]). SAGA also links other histone modifications with transcriptional processes: histone H3 methylation via Chd1[151]; and H2B deubiquination via Sgf11[83]. Thus, we expanded our screen to include additional SAGA subunits (**TABLE 3.1**). A majority of strains grew similarly to the wild-type strain on plates containing AT. In contrast, the *ada1*Δ and the *gcn5*Δ deletion strains showed poor growth on AT (**FIGURE 3.6A**). We next tested the consequence of these deletions on *CYC1* expression levels. Transcript levels in the *ada1*Δ and the *gcn5*Δ strains were similar to wild-type levels in the uninduced condition, but were compromised during induction. Deletion of *ADA2* or *ADA3* had little influence on *CYC1* transcript levels in the uninduced condition; during activation there was a slight decrease in the *ada2*Δ strain, and no significant effect in the *ada3*Δ strain (**FIGURE** 3.**6B**). Additionally, we found no significant effect upon deletion of *UBP8* and *SGF11* (data not shown), which provide the deubiquination activity of the SAGA complex[82-84]. Thus,

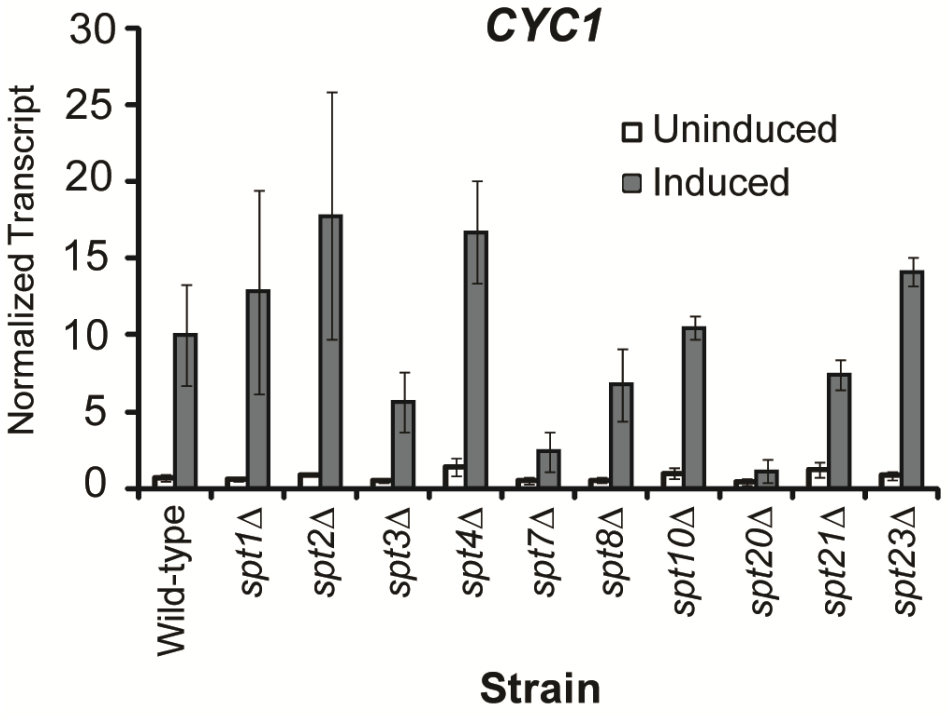

FIGURE 3.5. The *spt7*Δ and *spt20*Δ strains are defective for *CYC1* expression. Total RNA from indicated strains grown in glucose (uninduced; unfilled bars) and in ethanol (induced; filled bars) were analyzed via S1 nuclease assay using ^{32}P labeled *CYC1* and tryptophan tRNA probes. The tRNA^{w} signal was used as a loading control and to normalize transcript levels. In both panels, the mean ± SD of three separate biological samples is shown.

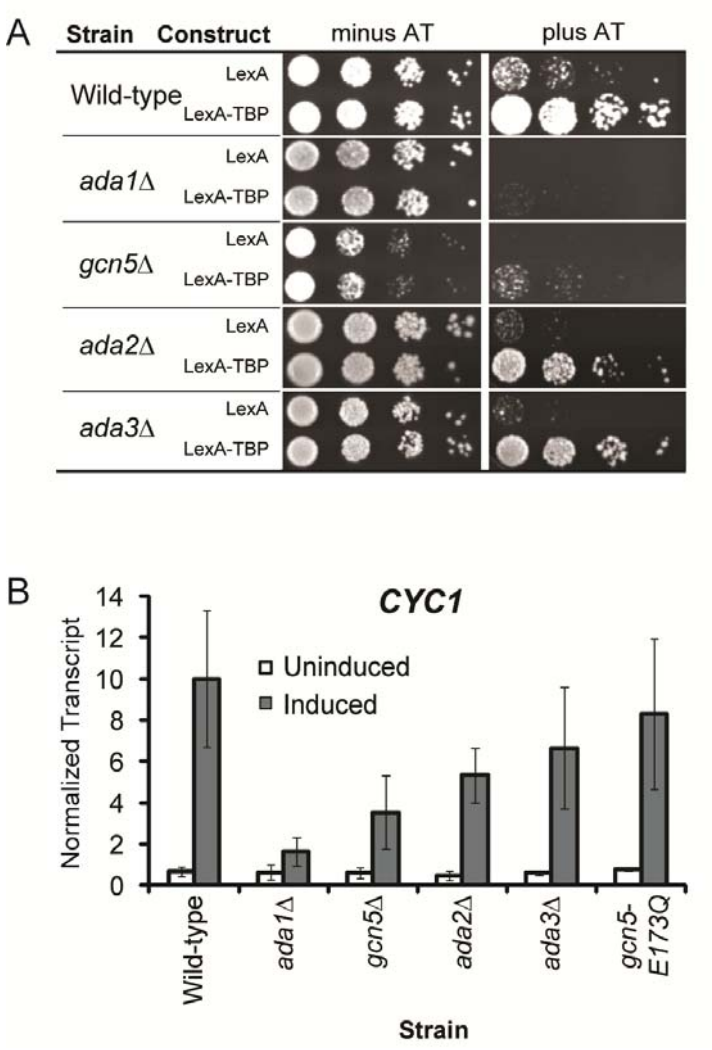

FIGURE 3.6. Additional SAGA subunits have postrecruitment functions. A) The wild-type strain and strains deleted for individual genes encoding representative subunits of the SAGA complex were assayed using the tethering system. Serial dilutions were spotted on media with and without AT, and incubated for 3 days. **B)** *CYC1* expression levels in the indicated strains during growth in glucose (uninduced; unfilled bars) and ethanol (induced; filled bars) were measured by S1 nuclease protection. Mean ±SD of 3 separate biological replicates (independent cultures) is shown.

four subunits of SAGA are critical for the postrecruitment regulation of *CYC1*, and three of those (Spt7, Spt20, and Ada1), are involved in the integrity of the complex[72,99].

3.3.4 The histone acetyltransferase activity of SAGA is not required for proper regulation of *CYC1:* Gcn5 is a histone acetyltransferase (HAT), which is an enzyme that transfers acetyl groups to histones[71-74]. To determine if the HAT activity of SAGA is important in the postrecruitment regulation of *CYC1*, we utilized a strain containing a Gcn5 derivative defective for histone acetylation[122], *gcn5*^{E173Q}. We found no change in *CYC1* expression in a strain with this mutant protein as compared to wild-type *GCN5* (**FIGURE 3.6B**). Thus, the HAT activity of the SAGA complex is not important for the activation of the preloaded complex on *CYC1*. Consistent with this finding, the deletion of the HAT-related SAGA subunits, Ada2 and Ada3, which together with Gcn5 comprise the catalytic core[152], has little effect on *CYC1* expression (**FIGURE 3.6B**).

3.3.5 *CYC1* is SAGA-dependent and TFIID-independent: To test for a direct role of SAGA in *CYC1* transcription, we performed chromatin immunoprecipitation assays to determine the occupancy of tagged derivatives of the Spt20, Gcn5 and Spt8 proteins both before and after activation of transcription. Importantly, the Spt8 subunit is present only in the SAGA complex, and not the related SLIK complex, unlike Spt20 and Gcn5[153]. Interestingly, Spt20, Gcn5 and Spt8 occupy the *CYC1* promoter in *both* the uninduced and induced state. Changes in occupancy do occur, however, with a drop in occupancy observed for each of the three subunits after activation of transcription (**FIGURE 3.7**). SAGA-dependent genes are largely TFIID-independent[154]. To determine if *CYC1* is TFIID-independent we examined the occupancy of Taf1, a TFIID-specific TAF[73]. We found occupancy of Taf1 was not greater than background, either before or after

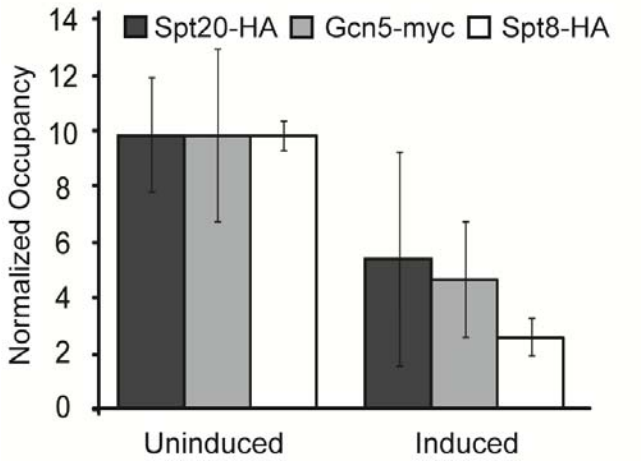

FIGURE 3.7. SAGA occupies the *CYC1* promoter. The indicated strains were grown under uninduced (glucose) or induced (ethanol) conditions. ChIP analyses was performed to measure the occupancy of Spt20-HA, Gcn5-myc, or Spt8-HA at the *CYC1* promoter region (-230 to +80 relative to the ATG). Normalized occupancy was calculated by determining the tagged derivative occupancy and dividing it by the occupancy observed in an untagged strain and setting the highest value for each strain to 10. The mean ± SD of 3 separate biological samples is shown. While the normalized occupancy value of Spt8-HA appears low in induced conditions, the IP value was higher than what is observed at an induced (galactose-grown) SAGA-dependent *GAL* promoter, suggesting this protein still occupies the *CYC1* promoter in induced conditions (data not shown).

activation (data not shown). These results are also consistent with the fact that *CYC1* is a TATA-containing gene, which are typically SAGA-dependent[154,155].

3.3.6 TBP recruitment function of SAGA is not required for *CYC1* regulation: Besides histone acetyltransferase activity, SAGA also has a well-characterized role in TBP delivery at recruitment-regulated promoters[93-98]. SAGA binds TBP[99] and transfers it to the TATA box[156]. A defining feature of the poised *CYC1* promoter is that it has TBP bound at the promoter in the uninduced state prior to activated levels of transcription[52,146-148]. The results above indicate that SAGA also occupies this poised promoter. To test whether loss of SAGA results in loss of TBP occupancy at the *CYC1* promoter, we performed chromatin immunoprecipitation assays for TBP occupancy in several deletion backgrounds (*spt7*Δ, *spt20*Δ, *gcn5*Δ, and *ada1*Δ) of SAGA. In all four deletion strains, TBP occupied the *CYC1* promoter similar to that of a wild-type strain in both the uninduced and induced condition (**FIGURE 3.8A**). Thus, the TBP recruitment function of SAGA is not involved in the regulation of *CYC1*. This is consistent with the observation that the *spt3*Δ and *spt8*Δ strains were not defective in any of the assays. We also found RNAPII occupies the *CYC1* promoter in the *spt7*Δ, *spt20*Δ, *gcn5*Δ, and *ada1*Δ strains during the uninduced condition to levels comparable to the wild-type strain (**FIGURE 3.8B**). Taken together, these results indicate that loss of SAGA does not impact preloading of TBP and RNAPII at the *CYC1* promoter, therefore, the *CYC1* promoter is still poised in the absence of this coactivator complex. Furthermore, SAGA plays a rate-limiting role downstream of the recruitment of both TBP and RNAPII at *CYC1*. This is in striking contrast to the recruitment-regulated *GAL1* gene. Like *CYC1*, *GAL1* is dependent upon SAGA for normal expression (**FIGURE 3.9A**). However, at *GAL1*, *SPT20* functions in TBP and RNAPII recruitment, as occupancy of these members of the transcription machinery are compromised in the *spt20Δ* strain (**FIGURE 3.9B**).

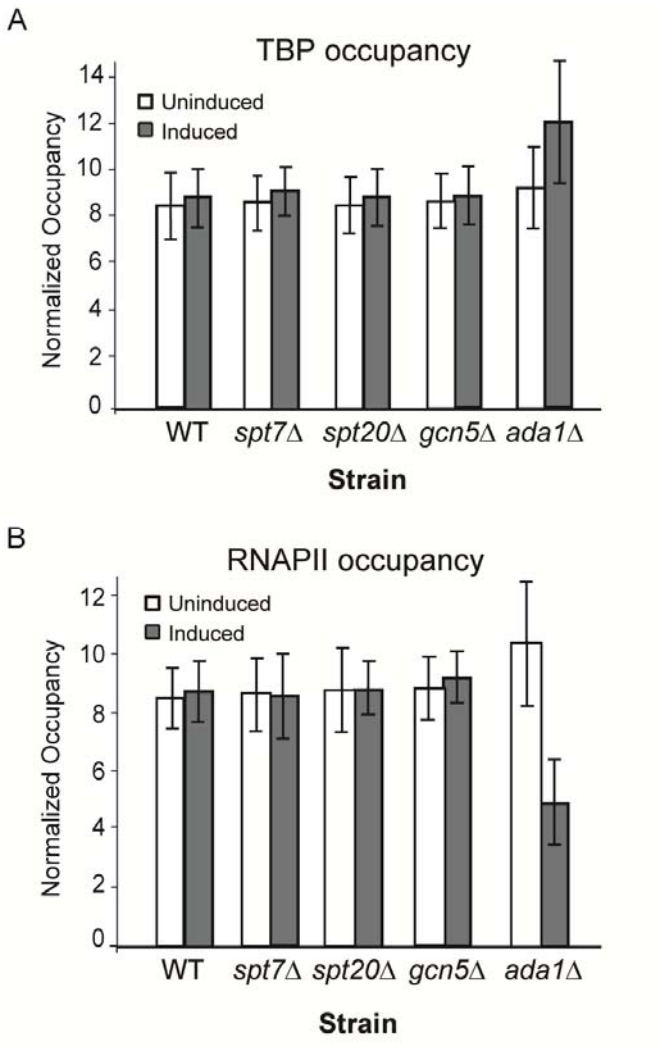

FIGURE 3.8. Occupancy of TBP and RNAPII at *CYC1* are unchanged in SAGA deletion strains compromised for activated transcription. **A)** The indicated strains were grown under uninduced (glucose; unfilled bars) or induced (ethanol; filled bars) conditions for the *CYC1* gene. ChIP analyses was performed to determine the occupancy of TBP at the *CYC1* promoter. The occupancy of TBP during uninduced and induced conditions at *CYC1* does not change in the deletion strains (mean ± SD). **B)** RNAPII occupancy at the *CYC1* promoter in the wild-type (BY4741) strain and strains with deletions in genes encoding SAGA subunits during uninduced (white bars) and induced (black bars) conditions. RNAPII occupancy does not change in the deletion strains (mean ± SD). The ratio between the immunoprecipitated sample and the input, minus background of no antibody control, was used to calculate the protein occupancy.

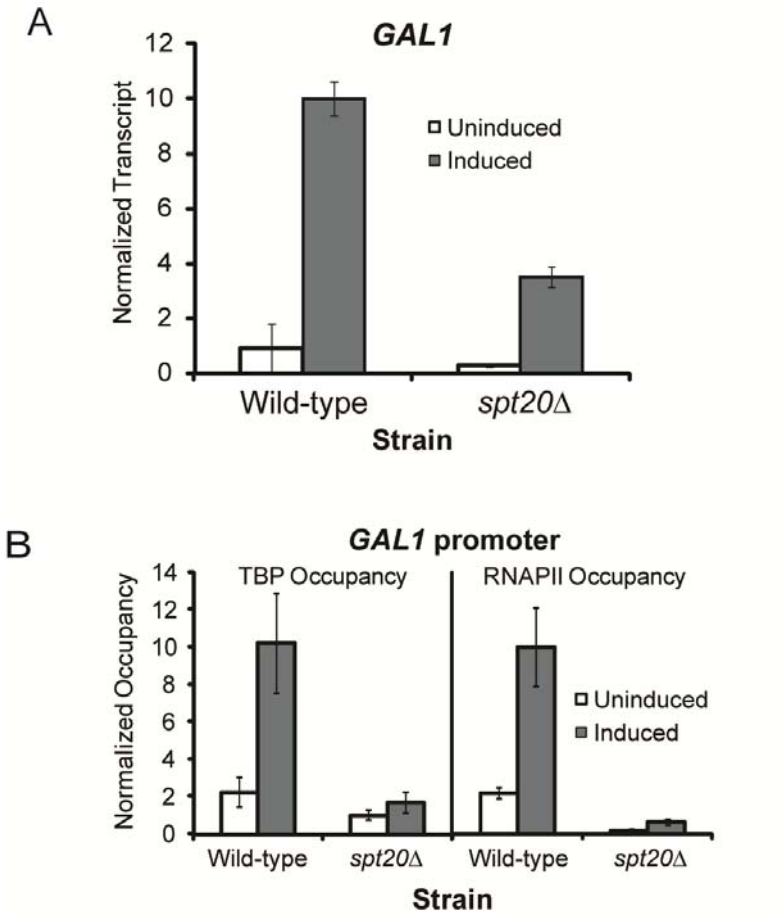

FIGURE 3.9. SAGA is required for TBP and RNAPII recruitment at the recruitment regulated *GAL1* gene. A) *GAL1* is dependent on the *SPT20* gene for normal levels of expression. *GAL1* expression levels in the indicated strains during growth in glucose (uninduced; unfilled bars) and galactose (induced; filled bars) were measured by S1 nuclease protection. Mean ±SD of 3 separate biological replicates (independent cultures) is shown. **B)** *SPT20* is required for TBP and RNAPII occupancy at the *GAL1* promoter. Occupancy of TBP and RNAPII in the indicated strains at the *GAL1* promoter.

3.3.7 Expansion of the tethering assay reveals an enrichment in Mediator subunits: As the tethering assay accurately revealed a postrecruitment function of the SAGA complex at the *CYC1* promoter, we expanded the screen to include other transcription-related factors. Specifically, we examined strains containing deletions of subunits of the Mediator complex, RNA polymerase II, activators, repressors, HDACs, ISW1/2 complexes, elongation factors, Swi/Snf, protein kinases, HAT components, the PAF complex, and others, for a role in postrecruitment regulation. The assay was highly selective, identifying only a few additional genes with putative postrecruitment functions (**TABLE 3.1**). Strikingly, out of 85 additional strains, only two strains (*med18*Δ and *med19* Δ) showed severe phenotypes in the tethering assay. *MED18* and *MED19* encode subunits of Mediator, an important multisubunit coactivator complex with both positive and negative roles in transcription[100,157-159].

3.3.8 Mediator is required for proper expression of *CYC1:* We next examined the role of the Mediator complex at the poised *CYC1* promoter. Mediator subunits can be classified into the head, middle, tail, and CDK8 module of the complex[115]. Under uninduced conditions, *CYC1* transcript levels were mildly diminished in strains containing deletions of the head and middle module subunits, and increases in expression were observed in strains containing deletions of the tail and CDK8 module subunits (**FIGURE 3.10**). Activation of *CYC1* transcription under inducing conditions was compromised in a number of strains, with the most significant effects associated with deletions of head module subunits (**FIGURE 3.10**). In all three strains tested (*med18*Δ*, med19*Δ and *med20*Δ*)* transcription was drastically compromised compared to wild-type. Other modules exhibited more complex patterns with deletions of particular subunits resulting in disparate effects. For example, deletion of the middle module subunit *MED31* abolished *CYC1* activation, whereas deletion of other members of this module

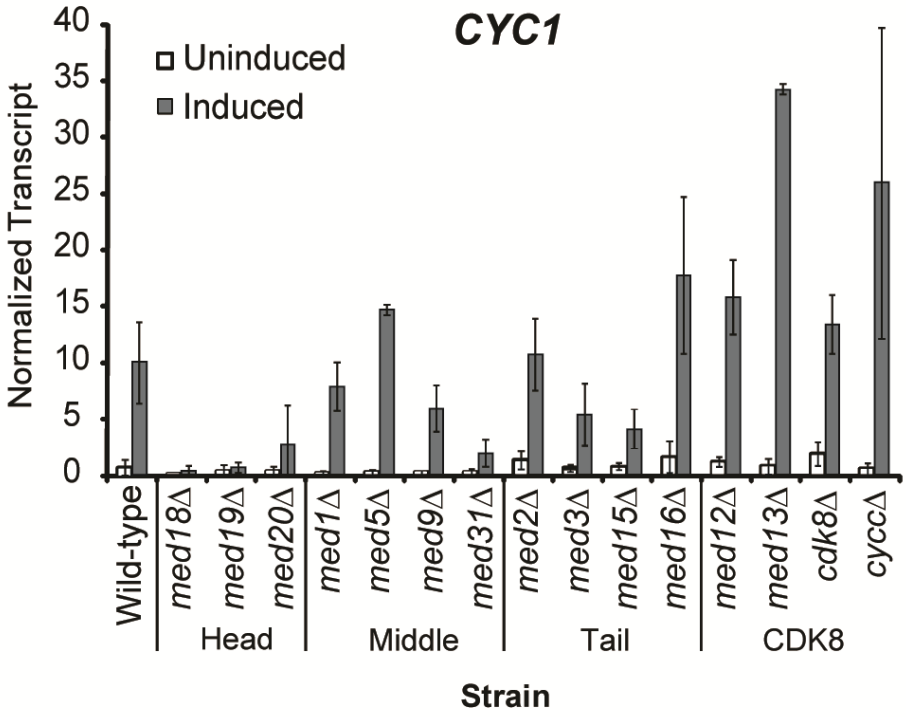

FIGURE 3.10. A functional Mediator complex is required for proper *CYC1* expression. *CYC1* transcript levels during growth in uninduced (glucose; unfilled bars) and induced (ethanol; filled bars) conditions in each strain harboring a deletion in a Mediator subunit. Strains are grouped according to the corresponding Mediator module. Total RNA from the indicated strains was analyzed via S1 nuclease assay using ^{32}P labeled *CYC1* and tryptophan tRNA probes as in **FIGURE 3.5**.

was well-tolerated. Enhanced transcriptional activation was observed in the CDK8 module deletions, which is entirely consistent with a role for this module in the negative regulation of gene expression[102-104]. Taken together, our results demonstrate that a functional Mediator complex is required for proper expression of *CYC1,* with a clear requirement for the head module and the Med31 subunit of the middle module in activation of the poised *CYC1* promoter.

