REGULATION OF TRANSCRIPTION ELONGATION FACTORS SPT2 AND SPT6 BY CASEIN KINASE II

Thèse

Abdul Wajid Bhat

Doctorat en Biologie Cellulaire et Moléculaire
Philosophiae Doctor (Ph.D.)

Québec, Canada

Résumé

Comme pour tous les autres processus en lien avec l'ADN, la structure de la chromatine lors de la transcription est dans un état de perpétuel changement. Ainsi, elle est ouverte pour permettre l'accès à l'ADN, pour ensuite se replier correctement. La dynamique de la structure chromatinienne est régulée finement par de multiples mécanismes qui agissent ensemble afin de rendre le processus hautement efficace. Ces mécanismes comprennent les modifications post-traductionelles des histones, le remodelage de la chromatine par les remodeleurs ATP-dépendants, l'incorporation des variants d'histones et l'assemblage/désassemblage des nucléosomes par les chaperons d'histones. En plus de ces activités, il y a un certain nombre de composantes non-reliées aux histones qui sont directement impliquées dans les modulations de la conformation de la chromatine associées à la transcription. Chez la levure, un de ces facteurs est la protéine *HMG-like* Spt2p, démontrée précédemment comme étant directement impliquée dans le réassemblage des nucléosomes dans le sillon de l'ARN polymérase II en déplacement le long du segment d'ADN transcrit. Dans la présente étude, nous démontrons que Spt2p est phosphorylée directement par la caséine kinase II (CKII) et que cette modification inhibe sa liaison à la chromatine. Nos résultats indiquent que la CKII altère l'interaction de Spt2p avec le chaperon d'histone Spt6p. Nous avons aussi trouvé que la phosphorylation directe de Spt6p par la CKII stimule l'association de ce facteur avec un autre partenaire, Iws1p. Cette association est absolument nécessaire pour le repliement correct des nucléosomes durant l'élongation. De plus, cette régulation positive du complexe Spt6p/Iws1p par la CKII module directement l'association de ce complexe avec la méthyltransférase de H3K36, Set2p. Finalement, nous avons montré que la phosphorylation de Spt6p par la CKII est essentielle à l'inhibition des promoteurs cryptiques et des erreurs de transcription. Dans l'ensemble, nos résultats suggèrent un nouveau mécanisme par lequel la CKII contrôle le repliement correct de la structure de la chromatine dans les régions codantes en modulant les interactions du chaperon d'histone essentiel Spt6p avec ses partenaires Spt2p, Iws1p et Set2p.

Abstract

Like any other DNA-related process, chromatin structure is in a state of constant flux during transcription, unfolded to get access to DNA and refolded back properly. The dynamics of chromatin structure are tightly regulated and multiple mechanisms act together to make the process highly efficient. These include modifications of histones, chromatin remodeling by ATP-dependent remodeling factors, incorporation of histone variants and nucleosome disassembly and reassembly by histone chaperones. In addition to these activities, there are a number of non-histone chromatin components that are directly involved in the modulation of chromatin associated with transcription. In yeast, one of these factors is the HMG-like protein Spt2p previously shown to participate directly in the process of nucleosome reassembly in the wake of RNA polymerase II movement along transcribed DNA. In this work, we show that Spt2p is directly phosphorylated by the casein kinase II (CKII) and we demonstrate that this modification inhibits its association with chromatin. Our findings indicate that CKII disrupts the interaction of Spt2p with the histone chaperone Spt6p. Interestingly, we also found that direct phosphorylation of Spt6p by CKII stimulates the association of this factor with another partner, Iws1p. This association is absolutely required for the refolding of nucleosomes during elongation. Furthermore, this positive regulation of the Spt6p/Iws1p complex by CKII modulates directly the association of this complex with the H3K36 methyltransferase Set2p. Finally, we show that phosphorylation of Spt6p by CKII is essential to the inhibition of cryptic promoters and spurious transcription. Taken together, our results suggest a new mechanism whereby CKII directs chromatin structure refolding in coding regions by modulating the interaction of the essential histone chaperone Spt6p with its partners Spt2p, Iws1p and Set2p.

Acknowledgments

To begin with, I would like to thank my supervisor Dr. Amine Nourani for his help, patience, guidance and valuable advice. He constantly supported and encouraged me throughout my graduate studies and at times when things were tough for me.

I would also like to thank all the present and past members of Nourani's Lab who have always been supportive. In particular I would like to thank Genevieve, Anne, Lynda, Marine, Phillip and David.

I am highly grateful to Dr. Richard Poulin for proofreading of my thesis. His suggestions and comments about my writing skills were helpful in improving the quality of my thesis.

I address my very special thanks to two special friends, Irfan and Altaf, for their support, encouragement and wonderful company in Quebec City. I will never forget the time we spent together. There are other friends who have constantly encouraged and supported me. Thank you Samir, Alejandro, Prakash, Ranjan, Bilal, Ajaz and Ashraf.

I would like to thank my wife Ambreen for being always there, in good and bad times. And last, but not least, I would like to thank my parents, my brothers and my sisters for their unconditional support throughout my life, for which the mere expression of my thanks will never be sufficient.

Table of contents

List of Tables

List of Figures

Abbreviations

ACF: ATP-utilizing chromatin assembly and remodeling factor

Act1: actin 1

ADA: adaptor

Asf1: anti-silencing function

ATP: adenosine triphosphate

bp: base pair

BRG1: brahma-related gene1

CAF1: chromatin assembly factor 1

CENP-A: centromeric protein A

Chd1: chromodomain helicase DNA binding 1

ChIP: chromatin immunoprecipitation

CKII: casein kinase II

CTD: C terminal domain

Ctk1: C terminal domain kinase 1

CYC1: cytochrome c, isoform 1

DNA: deoxyribonucleic acid

Eaf: Esa1-associated factor

FACT: facilitates chromatin transcription

Gcn5: general control non-depressible 5

GST: glutathione *S*-transferase

GTF: general transcription factor

HAT: histone acetyltransferase

HDAC: histone deacetylase

HMG: high-mobility group

HMT: histone methyltransferase

HP1: heterochromatin protein 1

HR: homologous recombination

IEF: isoelectric focusing

Ino80: inositol 80

ISWI: imitation SWI/SNF

Iws1p: interacts with Spt6

NAP1: nucleosome assembly protein 1

NuA4: nucleosome acetyltransferase of H4

ORF: open reading frame

PCR: polymerase chain reaction

PHD: plant homeodomain

PIC: pre initiation complex

RNA: ribonucleic acid

RNAPII: RNA polymerase II

Rpd3: reduced potassium dependency 3

RSC: remodels the structure of chromatin

SAGA: Spt, Ada, Gcn5 acetyltransferase

SAM: *S*-adenosylmethionine

SANT: Swi3, Ada2, N-Cor TFIIIB

Set: su (var), enhancer of zeste, trithorax

Sin: swi-independent

SLIDE: SANT-like ISWI domain

SNF: sucrose non fermentation

Spt: suppressor of Ty

SPN1: suppresses postrecruitment functions gene number 1

SWI: mating type switching

Swr1: Swi2/Snf2-related ATPase 1

TAP: TBP-associated factor

TAP: tandem affinity purification

TBP: TATA box-binding protein

TEV: tobacco etch virus

Ubp8: ubiquitin specific protease 8

WT: wild type

Chapter 1

Introduction

1.1 Chromatin structure: Role in gene expression

Genetic information in every living cell is in the form of one or several very long and continuous strings of DNA, which serves as a master blueprint for cellular architecture and for all of its functions. In order for these long strings of DNA to fit into the tiny nucleus, the DNA is compacted to nearly one hundred-thousandth of its linear dimension. This degree of compaction is achieved by several levels of folding with an approximately equal mass of proteins, forming a nucleoprotein complex called *chromatin* (Chakravarthy et al., 2005; Widom, 1989). The chromatin structure and dynamics are essentially similar in all eukaryotic organisms, from the simplest eukaryote yeast to the most complex human (Marino-Ramirez et al., 2006). Several hierarchies have been proposed to distinguish the multiple levels of organization of chromatin structure [reviewed in (Bassett et al., 2009; Chakravarthy et al., 2005; Li and Reinberg, 2011; Woodcock and Ghosh, 2010)]. A schematic representation of various levels of chromatin compaction is shown in Figure 1.1. The *first* level of organization is a linear array of nucleosomes separated by free DNA and often described as beads-on-a-string-like structure. Through short range internucleosomal interactions and linker histones, chromatin is further organized to a *secondary* level of organization known as the 30-nm fiber. The exact structure of the 30-nm fiber still remains uncertain despite the intensive efforts towards its investigation. A further, *tertiary* level of compaction is generated via short- and long-range interactions between regions of the secondary structure. The organization of this tertiary level varies due to various factors such as tissue type and cell cycle (Bonifer, 1999; Woodcock and Dimitrov, 2001). For all DNA-related processes, the genome is unfolded and then rapidly refolded on a time scale measured in seconds (Bucceri et al., 2006). This process is highly efficient due to the cooperative action of a number of specific factors. These include histone modifiers, ATP-dependent chromatin remodelers, histone variants and histone chaperones. Chromatin dynamics are tightly regulated and even a slight misregulation of these processes can lead to many diseases. Different diseases are linked with a dysfunctioning of the chromatin modulation machinery. Histone acetylase/deacetylase activities are strongly shifted toward histone hyperacetylation in patients with rheumatoid arthritis (Huber et al., 2007). Changes in chromatin structure are also linked to other disorders such as diabetes and heart disease (Antos et al., 2003; Han et al., 2011; Lawless et al., 2009; Liew and Dzau, 2004; Olson,

2006). Likewise, defects in the chromatin modulating machinery have been linked to various cancers (Avvakumov and Cote, 2007; Cairns, 2001; Chi et al., 2010; Clapier and Cairns, 2009; Wang et al., 2007).

All DNA-related processes within the cell (e.g. DNA replication, DNA repair and transcription) occur in the context of chromatin. By its very nature, the general organization of the genome in the form of chromatin is inhibitory to all these processes. Therefore, cells have developed various mechanisms to regulate the opening and closing of this barrier such as histone modifications, ATP-dependent chromatin remodeling and nucleosome disassembly. The studies described in this dissertation were done to understand the regulation of different factors that modulate chromatin structure during transcription elongation using the yeast *Saccharomyces cerevisiae* as a model system. The nucleosome is the basic unit of chromatin and these regulatory processes involve structural and/or chemical changes in the molecular composition of nucleosomes. I will first present an overview of the basic features of the nucleosome and of the general mechanisms used by cell to modify the structure of chromatin. This overview will then be followed by a description of the events occurring during transcription.

