DISSERTATION

REGULATION OF RNA POLYMERASE II TRANSCRIPTION BY THE SPT PROTEINS IN YEAST *SACCHAROMYCES CEREVISIAE*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LEI ZHANG ENTITLED REGULATION OF RNA POLYMERASE II TRANSCRIPTION BY THE SPT PROTEINS IN YEAST *SACCHAROMYCES CEREVISIAE* BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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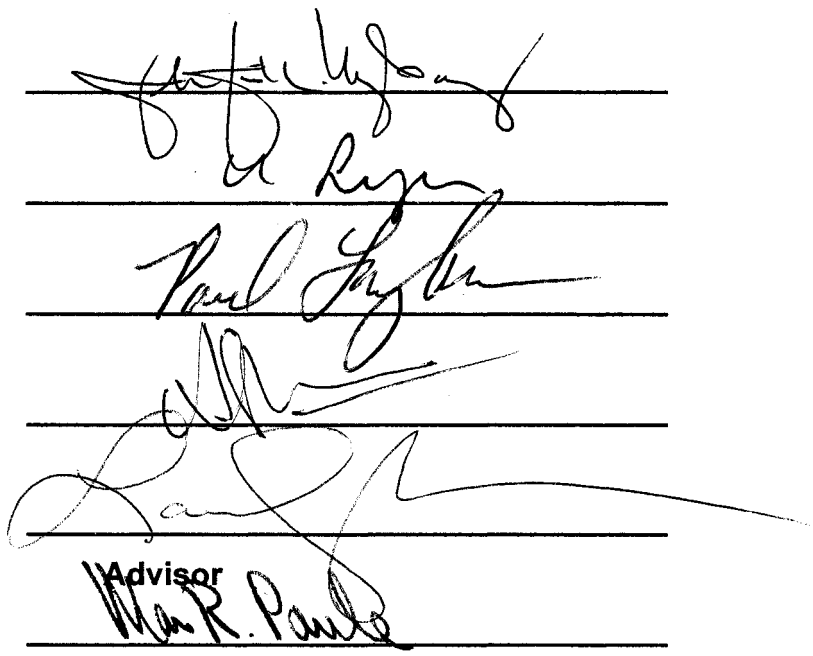

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ABSTRACT OF DISSERTATION

REGULATION OF RNA POLYMERASE II TRANSCRIPTION BY THE SPT PROTEINS IN YEAST *SACCHAROMYCES CEREVISIAE*

Transcription of protein–encoding genes by RNA polymerase II is a critical step in controlling cellular homeostasis, proliferation and differentiation, and requires concerted functions of a large number of transcription factors to precisely decipher the information packaged in the genome. While some factors regulate transcription by promoting the recruitment of TFIID/TBP or the assembly of the preinitiation complex, others functions at the post-recruitment steps. A group of transcription factors that are highly conserved throughout evolution, collectively known as Spt proteins, are particularly important for transcription regulation because their involvement in both recruitment functions and the post-recruitment functions.

The yeast *SPN1* is an essential and highly conserved SPT gene that has been implicated in regulating RNA polymerase II transcription by post-recruitment functions. To understand how Spn1 regulates RNA polymerase II transcription, a genetic screen was developed to target the functions of Spn1. The results of the genetic screen revealed the involvement of Spn1 in maintaining the chromatin structure and regulating the elongation.

Further analysis defined a critical role of Spn1 in coordinating recruitment of the chromatin-remodeling complex, Swi/Snf and the nucleosome reassembly factor, Spt6.

To get a better understanding of the functions of other Spt proteins during transcription, we analyzed the behavior of some non-essential Spt proteins and certain SAGA subunits in the artificial recruitment assays, and their effects on the transcription of genes that are regulated by the post-recruitment functions. Our initial results showed that while some Spt proteins function to regulate the recruitment of TBP/TFIID to the promoters, others affect the transcription after TBP recruitment. Furthermore, we showed that additional functions of the SAGA complex are required for post-recruitment regulation besides its roles in histone acetylation and TBP recruitment. These studies provide valuable information to understand the mechanistic functions of various Spt proteins in regulating RNA polymerase II transcription.

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The yeast SPT genes: key players in regulating chromatin structure and RNA polymerase II transcription

1.1 Significance of studying RNA polymerase II mediated transcription

1.1a Expression of protein-encoding genes is regulated at the transcription level

In all living organisms, most cellular and extra-cellular functions to maintain cellular homeostasis are carried out by different proteins. What distinguishes the various kinds of cells in higher eukaryotes is largely due to differences in the expression of genes that code for proteins. The cellular proliferation and differentiation processes required for proper development of all species are precisely controlled by programs to regulate the expression of protein-encoding genes packaged in the genome [1]. In response to a variety of endogenous and exogenous stimuli, living organisms utilize evolutionarily adapted strategies to change the gene expression either quantitatively or qualitatively. Therefore, regulation of gene expression is a vital step for the survival of all living species. In eukaryotes, protein-encoding genes are transcribed by RNA polymerase II (Pol II) in the nucleus as precursor RNAs; these precursor RNAs are then extensively processed before being translocated to the cytoplasm for

translation by the ribosome. This spatial and temporal separation between RNA and protein synthesis offers an immense opportunity for control and regulation. Thus the expression levels of any protein can be regulated at any of at least seven potential control steps: (1) chromatin structure, (2) transcription by RNA Pol II, (3) RNA processing, (4) transport to the cytoplasm, (5) translation of mRNA, (6) mRNA stability, (7) protein activity stability [2, 3]. Recent studies have firmly established that the transcription process is tightly coupled with RNA processing and exportation [4, 5], therefore, the crucial step in regulating gene expression is at the level of transcription: deciphering the protein-encoding information embedded in the chromatin structure by RNA Pol II transcription machinery with fidelity and efficiency. In vivo, RNA Pol II-mediated transcription is regulated by a large number of transcriptional factors during a multiple-step process including preinitiation complex formation, initiation, promoter clearance, elongation and termination [6, 7]. In humans, malfunctions in many transcriptional factors can cause aberrant transcription and uncontrolled expression levels of protein, which ultimately leads to tumorgenesis or many other diseases [8-10]. Studying the molecular basis of RNA Pol II transcription will provide valuable information to develop potential treatments of these diseases.

1.1b Yeast *Saccharomyces cerevisiae* as a model system

During the past five years, I focused my studies on the mechanism of RNA Pol II transcription activation in budding yeast *Saccharomyces cerevisiae* by characterizing a group of transcriptional factors, the yeast SPT (Suppressor of Ty) gene products [11]. There are several advantages of using yeast as a model system to study the eukaryotic transcription mechanisms. First, the yeast cell has a short life cycle of about 90 minutes and is easy to culture and handle. Thus yeast cells provide a quick and reliable material resource for any biochemical or genetic studies. Second, yeast has a small and sequenced genome of about 12,000 kb in size that can be maintained in both haploid and diploid states. This makes any genetic manipulations very easy in yeast. For the past 30 years, yeast genetics has been proven to be one of the most powerful tools to identify novel genes and their functions [12]. One of such example is the identification of the yeast SPT genes, the key players in regulating chromatin structure and transcription [13]. Third, the flood of information from large scale whole genome studies and various yeast databases provide ample resources for data referring and bioinformatics studies. Finally, although yeast is a single-celled species, it carries almost all the biological characteristics of higher eukaryotes. As the mechanisms of the fundamental processes such as transcriptional regulation, cell-cycle progression, DNA replication and many other processes are evolutionarily

conserved from yeast to human, information gained from yeast studies can be easily applied to human [14, 15]. However, none of these experimental advantages offered by the yeast system could negate the great contributions drawn from studies in other model organisms, which synergistically enriched our understanding in these fundamental biological processes.

1.2 Basics on RNA Pol II mediated transcription

Over the past 40 years, the search to understand the mechanisms of RNA Pol II mediated transcription has been a major topic in modern biological sciences. The combinatorial efforts of geneticists, biochemists and biophysicists enable us to envision the big picture in which the trans-acting transcriptional factors, working on the cis-elements of the DNA sequences embedded in the chromatin, aiding in the synthesis of RNA [5-7, 16, 17] . As there are numerous excellent reviews on almost every aspect of RNA Pol II transcription, and also due to limited ability to summarize the exploding information, I do not attempt to give a detailed review but to focus on the basics on Pol II mediated transcription in yeast that I feel will be helpful in understanding the focus of my PhD studies.

1.2a Structure of protein-encoding genes

The DNA sequences of protein-encoding genes can be typically divided into several regions: the regulatory elements, the core promoter elements, the protein coding region and the terminator region [18].

Regulatory elements are gene-specific sequences located upstream of the core promoter region and referred as upstream activation sequences (UAS) [19] or upstream repression sequences (URS) [20]. These sequences serve as the recognition and binding sites for gene-specific activators or repressors to control the rate of transcription initiation. The association of activators to UAS can facilitate the assembly of the preinitiation complex (PIC) by directly interacting with general transcriptional factors (GTFs) or indirectly interacting with coactivators such as chromatin modifying factors [21]. On the other hand, the URS-repressor association can repress transcription by several different mechanisms [22, 23]. The core promoter region contains the TATA element [24, 25], the initiator sequence (Inr) [26] and sometimes, a downstream promoter element (DPE) in TATA-less promoters [27]. The core promoter region defines the site for the assembly of the PIC. The TATA elements are the binding sites for TATA binding protein (TBP). The association of TBP to TATA element nucleates the assembly of PIC. In yeast *S. cerevisiae*, the TATA elements are usually located 40-120 bp upstream of the Inr elements [28]. The Inr elements are defined as sequences encompassing the transcription start site and can also nucleate the assembly of the PIC especially at TATA-less promoters [18, 29]. The DPE is usually located 30bp downstream of the Inr element and functions in conjunction with Inr element for TFIID binding at TATA-less promoters [30]. The sequences

that follow the coding region is referred as the terminator which contains important sequence elements for termination, the polyadenylation cleavage signal and a downstream pausing element [31, 32]. These elements when transcribe by Pol II into nascent RNA, can be recognized and cut by 3'-end processing enzymes for polyadenylation and triggers the termination of transcription [33, 34]. Other DNA elements such as silencers, enhancers, and insulators have also been implicated in transcription regulation [7].

1.2b Accessing the DNA template embedded in the chromatin structure

In vivo, DNA is packaged into nucleosomes and then into higher-order structure known as chromatin to fit in the nucleus [35, 36]. The packaging of DNA into chromatin structure was generally regarded as repressive on transcription and would impact probably every step of Pol II transcription process, because the efficient transcription requires access of the transcription factors to DNA template [16, 37, 38].

1.2b.1 Chromatin organization

The basic organizational unit of chromatin is the nucleosome [39], which consists of 146 bp of DNA wrapped 1.65 turns around a histone octamer core containing two copies each of four histone proteins: H2A, H2B, H3, and H4 [40] . Within the nucleosome, different DNA sequences are held in the surface of the nucleosomes by multiple interactions between histones and the phosphate backbone or the ribose moieties. The

amino-terminal tails of the histones protruding from the nucleosomes can interact with adjacent nucleosomes [40]. These nucleosomes can be further compacted into chromatin possibly via the linker histone H1 and the protruded amino-terminal tails of nucleosomal histones [41, 42]. However, not all the genome DNA sequences are packaged the same way: untranscribed regions of the genome are packaged into highly condensed "heterochromatin," while transcribed genes are present in more accessible "euchromatin" [43, 44].

1.2b.2 Chromatin modifying factors

The structural features of the chromatin assembly suggest that DNA template can be accessed by disrupting the histone-histone interactions in the chromatin and the DNA-histone contacts in the nucleosomes. To activate genes embedded in the chromatin, living cells adopt two strategies to make the DNA template accessible for the transcriptional factors: covalent modifications of histones and ATP-dependent chromatin remodeling [7, 45]. These are achieved by gene-specific activators to recruit coactivators that can modify the chromatin structure [46, 47].

Four classes of histone modifiers have been implicated in transcriptional regulation including the histone acetyltransferases (HATs) [48, 49], the histone deacetylases (HDACs) [50-52], the histone methyltransferases (HMTs) [53], and the histone kinases [7]. The best understood histone modification is histone acetylation. Recruitment of

HATs to promoters by activators results in the acetylation of lysine residues located in the N-terminal tails of histones and is crucial for the activation of many classes of gene. Acetylation of histone tails may disrupt nucleosome structure by neutralizing positively charged lysines, thus decreasing their affinity for DNA or neighboring nucleosomes [48, 49, 54]. This provides greater access to DNA for transcription apparatus and its regulators. In yeast, a number of protein complexes that have HAT activity have been identified including SAGA, ADA, and HAT1 complex, NuA3, NuA4, TFIID and Elongator.

In addition to covalent modifications of the histones, ATP–dependent chromatin-remodeling complexes can alter chromatin structure conformationally. The remodeling involves the breaking and reforming of histone–DNA contacts that results in the mobilization of nucleosomes in the chromatin template [55]. Several complexes have been identified including the Swi/Snf, RSC complexes, INO80, ISW1, ISW2, CHD1 in yeast and the NURF, CHRAC, and ACF complexes in drosophila [56]. All of these complexes contain an ATPase subunit that is essential for remodeling activity along with additional subunits that affect the efficiency and specificity [55]. The best-studied chromatin-remodeling complex is the yeast Swi/Snf complex [57, 58]. The yeast Swi/Snf complex is comprised of 11 subunits and has a molecular weight of approximate 2 MDa [59]. *SNF2* encodes an ATPase, the enzymatic subunit of the Swi/Snf complex [60].

Snf5 plays an essential role in maintaining the assembly of the Swi/Snf complex [61] . Further, the function of Snf2 requires both Snf5 and Snf6 [61-63]. The other subunits may play minor roles in regulating the functions of Swi/Snf complex. In vivo, the Swi/Snf complex remodel nucleosomes in an ATP-dependent fashion by sliding histone octamer on the same DNA template or transfer them to another DNA template [58, 64].

Among theses chromatin modifying factors, the SAGA complex (see below) and the Swi/Snf complex had been of great interest of mine during my PhD studies because of their links with Spt proteins during transcription.

1.2c The RNA Pol II transcription cycle

1.2c.1 Assembly of the preinitiation complex

A complete transcription cycle includes preinitiation complex formation, initiation, promoter clearance, elongation, and termination. As soon as the chromosomal template is made accessible for the transcription factor, TFIID comprised of TBP and its associated factors (TAFs) recognizes the TATA element, and then factors such as TFIIA (IIA), TFIIB (IIB), TFIIF (IIF), Pol II, TFIIE (IIE) and TFIIH (IIH) enter the promoter to form a preinitiation complex which is competent for transcription [6, 7, 65, 66]. Alternately, it has been suggested that the assembly of a pre-initiation complex might happen in a single concerted 'recruitment step' which brings to the promoter a large preassembled Pol II holoenzyme containing the

Mediator complex and most of the GTFs and sometimes even the Swi/Snf complex [67-69].

1.2c.2 Initiation and promoter clearance

Transcription initiates with the formation of an open complex between Pol II and the DNA in which the double-stranded DNA is melted into a single-stranded bubble in an ATP-dependent process by IIE and IIH [70-73]. The first phosphodiester bond of the nascent RNA strand is formed upon addition of the first two nucleoside triphosphates (NTPs) dictated by the DNA sequence. Before Pol II can enter the productive transcriptional phase (elongation), it must pass through a stage known as promoter clearance [74]. During this stage, the PIC is partially disassembled; A subset of GTFs remains at the promoter except IIF and serves as a scaffold for the formation of the next transcription initiation complex; IIF and Pol II complex escape from the promoter [75, 76]. The hallmark of this stage is the phosphorylation of the C-terminal repeat domain (CTD) of the largest subunit of Pol II, which is mediated by IIH and can be enhanced by the Mediator complex [77, 78]. Although it is still poorly understood how the CTD-phosphorylation triggers promoter clearance and thereby defines the initiation-to-elongation transition, in all likelihood, a major effect of CTD phosphorylation is thought to be the disruption of Pol II–GTF interactions and CTD–Mediator interactions, enabling 'recycling' of GTFs and the Mediator to a new initiating polymerase [79]. While leading to disruption of

one set of interactions, CTD-phosphorylation might concomitantly establish another set of interactions with elongation factors: RNA capping enzymes and [80-82], mRNA 3'-end processing factors [83] and even the RNA exportation factors [84] . Once the promoter is cleared, the next round of transcription can be reinitiated. Reinitiation of transcription is much faster than the initial round, and is responsible for the bulk of transcription in the cell [85-87].

1.2c.3 Elongation through chromatin template

1.2c.3.1 Factor affects the processivity of Pol II

The identification of a large number of factors important for Pol II to establish efficient elongation underscores the complicated nature of the process. In yeast, these factors include IIF [88], IIS [89], Elongins [90], Elongator [91], FCP1 [92], PTEF-b and DSIF [93]. Although the details of their contribution to elongation are still under intensive studies, accumulated results suggest that these factors exert their functions to help Pol II overcome the pausing, arresting sites and stimulate the transcription rate of Pol II [94]. Aside from its function in initiation, IIF diminishes the time Pol II is paused and stimulates the rate of transcriptional elongation [95]. In addition, the RAP74 subunit of IIF directly interacts with and stimulates the enzymatic activity of FCP1, a phosphatase that targets the CTD of Pol II and stimulates elongation in vitro [96-98]. FCP1 remains associated with the elongation-competent Pol II in yeast in vivo [92]. Further

experimentation identified that IIF associates with multiple elongation factors, including the Spt5 subunit of DSIF [99], and components of the PAF complex [100]. IIS promotes Pol II read through at transcriptional arrest sites [101, 102].

1.2c.3.2 Factors that regulate elongation via chromatin structure

The chromatin structure also has profound effects on transcriptional elongation. As Pol II escapes from the promoter and enters into to proceesive elongation, it would be impeded by the nucleosomes along the DNA template. Factors such as SAGA, Swi/Snf, Spt4/5(DSIF), Spt6, and FACT, the histone methyltransferases and Elongator play a not entirely defined role in dealing with chromatin during transcript elongation. Although the details of the connection between transcript elongation and histone acetylation are still elusive, it is evident that the process of elongation is tightly coupled to, and affected by, histone acetylation since the level of histone H3 acetylation across a gene is highly correlated with the level of transcription. Swi/Snf complex can be recruited to PIC by transcriptional activators. The purification of a large protein complex containing, among other factors, Pol II and Swi/Snf from both yeast and human cells suggested that Swi/Snf could hitch a ride with Pol II to remodel chromatin during elongation [67, 69]. Studies on heat-shock genes have shown that Swi/Snf is required for disruption of the first nucleosomes in front of promoter-proximally stalled Pol II [103]. However, it is still not clear

how Swi/Snf-mediated chromatin remodeling mechanistically facilitates elongation. The activity of FACT had been found to be required for promoter escape by Pol II on a chromatin template in vitro [104]. Both Spt6 and FACT can bind directly to histones [105, 106] and may enable a transient solubilization of nucleosomes so that they can be transferred out of the path of elongating Pol II and reassembled after the passage of transcribing Pol II [107-109]. The question remains of how Swi/Snf-mediated chromatin remodeling activities are coordinated with other known chromatin-remodeling elongation factors, such as FACT and Spt6.

1.2c.4 Transcription termination

The final step in the cycle is transcript termination. At this stage, the mRNA is cleaved, polyadenylated, and transported to the cytoplasm, where it will be translated. [83]. Although a lot is known about the earlier stages of the transcription process, much less is known about how transcription terminates. The mechanism of Pol II termination is intimately coupled to the maturation of mRNA 3' ends at the poly (A) site [110]. Earlier studies showed that the poly (A) consensus sequence is essential for triggering termination [111, 112]. Recent studies suggested that transcribing Pol II is 'torpedoed' by exonulease, which triggers the termination [34, 113]. As the nascent RNA is cut at the polyadenylation site, the exonulcease recruited by 3'-end processing factors associated

with phosphorylated CTD is loaded onto the free end of downstream RNA and rapidly degrade the RNA till it catches up with the transcribing Pol II. Thus transcription terminates. Evidence for this model is that inactivation of yeast Rat1, an exonulease, strongly stabilized the RNA fragment downstream from the cleavage/ polyadenylation site, indicating that Rat1 is responsible for degrading this region. Moreover, RNA Pol II could be chemically crosslinked to sequences in the encoding gene that are farther downstream from the cleavage site in the mutants than in the wild type, suggesting that without the proper function of Rat1, Pol II fails to stop when it should [34]. However, it is still not known how these nucleases stop Pol II from transcribing. The polymerase usually terminates in an untidy fashion at multiple positions situated hundreds or thousands of bases downstream from the poly (A) site after releasing the nascent transcript. The actual process of termination probably is even more complicated [17].

1.3 Recruitment and post-recruitment functions of transcription factors

1.3a Defining the recruitment and post-recruitment functions

The expression of most eukaryotic genes by Pol II is controlled at the level of transcription initiation [65, 114, 115]. In many cases, the recruitment of TBP (TATA binding protein) to promoters is in direct proportion to transcription activation [65, 116], and is therefore a rate-limiting step for transcription. A variety of transcription factors such as

activators, certain GTFs, TAFs (TBP-associated factors) and many others, regulate the binding of TBP either positively or negatively [6, 117]. Thus, the functions of these factors can be defined as "recruitment-functions". Because the recruited TBP/TFIID at the promoter nucleates the rest of the preinitiation complex and activates transcription, the recruitment-functions can therefore be extended to the functions that promote the assembly of the PIC. On the other hand, for some genes such as the yeast *CYC1, COX5a* and the *Drosophila* heat shock genes, the recruitment of TBP or Pol II or even the assembly of PIC at the promoter does not solicit transcription [65, 118, 119], suggesting certain functions that are required for transcription are missing. These functions of transcription factors are then defined as "post-recruitment functions" as they are required after the recruitment of TBP or the assembly of PIC. Such functions are generally required for transcription but not rate limiting as seen on many genes. However, these functions are rate limiting for the transcription of a growing number of genes we just began to know their regulatory mechanisms. Theoretically, such functions may be involved in promoter melting and promoter clearance, elongation, termination or even RNA processing. For many chromatin modifying factors, recruiting them to the promoter usually facilitate TBP binding or the assembly of the PIC as well as the transcription elongation, thus they could be defined as having both recruitment and post-recruitment functions. Defining the recruitment or

post-recruitment functions of different factors would provide valuable information on their mechanistic involvement during transcription.

1.3b Approaches to study the post-recruitment functions

Fusing TBP to the DNA binding domain of an activator can artificially recruit TBP to the promoter, which nucleates the rest of the transcriptional machinery and increases transcription [120-122]. This bypasses the functions that are required to bring TBP to the promoter. Thus the recruitment or the post-recruitment functions can be studied using the artificial recruitment assy. For example, let's assume that a gene product is involved in functions after TBP binds; deleting this gene would then abolish the functions of the product, therefore artificial recruiting TBP to DNA should fail to increase transcription. To the contrary, if a gene product is involved in functions for TBP recruitment, the mutant of this gene should still increase transcription in the artificial recruitment assay.