We next determined the occupancy of Mediator at *CYC1* using chromatin immunoprecipitation. The head, middle and tail modules of the complex are distributed in virtually identical patterns across the yeast genome[55], so we chose Med15 to represent this grouping since it has been successfully used in ChIP assays[96,160]. The CDK8 module is proposed to transiently associate with certain genomic locations[161], thus we also determined the occupancy of Med12 to represent this module. We found that both Med15 (**FIGURE 3.11A**) and Med12 (data not shown) exhibited low occupancy under noninducing conditions and high occupancy during growth in inducing conditions. Thus, whereas TBP, RNAPII, and SAGA occupy the poised *CYC1* promoter in the uninduced condition, Mediator has high occupancy only after transfer to inducing conditions. As such, Mediator recruitment correlates with the activation of the preloaded polymerase at the *CYC1* promoter.

3.3.9 SAGA is dispensable for recruitment of Mediator to *CYC1:* As SAGA and Mediator subunits were both over-represented in the tethering screen, and these complexes are mutually dependent at several promoters[96,144,162], we investigated their connection at *CYC1*. We examined Mediator recruitment to the *CYC1* promoter in a strain containing a deletion of the SAGA complex genes *GCN5* or *SPT20.* We found Med15-myc is recruited to the *CYC1* promoter in both the *gcn5*Δ and the *spt20*Δ strains (**FIGURE 3.11A**). Importantly, *SPT20* is absolutely required for the structural integrity of

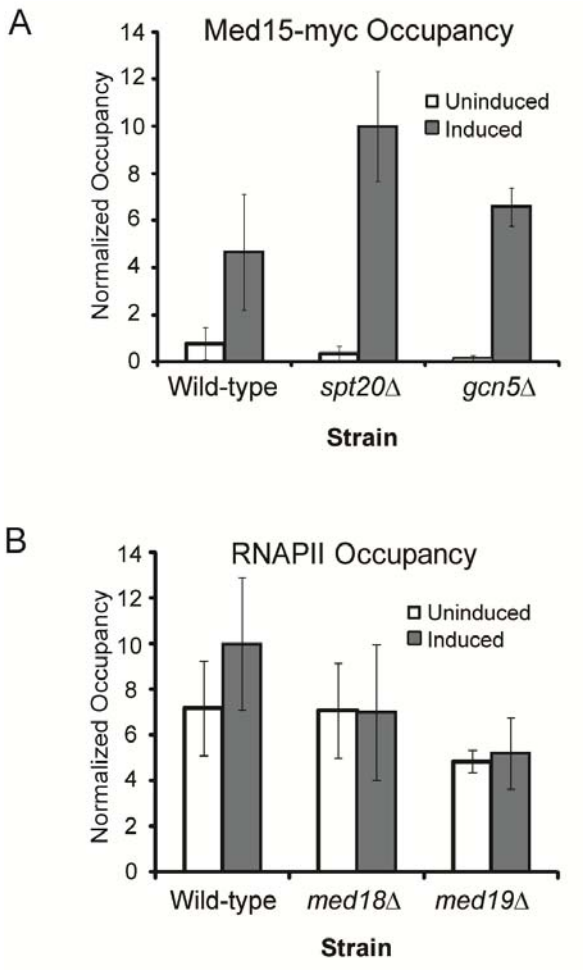

FIGURE 3.11. Mediator is recruited to the *CYC1* promoter upon induction in a SAGA-independent manner, and Mediator is not required for RNAPII occupancy. **A)** Chromatin immunoprecipitation of Med15 (Med15-myc) shows increased *CYC1* promoter occupancy after transfer to inducing conditions. An increase in occupancy is maintained in the *spt20*Δ strain or *gcn5*Δ strain, indicating core Mediator is recruited to this promoter independently of the SAGA subunits. Normalized occupancy was determined by subtracting occupancy at the *GAL1* promoter from occupancy at the *CYC1* promoter. **B)** Chromatin immunoprecipitation of RNAPII at the *CYC1* promoter in the wild-type, *med18*Δ, and *med19*Δ strains. Occupancy of RNAPII is independent of these two Mediator proteins. Occupancy is shown as fold over the occupancy at a telomere-proximal region on the right arm of Chromosome VI. In both panels the highest value is set to 10. Bars represent the average occupancy at *CYC1* (-150 to +40 relative to the ATG) of three biological replicates ± SD.

the SAGA complex[72,99], and also for function in the tethering assay and for *CYC1* transcription.

Although we found Mediator occupies the *CYC1* promoter only after activation, the Mediator complex has a well-established role in RNAPII recruitment[109,110]. To rule out the possibility that Mediator influences recruitment of RNAPII to the *CYC1* promoter, chromatin immunoprecipitation of RNAPII was performed in the *med18*Δ and *med19*Δ strains. We found RNAPII occupancy at the *CYC1* promoter is unaffected by deletion of either *MED18* or *MED19* (**FIGURE 3.11B**). Importantly, these two subunits of the Mediator complex both came out of the TBP-tethering assay and are crucial for *CYC1* gene expression. These results indicate that Mediator is not required for RNAPII preloading at the *CYC1* promoter. Thus, Mediator recruitment is independent of SAGA, and recruitment of Mediator is not sufficient for transcriptional activation of the poised promoter.

3.4 DISCUSSION

Transcription by RNAPII is a regulated and complex process that depends upon the coordinate activities of a large number of factors. Coactivators represent an important and highly conserved class of factors that mediate and integrate signals to arrive at the appropriate level of gene expression for a particular condition. Coactivators, like the multiprotein complexes SAGA, Mediator and TFIID, associate with the relevant promoter via protein-protein interactions with sequence-specific DNA binding proteins[43-47]. Coactivators facilitate transcription by recruiting RNAPII and/or enhancing the formation of the preinitiation complex by a variety of mechanisms[67,118,163,164]. Here we report that the SAGA and Mediator coactivator complexes also play essential and

distinct roles in the regulation of gene expression after the recruitment of TBP and RNAPII.

It is now clear that a growing number of genes are regulated at a step (or steps) after the recruitment of the general transcription machinery (for recent reviews see[133,134,165]). Such genes include the yeast *CYC1* gene, the *Drosophila* heat shock genes, and mammalian *c-myc* and HIV-1 genes[52,53,56,62,63,146]. Genome-wide studies also indicate that a large number of developmental and stress-inducible genes have RNAPII preloaded at promoter-proximal regions[59-61]. Our mechanistic understanding of these poised yet inactive promoters is woefully incomplete, and there is very little information on the regulatory factors that are required to activate these genes. As such, we set out to identify gene products with important roles in transcription after the recruitment of TBP and RNAPII.

We initially used a TBP-tethering screen with a large number of haploid deletion strains to search for gene products with required functions after TBP associates with the promoter. We observed a significant dependence on gene products in the SAGA and Mediator complexes for activity of the tethered TBP derivative (**TABLE 3.1**). Importantly, we also found that deletion of these gene products leads to diminished transcription of *CYC1*, an authentic postrecruitment regulated promoter.

Four genes encoding subunits of the SAGA complex were identified with potential roles in postrecruitment functions: *ADA1*, *SPT7*, *SPT20*, and *GCN5*. Three of these (*ADA1*, *SPT7* and *SPT20*) are required for the structural integrity of the SAGA complex [72,96,99], indicating that an intact complex is required for the activation functions. We therefore investigated the three well-established functions of SAGA for a role at *CYC1*: HAT activity[71-74], TBP recruitment[93-98], and histone H2B deubiquitination[82-84]. Our

data indicate that despite the SAGA-dependency we observe at *CYC1*, these traditional functions are not involved in the activation of the preloaded complex. Therefore, this promoter provides a useful tool for probing additional functions of this large complex.

The dependency on SAGA at *CYC1* may reflect a functional interaction with Mediator, since these two coactivators collaborate in regulating the expression of a wide array of recruitment-regulated promoters[96,162,166,167]. The connection between SAGA and Mediator functions is fairly well-established. Multiple genetic interactions between SAGA and Mediator subunits were shown over a decade ago[144]. Deletion of the SAGA subunit *SPT20* is lethal in combination with deletions in several non-essential genes encoding the Mediator subunits Med16 (Sin4), Med15 (Gal11), Med20 (Srb2), and Med18 (Srb5). Synthetic lethality was also observed with *spt20*Δ using a truncation mutation in the essential *MED14* gene[144]. Further, using synthetic genetic array and diploid-based synthetic lethality analysis on microarrays, negative genetic interactions are observed between the Spt3 and Spt8 subunits of SAGA and *MED16* (*SIN4*), *MED15* (*GAL11*), *MED2*, *MED3 (PGD1)*, *MED31* (*SOH1*), *MED20* (*SRB2*), and *MED5* (*NUT1*), which encompasses subunits within the head, middle, and tail modules of Mediator[168]. These genetic interactions give strong support for SAGA and Mediator acting in concert in the process of transcription. Indeed, we find that both complexes play important postrecruitment functions in the TBP-tethering assay and the native *CYC1* gene. However, there are significant differences in the functional roles for the two coactivators at *CYC1*. In contrast to SAGA (and TBP and RNAPII), we find that Mediator is recruited to the *CYC1* promoter only upon activation of the preloaded complex. Thus, Mediator occupancy correlates with transcriptional output of the poised *CYC1* promoter. This occupancy is not just fortuitous, but is functional, since Mediator subunits (Med18,

Med19, Med20, and Med31), are essential for transcriptional activation of the poised promoter.

Importantly, Mediator occupancy at *CYC1* is not dependent upon SAGA, since disruption of SAGA (in the *spt20*Δ strain), or alteration of SAGA (in the *gcn5*Δ strain) does not alter Mediator occupancy. This suggests that Mediator and SAGA are required for independent steps in the activation process of this poised promoter. It should be noted that particular recruitment-regulated genes require SAGA and Mediator for independent functions as well[70,166]. The mechanistic requirement of SAGA and Mediator at recruitment-regulated promoters is based on PIC formation, which is distinct from their requirement at the poised promoter.

Besides recruiting RNAPII and enhancing PIC formation, Mediator can also stimulate phosphorylation of the C-terminal domain (CTD) of RNAPII by the general transcription factor TFIIH[105,110]. Phosphorylation of the CTD at serine 5 is a prerequisite for the transition from initiation to elongation (reviewed in[169-171]). However, we have previously shown that Serine 5 is phosphorylated at the poised promoter prior to activation[53]. In keeping with this, TFIIH is also already present at the poised promoter before transcriptional activation[53]. Thus, Mediator does not function to stimulate this step in the process at the poised *CYC1* promoter.

What then is the functional activity provided by Mediator at the poised promoter? An involvement of Mediator in postrecruitment functions has been described at the mouse *Egr1* gene[172]. The authors suggest that an isomerization of the transcription complex may be the functional role of Mediator, and others concur[118,119]. It is interesting to note that the head module, which interfaces with RNAPII[116], plays a critical role in activation of the *CYC1* poised promoter (**FIGURE 3.10**), whereas it is not as uniformly

required for a recruitment-regulated promoter[166]. Furthermore, the Med31 subunit is also important for *CYC1* activation. Med31 belongs to the middle module, which also makes direct contact with RNAPII[115]. This subunit is well conserved across evolution[173], suggesting a possible role in activation of poised promoters in higher eukaryotes. Taken together, it is interesting to speculate that recruitment of Mediator results in a reorganization of the poised promoter into a transcriptionally active conformation. Consistent with this hypothesis, we see changes in the cross-linking pattern of occupancy of SAGA after activation of the poised promoter. Further work will be required to reveal the nature and extent of these changes, and how they lead to a productive RNAPII machinery.

TABLE 3.1. Growth phenotype[a] for strains with deletions in transcription-related genes.

STRAIN[b]	LEXA	LEXA-TBP	STRAIN	LEXA	LEXA-TBP	STRAIN	LEXA	LEXA-TBP
BY4741	+	+++	**Activators**			**Elongation Factors**		
SPTs			*asc1Δ*	+/-	+++	*dst1Δ*	+/-	+++
spt1Δ	+	+++	*bas1Δ*	-	+++	*ela1Δ*	+/-	+++
spt2Δ	+	+++	*gal4Δ*	+	+++	*elc1Δ*	+	+++
spt3Δ	+	+++	*gal80Δ*	+	+++	*elp2Δ*	++	++
spt4Δ	+	+++	*hpc2Δ*	+	+++	*elp3Δ*	+	+++
spt7Δ	+/-	-	*tbs1Δ*	+/-	+++	*elp4Δ*	++	+++
spt8Δ	+	+++	*mbf1Δ*	-	+++	*elp6Δ*	+	++
spt10Δ	+	+++	*met18Δ*	-	+++	*iki3Δ*	+	+++
spt20Δ	+/-	+	*mot3Δ*	-	+++	*nhp6aΔ*	-	+++
spt21Δ	+	+++	*swi5Δ*	+	+++	*rtf1Δ*	-	+++
spt23Δ	+	+++	*swi6Δ*	+	++	*thp1Δ*	+/-	+++
SAGA/ADA			**Repressors**			*thp1Δ*	-	+++
ada1Δ	-	+/-	*caf4Δ*	+	+++	**Swi/Snf**		
ada2Δ	-	++	*caf16Δ*	-	+++	*snf2Δ*	-	+++
ada3Δ	-	++	*caf17Δ*	-	+++	*snf5Δ*	-	+++
ahc1Δ	+	+++	*ccr4Δ*	+/-	+++	*snf6Δ*	-	++
chd1Δ	-	+++	*not3Δ*	-	+++	*snf11Δ*	+	+++
gcn5Δ	+/-	+	*not5Δ*	-	+++	*swi3Δ*	+	+++
sgf11Δ	+/-	++	*nrg2Δ*	+	+++	**Protein Kinase subunits**		
Mediator			*pop2Δ*	-	+++	*cka1Δ*	+	+++
med1Δ	+	++	*sig1Δ*	-	++	*cka2Δ*	+/-	+++
med3Δ	+/-	+++	*ssn6Δ*	-	+++	*ckb1Δ*	+	+++
med5Δ	+	+++	*sut1Δ*	+/-	+++	*ckb2Δ*	-	+++
med9Δ	-	++	*tup1Δ*	-	+++	*ctk1Δ*	+/-	+++
med12Δ	+	+++	**HDACs**			*ctk2Δ*	-	+++
med13Δ	+	+++	*hda1Δ*	+/-	+++	*ctk3Δ*	+/-	++
med15Δ	+	+++	*hos1Δ*	-	+++	**H4/H2A HAT complexes**		
med16Δ	+	+++	*hos2Δ*	-	+++	*eaf3Δ*	++	+++
med18Δ	+/-	+	*hos3Δ*	+	+++	*eaf6Δ*	+/-	+++
med19Δ	+/-	+	*hst1Δ*	+	+++	*hat1Δ*	-	++
med20Δ	+/-	++	*pho23Δ*	+/-	+++	*hat2Δ*	+	+++
med31Δ	+/-	++	*rpd3Δ*	-	+++	*taf14Δ*	+	++
cdk8Δ	+	+++	*sap30Δ*	+/-	+++	*yng1Δ*	-	+++
cyccΔ	+	+++	*sin3Δ*	-	+++	**Paf1 Complex**		
RNA Pol II subunits			**Subunits of ISW1/2**			*cdc73Δ*	+	+++
rpb4Δ	-	+++	*isw1Δ*	+	+++	*paf1Δ*	-	+++
rpb9Δ	-	+++	*isw2Δ*	-	+++	**Other**		
			itc1Δ	+	++	*mcm22Δ*	-	++
						mhr1Δ	+	+++
						rad26Δ	+	+++

[a]Relative growth rate on 20 to 40 mM AT of strains harboring the LexA or LexA-TBP expressing plasmid. Robust growth is scored as '+++' and is the result of *HIS3* gene activation. Intermediate to weak growth is indicated by '++', '+', or '+/-'.

[b]Strains assayed were wild-type (BY4741) or contained a deletion in the gene indicated. All strains were purchased from Research Genetics.

CHAPTER 4. THE HEAD MODULE OF MEDIATOR DIRECTS ACTIVATION OF THE PRELOADED *CYC1* GENE IN YEAST

In this chapter, I probe the requirement for the Mediator complex at the preloaded *CYC1* gene, and the recruitment-regulated *GTT2* gene during oxidative stress. This chapter will be submitted with Tyler Fara as an additional author. I wrote this chapter and contributed all of the figures. Tyler helped prepare RNA, which I analyzed in **FIGURES 4.7** and **4.11**. Dr. Alan Hinnebusch provided the Med15-myc and Med15-myc *med20*Δ strains.

4.1 INTRODUCTION

At postrecruitment regulated genes, RNAPII is preloaded at promoter DNA *prior* to gene activity. Therefore, there is high RNAPII occupancy when transcription is low. At these genes, RNAPII occupancy is not a marker for transcription, and steps after the recruitment of polymerase, or postrecruitment, are rate-limiting for the transcription reaction. Postrecruitment regulated genes have poised promoters, as they are ready (or poised) for future activation, and contain preloaded RNAPII. The best-characterized postrecruitment regulated gene in yeast is the *CYC1* gene. This type of regulation is prevalent in higher eukaryotes; the *Drosophila* heat shock genes and human c-myc genes are common examples[63,174].

The Hap2/3/4/5 activator targets the *CYC1* gene when yeast utilizes ethanol as a carbon source. Whole genome transcriptional profiling revealed that the preloaded

CYC1 gene is also activated in response to oxidative stress caused by exposure to the reactive oxygen species (ROS) hydrogen peroxide[12]. Oxidative stress is a damaging condition as ROS harm all cellular components[22,23]. The primary way that cells restore the balance between ROS and antioxidants during oxidative stress is through the increased expression of genes that encode proteins involved in cellular protection and detoxification. In yeast, the Yap1 activator is essential for driving this response, termed the oxidative stress response[31]. Yap1 contains a basic leucine zipper (b-ZIP) DNA binding domain and is regulated in an oxidation-reduction dependent manner[34,35]. Yap1 shuttles between the cytoplasm and nucleus, yet under normal conditions, it is predominantly cytoplasmic[34]. Yap1 contains two cysteine-rich domains (CRDs) that form intramolecular disulfide bonds upon oxidation, resulting in a conformational change in Yap1. This change in conformation masks the nuclear export recognition sequence from an exporter protein called Crm1, resulting in Yap1 nuclear localization during oxidative stress[37]. Once in the nucleus, Yap1 binds Yap1 Response Elements (YREs) in the promoter of target genes, which encode a variety of antioxidants, heat shock proteins, drug transporters, and enzymes involved in carbohydrate metabolism[38]. Once bound to promoter DNA, it is unclear how Yap1 orchestrates gene activation.

We previously identified a role for the yeast Mediator complex in postrecruitment regulation using a genetic screen and characterization of an endogenous preloaded gene (*CYC1*) (**Chapter 3**[48]). Mediator is a large coactivator complex that is conserved from yeast to humans[101]. Yeast Mediator is composed of 25 subunits and is described in terms of four modules called the head, middle, tail and CDK8[113-115,175,176]. Transcription of most RNAPII-dependent genes requires Mediator[100], which serves as a molecular bridge between activator proteins and RNAPII. In our previous work, we found subunits of the head module of Mediator (Med18, Med19, and Med20) are necessary for transcriptional

activation of the *CYC1* gene when stimulated by the Hap2/3/4/5 activator. Recent biochemical and structural data demonstrate that the Med18, Med19 and Med20 proteins are each involved in RNAPII interaction[117,177]. Yet, Med18, Med19 and Med20 are not required for RNAPII occupancy at this promoter (**CHAPTER 3**[48]). This demonstrates that at *CYC1*, Mediator does not recruit RNAPII, but is required for transcription at a postrecruitment step.

The aim of this study was to investigate the expression of the preloaded *CYC1* gene during oxidative stress, focusing on the role of the Mediator complex. We then compare the activation of *CYC1* to a recruitment-regulated gene, *GTT2*. Here, we show that oxidative stress stimulates *CYC1* expression rapidly via the Yap1 activator. This represents a distinct activation paradigm compared to previous work, which focused on expression of *CYC1* in response to growth in ethanol. Analysis of Mediator requirements during oxidative stress revealed that the Med18, Med19 and Med20 proteins of the Mediator complex are required for activation of *CYC1*. This demonstrates an important role of these proteins in the stimulation of the preloaded polymerase at this promoter. Finally, our analysis of a Yap1-dependent gene that does not contain a preloaded polymerase (*GTT2*) shows that Med18, Med19 and Med20 are not uniformly required for Yap1 activation, but are specifically required for stimulation of the polymerase at *CYC1*. Therefore, we have defined a functional submodule comprised of Med18, Med19 and Med20 that is required for activation of *CYC1*. Intriguingly, our data demonstrates that Med18, Med19 and Med20 are not required for Yap1 targeting of the Mediator complex, but are likely essential for activation of *CYC1* because they form a critical interaction surface with RNAPII itself.

4.2 RESULTS

4.2.1 Yap1 regulates *CYC1* gene expression during oxidative stress: Previous characterization of *CYC1* expression involved activation that occurs in response to growth in nonfermentable carbon sources[17,48,53,178]. Our previous investigation of Mediator requirements at *CYC1* during growth in nonfermentable carbon sources revealed a strong dependence on the Med18, Med19 and Med20 subunits for gene activation. These three proteins reside in the head module of Mediator, and interact with RNAPII[117,177]. Intriguingly, these three proteins are not required for RNAPII occupancy at this promoter; instead, they are essential for transcription at a postrecruitment step (**Chapter 3**[48]). To investigate the function of Mediator at the *CYC1* gene using a different activation paradigm, we took advantage of the observation that oxidative stress also induced *CYC1* expression in a whole genome transcriptional profiling study[12].

To examine *CYC1* transcript levels during oxidative stress, we analyzed RNA prepared from cells before and after treatment with sub-lethal levels of hydrogen peroxide (H_2O_2). We found that *CYC1* transcript levels are activated very quickly in response to this treatment. Indeed, peak levels occur just 10-20 minutes after the addition of H_2O_2 (**FIGURE 4.1A**). Transcript levels soon begin to decline, reaching pre-induction levels 50 minutes after the initial treatment with H_2O_2. Yap1 is a transcriptional activator protein that is important during oxidative stress in yeast[179]. Using a strain missing the Yap1 activator (*yap1Δ*), we found the expression of *CYC1* during oxidative stress is dependent on Yap1 (**FIGURE 4.1A**). Yap1 is essential for cellular survival during growth in oxidative stress-inducing conditions, as cells lacking the *YAP1* gene fail to grow on plates containing hydrogen peroxide **(FIGURE 4.1B)**.