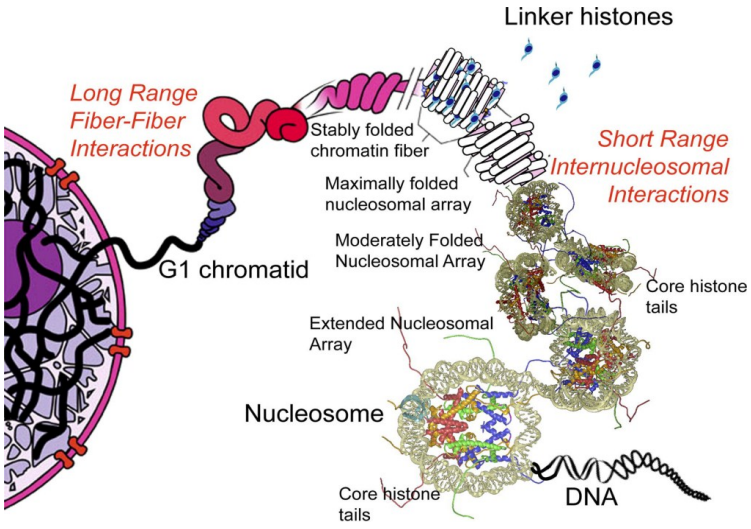

Figure 1.1 : Organization of chromatin
Schematic view of the different levels of chromatin packaging. Figure from Chakraverthy et al. (Chakravarthy et al., 2005).

1.1.1 Nucleosome

The basic unit of chromatin is the nucleosome, which consists of 147 base pairs (bp) of DNA wrapped 1.65 times around an octamer of histones, containing two copies of each of the histone species H2A, H2B, H3 and H4. The composition of the nucleosome core particle has been known since the pioneering studies of Roger Kornberg in the early 70s (Kornberg, 1977). In addition to core histones, the linker histone H1 is also an important component of chromatin but is localized outside the nucleosome core particle. The first high-resolution structures of the nucleosome core particle were published in 1997, thereby providing clear molecular details of nucleosome packaging (Figure 1.2) (Luger et al., 1997). The four core histones are strongly basic, with pI values between 10.5 and 11, and are highly conserved among eukaryotes. The bulk of the histone protein is folded into a helix-turn-helix motif known as a *histone fold*. This motif is optimized for efficient protein-protein interactions and dimerization. In addition to the histone fold, each core histone contains a highly dynamic N-terminal extremity rich in basic amino acids. The N-terminal section is unstructured, extends outwards from the nucleosome core particle and is subjected to a number of post-translational modifications (Kouzarides, 2007). The N-terminal end does not contribute to the core structure of the nucleosome but rather plays important roles in higher-order chromatin structure, which is regulated by different mechanisms of modification (Gelato and Fischle, 2008).

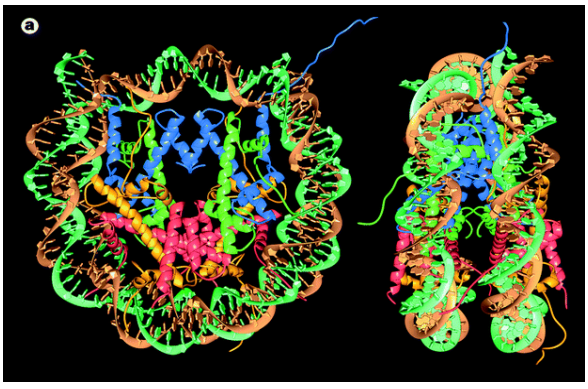

Figure 1.2 : Nucleosome structure
Top and side view of 146 base pairs of DNA in complex with the eight core histones. Two copies each of H2A (orange), H2B (pink), H3 (blue), and H4 (green) can be seen in the model on the left. In addition to the α-helical histone core domains, several histone "tails" can be seen protruding from the nucleosome core in both models. Figure from Luger et al. (Luger et al., 1997).

1.2 Different mechanisms regulate chromatin structure

The histone-DNA interactions are among the most stable interactions within the cell and the highly stable nucleosome structure blocks the access of other macromolecules to DNA. Cells have thus developed mechanisms to modulate chromatin structure and allow access to DNA for various essential processes. The different mechanisms that alter the structure of the nucleosome are briefly described below.

1.2.1 Histone modifications

One of the most important and extensively studied processes that change chromatin structure is the post-translational modification of the histones. Histone modifications primarily occur along the N-terminal extremity, although modifications in the core domains and the C-terminal tail of some histones have also been described (Kouzarides, 2007; Krebs, 2007; Turner, 2005). Different types of histone modifications have been reported. These include the methylation of lysine and arginine residues, the acetylation, ubiquitination or sumoylation of lysine, and the phosphorylation of serine, threonine and tyrosine residues. In addition to these modifications, isomerization, ADP-ribosylation and deimination have been reported and characterized (Kouzarides, 2007; Krebs, 2007; Turner, 2005). The characterization of histone modifying enzymes has been the focus of various recent studies. Enzymes catalyzing various histone modifications have been identified and in many cases, the enzymes that remove these modifications have also been characterized. The presence of enzymes that reversibly modify histones accounts for the highly dynamic nature of these modifications. The different histone modifications found in yeast and in some cases, the enzymes responsible for these modifications are shown in Figure 1.3. Histone modifications change chromatin structure by at least two different mechanisms. The first one is the weakening of histone-DNA interactions or interactions between different histones in adjacent nucleosomes. Belonging to the latter type of mechanism is *acetylation*, which can decrease histone-DNA interactions by neutralizing the positive charge of histones (Hong et al., 1993; Verdone et al., 2006). By using chemically modified recombinant histones, acetylation of lysine-16 of histone H4 has been shown to inhibit the

formation of compact 30-nm–like fibers (Shogren-Knaak et al., 2006). The second and best characterized mechanism of action for histone modification is the creation of new binding sites for the recruitment of other components of the chromatin modifying machinery. Chromatin remodeling requires the highly regulated and organized recruitment of a number of chromatin-modifying enzymes. These factors possess domains that recognize different histone modifications such as methylation and acetylation which recognize chromodomains and bromodomains, respectively. Several chromatin modulating factors with domains recognizing histone modifications have been characterized so far, such as the repressive Rpd3S histone deacetylase complex which recognizes methylation of H3K36 via the chromodomain-containing subunit Eaf3p and the PHD-finger-containing subunit Rco1p (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Li et al., 2007b). Three of the most important histone modifications associated with transcription elongation, namely methylation, acetylation and ubiquitination, are discussed in detail in section 1.5.1.

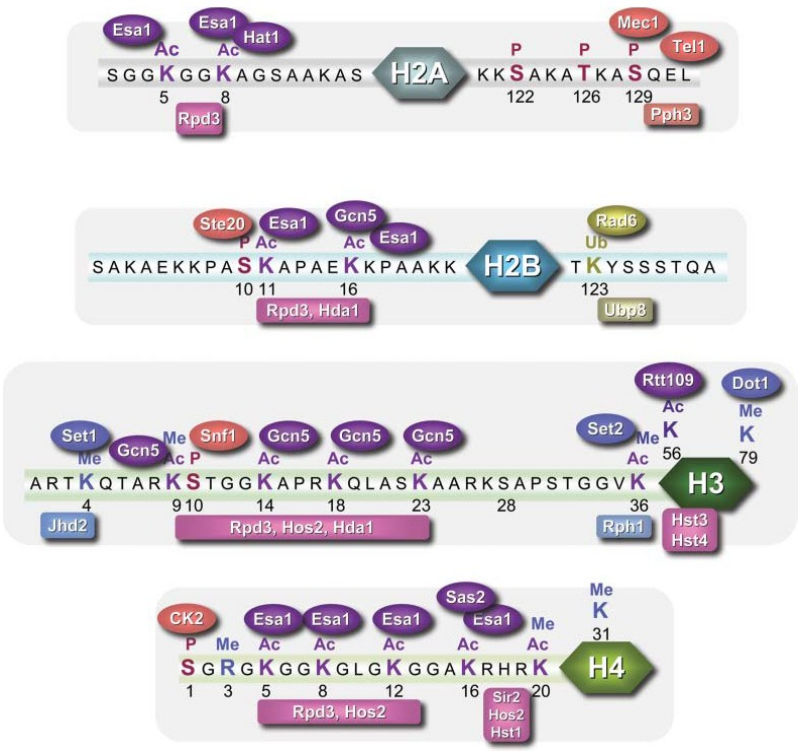

Figure 1.3 : Post-translational histone modifications in yeast
The four core histones are shown. The histone fold is represented by hexagons, and the sequences of N- and C-terminal tails are provided; please note that residues accompanied with a number indicate sites of modification. Known covalent modifications are indicated by different colors: **acetylation** (Ac) is shown in *purple*, **methylation** (Me) in *blue*,

phosphorylation (P) in *red* and **ubiquitination** (Ub) in *green*. Enzymes for some of these modifications are also shown. Enzymes that **add** modifications are shown in the *ovals* above the modification site, and enzymes that **remove** modifications are indicated below in *boxes*. The Figure is taken from Krebs (Krebs, 2007).

1.2.2 ATP-dependent chromatin remodeling

Chromatin remodelers, a set of multi-subunit protein complexes, utilize the energy of ATP hydrolysis to alter chromatin structure. All ATP-dependent chromatin remodelers contain a catalytic subunit with homology to superfamily 2 of DEAD/H-box nucleic acid-stimulated ATPase (Tsukiyama, 2002; van Vugt et al., 2007). These complexes fall into four families, which are classified by similarities in their ATPase subunits, i.e. the SWI2/SNF2, ISWI, CHD and INO80 families. The catalytic subunit of each family includes the highly conserved ATPase domain flanked by unique domains that allow their classification into four distinct families (Clapier and Cairns, 2009; Erdel et al.; Gangaraju and Bartholomew, 2007). ATP-dependent chromatin remodelers have been implicated in transcription, DNA repair as well as DNA replication. Along with histone modifications, ATP-dependent chromatin remodelers are the main contributors to chromatin dynamics (Becker and Horz, 2002; Clapier and Cairns, 2009; Gangaraju and Bartholomew, 2007; Saha et al., 2006). The ATP-dependent chromatin remodelers can induce changes in the chromatin structure by moving, ejecting, or restructuring the nucleosome. The different outcomes of chromatin remodeling are depicted in Figure 1.4. Different chromatin remodeling complexes are briefly described below.