Another way to study the post-recruitment functions is to examine the effects of a factor on transcription of certain genes such as the yeast *CYC1* or *COX5a*, the *Drosophila HSP* genes. The transcription levels of these genes remain low while TBP, Pol II or even the PIC have been already assembled at their promoters [65, 118], suggesting these genes are regulated by post-recruitment functions. Therefore, these genes provide a salient model to dissect the recruitment functions or the post-recruitment functions of a transcription factor. If a transcription factor

affected the transcription levels of these genes by perturbing the TBP occupancy level, this would suggest that the recruitment function of the transcription factor is required. If the transcription factor affected the transcription levels of these genes but not the occupancy level of TBP, this would indicate that the transcription factor exerts its function after TBP recruitment.

1.4 The Spt proteins: key players in regulating RNA Pol II transcription

As mentioned before, a group of transcription factors that are highly conserved throughout evolution, collectively known as Spt proteins, have been particularly interesting because they participate in almost every steps of transcription as well as many other diversified biological processes [11, 13]. In yeast, mutations in SPT genes shared the common phenotype of suppressing the Ty insertion mutation [13], but the roles of many Spt proteins are so distinct from each other and have been implicated in chromatin structure, initiation, elongation, RNA processing, suggesting these factors are the key players in regulating the RNA Pol II transcription. Studying the functions of these Spt proteins would provide insights on the mechanism of transcription.

1.4a Identification of Spt proteins

In the past 30 years, yeast genetic screening has been one of the most powerful tools in identifying novel genes and their functions. One

such example is the genetic screen taken by Winston and co-workers looking for suppressors that can restore transcription-defective mutants caused by insertion of the transposon Ty in the 5' or upstream region of *HIS4* or *LYS2*. These insertional mutations of *HIS4* or *LYS2* abolished expression of these genes, resulting in a His- or Lys- auxotrophy [123]. Selection for *spt* mutants in these strains therefore selects for His+ or Lys+ revertants and led to the identification of over a dozen of SPT genes [11, 124-126]. Later, certain mutants isolated from studies by other groups also shared the Spt- phenotype and are classified as SPT genes. The highly conserved Spn1 is one of such Spt proteins [127].

Further studies on these Spt proteins suggested that they possess partially overlapping but distinct roles *in vivo* [125]. For example, the *SPT11* and *SPT12* encode the histone H2A and H2B. Spt1, Spt10 and Spt21 are the regulatory factors that control the expression levels of histone proteins [128, 129]. *SPT15* encodes TBP [130, 131], and the functions of many Spt proteins are related to TBP function including Spn1 (aka Spt24), Spt3, Spt7, Spt8, and Spt20 [127-129, 132, 133]. Many key regulators of transcription and chromatin structure, such as DSIF, SAGA, and FACT complexes, all contain SPT gene products as essential subunits. Spt4/Spt5, the yeast homolog of human DSIF regulates transcription elongation through chromatin [134, 135]. Spt3, Spt7, Spt8, Spt20 are the subunits of SAGA complex, which regulates the recruitment

of TBP and acetylates chromosomal histones [128, 129, 132, 133]. Spt6 together with Spt16/Pob3 (the yeast FACT) have been implicated in maintaining the chromatin structure during elongation [105, 107, 109, 136]. The majority of these SPT genes genetically interact with SWI/SNF genes [137-139], although the functional mechanisms are still not clear. Based on their additional phenotypes and distinct functions in Pol II transcription, these Spt proteins have been categorized into different groups (Table 1).

1.4b Spn1, a novel Spt transcription factor

Work from this lab identified Spn1, a novel Spt factor. Unlike many other Spt proteins, Spn1 is essential for viability and highly conserved from yeast to human [127]; which implicates certain key functions of Spn1. Although the mechanism of how Spn1 regulates Pol II transcription is entirely unknown, the function of Spn1 is closely related to TBP and Spt6 [127, 140], two other essential Spt proteins. Understanding the function of Spn1 would provide valuable information of its mechanism in regulating Pol II transcription. With the identification of Spn1 involvement in post-recruitment functions, this prompted me to examine the post-recruitment functions of other Spt proteins.

1.4b.1 Isolation of yeast *SPN1*

As described before, the recruitment of TBP/TFIID by activators to the TATA element is rate-limiting for many of characterized Pol II

Table 1 Functions of Spt proteins

Group	Name	Essential	Functions	Reference
Histone group	Spt1	no	Regulates transcription of histone H2A, H2B, H3 and H4, recruits Swi/Snf to histone gene promoters; heterochromatic gene silencing.	
	Spt10	no	Histone acetylase, required for histone gene transcription with Spt21.	[128, 129, 133, 141]
	Spt11	no	One of two histone H2A subtypes; required for chromatin assembly and chromosome function.	[142-144]
	Spt12	no	One of two histone H2B subtypes; required for chromatin assembly and chromosome function.	[142-144]
	Spt21	no	Required for transcription at HTA2-HTB2, HHF2-HHT2 loci, associates with Spt10	[128, 129, 133, 141]
	Spt4	no	forms a complex with Spt5, mediates transcription elongation, pre-mRNA processing.	[134, 135, 145, 146]
	Spt5	yes	forms a complex with Spt4; mediates transcription elongation; pre-mRNA processing	[99, 134, 135, 145, 147]
	Spt6	yes	interacts with histones H3 and H4; nucleosome disassembly and reassembly during transcription elongation.	[107, 135, 148-150]
	Spn1	yes	Interacts with Spt6, involved in chromatin remodeling and transcription elongation.	[99, 127, 151, 152]
	Spt16	yes	Subunit of the FACT complex (Spt16p-Pob3p), facilitates RNA Pol II transcription elongation by destabilizing and then reassembling nucleosome structure.	[104, 108, 109, 136, 153, 154]
TBP group	Spt3	no	Subunit of the SAGA and SLIK complexes, interacts with Spt15p.	[124, 155-158]
	Spt7	no	SAGA subunit, involved in complex structural integrity; C-terminally truncated form presents in SLIK.	[156-160]
	Spt8	no	SAGA subunit; not present in SLIK, required for SAGA-mediated inhibition at some promoters.	[156-158]
	Spt15	yes	TATA-binding protein (TBP)	[131, 161-163]
	Spt20	no	Subunit of the SAGA complex; required for structural integrity of the complex.	[156-158, 164, 165]
other	Spt2	no	Interacts with histones and SWI-SNF components, similar to HMG1 proteins	[166-168]
	Spt13	no	Mediator complex component; interacts with Pol II and TFIIE to form the holoenzyme.	[169-172]
	Spt23	no	Associates with Mga2p, involved in OLE1 transcription; inactive form dimerizes and is activated by ubiquitin/proteasome-dependent processing followed by nuclear targeting.	[173-176]

transcribed genes [65, 116]. But there are genes regulated at the steps after TBP recruitment. For example, on the yeast *CYC1* gene or the heat shock genes in *Drosophila*, TBP and Pol II have already been recruited while the levels of transcription remain low [65, 118, 119], suggesting that certain functions necessary for transcription are missing. To identify such "post-recruitment" components that are necessary for high levels of transcription, a genetic screen was conducted using a post -recruitment defective TBP mutant allele, tbp^{F237D} of which a phenylalanine at position 237 was mutated to aspartic acid [121]. This particular allele could bind TATA element with wild type affinity and interact weakly with TFIIA and TFIIB, but failed to activate transcription in the presence of activators such as Gcn4, Gal4 and Ace1 [121]. Even when it was artificially recruited to the *HIS3* reporter gene, it still could not increase transcription, and the mutant cells could not grow on medium containing 3-aminotriazole (a competitive inhibitor of the *HIS3* gene product) [121]. Using this TBP allele, a genetic screen was performed to look for suppressors that can activate the transcription of this post-recruitment defective TBP allele. Several suppressors were isolated and one of the suppressors was determined to be a K192N allele of *SPN1* (Suppresses Post-recruitment functions gene Number 1) [127]. Further genetic analysis showed that the *SPN1* mutant also suppressed the Ty insertional mutation thus defined it as a novel SPT gene [99, 127].

1.4b.2 Possible functions of Spn1 in regulating Pol II transcription

Functional characterization of Spn1 in our lab showed that Spn1 affected the transcription of several Pol II genes. More significantly, Spn1 repressed the activation of *CYC1* gene, a gene that is regulated by post-recruitment functions. The *spn1*^{K192N} allele also suppressed other TBP mutants, thus is not allele-specific. Spn1 was not associated with TBP or any known TAFs. Therefore, it did not appear that Spn1 exerted it function through TFIID directly [127]. These results pointed out the post-recruitment functions of Spn1 in transcription. About the same time, studies from other lab had showed that Spn1 was associated with Spt6 and certain kinase subunits [140] and was crosslinked to different regions of several genes during transcription [141]. This linked the functions of Spn1 to Spt6.

Spt6 was originally identified in the same genetic screen that uncovered Spt4, Spt5, and Spt16 [125, 142] The early genetic connections between Spt6 and DSIF (Spt4 and Spt5), implicating the participation of Spt6 in elongation [134]. Spt6 specifically colocalizes to actively transcribed regions together with the elongating form of Pol II [135, 143, 144]. Results from in vitro elongation assays on naked DNA showed that human Spt6 promoted the transcription rate of Pol II both autonomously or with DSIF [145]. Another totally different genetic screen for suppressors of *swi2/snf2* led to the discovery of *SSN20* (for suppressor of sn*f2*), which is

identical to *SPT6* [139]. Later research has demonstrated that mutations in *SPT4*, *SPT5*, *SPT6*, *SPT11*, *SPT12*, and *SPT16* also suppress *SNF2/SWI2*, and in most cases, *SNF5* and *SNF6* mutants. These genetic interactions between *SPT6* with SWI/SNF genes foretold the involvement of Spt6 in the maintenance of chromatin structure. Further studies show that Spt6 promotes nucleosome assembly in vitro and interacts with histone H3 and H4, preferentially histone H3 [105]. Consistent with these findings, Spt6 mutant strains display alterations in chromatin structure in vivo [105]. Mutations in the *SPT6* gene lead to transcription initiation from cryptic start sites within the coding sequences [107]. These results support the role of Spt6 in maintaining chromatin structure during elongation.

1.4b.3 Specific aims to target the mechanistic functions of Spn1 during transcription

The association of Spn1 with Spt6 postulated possible involvement of Spn1 in chromatin structure and the elongation process, but this association does not reveal any mechanistic aspects of Spn1 function. Unlike *SPT6*, mutations in *SPN1* did not display sensitivity to 6-azauracil, a drug that reduces intracellular NTP concentrations and thus is expected to decrease the rate of transcript elongation [146]. This suggested a functional difference between Spn1 and Spt6. Given the fact of the diversified roles of the Spt proteins in regulating transcription and the

uncharacterized nature of Spn1, it is possible that Spn1 could be involved in every step of Pol II transcription.

To understand the functions of Spn1 in regulating Pol II transcription, I proposed a targeted genetic screen by taking the advantage of the well-characterized *spn1* ^{K192N} allele. In the genetic screen, the *spn1*^{K192N} allele was directly combined with the mutants of many other transcription factors. The functions of these transcription factors have been characterized, and are involved in not only the post-recruitment steps but also the recruitment steps. Therefore, the genetic interactions we found through the screen would directly link Spn1 to the regulatory steps during Pol II transcription. Further characterization of these new functional interactions of Spn1 will provide valuable information on its mechanism.

1.4c Spt16 and the yeast FACT complex

Experiments designed to identify factors that support Pol II transcription on chromatin templates led to the discovery of FACT (facilitates chromatin transcription) [104]. The FACT complex is highly conserved among eukaryotes, functions after transcription initiation to allow Pol II transcription on nucleosome templates, and acts independently of IIF, IIS, and ATP [147]. The yeast FACT components, Spt16/Cdc68 and Pob3, are encoded by essential genes and are implicated in regulating elongation and chromatin structure [148]. Mutations in SPT16 caused

sensitivity to 6-azauracil and synthetically interact with IIS, Spt4/Spt5, Spt6, SWI/SNF, Chd1, and the Paf1 complex [136, 149-151]. Spt16/Pob3 also physically associates with Spt4/Spt5, Spt6, Paf1, Chd1, nucleosome and histone H2A-H2B dimer [109, 141, 151, 152]. Changes of chromatin structure during active transcription are accompanied by specific loss of H2A-H2B dimer [153]. FACT was shown to facilitate the loss of H2A/H2B dimers in assays using immobilized nucleosomal templates [109]. ChIP analyses showed that Spt16 is localized immediately downstream of the promoter region at active genes upon induction [154]. Yeast cells containing mutations in histone H4 that disrupt associations with H2A/H2B dimers exhibit phenotypes similar to those of Spt16 mutant strains [136, 148]. Furthermore, similar to Spt6, Spt16/Pob3 facilitates the deposition of histones onto DNA in vitro [109]. Genetic studies showed that mutations in *SPT16* and *POB3* result in cell survival being dependent on the Hir/Hpc nucleosome assembly pathway, suggesting that FACT plays a role in nucleosome reassembly after Pol II passage [136]. Taken together, these observations suggest that Spt16/Pob3 functions as a modulator of chromatin structure during Pol II transcription.

1.4d The SAGA Spts: Spt3, Spt7, Spt8, Spt20 and their functions

Based on their common mutant phenotypes, a subset of five *SPT* genes is grouped together. This group includes *SPT15*, which encodes TBP [130, 131]. The other four members of this group encode the Spt

proteins contained in SAGA [155] complex includes Spt3, Spt7, Spt8 and Spt20 [156]. Spt3 and Spt8 in particular seem to have the most direct relationship to TBP, since specific *spt3* and *spt15* mutations suppress each other in an allele-specific fashion [157], implying possible physical interaction. However, protein-protein interaction studies and two-hybrid analysis have not shown any direct interaction between Spt3 and TBP [158, 159]. The intact SAGA was found to interact with TBP and this interaction required Spt8 but was independent of Spt3 [160, 161]. Furthermore, it is proposed that Spt8 may sterically interfere with TFIIA in binding TBP in the preinitiation complex both positively or negatively affect transcription [162]. The Spt7, Spt20 functions as the core components for the structural integrity of SAGA and mutant strains that did not express one of these subunits lack intact SAGA complex and exhibit severe phenotypes [155, 161, 163, 164]. Further biochemical characterization suggests that Spt7 plays a key role in SAGA assembly and in maintaining normal levels of SAGA [164].

Besides the Spt proteins, the yeast SAGA also contains products of several other distinct classes of genes. The Ada proteins (Ada1, Ada2, Ada3 and Gcn5), serve for the "adaptors" of the gene-specific activator Gcn4 and the activation domain of herpes simplex virus VP16. Like Spt7 and Spt20, the Ada1 subunit also functions to maintain the structural integrity of SAGA. Gcn5 is the catalytic subunit for the HAT activity of

SAGA and forms a subcomplex with Ada2 and Ada3 subunits. A subset of TBP-associated factors (TAFs; TAF5, TAF6, TAF9, TAF10 and TAF12) was found in the SAGA complex. The functions of these TAFs have been implicated in interacting with activators and forming histone-fold domain (HFD)-containing heterodimer pairs (TAF6–TAF9, TAF10–Spt7 and TAF12–Ada1) to maintain SAGA structure [165]. The product of the essential *TRA1* gene is a target for several activators including Gcn4, VP16 and Gal4 [166]. In addition to these subunits, novel SAGA components have recently been described: Ubp8, a ubiquitin-specific protease; Sgf11, required for anchoring of Ubp8; Sgf29 and Sgf73, with unknown function; and Sus1, which is also part of the nuclear pore-associated mRNA export machinery [167-169]. Recently, a variant of the SAGA complex, named SALSA or SLIK, which lacks Spt8 and contains a truncated form of Spt7, has been isolated, but its functional role is unclear [170, 171]. In vivo, yeast SAGA is required for the normal transcription of approximately 10% of genes [172].

1.4e Roles of Spt4, Spt5 in transcription elongation

Spt4 and Spt5 form a complex that is conserved from yeast to mammalian cells [134, 144, 173]. The mammalian and *Drosophila* Spt4–Spt5 complex, termed DSIF (DRB sensitivity-inducing factor), is involved in both the positive control of transcript elongation and in NELF-mediated Pol II pausing at the promoter before attaining a mature

elongation complex [93, 173, 174]. In *S. cerevisiae*, some *spt4* and *spt5* mutations render yeast sensitive to the drug 6-azauracil [135]. In addition, some phenotypes with *spt4* and *spt5* mutations are either suppressed or enhanced by mutations in two of the largest subunits of Pol II and in elongation factor IIS [135]. Genetic studies in yeast and in vitro transcription assays implicate Spt4 as a positive elongation factor [175]. Spt4 antagonizes the negative effects of Pol II pausing imposed by the chromatin-remodeling yeast factor Isw1p [176]. DSIF genetically and physically associates with IIF, CSB (Rad26), Spt6, FACT, Chd1, and the PAF1 complex [99, 135, 145, 150, 152, 177, 178]. Biochemical analyses suggest that DSIF, together with NELF, directly bind Pol II to repress elongation, while P-TEFb, a cyclin-Cdk pair with DRB-sensitive protein kinase activity, reverses the repression by phosphorylating the C-terminal domain (CTD) of the Pol II largest subunit [179]. Spt5 can be methylated at arginine residues by PRMT1 and PRMT5 in vitro [180]. Methylation of Spt5 together with P-TEFb-mediated phosphorylation of Spt5 and the CTD of Pol II seem to constitute modifications requisite for productive elongation.

1.4f Spt1, Spt10, Spt21, Spt11, and Spt12: regulating the histone levels

In yeast, each of the four histone genes (H2A, H2B, H3, and H4) is duplicated [181, 182], and therefore deletion of one copy of a gene is not lethal. However, disruption of histone genes, or overproduction of histones

can change the nucleosome patterns and lead to changes in the level of transcription at several loci, including at Ty insertion sites [13, 183, 184]. This is one of the earliest notion that chromatin regulates gene expression. The histones H2A and H2B are encoded by *SPT11* and *SPT12* respectively [181]. *SPT1* is identical to *HIR2* (histone regulatory) [185]. Spt1/Hir2 and another protein (Hir1) form a repressor complex that affects expression of histone genes [186]. Mutations in two other *SPT* genes, *SPT10* and *SPT21* [128], display similar phenotypes to mutations in *SPT1* [185]. These gene products also appear to be involved in histone gene expression [129]. Notably, Spt10 itself has HAT activity, it physically associates with Spt21 and regulate the transcription levels of one copy of H2A, H2B and H4 genes [133].

1.4g Other Spt proteins

SPT2 is identical to *SIN1* (for switch independent), which was isolated in a screen for suppressor mutations of *swi1* mutants [187]. *SPT2* encodes for an HMG1-like protein that genetically interacts with the Pol II CTD [188], histones H3 and H4 and physically with Swi/Snf complex [189]. However, it is still not clear the exact role of Spt2 in regulating chromatin structure.

SPT13 is identical to *GAL11* [190], which is a component of the Mediator complex that binds to the Pol II CTD[191]. Spt13 physically associates with Gal4, and is required for efficient utilization of galactose and for the Gal4-mediated expression of *GAL1*, *GAL7* and *GAL10* [192].

Unlike other SPT genes, *SPT23* was identified as a multi-copy suppressor of the Ty-induced promoter mutations of *HIS4* and *LYS2* and spt23 mutations do not confer an Spt- phenotype [193]. In vivo, Spt23 together with Mga2 are associated with ER membrane and required for transcription of fatty acid desaturase gene *OLE1* and transcription silencing [194, 195].

1.4h Understanding the involvement of other Spt transcription factors in post-recruitment functions

With the identification of Spn1 involvement in post-recruitment functions, this prompted me to examine the functions of other Spt proteins after TBP/TFIID recruitment. Taking the yeast SAGA complex for example, a lot of effort in characterization of SAGA functions during the past have been focused on the roles of SAGA in acetylating promoter nucleosomes and delivering TBP to TAF-independent promoters at the initiation stage [161, 196-198]. Less is known about how each of these SAGA subunits functions after the recruitment of TBP. Another example is Spt5. Recently, Spt5 was found to be involved in the regulation of pre-mRNA processing, as it interacts with the capping enzyme and stimulates its activity [99, 199] Spt5 (NELF) induced arrest of early elongation at promoter proximal positions was proposed to provide a frame of time during which recruitment of the capping enzymes occurs [93, 174]. Therefore, understanding the post-recruitment involvement of these Spt proteins would provide further

mechanistic information during Pol II transcription.

During my PhD studies, the behavior of all the non-essential SPT genes products were analyzed by artificial recruitment assay; I also studied their effects on post-recruitment regulated genes. These studies were extended to the non-Spt subunits of SAGA complex as described later in this dissertation. Our initial results have laid the foundation for the idea that the SAGA complex functions during the later stages of Pol II transcription.

1.5 Significance and thesis layout

As described above, the post-recruitment functions have important implications in addressing the regulatory mechanism of Pol II transcription. To get a better understanding of these post-recruitment functions, I focused my research on two aspects: 1. Characterize the functions of Spn1; 2. Define the post-recruitment functions of other Spt proteins during Pol II transcription.

To characterize the functions of Spn1 during Pol II transcription, a targeted genetic screen was designed to identify additional transcriptional factors that interact with Spn1. Through this genetic screen, two new functional interactions of Spn1 were uncovered and linked Spn1 chromatin remodeling and transcription elongation processes. The design and the results of the genetic screen are reported in Chapter 2.

Upon finishing the genetic screen, I focused my studies on the role of Spn1 in maintaining the chromatin structure. Using the *CYC1* gene as a

model, I was able to define the functional relationship among Spn1, Spt6 and the Swi/Snf complex during Pol II transcription. A model was proposed in which Spn1 coordinates the recruitment of chromatin remodeling activities of Spt6 and the Swi/Snf complex. This model was further tested by ChIP analysis. These studies are described in Chapter 3.

To further look at the functions of Spn1 during transcription, I compared the behavior of Spn1 with Spt6. The results from this part of study has shown the distinct functions of Spn1 from Spt6, proposing a possible role of Spn1 in transcription start sites selection as desribed in Chapter 4.

I also examined the post-recruitment functions of other Spt proteins during transcription. The initial results had led me to expand the focus on the post-recruitment functions of the yeast SAGA complex. These results suggested additional functions of SAGA complex during later stages of Pol II transcription as discussed in Chapter 5.

In Chapter 6, I summarized the conclusions drawn from my studies and tried to point out the future perspectives with respect to Spn1 functions and the post-recruitment functions of the SAGA complex.

In collaboration with Gayatri Yatherajam, a former graduate student in the Stargell Lab, I mapped the protein-protein interaction network in the yeast TFIID complex. This work resulted in a successful publication, which became a part of Dr. Yatherajam's dissertation. The abstract of the paper

is attached in the appendix.

I think that the results I gained from these studies have provided a basis toward a better understanding about the regulatory mechanism of RNA Pol II mediated transcription.