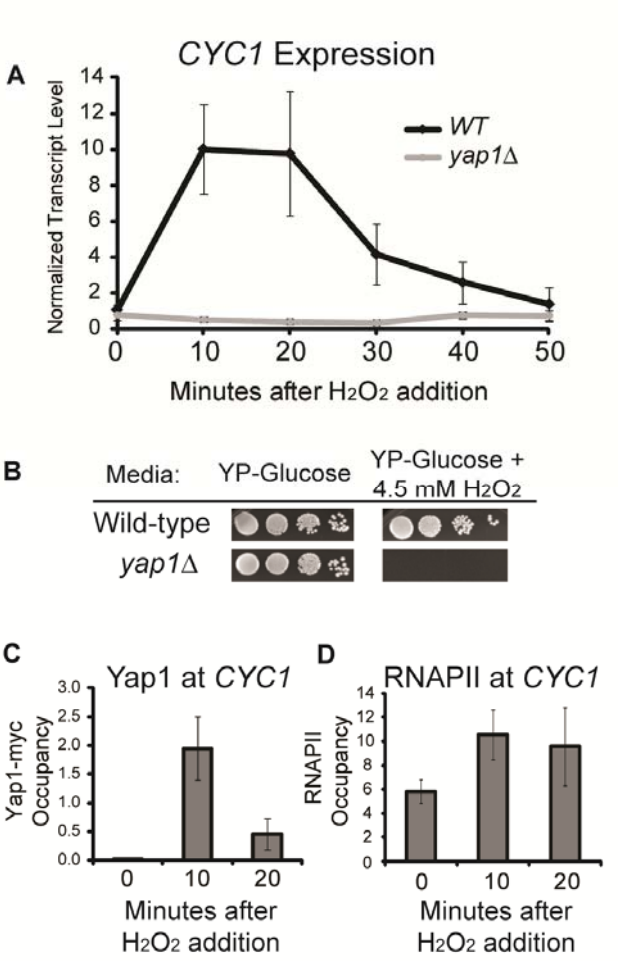

FIGURE 4.1. **Yap1 directly regulates *CYC1* during oxidative stress. A)** *CYC1* transcript levels are rapidly induced in response to oxidative stress. An S1 nuclease protection assay was performed with RNA isolated from wild-type and *yap1*Δ cells before exposure to H_2O_2 and in 10 minute intervals after H_2O_2 (0.3 mM) addition. Transcript levels are not induced in the *yap1*Δ strain. A probe for *tRNA*^{w} was used as a loading control in the assays, and the transcript level in the wild-type strain 10 minutes after H_2O_2 exposure was set to 10. Points represent the average of at least three biological replicates ±SD. **B)** Serial spot dilutions of the wild-type and *yap1*Δ strains on YP-Glucose and on YP-Glucose containing 4.5 mM H_2O_2. Plates were incubated at 30^0C for 3 days. **C)** Yap1 directly regulates *CYC1*, as Yap1-myc occupies the *CYC1* promoter during oxidative stress. Occupancy was determined with a ChIP using a Yap1-myc strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *CYC1*. Bars represent the average ±SD of three samples processed independently from a master culture. **D)** RNAPII occupies the *CYC1* promoter prior to gene activation, and occupancy does not change significantly after gene expression is detected. Occupancy at a region proximal to the telomere on chromosome VI was subtracted as background from the occupancy at *CYC1*. Bars represent the average ±SD of three samples processed independently from a master culture.

We next wanted to determine if the transcriptional dependence on Yap1 is a direct result of promoter occupancy by this activator, or due to indirect effects. A time course chromatin immunoprecipitation assay (ChIP) was performed using a strain containing a myc-tagged version of Yap1 (Yap1-myc). In this experiment, an aliquot of cells was removed from an uninduced culture, and 10 and 20 minutes after the addition of H_2O_2. These time points were chosen based on the transcript profile of *CYC1* during oxidative stress (**FIGURE 4.1A**). Cells were cross-linked by addition of formaldehyde at the time indicated. We found no significant occupancy of Yap1-myc at the *CYC1* promoter in uninduced conditions. However, Yap1-myc occupancy increases greatly after induction with H_2O_2. Maximum occupancy of Yap1 was found 10 minutes after the addition of H_2O_2 (**FIGURE 4.1C**), which is consistent with the peak level of *CYC1* transcript. Therefore, Yap1 is directly involved in the rapid activation of the poised *CYC1* promoter during the oxidative stress response in yeast.

4.2.2 Yap1 does not preload the *CYC1* promoter: During normal conditions, interaction with the nuclear exporter Crm1 maintains Yap1 primarily in the cytoplasm[180]. During oxidative stress this interaction is masked due to oxidant-induced changes in the structure of the Yap1 protein itself, and Yap1 accumulates in the nucleus[34,181]. We and others have noted previously that the *CYC1* promoter contains preloaded RNAPII[48,52,146,148]. Thus, the RNAPII enzyme occupies the promoter region prior to gene activity, and occupancy only modestly changes upon activation (**FIGURE 4.1D**). Given that the Yap1 protein shuttles into and out of the nucleus, it is formally possible that low levels of Yap1 that escape the export machinery could be responsible for recruiting the inactive RNAPII at the *CYC1* promoter during uninduced conditions. To test this, we analyzed RNAPII occupancy in the wild-type and *yap1Δ* strain under normal conditions. While there is a slight drop in RNAPII occupancy in the *yap1Δ* strain, RNAPII still

occupies this promoter well above background levels when Yap1 is missing (**FIGURE 4.2**). This indicates that Yap1 is not responsible for preloading the promoter with RNAPII, but instead functions to trigger an inactive RNAPII at the *CYC1* promoter during activation.

4.2.3 Distinct activators drive *CYC1* expression during growth in nonfermentable carbon sources versus oxidative stress: As previously mentioned, *CYC1* transcription is activated in response to cell growth in media containing nonfermentable carbon sources, such as glycerol or ethanol[12,178]. This carbon-source dependent activation requires the evolutionarily conserved Hap2, Hap3, Hap4, Hap5 complex of proteins, or the Hap complex[182,183]. The Hap complex activates yeast genes involved in cellular respiration (such as *CYC1*, which encodes an isoform of cytochrome c)[17]. The Hap2, Hap3 and Hap5 proteins are required for binding target sequences in gene promoters. In contrast, the Hap4 protein does not directly associate with DNA; instead, it interacts with the Hap2/3/5 proteins and provides the transcriptional activation function[13]. In order to characterize *CYC1* transcriptional activation further, we next wanted to determine if the transcriptional response to nonfermentable carbon sources was biologically distinct from the response to oxidative stress. In other words, does Yap1 also play a role in activation of *CYC1* during growth in nonfermentable carbon sources?

To investigate this, we analyzed transcript levels in the wild-type, *yap1Δ*, and *hap4Δ* strains grown in glucose (uninduced) and ethanol (induced). *CYC1* activates normally in response to nonfermentable carbon sources in the *yap1Δ* strain, as we found transcript levels are comparable to a wild-type strain (**FIGURE 4.3**). Therefore, the response to nonfermentable carbon sources is Yap1-independent. As expected, we found that ethanol-induced activation is dependent upon the Hap complex, as transcript

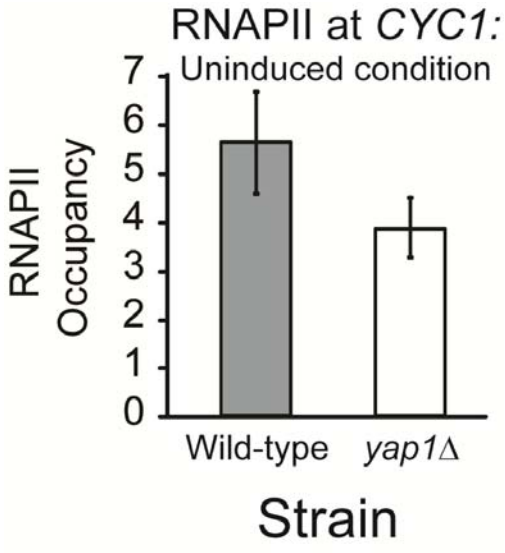

FIGURE 4.2. Yap1 is not required for preloading the *CYC1* promoter. Occupancy of RNAPII during growth in YP-Glucose (uninduced condition) in the wild-type (gray bar) and *yap1*Δ (unfilled bar) strains. Occupancy at a region proximal to the telomere on chromosome VI was subtracted as background from the occupancy at *CYC1*. Bars represent the average ±SD of three samples processed independently from a master culture.

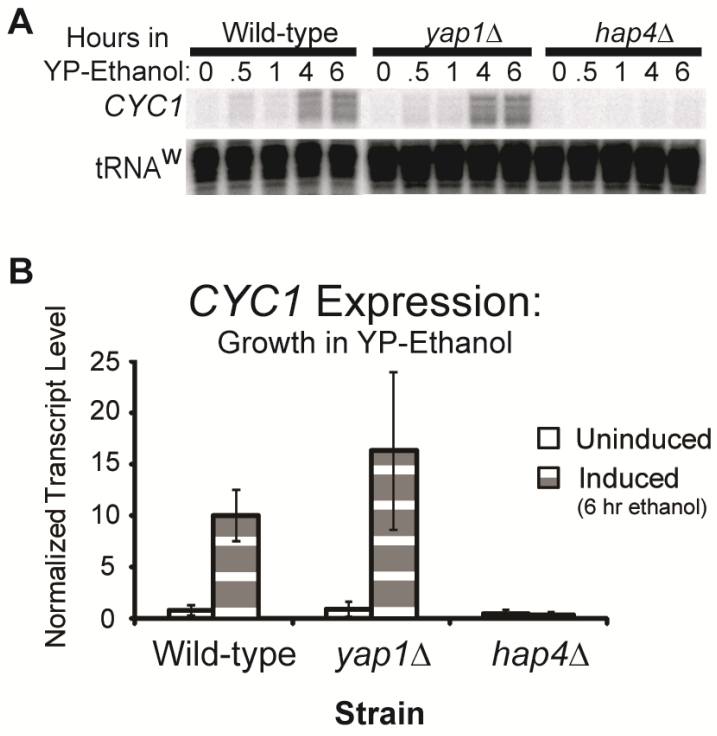

FIGURE 4.3. *CYC1* activation during growth in nonfermentable carbon sources (YP-Ethanol) is Yap1-independent but Hap4-dependent. A) Transcript levels were analyzed with RNA prepared from the wild-type, *yap1*Δ, and *hap4*Δ strains after cells were transferred to media containing 3% ethanol as the carbon source. A *tRNA*^{w} probe was used as a loading control in the S1 nuclease protection assays. Representative gel is shown. The wild-type reactions were run on a separate gel than the *yap1*Δ and *hap4*Δ reactions, the picture was cropped with Photoshop. **B)** Quantification of *CYC1* transcript levels in each strain. Level in the wild-type strain 6 hours after cells were transferred to ethanol-containing media was set to 10. Bars represent the average of three biological replicates ±SD.

levels did not increase during growth in nonfermentable carbon sources in the *hap4Δ* strain (**FIGURE 4.3**).

We also wanted to test the converse relationship: is the Hap complex involved in the response to oxidative stress? As previously shown, the response to oxidative stress is Yap1-dependent (**FIGURE 4.1**). We found the response to oxidative stress is independent of the Hap4 protein (**FIGURE 4.4**). This finding shows that the Hap complex is not involved in the oxidative stress-dependent activation of *CYC1*. Therefore, two distinct activators govern *CYC1* transcription.

4.2.4 Mediator subunits are required during oxidative stress and growth on nonfermentable carbon sources: We next wanted to determine if Mediator proteins are required for cell growth during conditions that induce *CYC1* transcription. To test this, we analyzed phenotypes of 11 Mediator deletion strains through serial spot analysis on media containing glucose, on media supplemented with H_2O_2, and on media containing a nonfermentable carbon source (ethanol/glycerol). We found that strains missing subunits within the head region of Mediator show diminished cell growth on both types of plates compared to growth on glucose (**FIGURE 4.5**). This suggests the proteins within the head module (Med18, Med19 and Med20) are important for expression of genes that help the cell survive during growth on both oxidative stress inducing conditions and nonfermentable carbon sources. In contrast, the *med1*Δ, *med5*Δ, and *med9*Δ strains are not sensitive to growth on these two conditions (**FIGURE 4.5**). The *med31*Δ strain shows slight sensitivity compared to the wild-type strain. Strains missing genes encoding subunits of the tail module also show disparate effects. The *med2*Δ and *med15*Δ strains each show sensitivity to oxidative stress, with a more pronounced phenotype in the *med15*Δ strain. The *med3*Δ and *med16*Δ strains grow similarly to wild-

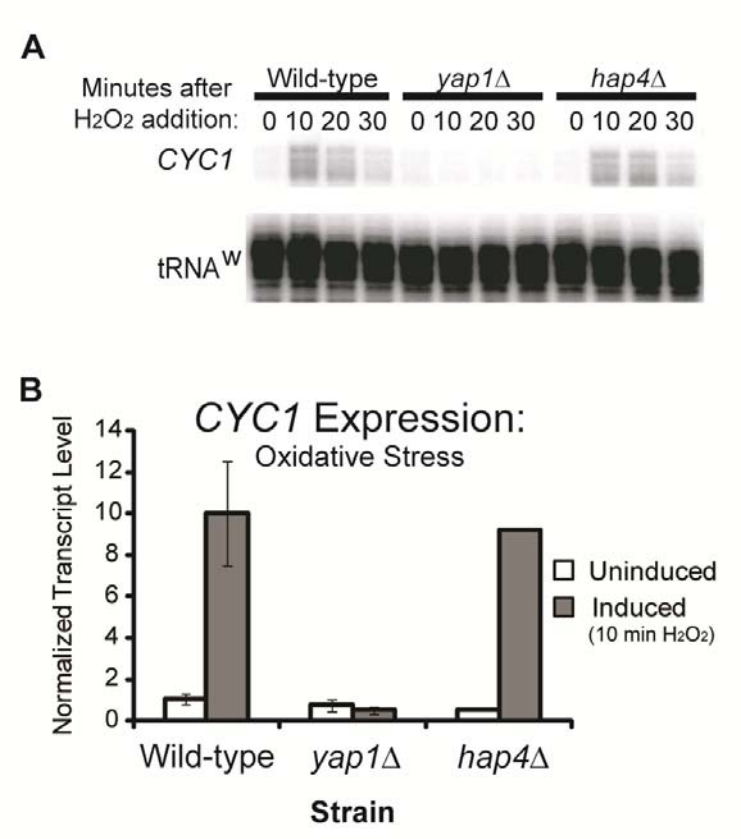

FIGURE 4.4. *CYC1* activation during oxidative stress is Hap4-independent. A) *CYC1* transcript levels were analyzed using an S1 nuclease protection assay with RNA prepared from the wild-type, *yap1*Δ and *hap4*Δ strains. A *tRNA*^{w} probe was used as a loading control in the S1 nuclease protection assays. Representative gel is shown. **B)** Quantification of *CYC1* transcript levels in each strain. *CYC1* expression in the wild-type strain 10 minutes after cells were exposed to H_2O_2 was set to 10; bars represent the average of three biological replicates ±SD.

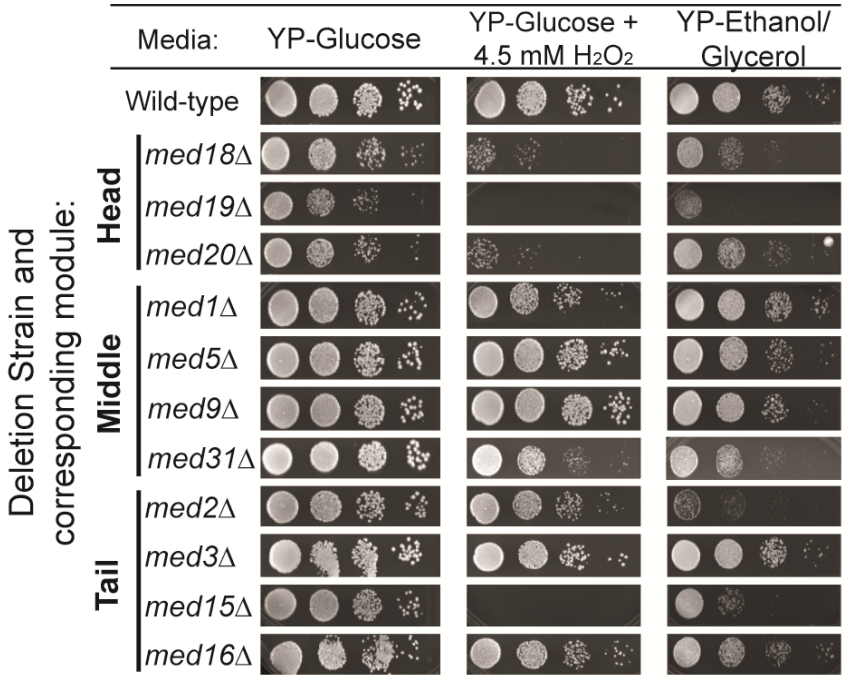

FIGURE 4.5. Med18, Med19 and Med20 are required during oxidative stress and growth on nonfermentable carbon sources. Serial spot dilutions of the wild-type strain and strains containing deletions of 11 Mediator subunits on YP-Glucose, YP-Glucose supplemented with H_2O_2, and YP-Ethanol/Glycerol. Plates were incubated at 30^0C for 2-5 days before photographing.

type on these conditions. Meanwhile, the *med2*Δ and *med15*Δ strains are also sensitive to growth on plates containing nonfermentable carbon sources. This analysis reveals an important role of the subunits of the head module of Mediator (Med18, Med19 and Med20) for normal growth during sub-optimal conditions, as well as Med31, Med2, and Med15. Therefore, these proteins are only "non-essential" during optimal conditions (YP-Glucose), and are required for cell growth when yeast experience sub-optimal conditions. This finding is consistent with the proposal that Mediator largely functions in the induction of stress-inducible genes[184], as these phenotypes suggest these subunits of Mediator are required for proper expression of genes involved in cell survival in the conditions assayed.

4.2.5 Oxidative stress results in Mediator recruitment to *CYC1*: Having established that oxidative stress induces *CYC1* via Yap1, and Mediator subunits are required for cell growth during oxidative stress, we next explored the role of Mediator at *CYC1* during this condition. First, we performed a ChIP assay to determine if Mediator occupies the *CYC1* promoter during oxidative stress. We determined the occupancy of two tagged subunits (Med14-HA and Med15-myc) of the complex before induction and 10 and 20 minutes after induction with H_2O_2 (to correlate with maximum transcript levels and Yap1 occupancy). We found that occupancy of both subunits increases greatly upon gene induction with oxidative stress (**FIGURE 4.6A and B**), demonstrating that Mediator does not occupy the promoter in the uninduced condition, but is recruited to *CYC1* during Yap1-dependent induction.

4.2.6 Mediator requirements at *CYC1* during activation by Yap1: We next wanted to test whether the Mediator subunit requirements when Yap1 controls *CYC1* (during oxidative stress) were similar to the subunit requirements when the Hap complex

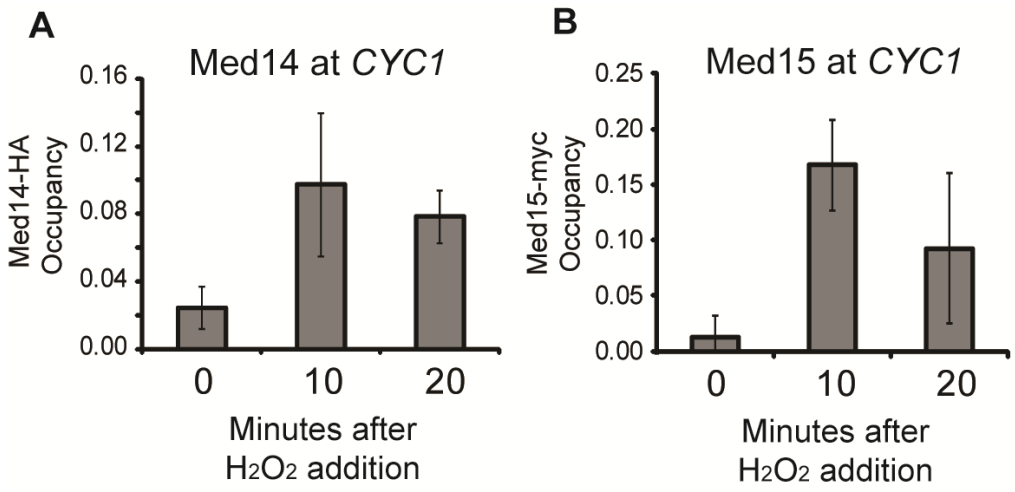

FIGURE 4.6. **Mediator is recruited to the *CYC1* promoter upon activation**. Occupancy of two tagged proteins within the Mediator complex increases upon gene activation. Occupancy of Med14-HA (**A**) and Med15-myc (**B**) was determined with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted as background from the occupancy at *CYC1*. Bars represent the average ±SD of three samples processed independently from a master culture (Med14-HA), or the average ±SD of six samples processed independently from two master cultures (Med15-myc).

controls *CYC1* (during growth in nonfermentable carbon sources). RNA was prepared from strains containing deletions of the non-essential Mediator subunits, and probed for *CYC1* transcript. We found that *CYC1* transcript levels during oxidative stress are greatly diminished in strains containing deletions of the *MED18, MED19*, and *MED20* genes (**FIGURE 4.7**). These genes encode proteins that reside in the head module of the Mediator complex, and are the only non-essential proteins within this module. The transcriptional defect of *CYC1* is largely specific for the subunits within the head module since transcript levels are unaffected in strains containing deletions of the middle module subunits of Mediator (Med1, Med5, Med9, and Med31) (**FIGURE 4.7**). Med15 (from the tail module) is also important for activation of *CYC1* during this stress, although the transcriptional defect seen in the *med15*Δ strain is not as severe as deletion of the head module subunits.

This analysis reveals that *CYC1* expression is Mediator dependent during oxidative stress. Specific subunits of the large complex are more important for its expression than others. The Yap1-dependent Mediator subunit usage is strikingly similar to what we found when the Hap complex activates this gene during ethanol induction (**CHAPTER 3**[48]). During ethanol induction, Med18, Med19 and Med20 were each essential for *CYC1* transcription. Together, the transcriptional profiling at *CYC1* during two conditions reveals the requirement for Med18, Med19 and Med20 is activator-independent; they are required regardless of the activator protein that is conscripting the Mediator complex for *CYC1* activation.