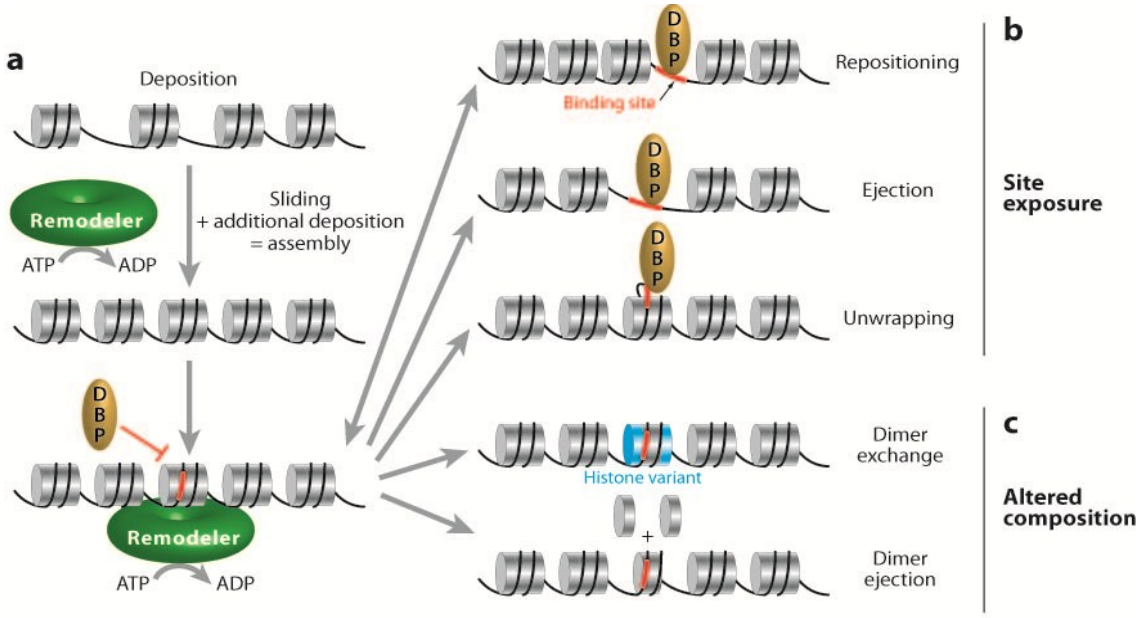

Figure 1.4 : ATP-dependent chromatin Remodeling
Different outcomes of chromatin remodeling, Remodelers (*green*) can assist in chromatin assembly by moving already deposited histone octamers, generating room for additional deposition (a). Remodeler action on a nucleosome array results in various products that can be classified in two categories: (b) **site exposure**, in which a site (*red*) for a DNA-binding protein (DBP), initially occluded by the histone octamer, becomes accessible by nucleosomal sliding (*repositioning*), or nucleosomal eviction (*ejection*), or localized *unwrapping*, and (c) **altered composition**, in which the nucleosome content is modified by dimer replacement [exchange of a H2A-H2B dimer with an alternative dimer containing a histone variant (*blue*)] or through dimer ejection. From Clapier & Cairns (2009) (Clapier and Cairns, 2009).

1.2.2.1 SWI/SNF

The SWI/SNF family chromatin remodelers are characterized by the presence of bromodomains in the catalytic ATPase subunit, as shown in Figure 1.5 (Clapier and Cairns, 2009; Gangaraju and Bartholomew, 2007). This family of chromatin remodelers is conserved among eukaryotes and includes yeast SWI/SNF, yeast RSC, the drosophila Brahma complex as well as mammalian BRM (Brahma) and BRG1 (Brahma-related gene) complexes (Muchardt and Yaniv, 1999; Wang, 2003; Winston and Carlson, 1992). SWI/SNF was the first chromatin remodeling complex discovered in *S. cerevisiae*. Initially, members of this complex were identified in independent genetic screens. SWI genes were first identified from mutants defective in the expression of the *HO* gene, which is required

for mating type switching (SWI= switching defective) (Stern et al., 1984; Winston and Carlson, 1992), and SNF genes were identified from mutations in the expression of the *SUC2* gene, which is required for growth on sucrose (SNF = sucrose non-fermenting) (Neigeborn and Carlson, 1984; Winston and Carlson, 1992). The *SNF2* gene encodes the catalytic ATPase subunit, which was purified as a complex containing the gene products of other SWI and SNF genes, hence called SWI/SNF complex (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994).

Earlier genetic and biochemical analysis showed a link between the SWI/SNF complex and chromatin. Mutations in the *swi/snf* genes were found to be suppressed by mutations in the histone genes and other chromatin components (Kruger et al., 1995; Perez-Martin and Johnson, 1998a; Recht and Osley, 1999). Subsequently, several in vitro and in vivo studies showed that the SWI/SNF complex alters chromatin structure. SWI/SNF disrupts nucleosome structure and increases DNA accessibility to activators in an ATP-dependent manner (Cote et al., 1994). Additionally, the SWI/SNF complex was shown to be important for the transcriptionally active structure of chromatin in the promoter of the *SUC2* gene (Matallana et al., 1992; Sudarsanam and Winston, 2000; Wu and Winston, 1997). In addition to its role in transcription activation, the SWI/SNF chromatin remodeling complex is also required for the repression of transcription of some genes (Martens and Winston, 2002, 2003). Moreover, SWI/SNF complex is required for ribosomal DNA and telomere silencing in *S. cerevisiae* (Dror and Winston, 2004).

RSC (Remodels the Structure of Chromatin) is another member of the SWI/SNF family of chromatin remodeling complexes in *S. cerevisiae*. Initially, it was isolated as a 15-subunit complex from yeast on the basis of its homology to SWI/SNF (Cairns et al., 1996). Sth1p, the catalytic subunit of RSC, is homologous to Snf2p, and both proteins have similar domain compositions (Laurent et al., 1992). RSC was later found to exist in two isoforms that are distinguished by the presence or absence of Rsc1p and Rsc2p subunits (Cairns et al., 1999). In contrast to SWI/SNF, several of the RSC subunits are essential for viability in yeast (Cairns et al., 1996; Cairns et al., 1999). Depletion of Sth1p deregulates about 40% of all yeast genes, which suggests a general requirement of RSC for global transcription (van Vugt et al., 2007).

1.2.2.2 ISWI

The Imitation SWItch, or ISWI, family of chromatin-remodeling complexes in yeast is represented by Isw1p and Isw2p, which are part of distinct complexes that do not share subunits (Tsukiyama et al., 1999). The ISWI family also includes the catalytic ATPase subunits Iwp1p and Iws2p, which are similar to SWI/SNF-type ATPases, but lack bromodomains. Instead, these subunits are characterized by SANT and SLIDE domains, which are involved in histone tail and linker DNA binding, respectively (Figure 1.5) (Clapier and Cairns, 2009; Gangaraju and Bartholomew, 2007). In *S. cerevisiae*, genetic and biochemical studies show that ISWI complexes are involved in transcriptional repression in vivo (Corona and Tamkun, 2004; Goldmark et al., 2000). ISWI complexes primarily organize and position nucleosomes into ordered arrays and inhibit transcription by restricting access to the underlying DNA (Gangaraju and Bartholomew, 2007; van Vugt et al., 2007). On the other hand, some studies have shown that ISWI might also promote transcription (Corona and Tamkun, 2004; Morillon et al., 2003). Nucleosome repositioning by Isw2p is directional, and this activity is important for the suppression of antisense transcription (Whitehouse et al., 2007). Isw2p positions the nucleosomes bordering the promoter at the interface between genic and intergenic regions. This activity is directional and results in increased nucleosome occupancy in the intergenic region, thus forcing transcription in a single direction (Whitehouse et al., 2007).

1.2.2.3 CHD family

The only CHD (chromodomain helicase DNA-binding)-type ATPase present in yeast is Chd1p. It is characterized by the presence of two chromodomains in addition to the Snf2p-like ATPase domain (Figure 1.5) (Clapier and Cairns, 2009; Gangaraju and Bartholomew, 2007; Woodage et al., 1997). The presence of chromodomains suggests that Chd1p is targeted to chromatin through interactions with methylated lysine residues (Pray-Grant et al., 2005). Chd1p interacts with transcriptional coactivators, suggesting a potential role in transcriptional activation (Pray-Grant et al., 2005). However, Chd1p has been shown to localize to transcribed genes, and shows physical and functional interactions with transcription elongation factors such as FACT, the Paf1p1p complex and Spt4p-Spt5p

(Krogan et al., 2002; Simic et al., 2003). These studies suggest a role of Chd1p in transcription elongation.

1.2.2.4 INO80

The INO80 family is the most recently identified among the ATP-dependent chromatin remodeling complex families. In yeast, the INO80 family is represented by the INO80 and SWR1 complexes. The catalytic subunits (Ino80p and Swr1p) of these complexes are characterized by an ATPase domain partitioned by a large spacer region (Figure 1.5) (Clapier and Cairns, 2009; Gangaraju and Bartholomew, 2007). The INO80 complex is composed of 15 subunits in yeast (Jonsson et al., 2004; Shen et al., 2000). Two of these subunits, Rvb1p and Rvb2p, share homology with the bacterial Holliday junction helicase RuvB (Shen et al., 2000). The INO80 complex is the only ATP-dependent chromatin remodeling complex endowed with helicase activity, and is involved in a variety of nuclear processes, including transcriptional regulation, DNA repair and DNA replication [reviewed in (Bao and Shen, 2007; Conaway and Conaway, 2009; Morrison and Shen, 2009)]. The SWR1 complex is responsible for the incorporation of histone H2A.Z into nucleosomes. SWR1 interacts with H2A.Z-H2B dimers and catalyzes the substitution of canonical H2A/H2B dimers with H2A.Z/H2B dimers (Kobor et al., 2004; Mizuguchi et al., 2004).

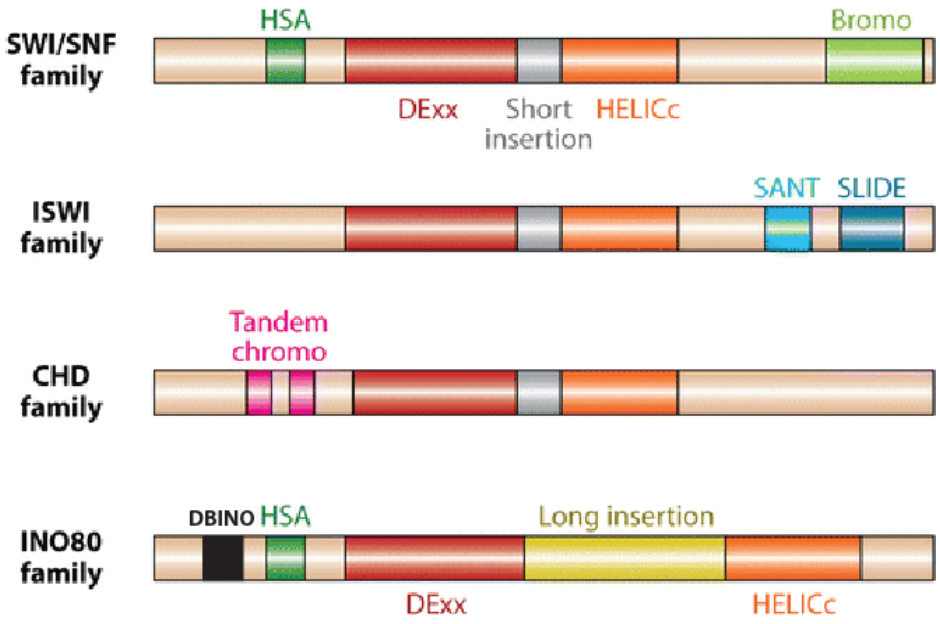

Figure 1.5 : ATPase structure in an ATP-dependent chromatin remodeling complex
All ATP-dependent chromatin remodelers contain ATPase subunits characterized by an ATPase domain that is split into two parts: DExx (*red*) and HELICc (*orange*). The ATPase domain of CHD, ISWI and SWI/SNF families is interrupted only by a short protein sequence (*gray*), whereas the ATPase domain of INO80 family members is partitioned by a long insertion (*yellow*). Although ATPase subunits of different remodelers share a common catalytic domain, they contain unique flanking domains: the SWI/SNF family members contain a bromodomain (*light green*) and a HSA (helicase-SANT) domain (*dark green*), the ISWI family is characterized by a SANT-SLIDE module (*blue*), CHD family members have tandem chromodomains (*magenta*), the INO80 family is characterized by the presence of an HSA domain (*dark green*) and a DBINO domain (*black*). Figure adapted from Clapier and Cairns (Clapier and Cairns, 2009).