Chapter 2

SPN1 genetically interacts with the SWI/SNF genes, *RTF1* and TFIIS

One way to understand the mechanism of how Spn1 regulates RNA Pol II transcription is to find additional transcription factors that impact *SPN1* function. A yeast genetic screen was designed to directly target the transcription factors functionally interacting with *SPN1*. A variety of Pol II transcription factors were screened. The design and the results of the genetic screen are described and discussed in the following manuscript entitled as:

***SPN1* genetically interacts with the SWI/SNF genes, *RTF1* and TFIIS**

Lei Zhang and Laurie Stargell

SPN1 genetically interacts with the SWI/SNF genes, *RTF1* and TFIIS

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2.1 Abstract

SPN1 is an essential gene with a critical, although not well-defined role in regulating RNA polymerase II transcription. A genetic screen was developed to target the functions of Spn1 during transcription. After combining $spn1^{K192N}$, an *SPN1* mutant allele, with over 80 transcription factor mutants, we found that the $spn1^{K192N}$ allele weakly exacerbates the phenotypes of the deletion mutations of *RTF1*, TFIIS (*DST1*), suggesting a positive effect of *SPN1* in regulating transcription elongation. The $spn1^{K192N}$ allele strongly suppresses the deletion mutations of the key subunits of the Swi/Snf complex: *SNF2*, *SNF5* and *SNF6*. In addition, these three mutants suppress the temperature sensitive phenotype of the $spn1^{K192N}$ allele. There are no genetic interactions observed between *SPN1* and many other chromatin remodeling factors tested, thus the genetic interactions between *SPN1* and these SWI/SNF genes are specific and point out a role of *SPN1* in the chromatin remodeling process.

2.2 Introduction

Transcription by RNA Polymerase II (Pol II) initiates with the assembly of a preinitiation complex (PIC) at the promoter termed the transcription machinery. A large number of proteins are included in this PIC including general transcriptional factors (GTFs) TFIID, TFIIB, TFIIF, TFIIH, TFIIS, SRB/mediators and certain chromatin modifying factors [6, 7] For most of Pol II transcribed genes, recruitment of TFIID to the TATA element is a rate-limiting step [115, 121, 200]. Various activators enhance the PIC formation by directly interacting with TATA-binding protein (TBP), GTFs, and RNA Pol II [65, 116], certain co-activators such as TFIIA [201], TAFs [202], SRB/mediators [203]. Activators can also alter the locations or structures of nucleosomes by recruiting two classes of co-activators. One class of co-activators uses the energy of ATP-hydrolysis to displace nucleosomes and thereby expose or obscure protein-binding sites in the promoter. In *Saccharomyces cerevisiae*, these co-activators include Swi/Snf [204], RSC [205], ISW1 [206], ISW2 [207] and INO80 complexes [208]. Another class of co-activators alters chromatin structure by acetylating the lysine residues in the N-terminal tails of core histones. Such HAT complexes in yeast are known as the SAGA complex [161], NuA4 complex [209] and NuA3 complex [210]. Elongation provides another important aspect for the regulation of transcription by Pol II. Some factors affect elongation by overcoming transcriptional pausing or arrest of Pol II;

and others regulate through the chromatin structure. These factors include TFIIS, TFIIF, TFIIH, elongin, histones, Swi/Snf complex and some SPT genes such as *SPT4, SPT5, SPT6* and *SPT16* [94, 135, 211, 212].

SPN1 is an essential gene in yeast and conserved throughout evolution [127]. Previous studies from this lab have shown that a K192N mutation in *SPN1* suppresses the post-recruitment defects of a TBP mutant allele and affect the transcription of many Pol II genes differently. [127]. These results suggest that Spn1 plays important roles in regulating Pol II transcription. But the mechanism of how Spn1 functions is not clear. In this study, we set out to identify transcription factors that genetically interact with *SPN1*. The multiple steps of Pol II transcription are regulated by various transcriptional factors and many of these factors are encoded by non-essential genes. For example, Dst1 and Elp3 are involved in elongation [213]. Ada2, Gcn5, Snf2 and Snf5 are components of the chromatic altering complexes that are involved both initiation and the elongation steps [214, 215]. Components of the Srb/Mediators complex like Srb2, Sin4 and Nut1 modulate the transcriptional machinery functions on a subset of genes [216]. The identification of *SPN1* as an SPT gene [127] made it interesting to study the genetic connections between *SPN1* and other SPT genes since many of these SPT gene products affect chromatin structure and participate in multiple steps of the transcription [11, 107].

The completion of "Yeast Deletion Project" produced a unique collection comprised of 4787 individual yeast mutant strains in which each strain has one non-essential gene deleted [217]. From this collection, we chose over 80 strains that each had one Pol II transcriptional factor deleted, to study their genetic interactions with *SPN1*. The transcription factors include different activators, chromatin modifying factors, subunits of Mediator complex, elongation factor and many more. Since the functions of these factors have been characterized and are involved in different steps of Pol II transcription, combining this subset of mutants with the $spn1^{K192N}$ allele allowed us to define *SPN1* function during Pol II transcription. We found that *SPN1* genetically interacts with *RTF1,* TFIIS and some SWI/SNF genes, which links to the functions of *SPN1* to elongation and the chromatin remodeling processes.

2.3 Materials and methods

2.3a Yeast strains

The deletion collection strains [217] and the parental BY4741 strain (*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*) were purchased from Research Genetics. A subset of these deletion strains was chosen to study the genetic interactions between *SPN1* and the genes deleted in these strains. The *snf2Δ* strain (*MATα ada2Δ ada3Δ leu2Δ1 ura3Δ0 snf2::Kan*^{r}) was kindly provided by Dr. Karoline Cane [218]. The *spt16* (*MAT***a** *trp1 leu2 ura3 his3 spt16-11)* and *pob3* (*MAT***a** *trp1 leu2 ura3 his3 pob3-7)* mutant

strains and their parental strain (*MATa trp1 leu2 ura3 his3)* were provided by Dr. Timothy Formosa [106].The names of the genes that were mutated or deleted in these strains were listed in Table 2.1.

2.3b Yeast medium

Yeast medium used to analyze phenotypic changes were made according to literature [219]. The 5-fluoroortic acid (FOA) plates were made as described [220]. YPGal, YPEG plates were made by substituting dextrose of YPD with 2% galactose, 2% ethanol +2% glycerol. Plates containing sorbitol, NaCl, H_2O_2 were made by supplementing YPD medium with 1M sorbitol, 1M NaCl and 4mM H_2O_2. Medium lacking inositol (Ino^-) was made as described [221]. MPA plates were made by supplementing SC-U plates with 20 ug/ml mycophenolic acid.

2.3c Plasmid construction

A 2.2 kb fragment of *LEU2* gene containing its promoter, coding region and terminator was amplified from yeast genomic DNA and subcloned into pJF201 (*TRP1, CEN*) plasmid [127] to replace the *SPN1* open reading frame (ORF). The resulting plasmid pLT-1 has *LEU2* gene flanked by *SPN1* promoter and terminator, and was used to produce the *SPN1*::*LEU2* fragment for genomic *SPN1* deletion. An *SPN1* covering plasmid (pUS-1) was created by ligating *TOA1* promoter, *SPN1* ORF and *TOA1* terminator together and subcloned it into pRS316 plasmid *(URA3, CEN)*. Two 1.7 kb fragments containing either wild type *SPN1* gene or

spn1^{K192N} were isolated from pJF201 or pJF202, and subcloned into pRS313 *(HIS3, CEN)* to generate pHS-1, pHS-2 respectively.

2.3d Genetic screen

Briefly, both BY4741 and the deletion collection strains were first covered by pUS-1 by lithium acetate transformation. Then, 200ng of *SPN1::LEU2* fragment was used to replace the *SPN1* open reading frame (ORF) in the genome. Yeast cells with genomic *SPN1* deletion were unable to grow on 5-FOA medium and the deletion was further confirmed by genomic PCR. Finally, *HIS3* marked *SPN1* and *spn1*^{K192N} plasmids were shuffled into these genomic *SPN1* knockout strains and the engineered *SPN1* covering plasmid was cured by growing *HIS3* plus cells on 5-FOA containing media (Figure 2.1). To assay the genetic interactions of *SPN1* with different Pol II transcriptional factors, the strains that have genomic *SPN1* knocked out and covered by pHS-1 and pHS-2 were grown at different conditions. Phenotypic changes were scored by comparing the difference in growth between strains covered by *SPN1* and strains covered by *spn1*^{K192N}.

2.4 Results

2.4a Design of the genetic screen

To combine the *spn1*^{K192N} allele with the deletion mutations of various Pol II transcriptional factors, we used a one-step knockout method to delete the genomic *SPN1* ORF by *LEU2* replacement through

homologous recombination. The *spn1*^{K912N} allele was then brought in the collection strains by plasmid shuffling. Because *SPN1* is essential for viability, an alternate copy of *SPN1* must be transformed into these strains before deleting the genomic *SPN1*. We had used a *URA3* marked *SPN1* plasmid that had its native promoter and terminator. But the efficiency of genomic *SPN1* deletion was very low because the *LEU2* fragments typically integrated into the *SPN1* covering plasmid. To avoid this problem, two modifications were taken to facilitate the deletion of genomic *SPN1* (Figure 2.2A). First, an engineered *SPN1* plasmid in which *SPN1* ORF was controlled by *TOA1* promoter and terminator was used to cover the genomic *SPN1* deletion. This engineered *SPN1* covering molecule has no homologous region with the *SPN1::LEU2* fragment, thus ensures the efficient deletion of the genomic *SPN1*. Second, we increased the homologous region between *SPN1::LEU2* fragments and genomic *SPN1* locus to over 100 bp by flanking the *LEU2* gene with *SPN1* promoter and terminator. These modifications increased efficiency of genomic *SPN1* knockout by almost 200 fold and therefore made the genetic screen more successful. The deletion of genomic *SPN1* was be further confirmed by genomic PCR (Figure 2.2B). For final strain creation, *SPN1* and *spn1*^{K192N} molecules under native promoter and terminator on a pRS313 (*HIS3*, CEN) plasmid were shuffled in and the engineered *SPN1* covering molecule was cured by 5-FOA counter-selection.

Figure 2.1

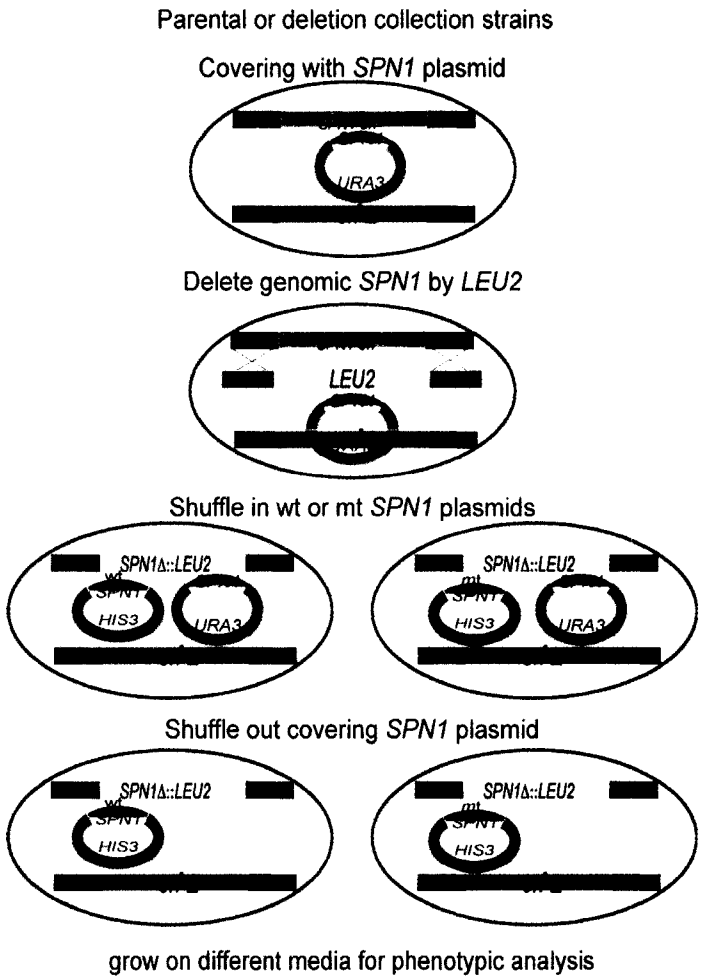

Figure 2.1 Design of the genetic screen. Schematic diagram of the genetic screen shows the process of combining mutant *SPN1* allele with deletion collection of different Pol II transcriptional factors.

Figure 2.2A

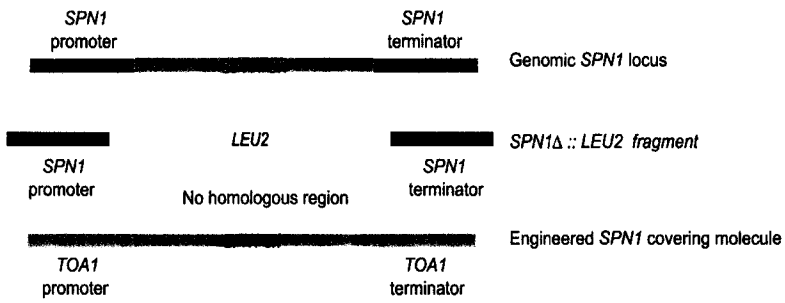

Figure 2.2A Schematic diagram shows the modifications that made the genomic *SPN1* deletion efficient A *URA3* marked *SPN1* plasmid to cover the genomic deletion is engineered, in which the *SPN1* open reading frame is controlled by *TOA1* promoter and terminator. The *SPN1::LEU2* fragment to replace the genomic *SPN1* has both ends flanked by over 100 bp sequences that are homologous to *SPN1* promoter and terminator in the genomic locus.

Figure 2.2B

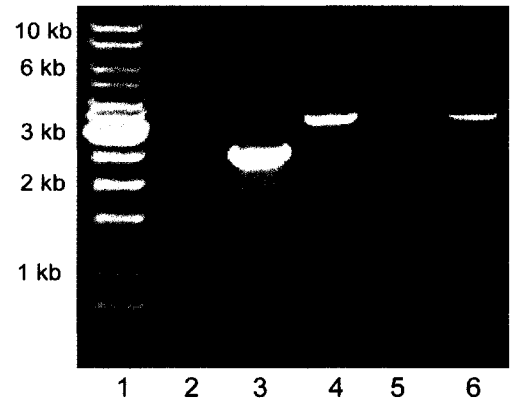

Figure 2.2B Confirmation of the genomic *SPN1* deletion by PCR Genomic DNA from different strains was subjected to PCR analysis. Successful integrating *LEU2* fragment into *SPN1* locus increases the size of *SPN1* loci by 720 bp. Primers that can encompass the integration region were used. Shown here is the picture of the PCR products separated on 1% agrose gel staining with ethidium bromide. Lane 1, 1Kb DNA ladder. Lane2, no templates control. Lane 3, BY4741. Lane 4, BY4741 *spn1Δ::LEU2*/pUS-1. Lane 5, BY4741 *spn1Δ::LEU2*/pHS-1. Lane 6, BY4741 *spn1Δ::LEU2*/pHS-2.

2.4b The *spn1*^{K192N} confers a temperature phenotype to the parental strain, BY4741

The behavior of the *spn1*^{K192N} allele in the parental BY4741 strain under a number of different conditions was tested. The *spn1*^{K192N} mutant strain only exhibited a temperature sensitive (TS) phenotype (Figure 2.3A). This TS phenotype is not due to the under-expression levels of K192N *SPN1* since protein blotting confirmed that expression of Spn1 and spn1^{K192N} from the plasmids were comparable to that from the genomic *SPN1* (Figure 2.3 B).

2.4c Genetic interactions between *SPN1* and Pol II transcriptional factors

After combining the *spn1*^{K192N} with the mutant alleles of over 80 Pol II transcription factors, these strains were grown under 11 different conditions to assay the phenotypic changes, which would indicate the genetic interactions between *SPN1* the deleted genes. The deletion mutants of *DPB4, PBP1* and *MSL1* were used as controls for the functions related to Pol II transcription process, since the gene products of them are implicated in DNA replication (Dpb4)[222], mRNA processing (Pbp1 and Msl1) [223, 224]. The summary of theses genetic interactions is shown in Table 2.1. We observed the phenotypic changes upon combining *spn1*^{K192N} with the deletion mutants of three Swi/Snf subunits, *snf2Δ*, *snf5Δ* and

Figure 2.3A

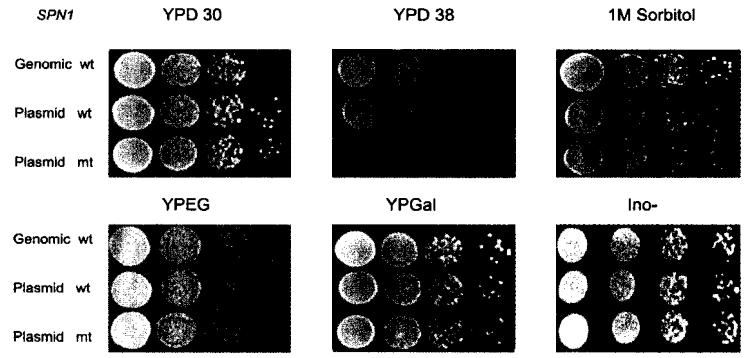

Figure 2.3A The *spn1*^{K192N} exhibited a temperature sensitive phenotype in BY4741 parental strain. Parental BY4741 strain with intact genomic *SPN1* or has the genomic *SPN1* and covered by plasmid-borne either wild type (wt) *SPN1* or *spn1*^{K192N} (mt) were assayed under different growing conditions. Similar amount of yeast cells were diluted serially and plated onto medium as indicated. Pictures were taken after growing the cells at 30°C for 2~3 days and at 38°C on YPD for 3 days. Picture showed that the *spn1*^{K192N} (mt) allele conferred a temperature sensitive phenotype to the parental strain; plasmid-borne *SPN1* molecule did not affect the behavior of the parental strain.

Figure 2.3B

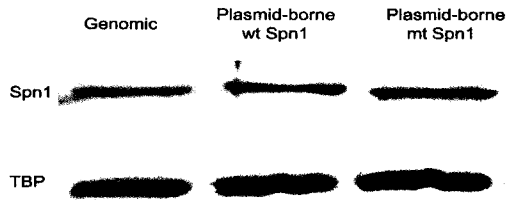

Figure 2.3B Protein levels of Spn1 from plasmid borne *SPN1*. Protein extracts prepared from strains as shown in figure 3A were subjected to western blot analysis using anti-Spn1 and anti-TBP antibodies. Picture shows comparable expression levels of plasmid borne *SPN1* and *spn1*^{K192N} to that of the genomic *SPN1*. TBP levels were used for protein loading control.

snf6Δ, under several conditions tested. We also observed the phenotypic changes after combining *spn1*^{K192N} with *rtf1Δ*, and with *dst1Δ*, which encodes the general transcription factor TFIIS. The genetic interactions between *SPN1* with these genes are further described in the following sections. However, we did not observe any phenotypic changes upon combining *spn1*^{K192N} with the other deletion mutants of the transcription factors such as activator, the Mediator complex subunits, Pol II associated factors and different histone acetylation factors (Table2.1). Although the casein kinase subunits were found in association with Spn1 [140], none of them genetically interacted with *SPN1* (Table 2.1).

2.4d The *spn1*^{K192N} allele suppresses the deletions of *SNF2, SNF5* and *SNF6*.

After combing *spn1*^{K192N} allele with 7 Swi/Snf mutant strains, we found that *spn1*^{K192N} strongly suppressed the phenotypes caused by deletions of three subunits of Swi/Snf complex, *SNF2*, *SNF5* and *SNF6*. (Table 2.1 and Figure 2.4A). Deletion of *SNF2* caused severe growth defects of yeast cells on all assaying conditions. But *snf5Δ* and *snf6Δ* mutants only exhibited growth defects on YPGal, YPEG, Ino$^-$ plates and H_2O_2, MPA and AT supplemented medium. All these growth defects were suppressed by further mutating *SPN1* in these strains. More strikingly, these SWI/SNF mutants also suppressed the TS phenotype of *spn1*^{K192N}.

Table 2.1 Summary of genetic interactions of between *SPN1* and the RNA polymerase II transcriptional apparatus

	Phenotypic conditions assayed [a]										
Genes deleted	30°	38°	14°	1M NaCl	1M sorbitol	4 mM H_2O_2	Ino⁻	YPG	YPEG	50mM AT	MPA
Swi/Snf genes											
SNF2	Sup	Sup	N	Sup	Sup	Sup	Sup	Sup	Sup	Sup	Sup
SNF5	N	Sup	N	N	N	Sup	Sup	Sup	Sup	Sup	Sup
SNF6	N	Sup	N	N	N	Sup	Sup	Sup	Sup	Sup	Sup
SNF11	N	N	N	N	N	N	N	N	N	N	N
SWI3	N	N	N	N	N	N	N	N	N	N	N
SWI5	N	N	N	N	N	N	N	N	N	N	N
SWI6	N	N	N	N	N	N	N	N	N	N	N
SAGA components											
SPT3	N	N	N	N	N	N	N	N	N	N	N
SPT7	N	N	N	N	N	N	N	N	N	N	N
SPT8	N	N	N	N	N	N	N	N	N	N	N
SPT20	N	N	N	N	N	N	N	N	N	N	N
ADA2	N	N	N	N	N	N	N	N	N	N	N
AHC1	N	N	N	N	N	N	N	N	N	N	N
NGG1	N	N	N	N	N	N	N	N	N	N	N
NIF3	N	N	N	N	N	N	N	N	N	N	N
HFI1	N	N	N	N	N	N	N	N	N	N	N
GCN5	N	N	N	N	N	N	N	N	N	N	N
Elongation factors											
DST1	N	N	N	Ex	Ex	Ex	N	Ex	Ex	Ex	Ex
RTF1	N	N	N	Ex	Ex	Ex	N	Ex	Ex	Ex	Ex
ELP2	N	N	N	N	N	N	N	N	N	N	N
ELP3	N	N	N	N	N	N	N	N	N	N	N
ELP4	N	N	N	N	N	N	N	N	N	N	N
ELP6	N	N	N	N	N	N	N	N	N	N	N
IKI3	N	N	N	N	N	N	N	N	N	N	N
ELA1	N	N	N	N	N	N	N	N	N	N	N
ELC1	N	N	N	N	N	N	N	N	N	N	N
NuA HAT factors											
HAT1	N	N	N	N	N	N	N	N	N	N	N
HAT2	N	N	N	N	N	N	N	N	N	N	N
EAF3	N	N	N	N	N	N	N	N	N	N	N
YNG1	N	N	N	N	N	N	N	N	N	N	N
HDAC factors											
HOS1	N	N	N	N	N	N	N	N	N	N	N
HOS2	N	N	N	N	N	N	N	N	N	N	N
HOS3	N	N	N	N	N	N	N	N	N	N	N
HST1	N	N	N	N	N	N	N	N	N	N	N
HDA1	N	N	N	N	N	N	N	N	N	N	N
RPD3	N	N	N	N	N	N	N	N	N	N	N
SIN3	N	N	N	N	N	N	N	N	N	N	N
SAP30	N	N	N	N	N	N	N	N	N	N	N
Nucleosome remodeling factors											

ISW1	N	N	N	N	N	N	N	N	N	N	N
ISW2	N	N	N	N	N	N	N	N	N	N	N
RAD26	N	N	N	N	N	N	N	N	N	N	N
MCM22	N	N	N	N	N	N	N	N	N	N	N
MGA2	N	N	N	N	N	N	N	N	N	N	N
NRG2	N	N	N	N	N	N	N	N	N	N	N
ITC1	N	N	N	N	N	N	N	N	N	N	N
CHD1	N	N	N	N	N	N	N	N	N	N	N
SPT16	N	N	N	N	N	N	N	N	N	N	N
POB3	N	N	N	N	N	N	N	N	N	N	N
SPT21	N	N	N	N	N	N	N	N	N	N	N
SPT23	N	N	N	N	N	N	N	N	N	N	N
Casein Kinase II subunits											
CKA1	N	N	N	N	N	N	N	N	N	N	N
CKA2	N	N	N	N	N	N	N	N	N	N	N
CKB1	N	N	N	N	N	N	N	N	N	N	N
CKB2	N	N	N	N	N	N	N	N	N	N	N
RNA polymerase II associated factors											
CDC73	N	N	N	N	N	N	N	N	N	N	N
MHR1	N	N	N	N	N	N	N	N	N	N	N
RPB9	N	N	N	N	N	N	N	N	N	N	N
Pol II holoenzyme Srb/Med factors											
ANC1	N	N	N	N	N	N	N	N	N	N	N
GAL11	N	N	N	N	N	N	N	N	N	N	N
ROX3	N	N	N	N	N	N	N	N	N	N	N
NUT1	N	N	N	N	N	N	N	N	N	N	N
SIN4	N	N	N	N	N	N	N	N	N	N	N
SRB2	N	N	N	N	N	N	N	N	N	N	N
SRB5	N	N	N	N	N	N	N	N	N	N	N
SRB8	N	N	N	N	N	N	N	N	N	N	N
SRB9	N	N	N	N	N	N	N	N	N	N	N
SSN3	N	N	N	N	N	N	N	N	N	N	N
Transcriptional activators and other SPT genes											
GAL4	N	N	N	N	N	N	N	N	N	N	N
GAL80	N	N	N	N	N	N	N	N	N	N	N
HPC1	N	N	N	N	N	N	N	N	N	N	N
STB5	N	N	N	N	N	N	N	N	N	N	N
MOT3	N	N	N	N	N	N	N	N	N	N	N
NHP6A	N	N	N	N	N	N	N	N	N	N	N
BAS1	N	N	N	N	N	N	N	N	N	N	N
RLR1	N	N	N	N	N	N	N	N	N	N	N
PHO23	N	N	N	N	N	N	N	N	N	N	N
TBS1	N	N	N	N	N	N	N	N	N	N	N
MBF1	N	N	N	N	N	N	N	N	N	N	N
SPT1	N	N	N	N	N	N	N	N	N	N	N
SPT2	N	N	N	N	N	N	N	N	N	N	N
Controls											
DPB4	N	N	N	N	N	N	N	N	N	N	N
PBP1	N	N	N	N	N	N	N	N	N	N	N
MSL1	N	N	N	N	N	N	N	N	N	N	N

[a]: mutant strains (as indicated) were combined with the K192N derivative of *SPN1* and alterations in growth properties under 11 different conditions were assayed. N denotes no change in phenotype from that observed in either of the two parental strains (the knock out strain or the K192N strain). Phenotypes that were exacerbated in the double mutant strains are denoted by "Ex". Strains in which we observed suppression of mutant phenotypes in the double mutant background are denoted by "Sup".