4.2.7 Mediator recruitment via Yap1 is independent of Med20: It is possible that Med18, Med19 and Med20 are required for Yap1-dependent Mediator occupancy at the *CYC1* promoter? In other words, Yap1 could utilize Med18, Med19 and Med20 for

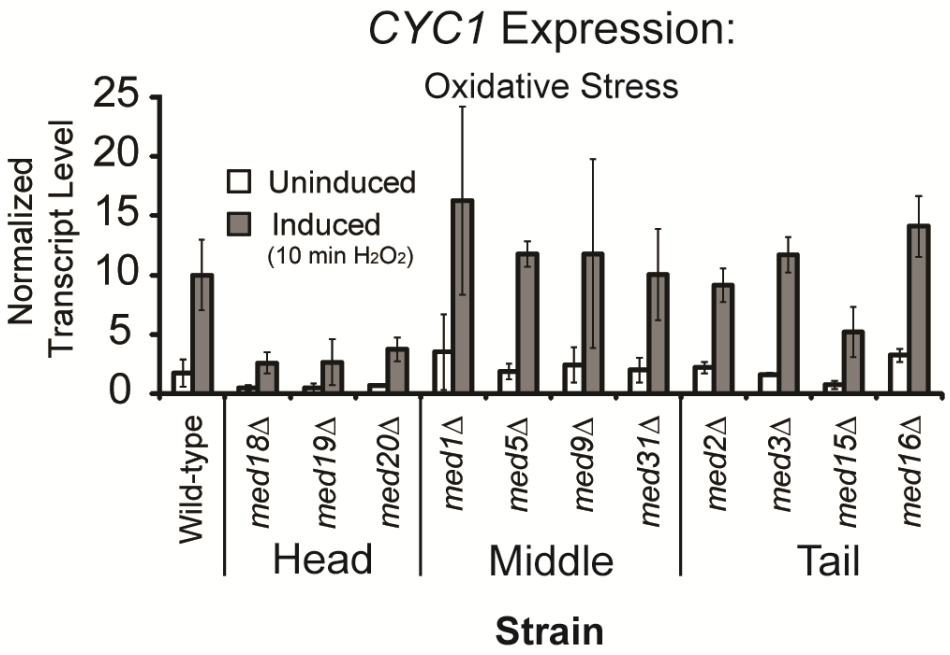

FIGURE 4.7. *CYC1* activation during oxidative stress is dependent upon certain subunits of the Mediator complex. Transcript levels were analyzed with RNA prepared from the wild-type strain and strains containing deletions of the indicated Mediator subunit in both uninduced (YP-Glucose, unfilled bars) and induced (10 min H_2O_2 treatment, dark gray bars) conditions. *CYC1* transcript level was normalized to a $tRNA^w$ probe in the S1 nuclease protection assays. Expression level in the wild-type strain 10 minutes after activation was set to 10. The average ±SD of three biological replicates is shown.

Mediator recruitment to *CYC1*- this would explain their requirement for transcription. In order to test this hypothesis, we examined Mediator occupancy at the *CYC1* promoter in a strain missing one of these three proteins. It is important to note that Med18, Med19 and Med20 are not implicated in complex integrity. Deletion of one of these subunits does not result in failure of the Mediator complex to form[44,185,186]. Therefore, we can test if the complex is recruited in the absence of one of these proteins. Thus, we performed a ChIP for the Med15-myc protein in a wild-type strain, and a strain missing the Med20 protein (*med20*Δ).

If Med20 is required for occupancy of the rest of the Mediator complex at *CYC1*, this would suggest that Yap1 utilizes this protein for recruitment of the complex to the *CYC1* promoter. The *med20*Δ background was used because this strain is the healthiest strain with a defect in *CYC1* activation. The ChIP revealed that Mediator (assayed with Med15-myc) does not occupy this promoter before induction, but is recruited to *CYC1* upon activation even when the Med20 protein is absent (**FIGURE 4.8**). Therefore, Mediator recruitment to *CYC1* is independent of the Med20 protein. Yet, loss of Med20 compromises *CYC1* transcription. This indicates that in the *med20*Δ background, the Mediator complex occupies the promoter, but the gene is in an inactive state. Med20 is necessary for activation of the polymerase in a postrecruitment step, pointing to the intriguing model that Med18, Med19, and Med20 are required for triggering the inactive RNAPII at *CYC1* into an active one. If true, then these subunits may be dispensable for activation at a recruitment-regulated gene.

4.2.8 *GTT2* is a Yap1-dependent, recruitment-regulated gene: To test if loss of Med18, Med19 and Med20 impacts a recruitment-regulated Yap1-dependent gene, we

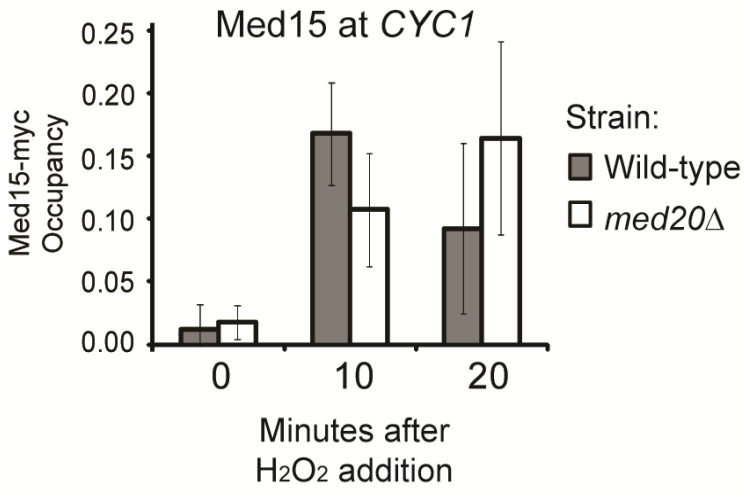

FIGURE 4.8. **Med20 is not required for Mediator recruitment to the *CYC1* promoter region.** Occupancy of Med15-myc in a wild-type (filled bars) and *med20*Δ (unfilled bars) strains was determined with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted as background from the occupancy at *CYC1*. Bars represent the average ±SD of three samples processed independently from a master culture.

broadened our analysis to include *GTT2*. The *GTT2* gene encodes a glutathione s-transferase, a protein involved in cellular detoxification during oxidative stress[187]. We chose to analyze expression of this gene for two reasons. First, H_2O_2 induces *GTT2* transcript levels[12], and second, the promoter does not appear to have preloaded RNAPII, as assayed by a genome-wide ChIP study[188]. We analyzed *GTT2* transcript levels in our wild-type and *yap1*Δ strains and found that transcription of this gene during oxidative stress is completely dependent upon the Yap1 activator (**FIGURE 4.9A**). The transcript profile for *GTT2* was very similar to *CYC1*, with peak levels occurring 10 minutes after the addition of H_2O_2 to the culture, and transcripts reaching pre-induction levels 50 minutes after the addition of H_2O_2. Further, the Yap1-dependency is direct, as Yap1 occupies the promoter region of this gene only after activation (**FIGURE 4.9B**). Occupancy of the Yap1 transcription factor correlates positively with transcriptional activation, demonstrating that Yap1 is responsible for the oxidative stress induction of this gene.

We next wanted to know if the *GTT2* promoter contains preloaded polymerase, like *CYC1*. Using a time course ChIP assay, it was found that RNAPII does not occupy the promoter prior to activation, consistent with the previous observation[188], and occupancy increases about 10-fold during activation (**FIGURE 4.9C**). This is consistent with recruitment-regulation for *GTT2* and is in striking contrast to what is observed at the *CYC1* promoter.

Having characterized *GTT2* as a Yap1-dependent recruitment-regulated gene, we next tested Mediator recruitment during activation. A ChIP assay was performed examining Med14-HA and Med15-myc occupancy during the uninduced condition and after a 10 and 20-minute treatment with H_2O_2 (when transcript levels were high). Similar

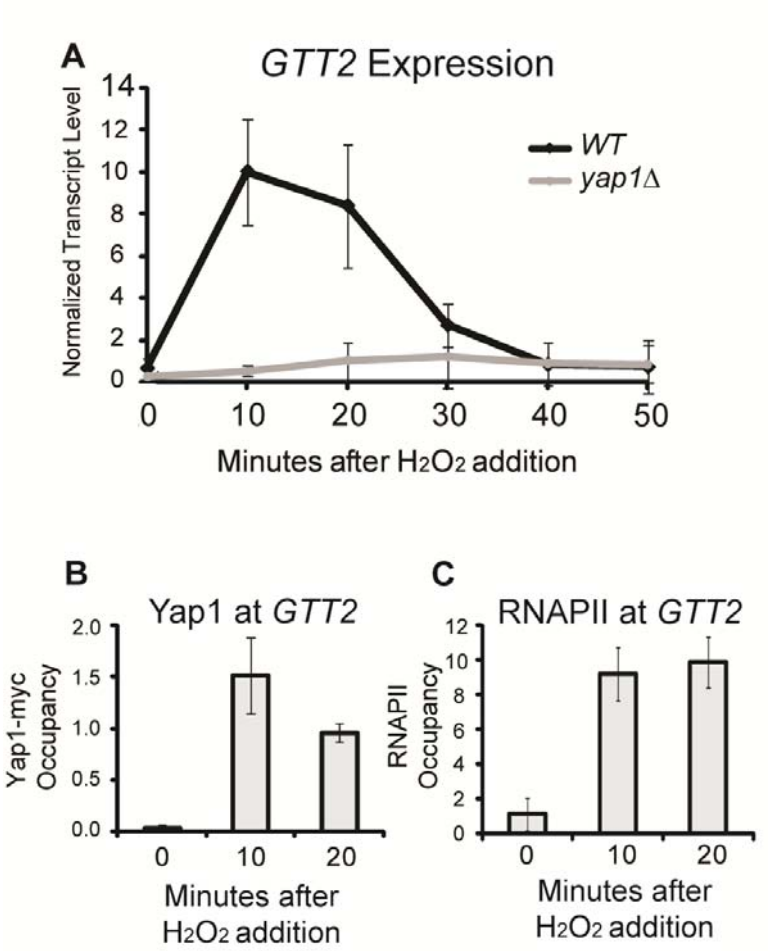

FIGURE 4.9. *GTT2* is a Yap1-dependent gene without preloaded RNAPII. A) *GTT2* transcript levels are rapidly induced in response to oxidative stress. An S1 nuclease protection assay was performed with RNA isolated from wild-type and *yap1*Δ cells before exposure to H_2O_2 and in 10 minute intervals after H_2O_2 exposure (0.3 mM). Increased expression is Yap1-dependent, as transcript levels are not induced in the *yap1*Δ strain. A probe for *tRNA*^{w} was used as a loading control in the S1 nuclease protection assays and the transcript level in the wild-type strain 10 minutes after H_2O_2 exposure was set to 10. Points represent the average ±SD of at least three biological replicates for the wild-type, and two biological replicates for the *yap1*Δ strain. **B)** Yap1 directly stimulates *GTT2* transcription, as Yap1-myc occupies this promoter during oxidative stress. Occupancy was determined with a ChIP of a Yap1-myc fusion protein, and correlates with peak *GTT2* transcript levels. Occupancy at the *GAL10* promoter region was subtracted as background from the occupancy at *GTT2*. Bars represent the average ±SD of three samples processed independently from a master culture. **C)** RNAPII does not occupy the *GTT2* promoter prior to gene activation, but is recruited upon activation. RNAPII occupancy at a region proximal to the telomere on chromosome VI was subtracted as background from the occupancy at *GTT2*. Bars represent the average ±SD of three samples processed independently from a master culture.

to *CYC1*, we found Mediator does not occupy this promoter in the uninduced condition, but occupancy increases greatly upon activation (**FIGURE 4.10A and B**).

4.2.9 Med18, Med19 and Med20 are not required for activation of the recruitment-regulated *GTT2* gene: Given the finding that Med18, Med19 and Med20 are required for stimulating the preloaded polymerase at *CYC1*, we next wondered if this requirement was specific for genes containing preloaded RNAPII at the promoter region. To test this, we examined *GTT2* expression levels during oxidative stress in strains containing deletions of *MED18, MED19* and *MED20*. We found *GTT2* transcript levels were not nearly as sensitive to deletion of the head module subunits as *CYC1* (**FIGURE 4.11**). At *GTT2*, transcript levels in the most sensitive strain (*med18*Δ) reached 60% of the wild-type level. However, at *CYC1* there is a drastic diminishment in this strain, with transcript levels reaching only 25% of the wild-type level. The relative difference between *GTT2* and *CYC1* transcription in the *med18*Δ strain compared to their transcription in the wild-type strain is statistically significant ($p=0.008$ with a Student's T Test). These results suggest that the functional submodule we identified at *CYC1* (Med18, Med19 and Med20) is specifically required for stimulation of the preloaded polymerase at this promoter, and is not required for activation of a recruitment-regulated gene driven by the same activator protein.

4.3 DISCUSSION

The Mediator complex is a multisubunit coactivator involved in the transcription of most RNAPII-dependent genes. Here we show Mediator is required at the phenotypic level during oxidative stress, and during growth on nonfermentable carbon sources. We also identified a functional submodule of the Mediator complex (Med18, Med19 and

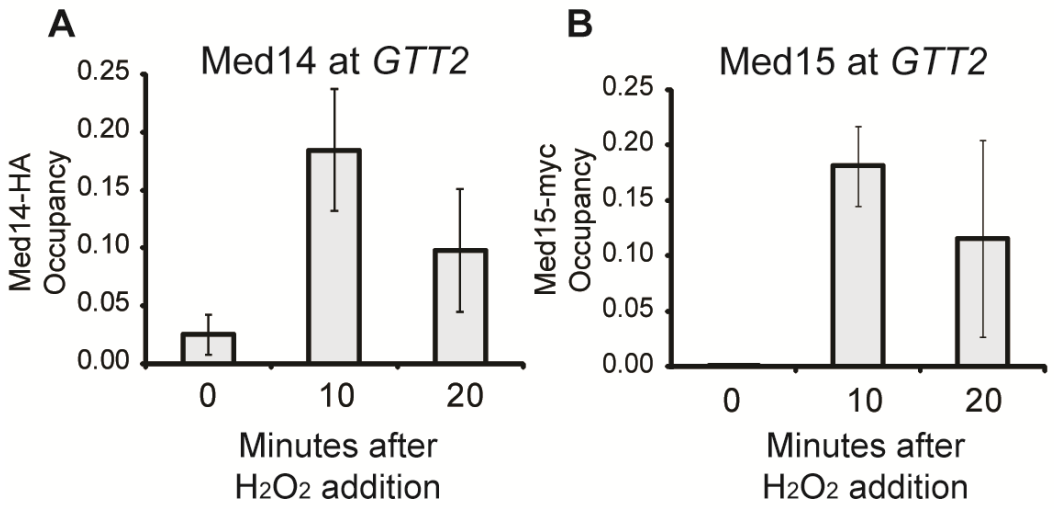

FIGURE 4.10. **Mediator is recruited to the *GTT2* promoter upon activation.** Occupancy of two proteins within the Mediator complex increases upon gene activation. Occupancy of Med14-HA (**A**) and Med15-myc (**B**) was determined with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted as background from the occupancy at *CYC1*. Bars represent the average ±SD of three samples (Med14-HA) processed independently from a master culture, or six samples processed independently from two master cultures (Med15-myc).

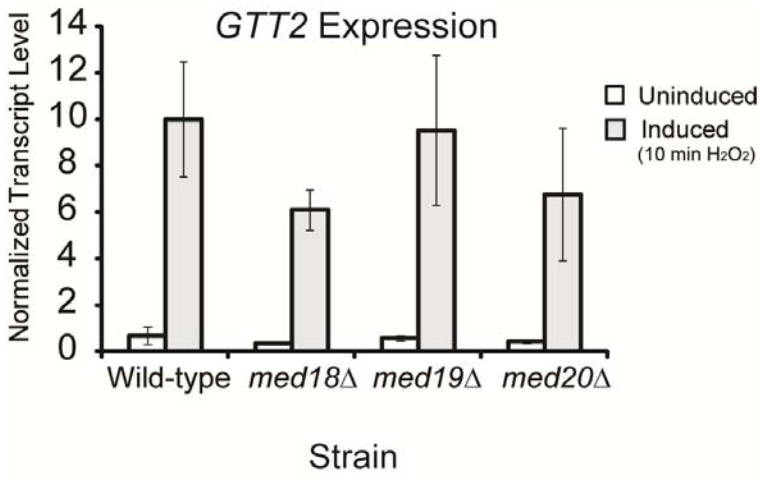

FIGURE 4.11. *GTT2* activation is largely independent of the Med18, Med19 and Med20 proteins. Transcript levels were analyzed with RNA prepared from the wild-type strain and strains containing deletions of the indicated Mediator subunit in both uninduced (YP-Glucose, unfilled bars) and induced (10 min H_2O_2 treatment, light gray bars) conditions. *GTT2* transcript was normalized to a *tRNA*^{w} probe in the S1 nuclease protection assays. Expression level in the wild-type strain 10 minutes after activation was set to 10. The average ±SD of three biological replicates is shown.

Med20) that is involved in stimulating transcription of genes with preloaded RNAPII at the promoter region.

Our study of the regulation of *GTT2* transcription revealed that a different regulatory strategy operates at this promoter compared to *CYC1*. This gene is recruitment-regulated, and does not rely on the Med18, Med19 and Med20 submodule, suggesting this submodule is specific to activation of *preloaded* complexes. This finding reveals that genes within the same regulon (Yap1 controls both genes) can be governed in fundamentally different ways. This is an important distinction, as it is broadly assumed that activators regulate target genes in a similar fashion. Instead, we show that Yap1 orchestrates transcription of genes within the two broad classes of regulation: recruitment and postrecruitment. This features the versatility of activator proteins. At *CYC1*, Yap1 stimulates a preloaded polymerase that is at the promoter DNA when Yap1 arrives after oxidative stress. At *GTT2*, Yap1 recruits polymerase to the promoter to initiate transcription. Clearly, this activator is elastic in its interactions. Indeed, proteomic studies of yeast cells show Yap1 physically interacts with over 60 proteins[42,189-193]. The activator presumably needs this repertoire of physical interactions to drive transcription at its target genes. Yap1 controls approximately 50 yeast genes in addition to *CYC1* and *GTT2*. Our data suggest that within this group of genes there are likely additional recruitment and postrecruitment regulated genes.

The polymerase at the *CYC1* promoter must be fundamentally different from the polymerase at the *GTT2* promoter, either in composition or in conformation. At *GTT2*, the polymerase is able to initiate transcription upon arrival (**FIGURE 4.12A**). At *CYC1*, we show the Med18, Med19 and Med20 submodule is required to stimulate the polymerase in some way prior to efficient transcription (**FIGURE 4.12B**).

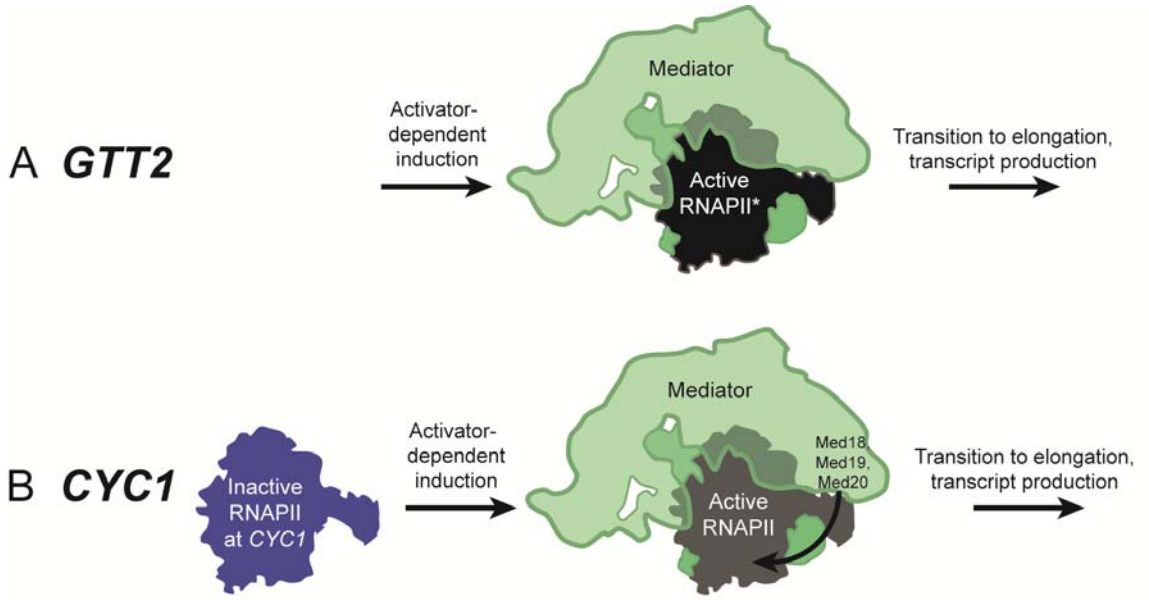

FIGURE 4.12. **Model highlighting the differences in the RNAPII at the *GTT2* and *CYC1* promoters. A**) Transcriptionally competent RNAPII is recruited to the *GTT2* promoter (RNAPII*, black) along with Mediator during induction. **B)** The inactive RNAPII (blue) at the *CYC1* promoter is converted into an active one (gray) in a Med18, Med19, Med20-dependent manner.

Why is the submodule not required at *GTT2* (**FIGURE 4.13A**)? Studies in both yeast cells and human cells support the idea that there are multiple populations of RNAPII-containing complexes[194,195]. Perhaps the specific context of each promoter results in a variation in the type of polymerase-containing complex that is recruited to each location. Characterizing the composition of the complexes at each promoter (*CYC1* and *GTT2*) in more detail will allow us to determine if different polymerase populations play a role in the regulation outlined in this paper.