1.2.3 Histone variants

In addition to histone modification and chromatin remodeling, an important way to alter chromatin structure is through a change in nucleosome composition by the incorporation of histone variants. Canonical histones are highly conserved among eukaryotes and are expressed mostly in the S-phase of the cell cycle. In contrast, non-allelic histone variants exhibit significant differences in primary sequence and are expressed throughout the cell cycle. Histone variants differ from canonical histones by their primary sequence (Malik and Henikoff, 2003). Some variants have distinct biophysical characteristics that are thought to alter the properties of nucleosomes, while others localize to specific regions of the genome

(Kamakaka and Biggins, 2005). Since histone variants are expressed throughout the cell cycle, histone variants can be incorporated into chromatin in a DNA replication-independent manner. The two major variants of histone H3 are centromeric H3 (CenH3) and H3.3. The CenH3 variant is also known as CENP-A in humans and chromosome segregation protein 4 (Cse4p) in *S. cerevisiae*. CenH3 replaces histone H3 in the centromere and is required for kinetochore formation (Stoler et al., 1995; Van Hooser et al., 2001). Histone variant H3.3 is the best characterized among the H3 variants and differs from H3 at four residues (Ahmad and Henikoff, 2002). It is deposited in regions of active transcription independently of replication (Henikoff and Ahmad, 2005; Mito et al., 2005). In yeast, only one non-centromeric H3 (similar to H3.3) is present and is deposited on chromatin in both replication-dependent and replication-independent manners. H2A has the largest number of variants, which include H2A.Z, macroH2A, H2A-BbD, H2AvD and H2AX [reviewed in (Ausio et al., 2001; Kamakaka and Biggins, 2005)]. Of note, among these H2A variants, H2AX and H2AZ are highly conserved from *S. cerevisiae* to human (Redon et al., 2002). These histone variants have been implicated in various processes such as transcription regulation, heterochromatin establishment and DNA damage response (Kamakaka and Biggins, 2005). The functions of some of the major histone variants are summarized in Table 1.1.

Table 1.1 : Histone variants and their function

Variant	Species	Chromatin effect	Function
MacroH2A	Vertebrate	Condensed chromatin	X-chromosome inactivation
H2ABbd	Vertebrate	Open chromatin	Transcription activation
H2A.X	Ubiquitous	Condensed chromatin	DNA repair/recombination/transcription repression
H2A.Z	Ubiquitous	Open/closed chromatin	Transcription activation/repression, chromosome segregation
SpH2B	Sea urchin	Chromatin condensation	Chromatin packaging
CenH3	Ubiquitous		Kinetochore formation/function
H3.3	Ubiquitous	Open chromatin	Transcription

Table adapted from Kamakaka and Biggins (Kamakaka and Biggins, 2005). The species distribution and probable function of the different histone variants are shown.

1.2.4 Histone chaperones

Histone-DNA interactions are among the most stable interactions within the cell. Histones are highly basic and have a high affinity for DNA. Histones have a tendency to form insoluble aggregates when mixed with negatively charged DNA under physiological conditions. To prevent the non-specific aggregation of histones with DNA during chromatin assembly, acidic proteins are required to bind free histones and shield their positive charges, so that ordered nucleosome assembly may occur. This role is played by a special group of acidic proteins called **histone chaperones**. Nucleoplasmin was the first protein found to play a role as chaperone (Laskey et al., 1978). With time, the number of proteins with the characteristics of histone chaperone has kept increasing and several other proteins/protein complexes (e.g. Nap1p, CAF-1, FACT, Spt6p and Asf1p) were found to act as histone chaperone. These proteins play important roles in nucleosome assembly and disassembly during DNA transcription, replication and repair [reviewed in (De Koning et al., 2007)]. The role of histone chaperones is to preserve chromatin structure during various cellular processes such as DNA replication and transcription. Both DNA replication and transcription require nucleosome disassembly ahead of DNA and RNA polymerases, respectively, and nucleosome reassembly following polymerase action on naked DNA. Since the fundamentals of both processes are similar, many histone chaperones appear to be active in both replication and transcription as well as in both nucleosome assembly and disassembly. For instance, histone chaperones Asf1p, Nap1p, and FACT (Spt16p-Pob3p) physically and genetically act in conjunction with transcription as well as replication components (Biswas et al., 2005; Park and Luger, 2006; Rocha and Verreault, 2008; Rufiange et al., 2007; VanDemark et al., 2006). Other chaperones such as CAF-1 and Spt6p appear to be replication- or transcription-specific, respectively (Mello and Almouzni, 2001; Rocha and Verreault, 2008).

1.3 Transcription in the context of chromatin

Transcription is a multistage event. Transcription starts by the stepwise recruitment of transcriptional machinery including general transcription factors (GTFs), gene-specific transcription factors and RNA polymerase II. The presence of nucleosomes and the overall state of the chromatin are strong determinants for the accumulation of pre-initiation

complex (PIC) constituents. When critical protein-binding sites are buried within the nucleosome, cells use different strategies to expose these sites in order to achieve proper binding. Eukaryotic cells tend to position sequence-specific transcription factor-binding sites within accessible regions. Genome-wide studies show that nucleosome density is lower in the promoter than in the coding region (Bernstein et al., 2004; Lee et al., 2004; Sekinger et al., 2005). Yeast genes transcribed by RNA polymerase II (RNAPII) generally have a nucleosome-free region in the promoter that is flanked by two well-positioned nucleosomes (Jiang and Pugh, 2009; Rando and Chang, 2009). These two well-positioned nucleosomes preferentially carry histone variant H2A.Z (Htz1p) instead of H2A (Guillemette et al., 2005; Li et al., 2005a). The Htz1p-containing nucleosome is less stable and is susceptible to histone loss or ejection, suggesting that the presence of Htz1p poises the repressed, basal states of genes for full activation (Zhang et al., 2005). Upon activation, activators bind to specific DNA sequences and trigger a cascade of recruitments of coactivator complexes (chromatin-remodeling complexes, histone-modification enzymes and Mediator) (Figure 1.6). Coactivators can either interact directly with RNAPII or GTFs and regulate gene expression, or they can remodel nucleosomes or covalently modify chromatin, thus changing the chromatin architecture of the gene (Fuda et al., 2009; Li et al., 2007a). Chromatin remodeling by coactivators influences transcription factor binding and the transcriptional status of RNAPII. The first complex to bind is TFIID, which contains TATA-binding protein TBP and TBP-associated factors which are targets for further activation of transcription (Fuda et al., 2009; Li et al., 2007a). RNAPII, in association with different transcription factors, then begins to form the pre-initiation complex. The last transcription factor to enter this complex is TFIIH and its stimulatory factor THIIE. With the helicase activity of TFIIH, the DNA double helix is unfolded, the pre-initiation complex opens and transcription starts via RNAPII (Fuda et al., 2009; Ohkuma, 1997). During transcription initiation, the C-terminal domain (CTD) of RNAPII is unphosphorylated, which keeps components of the PIC attached to RNAPII, resulting in transcription arrest near the promoter. TFIIH-mediated phosphorylation of Ser5 in the CTD of RNAIIP facilitates escape from the core promoter and causes the elongating complex to pause in the promoter-proximal region. Recruitment of the cyclin-dependent kinase P-TEFb (CTDK-I in yeast) and phosphorylation at the Ser-2 position of the YSPTSPS heptade repeats present in

the CTD of RNAPII triggers the productive elongation phase. Different factors important for transcription elongation bind to the latter RNAPII isoform, and move along with RNAPII during elongation (Fuda et al., 2009; Nechaev and Adelman, 2011; Phatnani and Greenleaf, 2006; Selth et al., 2010; Shandilya and Roberts, 2012). After productive elongation and completion of mRNA synthesis, RNAPII dissociates from the DNA template, which marks the end of the transcription cycle (Shandilya and Roberts, 2012).

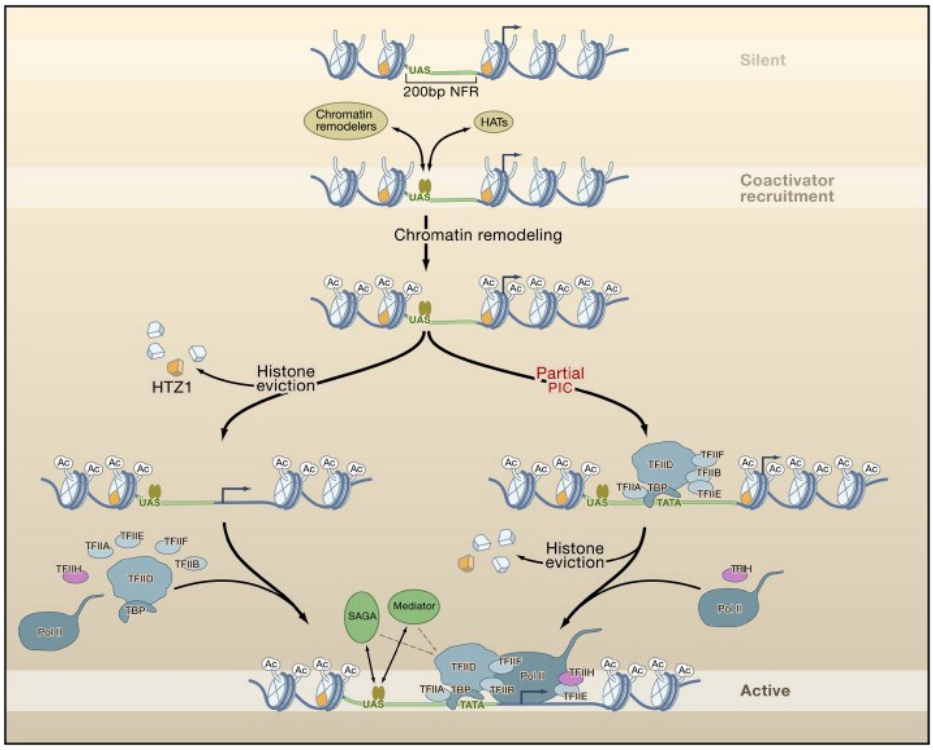

Figure 1.6 : Transcription initiation
Activators recruit various coactivators (such as SWI/SNF or SAGA). Such recruitment further increases the binding of activators, particularly those that are bound within nucleosomal regions. Histones are acetylated in promoter-proximal regions. In one model (*left*), a combination of acetylation and chromatin remodeling directly results in the loss of Htz1p-containing nucleosome, thereby exposing the entire core promoter to the GTFs and RNAPII. SAGA and Mediator then facilitate PIC formation through direct interactions. In the second model (*right*), a partial PIC is assembled at the core promoter without loss of Htz1p. Binding of RNAPII and TFIIH then leads to the displacement of Htz1p-containing nucleosomes and the full assembly of the PIC. Figure from Li et al. (Li et al., 2007a)