Figure2.4A

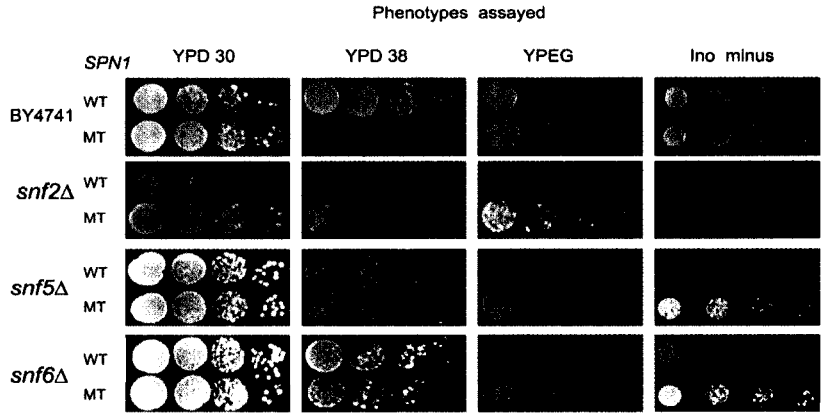

Figure 2.4A Suppressive effects between *SPN1* and the Swi/Snf genes Similar amount of yeast cells of the strains as indicated were diluted serially and plated onto medium shown above. Pictures were taken after growing the cells at 30°C for 2~3 days and at 38°C on YPD for 3 days. Figure shows that the TS phenotype of $spn1^{K192N}$ was suppressed by *snf2Δ*, *snf5Δ* and *snf6Δ*, and the growth defects of the SWI/SNF mutants were suppressed by $spn1^{K192N}$.

Figure 2.4B

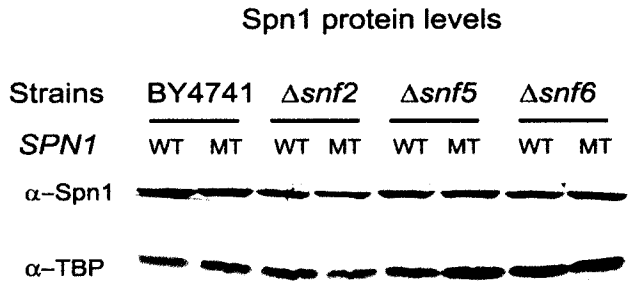

Figure 2.4B, Comparable Spn1 protein levels in the SWI/SNF mutant strains Protein extracts prepared from strains as shown were subjected to western blot analysis using anti-Spn1 and anti-TBP antibodies. Both Spn1 and $spn1^{K192N}$ levels from all strains tested are similar. TBP levels in these strains were used for protein loading control.

The suppressive effects between *SPN1* and the SWI/SNF genes were not due to the abnormal expression levels of Spn1, since the expression levels of Spn1 and spn1^{K1292N} in these strains were quite similar to that in the parental strain (Figure2.4B).

2.4e *SPN1* weakly interacts with *RTF1* and TFIIS.

Other than the strong genetic interactions described above, we found some weak genetic interactions between *SPN1* and two other RNA Pol II transcriptional factors, *RTF1* and *DST1* (Table 2.1, Figure 5.A). Deletion of *RTF1* only led to minor growth defects under most of the conditions used in this study as compared to the parental strain. Combing the *spn1^{K192N}* allele with the *RTF1* deletion mutation exacerbated these growth defects. *DST1* encodes the general transcription factor TFIIS that has been implicated in regulating transcription elongation [225, 226]. Deletion of TFIIS did not produce any observable growth defects under most growing conditions except a MPA sensitive phenotype. Further mutating *SPN1* to *spn1^{K192N}* in *dst1Δ* strain led to several observable growth defects (Figure 2.5A) and the MPA sensitive phenotype was further worsened (Table 2.1). Again, the protein levels of both wild type and the mutant Spn1 in these mutant strains are comparable to those in the parental strains (Figure 2.5B). Unlike the strong suppressive effects between s*pn1^{K192N}* and deletion mutants of the Swi/Snf complex, the

Figure 2.5A

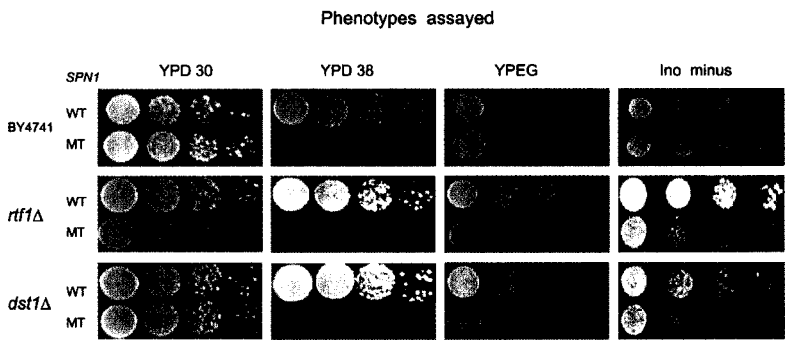

Figure 2.5A *spn1*^{K192N} exacerbates the phenotypes of *rtf1Δ* and *dst1Δ*
Similar amount of yeast cells of strains were diluted serially and plated onto YPD, YPEG plates and plates lacking inositol (Ino minus). Genotypes of the strains are indicated to left of the picture. Pictures were taken after growing the cells at 30°C for 2~3 days and at 38°C on YPD for 3 days. Figure shows that the growth defects of *rtf1Δ* and *dst1Δ* were exacerbated by further mutating *SPN1* in these strains

Figure 2.5B

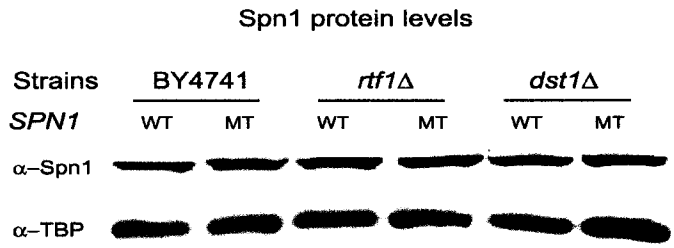

Figure 2.5B Spn1 protein levels were similar in *rtf1Δ* and *dst1Δ* strains
Protein extracts prepared from strains as shown were subjected to western blot analysis using anti-Spn1 and anti-TBP antibodies. Both Spn1 and spn1^{K192N} levels from all strains tested are similar. TBP levels in these strains were used for protein loading control.

genetic interactions of *SPN1* with *RTF1* or *DST1* are exacerbation and weak.

2.5 Discussion

In this study, we set out to identify transcriptional factor that functionally interact with *SPN1*. The modifications we took to facilitate the deletion of genomic *SPN1* expedited the genetic screen dramatically. The design of the genetic screen ensures that the $spn1^{K192N}$ allele was combined with deletion mutants of different Pol II transcription factors we chose to study, therefore directly target the genetic interactions between them. This method can be easily applied to study the genetic interactions of *SPN1* with factors in many other biological processes. Two lines of evidence suggested that the genetic interactions we identified are specific. First, we only identified the genetic interactions of *SPN1* with 5 transcription factors from over 80 factors we tested. Second, we observed that *SPN1* only genetically interacts with one ATP-dependent chromatin-remodeling complex, the Swi/Snf complex; but not with other ATP-dependent chromatin remodeling factors such as ISW1, ISW2 and Chd1 we tested (Table 2.1).

2.5a Implications of Spn1 in affecting chromatin-remodeling activity

The strong and specific genetic interactions of *SPN1* with the SWI/SNF genes linked Spn1 to the chromatin-remodeling process. Swi/Snf complex is comprised of 11 subunits and has a molecular weight of

approximate 2 MDa [59]. *SNF2* encodes an ATPase, the enzymatic subunit of the Swi/Snf complex [60]. Snf5 plays an essential role in maintain the assembly of the Swi/Snf complex [61]. Further, the function of Snf2 requires both Snf5 and Snf6 [61-63]. The other subunits may play minor roles in regulating the functions of Swi/Snf complex. In vivo, the Swi/Snf complex remodel nucleosomes in an ATP-dependent fashion by sliding histone octamer on the same DNA template or transfer them to another DNA template [58, 64]. The suppressive effects of $spn1^{K192N}$ on the deletion mutants of the Swi/Snf subunits suggest that Spn1 may counteract the chromatin-remodeling activities of the Swi/Snf complex. Krogan et al has identified that Spn1 (Iws1) directly interact with Spt6 [141]. Spt6 was shown to interact with histone H3 and capable of nucleosome assembly in vitro [105]. Mutations in *SPT6* not only suppress the transcription defects but also change the chromatin structure in Swi/Snf mutant strains [139]. Recent studies show that Spt6 facilitates transcription elongation through chromatin by disassembling and reassembling nucleosomes [107]. Therefore, Spn1 may serve as a modulator, regulating the nucleosome disassembly and reassembly activities of Spt6 to counteract the nucleosome remodeling activities of the Swi/Snf complex.

2.5b Involvement of Spn1 in transcription elongation

Since $spn1^{K192N}$ exacerbated the growth defects of both *dst1Δ* and *rtf1Δ* strains, this suggests that Spn1 may function in the same pathway in

regulating Pol II transcription with Rtf1 and Dst1 (TFIIS). *RTF1* was originally identified from a screen in which a mutant of *RTF1* suppressed a TBP mutant, *spt15-122*, by altering the transcription initiation [227]. Rtf1 was also identified as a member of the Paf1 complex that associates with Pol II [177]. *RTF1* genetically interacts with a range of transcriptional factor such as *SRB5, CTK1, FCP1, POB3, SPT4, SPT5, SPT6* and *SPT16*, and products of all these genes have been implicated in elongation [135, 150, 151, 211, 228, 229]. TFIIS has long been known for its role in transcriptional elongation [89, 101, 225, 226]. TFIIS rescues arrested polymerase at pausing sites by stimulating polymerase to cleave its nascent transcript and read through the elongation blocks [102, 226, 230]. The genetic interactions of *SPN1* with these two elongation factors indicate that Spn1 has a positive role in transcriptional elongation. Results of others also suggest that Spn1 may regulate the elongation process. For example, Spn1 colocalizes with elongating Pol II [99]. However, the weak interactions of Spn1 with the elongation factors suggest that Spn1 may only have a minor or an indirect effect in elongation. In addition, the $spn1^{K192N}$ does not display 6-AU or MPA sensitivity [127], a common phenotype shared by mutants of many transcriptional elongation factors [231]. Studies also showed that mutants of *SPT6* and the Swi/Snf complex were synthetically lethal with TFIIS null mutant [135, 218]. We think that the involvement of Spn1 in elongation may be related from its role in

affecting the chromatin remodeling activities since chromatin structure also has a profound effect on elongation [16, 87].

Our genetic screen successfully identified new transcription factors that functionally interact with Spn1. These interactions implied a role for Spn1 in regulating RNA Pol II transcription by affecting chromatin structure and the elongation process.

Chapter 3

Spn1 coordinates the recruitment of Spt6 and the Swi/Snf complex to the *CYC1* gene

The suppressive effects between *SPN1* and the Swi/Snf genes identified from the genetic screen suggest a role of Spn1 in regulating the chromatin remodeling process during Pol II transcription. Therefore, I looked at functional relationship between Spn1 and the Swi/Snf complex during *CYC1* activation. The results are reported and discussed in this chapter in the following written manuscript.

Spn1 coordinates the recruitment of Spt6 and the Swi/Snf complex to the *CYC1* gene: Lei Zhang and Laurie Stargell

Spn1 coordinates the recruitment of Spt6 and the Swi/Snf complex to the *CYC1* gene

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3.1 Abstract

Previous studies suggest that Spn1, which is encoded by a conserved and essential gene in yeast, regulates transcription by RNA polymerase (pol) II after recruitment of TATA-binding protein (TBP). We also observed strong antagonizing genetic interactions between Spn1 and the chromatin remodeling complex Swi/Snf, and direct physical interactions with Spt6, a chromatin-remodeling factor, have been observed. The mechanistic interactions between Spn1, Spt6, and the Swi/Snf complex were investigated on the *CYC1* gene, which is an excellent model system for genes transcriptionally regulated at steps after TBP and Pol II binding. We find that Spn1 is constitutively recruited to the *CYC1* promoter under both partially repressed and fully activated conditions. This recruitment is likely due to the fact that Spn1 associates with Pol II. In contrast, both Spt6 and Swi/Snf appear at *CYC1* only after activation. An Spn1 mutant that is defective for interaction with Pol II, is no longer recruited to the

CYC1 promoter. In addition, Spt6 is absent from the promoter under both partially repressed and activated conditions. In contrast, Swi/Snf is now constitutively recruited to *CYC1*. The overall affect of loss of Spn1 recruitment and constitutive Swi/Snf occupancy is an increase in *CYC1* expression under partially repressed conditions. These observations support a model whereby Spn1 negatively regulates recruitment of Swi/Snf to the *CYC1* promoter, and this inhibition is abrogated by the Spn1-Spt6 interaction. This model predicts that upon activation in a wild type cell, Spt6 should be recruited to the promoter prior to the Swi/Snf complex. A time course during activation reveals that Spt6 significantly precedes Swi/Snf occupancy at the *CYC1* promoter. Taken together, these results indicate that Spn1 serves a critical role in regulating Pol II transcription by coordinating the recruitment of chromatin remodeling factors.

Spn1 is a conserved RNA Polymerase II (Pol II) transcriptional factor identified recently by others and us [99, 127, 141]. The genetic interaction of Spn1 with TBP [127], its co-localization with Pol II during transcription and potential association with capping enzyme [99, 141], its physical association with Spt6 [140, 141] suggest that Spn1 plays a role in transcription initiation, elongation and mRNA processing, although the functional mechanisms of how Spn1 regulates these processes are entirely unknown.

To understand the role of Spn1 during RNA Pol II transcription in *S.cerevisiae*, a recessive and temperature sensitive *SPN1* allele [127], $spn1^{K192N}$ was utilized in a genetic screen to identify additional transcriptional factors that interact with Spn1. Strong antagonizing interactions between *SPN1* and *SNF2*, *SNF5* and *SNF6,* respectively, were uncovered. The $spn1^{K192N}$ allele suppressed the severe growth defects of *snf2Δ, snf5Δ* and *snf6Δ* on nonfermental carbon source medium; in addition, these SWI/SNF mutants all suppressed the temperature sensitive phenotype of $spn1^{K192N}$ [232]. Snf 2, 5 and 6 are the core subunits of the Swi/Snf complex, a conserved ATP-dependent chromatin-remodeling factor that can facilitate Pol II transcription by altering the nucleosomal structure [58, 63, 233]. Snf2 harbors an ATPase activity [234]; Snf5 and Snf6 are involved in maintaining the integrity of the Swi/Snf complex [61, 63]. Therefore, the strong genetic interactions between *SPN1* and the SWI/SNF genes suggested that Spn1 might regulate Pol II transcription by antagonizing the function of the Swi/Snf complex *in vivo.*

3.2 The Swi/Snf complex affects *CYC1* transcription

To further characterize the mechanistic interactions between Spn1 and the Swi/Snf complex, we focus our studies on a particular yeast gene *CYC1*. Unlike the majority of other Pol II transcribed genes, of which the transcription levels are closely correlated to the levels of TBP occupancy [65, 115]; *CYC1* transcription levels remains low even when TBP and Pol II

are already recruited to the promoter [65, 118]. Like *CYC1*, there are some other genes that are repressed similarly [65, 119]. However, little is known about the mechanism of how these genes are regulated. Previous study from this lab showed that *CYC1* transcription levels were increased even when the $spn1^{K192N}$ cells were grown in partial repressed conditions, and there was an additional 5-fold increase upon activation [127]. This result suggested a negative effect of Spn1 on *CYC1* transcription. The genetic interaction between Spn1 and Swi/Snf complex promotes us to explore whether Swi/Snf was also involved in *CYC1* transcription regulation. Using S1 nuclease assay, *CYC1* transcripts were measured in the parental and $spn1^{K192N}$ strains, *snf5*Δ and *snf6*Δ strains, $spn1^{K192N}$ *snf5*Δ *and* $spn1^{K192N}$ *snf6*Δ double mutant strains (Figure 3.1A and B). Consistent with our previous studies, the K192N mutation in *SPN1* resulted in an additional 5-fold increase in *CYC1* transcription levels upon activation [127]. Deletions of *SNF5* or *SNF6*, the two key Swi/Snf subunits, caused a 50 or 35 percent decrease in *CYC1* transcription level as compared to wild type parental strain. This indicated that the function of the Swi/Snf complex was required for normal levels of *CYC1* transcription. Further mutating *SPN1* in these Swi/Snf deletion strains restored the *CYC1* transcription close to normal levels (Figure 3.1A and B). These results supported our genetic findings

Figure 3.1A

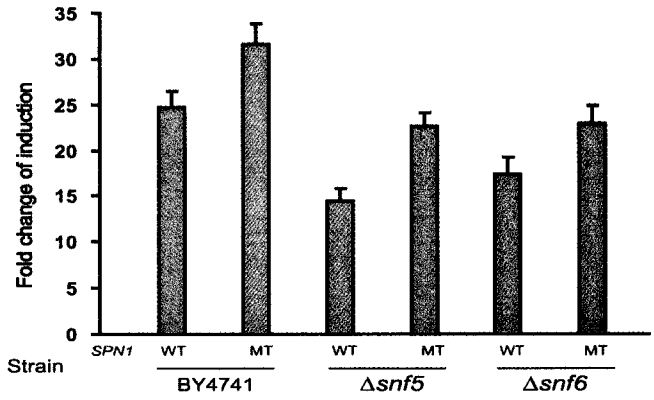

Figure 3.1B

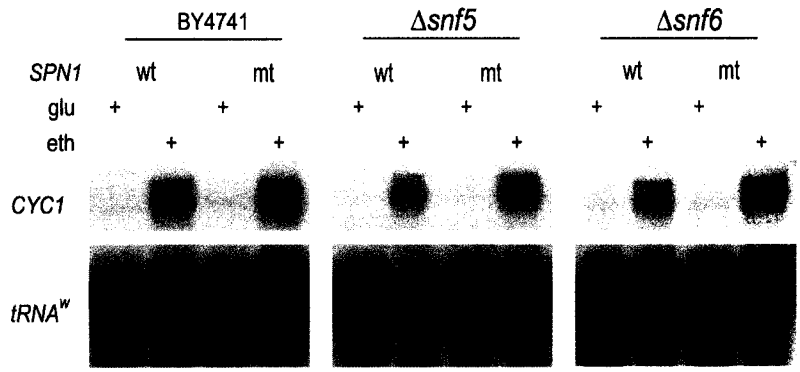

Figure 3.1 The Swi/Snf complex is required for full activation of the *CYC1* gene (**A**) Quantification of the effects of Swi/Snf mutants on *CYC1* transcription. Bar graphs showed fold changes (mean ±SD, $p<0.005$) of *CYC1* levels upon activation versus partial repression from each strain of 4 separate experiments. The induction fold changes were calculated by dividing the signals of *CYC1* transcripts upon activation by those during partial repression. (**B**) Representative picture of S1 nuclease assay results. Total RNA from strains as indicated grown under partial repressed and activated conditions were analyzed by S1 nuclease assay. Protected nucleic acids were resolved on denaturing gel. $tRNA^W$ signal was used as a load control to normalize signals of *CYC1* transcripts.

and suggested a counteracting effect between Spn1 and Swi/Snf complex at a molecular level.

3.3 Spn1, Spt6 and the Swi/Snf complex were all present on the *CYC1* promoter

Spn1 physically associated with Spt6 [140, 141, 235]. More interestingly, certain mutations in *SPT6* also suppressed mutations of *SNF2, SNF5* and *SNF6* [137, 139, 236]. Since Spn1 and the Swi/Snf complex both affected *CYC1* transcription, it is possible that these three factors may directly regulate *CYC1* transcription. To test this hypothesis, we examined the occupancy levels of these factors on the *CYC1* gene during transcription by chromatin immunoprecipitation (ChIP) analysis.