What is the mechanistic function of the Med18, Med19, Med20 submodule at *CYC1*? Two scenarios are most likely. First, Med18, Med19 and Med20 could be required for recruitment of a missing factor involved in *CYC1* activation. However, deletion of proteins that could be involved in later step in the transcription process such as Swi/Snf and Spt4 do not result in transcript levels as low as deletion of Med18, Med19 and Med20 ([53] and our unpublished observations). This suggests these two complexes are not the missing factor. SAGA is also required for activation of the *CYC1* gene (**CHAPTER 3**[48], and **CHAPTER 5**). Yet, this complex occupies promoter DNA prior to Mediator recruitment (**CHAPTER 3**[48]), suggesting SAGA is not the missing factor. Future studies aimed at further characterizing the complex at the *CYC1* promoter will shed more light on the possibility that Med18, Med19 and Med20 are necessary for recruiting another factor. Second, Med18, Med19, and Med20 could be required for affecting a conformational change in RNAPII, shifting it to a more active conformation (**FIGURE 4.13B**). Electron microscopy analysis of Mediator particles with RNAPII revealed an extensive interface between Mediator and polymerase[113,114], and further work demonstrated that this interface is localized to the head and middle modules of Mediator[115,116]. More recent structural advances show that polymerase subunits bind in

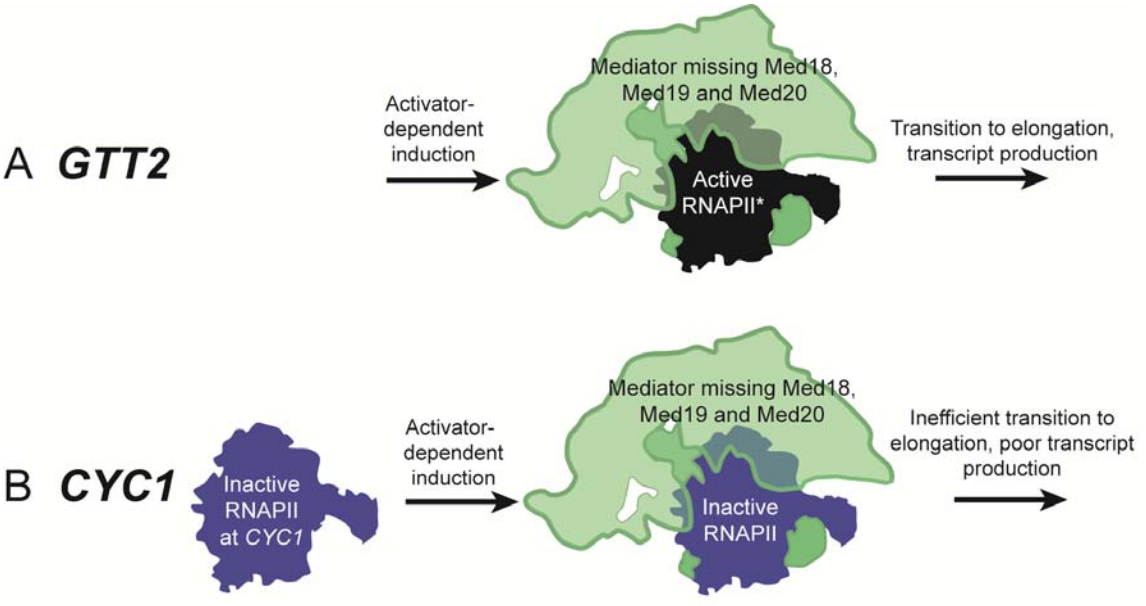

FIGURE 4.13. **Possible model for the differences in Med18, Med19 and Med20 requirement at *GTT2* and *CYC1.* A**) The RNAPII at the *GTT2* promoter does not rely on Med18, Med19 and Med20 for normal transcription. **B**) The RNAPII at the *CYC1* promoter requires Med18, Med19 and Med20 for the transition into elongation and transcript production.

close proximity to the location of the Med18 and Med20 subunits within the Mediator head[117]. Med18 and Med20 also physically interact with polymerase subunits, and display negative genetic interactions with them as well. Med19 is also important for RNAPII interaction. A purified Mediator complex missing only Med19 is unable to interact with RNAPII in a co-IP assay[177]. Together, this data highlights the importance of these proteins and suggests that they sit poised to play an essential function of interacting with, and perhaps stimulating, RNAPII by contributing to an active conformation of the enzyme. It is intriguing to speculate that at the *CYC1* promoter, interactions between Mediator and RNAPII result in the formation of a transcriptionally competent polymerase. The functional data we presented here considered with recent structural advances of the Mediator-polymerase holoenzyme strongly suggest that this is the case. Exactly how Med18, Med19 and Med20 affect the polymerase and allow transcription to begin at *CYC1* is an outstanding question. As postrecruitment regulation and the Mediator complex are conserved from yeast to higher eukaryotes, it is likely that Med18, Med19 and Med20 play key roles in the activation of poised promoters across the evolutionary spectrum. We therefore believe future investigation into the regulation of poised promoters and the role of the Mediator complex has the potential to transform our current view of transcription.

CHAPTER 5. DIFFERENTIAL CONTROL OF YAP1-DEPENDENT, OXIDATIVE STRESS RESPONSE GENES IN YEAST

In this chapter, I probe the regulation of a group of four Yap1-dependent oxidative stress response genes. I wrote this chapter and contributed all of the figures. I would like to thank Tyler Fara for helping prepare RNA, which I analyzed in **FIGURE 5.8**. Dr. Alan Hinnebusch provided the Med15-myc and Med15-myc *spt20*Δ strains.

5.1 INTRODUCTION

Aerobic organisms are continuously challenged with the formation of reactive oxygen species (ROS) generated via respiration. Exposure to ROS can lead to oxidative stress, which occurs when an imbalance between oxidants and antioxidants exists in favor of oxidants[21]. This condition is damaging, as ROS harm DNA, lipids, and proteins[23]. Yeast cells respond to oxidative stress induced by the ROS hydrogen peroxide with a dramatic and quick change in transcriptional programming[12]. During this biological response, the Yap1 transcriptional activator is responsible for the activation of approximately 50 yeast genes, comprising the Yap1 regulon.

The Yap1 protein contains a nuclear import and export sequence, and is regulated primarily by cellular localization[34]. During normal conditions, interaction with the nuclear exporter protein Crm1 maintains Yap1 primarily in the cytoplasm[180]. During oxidative stress the Yap1-Crm1 interaction is masked due to oxidant-induced changes in

the structure of the Yap1 protein itself, and Yap1 accumulates in the nucleus[34,181]. Once in the nucleus, Yap1 binds Yap1 Response Elements (YREs) in the promoter of target genes. These target genes encode gene products that act as antioxidants, heat shock proteins, drug transporters, and enzymes involved in carbohydrate metabolism[38]. While quite a bit is known about the regulation of the nuclear localization of Yap1, our understanding of the means by which it brings about activation is lacking. For instance, once in the nucleus, how does it generate rapid transcriptional activation?

The cell utilizes two basic regulatory strategies to control gene transcription; these are termed recruitment regulation and postrecruitment regulation. The timing of RNAPII occupancy at promoter DNA distinguishes the two types of regulation. We previously observed that Yap1 controls genes in each regulatory category (*GTT2* and *CYC1*, respectively, **CHAPTER 4**). At *GTT2*, Yap1 recruits RNAPII. At *CYC1*, Yap1 activates a preloaded RNAPII. As the majority of genes in yeast are thought to be recruitment-regulated (i.e. do not contain preloaded polymerase), it is unclear if additional postrecruitment regulated genes exist within the Yap1 regulon. Here, we present evidence that two additional Yap1-regulated genes, *TRX2* and *FLR1*, are postrecruitment regulated.

The means by which Yap1 activates transcription are poorly understood. We previously showed that Yap1 relies on the coactivator Mediator to control transcription of the *CYC1* target gene (**CHAPTER 4**). Does Yap1 also utilize other coactivators? Comparing genome-wide microarray data, Huisinga and Pugh found enrichment for SAGA-dependent genes within the group of genes upregulated by different types of stress[154]. This suggests that stress-inducible genes rely on the SAGA coactivator for expression. Furthermore, in the yeast *Candida albicans,* genome-wide chromatin immunoprecipitation (ChIP) revealed SAGA binds in the vicinity of stress-inducible

genes. This binding is functional, as several oxidative stress inducible genes in *Candida* were dependent on the Ada2 subunit of the SAGA complex for expression[196]. These reports are consistent with our previous finding that SAGA constitutively occupies the promoter region of the *CYC1* gene, and is required for *CYC1* expression during growth in ethanol (**CHAPTER 3**[48]). Finally, proteomic studies suggest several subunits of SAGA physically interact with Yap1 in budding yeast[189,191,197,198]. These findings prompted us to investigate the role of the SAGA coactivator at Yap1 dependent genes in budding yeast. Here, we show that the SAGA coactivator is required during oxidative stress in budding yeast, and the Yap1 activator utilizes this complex for driving expression of its target genes. SAGA genetically interacts extensively with the Mediator complex[99,144,162,168,199,200], suggesting a functional interplay between these two complexes. We found Mediator recruitment to each Yap1 target gene, but this recruitment is independent of the SAGA complex. Together, this study reveals a complex regulation of the Yap1 gene network; however, a commonality is the dependence on the SAGA coactivator for expression.

5.2 RESULTS

5.2.1 Yap1 controls *GTT2*, *TRX2*, *FLR1*, and *CYC1* transcription in response to oxidative stress: To understand how Yap1 brings about transcriptional activation of its target genes, we first examined the kinetics of activation and Yap1-dependency of four genes, *GTT2*, *TRX2*, *FLR1* and *CYC1*. We found that the transcriptional response to H_2O_2 is very fast; each gene analyzed exhibited maximal levels of transcript 10-20 minutes after the addition of H_2O_2 to the cell culture (**FIGURE 5.1**). RNA analysis of the wild-type strain and a strain missing the Yap1 activator (*yap1*Δ) demonstrates each gene responds to oxidative stress in a Yap1-dependent manner (**FIGURE 5.1**). In contrast to *GTT2*, *FLR1* and *CYC1*, the *TRX2* gene also has a Yap1-independent response to

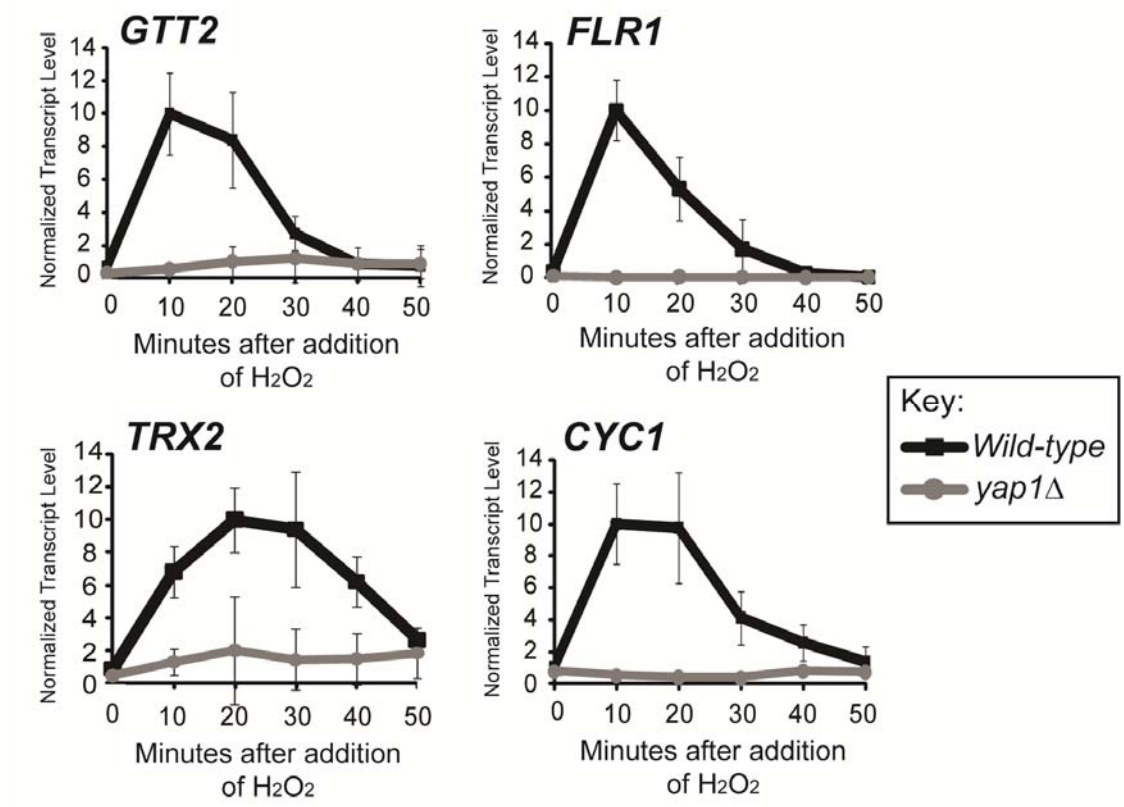

FIGURE 5.1. Gene expression profiles during oxidative stress. Transcript levels of each gene are rapidly induced in response to oxidative stress (black line). An S1 nuclease protection assay was performed with RNA isolated from wild-type and *yap1*Δ cells before exposure to H_2O_2 and in 10 minute intervals after H_2O_2 was exposure (0.3 mM). Increased expression of each gene is Yap1-dependent, as transcript levels are not induced in the *yap1*Δ strain (gray line), or induced at very low levels (*TRX2*). A probe for *tRNA*^{w} was used as a loading control in the S1 nuclease assays and the transcript level in the wild-type strain 10 or 20 minutes after H_2O_2 exposure was set to 10. For the wild-type transcript level, points represent the average of at least three biological replicates ±SD. For the transcript level in the *yap1*Δ strain, points represent the average of two biological replicates ±range.

H_2O_2. Transcript levels of *TRX2* increased slightly in the strain deleted for Yap1 (*yap1*Δ). The response is greatly impaired, reaching only 8-20% of the wild-type transcript level, but it does demonstrate a Yap1-independent response to oxidative stress at *TRX2*.

In order to determine if the Yap1-dependent transcription is a direct result of Yap1 promoter occupancy, a ChIP was performed to investigate Yap1 occupancy at each promoter region before and after induction with H_2O_2. Yap1 does not occupy promoter DNA prior to oxidative stress at each gene tested (**FIGURE 5.2**). This is consistent with the current model of Yap1 regulation, in which the protein is localized to the cytoplasm during normal conditions. After the addition of H_2O_2, Yap1 occupies each promoter region. This demonstrates that Yap1 directly regulates each gene, as we previously showed for *CYC1* and *GTT2* (**CHAPTER 4**). In all cases Yap1 promoter occupancy occurs very quickly, reaching maximal occupancy at just 10 minutes after induction with H_2O_2 (**FIGURE 5.2**).

5.2.2 Prevalence of postrecruitment regulation within the Yap1 regulon: Recent studies demonstrate that postrecruitment regulation is widespread[59-61]. However, the majority of genes in yeast are still thought to be recruitment-regulated[52,201]. Our previous observation that Yap1 controls genes in each regulatory category (*CYC1* and *GTT2*, **CHAPTER 4**) led us to question if other postrecruitment regulated genes exist within the Yap1 regulon, or if *CYC1* is unique. To answer this question, we examined two additional Yap1-dependent genes, *TRX2* and *FLR1*. To classify a gene as recruitment or postrecruitment regulated, we examined two features: RNAPII occupancy in the uninduced and induced conditions, and the change in transcript level upon induction.

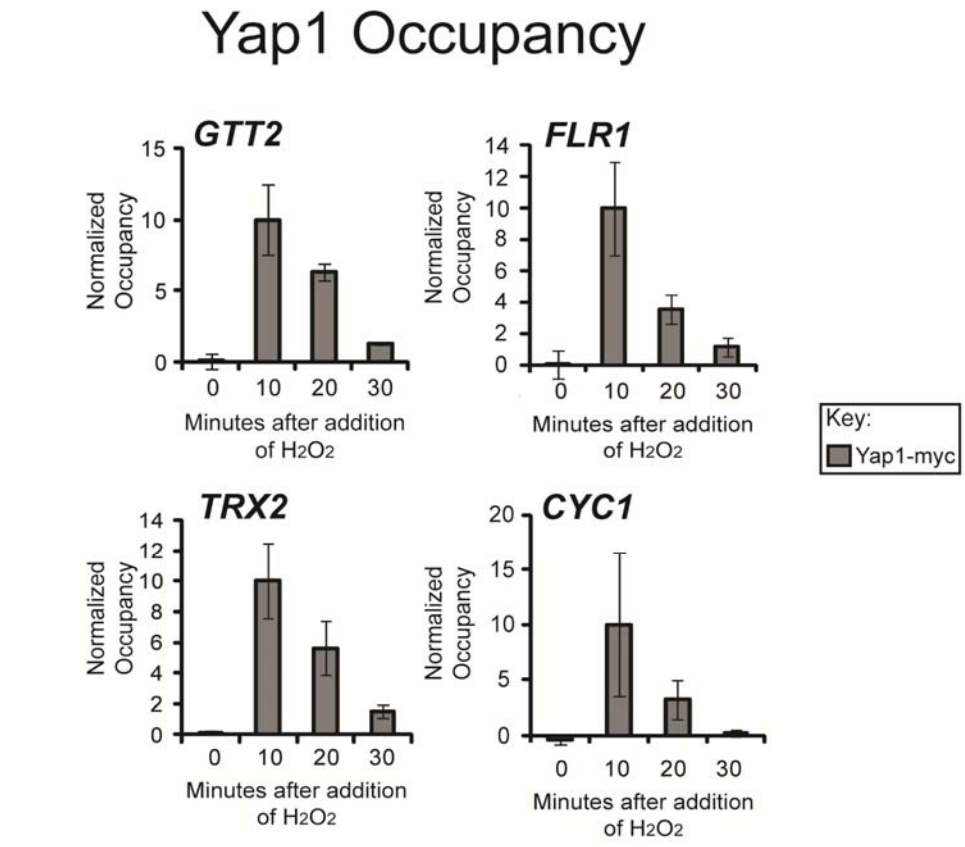

FIGURE 5.2. Yap1 occupies the *GTT2*, *TRX2*, *FLR1* and *CYC1* promoter regions during oxidative stress. Occupancy was determined with a ChIP using a Yap1-myc strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at each gene to remove non-specific signal. The occupancy at the 10 minute time point was then set to 10. Bars represent the average ±SD of three samples processed independently from a master culture.

RNAPII occupancy was determined before and after induction at the promoter regions of *GTT2*, *TRX2*, *FLR1* and *CYC1* (as a control). In addition to examining the level of RNAPII occupancy at these genes, we also looked at RNAPII occupancy at two non-transcribed regions of the genome. These areas represent background or non-specific binding. The non-transcribed regions are an area proximal to the telomere of the right arm of Chromosome VI, and the repressed *GAL10* promoter. Occupancy of RNAPII at the promoter regions of *TRX2*, *CYC1*, and *FLR1* is significantly higher than occupancy detected at these two regions (**FIGURE 5.3**).

Therefore, these genes are marked by high levels of RNAPII prior to Yap1-dependent activated transcription (no H_2O_2). The second feature we examined to classify each gene was the fold change in occupancy upon induction. By definition, the promoter regions of postrecruitment regulated genes undergo small changes in polymerase occupancy during induction[202]. These changes are significantly smaller than the change in transcript level observed from the gene. At *TRX2*, *FLR1* and *CYC1* the change in RNAPII occupancy is quite small (<3-fold) upon induction (**FIGURE 5.4**). This is despite large changes (>10-fold) in transcript production at each gene (summarized in **TABLE 5.1**). Therefore, the change in transcript level is inconsistent with the change in RNAPII occupancy. Given that *TRX2*, *CYC1,* and *FLR1* have RNAPII present before induction (with little/no transcripts present), and the change in occupancy upon induction is inconsistent with the change in transcript level, these genes are postrecruitment regulated.

GTT2 has a distinct regulatory mechanism that is indicative of recruitment regulation. Prior to induction, *GTT2* transcript levels are quite low, and very low levels of RNAPII are present at this promoter. In fact, occupancy is not significantly different from

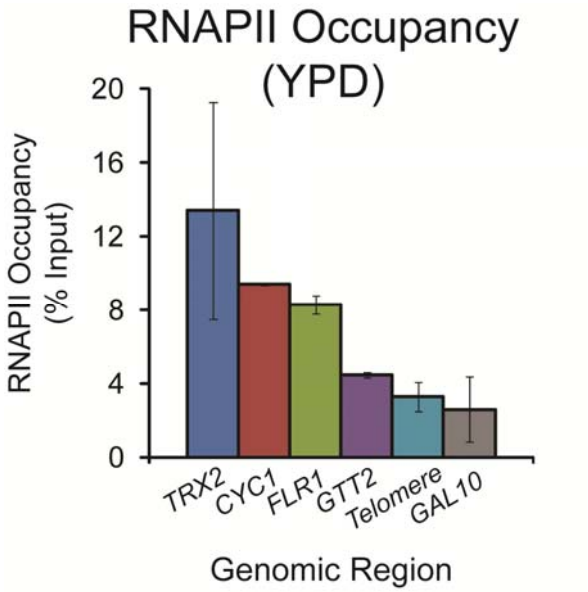

FIGURE 5.3. RNAPII occupancy at six genomic regions suggests preloading at *TRX2*, *CYC1* and *FLR1*. Occupancy at the indicated genomic region in uninduced conditions (growth in YP-glucose) was calculated using the % Input method. The *TRX2*, *CYC1* and *FLR1* promoter regions have higher RNAPII occupancy than the *GTT2* promoter, a region proximal to the telomere on chromosome VI, and the *GAL10* promoter region. The difference in occupancy at the *FLR1* and *GTT2* promoters is statistically significant (p=0.0003 using a two-tailed unpaired T-test). The difference in occupancy at the *GTT2* and telomere or *GAL10* promoter region is not statistically significant (p=0.076 and 0.146, respectively). Bars represent the average ±SD of three samples processed independently from a master culture.

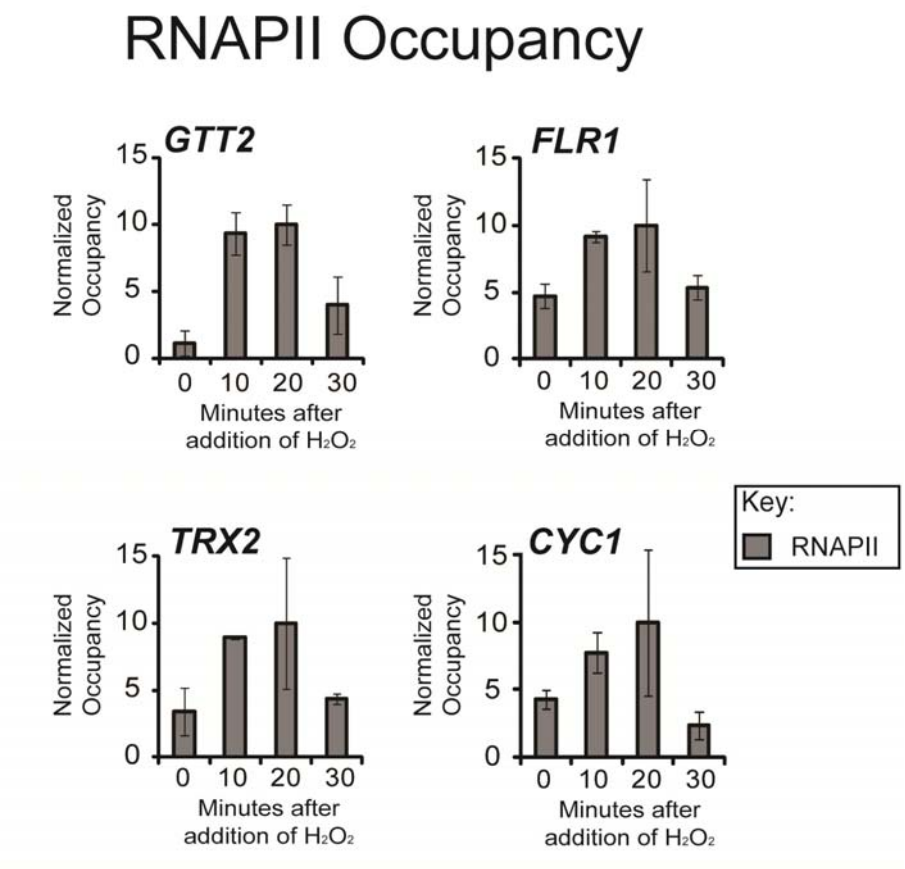

FIGURE 5.4. RNAPII occupancy profile at *GTT2*, *TRX2*, *FLR1* and *CYC1*. Occupancy was determined with a ChIP of the wild-type strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the telomere proximal region was subtracted from the occupancy at each gene to remove non-specific signal. The occupancy at the 10 minute time point was then set to 10. Bars represent the average ±SD of three samples processed independently from a master culture.

the telomere or *GAL10* control regions of the genome (**FIGURE 5.3**). Upon induction, the amount of RNAPII present at the promoter changes greatly (>8-fold) (**FIGURE 5.4**), along with transcript levels (>10-fold) (summarized in **TABLE 5.1**). The regulatory strategy at *GTT2* is similar to the well-characterized recruitment-regulated *GAL10* gene. At *GAL10*, RNAPII does not occupy promoter DNA prior to induction, but is recruited during activation with a concomitant increase in transcript levels (**CHAPTER 3**[48]).