1.4 Transcription elongation within chromatin

Until fairly recently, the recruitment of RNAPII and formation of the PIC was considered to be the only rate-limiting step in transcription that could be regulated. It has now become clear that regulation of transcription elongation is equally important. In fact, in some genes, the recruitment of transcription elongation factors and chromatin remodeling in the early stages of transcription elongation are the rate-limiting steps of transcription, e.g. as in the case of the *CYC1* gene in *S. cerevisiae* (Martens et al., 2001; Ni et al., 2008; Saunders et al., 2006). *CYC1* encodes iso-1-cytochrome *c*, a nuclear-encoded protein involved in mitochondrial electron transport chain. This gene is highly expressed in media containing a non-fermentable carbon source (e.g. ethanol). In contrast, *CYC1* expression is very low in the presence of a fermentable carbon source (e.g. dextrose). In spite of the fact that *CYC1* expression is very low in dextrose-containing media, its promoter is preloaded with RNAPII, TBP, TFIIH, the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex and Spn1p. Moreover, even the CTD of RNAPII is phosphorylated at Ser-5, a hallmark of early elongation [reviewed in (Yearling et al., 2011)]. Upon *CYC1* induction (e.g. via transfer to a non-fermentable carbon source), a number of new factors, including the Mediator complex, the SWI/SNF complex and Spt6p, are recruited, which helps in the transition to an actively transcribing unit and an increase in transcriptional output (Lee et al., 2010; Yearling et al., 2011).

Regardless of what constitutes the rate-limiting step in transcription, nucleosomes represent a strong barrier for elongating RNAPII in all genes. How cells control this barrier has been the focus of recent studies. Various factors have been identified that help elongating RNAPII to suppress this barrier. The outcome of this process might be the nucleosome disassembly that occurs ahead of elongating RNA polymerase in highly transcribed genes (Schwabish and Struhl, 2004; Varv et al., 2007). On the other hand, there is strong evidence that RNAPII can cross a nucleosome barrier even without complete nucleosome disassembly. In vitro studies have shown that RNAPII can pass through nucleosomes without completely disassembling them (Kireeva et al., 2002; Kulaeva et al., 2010). However, a second round of transcription through the same template results in complete disassembly of the nucleosomes initially overridden (Kulaeva et al., 2010), suggesting that nucleosomes can be partly or completely disassembled during transcription, depending on

RNAPII density (Figure 1.7). The latter corollary is in accordance with in vivo data suggesting the existence of at least two distinct mechanisms for altering chromatin structure during transcription elongation, namely loss of nucleosomes and a histone acetylation-dependent mechanism with little or no net loss of nucleosomes (Kristjuhan and Svejstrup, 2004). Not surprisingly, two situations also arise regarding histone exchange during transcription, i.e. low rate of histone exchange in weakly expressed genes and a high rate of exchange in highly expressed genes (Dion et al., 2007; Rufiange et al., 2007). Since H2A/H2B dimers are in a peripheral position in the nucleosome, they are globally exchanged at a more rapid rate, whereas H3/H4 exchange necessitates the eviction of all four core histones, a process which occurs only in highly transcribed genes [reviewed in Das and Tyler (Das and Tyler, 2012)].

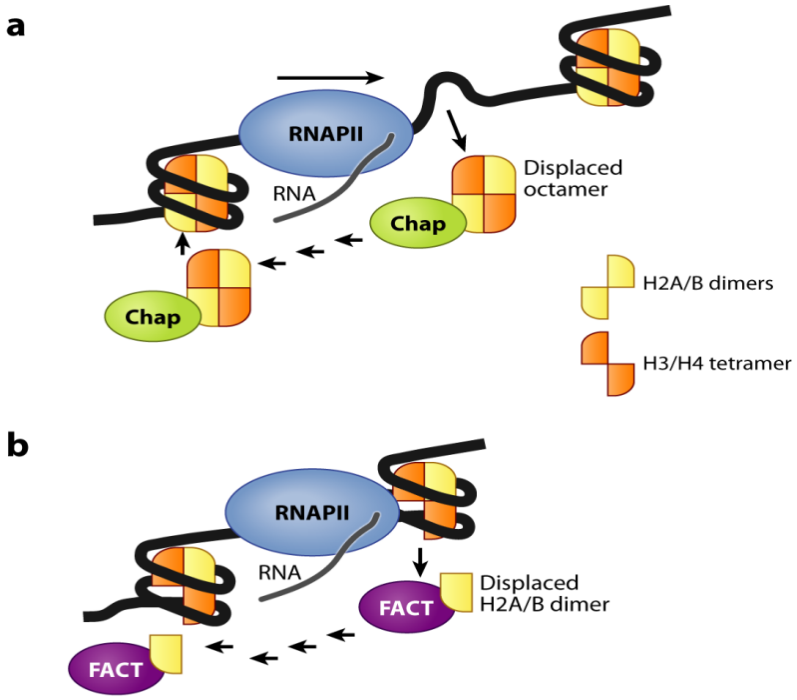

Figure 1.7 : Transcription through chromatin
(A) Transcription through a nucleosome can result in the release of histone proteins from DNA and their association with histone chaperones (Chap). However, as indicated in panel (B), progression of RNAPII through a nucleosome may also occur without complete histone displacement from DNA. In this case, only an H2A/H2B dimer is likely to be displaced. FACT, and possibly other histone chaperones, can attach to this dimer and presumably reload it in the wake of the progressing polymerase. Figure from Selth et al. (Selth et al., 2010).

1.5 Factors regulating chromatin structure during transcription Elongation

Native chromatin structure is intrinsically incompatible with elongating RNAPII. During transcription elongation, chromatin structure is modulated in order to allow access to elongating RNAPII. However, chromatin must be refolded properly after transcription by RNAPII. The latter process is of utmost importance to the cell and detrimental consequences result from its deficiency. For instance, genetic and molecular studies show that defects in chromatin refolding result in a stable, transcriptionally permissive state of the chromatin along the transcribed regions, along with the loss of nucleosomes (Kaplan et al., 2003). This permissive structure allows the spurious initiation of transcription from cryptic sites within coding regions (Figure 1.8). Cryptic transcription is widespread in yeast, and is occurs in ≥1,000 genes (Cheung et al., 2008). A recent study in mammalian cells has also demonstrated the presence of cryptic transcripts resulting from defects in chromatin structure during transcription elongation (Lin and Workman, 2011; Xie et al., 2011).

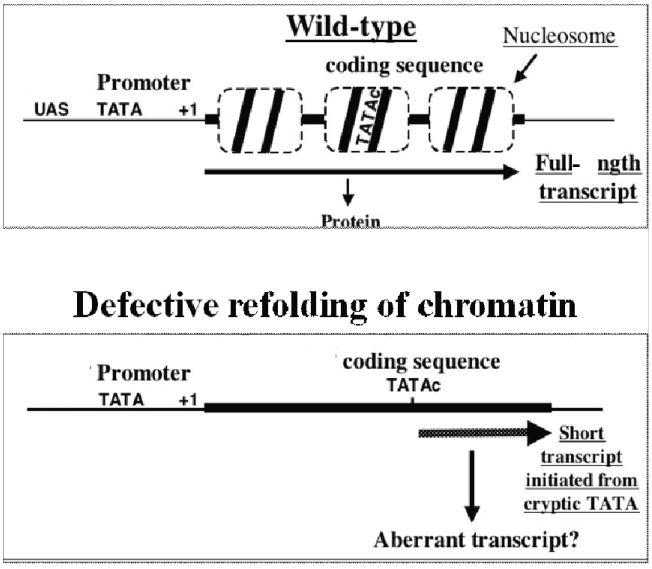

Figure 1.8 : Spurious transcription results from defective refolding of chromatin
A cryptic promoter located in the coding region is not accessible to the basal transcriptional machinery. Defects in proper refolding of chromatin during transcription elongation results cryptic promoter being accessible to transcriptional machinery and transcription can start from this cryptic promoter. **UAS**, upstream activating sequence.

Cells has evolved intricate mechanisms to modulate chromatin structure during transcription elongation, allowing RNAPII to cross the nucleosome barrier while refolding the chromatin after completion of transcription via RNAPII. Different groups of peptidic factors act together in a highly concerted manner towards transforming chromatin structure into a state suitable for transcription elongation. These factors include histone modifiers, ATP-dependent chromatin remodelers and histone chaperones. The events that alter chromatin structure during transcription elongation are depicted in Figure 1.9 [reviewed by Selth et al. (Selth et al., 2010)]. Briefly, during that process, the nucleosome immediately ahead of elongating RNAPII is acetylated by an acetyltransferase bound to RNAPII. Histone chaperones then disassemble the nucleosome ahead of RNAPII and reassemble it after transcription of the associated DNA by the polymerase. This newly assembled nucleosome is not only hyperacetylated, but is also methylated by histone methyltransferase Set2p (which is bound to elongating RNAPII). Set2p methylates the K36 residue of histone H3. The latter methylation is recognized by the histone deacetylase complex Rpd3S, which deacetylates the nucleosome, thereby resulting in completely refolded chromatin. The various factors involved in this process along with their roles are described in the following section.