While there were comparable levels of wild type Spn1 occupied on the *CYC1* promoter during both partial repression and activation; the occupancy levels of mutant Spn1 were diminished under both conditions and was only 10% of that of the wild type Spn1 (Figure 3.2A and B). Lower levels of Spt6 occupied on the *CYC1* gene during partial repression; and were increased by more than five-fold upon activation. In the K192N mutant *SPN1* background, very little Spt6 was recruited to *CYC1* under both partial repressed and activated conditions; the occupancy level of Spt6 under both conditions in mutant *SPN1* strain is comparable to that in the wild type *SPN1* strain under partially repressed condition (Figure 3.2A and B). On the other hand, the Swi/Snf complex occupancy levels were

Figure 3.2A

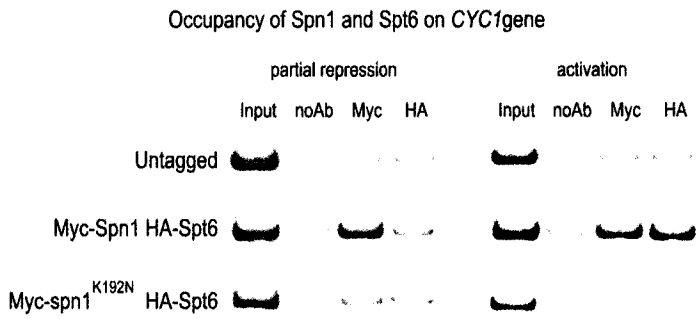

Figure 3.2B

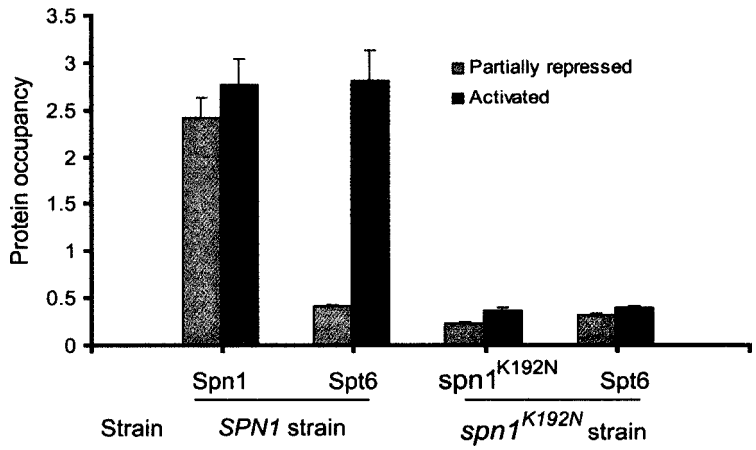

Figure 3.2A and B Occupancy of Spn1 and Spt6 on *CYC1* promoter during activation. ChIP analysis was performed on strains as indicated grown under either partial repression or activation to measure the occupancy levels of Spn1 and Spt6. Figures show wild type Spn1 constantly occupies the *CYC1* gene while mutant spn1 diminished from the *CYC1* gene. Spt6 is recruited upon activation only in *SPN1* strain but not in *spn1*^{K192N} strain. (**A**) Representative picture of gel shows the occupancy of Spn1 and Spt6 on *CYC1* gene during partial repression and activation. (**B**) Bar graph showed quantifications of the relative Spn1 and Spt6 occupancy levels upon activation versus those during partial repression (n=4, $p<0.005$). Protein occupancy was represented by ratio of signal from IP samples versus that of the input after minus the signal of no antibody control.

fairly low during partial repression; upon *CYC1* activation, the occupancy levels of the Swi/Snf complex were increased by more than 5 fold (Figure 3.2C and D). This is rather similar to the occupancy pattern of Spt6 in wild type *SPN1* strain. However, in the mutant *SPN1* strain, unlike the diminished occupancy levels of Spt6 or mutant Spn1, the Swi/Snf complex constitutively occupies the *CYC1* promoter in levels that was comparable to that in the wild type *SPN1* strain during activation (Figure 3.2C and D). The presence of Spn1, Spt6 and the Swi/Snf complex on the *CYC1* gene clearly suggested the direct involvement of these factors in regulating *CYC1* transcription.

Recent studies have shown that Spt6 promotes nucleosome assembly in vitro and interacts with histones [105]. Consistent with these findings, *SPT6* mutant strains display alterations in chromatin structure in vivo [105]. Mutations in the *SPT6* gene lead to transcription initiation from cryptic start sites within the coding sequences [107]. These results support the role of Spt6 in remodeling the chromatin structure and lead to the argument that Spt6 is involved in reassembling nucleosomes after the passage of Pol II during transcription [107, 237, 238]. Indeed, it has been shown that nucleosomal patterns of *CYC1* coding region remains unchanged during activation [118], which firmly suggests that the coordinated functions of the chromatin remodeling and reassembly factors are required in maintaining normal level of *CYC1* transcription.

Figure 3.2C

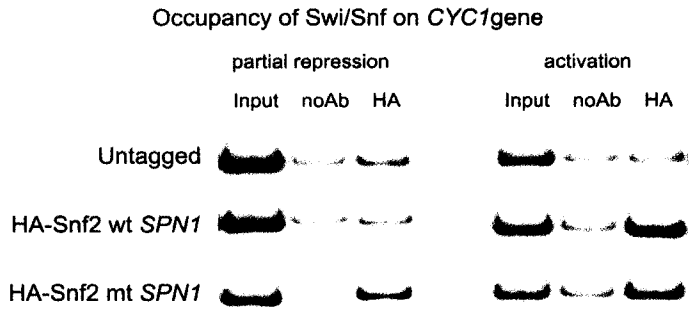

Figure 3.2D

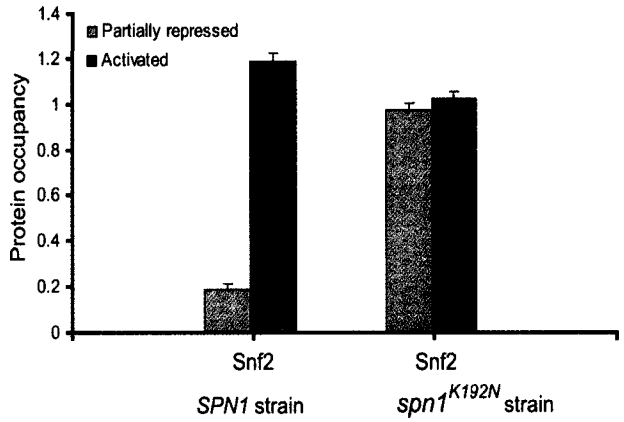

Figure 3.2C and D The Swi/Snf complex is recruited to the *CYC1* promoter only upon activation in *SPN1* strain but is constantly recruited in *spn1*^{K192N} strain Occupancy levels of the Swi/Snf complex in strains grown under either partial repressed or activated conditions *CYC1* gene were measured by ChIP analysis. Figure showed increased Swi/Snf occupancy upon activation in wild type *SPN1* strain and constitutively enhanced occupancy of Swi/Snf complex in the mutant strain under both conditions. (**C**) One representative gel showed the signals of Swi/Snf occupancy levels by ChIP analysis. (**D**) Changes of Swi/Snf occupancy on the *CYC1* gene in *SPN1* and *spn1*^{K192N} strains. Bar graph showed the Swi/Snf complex occupancy on *CYC1* promoter during partial repressed and activated conditions (n=4, $p<0.005$).

3.4 Spt6 was recruited earlier than the Swi/Snf complex during *CYC1* activation

Since Spn1 constitutively occupies the *CYC1* promoter and the loss of Spn1 occupancy resulted in loss of Spt6 occupancy, these suggest that Spt6 may be recruited through its interaction with Spn1. Because the diminished occupancy levels of Spn1 led to the increased occupancy levels of the Swi/Snf complex, and Spn1 functionally antagonized the Swi/Snf complex, these results suggest that Spn1 negatively regulates the recruitment of the Swi/Snf complex to the *CYC1* gene. Taken together, we propose a model in which Spn1 blocks recruitment of the Swi/Snf complex during partial repression of *CYC1* and it recruits Spt6 to the *CYC1* gene upon activation. Furthermore, we think that the interactions between Spt6 and Spn1 on the *CYC1* gene may relieve Spn1 inhibition on the Swi/Snf complex recruitment. This model predicts a time line in which Spt6 precedes Swi/Snf occupancy during *CYC1* activation. To test this hypothesis, we performed time courses on the occupancy levels of both Spt6 and the Swi/Snf complex during *CYC1* activation.

The *CYC1* gene activation reached its maximum level in about 6 hours after growing the yeast cells in medium containing ethanol (Figure 3.3A). As expected, Spt6 was recruited quickly upon activation. At 1 hour after activation, Spt6 occupancy levels reached over 70% of its maximum

occupancy level (Figure 3.3B). The Swi/Snf complex was recruited much slower than Spt6; its occupancy levels were not increased until 2 hours after activation and reached its maximum level at about the 4 hr-time point (Figure 3.3C and D). The fact that Spt6 was recruited earlier than the Swi/Snf complex upon activation supports our model that the interacting of Spt6 with Spn1 lifts the block on Swi/Snf recruitment.

3.5 Spn1 and the Swi/Snf complex are recruited to the *CYC1* gene by interacting with Pol II and exist in two populations of Pol II complexes

In our model, we think that Spt6 is recruited by its interaction with Spn1, which constantly occupies the *CYC1* gene. However, it is not known how Spn1 is recruited. Studies have shown that TBP and Pol II are already recruited to the *CYC1* gene even when it is repressed [118], and that Spn1 does not associate with TBP or TFIID in vivo [127]. Therefore, one possibility is that Spn1 may be recruited by interacting with Pol II. To test this hypothesis, we performed the coimmunoprecipitation assay to examine the association of Spn1 with Pol II. We found that Spn1 was co-precipitated with Pol II but the mutant Spn1 was not (Figure 3.4A). This suggests that Spn1 is indeed recruited by interacting with Pol II. The finding that mutant Spn1 lost its association with Pol II well explained the diminished occupancy levels of $spn1^{K192N}$. It also explained the diminished Spt6 occupancy on the *CYC1* gene in $spn1^{K192N}$ strain since Spt6 was recruited by interacting with Spn1. Similarly, it is possible that the Swi/Snf complex

Figure 3.3A

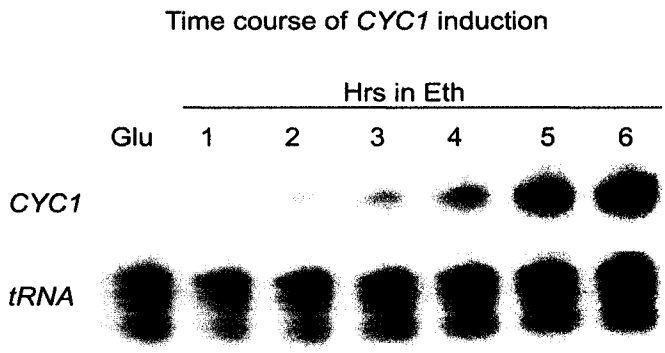

Figure 3.3B

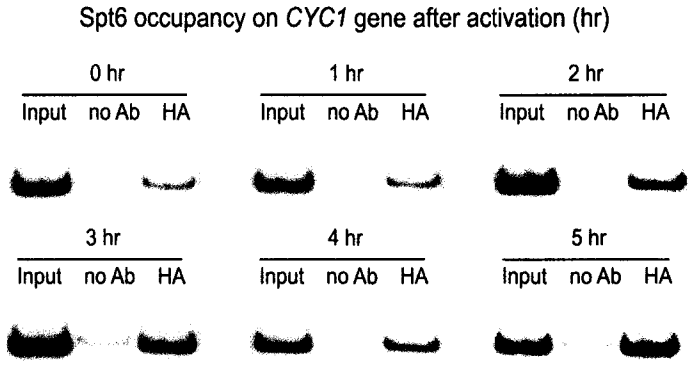

Figure 3.3A and B Time course of *CYC1* activation and Spt6 occupancy during *CYC1* transcription (**A**) Time course of *CYC1* activation reaches its peak after induction for 6 hours. S1 nuclease assay was used to detect *CYC1* transcripts after activated for 0 hr (cells were grown in glucose-containing medium) and 1~5 hours (cells were grown in medium containing ethanol). tRNA signal was used as loading control. (**B**) Changes of Spt6 occupancy on *CYC1* gene after activation. Shown here is a representative picture of Spt6 occupancy after 0~5 hours of activation.

Figure 3.3C

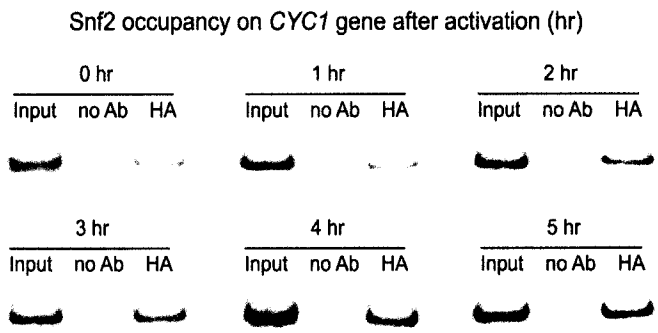

Figure 3.3D

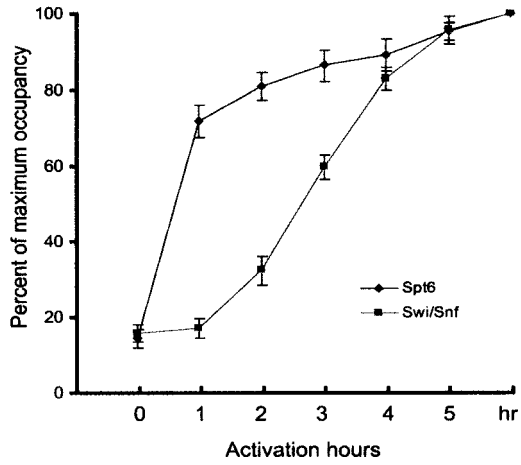

Figure 3.3C and D Spt6 is recruited earlier than the Swi/Snf complex upon *CYC1* activation (**C**) Changes of Swi/Snf complex occupancy on *CYC1* gene after activation. Shown here is a representative picture signals of Swi/Snf occupancy after 0~5 hours of activation. (**D**) Time course of Spt6 and Swi/Snf complex occupancies on *CYC1* upon activation. The levels of Spt6 and Swi/Snf complex occupancies at 6 hours after activation were set as 100%. The occupancy levels of both factors at each time point were converted to the percentage of their maximum occupancy levels and graphed (n=3, $p<0.05$).

may also be recruited by interacting with Pol II since many studies have observed the association of the Swi/Snf complex with Pol II [67, 68]. Consistent with these findings, we found that in both wild type and mutant *SPN1* strain background, the Swi/Snf complex co-immunoprecipitated with Pol II (Figure 3.4B).

Spn1 and the Swi/Snf complex might be in the same Pol II complex. However, this was not the case since we found that neither wild type Spn1 nor the mutant co-precipitated with the Swi/Snf complex (Figure 3.4C). This indicates that there are two populations of Pol II complexes, one contains Spn1 and the other contains the Swi/Snf complex. More likely, upon the recruitment of the Swi/Snf complex, Spn1 may become loosely associated with Pol II or exist in the vicinity of the Pol II complex such that it can be detected by ChIP analysis but not by the coimmunoprecipitation assay. Nonetheless, the existence of Spn1 and the Swi/Snf complex in two different pol II complexes may suggests that Spn1 and the Swi/Snf complex may mutually exclude each other. This model was also supported by the observations of *CYC1* transcription in mutant *SPN1* strain. Diminished Spn1 occupancy led to increased recruitment of the Swi/Snf complex, which caused the depression during partial repression and the elevated transcription upon activation of the *CYC1* gene.

Figure 3.4A

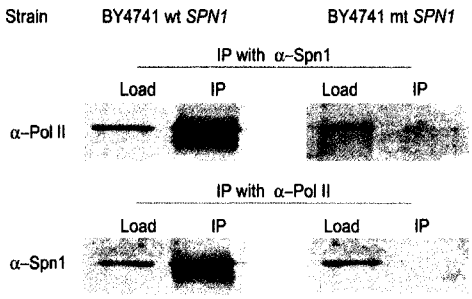

Figure 3.4B

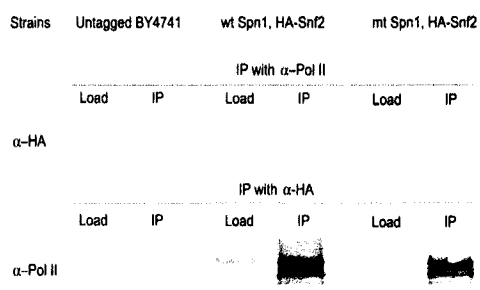

Figure 3.4C

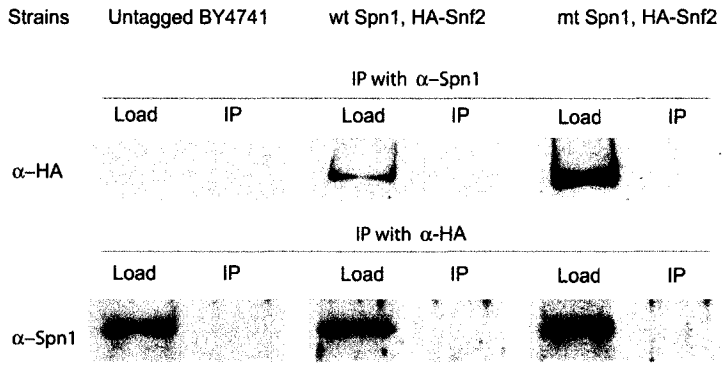

Figure 3.4 Physical associations among Spn1, the Swi/Snf complex and Pol II Protein extracts from strains indicated were precipitated with protein-A sepharose beads coupled with different antibodies as indicated. Protein of interest was detected by western blot using corresponding antibodies. (**A**) Spn1 associates with Pol II and spn1^{K192N} loses the association with Pol II (**B**) The Swi/Snf complex associates with Pol II in both *SPN1* and *spn1*^{K192N} strains. Untagged BY4741 strain was used as a negative control. (**C**) The Swi/Snf complex does not associate with either Spn1 or spn1^{K192N}. Untagged BY4741 strain was used as a negative control.

3.6 The role of Spn1 in regulating the *CYC1* gene transcription

Our results showed that Spn1, a highly conserved RNA Pol II transcription factor, associates with Pol II complex and represses the recruitment of chromatin remodeling complex, Swi/Snf. It also recruits Spt6, an essential chromatin reassembly factor and then facilitates the recruitment of Swi/Snf complex. Therefore, Spn1 plays a central role in coordinates the recruitment of the chromatin remodeling factor and chromatin reassembly factors. Furthermore, we showed that it was not the chromatin remodeling factors but the reassembly factors being recruited first. This fact has important implications for the maintenance of chromatin structure, as more and more evidences suggest that normal chromatin structure is maintained during transcription [107, 108, 136]. The concerted functions of chromatin remodeling and reassembly factors will probably ensure the genes being transcribed at precisely levels.

3.7 Materials and methods

3.7a Yeast strains

The yeast strains used in this study are listed in Table 3.1

3.7b Transcriptional assays

S1 nuclease assay were conducted as described[127]. For *CYC1* induction, cultures grown overnight in rich medium containing 2% glucose

Table 3.1 Yeast strains used in this study

Strains	Genotype
BY4741	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0*
LZY001	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 spn1Δ:: LEU2/PRS313- SPN1*
LZY002	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 spn1Δ:: LEU2/PRS313- spn1^{K192N}*
snf5Δ	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf5Δ::Kan^{r}*
LZY103	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf5Δ::Kan^{r} spn1Δ:: LEU2/PRS313- SPN1*
LZY104	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf5Δ::Kan^{r} spn1Δ:: LEU2/PRS313- spn1^{K192N}*
snf6Δ	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf6Δ::Kan^{r}*
LZY105	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf6Δ::Kan^{r} spn1Δ:: LEU2/PRS313- SPN1*
LZY106	*MATa his3Δ1 ura3 leu2Δ0l met15Δ0 snf6Δ::Kan^{r} spn1Δ:: LEU2/PRS313- spn1^{K192N}*
snf2Δ	*MATα ada2Δ ada3Δ leu2Δ1 ura3Δ0 snf2Δ::Kan^{r}*
LZY101	*MATα ada2Δ ada3Δ leu2Δ1 ura3Δ0 snf2Δ ::Kan^{r} spn1Δ:: LEU2/ YCp50-SPN1*
LZY102	*MATα ada2Δ ada3Δ leu2Δ1 ura3Δ0 snf2Δ ::Kan^{r} spn1Δ:: LEU2/ YCp50-spn1^{K192N}*
LZY201	*MATa his3Δ1 ura3 leu2Δ01 met15Δ0 SNF2-3HA::HIS3 spn1Δ::LEU2 /YCp50-SPN1*
LZY202	*MATa his3Δ1 ura3 leu2Δ01 met15Δ0 SNF2-3HA::HIS3 spn1Δ::LEU2 /YCp50-spn1^{K192N}*

were washed three times in medium lacking glucose, diluted into medium containing 3% ethanol, and cultured at 30°C for 6 hr. For uninduced samples, cells were grown in YPD for 6 hours at 30°C till optical density reached 0.8~1.0. Yeast cells were then harvested and total RNA was isolated by hot phenol extraction. Hybridizations with excess probe were normally done with 25–30 μg of RNA samples were hybridized with excess ^{32}P labeled probe overnight at 55°C. S1 nuclease digestion was performed on the hybridized samples for 30–45 min at 37°C. Band intensity was normalized to the intensity of the $tRNA^{W}$ band.

3.7c Co-immunoprecipitation experiments

Co-immunoprecipitation experiments were performed as described previously with a few modifications [239]. Cultures were grown to an optical density (600 nm) of about 1.0 in rich medium containing 2% dextrose. Cell extracts (300 μg) were used immediately following preparation and were precleared by incubation with a 50 μl of plain protein A-sepharose beads (Pharmacia) for 1 hr at 4°C. A small sample was taken after the preclear step to provide a load control. Anti-HA, polyclonal anti-Spn1 and anti-Rpb1 antibodies were coupled to protein A-sepharose beads, and the remaining extract was incubated with 50 μl of these coupled beads for 2 hr at room temperature with occasional stirring. After six washes, the beads were boiled in loading buffer and 15 μl was loaded for SDS-PAGE, followed by immunoblot analysis.

3.7d Chromatin immunoprecipitation analysis

Chromatin immunoprecipitations were performed as described [240] with few modifications. Cells (150 ml) were grown to OD 600 of 0.8~1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 minutes with occasional swirling of the flasks at intervals of 5 minutes. Glycine was added to a final concentration of 125mM at room temperature for 5 minutes to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were resuspended in FA-lysis buffer (500 µl of FA-Lysis buffer for a total of 50ml of cell culture). Chromatin was sheared by sonication using a Branson W-350 model of sonifier (10 times at 10seconds each on continuous pulse at a microtip power setting of 6). A 10% of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. About 500 µl of the chromatin material was incubated with approximately 5µl of either anti-Spn1 or anti-HA antibodies by rotation overnight at 4°C. A 50µl of protein-A sepharose beads (Pharmacia-prepared as slurry as per the manufacturers directions) was further incubated with the chromatin material for 2 hours at 4°C. The beads were spun down and the Antigen-Antibody complexes bound to the beads were recovered and further treated with TE/SDS buffer for 15 minutes at 65°C to elute the complexes. Protein-DNA crosslink were reversed by incubation

overnight at 65°C and the DNA was purified by phenol-chloroform extraction and used for the PCR analysis.