TABLE 5.1. Summary of gene characteristics leading to their classification as recruitment or postrecruitment regulated.

GENE	FOLD CHANGE IN TRANSCRIPT LEVEL[a]	FOLD CHANGE IN RNAPII OCCUPANCY[b]	CLASSIFICATION
GTT2	26-fold	8.8	Recruitment
TRX2	30-fold	2.9	Postrecruitment
FLR1	30-fold	2.1	Postrecruitment
CYC1	10-fold	2.3	Postrecruitment

[a] Fold change in gene transcript level determined using an S1 nuclease assay. Transcript level after induction was divided by the transcript level before induction to calculate fold change.

[b] Fold change in RNAPII occupancy determined with a chromatin immunoprecipitation assay. Occupancy after induction was divided by the occupancy before induction to calculate fold change.

5.2.3 SAGA subunits are required for cell growth on plates containing H_2O_2: SAGA is a conserved coactivator composed of 20 subunits[46,150,203]. The complex is a target of activator proteins, and serves as a classic coactivator by facilitating activated transcription at many genes[46]. Several subunits of SAGA physically interact with Yap1[189,191,197,198] (**TABLE 5.2**). SAGA also directly interacts with members of the general transcription machinery such as TPB[99]. The complex contains two chromatin-modifying functions. The Gcn5 protein is a histone acetyltransferase, and the Ubp8 protein deubiquitinates histone H2B. The histone-modifying functions of the SAGA complex

contribute to its function in transcription by promoting classic marks of active chromatin[87].

Genetic and biochemical analysis of the SAGA complex has resulted in classification of its 20 subunits into various functional categories (**TABLE 5.2**).

TABLE 5.2. Functional classification of yeast SAGA subunits.

FUNCTIONAL CATEGORY	SUBUNIT NAME	PHYSICAL INTERACTION WITH YAP1
Structural integrity of the complex	Spt7	✓[a]
	Spt20	✓
	Ada1	✓
Histone acetyltransferase activity	Gcn5	✓
	Ada2	✓
	Ada3	
Histone deubiquitination activity	Ubp8	
	Sgf11	
	Sgf73	
	Sus1	
Unknown	Sgf29	✓
Chromatin remodeling	Chd1	
Interaction with basal transcription machinery	Taf5*[b]	
	Taf6*	✓
	Taf9*	
	Taf10*	
	Taf12*	
	Spt3	✓
	Spt8	
Activator interaction	Tra1*	✓

[a] Proteins that physically interact with Yap1 are indicated with a check mark, interactions were downloaded from the BioGrid repository May 6, 2009 (www.thebiogrid.org).

[b] Subunits marked with an asterisk are essential for viability.

We tested the global requirement for SAGA during oxidative stress by analyzing cell growth of strains containing a deletion in one of the 14 non-essential SAGA subunits on plates containing a range of H_2O_2 concentrations. This phenotypic analysis revealed several strains missing subunits of the SAGA complex are sensitive to H_2O_2 (**FIGURE 5.5**).

Strains containing deletions in genes required for the structural integrity of the SAGA complex were the most sensitive to growth on plates containing H_2O_2. In each of these three strains (*spt7*Δ, *spt20*Δ, and *ada1*Δ), the complex fails to form, rendering them functionally SAGA-null[72,96,99]. While each was sensitive to H_2O_2, the *ada1*Δ strain had the most dramatic phenotype, showing growth sensitivity on plates with the lowest concentration of H_2O_2. The *spt20*Δ strain is also sensitive to oxidative stress, but grows slightly better when compared to the *ada1*Δ strain. The *spt7*Δ strain is also sensitive to oxidative stress, but this is only evident on plates with higher concentrations of H_2O_2.

The remaining SAGA subunits are not required for the integrity of the complex (**Table 5.2**). However, deletion of individual subunits compromises specific functions of SAGA. Spt3 and Spt8 contact the basal machinery, and deliver TBP to promoter DNA at a variety of promoters[93-98]. Strains missing either of these two proteins showed only slight sensitivity to growth on H_2O_2. The HAT module of SAGA is comprised of Gcn5, the acetyltransferase enzyme, and accessory factors Ada2 and Ada3[152]. These strains are also slightly sensitive to growth during oxidative stress, with *ada3*Δ showing the most sensitivity. SAGA also contains histone H2B deubiquitination activity, encoded by the Ubp8 subunit. The in vivo activity of Ubp8 relies on the Sgf11, Sus1 and Sgf73 proteins[85,86]. These subunits form a highly interconnected subcomplex within SAGA[204].

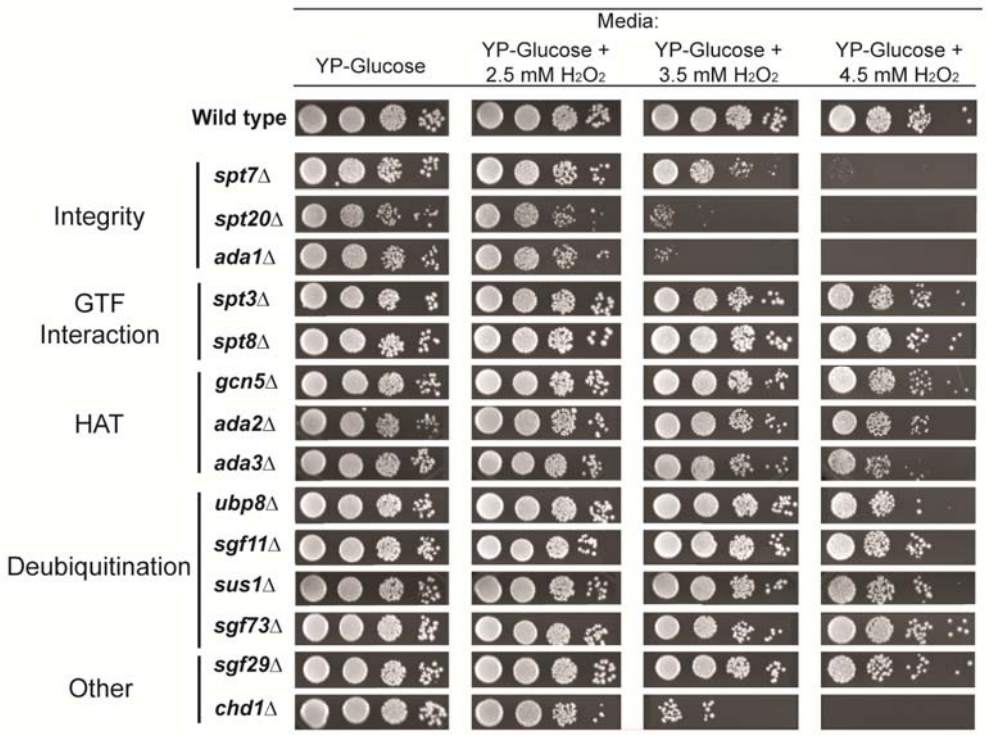

FIGURE 5.5. SAGA is essential for growth during oxidative stress. Serial spot dilutions of the wild-type strain and strains containing deletions of 14 SAGA subunits on YP-Glucose and on YP-Glucose supplemented with three concentrations of H_2O_2. Plates were incubated at 30^0C for three days before photographing. Two to three independent biological samples were spotted with little difference between samples. Representative spots shown here.

Strains containing deletions in each of these subunits show very slight sensitivity to the highest concentration of H_2O_2. The strain containing a deletion of the chromatin remodeler Chd1 protein was very sensitive to H_2O_2. The *sgf29*Δ strain contains a deletion of a protein with an unknown function within the SAGA complex. This strain also shows slight sensitivity to growth on H_2O_2.

Taken together, this phenotypic analysis reveals a very important role of the subunits involved in the integrity of the SAGA complex (Spt7, Spt20, and Ada1) for normal growth during oxidative stress. This is consistent with the finding that stress-inducible genes tend to be SAGA-dependent[154], but beyond this, demonstrates a function of SAGA in regulating genes important for cell survival during oxidative stress. A number of the strains containing mutations that do not result in abolishing SAGA integrity have intermediate sensitivities. These intermediate phenotypes suggest there could be different degrees of transcriptional defect at oxidative stress response genes in these strains.

5.2.4 SAGA is involved in activation of *GTT2*, *TRX2*, *FLR1* and *CYC1* during oxidative stress: SAGA is clearly required at a global level during oxidative stress. However, the gross phenotypes we observed can result from the combined transcriptional defects of many oxidative stress response genes. To determine if SAGA plays a role at the *GTT2*, *TRX2*, *FLR1* and *CYC1* genes, we determined SAGA occupancy and transcriptional dependence of these genes during oxidative stress.

To determine SAGA occupancy, a ChIP assay was performed detecting Spt8 occupancy. The Spt8 protein is present only in the SAGA complex, and not in a related complex termed SLIK[153]. Therefore, Spt8 occupancy is a direct test of SAGA occupancy.

The pattern of Spt8 occupancy observed differed among the promoters (**FIGURE 5.6**). At *FLR1* and *CYC1*, SAGA occupancy only changed 2-fold during activation. This is consistent with our previous demonstration that SAGA constitutively occupies the *CYC1* promoter (**CHAPTER 3**[48]). At *GTT2* and *TRX2*, occupancy changed about 5-fold. Therefore, SAGA occupies each gene promoter, but it appears to occupy the *FLR1* and *CYC1* preloaded promoters prior to, and during, gene activity.

To test if SAGA is required for transcription of Yap1 dependent genes, we analyzed transcript levels of *GTT2*, *TRX2*, *FLR1* and *CYC1* in strains missing components of the SAGA complex. We found that each gene analyzed is SAGA-dependent (assayed via the transcript production in the strain missing the SAGA integrity subunit Spt20). However, the role of SAGA is gene-specific, as demonstrated by the differences in protein dependencies at each gene.

GTT2 expression during oxidative stress is SAGA-dependent, since deletion of the *SPT20* gene results in lower transcript levels compared to the wild-type strain (**FIGURE 5.7**). Analysis of several strains containing subunit deletions reveals *GTT2* relies on Spt3 and Spt8 for full expression. The Spt3 and Spt8 proteins interact with TBP and deliver TBP to some promoters in yeast[93-98]. *GTT2* is also dependent on the SAGA subunits controlling the HAT activity of the complex. Transcript levels were compromised in the strains missing the Gcn5, Ada2 and Ada3 proteins. This suggests that *GTT2* expression requires the histone acetyltransferase activity of the SAGA complex for full activation. Expression did not require the Ubp8 subunit of SAGA as transcript levels in the *ubp8*Δ strain looked very similar to the wild-type strain. Therefore, *GTT2* is strongly dependent on the SAGA complex for transcriptional activation during oxidative stress. This catalog of subunit dependencies suggests that *GTT2* expression relies on the function of Spt3 and Spt8, and the HAT activity of the complex for expression.

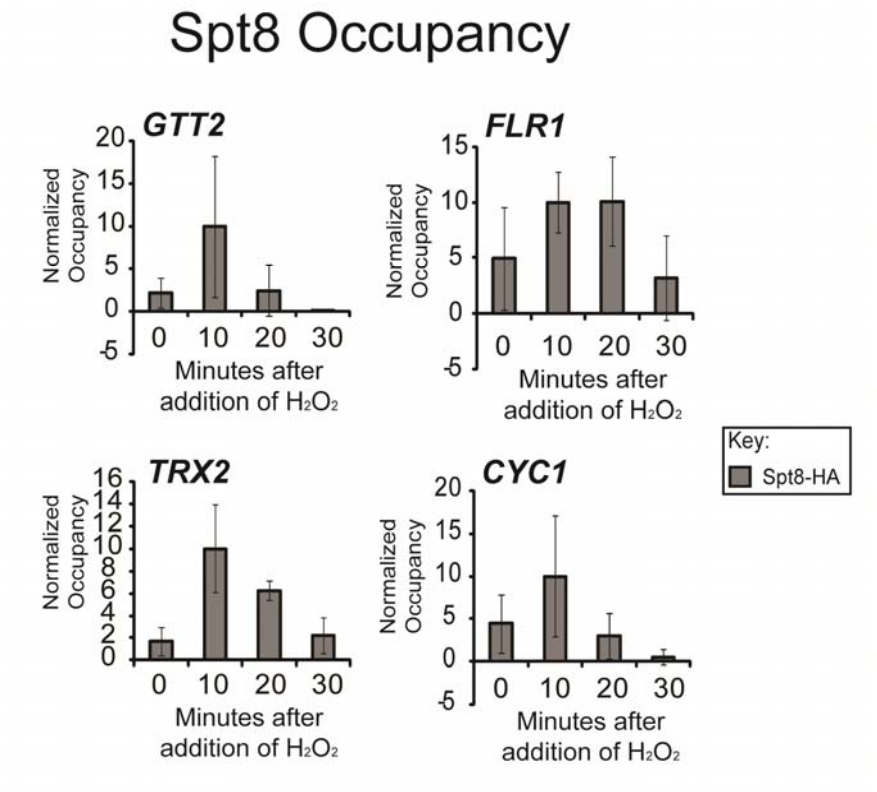

FIGURE 5.6. SAGA occupancy profile at *GTT2*, *TRX2*, *FLR1* and *CYC1*. Occupancy of Spt8 was determined with a ChIP of the Spt8-HA strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at each gene to remove non-specific signal. The occupancy at the 10 minute time point was then set to 10. Bars represent the average ±SD of three samples processed independently from a master culture.

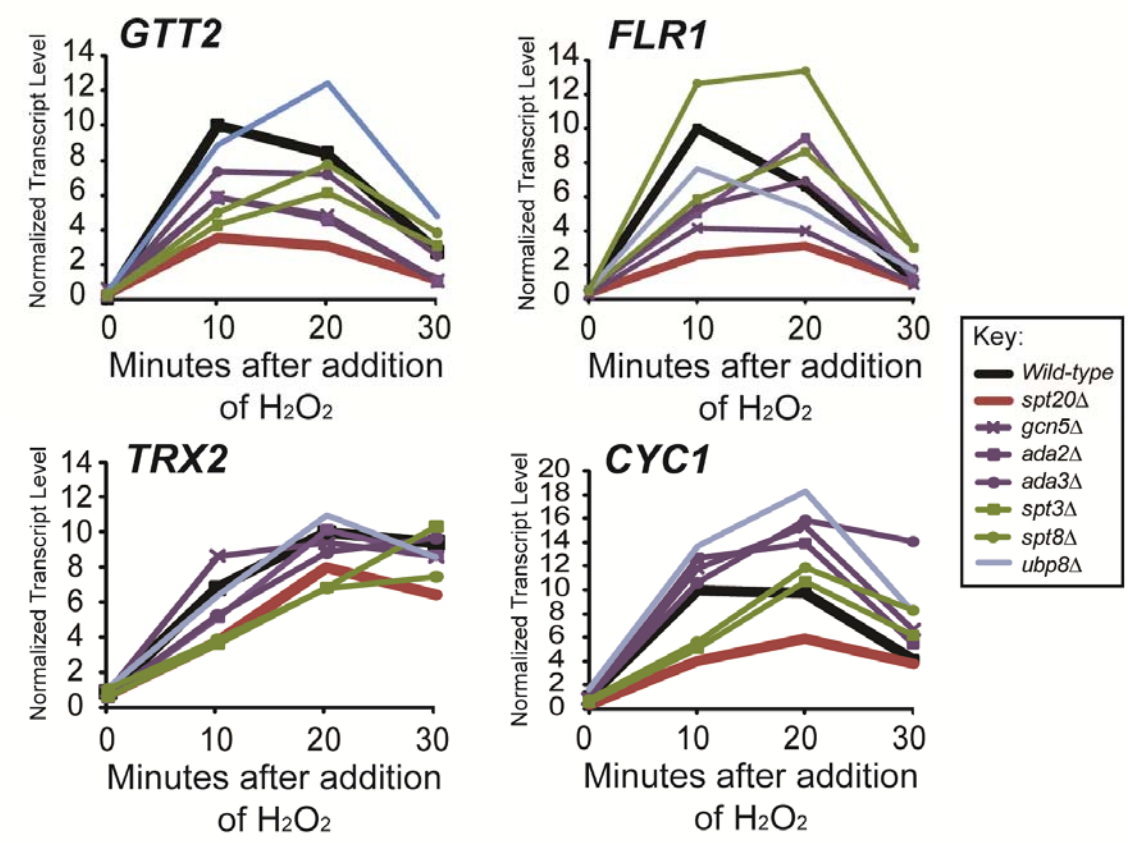

FIGURE 5.7. SAGA is required for gene expression during oxidative stress. An S1 nuclease protection assay was performed with RNA isolated from the wild-type strain and strains containing deletions of seven SAGA subunits. Samples were taken before exposure to H_2O_2 and in 10 minute intervals after H_2O_2 was added to liquid media at a final concentration of 0.3 mM. A probe for *tRNA*^{w} was used as a loading control in the S1 nuclease assays and the transcript level in the wild-type strain 10 or 20 minutes after H_2O_2 exposure was set to 10. For the wild-type transcript level, points represent the average of at least three biological replicates. For the transcript level in the SAGA deletion strains, points represent the transcript level from one biological replicate, except for the following. At *FLR1*, transcript levels in the *gcn5*Δ and *spt20*Δ strains represent the average of two biological replicates. For *CYC1*, transcript levels at the 0 and 10 minute time point represent the average of three biological replicates.

TRX2 expression is partially SAGA-dependent (**FIGURE 5.7**). Transcript levels in the *spt20*Δ strain are decreased compared to the wild-type strain, however, levels are not completely abolished. *TRX2* expression in the strains missing the Spt3 and Spt8 proteins match the level found in the *spt20*Δ strain, suggesting that the primary function of SAGA at *TRX2* involves Spt3 and Spt8. *TRX2* transcript levels are completely independent of Gcn5 and Ubp8. This demonstrates *TRX2* does not require the chromatin modifying activities of the SAGA complex for expression.

FLR1 expression during oxidative stress is also SAGA-dependent (**FIGURE 5.7**). The HAT module (Gcn5, Ada2 and Ada3) is important for *FLR1* expression, as transcript levels are down in strains containing deletions in these three subunits. *FLR1* is not dependent on the Ubp8 subunit of SAGA for expression. Transcript levels were also not dependent on the Spt3 subunit. The other TBP-interacting SAGA subunit, Spt8, may play a repressive role at *FLR1* as deletion of Spt8 results in an increased amount of *FLR1* transcript. This occurs only after activation, and not in the uninduced condition. Therefore, *FLR1* is SAGA-dependent, and relies primarily on the SAGA subunits responsible for HAT activity for full expression.

CYC1 expression during oxidative stress is dependent upon the SAGA complex for full activation (**FIGURE 5.7**). Deletion of *SPT20* results in diminished levels of *CYC1* transcript, both in the uninduced and induced conditions. Expression is not dependent on the Gcn5 protein during oxidative stress, as transcript levels were similar to wild-type in the *gcn5*Δ strain. Further, the histone acetyltransferase accessory factors (the Ada2 and Ada3 proteins), are also not required for normal transcription of this gene. Together,

this shows *CYC1* does not rely on the HAT activity of SAGA during oxidative stress. Similarly, Ubp8 is not required for transcription, demonstrating the histone deubiquitination activity of the SAGA complex is not important for *CYC1* transcription. We also analyzed *CYC1* expression in several additional SAGA deletion strains (**FIGURE 5.8A and B**). This expanded probing revealed that in addition to its dependence on Spt20, *CYC1* also requires the other SAGA integrity subunits for expression (Spt7 and Ada1). The deubiquitination accessory proteins (Sgf11, Sus1, Sgf73), the chromatin remodeler (Chd1), and protein of unknown function (Sgf29) are not required for expression of *CYC1* during oxidative stress.

5.2.5 SAGA is not required for Mediator occupancy at Yap1-dependent genes: Mediator is a coactivator that interacts extensively with RNAPII[115,117]. There are extensive genetic interactions between subunits of the SAGA complex and Mediator complex (see **APPENDIX I**)[99,144,162,168,199,200]. Mediator also collaborates with SAGA to regulate the expression of wide array of promoters[96,162,166,167]. Previously, we found Mediator is recruited to the *CYC1* and *GTT2* promoter regions during oxidative stress (**CHAPTER 4**), and is required for activation of the *CYC1* gene during oxidative stress (**CHAPTER 4**), and during growth in ethanol (**CHAPTER 3**).

To test if Mediator occupancy is a common feature of Yap1-dependent genes, we performed a ChIP for Mediator (using a tagged allele of Med15) in uninduced conditions and after activation. We found Mediator does not occupy the promoter region of *GTT2*, *TRX2*, *FLR1* or *CYC1* prior to activation (**FIGURE 5.9**). Upon induction, Mediator occupancy at each promoter increased, demonstrating Mediator recruitment during activation. It is interesting to note that this data demonstrates that Mediator is a better

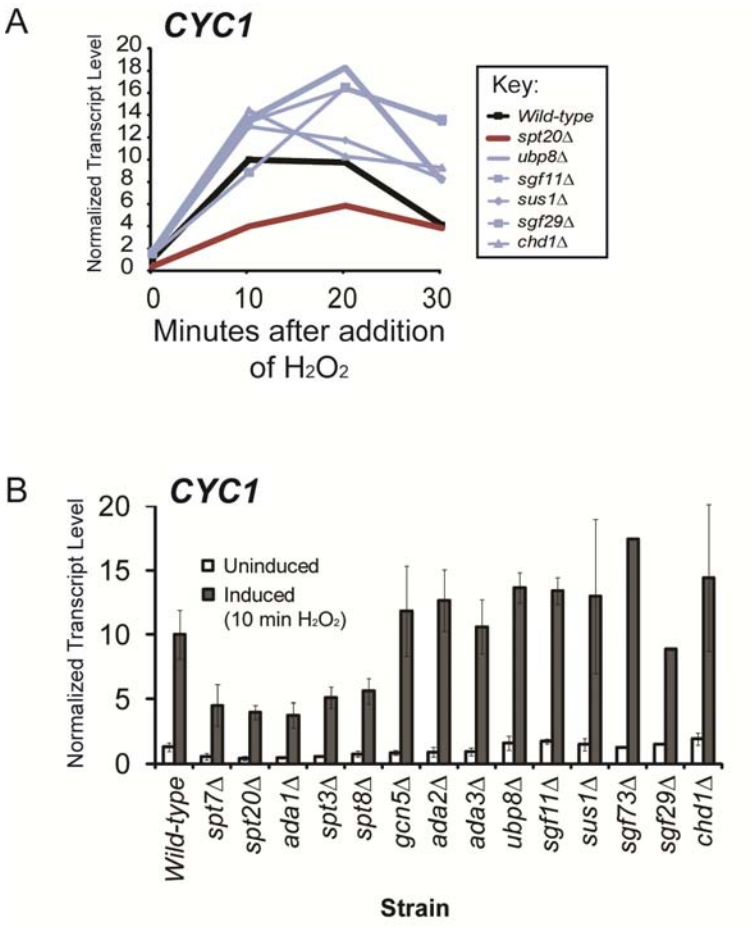

FIGURE 5.8. SAGA is required for gene expression of *CYC1*. S1 nuclease protection assays were performed with RNA isolated from the wild-type strain and strains containing deletions of SAGA subunits. A probe for *tRNA*^{w} was used as a loading control in the S1 nuclease assays and the transcript level in the wild-type strain 10 minutes after H_2O_2 exposure was set to 10. **A)** Time course analysis of *CYC1* expression in strains containing deletions in subunits involved in the deubiquitination activity of SAGA. Samples were taken before exposure to H_2O_2 and after 10, 20 and 30 minutes of exposure to H_2O_2. **B)** *CYC1* expression before and after induction with H_2O_2. Bars represent the average ±SD of three biological replicates for each strain except *sgf73*Δ and *sgf29*Δ, which were performed on only one replicate.