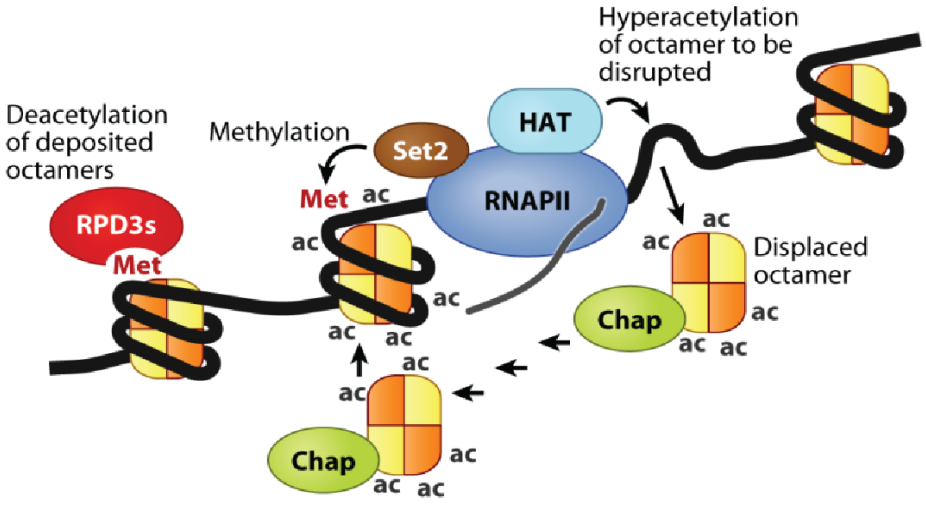

Figure 1.9 : Transcription elongation along chromatin
Histone acetyltransferases (HATs) acetylate histones as the polymerase moves along chromatin. This stimulates nucleosome dissociation and chaperoning (Chap) histone

proteins. Upon reloading, the hyperacetylated nucleosome also becomes methylated by Set2p (bound to elongating RNAPII via the hyperphosphorylated C-terminal repeat domain). This, in turn, leads to the recruitment of the histone deacetylase complex Rpd3S, which specifically associates with methylated nucleosomes and deacetylates them, so that the initial chromatin structure is restored, completing a cycle. Figure from (Selth et al., 2010) .

1.5.1 Histone modifications and transcription elongation

Histone modifications that are associated with active transcription and transcription elongation include H3 and H4 acetylation, H3K4 and H3K36 methylation and H2B monoubiquitination. These modifications and their role in transcription are briefly described below.

1.5.1.1 Acetylation

Histone acetylation was the first covalent histone modification to be identified and remains the most common. The four core histone proteins H2A, H2B, H3, and H4 are acetylated at multiple residues in vivo (Figure 1.3). Histones are acetylated almost exclusively in their N-terminal end, although modifications within the histone core domain have also been identified [reviewed in Kouzarides (Kouzarides, 2007)]. Acetylation of histones is carried out by a group of enzymes known as histone acetyltransferases (HATs). Individual HATs often possess the ability to modify multiple lysine residues in histones (Lee and Workman, 2007; Roth et al., 2001). Acetylation is generally associated with active transcription. The role of acetyltransferases as coactivators at the promoter of active genes is well documented (Brown et al., 2000; Kingston and Narlikar, 1999; Kuo et al., 1998; Larschan and Winston, 2001). The role of histone acetylation in transcription elongation is not fully understood. Although ORFs of transcriptionally active genes are acetylated, their acetylation levels are lower than in promoters (Kouskouti and Talianidis, 2005). Nevertheless HATs and deacetylases (HDACs) are associated with coding regions of transcriptionally active genes (Carrozza et al., 2005; Govind et al., 2007; Keogh et al., 2005), suggesting a possible role for histone acetylation and deacetylation in transcription

elongation. Furthermore, transcription levels correlate with the acetylation levels of coding regions for several genes (Kristjuhan et al., 2002).

HATs involved in the acetylation of coding regions have not been thoroughly studied. Elongator is an acetyl transferase thought to have role in transcription elongation, although its actual function remains poorly documented. Elongator was first identified as a factor associated with elongating RNAPII (Otero et al., 1999), and was shown to stimulate transcription of chromatin by RNAPII in vitro (Kim et al., 2002). Further work should help to establish its putative role in transcription elongation. Gcn5p, a member of the SAGA coactivator complex, is another acetyltransferase with a potential role in elongation. Members of the SAGA complex show physical and genetic interactions with transcription elongation factors (Milgrom et al., 2005; Wery et al., 2004). More importantly, mutations of the *GCN5* gene lead to reduced levels of acetylation in the coding region of some yeast genes, resulting in transcription elongation defects (Govind et al., 2007; Kristjuhan and Svejstrup, 2004; Kristjuhan et al., 2002). NuA4, the major H4 lysine acetyltransferase complex in *S. cerevisiae*, is also recruited to the coding regions of transcriptionally active genes (Ginsburg et al., 2009). NuA4 acetylates H4, which is required for normal elongation by RNAPII. Acetylation of H4 by NuA4 is also required for RSC recruitment and subsequent nucleosome eviction (Ginsburg et al., 2009). This is consistent with in vitro studies showing that acetylation enhances RSC binding to the nucleosome (Carey et al., 2006).

Histone acetylation is actively counteracted by enzymes known as HDACs. These proteins generally function as gene repressors (Kouzarides, 2007). Accumulation of histone acetylation within ORFs results in defective refolding of chromatin after transcription elongation, which in turn leads to transcription from cryptic promoters within the coding region of some genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Histone acetylation within coding regions of various genes is removed by the HDAC complex Rpd3S, which is recruited to the coding region through its interaction with methylated H3K36 (cf. next section) (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Lee and Shilatifard, 2007; Li et al., 2007b)

1.5.1.2 Methylation

Histone methylation plays an important role in gene regulation and chromatin structure. Histones are methylated either on arginine or lysine residues. The free terminal amino group of lysine residues can be mono-, di- or trimethylated, whereas the guanidinium moiety of arginine can be either mono or dimethylated (Pavri et al., 2006; Zhang and Reinberg, 2001). Methylation is indicative of either active transcription (H3K4, H3K36) or transcriptionally repressed chromatin (K9) (Pavri et al., 2006). The important hallmarks of transcription elongation are the di- and/or trimethylation of H3K4 and H3K36. The enzyme responsible for methylation of H3K4 is Set1p. Set1p is a component of the multiprotein complex COMPASS (complex proteins associated with Set1) and is active only when present in that complex (Miller et al., 2001; Schneider et al., 2005). COMPASS travels with RNAPII in early elongation, and its association is regulated by the Paf1p1p complex (Krogan et al., 2003a; Wood et al., 2003) and Ser-5 phosphorylation of CTD by TFIIH (Ng et al., 2003). Recent genome-wide studies on histone modification in yeast are consistent with an association of COMPASS with the early elongation form of RNAPII, and show a peak of H3K4 methylation in the 5'-most end of coding regions (Rando and Chang, 2009). Although the pattern of H3K4 methylation is highly conserved among eukaryotes, its role is not fully understood. A recent report provides evidence for the recruitment of the Set3p HDAC complex by H3K4Me2 to the 5′-most transcribed regions of genes (Kim and Buratowski, 2009)

The best characterized histone modification with a role in transcription elongation is H3K36 methylation. H3K36 is methylated by Set2p. The elongating form of RNAPII is phosphorylated at Ser-2 of the heptad repeat (YSPTSPS) by Ctk1p, and this modification is specifically recognized by Set2p (Krogan et al., 2003b; Li et al., 2003). Set2p trvels along with elongating RNAPII, resulting in increased K36 methylation in the coding region of transcriptionally active genes (Rando and Chang, 2009). H3K36 methylation plays an important role in the proper refolding of chromatin in the wake of actively transcribing RNAPII. After transcription by RNAPII, chromatin is still hyperacetylated and cannot fully refold, and thus needs to be deacetylated for correct refolding. Methylated H3K36 is recognized by the Rpd3S HDAC complex via two of its subunits, i.e. the chromodomain-

containing Eaf3p and the PHD domain-containing Rco1p, resulting in histone deacetylation and full chromatin refolding (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Lee and Shilatifard, 2007; Li et al., 2007b).

1.5.1.3 Ubiquitination

Another histone modification associated with transcription is ubiquitination. In general, proteins can be mono- or polyubiquitinated. Polyubiquitination targets proteins for degradation, whereas monoubiquitination regulates the activity of various cellular proteins (Hicke, 2001; Varshavsky, 2005). Monoubiquitination of H2B is associated with both transcription initiation and transcription elongation. Histone H2B is monoubiquitinated at K123 by the Rad6p/Bre1p/Lge1p complex. The complex may be recruited to the promoter of transcriptionally active genes through interaction with activators, following which the complex moves along the transcribed gene, together with elongating RNAPII, via its association with the Paf1p complex [reviewed in Laribee et al., Selth et al., and Weake and Workman (Laribee et al., 2007; Selth et al., 2010; Weake and Workman, 2008)]. Monoubiquitination regulates the early stages of transcription via its modulation of H3K4 methylation. Monoubiquitination of H2B is essential for the integrity of COMPASS (as Set1p contains the H3K4 methylation complex). Defective H2B ubiquitination results in the loss of Cps35p (a subunit of COMPASS), which is required for the di- and trimethylation of H3K4 (Lee and Shilatifard, 2007). More recently, a role for H2B ubiquitination in transcription elongation, independently from H3K4 methylation, has been proposed. In cooperation with FACT, H2B ubiquitination plays an important role in transcription elongation and in the proper refolding of chromatin in the wake of elongating RNAPII (Fleming et al., 2008; Pavri et al., 2006; Tanny et al., 2007).

1.5.2 ATP-dependent chromatin remodeling and transcription elongation

ATP-dependent chromatin remodelers use energy from ATP hydrolysis to change chromatin structure. As in the case of histone acetylation, the role of ATP-dependent chromatin remodeling factors in modifying promoter structure and in transcription initiation has been extensively studied. Mounting evidence also suggests a role of ATP-dependent chromatin remodeling factors in transcription elongation. The chromatin-

remodeling complex SWI/SNF shows functional interaction with elongation factor TFIIS (Biswas et al., 2005). SWI/SNF is also required for the induction of the *CYC1* gene upon RNAPII pausing at an early elongation step (Zhang et al., 2008). Furthermore, SWI/SNF associates with the coding regions of transcriptionally active genes and is important for histone eviction within coding regions in a FACT-depleted background (Park and Luger, 2006). Among other chromatin remodelers, RSC, a member of the SWI/SNF family, is also linked with transcription elongation. RSC helps elongating RNAPII to cross over nucleosomes in vitro, an effect which is stimulated by histone acetylation (Carey et al., 2006). Recently, RSC was shown to bind the coding regions of stress-activated genes (*STL1* and *CTT1*) upon osmotic stress and help RNAPII to progress through the coding region of these genes (Mas et al., 2009).

Members of the CHD family of chromatin remodeling factors in different species have been implicated in transcription elongation. In yeast and drosophila, the chromatin remodeling protein Chd1p (CHD1 in *Drosophila* spp.) localizes to the coding regions of some transcriptionally active genes (Simic et al., 2003). In addition, Chd1p shows genetic and physical interactions with transcription elongation factors like the Spt4p-Spt5p, FACT, and Paf1p1p complexes (Krogan et al., 2002; Simic et al., 2003). Mammalian CHD8 belongs to the CHD family of chromatin-remodeling factors. A recent study shows that this factor interacts with the hyperphosphorylated elongating form of RNAPII in vivo. Moreover, CHD8-depleted cells are hypersensitive to low concentrations of transcription elongation inhibitors like 5,6- dichloro-1-ß-D-ribofuranosyl benzimidazole (DRB) and flavopiridol (Rodriguez-Paredes et al., 2009). The *Drosophila* protein Kismet-L (KIS-L), a member of the CHD subfamily of chromatin-remodeling factors, is also linked to transcription elongation. Interestingly, kis mutants have strongly reduced levels of Ser-2-phosphorylated RNAPII whereas phosphorylation at Ser-5 remains unaffected (Srinivasan et al., 2005). In addition, association of SPT6 and CHD1 with chromatin in drosophila is significantly reduced in *kis* mutants (Srinivasan et al., 2005). Recently, two reports have shown that the chromatin remodelers Iwp1p and Chd1p are important to maintain chromatin structure and prevent histone exchange during transcription (Smolle et al., 2012; Venkatesh et al., 2012).