PCR reactions were carried out in a total volume of 25 µl. Each reaction contains 1µl of 1/100 dilution of 10mCi/ml ^{32}P labeled dATP. Different dilutions of each input and immunoprecipitated samples were used to determine the linear range of the PCR reaction. The PCR products were run on 5% native polyacrylamide gels in 0.5X TBE buffer. The gels were dried and exposed top phosphor-image screen. Images were scanned by STORM and quantified using image quant software analysis to detect the strengths of various signals. No antibody samples were used as controls. Primers for the PCR were designed at the promoter region of the *CYC1* gene and amplified a product of 312 bp [118]. The signal strength ratio between the IP sample and the input after minus the signals of no antibody control was used as an indication of the occupancy of the protein.

Chapter 4

Distinct functions of Spn1 and Spt6 during RNA polymerase II transcription

From the previous studies, Spn1 was found in association with Spt6 and RNA polymerase II. In this Chapter, I focused on the roles of Spn1 and Spt6 on transcription after they are recruited to the *CYC1* promoter. Part of the work was done in collaboration with David Goldstrohm, a fellow graduate student in our lab and with Vanessa Cheung from Fred Winston's lab at Harvard Medical School. They contributed Figure 4.4B and Figure 4.2B of this chapter. The results were presented in the following manuscript entitled:

Distinct functions of Spn1 and Spt6 during RNA polymerase II transcription: Lei Zhang, David Goldstrohm, Vanessa Cheung, Fred Winston and Laurie Stargell

Distinct functions of Spn1 and Spt6 during RNA polymerase II transcription

Lei Zhang, David Goldstrohm, Vanessa Cheung*, Fred Winston* and Laurie Stargell

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4.1 Abstract

In this study, we show that Spn1 associates in a 1:1 ratio with Spt6. A truncated Spt6 with 1037 aa of the N-terminus can still interact with Spn1. In contrast to the enhanced *CYC1* transcription by the K192N allele of *SPN1*, the 1004 allele of *SPT6* decreases *CYC1* transcription. Unlike the *spt6-1004* allele, the $spn1^{K192N}$ allele does not allow transcription of *FLO8* gene from cryptic starting site. While the $spn1^{K192N}$ allele supports transcription initiated from unfavorable TATA elements in *CYC1* gene, the *spt6-1004* allele does not. Taken together, these results suggest that each subunit of the Spn1/Spt6 complex plays a distinct role during RNA polymerase II transcription.

4.2 Introduction

In yeast *Saccharomyces cerevisiae* as well as in higher eukaryotes, transcriptional activation by RNA polymerase II (Pol II) requires many proteins to decode the genomic information embedded in the chromosomal DNA. To overcome the repressive effects of chromatin structure on transcription, gene specific activators recruit different chromatin modifying factors to alter the locations or structures of nucleosomes and make the template DNA accessible for transcription factors [47, 241]. During initiation, Pol II, general transcriptional factors (GTFs) TFIIA, -B, D, -E, -F, H, coactivators such as SRB/mediators, chromatin modifiers are assembled into a preinitiation complex (PIC) at the promoter [6, 7, 16]. During the elongation phase, nucleosomes in front of the transcribing Pol II are remodeled by the chromatin modifying factors such as Swi/Snf complex, SAGA complex that may hitch a ride with Pol II, and are reassembled after the passage of Pol II [16, 87, 242, 243]. Studies suggest that the yeast Spt6 protein plays a critical role in regulating elongation through chromatin [107, 238]. Spt6 was shown to interact with Histone H3 and capable of nucleosome assembly in vitro [105]. This function of Spt6 had been implicated in reassembling nucleosomes after the passage of RNA Pol II during elongation [107]. Spt6 also interacts genetically or biochemically with a plethora of different transcription factors such as Pol II

[99], Spt4, Spt5 [135], Swi/Snf complex [137]. However, the exact role of Spt6 during transcription is still elusive.

Studies by others and us have shown that Spt6 directly associates with Spn1, an essential and conserved transcriptional factor [127, 140, 235]. But it is unclear how Spn1 and Spt6 are associated in this complex. Both Spn1 and Spt6 have been found in association with RNA Pol II complex [99, 244]. On the yeast *CYC1* gene, Spn1 associates with RNA Pol II and recruits Spt6 to the promoter. Recruitment of Spt6 by Spn1 further regulates the recruitment of the Swi/Snf complex, an ATP-dependent chromatin-remodeling factor [244]. This finding suggests a pivotal role of Spn1 in maintaining the chromatin structure during transcription by coordinating the chromatin remodeling and reassembly activities. However, it is not clear how each subunit of this Spn1 /Spt6 complex affects the functions of RNA Pol II after being recruited.

In this study, by comparing two distinct mutants of this complex, we were able to dissect the differential contributions of Spn1 and Spt6 to Pol II transcription.

4.3 Material and methods

4.3a Yeast strains

The yeast strains used in this study were listed in Table 4.1

Table 4.1 Yeast strains used in this study

Strain	Genotype	Reference
SK1	*MATα ura3-52trp1-Δ63 his3Δ200 ura3 leu2Δ2::PET56 LexAOp::HIS3*	[127]
JF10	SK1 *spn1*^{K192N}	[127]
JF26	SK1 *SPT6-3HA::kanMX*	This study
LZY301	SK1 *SPT6-3HA::kanMX* + YCp22-3MYC-SPT6 (*TRP1*)	This study
FY2134	*MATa his4-912δ lys2-128δ leu2Δ1RPB3-HA::LEU2 SPT6-FLAG CTR9-9MYC::kanMX*	[107]
FY2135	*MATa his4-912δ lys2-128δ leu2Δ1RPB3-HA::LEU2 spt6-1004-FLAG CTR9-9MYC::kanMX*	[107]
FY2181	*MATa his4-912δ lys2-128δ leu2Δ1*	[107]

4.3b Co-immunoprecipitation experiments

Co-immunoprecipitation experiments were performed as described previously with a few modifications [239]. Cultures were grown to an optical density (600 nm) of about 1.0 in rich medium containing 2% dextrose. Cell extracts (300 µg) were used immediately following preparation and were precleared by incubation with a 50 µl plain protein A-sepharose beads (Pharmacia) for 1 hr at 4°C. A small sample was taken after the preclear step to provide a load control. Anti-HA, polyclonal anti-Spn1, anti-Myc and anti-FLAG antibodies were coupled to protein A-sepharose beads, and the remaining extract was incubated with a 50 µl of these coupled beads for 2 hr at room temperature with occasional stirring. After six washes, the beads were boiled in loading buffer and 15 µl was loaded for SDS-PAGE, followed by immunoblot analysis.

4.3c S1 nuclease assay

S1 nuclease assay were conducted as described [115]. For *CYC1* induction, cultures grown overnight in rich medium containing 2% glucose were washed three times in medium lacking glucose, diluted into medium containing 3% ethanol, and cultured at 30°C for 6 hr. For uninduced samples, cells were grown in rich medium containing 2% glucose for 6 hours at 30°C till optical density reached 0.8~1.0. Yeast cells were then harvested and total RNA was isolated by hot phenol extraction. Hybridizations with excess probe were normally done with 25–30 µg of

RNA samples were hybridized with excess ^{32}P labeled probe overnight at 55°C. S1 nuclease digestion was performed on the hybridized samples for 30–45 min at 37°C. Band intensity was normalized to the intensity of the $tRNA^{W}$ band.

4.3d Northern Blotting

Northern blotting was performed as described [245]. Total RNA was extracted from wild type or mutant *SPN1* and *SPT6* strains grown at 30°C or after an 80-minute shift to 39°C. Probe for *FLO8* was PCR-amplified from genomic DNA and covers +1595 to +2349 relative to the *FLO8* ATG.

4.3e Primer extension assay

Total RNA for *CYC1* transcription was prepared by hot phenol extraction. Primer extension assay was performed as described [246]. The probe used to map the 5' end of *CYC1* mRNA anneals to the *CYC1* gene from +79 to +56 with a sequence as " 5'-GTGTGGCATTGTAGACACTC TAGT-3'". In brief, 80ug of RNA sample was resuspended in 20 μl of hybridization buffer (250 mM KCl, 10mM Tris /HCl (pH 7.6), 1 mM EDTA and 1 pM ^{32}P labeled *CYC1* probe; the hybridization buffer also contained 1 pmole labeled primer. The mixtures were covered with 40 μl paraffin oil, heated to 80°C for 3min, and the nucleic acids were annealed for 2h at 45°C. the chilled reactions were mixed with 40 μl of reverse transcriptase mixture (112.5 mM Tris/HCl (pH7.6) 22.5 mM dithiotheriotol, 18 mM $MgCl_2$, 15 units of RNasin, a 750 μM concentration of each of the four

deoxynucleoside triphosphates, and 200 units of Moloney murine leukemia virus reverse transcriptase. The primer extension reaction was performed at 37°C for 1 h. Reactions were stopped by extraction, and the ethanol-precipitated nucleic acid were resuspended in 10 ul of PAGE loading buffer and resolved on 7% polyacrylamide gel containing 8M urea. Gels were dried and exposed to phosphor-screen. Signals were visualized on STORM phosphoimager.

4.4 Results

4.4a Spt6 associates with Spn1 in 1:1 ratio in a complex

We and others have shown that Spt6, an essential protein in yeast exists in a complex with Spn1 [140, 141, 235]. But how Spt6 and Spn1 associate in this complex is not clear. To address this question, we created a yeast strain containing two copies of *SPT6*, a genomic *SPT6* tagged at the C-terminus with 3 HA module and an *SPT6* tagged at the N-terminus with 3 Myc module on a *TRP1* plasmid. These tagged *SPT6* genes were expressed similarly and did not affect the behavior of this strain as compared to its parent. This allowed us to determine the ratio of Spt6 to Spn1 in the complex in its native environment by co-immunoprecipitation (CoIP) assay. Spn1 was associated with only one-type of tagged Spt6 and there was no self-association between two differently tagged Spt6 protein (Figure 4.1A). Similar studies on Spn1 showed no association between two differently tagged Spn1 molecules in vivo (Julie Fischbeck, personal

communication). Thus, Spt6 and Spn1 associate with each other in a 1:1 ratio in a complex.

We also tried to map the interaction domains of Spt6 with Spn1 using N-terminal Myc-tagged Spt6 truncations in CoIP studies. The full length Spt6 has 1451 amino acid residues. We found that a truncated form of Spt6 containing amino acid residues from 1-1037, Spt6 (1-1037aa), still associates with Spn1 (Figure 4.1B). Other Spt6 derivatives that were created including Spt6 (1-215aa) and Spt6 (1-605) were not stable in yeast, and thus could not be assayed. This indicated that the 400 amino acid residues of the C-terminus of Spt6 were not required for interacting with Spn1.

4.4b Distinct effects of Spt6 and Spn1 on RNA Pol II transcription

It has been shown that Spn1 recruits Spt6 through its direct interaction with Spt6 to the *CYC1* promoter, implicating that these two proteins function as a complex during transcription [244]. Our previous study had shown that a K192N allele of *SPN1* enhanced *CYC1* transcription, suggesting a repressive role of Spn1 on the *CYC1* gene [127]. If the Spn1/Spt6 complex regulated transcription by affecting the same function, a mutant of either subunit would affect transcription similarly. To test this hypothesis, we measure the effect of a well-characterized *SPT6* allele, *spt6-1004* on *CYC1* transcription. Unlike the

Figure 4.1A

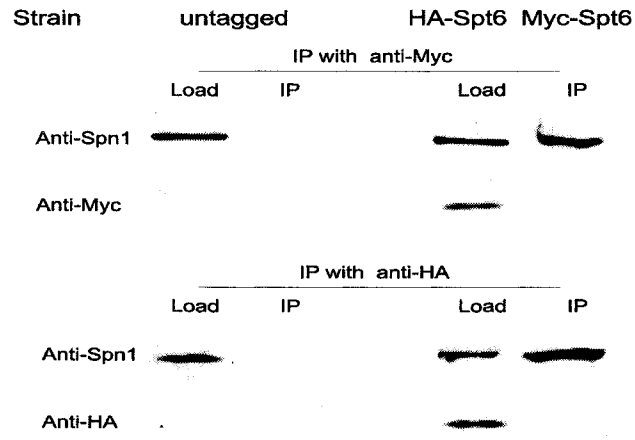

Figure 4.1B

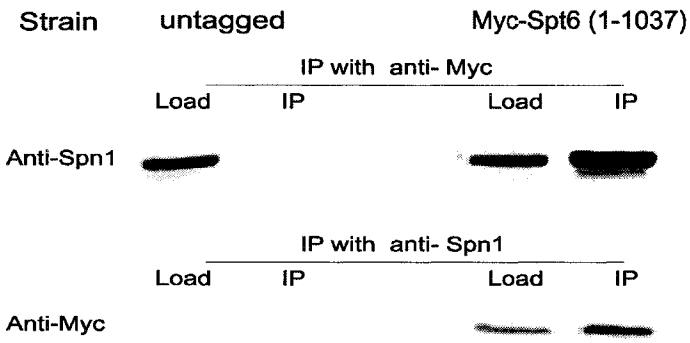

Figure 4.1 The association of Spn1 with Spt6 in whole cell extracts Whole cell extracts from strains as indicated were precipitated with anti-HA or anti-Myc antibody coupled protein-A sepharose beads. Protein bound to the beads was detected by western blot using corresponding antibodies (**A**) Spn1 associates with only one-type of tagged Spt6 in cell extracts. (**B**) Spn1 interacts with truncated Spt6 (1-1037).

enhanced *CYC1* transcription by the *spn1*^{K192N} allele [127], the activation of *CYC1* in this *SPT6* mutant was decreased by 35% (Figure 4. 2A). The diminished levels of *CYC1* transcription by the *spt6-1004* allele suggest a positive role of Spt6 in regulating *CYC1* transcription.

Previous studies had shown that this *spt6-1004* mutant allowed transcription to be initiated from cryptic TATA sites in the *FLO8* gene, which was due to the defects of this allele in maintaining normal chromatin structure during transcription elongation [107]. The association of Spt6 with Spn1 prompted us to test whether the *spn1*^{K192N} allele would also allow Pol II to initiate transcription from the cryptic start site in the *FLO8* gene. Surprisingly, under both permissive and non-permissive temperatures, no short cryptic *FLO8* transcripts were observed in the *spn1*^{K192N} strain (Figure 4.2B).

4.4c spt6-1004 interacts with Spn1

The spt6-1004 mutant has an internal 64 amino acids deletion from 931-994 [107]. We had shown that the recruitment of Spt6 by Spn1 also regulated the recruitment of Swi/Snf complex, which is required for *CYC1* activation [244]. The diminished levels of *CYC1* transcripts in spt6-1004 allele could be due to that spt6-1004 loses interaction with Spn1, which in turn fails to recruit the Swi/Snf complex and leads to the decreased *CYC1* activation. We performed co- immunoprecipitation assays to test whether

Figure 4.2A

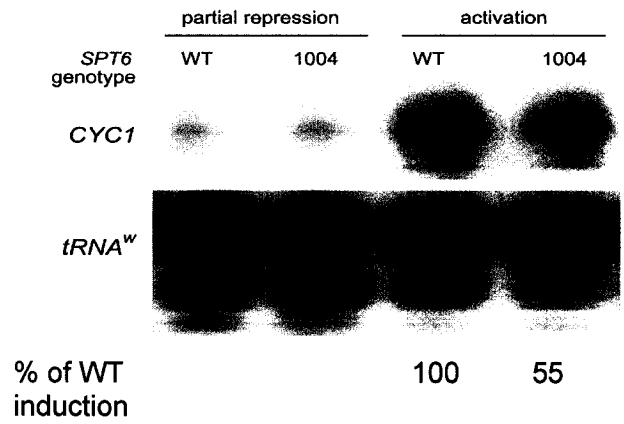

Figure 4.2B

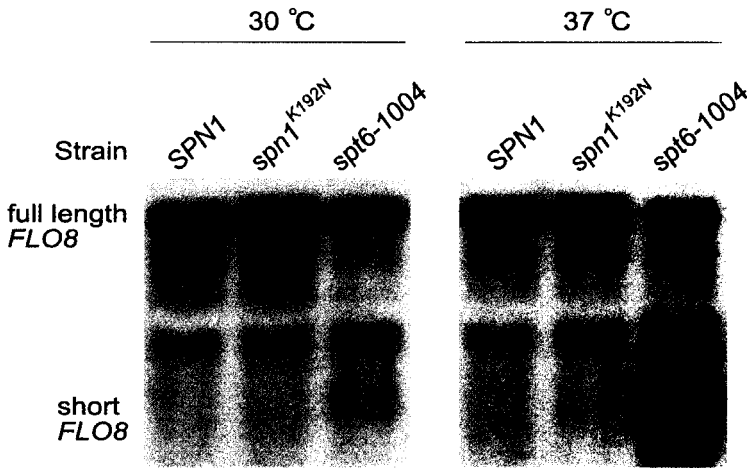

Figure 4.2 Different effects of *SPT6* and *SPN1* on *CYC1* and *FLO8* transcription (A) The *spt6-1004* allele decreases *CYC1* activation. Total RNA from wild type *SPT6* and *spt6-1004* strains grown under partial repressed and activated conditions were analyzed by S1 nuclease assay to measure *CYC1* transcripts. $tRNA^{w}$ signal was used as a load control to normalize signals of *CYC1* transcripts. Numbers to the bottom show the percentage of *CYC1* induction fold upon activation. The induction fold in the wild type *SPT6* strain (WT) was set to 100%. (**B**) The $spn1^{K192N}$ allele does not allow the cryptic *FLO8* transcripts Total RNA from wild type and $spn1^{K192N}$ strains, grown at 30 °C or after an 80-minute shift to 37°C, was subjected to northern blot analysis for *FLO8* RNA. The *spt6-1004* strain was used as a positive control to show the short transcripts of *FLO8.*

spt6-1004 interacts with Spn1 in protein extracts from FLAG tagged Spt6 strains. We found that spt6-1004 still associated with Spn1 in the protein extracts (Figure 4.3). The association of spt6-1004 with Spn1 indicated that this mutant could be recruited to the *CYC1* promoter. Therefore, the distinct effects of Spn1 and Spt6 on the *CYC1* gene suggest the different functions of these two after they are recruited.

4.4d *CYC1* transcripts initiated from abnormal starting sites in the *spn1*^{K192N} strain

On the *CYC1* promoter, there are at least 5 TATA or TATA-like elements, which directs the transcription to initiate from different start sites [247, 248] (Figure 4.4A). Of these TATA elements, the -178 and the -123 TATA account for most of the transcription of the *CYC1* gene and the downstream TATA sites are not used unless the two upstream TATA sites are rendered nonfunctional [249]. The different effects of *SPN1* and *SPT6* mutants on *CYC1* transcription may result from the different usage of the TATA sites in these strains. Using primer extension assays, we were able to map the start sites of the *CYC1* transcripts. Upon activation, *CYC1* transcripts in the *spn1*^{K192N} strain directed by -178 and -123 TATA sites were slightly higher than that in the wild type *SPN1* strain; but the transcripts directed by those unfavorable TATA sites were increased dramatically (Figure 4.4B). The starting sites of these new transcripts were around the -28 position. These results suggest that Spn1 might suppress

Figure 4.3

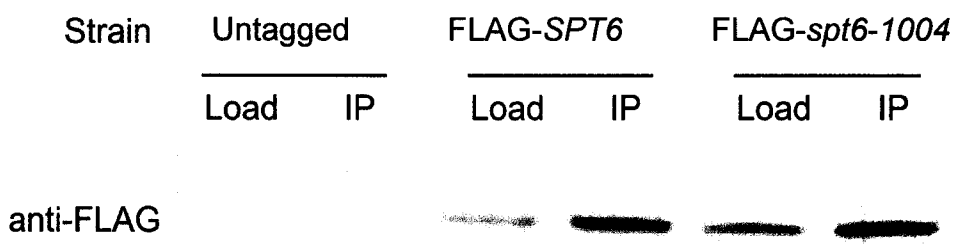

Figure 4.3 spt6-1004 associates with Spn1 in whole cell extracts 400 ug of whole cell extracts from FLAG-tagged wild type *SPT6* (FY2134), spt6-*1004* (FY2135) and untagged FY2181 strains were precipitated with polyclonal anti- Spn1 antibody coupled protein-A sepharose beads. The presence of Spt6 in the precipitates was detected by western blot using anti-FLAG antibody.

transcription initiated at the unfavorable TATA elements in the *CYC1* promoter. Consistent with the decreased levels of *CYC1* RNA levels in the *SPT6* mutant strain, transcripts from all the TATA elements were decreased; there were low levels of *CYC1* transcripts directed by the unfavorable TATA sites.

4.5 Discussion

4.5a Spn1 and Spt6 regulate Pol II transcription differently

We reported the role of Spn1 in coordinating the ordered recruitment of Spt6 and the chromatin remodeling complex Swi/Snf [244]. Since Spn1 is associated with Spt6 at the *CYC1* promoter, a further question is how they affect the transcription after the recruitment. spn1^{K192N} lost it association with Pol II and failed to recruit Spt6 to the promoter. This created a situation where Pol II was transcribing without the influence of Spn1/Spt6 complex. We had found that *CYC1* activation was elevated in this situation [127], which indicated a repressive effect of this complex on Pol II transcription. On the other hand, an *SPT6* mutant, spt6-1004, which has defects in depositing histones back on the DNA template but is still capable of being recruited by Spn1; would allow us to see the effects of Spt6 on transcription. We found that *CYC1* activation was decreased in *spt6-1004* strain suggesting a positive effect of wild type Spt6. If the Spn1/Spt6 complex functions in maintaining the chromatin structure, the effects of the *spn1^{K192N}* allele and the spt6-1004 allele on transcription

Figure 4.4A

```
                                        -178 TATA
-200
   TTTAGTGCTG  ACACATACAG  GCATATATAT  ATGTGTGCGA  CGACACATGA
   AAATCACGAC  TGTGTATGTC  CGTATATATA  TACACACGCT  GCTGTGTACT
                                    -123 TATA
-150
   TCATATGGCA  TGCATGTGCT  CTGTATGTAT  ATAAAACTCT  TGTTTTCTTC
   AGTATACCGT  ACGTACACGA  GACATACATA  TATTTTGAGA  ACAAAAGAAG
          -93 TATA                -69 TATA                -56 TATA
-100
   TTTTCTCTAA  ATATTCTTTC  CTTATACATT  AGGTCCTTTG  TAGCATAAAT
   AAAAGAGATT  TATAAGAAAG  GAATATGTAA  TCCAGGAAAC  ATCGTATTTA
-50
   TACTATACTT  CTATAGACAC  GCAAACACAA  ATACACACAC  TAAATTAATA
   ATGATATGAA  GATATCTGTG  CGTTTGTGTT  TATGTGTGTG  ATTTAATTAT
+1
   ATGACTGAAT  TCAAGGCCGG  TTCTGCTAAG  AAAGGTGCTA  CACTTTTCAA
   TACTGACTTA  AGTTCCGGCC  AAGACGATTC  TTTCCACGAT  GTGAAAAGTT
+50
   GACTAGATGT  CTACAATGCC  ACACCGT
   CTGATCTACA  GATGTTACGG  TGAGGCA
               primer
```

Figure 4.4B

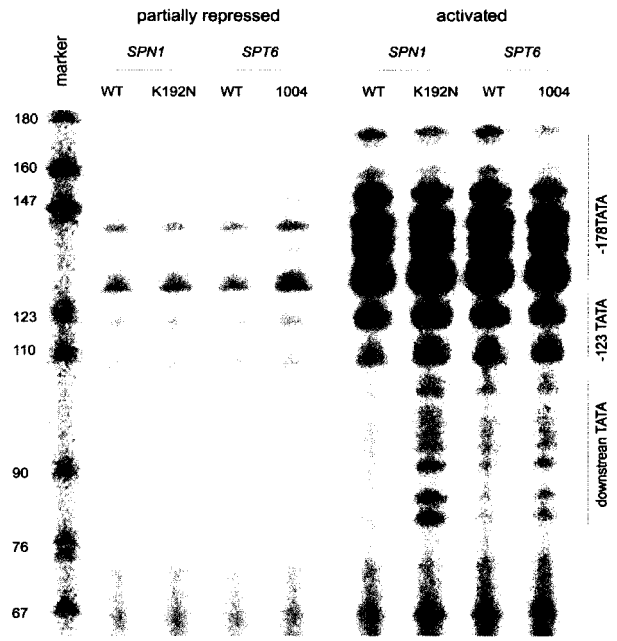

Figure 4.4 Effect of Spn1 and Spt6 on TATA usage selection (**A**) TATA positions in the *CYC1* core promoter. DNA sequence shows the core promoter region of *CYC1* gene. The positions of the five TATA elements were indicated above the highlighted in boldface letters in the DNA sequence. The sequence underlined shows sequence and the position of the primer used in the primer extension assay. (**B**) An 80 ug of total RNA from wild type or mutant *SPN1* and *SPT6* strains grown under partial repressed and activated conditions were analyzed by primer extension assay. Markers to the left indicated the length of *CYC1* transcripts. TATA sites from which these transcripts were initiated were labeled to the right.

should be quite similar. The totally opposite effects of the two mutants on *CYC1* transcription indicated there might be some additional unknown functions of this Spn1/Spt6 complex. Additional Spt6 mutants that lose interactions with Spn1 would help to define the unknown functions.