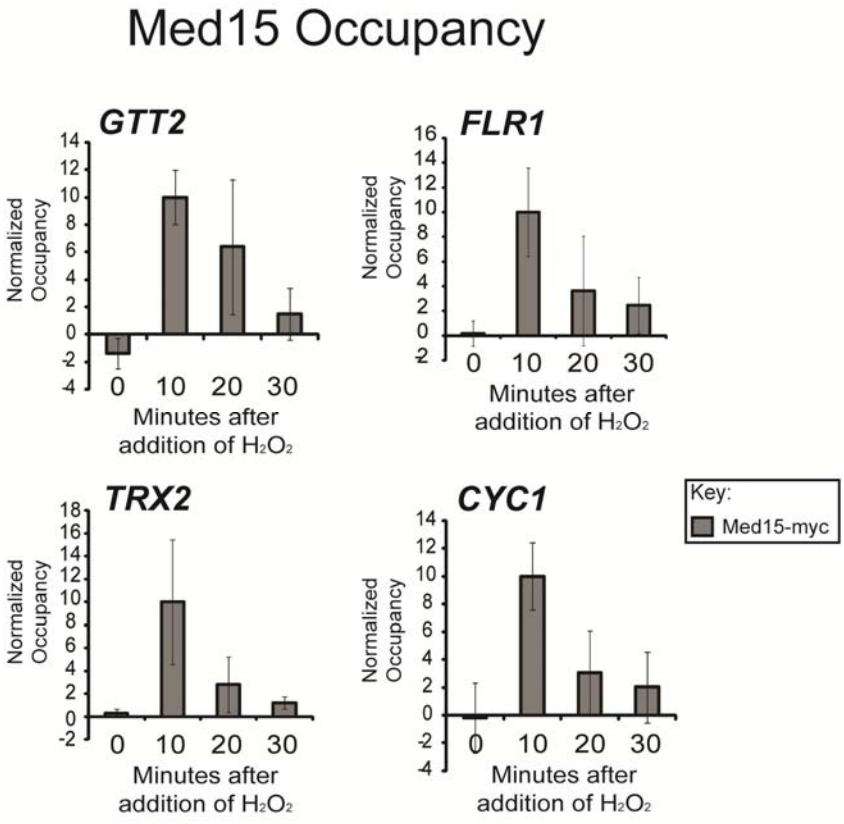

FIGURE 5.9. Mediator is recruited during oxidative stress, and is not present with RNAPII at preloaded genes prior to induction. Occupancy of Med15 was determined with a ChIP of the Med15-myc strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at each gene to remove non-specific signal. The occupancy at the 10 minute time point was then set to 10. Bars represent the average ±SD of three samples processed independently from a master culture.

indicator of active transcription than RNAPII itself, as an inactive RNAPII occupies *TRX2*, *FLR1* and *CYC1* prior to activation (**FIGURE 5.3**).

SAGA and Mediator cooperate with each other to regulate the expression of many promoters in yeast[96,162,166,167], and in humans[167]. Indeed, another aspect of SAGA function is its interaction with Mediator. For instance, at the Gcn4-regulated *ARG4* and *SNZ1* genes, SAGA and Mediator occupancy is co-dependent. In other words, Mediator occupancy at these promoters is decreased in strains containing SAGA deletions[96]. We know that SAGA **(FIGURE 5.7)** and Mediator (**CHAPTER 4**) are both important for activation of many Yap1-dependent genes, so we were curious to investigate if Mediator recruitment is dependent on SAGA. To test this, we looked for Mediator (Med15-myc) occupancy in a wild-type strain and a strain containing a deletion of the *SPT20* gene. Spt20 is an important SAGA subunit that is required for integrity of the entire complex[72,96,99]. Indeed, *GTT2*, *TRX2*, *FLR1* and *CYC1* each require the Spt20 protein for expression (**FIGURE 5.7**). We found Mediator is still recruited to each promoter region even in the absence of SAGA (**FIGURE 5.10**). This is in stark contrast to the reported case at the Gcn4-regulated *ARG4* and *SNZ1* genes[96]. However, it is consistent with our previous observation that Mediator recruitment to the *CYC1* gene during growth in ethanol is SAGA-independent (**CHAPTER 3**[48]).

5.3 DISCUSSION

The oxidative stress response involves the rapid activation of genes involved in cellular detoxification, heat shock proteins, drug transporters, and enzymes involved in carbohydrate metabolism[38]. We previously found that the Yap1 transcriptional activator controls the *CYC1* and *GTT2* genes during oxidative stress (**CHAPTER 4**). Furthermore, the *CYC1* gene is postrecruitment regulated while the *GTT2* gene is recruitment-

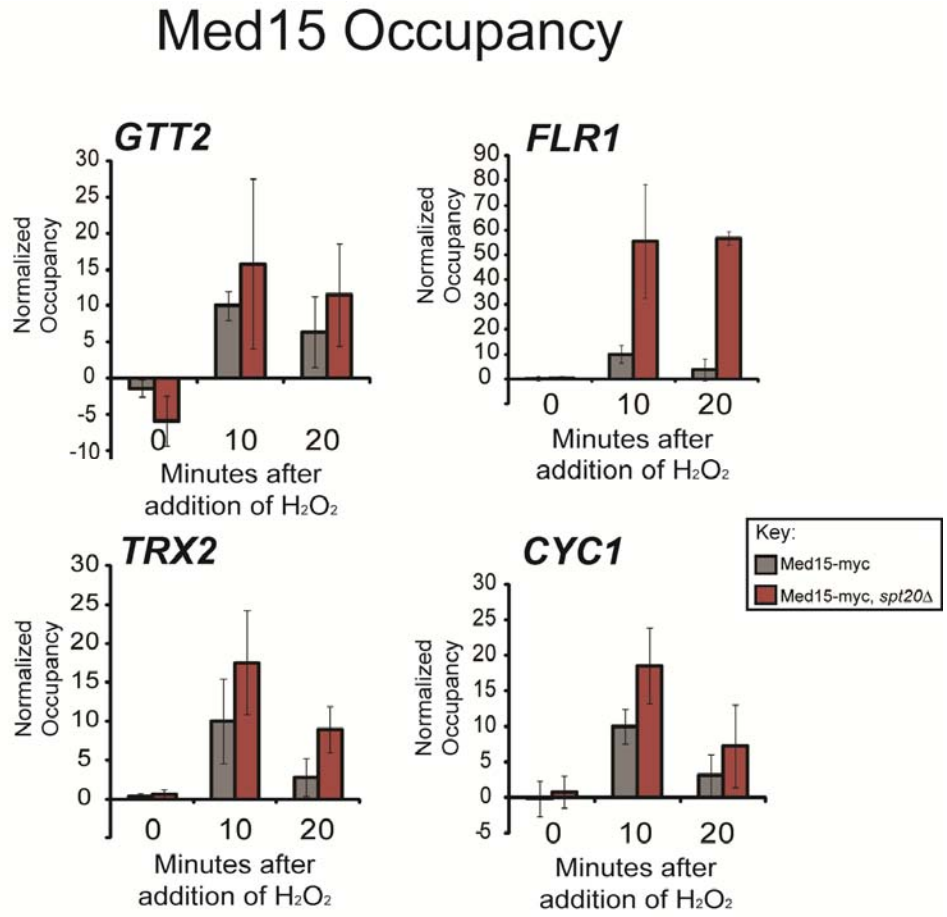

FIGURE 5.10. Mediator occupancy does not require SAGA. Occupancy of Med15-myc was determined with a chromatin immunoprecipitation in the wild-type strain (Med15-myc) and *spt20*Δ strain (Med15-myc *spt20*Δ). Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the x-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at each gene to remove non-specific signal. The occupancy at the 10 minute time point was then set to 10. Bars represent the average ±SD of three samples processed independently from a master culture.

regulated (**CHAPTER 4**). Here, we investigated the regulatory strategy controlling the expression of two additional oxidative stress response genes: *TRX2* and *FLR1*. We also expanded our characterization of *CYC1* and *GTT2*, and found that all four Yap1 dependent genes rely on the SAGA coactivator complex for expression, but SAGA is not required for Mediator recruitment.

Despite each gene being dependent on the SAGA complex for expression, we found distinct functions of this multiprotein complex are required at each gene. Our finding that specific genes require distinct SAGA subunits for activation is particularly interesting given that the same activator protein controls each gene. This demonstrates the fluidity in transcriptional regulation; even within a group of genes controlled by the same activator, differences in coactivator requirements exist.

One well-characterized function of the SAGA complex is acetylation of histone tails via the Gcn5 protein. Histone acetylation is strongly correlated with transcriptional activation[75-77]. Only two of the oxidative stress response genes analyzed here requires the subunits containing HAT activity for expression: *GTT2* and *FLR1*. Both of these genes required each member of the HAT module (Gcn5, Ada2 and Ada3) for normal expression, suggesting that the HAT function of SAGA is required for expression of these genes. Meanwhile, *TRX2* and *CYC1* expression was not responsive to deletion of any of these subunits (Gcn5, Ada2 or Ada3). Therefore, the HAT activity of the complex is not required for proper expression of these two Yap1 target genes. This is interesting given the strong correlation between acetylation and transcription across the genome. Further, SAGA occupies the promoter region of each of these genes. Therefore, Gcn5 is positioned to play a role at *TRX2* and *CYC1*, but this role is clearly not required for normal expression during oxidative stress.

SAGA also cleaves monoubiquitin from lysine 123 of histone H2B. The Ubp8 subunit is responsible for this enzymatic function. In vivo, Ubp8 relies on the Sgf11, Sgf73 and Sus1 accessory proteins for activity. Deubiquitination of histone H2B plays a role in transcriptional activation of the yeast *GAL1* gene[87]. However, none of the genes analyzed here required the Ubp8 subunit for normal expression. Therefore, the deubiquitination activity of SAGA is not globally required for activated gene expression, but is gene-specific. Perhaps genes highly regulated by chromatin structure are more dependent upon this histone-modifying function. While the four oxidative stress response genes analyzed here do not require the deubiquitination activity of SAGA for expression, phenotypic analysis of the strains missing the Ubp8, Sgf11, Sgf73 and Sus1 proteins revealed slight H_2O_2 sensitivity. This suggests that deubiquitination of H2B plays a role at other genes that play a role in normal growth on plates containing H_2O_2.

The *CYC1* gene was very sensitive to deletion of the subunits required for SAGA integrity (Spt7, Spt20 and Ada1). In addition to these proteins, the only other subunits important for *CYC1* expression were Spt3 and Spt8. Spt3 and Spt8 interact with TBP and have been shown to deliver TBP to some target promoters in yeast[93-98]. However, we previously showed that Spt3 and Spt8 are not required for TBP or RNAPII occupancy at the *CYC1* promoter (**CHAPTER 3**[48]). What is the function of Spt3 and Spt8 at *CYC1* if they are not involved in TBP delivery? Recent studies revealed a functional linkage between Spt3 and Spt8 with the TFIIA general transcription factor. Both Spt proteins genetically interact with TFIIA[205,206]. Further, using site-specific protein cross-linking, Spt8 was found in close proximity to the N-terminal domain of TFIIA in assembled PICs[206]. Therefore, one possible explanation for the transcriptional dependence on Spt3 and Spt8 at *CYC1* is that they could function in TFIIA recruitment or stability at promoter DNA. Interestingly, the Yap1 activator protein interacts with TFIIA in a two-hybrid

interaction assay, and strains containing mutants of TFIIA display defects in transcription of genes regulated by Yap1[42].

Another aspect of SAGA function is Mediator interaction. SAGA genetically interacts with Mediator (see **APPENDIX I** and[99,144,162,168,199,200]), and is required for recruitment of Mediator at some genes[96]. However, we found that SAGA is not required for Mediator recruitment to *GTT2*, *TRX2*, *FLR1* and *CYC1*. This demonstrates that Yap1 can recruit Mediator independently of SAGA. Strikingly, deletion of SAGA (*spt20*Δ) led to increased Mediator recruitment at all of the target genes tested (*GTT2*, *TRX2*, *FLR1* and *CYC1*). There are several explanations for this finding. First, deletion of *SPT20* could result in a mis-localization of Mediator across the genome. We think this is unlikely given that occupancy of Mediator did not increase concomitantly at two negative control regions tested: the *GAL10* promoter and *AIM13* promoter (**FIGURE 5.11**). These two genes are not expressed during oxidative stress and serve as negative controls in this experiment. Another possibility is that when SAGA is present, Med15 ChIPs with a lower efficiency because of epitope masking. However, if this were the case, an increase in Mediator occupancy would be expected at *ARG4* and *SNZ1* genes, as they also contain SAGA. Another possibility is that SAGA negatively regulates the association of Mediator at the genes tested. This hypothesis can be tested by examining Mediator occupancy in a strain containing a deletion in a SAGA subunit that does not result in loss of the complex (like *gcn5*Δ). If Mediator occupancy is similar to the wild-type in this strain, this suggests that an Spt20-specific function is involved in maintaining proper amounts of Mediator occupancy. If occupancy is still higher than the wild-type strain, this suggests that the SAGA complex is involved in keeping Mediator occupancy low at the promoters we test. This would be an interesting function of the complex, which has only been implicated in the positive regulation of Mediator thus far.

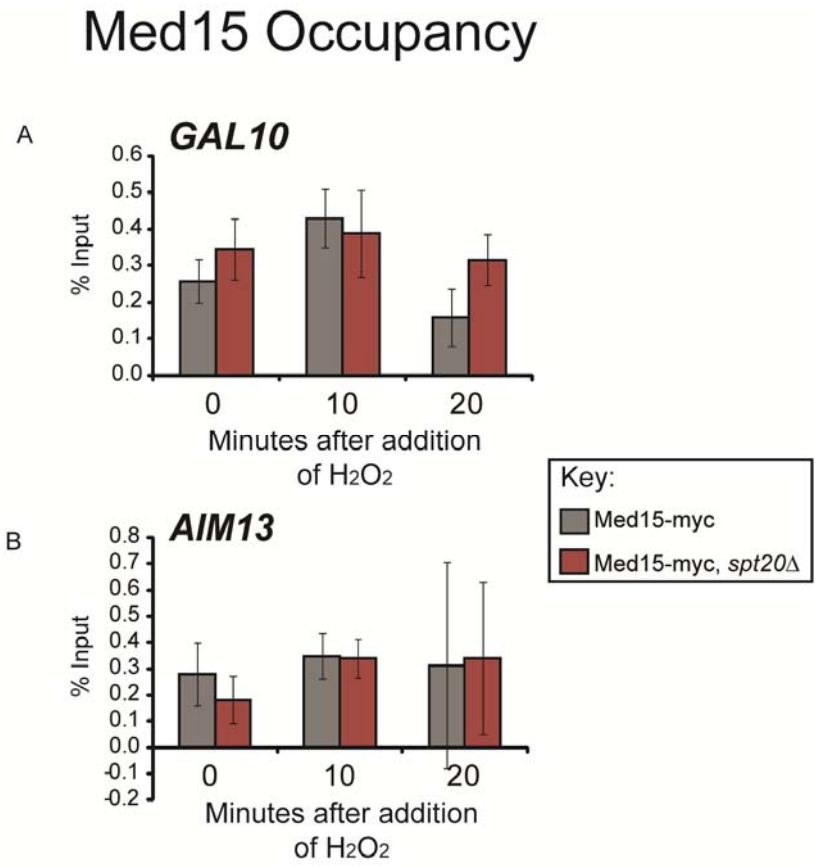

FIGURE 5.11. SAGA disruption does not result in Mediator mislocalization across the genome. Occupancy of Med15-myc was determined with a ChIP in the wild-type strain (Med15-myc) and *spt20*Δ strain (Med15-myc *spt20*Δ). **A)** Occupancy at the repressed *GAL10* promoter region is shown as % Input in each strain. **B)** Occupancy at the *AIM13* promoter is shown as % Input in each strain. *AIM13* is not induced during oxidative stress.

In the course of this study, we discovered two additional yeast genes are preloaded. This is intriguing, given the widely circularized idea that the majority of genes in yeast are recruitment-regulated (i.e. do not contain preloaded polymerase)[52,201]. However, comprehensive studies aimed at characterizing the regulatory strategy at a large number of genes have not been performed. While genome-wide RNAPII localization during optimal growth conditions is known[188], this has not been done under inducing conditions for specific genes. Therefore, we do not know the fold change in RNAPII occupancy upon induction, and the change in transcript level upon induction. These parameters are an important aspect of classifying genes as recruitment or postrecruitment regulated[48,202]. Only after this type of study is done will we be able to further understand the conservation of recruitment and postrecruitment regulation across the yeast genome.

CHAPTER 6. FUTURE DIRECTIONS

6.1 MEDIATOR-RNAPII INTERACTION

The Med18, Med19 and Med20 proteins of the Mediator complex form a functional submodule that is critical for activation of the preloaded polymerase at the *CYC1* promoter (**CHAPTER 4**). Mediator acts as a molecular bridge between activator proteins and RNAPII. I found that Med18, Med19 and Med20 do not function as an activator-interface, and are likely essential for activation of *CYC1* through their interaction with RNAPII.

The Med18, Med19, and Med20 proteins stimulate the preloaded polymerase at *CYC1* via changing the composition of the PIC, or affecting a conformational change in the PIC (likely in RNAPII itself, see below). We can further probe the requirement for these proteins at *CYC1* by testing the composition of the PIC in a strain missing a Mediator subunit. We are currently generating strains to test if the chromatin remodeler Swi/Snf and the general transcription factor TFIIH are recruited to the *CYC1* promoter in the absence of the Med20 protein. We are interested in these complexes as we know that Swi/Snf is important for *CYC1* activation[53], and TFIIH interacts with Mediator[110]. These tests will reveal if recruitment of either of these two complexes relies on Mediator (specifically Med20).

The head module of Mediator is structurally conserved, and plays an important role in RNAPII interaction[116]. Five of the eight proteins that compose the module are

essential (Med17, Med6, Med22, Med11, Med8) and three are non-essential (Med18, Med19, and Med20). Imaging recombinant head modules using EM analysis revealed three conformations of the module that differ by the angle at which a movable jaw is attached to the rest of the complex[117]. Imaging recombinant head preparations from strains missing Med18 and Med20 or just Med20 (using the *med18*Δ and *med20*Δ deletion strains, respectably) revealed that the movable jaw corresponds to the Med18 and Med20 subunits[117].

While the significance of the Med18 and Med20 movable jaw within the head module is still unclear, it is known that Med18 and Med20 are important for interaction with TBP[117,207] and RNAPII[117]. Specifically, Med18 and Med20 physically and genetically interact with the Rpb4 and Rpb7 subunits of RNAPII[117,208]. Rpb4 is a non-essential subunit of the polymerase. It is thought to be important during stress responses and diverse processes such as mRNA export and transcription coupled repair (for review, see[209]). As it is non-essential, we can test if Rpb4 is required for transcription of *CYC1* (and the other preloaded genes *TRX2* and *FLR1*). Given their interaction with Med18 and Med20[117], I hypothesize that this polymerase subunit will be required for transcription of *CYC1*. If it is required, how does this further our understanding of the mechanism of Mediator activation of preloaded polymerases? Rpb4 and Rpb7 form a heterodimer, and some have speculated that the N-terminus of Rpb7 could modulate active site conformation of RNAPII through interactions with the clamp of the polymerase[210,211]. It is tempting to speculate that the requirement of Med18, Med19 and Med20 at the *CYC1* gene involves stimulation of RNAPII mediated through the Rbp4 and Rpb7 subunits (**FIGURE 6.1**). Perhaps movement by the Med18/Med20 jaw facilitates this interaction with Rpb4 and Rpb7.

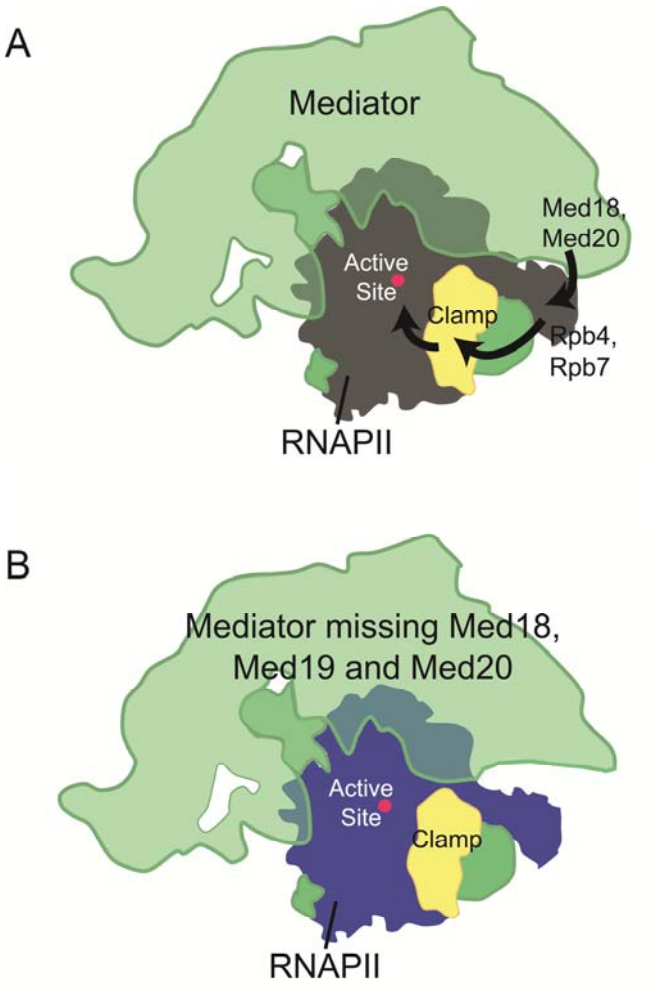

FIGURE 6.1. Possible model for the Med18/Med20 stimulation of RNAPII via the Rbp4 and Rpb7 polymerase subunits. **A)** In cells with wild-type Mediator, Med18 and Med20 interact with Rpb4 and Rpb7, this signal (arrow) is translated to the clamp (yellow) of polymerase, which controls the active site of the enzyme (arrow). **B)** In cells with mutant Mediator, Rpb4 and Rpb7 fail to productively interact with the coactivator, and do not affect the clamp (yellow) of polymerase, and thus the active site.