1.5.3 Histone chaperones and transcription elongation

FACT and Spt6p are two essential histone chaperones that are important for transcription elongation and are discussed briefly below.

1.5.3.1 FACT

FACT (facilitates chromatin transcription), as the name suggests, was one of the first factors identified to facilitate transcription elongation through chromatin templates in vitro (LeRoy et al., 1998; Orphanides et al., 1998). FACT is composed of two subunits that are evolutionarily conserved in all eukaryotes. The largest subunit is Spt16p whereas the smaller subunit is Pob3p in yeast (SSRP1 in mammals). The N-terminal part of SSRP1 is structurally similar to Pob3p except that it possesses a unique high-mobility group (HMG) fold in its CTD. In yeast, this domain is functionally replaced by the small HMG protein Nhp6p, which interacts with yeast FACT in vivo (Brewster et al., 2001).

Several reports suggest that Spt16p might play a role as a histone chaperone. FACT binds specifically to nucleosomes and H2A/H2B dimers (Orphanides et al., 1999). Furthermore, FACT possesses intrinsic histone chaperone activity and is able to stimulate nucleosome assembling in vitro. It alters chromatin structure by specifically removing H2A/H2B dimers during transcription elongation (Belotserkovskaya et al., 2003). Another important histone chaperone with a role in transcription elongation is Spt6p. The regulation and role of Spt6p are the focus of this work, and Spt6p is discussed in detail below.

1.5.3.2 Spt6p

Spt6p is an essential histone chaperone that is important for maintaining chromatin structure during transcription elongation. The *SPT6* gene was initially identified in a genetic screen that was aimed at identifying mutations that suppress insertion of Ty or δ transposable elements into the 5' regions of the *S. cerevisiae HIS4* gene (Winston et al., 1984), hence its name (suppressor of Ty). Two screens by other teams also identified *SPT6* in parallel studies. In one screen, *SPT6* (under the name *SSN20*) was identified as a group of mutations that suppress loss of the ATP-dependent chromatin remodeling complex

SWI/SNF (Neigeborn et al., 1986). The SWI/SNF chromatin remodeling complex is required for de-repression of the *SUC2* gene which encodes invertase in response to glucose deprivation (Sarokin and Carlson, 1985). The SWI/SNF chromatin remodeling complex acts in the upstream regulatory region of *SUC2*, and therefore, Spt6p probably also acts near the promoter. This can be seen as among the earliest evidence that Spt6p can restore nucleosome structure that has been remodeled by SWI/SNF or other chromatin remodeling complexes. Another genetic screen identified a Spt6p mutant allele, *cre2-1*, that bypasses the requirement for the transcription factor Adr1p for *ADHII* expression (Denis, 1984).

1.5.3.2.1 Spt6p as a transcription elongation factor and histone chaperone

Various genetic and biochemical evidence suggests that Spt6p plays a role in transcription elongation. Spt6p genetically interacts with the transcription elongation factor TFIIS (Hartzog et al., 1998). In addition, Spt6p interacts with mammalian transcription elongation factor DSIF (Spt4p/Spt5p in yeast) and stimulates transcription elongation in vitro (Endoh et al., 2004). Furthermore Spt6p has been shown to interact physically with the elongation form of RNAPII (Yoh et al., 2007). It is now established that the diverse roles played by Spt6p are due to its ability to modify chromatin structure. As mentioned earlier, the first evidence on the latter function came from its possible role in restoring upstream promoter elements disrupted by SWI/SNF. Mounting evidence was later obtained in support of the idea that Spt6p modulates chromatin structure by acting as a histone chaperone. First, it was shown that Spt6p genetically and physically interacts with histones. Overexpression of H3-H4 suppresses the lethality of *spt6*, and *spt6* mutants show similar chromatin defects as *hta1-htb1* mutants as observed by the pattern of micrococcal nuclease digestion (Bortvin and Winston, 1996). Moreover, the *spt6-1004* yeast mutant shows significant hypersensitivity to micrococcal nuclease over the coding regions of several genes when shifted to the non-permissive temperature (Kaplan et al., 2003). Furthermore, Spt6p directly interacts with histones (as shown by GST pulldown assays) and exhibits an intrinsic property of nucleosome assembling in vitro (Bortvin and Winston, 1996). The ability of Spt6p to act as a histone chaperone and modulate chromatin structure could explain its role as a transcription elongation factor.

1.5.3.2.2 Structural features of Spt6

Structurally, Spt6p has three distinct regions with distinct features, namely the N-terminal region, a central core and the C-terminal region (Figure 1.10) (Close et al., 2011; Diebold et al., 2010). The N-terminal part of Spt6p (residues 1-298) is predicted to be unstructured but contains regions that are important for Spt6p function. The most important region spans amino acid residues 239 to 268. It is the latter region which is responsible for binding to another essential protein, Iws1p. This interaction is important for transcription and chromatin structure (Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Another important feature of this region is the presence of phosphoserine residues. All phosphorylated residues identified so far in yeast Spt6p reside in the N-terminal region. The central core region is homologous to the bacterial Tex protein which is implicated in transcription in prokaryotes (Close et al., 2011; Fuchs et al., 1996; Ponting, 2002). The core region contains several recognizable domains, namely a helix–turn–helix domain, a YqgF homologous domain, a helix–hairpin–helix domain, a death-like domain and an S1 domain. When isolated, the central core region is able to bind DNA *per se*. The N-terminal region inhibits the DNA-binding property of the central core region, and the ability to bind DNA is lost in the full-length protein. The C-terminal region is also rather flexible and contains tandem SH2 domains. The tSH2 domain of Spt6p has been shown to bind the RNAPII CTD phosphorylated at the Ser-2 position of the heptad repeats (Yoh et al., 2007).

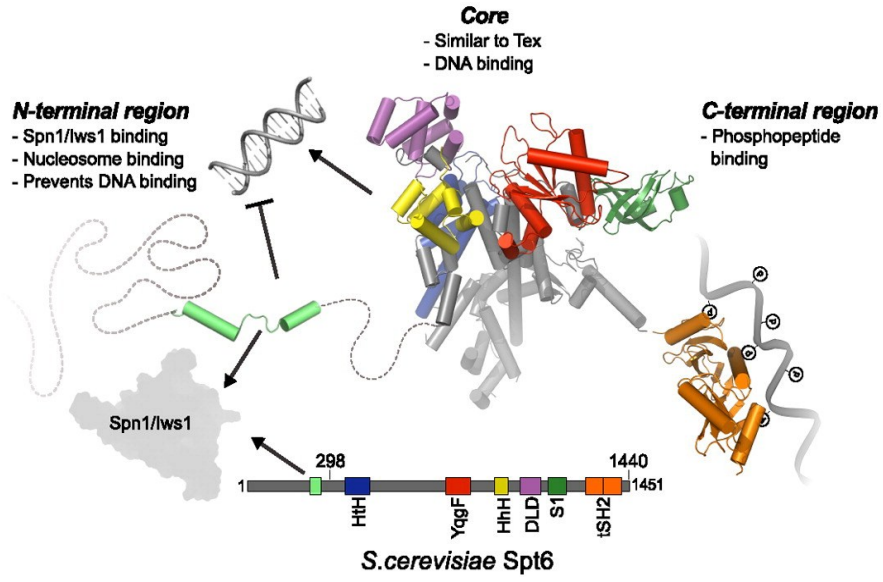

Figure 1.10 : Schematic representation of the three domains of Spt6p

Different regions of Spt6p with possible functions are shown. The N-terminal region is unstructured. It contains the region (*light green*) that interacts with Iws1p and the nucleosome. The N-terminal region also includes phosphoserines. The central core contains various domains (helix-turn-helix (HtH), YqgF, helix-hairpin-helix (HhH), death-like domain (DLD), and S1). This region can bind to DNA in isolation. The C-terminal part contains tandem SH2 domains that bind to the CTD of RNAPII in its isoform phosphorylated at the Ser-2 position of the heptad repeats in the CTD. Figure adapted from Close et al. (Close et al., 2011).

1.5.3.2.3 Spt6p interacts with other factors to regulate chromatin Structure

Spt6p shows physical and functional interactions with different elongation factors. Proteomics studies have found that Spt6p exists in complex with other elongation factors like Spt5p, Spt4p, FACT and Iws1p (Krogan et al., 2002; Lindstrom et al., 2003). The only protein that copurifies consistently with Spt6p under different conditions is Iws1p (interacts with Spt6) (Krogan et al., 2002). Iws1p is also known as SPN1 (suppresses post recruitment functions gene number 1) as it was also identified in a genetic screen to find suppressors of a postrecruitment-defective TBP allele (Fischbeck et al., 2002). Iws1p is an essential protein that is highly conserved among eukaryotes and plays an important role in chromatin structure. Mutations in Iws1p lead to an SPT (i.e. suppression of Ty) phenotype as well as synthetic lethality with mutations in the transcription elongation factor genes *SPT6*, *SPT4*, or *SPT5* (Lindstrom et al., 2003). The interaction between Spt6p and Iws1p is required for different steps of transcription as well as K36 methylation (Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008).

1.5.4 Other Spt proteins and non-histone chromatin components

An elegant genetic screen aimed at identifying mutations that suppress the effects of insertion of Ty or δ transposable elements into the 5'-region of the *S. cerevisiae HIS4* gene led to the identification of a set of genes collectively known as SPTgenes (Winston et al., 1984). With time, gene products of this class have been shown to be critical components and play roles in every step of transcription and in other processes [reviewed in Yamaguchi et al. (Yamaguchi et al., 2001)]. Two of the most important factors of this class, Spt6p and Spt16p (one of the two subunits of the heterodimeric complex FACT) have been described above. Another important member of this group is Spt2p. Spt2p is functionally linked with

Spt6p. As for Spt6p, regulation of Spt2p is also the focus of this work and is discussed briefly below.

1.5.4.1 Spt2p modulates chromatin structure during transcription elongation

Another important member of *SPT* genes is *SPT2*. *SPT2* is also called *SIN1* (Swi independent1) as mutations of *SPT2* suppress the loss of the SWI/SNF chromatin-remodeling complex (Pollard and Peterson, 1997). Mutations in *SPT2* suppress the mutant phenotypes associated with mutations in the SAGA complex and with partial deletions within the CTD of the largest subunit of RNAPII, Rpb1p (Perez-Martin and Johnson, 1998b; Peterson et al., 1991; Pollard and Peterson, 1997). These observations strongly suggest a negative role for Spt2p in transcription initiation.