The effects of these two mutants on *FLO8* transcription were in contrast to what we saw on the *CYC1* gene. There are several questions needed to be answered before we can fully understand these totally opposite effects. First, do both Spn1 and Spt6 directly regulate *FLO8* transcription? If Spn1 recruits Spt6 to the *FLO8* gene also, we should see short *FLO8* transcripts in *SPN1* mutant strain as we saw in spt6-1004 strain. These short transcripts of *FLO8* were transcribed by Pol II initiated from the cryptic internal TATA element due to the defects of SPT6 mutant to restore the chromatin structure in the wake of Pol II elongation [107]. We found that Spn1 affected neither the normal nor the short cryptic transcripts of *FLO8* gene under both permissive and non-permissive conditions. Examining the occupancy of both Spn1 and Spt6 would help to explain these distinct effects on *FLO8* transcription.

4.5b The concerted functions of Spn1 and Spt6 in regulating transcription initiation

We observed the *CYC1* transcripts from normally unused TATA sites in *spn1*^{K192N} strain but not in the *spt6-1004* strain. This suggested a role of Spn1/Spt6 complex in regulating the selection of transcription start

site. As Spn1, like TBP and RNA Pol II, constantly occupies the *CYC1* promoter under both partially repressed and fully activated conditions [65, 118]. One possibility is that these relatively short *CYC1* transcripts could be the results of mRNA processing because both Spn1 and Spt6 were implicated in mRNA processing. But this is probably not true for the following reasons. The new *CYC1* transcripts were seen only in mutant *SPN1* strain during activation but not the wild type *SPN1* strain; the starting sites of the new *CYC1* transcripts matched with those directed by the unfavorable TATA elements reported previously. This indicated that they could be resulted from new transcription starting sites. Another possibility is that these new *CYC1* transcripts were produced because of the permissive chromatin structure *CYC1* gene of the $spn1^{K192N}$ strain. Although *CYC1* is a short gene with a coding region of only 310 bp, the coding region is still wrapped in the chromatin structure [118]. Normally, the closeness of the downstream TATA sites to the coding region makes the assembly of the initiation complex at these down stream TATA sites very difficult because of the steric hindrance of nucleosome in the coding region. Spn1 coordinates the recruitment of Spt6 and Swi/Snf complex. The recruited Spt6 and Swi/Snf complex can maintain the proper chromatin structure during elongation. $spn1^{K192N}$ fails to recruit Spt6 but leads to the recruitment of Swi/Snf complex. Without Spt6, the chromatin structure could not be restored during transcription elongation. This would make not

only the transcription but also the assembly of the preinitiation complex at the down stream TATA sites easier. Therefore, we observed the new *CYC1* transcripts in the *spn1*^{K192N} strain. As in the Spt6 mutant strain, we did see the very small amount of the new *CYC1* transcripts, further supporting the role of chromatin structure in starting sites selection. But the compromised function of spt6-1004 could make the changes of the chromatin structure much slower, which would ultimately decrease the efficiency of transcription.

In this study, we determined the ratio of Spn1 and Spt6 association in a complex. The distinct effects of the two mutants on transcription elongation revealed that there might be additional unknown functions of this Spn1/Spt6 complex. The concerted functions of Spn1 and Spt6 suggested a role of this complex in selection of the starting sites during transcription.

Chapter 5

Post-recruitment functions of the SAGA complex during RNA polymerase II transcription

In this chapter, I focused on the identification of post-recruitment functions of the other Spt proteins during Pol II transcription. Our initial efforts have revealed clear involvement of Spt7 and Spt20 during the post-recruitment steps. The essentiality of Spt7 and Spt20 in maintaining the structural integrity of SAGA complex led me to extend the research to other SAGA subunits. We found that while both the histone acetylation activity and structural integrity of SAGA were not required for TBP recruitment, SAGA was still required for transcription. These studies suggest that additional unknown functions of SAGA complex beyond its role in histone acetylation and TBP recruitment are rate limiting for post-regulated genes. Julie Fischbeck, our previous lab technician developed artificial recruitment assay. The results of this work are presented here in the form of a manuscript entitled:

Post-recruitment functions of the SAGA complex during RNA polymerase II transcription: Lei Zhang, Julie Fischbeck and Laurie Stargell

Post-recruitment functions of the SAGA complex during RNA polymerase II transcription

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5.1 Abstract

Little is known about how different transcription factors regulate gene expression after the recruitment of TBP to promoters. Using an artificial recruitment assay as well as transcription assays on post-recruitment regulated genes, we investigated the roles of SPT genes in these processes. We found that the functions of Spt4, Spt7 and Spt20 are required for post-recruitment functions. These studies were extended to other subunits of the SAGA complex; we found that besides its role in histone acetylation and delivering TBP to certain promoters, SAGA must contain some additional functions exerted after recruitment of TBP and RNA polymerase II. Taken together, these results underscore the dual nature of many factors involved in transcription mechanisms.

5.2 Introduction

Transcription by RNA polymerase II (Pol II) requires the concerted functions of a large number of transcription factors including Pol II itself [5-7]. In many cases, the recruitment of TBP (TATA binding protein) to promoters is in direct proportion to the levels of gene expression observed. [65, 116], and is therefore an important step for transcription. But there are some genes such as the yeast *CYC1, COX5a* and the *Drosophila* heat shock genes, the recruitment of TBP or Pol II or even the assembly of PIC at the promoter does not solicit transcription [65, 118, 119]. Thus certain functions required for transcription are absent until an as yet uncharacterized triggering event occurs. These functions of transcription factors are then defined as "post-recruitment functions" as they are required after TBP recruitment. Such functions are generally required for transcription as seen on many genes. But they are not rate limiting. However, these functions are rate limiting for transcription of a growing number of genes we just began to know their regulatory mechanisms. Theoretically, these functions may be involved in promoter melting and promoter clearance, elongation, termination or even RNA processing. Thus, uncovered these potential rate-limiting steps after TBP recruitment would add to our understanding of transcription mechanisms.

Fusing TBP to the DNA binding domain can artificially recruit TBP to the promoter, which bypasses the functions that are required for TBP

recruitment to the promoter and increases the level of transcription [120-122]. If the functions of a transcription factor are required after TBP recruitment, disrupting these functions would cause the artificial recruited TBP fail to activate. Therefore, the recruitment and the post-recruitment functions of a transcriptional factor can be separated by the artificial recruitment assay. These functions can be further studied by examining the effects of a transcription factor on transcription of certain genes that are regulated by post-recruitment functions such as the yeast *CYC1* gene.

The products of yeast SPT (Suppressor of Ty) genes represent over 20 transcription factors that have been implicated in various processes such as maintaining chromatin structure, initiation, elongation and RNA processing [11, 125, 126, 250]. *SPT15* encodes TBP [131], and the functions of many Spt proteins are related to TBP function including Spt3, Spt7, Spt8, and Spt20 [156, 157, 161, 172 , 251]. The *SPT11* and *SPT12* encode the histone H2A and H2B [181, 182]. Spt1, Spt10 and Spt21 are the regulatory factors that control the expression levels of histone genes [128, 129, 132, 133]. Many key regulators of transcription and chromatin structure, such as DSIF, SAGA, and FACT complexes, all contain *SPT* gene products as essential components. Spt4/Spt5, the yeast homolog of human DSIF regulates transcription elongation through chromatin [134, 135]. Spt3, Spt7, Spt8, Spt20 are the subunits of SAGA complex, which regulates the recruitment of TBP and acetylates chromosomal histones

[128, 129, 132, 133]. Other SPT genes such as *SPT2,* which encodes for an HMG1-like protein that genetically interacts with the Pol II CTD [188], histones H3 and H4 and physically with Swi/Snf complex [189]. *SPT13,* which encodes a component of the Mediator complex that binds to the Pol II CTD [190, 191]. Spt23 together with Mga2 are associated with ER membrane and required for transcription of fatty acid desaturase gene *OLE1* and transcription silencing [194, 195].

Recent work from this lab has isolated Spn1, a highly conserved and essential factor that regulates Pol II transcription though post-recruitment functions was also an Spt protein [127, 232, 244]. This brought our attention to post-recruitment functions of the other Spt factors. In this study, we analyzed the behavior of all the non-essential Spt protein in artificial recruitment assay and their effects on post-recruitment regulated gene transcription. We also expanded these studies on non-Spt subunits of the SAGA complex.

5.3 Material and methods

5.3a Yeast strains, medium and DNA

The deletion mutant strains and its parental strain (BY4741, *MATa his3Δ1 ura3Δ0 leu2Δ01 met15Δ01)* used in this study were purchased from Research Genetics Inc. The names of the gene deleted are *SPT1, 2, 3, 4, 7, 8, 10, 13, 20, 21, 23, GCN5, ADA1, 2, 3.* The yeast complete and synthetic complete (SC) medium was made according to the literature

[219]. The 3-AT (3-aminotriazol, competitive inhibitor of *HIS3* gene product) containing plates were made by supplementing AT to SC-ULH medium. To create the *HIS3* reporter plasmid LexAopHIS3, *HIS3* gene in SK1 strain [127] that has its Gcn4 binding sites replaced by LexA operator was amplified by PCR and subcloned into YCp111 plasmid *(LEU2, CEN).* LexA and LexA fused TBP derivatives cloned into pRS316 *(URA3, CEN)* plasmids were obtained from previous studies [121]. All the LexA derivatives had HA module in front of LexA-protein fusion sequence for testing the expression levels of the fusion proteins.

5.3b Plasmid based artificial recruitment assay

Yeast cells of the parental and the deletion strains were transformed with the plasmids using standard procedures [252]. Briefly, yeast cells were first transformed with the LexAopHIS3 plasmid DNA, then the *LEU2*+ yeast cells were further transformed with LexA, and LexA-TBP fusion constructs in *URA3* marked plasmids; finally the *LEU2*+ *URA3*+ yeast cells were streaked onto SC-UL medium and SC-ULH medium containing 3-AT. Cell growth was scored as ranging from "-" to "++++". The "-" means little or no growth and the "+" means robust growth.

5.3c Western analysis

10ml of yeast cells grown in SC-UL with an OD600 about 0.8 were harvested, washed once with sterile water, resuspended in 200ul lysis buffer (0.5M phosphate buffer, pH 7.5). Whole cell extract were prepared

by vigorous bead beating. Cellular debris were removed by spinning the extracts at 10, 000g at 4°C for 10 min. Protein concentrations were determined by Bradford assay (Bio-Rad). Equal amount of whole cell extracts were separated on 10%SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used at the given concentrations: anti-HA (12CA5, from Covance Inc), 1:250), homemade anti-Toa1 (1:10,000). Horseradish peroxidase (HRP) conjugated second antibodies were used at 1:20,000 dilution and protein bands were detected by western blotting detection reagents from Amersham Biosciences.

5.3d Transcription assay

S1 nuclease assays were conducted as described[115] . For *CYC1* induction, cultures grown overnight in rich medium containing 2% glucose were washed three times in medium lacking glucose, diluted into medium containing 3% ethanol, and cultured at 30°C for 6 hr. For uninduced samples, cells were grown in YPD for 6 hours at 30°C till optical density reached 0.8~1.0. Yeast cells were then harvested and total RNA was isolated by hot phenol extraction. Hybridizations with excess probe were normally done with 25~30 μg of RNA samples were hybridized with excess ^{32}P labeled probe overnight at 55°C. S1 nuclease digestion was performed on the hybridized samples for 30~45 min at 37°C. The hybridization signals were visualized by phosphoimager. mRNA loading was normalized to the intensity of the $tRNA^{W}$ band.

5.3e Chromatin immunoprecipitation analysis

Chromatin immunoprecipitations were performed as described [240] with few modifications. Cells (150 ml) were grown to OD 600 of 0.8~1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 minutes with occasional swirling of the flasks at intervals of 5 minutes. Glycine was added to a final concentration of 125mM at room temperature for 5 minutes to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were resuspended in FA-lysis buffer (500 µl of FA-Lysis buffer for a total of 50ml of cell culture). Chromatin was sheared by sonication using a Branson W-350 model of sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). 10% of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. 500 µl of the chromatin material was incubated with approximately 5µl of either homemade anti-TBP, anti-Pol II (8WG16, Covance Inc.) or anti-HA (Covance Inc.) antibodies by rotation overnight at 4°C. 50µl of protein-A sepharose beads (Pharmacia-prepared as slurry as per the manufacturers directions) was further incubated with the chromatin material for 2 hours at 4°C. The beads were spun down and the Antigen-Antibody complexes bound to the beads were recovered and further treated with TE/SDS buffer for 15 minutes at 65°C to elute the complexes. Protein-DNA crosslink were reversed by incubation overnight at 65°C and the DNA was purified by

phenol-chloroform extraction and used for the PCR analysis. PCR reactions were carried out in a total volume of 25µl. Each reaction contains 1 ul 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. The samples were run on 5% native polyacrylamide gels in 0.5XTBE buffer. The gels were dried and exposed to the phosphor-image screen. Image was scanned on STORM and quantified using image quant software analysis to detect the strengths of various signals. No antibody samples were used as controls. Primers for the PCR were designed at the promoter region of the *CYC1* gene and amplified a product of 312 bp [118]. For analysis of the occupancy of the LexA derivative, primers were designed to encompass the whole engineered *HIS3* reporter promoter region and amplified a product of 646 bp. The signal strength ratio between the precipitated sample and the input after minus the signals of no antibody control was used as an indication of the protein occupancy.

5.4 Results

5.4a Different behavior of SPT mutants in the artificial recruitment assay

A plasmid-based artificial recruitment assay was developed to identify the post-recruitment functions of the non-essential SPT proteins. A reporter *HIS3* gene was engineered by replacing the Gcn4 binding sites with LexA operator and cloned into a *LEU2* marked plasmid. TBP fused to

the DNA-binding domain of LexA was cloned into a *URA3* marked plasmid. By transforming these two plasmids into the SPT mutant strains, TBP can be artificially recruited to the reporter *HIS3* gene. If these SPT mutants were defective for TBP recruitment, then artificially recruiting TBP should be able to correct these defects and *HIS3* gene would be transcribed similarly as in wild type strain. If they were defective for the functions after TBP recruitment, these defects would not be corrected by the artificial recruitment assay. Therefore the behavior of the mutant reflects the involvement of the wild type protein during the post-recruitment regulation. In the artificial recruitment assay, while the growth of the deletion mutants of *SPT1, 2, 3, 8, 10, 13, 21* and *23* on AT medium were quite similar to that of the wild type strain (Table 5.1); the deletion mutants of *SPT4, 7* and *20* grew poorly on medium containing AT (Table 5.1 and Figure 5.1). However, the poor growth of *SPT4, 7* and *20* mutants could be due to low expression levels of LexA-TBP fusion protein. To test this possibility, we examined the expression levels of LexA-TBP protein levels in strains that showed normal or poor growth 3-AT plates. We found that the expression levels of LexA-TBP in all strains tested are quite comparable (Figure 5.2A). Another possibility of the poor growth of *SPT4, 7* and *20* mutants on 3-AT plate could be that the LexA-TBP protein was not recruited to the promoter of *HIS3* reporter gene. To test this hypothesis, we performed ChIP analysis to look at the occupancy of LexA-TBP on the promoter of *HIS3* reporter

Table 5.1

Results of plasmid based artificial recruitment assay

Strains	LexA	LexA-TBP	[AT] used	Days of growth
BY4741	+	++++	40	3
Δ*spt1*	+	++++	40	3
Δ*spt2*	+	++++	40	3
Δ*spt3*	+	++++	40	3
Δ*spt4*	+	+++	40	3
Δ*spt7*	+/-	+	20	4
Δ*spt8*	+	++++	40	3
Δ*spt10*	+	+++	40	3
Δ*spt13*	+	++++	40	3
Δ*spt20*	+/-	++	20	4
Δ*spt21*	+	++++	40	3
Δ*spt23*	+	++++	40	3

The yeast cells transformed with the plasmid-based artificial recruitment system were grown on SC-UL and SC-ULH 3-AT plates at 30 °C. The growth of cells was scored as ranging from "+/-" to "++++". The "-" means no growth at all and the "++++" robust growth.

Figure 5.1

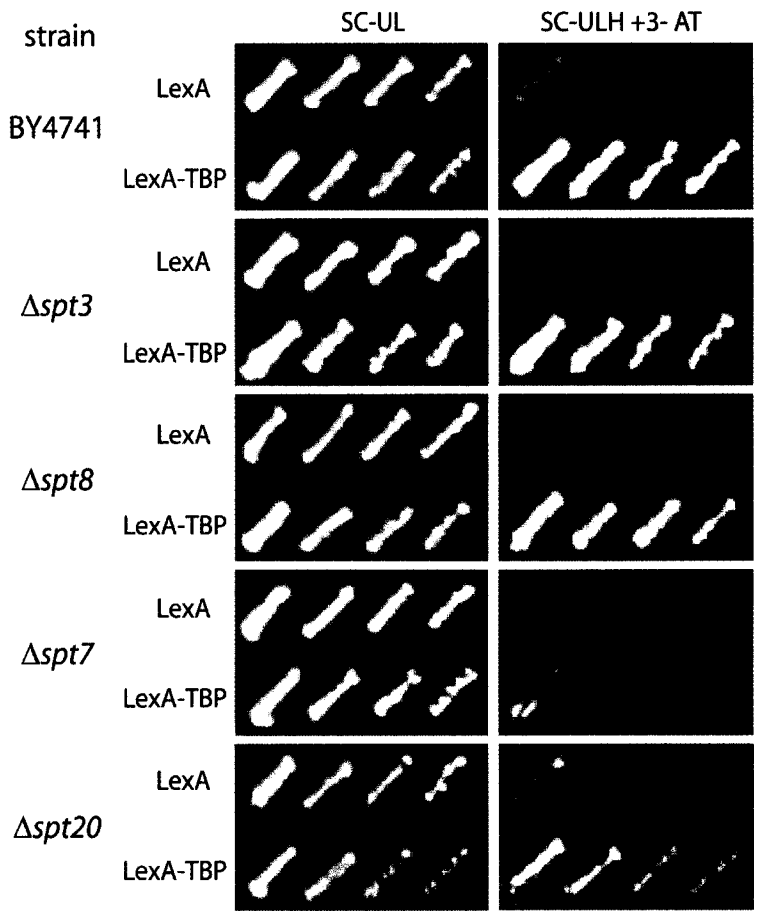

Figure 5.1 Representative pictures of the results of plasmid-based artificial recruitment assay The parental strain and strains that had its SPT gene deleted as indicated to the left were transformed with *HIS3* reporter gene plasmid and with either LexA alone or LexA-TBP containing plasmid, and similar amount of yeast cells of these strains were streaked four times on to both SC-UL and SC-ULH +3-AT medium. Pictures were taken after growing for 3~7 days. Strains transformed with LexA alone plasmids were used as controls.

gene. We showed that in both *SPT7* and *SPT20* deletion strains, LexA-TBP was indeed recruited to *HIS3* reporter gene promoter (Figure 5.2B)

5.4b Transcriptional defects of SPT mutants after TBP and Pol II recruitment

Results from the artificial recruitment assay showed the different behavior of these non-essential Spt factor. To further elucidate at which stage these SPT mutant affect transcription, we chose to study the effects of these SPT mutants on transcription of the yeast *CYC1* gene. *CYC1* is a native gene regulated by post-recruitment functions [65, 118]. Therefore, regulation of *CYC1* gene provides an excellent model to study the post–recruitment functions. The transcription levels of *CYC1* in these SPT mutants were measured by S1 nuclease assay. While the *CYC1* activation levels were only slightly decreased in *spt1, 2, 3, 8, 10, 13, 21* and *23* mutant strains, they were moderately decreased in *spt4* deletion strain and almost abolished in *spt7* and *spt20* deletion strains (Figure 5.3A).

5.4c Deletions of *SPT7* and *SPT20* did not affect the levels of TBP and Pol II occupancy on the *CYC1* gene.

Results form both assays consistently showed the involvement of Spt7 and Spt20 in post-recruitment functions. However, reminiscent to the artificial recruitment assay, one possibility of the full dependence of *CYC1*

Figure 5.2A

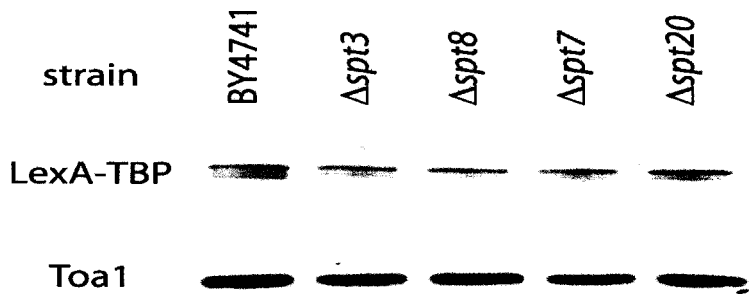

Figure 5.2A Expression levels of LexA-TBP protein were similar in SPT deletion strains Protein extracts from strains transformed with LexA-TBP plasmids were separated on SDS-PAGE and subjected to western blot analysis. Levels of LexA-TBP were detected by anti-HA antibody against HA tag on the N-terminus of the fused protein. Anti-Toa1 antibody was used to detect the internal Toa1 levels for loading control.