6.2 WHAT IS THE FUNCTION OF SAGA AT THE *CYC1* GENE?

While I found Mediator has a key role in stimulating preloaded polymerases presumably through an interaction with RNAPII, the function of SAGA in transcription of preloaded genes remains elusive. During our analysis of SAGA function at the *CYC1* gene, I found there are differences in the requirement for SAGA during two types of induction. During growth in ethanol, *CYC1* is dependent on the integrity subunits of the complex (therefore it is "SAGA-dependent"), but it also requires the Gcn5 protein for full expression. We looked at expression during growth in ethanol in a HAT-deficient strain (*gcn5*^{E173Q}), and found that expression was relatively normal. Therefore, a function other than the HAT activity of Gcn5 is required for *CYC1* expression during growth in ethanol. We can test if the bromodomain or Ada2-interaction domain are required using plasmids encoding these Gcn5 derivatives we currently have in the lab.

Intriguingly, during oxidative stress, Gcn5 is dispensable for *CYC1* activation (**CHAPTER 5**). This demonstrates that the function of Gcn5 during growth in ethanol can be bypassed during oxidative stress. Hap4 and Yap1 both have two acidic activation domains. Despite this broad similarity, they are different activators and have a different profile of protein-protein interactions. It is possible that Yap1 recruits proteins to this promoter that can by-pass the need for Gcn5 during oxidative stress.

The Spt3 and Spt8 subunits of SAGA interact with TBP, and are required for recruitment and stability of TBP at the promoters of several genes. We found that SAGA is not required for TBP occupancy at the *CYC1* promoter (**CHAPTER 3**), but *CYC1* does require Spt3 and Spt8 for full expression. What is the nature of this requirement if TBP still occupies the promoter in their absence? Spt3 and Spt8 also functionally interact with the general transcription factor TFIIA. Could these proteins be involved in recruitment of

TFIIA to the *CYC1* promoter? This can be tested by examining TFIIA occupancy in the *spt3*Δ and *spt8*Δ strains.

SAGA could also play a role in stabilization of the general transcription machinery at *CYC1*. Perhaps in the absence of SAGA, TBP (and/or TFIIA) interaction with the *CYC1* promoter is unstable and unproductive. The ChIP assay involves using formaldehyde to crosslink protein-DNA and protein-protein interactions. This crosslinking traps even transient associations. If deletion of SAGA results in a more dynamic (and non-functional) TBP/TFIIA interaction with the *CYC1* promoter, occupancy of these factors could still be detected. We can test how dynamic or stable the TBP/TFIIA association is with the *CYC1* promoter using an exchange experiment that is currently being developed in the lab.

6.3 GCN5-DEPENDENT TRANSCRIPTION

The Gcn5 protein is also required for transcription of *GTT2* and *FLR1* (**CHAPTER 5**). We can next test if the HAT activity of Gcn5 is required with a strain containing a HAT-defective allele of *GCN5* (*gcn5*^{E173Q}). We can also test expression in strains containing the other Gcn5 derivatives we have in the laboratory. This will reveal the function of the Gcn5 protein at these two promoters during oxidative stress. It would also be interesting to test H3K9 and H3K14 acetylation at the promoter regions of all of the genes tested. The Gcn5 protein generates these two chromatin marks. A genome-wide ChIP of H3K14 acetylation suggests that the *FLR1* promoter is pre-acetylated[76], while the *GTT2* promoter does not contain H3K14 acetylation in the absence of induction. This is consistent with our finding that SAGA occupies *FLR1* before induction, but not the *GTT2* promoter region (**CHAPTER 5**).

6.4 WHAT IS THE NATURE OF THE PRELOADED POLYMERASE?

My data does not address the nature of the preloaded polymerase at the *TRX2*, *FLR1*, and *CYC1* promoters. Indeed, we use the term "preloaded" because it is inclusive of the two major possibilities regarding the RNAPII interaction at these promoters: bound but not initiated, and initiated.

First, the polymerase could be associated with promoter DNA, but failed to initiate transcription. In other words, the DNA may not be melted, even though RNAPII is present. Others in our lab have found that TFIIH, the complex involved in promoter melting, is present at the *CYC1* promoter in uninduced conditions[53]. This finding suggests that this scenario (bound, but not melted) is unlikely, as the enzyme involved in promoter melting is already present in uninduced conditions. It will be revealing to test for TFIIH occupancy at the other preloaded genes identified in this work (*TRX2* and *FLR1*).

Second, the polymerase could be bound to promoter DNA, and initiated transcription. Here, we would expect an open transcription bubble of unduplexed DNA. The presence of a transcription bubble can be detected with permanganate footprinting. We can also detect if the polymerase is initiated, but paused, using nuclear run-on assays. Here, RNAPII initiation is prevented, but previously initiated polymerases are free to transcribe. If we detect transcript from *TRX2*, *FLR1* or *CYC1* in the run-on assay, this suggests that the polymerase at their promoter regions is initiated. This would reveal that initiation is rapid at preloaded genes such as *CYC1*, but there is a slow step downstream of initiation, before elongation.

How can we further probe the nature of the preloaded polymerase molecules? As I mentioned above, there are several experiments that can distinguish between initiated and non-initiated forms of RNAPII. Performing potassium permanganate assays and run-on transcription assays in vivo will detect if preloaded genes have melted regions in their promoter, and if polymerase has already started making a transcript, but is stalled. Using an exchange system, we are currently investigating how dynamic the association of the PIC is on promoter DNA. Is this association very dynamic, with RNAPII molecules associating and leaving quickly? Alternatively, is the interaction with promoter DNA a very stable one? While this experiment will not distinguish between initiated and non-initiated polymerase, it will give us a better understanding of the features of these promoters.

6.5 PERSPECTIVES

The work presented in this dissertation illustrates essential functions of the SAGA and Mediator coactivator complexes at postrecruitment regulated genes. This is an important finding, as this was a previously unknown aspect of coactivator function prior to this investigation. It also allows us to build on the model figure presented in **CHAPTER 1** (**FIGURE 1.3B**). We can now add both coactivators into this simple model of postrecruitment regulation (**FIGURE 6.2**). Furthermore, my work revealed that SAGA and Mediator are essential in the gene expression changes that occur during oxidative stress, furthering our understanding of how yeast cells respond to this biologically important assault. Finally, my identification of additional preloaded genes in yeast, as well as a recruitment-regulated gene, gives us the ability to perform experiments aimed at furthering our understanding of postrecruitment regulation using multiple promoters (*TRX2*, *FLR1*, and *CYC1*), including a negative control (*GTT2*). Among other things, studying this panel of genes has already revealed that all postrecruitment regulated

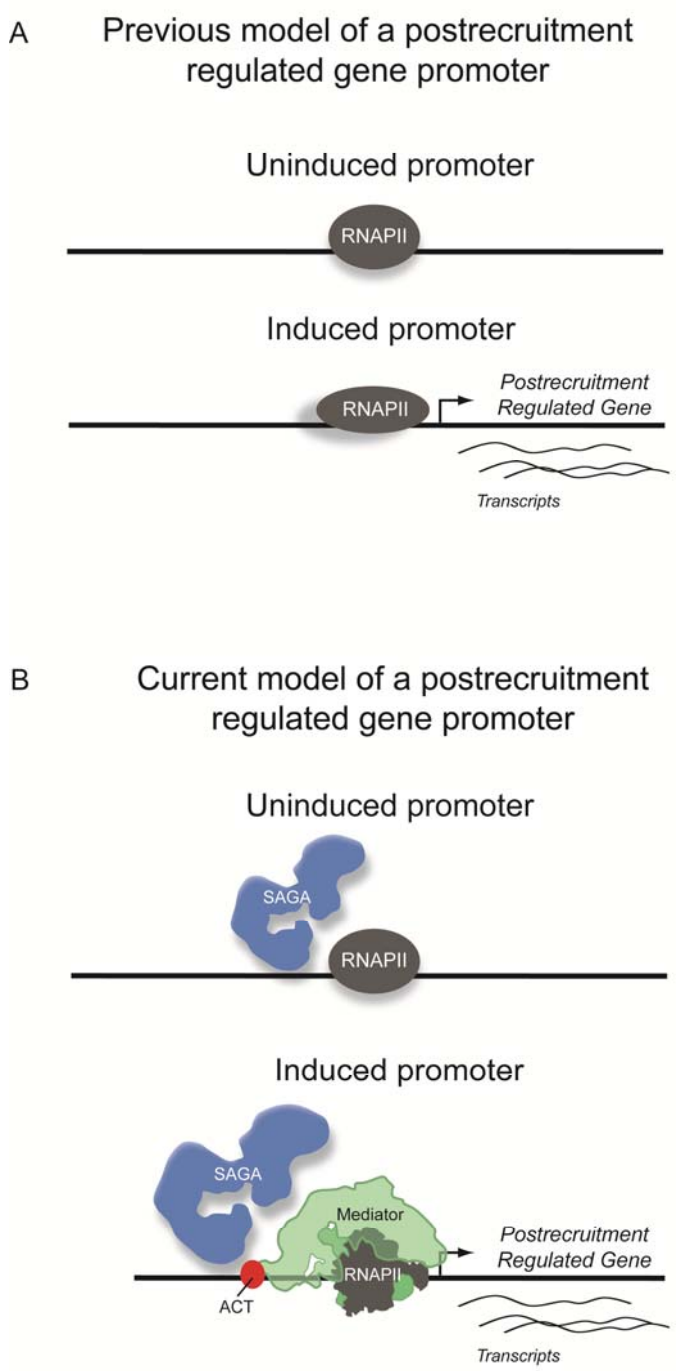

FIGURE 6.2. Advancement in our understanding of postrecruitment regulation. **A)** Previous model of a postrecruitment regulated gene, knowledge was limited to the presence of RNAPII at promoter DNA prior to induction. **B)** Current model of a postrecruitment regulated gene based on the work presented in this dissertation. In the uninduced state, RNAPII occupies promoter DNA. SAGA also occupies the promoter region of some postrecruitment regulated genes. During induction, an activator protein utilizes SAGA and Mediator to control the polymerase at the promoter and the transition to productive transcription occurs.

genes do not experience identical regulation, as they have disparate SAGA requirements (**CHAPTER 5**), and analysis of the recruitment-regulated *GTT2* gene revealed that preloading does not confer a transcriptional kinetic advantage. Thus, the investigation detailed in this dissertation not only furthers our understanding of transcriptional regulation in vivo, but also provides valuable tools that can be exploited for future investigations.

APPENDIX I

GENETIC CONNECTIVITY BETWEEN SAGA AND MEDIATOR

Just as proteins interact with each other, genes also interact. Genetic interaction is classically defined as an unexpected phenotype arising by combining the effects of individual gene variants[212]. Genetic interaction analysis involves comparing the phenotype of a strain containing a single mutation to the phenotype of a strain containing a double mutation (**FIGURE 1**). The goal of studying genetic interactions is to discern biological function. Particularly informing interactions are those in which two mutations alleviate or enhance each other's defects. A double mutant strain with no discernable phenotype (it is neither healthier nor sicker than the expected combination of the two single mutant strains) does not display a genetic interaction.

Genetic interactions are classified as either positive or negative (reviewed in[213]). Positive genetic interactions are alleviating. These include phenotypic suppression, synthetic rescue, dosage lethality, and dosage growth defects. These interactions describe cases where a double mutation results in a healthier strain than a single mutation. For instance, the *med13*Δ*spt20*Δ double deletion strain is unexpectedly healthier than strains containing single deletions of either *med13*Δ or *spt20*Δ. At the biological level, positive genetic interactions suggest the two interacting genes have opposing goals and act in conflicting pathways[214] (**FIGURE 2A**).

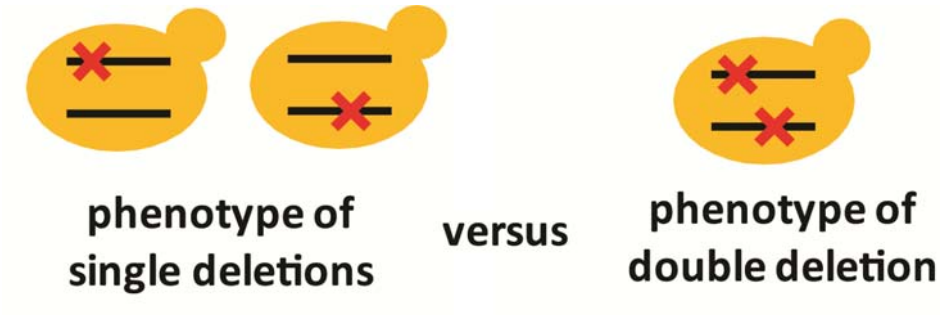

FIGURE 1. Genetic interaction analysis of yeast strains. Genetic interaction analysis involves comparing the phenotypes of two single mutant strains to the phenotypes of a strain containing deletions in both genes. Figure modified from[213].

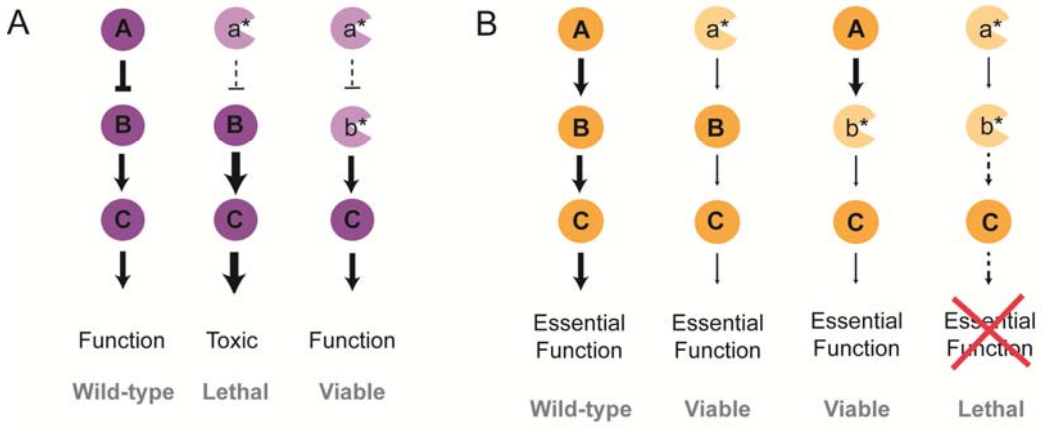

FIGURE 2. Genetic interactions suggest biological function. A) One model to explain positive genetic interactions. Gene A and Gene B have opposing goals. **B)** A model to explain negative genetic interactions. Gene A and Gene B have similar goals, loss of both is lethal in this model. Figure modified from[213].

Negative genetic interactions are aggravating. These include phenotypic enhancement, dosage rescue, synthetic growth defect, synthetic haploinsufficiency, and synthetic lethality. These categories each describe cases where a double mutation (strain contains two mutant genes) results in an enhancement of the phenotype associated with a single mutation. For instance, the *med15*Δ*spt20*Δ double deletion strain is sicker than strains containing single deletions of either *med15*Δ or *spt20*Δ. Negative genetic interactions between genes suggest the genes are involved in parallel pathways, often with redundant functions and a similar goal[214] (**FIGURE 2B**).

In order to investigate the genetic interaction profile between subunits of the Mediator and SAGA complexes, I utilized gene interaction datasets from several publications[99,144,162,168,199,200,215,216]. Here, the phenotypes of double deletion strains were compared to single deletion strains, and interactions were classified as described above. The reported genetic interactions between the non-essential subunits of the Mediator and SAGA complexes were downloaded from the BioGrid website (http://www.thebiogrid.org/) in August of 2009. The BioGrid is a repository for physical and genetic interactions between proteins in several model organisms[217]. I organized the data into table format with the Mediator deletion strains listed along the vertical, and SAGA deletion strains listed along the horizontal (**FIGURE 3**). If a negative genetic interaction was reported in the literature between two subunits, the corresponding box on the grid was colored red. If a positive genetic interaction was reported in the literature between two subunits, the corresponding box was colored blue. Black boxes indicate no genetic interaction reported between the two corresponding subunits.

Extensive genetic interactions between the Mediator and SAGA complexes exist. The majority of these interactions are negative (aggravating). The most severe negative

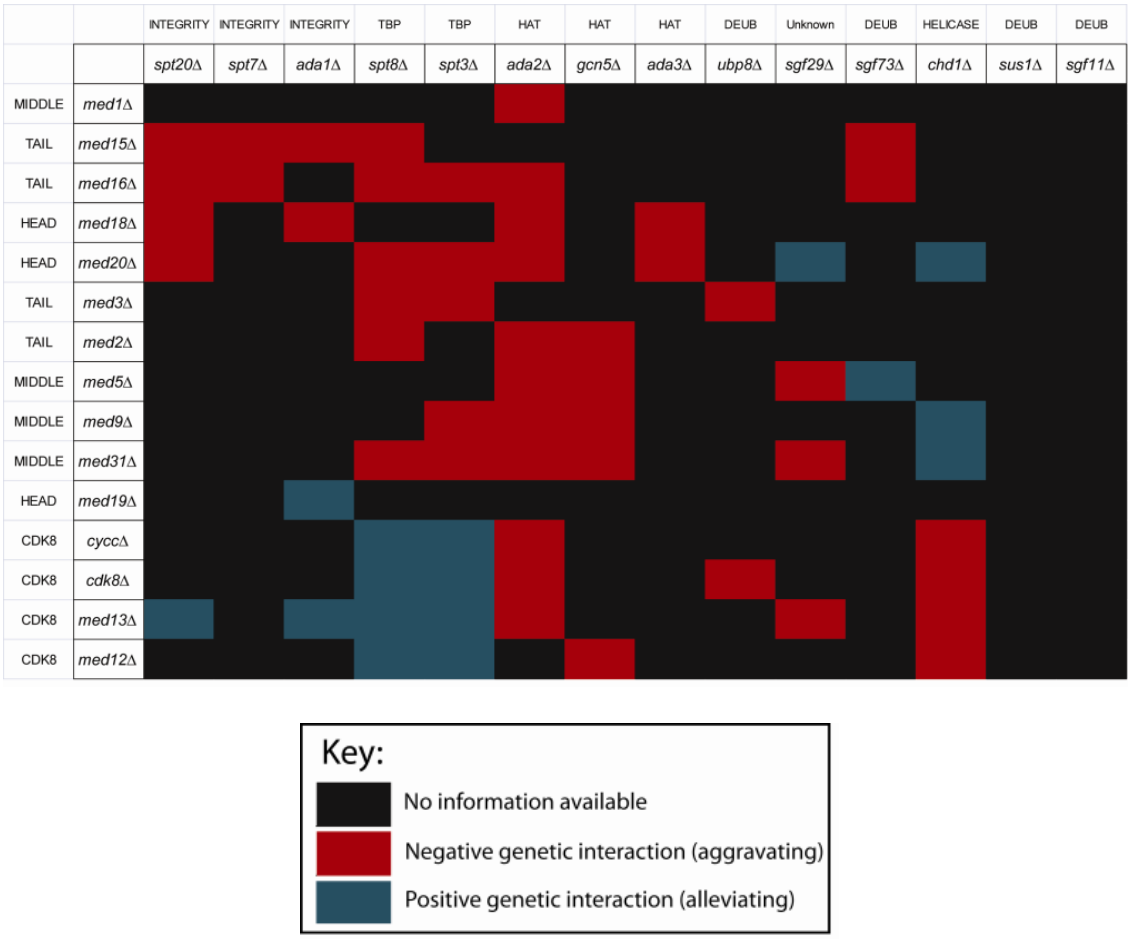

FIGURE 3. **Mediator-SAGA genetic interaction map.** Non-essential subunits in both complexes are grouped based on the genetic interaction profile, and are not necessary grouped by submodule or established function. Mediator deletion strains are listed vertically, SAGA deletion strains are listed horizontally. Squares were left black if no genetic interaction data exists for a particular gene pair. Red squares represent gene pairs with a negative genetic interaction.

genetic interaction is synthetic lethality. Here, a deletion of two non-essential proteins results in lethality. Several double deletion strains are inviable, including *med18*Δ*spt20*Δ, *med18*Δ*ada1*Δ, *med20*Δ*spt20*Δ, *med1*Δ*ada2*Δ, *med2*Δ*ada2*Δ, *med15*Δ*spt20*Δ, *med15*Δ*spt7*Δ, *med15*Δ*ada1*Δ, *med16*Δ*spt20*Δ, and *med16*Δ*spt7*Δ. These interactions suggest SAGA and Mediator act in parallel pathways with similar goals. They also suggest somewhat redundant functions of the two complexes, as single deletions in these genes are non-lethal, but a combination is lethal.

The HAT function of SAGA resides in three non-essential subunits, Gcn5, Ada2, and Ada3. These subunits have many negative interactions with Mediator, centering on the head and middle submodules of Mediator. Oddly, the *ada2*Δ strain also has negative (aggravating) genetic interactions with the CDK8 submodule of Mediator, which is the submodule with largely repressive functions in transcription regulation. This suggests Ada2 may also play a repressive role in the process of transcription. The TBP-interacting subunits of SAGA, Spt3 and Spt8, have several negative interactions with Mediator, centered on the middle and tail submodules of the complex. Spt3 and Spt8 interact positively (alleviating) with the CDK8 module, suggesting opposing functions of these subunits. Interestingly, the Chd1 subunit of SAGA interacts negatively with the CDK8 module of Mediator, and positively with *med20*Δ, *med9*Δ, and *med31*Δ. This suggests Chd1 has a repressive role in transcription.

Overall, the genetic interactions that exist between subunits of Mediator and SAGA demonstrate a tight relationship between these two complexes. Furthermore, core Mediator (the head, middle and tail modules) genetically interacts very negatively with subunits of the SAGA complex, suggesting these complexes have largely similar functions, and act in parallel pathways. The positive (alleviating) genetic interactions between these complexes are confined mostly to interactions with the CDK8 module of Mediator. The CDK module is a repressive module that interacts transiently with the

complex[102-104,161]. As this module is repressive of transcription, the positive (alleviating) interactions seen with subunits of SAGA (*spt20*Δ*, ada1*Δ*, spt8*Δ*, spt3*Δ) suggest SAGA is acting in a positive sense for transcription regulation. The insights gained from investigating the genetic interactions between these two coactivators are consistent with our view of Mediator and SAGA at a functional level. My transcriptional analysis of several oxidative stress response genes shows Mediator and SAGA are functionally very important for transcription. Several strains containing deletions in subunits of these complexes exhibit drastically reduced transcript levels of Yap1-dependent genes (**CHAPTER 5**).

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