Figure 5.2B

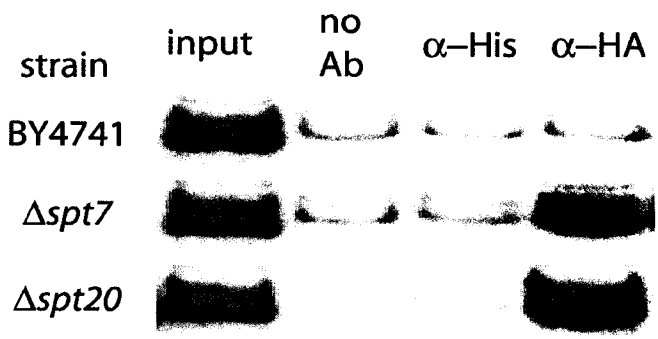

Figure 5.2B LexA-TBP is artificially recruited to the reporter *HIS3* gene Strains as indicated were transformed artificial recruitment assay plasmids and subjected to ChIP analysis to examine the occupancy level of LexA-TBP on the reporter gene promoter. Anti-HA antibody was used to immunoprecipitate LexA-TBP, anti-His antibody was used as irrelevant antibody control.

gene activation could be that Spt7 or Spt20 are required to recruit TBP and Pol II to its promoter. To test this hypothesis, we performed ChIP analysis to measure the TBP and pol II occupancy levels in both *spt7* and *spt20* mutant strains during partial repression and activation of *CYC1* gene. We found that the occupancy levels of both TBP and Pol II remained changed in either mutant under both conditions (Figure 5.3B and C). These results indicated that Spt7 and Spt20 did not affect TBP and Pol II occupancy levels on *CYC1* gene. Because spt4 mutant only has moderate effects in both artificial recruitment assay and on the *CYC1* transcription, its involvement in post recruitment steps would be pursued in another study.

5.4d Behavior of non-Spt SAGA subunits in the artificial recruitment assay and their effects on *CYC1* transcription

Since both Spt7 and Spt20 are the subunits of the yeast SAGA complex [243, 253]. Studies had shown that Spt7, Spt20 together with Ada1 and several TAFs are the core subunits of the SAGA complex and deletion of any of these subunits would disrupt the structural integrity of the SAGA complex [156, 164, 254-256]. Our findings that both Spt7 and Spt20 were involved in post-recruitment functions prompted us to look at the post-recruitment functions of other SAGA subunits. The yeast SAGA complex is a multiple subunits complex comprised of at least 14 peptides including TAFs, Ada proteins and Spt proteins. SAGA complex has been implicated in delivering TBP to TAF-independent promoters, acetylating nucleosome

Figure 5.3A

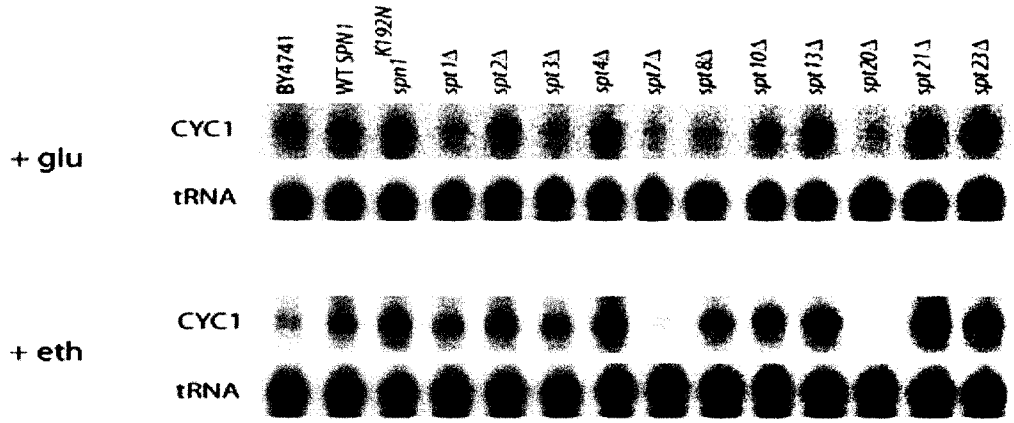

Figure 5.3A Deletion mutants of *SPT4*, *SPT7* and *SPT20* are defective for *CYC1* activation Total RNA from both wild type and different *spt* mutant strains grown under partial repressed and activated conditions were analyzed by S1 nuclease assay. Protected nucleic acids were resolved on denaturing gel. $tRNA^{w}$ signal was used as a load control to normalize signals of *CYC1* transcripts. Figure shows that the *spt4Δ* and *spt10Δ* alleles decrease *CYC1* activation moderately; the *spt7Δ* and *spt20Δ* alleles abolish *CYC1* activation.

Figure 5.3B

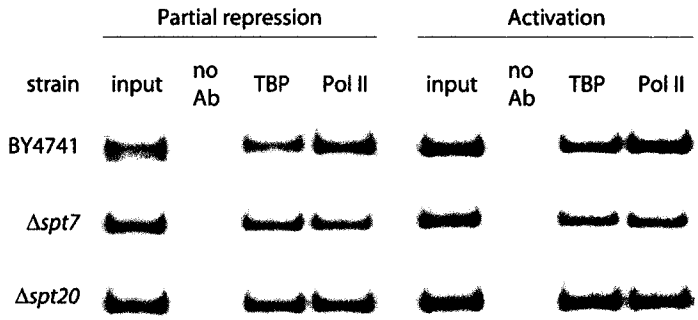

Figure 5.3C

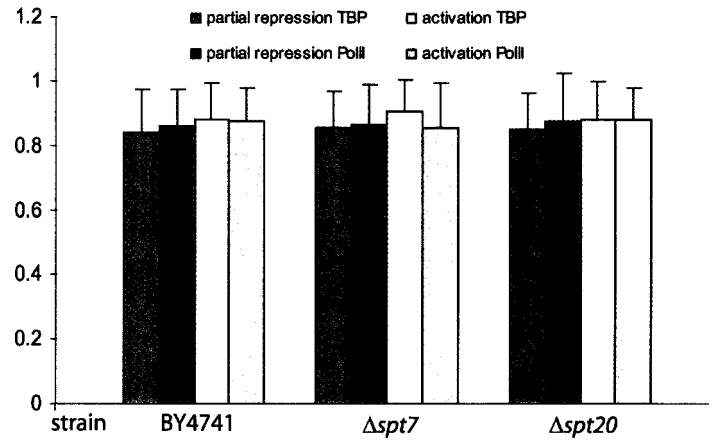

Figure 5.3B and C Occupancy levels of TBP and Pol II on the *CYC1* gene during activation were not changed in both *SPT7* and *SPT20* deletion strains (**B**) Strains as indicated were grown under partial repressed or activated conditions of *CYC1* gene. ChIP analysis was performed to measure the occupancy levels of TBP and Pol II. Shown here is one representative picture of three independent experiments. (**C**) Bar graph showed similar levels of TBP and Pol II occupancy upon activation to those during partial repression (mean±SD, n=3, and p <0.005). Protein occupancy was represented by ratio of signal from IP samples versus that of the input after minus the signal of no antibody control.

histone tails [196, 243, 253]. Gcn5 is the HAT subunit of the SAGA complex and its function is modulated by two other subunits Ada2 and Ada3 [163, 257-260]. Our results above suggest deletion of Spt7 and Spt20 did not affect the recruitments of TBP and Pol II but still diminished *CYC1* transcription (Figure 5.3 A, B and C). This could be resulted from the fact that the HAT activity of SAGA was lost since deletion of *SPT7* or *20* disrupted the SAGA complex and also abolishes the HAT activity of SAGA complex [164, 254]. To test this possibility, we examined the behaviors of *GCN5* deletion mutant in artificial recruitment assay and its effect on *CYC1* transcription. Since deletion of the enzymatic Gcn5 subunit only results in a SAGA complex without HAT activity [164], this would discriminate the difference in the requirement for Spt7 and Spt20 or the HAT activity in the post-recruitment steps. The deletion mutants for three additional subunits Ada1, Ada2 and Ada3 were also included in the experiments. Deletion of *ADA1* caused a similar effect on *HIS3* activation to that observed in *SPT7* deletion strain; but unlike the deletions of *SPT7* and *SPT20*, deletion of the *GCN5*, *ADA2* and *ADA3* caused a moderate or only slight decrease in reporter *HIS3* gene activation (Figure 5.4). As to activation of *CYC1* gene, *CYC1* activation was almost abolished in ADA1 deletion strain, while deletion of *GCN5* caused 45% decrease in *CYC1* activation and deletions of *ADA2* and *ADA3* had only minor effects on *CYC1* activation levels (Figure 5.5A and B). However, the occupancy

Figure 5.4

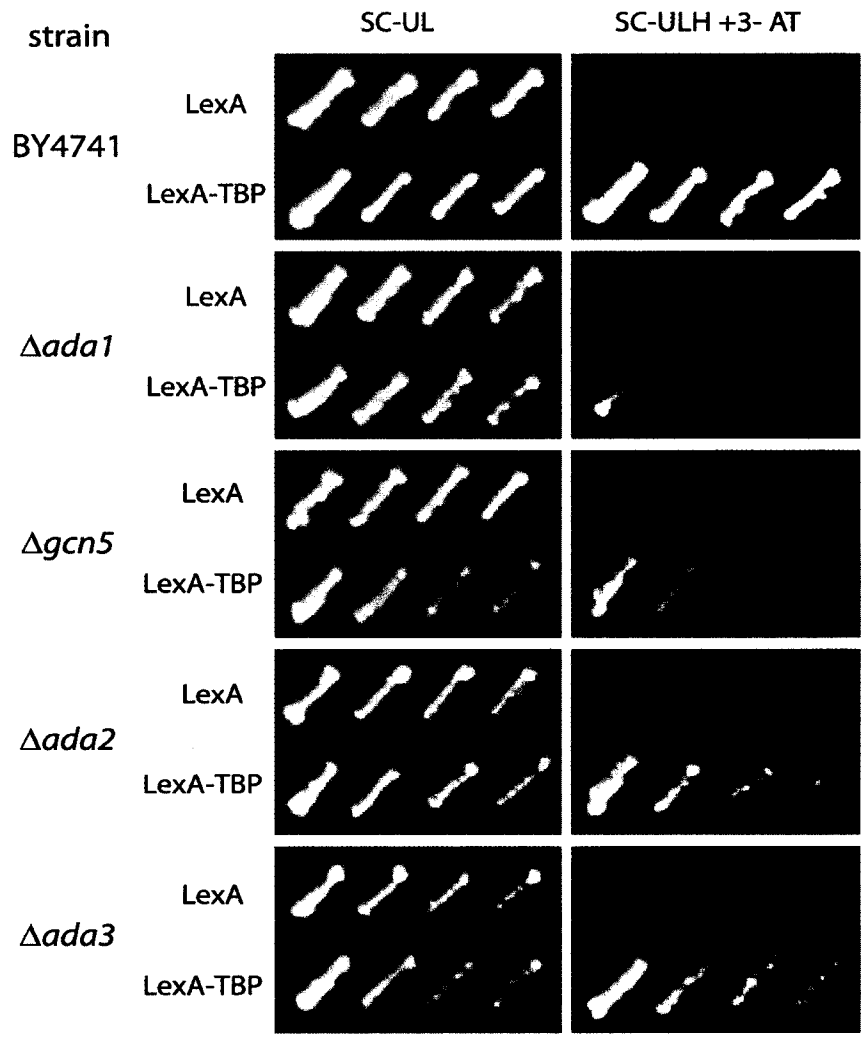

Figure 5.4 Results of plasmid-based artificial recruitment assay in non-Spt SAGA deletion strains The parental strain and strains that had their SAGA subunit genes deleted as indicated to the left were transformed with the *HIS3* reporter gene plasmid and with either LexA alone or LexA-TBP containing plasmid, and similar amount of yeast cells of these strains were streaked four times on to both SC-UL and SC-ULH +3-AT medium. Pictures were taken after growing for 3~7 days. Strains transformed with LexA alone plasmids were used as controls.

Figure 5.5A

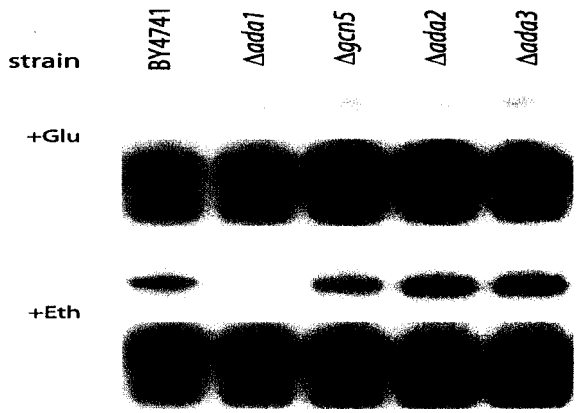

Figure 5.5B

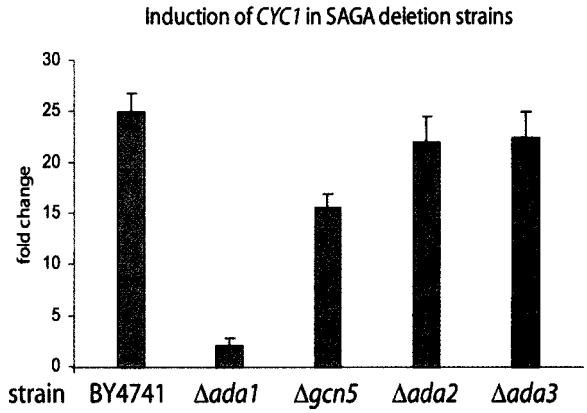

Figure 5.5A and B Regulation of *CYC1* transcription by non-Spt subunits of SAGA complex (**A**) Total RNA from strains as indicated grown under partial repressed) and activated conditions were analyzed by S1 nuclease assay using ^{32}P labeled *CYC1* and tryptophan tRNA probes. Protected nucleic acids were resolved on denaturing gel. $tRNA^{w}$ signal was used as a load control to normalize signals of *CYC1* transcripts. (**B**) Bar graphs showed fold changes (mean ±SD, $p<0.005$) of *CYC1* levels upon activation versus partial repression from each strain of 3 separate experiments. The induction fold changes were calculated by dividing the signals of *CYC1* transcripts upon activation by those during partial repression. Figures show that while the *ada1Δ* allele diminishes *CYC1* activation, the *gcn5Δ* allele moderately, the *ada2Δ* and *ada3Δ* alleles slightly affect *CYC1* transcription.

Figure 5.6A

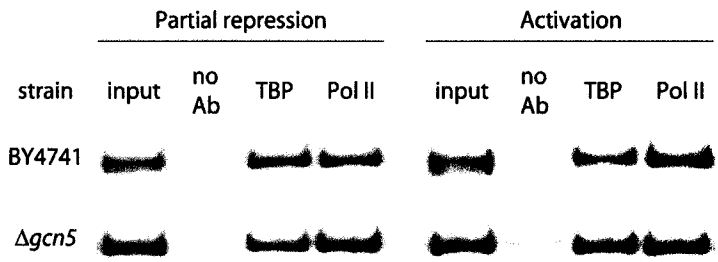

Figure 5.6B

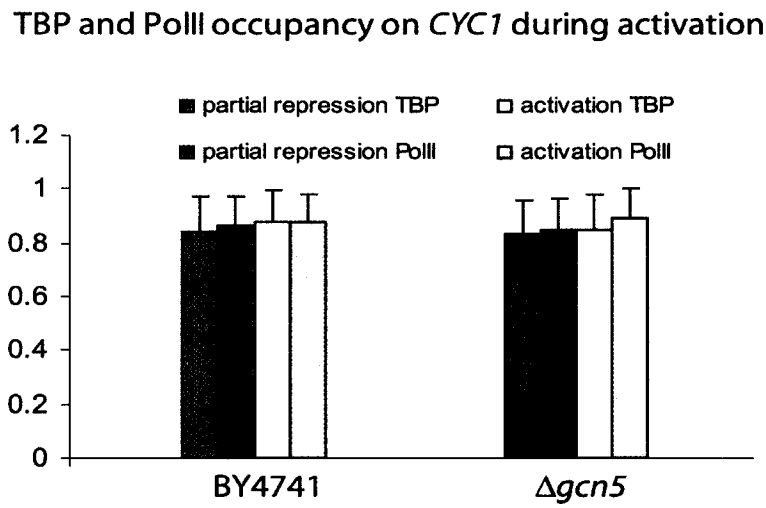

Figure 5.6A and B Gcn5 does not affect the occupancy levels of TBP and Pol II **on *CYC1* gene during activation (A)** Both parental and *gcn5* deletion strains were grown under partial repression or activation of *CYC1* gene. ChIP analysis was performed to measure the occupancy levels of TBP and Pol II. Shown here is one representative picture of three independent experiments. (**B**) Bar graph showed the relative occupancy levels of TBP and Pol II during both partial repression and activation of *CYC1* gene (mean±SD, n=3, $p<0.005$). Protein occupancy was represented by ratio of signal from IP samples versus that of the input after minus the signal of no antibody control.

levels of both TBP and Pol II were quite comparable in *GCN5* deletion strain with that in the parental strain (Figure 5.6 A and B).

5.5 Discussion

In this study, we looked at the post-recruitment functions of all the non-essential Spt proteins during transcription. Our results from both artificial recruitment and transcription assays clearly pointed out the post-recruitment functions of Spt4, Spt7 and Spt20. Spt4 has long been known for its role in regulating transcription elongation [135, 178]. Genetic studies in yeast and in vitro transcription assays implicate Spt4 as a positive elongation factor [175]. Spt4 antagonizes the negative effects of Pol II pausing imposed by the chromatin-remodeling yeast factor Isw1p [176]. The role of Spt4 in regulating transcription elongation fits well in the post-recruitment regulation steps and our results on *spt4* mutant also implied that Spt4 probably was not involved in recruitment functions. For the other Spt proteins we tested in both assays, their functions during post-recruitment regulations might be subtle since those Spt proteins that only slightly affected *CYC1* transcription and can be bypassed by artificial recruitment of TBP.

The requirement of Spt7, Spt20 and Ada1 for transcription after TBP recruitment links the SAGA complex to post-recruitment steps since Spt7, Spt20 together with Ada1 and several TAFs are the core subunits to maintain the structural integrity of the SAGA complex [156, 164, 254-256].

However, the nature of these SAGA post-recruitment functions is still unknown and different from its currently known functions of histone acetylation and TBP recruitment. There were several lines of evidence to support this argument. First, the functions of Spt7, Spt20 and Ada1 were not bypassed by artificial recruitment of TBP. Second, although the SAGA complex is required for delivering TBP to certain genes, it was not required for TBP recruitment at *CYC1* gene since disrupting SAGA did not affect TBP occupancy at *CYC1* promoter. Third, deletion of the enzymatic subunit *GCN5* only moderately affected *CYC1* transcription while disrupting the SAGA complex abolished transcription. Fourth, the HAT activity of SAGA did not affect the TBP recruitment on *CYC1*. These results suggested that the HAT activity of SAGA complex were probably partially required for *CYC1* activation after TBP recruitment, while the intact SAGA was required for fully post-recruitment regulation of *CYC1* gene transcription. Taken together, our data suggested that, in addition to its roles in histone acetylation and delivering TBP to certain promoters, there must be some other unknown functions of SAGA complex in the late stages of transcription process.

Chapter 6

Perspectives and Future directions

The work presented in this dissertation was initially focused on characterizing the functions of a newly identified RNA Pol II transcription factor Spn1, which regulates Pol II transcription in post-recruitment steps. From a genetic screen, two opposite types of genetic interactions of *SPN1* were identified: *SPN1* weakly interacts with *RTF1*, TFIIS which points out a positive role of Spn1 in regulating elongation; *SPN1* strongly suppresses the functions of Swi/Snf indicating its role in counteracting the chromatin-remodeling activity. These findings further support the involvement of Spn1 in post-recruitment regulation. But it is still not clear how Spn1 affects elongation. Using in vitro transcription assay reconstituted on the chromatin template would help to answer this question.

In the following-up studies, I only focused on the role of Spn1 in counteracting the functions of the Swi/Snf complex. The results I obtained suggested a model in which Spn1 in association with Pol II complex blocks the recruitment of the Swi/Snf complex and can also induced the recruitment of the Swi/Snf complex upon interacting with Spt6. As part of the evidence, Spt6 is recruited earlier than the Swi/Snf complex. This is the

first time that a chromatin reassembly factor facilitates the recruitment of a chromatin-remodeling factor. A good genetic test of this model would be to find an spt6 mutant allele that is defective for interacting with Spn1 thus would totally abolish the recruitment of the Swi/Snf complex. Furthermore, testing the direct effects of Spn1 or the Spn1/Spt6 complex on the chromatin structure using reconstituted in vitro system would provide firm evidence for this model.

The concerted action of Spn1, Spt6 and the Swi/Snf complex during *CYC1* transcription suggests a role of Spn1 transcription start site selection or in TATA box usage. This also links Spn1 with recruitment functions. Further analysis of the occupancy of other important transcriptional factors such as TFIIH, RNA processing factors etc. would provide valuable functions of Spn1 during the transcription process.

Finally, my studies to defining the post-recruitment functions of other different Spt proteins brought my attention to the SAGA complex. If we determined the direct occupancy of SAGA on the *CYC1* gene, the current results would clearly predict the additional unknown functions of the SAGA complex during post-recruitment regulation. I only looked at the occupancy of TBP and Pol II in SAGA disrupted strains, it will be logical that we continue to look at the occupancy of other factors that come to promoter at the late initiation stage such as TFIIH, TFIIF, CTD kinases, Capping enzyme etc.

I think that the work I presented in this dissertation provides valuable information for us to understand the mechanistic functions of Spn1 and the SAGA complex during RNA polymerase II transcription. The ideas propose here, if carried out, would further enrich our knowledge on the mechanisms of RNA polymerase II mediated transcription.

Appendix

Protein-Protein interaction map for TFIID

During my first year of my Ph.D. studies in Dr. Stargell's lab, in collaboration with Dr. Gayatri Yatherajam and Dr, Susan Kraemer, I tried to map the interaction network in yeast TFIID complex which resulted in my first publication as a graduate student. The paper was published in Nucleic Acids Research. I contributed Table 2 and Table 3 of the paper. Here I attached the abstract of the paper entitled:

Protein-Protein Interaction map for TFIID Yatherajam,G., Zhang,L., Kraemer,S.M., and Stargell,L.A. *Nucleic Acids Res.* 2003 Feb 15; 31(4): 1252-60.

Protein-Protein Interaction map for TFIID

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A.1 Abstract

A major rate-limiting step in transcription initiation by RNA polymerase II is recognition and binding of the TATA element by the transcription factor TFIID. TFIID is composed of TATA binding protein (TBP) and approximately a dozen TBP-associated factors (TAFs). Emerging consensus regarding the role of TAFs is that TFIID assumes a gene specific activity that is regulated by interaction with other factors. In spite of many studies demonstrating the essential nature of TAFs in transcription, very little is known about the subunit contacts within TFIID. To understand fully the functional role of TAFs, it is imperative to define TAF-TAF interactions and their topological arrangement within TFIID. We performed a systematic two-hybrid analysis using the thirteen essential TAFs of the *Saccharomyces cerevisiae* TFIID complex and TBP. Specific interactions were defined for each component, and the biological significance of these interactions is supported by numerous genetic and biochemical studies. By combining the interaction profiles presented here and the available studies

utilizing specific TAFs, we propose a working hypothesis for the arrangement of components in the TFIID complex. Thus these results serve as a foundation for understanding the overall architecture of yeast TFIID.

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