Recent studies have shown that Spt2p plays an important role in maintaining chromatin structure during transcription elongation similarly to Spt6p. Spt2p is recruited to coding regions of transcriptionally active genes and plays a role in regulating the levels of histone H3 across transcribed regions (Nourani et al., 2006). Spt2p shows functional interactions with different transcription elongation factors like the Paf1p1p and HIR/HPC complexes. This interaction is important to restore a proper chromatin structure during transcription elongation and for the suppression of cryptic transcription (Nourani et al., 2006). Spt2p is also functionally linked with the histone chaperone Spt6p. Recruitment of Spt2p to coding regions of transcriptionally active genes is dependent on Spt6p (Nourani et al., 2006). Recently, we have shown that along with Spt6p, Spt2p is important to maintain a proper repressive chromatin structure in the *SER3* promoter at the *SRG1-SER3* locus (Hainer et al., 2011; Thebault et al., 2011) (Annex 2). At the *SRG1-SER3* locus, *SER3* transcription is repressed by competing transcription of the adjacent non-coding region *SRG1*, a phenomenon known as transcription interference. This repression is achieved through two events, namely (i) the passage of RNAPII through the *SER3* promoter when transcribing *SRG1*, and (ii) the proper secondary deposition of nucleosomes after the passage of RNAPII through the *SER3* promoter. Spt2p plays an important role in the deposition of nucleosomes after transcription of *SRG1* by RNAPII after it passes through the *SER3* promoter (Thebault et al., 2011). Spt2p is important for 3'-end mRNA synthesis and plays a significant role in genome stability and in transcription-dependent gross

chromosomal rearrangements (GCRs) (Hershkovits et al., 2006; Nourani et al., 2006; Sikdar et al., 2008). Sequence analysis of Spt2p has revealed several important structural features. Spt2p has two HMG-like domains found in several proteins involved in the regulation of chromatin structure and gene expression, as well as a C-terminal tail enriched in polar residues (Kruger and Herskowitz, 1991; Lefebvre and Smith, 1993) (Figure 1.11). Spt2p binds to four-way junction DNA, a structure similar to that found at the entrance/exit point of DNA into/from a nucleosome. The latter binding characteristic is a common feature of HMG proteins (Novoseler et al., 2005; Zlatanova and van Holde, 1998).

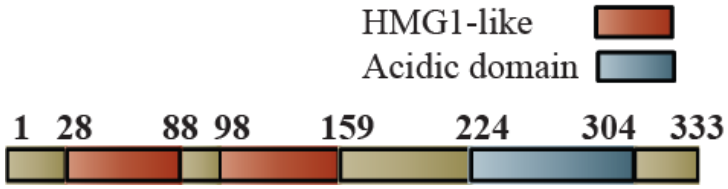

Figure 1.11 : Features of Spt2p sequence
Spt2p contains two regions of sequence similarity with the HMG-box motif (amino acids 28-88 and 98-159) and a highly polar, acidic domain in the C-terminal region of Spt2p (amino acids 224-304).

1.6 Posttranslational modification of chromatin regulators

Chromatin dynamics is a tightly regulated process. The different players of the chromatin modulation to machinery must be tightly regulated. Post-translational modifications are increasingly recognized as playing an important role in the regulation of various chromatin remodelers. Phosphorylation regulates the activity of various ATP-dependent chromatin remodeling complexes such as human SWI/SNF, drosophila Mi-2 and yeast Ino80p [reviewed in (Clapier and Cairns, 2009)]. Various reports also show that phosphorylation regulates the function of histone chaperones. Phosphorylation by the protein kinase casein kinase (CKII) controls the nuclear import and function of histone chaperone Nap1p (Calvert et al., 2008). Recently, it has been reported that phosphorylation by cell-cycle regulated Tousled-like kinases positively regulates drosophila ASF1 and human ASF1a (a nucleosome assembly factor) protein levels (Pilyugin et al., 2009). Phosphorylation by

CKII regulates the DNA binding activity of SSRP1, a subunit of human FACT (Krohn et al., 2003; Li et al., 2005b). DEK, an important factor in maintaining the balance between heterochromatin and euchromatin in vivo, has recently been shown to have histone chaperone activity and the histone chaperone activity of drosophila DEK and human DEK is dependent on phosphorylation by CKII (Kappes et al., 2011; Sawatsubashi et al., 2010).

1.7 Casein Kinase II

CKII or CK2 (casein kinase 2) is one of the first protein kinases discovered, together with casein kinase 1 (CK1/CKI) (Burnett and Kennedy, 1954). It is ubiquitously found in a variety of eukaryotic tissues and organisms. CKII is a highly conserved, essential protein kinase, which phosphorylates >300 substrates (Lee and Shilatifard, 2007; Lee et al.; Meggio and Pinna, 2003). CKII is a tetramer composed of two catalytic subunits (α) and two regulatory subunits (β). Different isoforms of the catalytic and regulatory subunits have been identified. In *S. cerevisiae*, two catalytic subunits (α and α') and two regulatory subunits (β and β') are encoded by the genes *CKA1, CKA2, CKB1* and *CKB2*, respectively (Glover, 1998; Poole et al., 2005). In human, three isoforms of the catalytic subunits (α, α' and α'') and a single regulatory subunit (β) have been identified (Chester et al., 1995; Shi et al., 2001). CKII phosphorylation sites always show acidic features, with the typical CKII consensus site being Ser/Thr-X-X-Glu/Asp (Yearling et al., 2011). The most crucial specificity determinant is an acidic residue (Glu, Asp) at the +3 position. The Glu and Asp residues at position +3 can be efficiently replaced with phosphorylated amino acids. Moreover, additional acidic residues at positions spanning from -2 to +7 also act as positive specificity determinants for CKII, whereas basic residues at these positions, a prolyl residue at position +1, or a bulky hydrophobic doublet at positions +1 and +2, are strong negative determinants (Lee and Shilatifard, 2007; Yearling et al., 2011). CKII is primarily a serine/threonine kinase, but there are reports showing that CKII might actually be a dual kinase that can also phosphorylate tyrosine residues in yeast as well as in mammalian cells (Marin et al., 1999; Vilk et al., 2008; Wilson et al., 1997; Zhu et al., 2000). Tyrosine phosphorylation by CKII might be of more significance in yeast, which does not have *bona fide* tyrosine kinases in its genome. In yeast, deletion of both catalytic subunits is lethal

while deletion of single catalytic subunits does not lead to obvious growth defects, suggesting some level of functional redundancy (Padmanabha et al., 1990).

There is growing evidence that CKII-mediated phosphorylation may play a key role in the regulation of various factors important for chromatin refolding during transcription elongation. Proteomic studies have detected peptides of the various subunits of protein kinase CKII in purified fractions of some transcription elongation factors like Spt6p, Spt2p, Spt4p/Spt5p and Spt16p (Krogan et al., 2002) (Chapters 2 and 3).

The main objective of this thesis is to study the regulation of transcription elongation factors Spt2p and Spt6p through post-translational modifications by CKII.

1.8 Doctoral Project

Chromatin refolding after the passage of RNAPII in the elongation phase of transcription is crucial. Failure of a proper refolding can have several consequences, including the initiation of transcription from cryptic promoters within the coding region of genes (Carrozza et al., 2005; Kaplan et al., 2003; Keogh et al., 2005). Different factors are important to suppress the cryptic transcription, including Spt6p, Spt16p, Set2p, Rtt106p and Spt2p (Cheung et al., 2008; Imbeault et al., 2008; Nourani et al., 2006). While the role of these factors in chromatin structure dynamics during transcription has been well established, the mechanistic details and the regulation of many of these factors is not yet fully understood. The initial goal of my project was to understand the function and regulation of one such factor, Spt2p. I used different purification strategies under stringent conditions to identify binding partners of Spt2p and found that all four subunits of CKII show strong physical interaction with Spt2p. Based on these observations, we hypothesized that CKII phosphorylates and regulates the function of Spt2p. I used several biochemical, molecular and genetic approaches to test our hypothesis (discussed in Chapter 2). I studied the phosphorylation of Spt2p by CKII and the functional significance of this phosphorylation.

While investigating the functional significance of Spt2p-CKII interaction, we found that depletion of CKII activity results in severe chromatin defects. Although our studies showed that CKII phosphorylates and regulates the function of Spt2p, the chromatin defects associated with CKII mutants could not be explained by Spt2p phosphorylation alone. This suggested an additional role of CKII in the modulation of chromatin structure. Previously, CKII subunits were found in purified fractions of various transcription elongation factors such as Spt6p/Iws1p, Spt16p, Chd1p and Spt5p (Krogan et al., 2002). This would suggest that the function of CKII in chromatin integrity might be mediated via the phosphorylation of one or more of these factors. I used genetic analysis and found that CKII and Spt6p are epistatic and act in the same pathway. Interestingly, Spt6p is shown to be phosphorylated in vivo at CKII consensus sites (Krogan et al., 2002). This led us to hypothesize that CKII regulates chromatin by modulating the function of histone chaperone Spt6p. In Chapter 3, I studied the phosphorylation of Spt6p and investigated the significance of phosphorylated residues with respect to Spt6p function and its interaction with different binding partners

Chapter 2

Casein Kinase II Modulates the Function of Elongation Factor Spt2p

Wajid Bhat[1], Geneviève Boutin[1], Anne Ruffiange[1] and Amine Nourani[1]*

1. Centre de recherche en cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec (CHUQ), 9 McMahon Street, Quebec City, Qc G1R 2J6 Canada

*To whom correspondence should be addressed

E-mail: Amine.Nourani@crhdq.ulaval.ca

2.1 Foreword

This Chapter is in the form of a manuscript. I performed most of the experiments presented in the manuscript. Geneviève Boutin did the chromatin immunoprecipitation experiments represented in Figures 2.8 and 2.9. I also co-wrote the manuscript.

2.2 Résumé

Spt2p (Sin1p) est une protéine de liaison à l'ADN qui possède des domaines « HMG-like » et joue un rôle dans les modulations de la chromatine associées à l'élongation de la transcription chez *S. cerevisiae*. Afin de mieux comprendre le rôle de ce facteur dans les modifications de la chromatine associes à la transcription, nous avons utilisé des approches biochimiques pour identifier ses partenaires d'interaction. En utilisant différentes méthodes de purification, nous avons montré que toutes les sous-unités de la caséine kinase (CKII) sont fortement associées à Spt2p. Nous avons par la suite trouvé que CKII phosphoryle Spt2p in vitro et in vivo. De plus, nous avons trouvé que la phosphorylation de Spt2p par CKII s'effectue sur des résidus sérine et tyrosine, confirmant ainsi l'observation que CKII est une kinase à double spécificité. Finalement, nous avons identifié les résidus phosphorylés et trouvé qu'en imitant la phosphorylation constitutive de ces résidus on réduit de façon significative la fonction de Spt2p et son recrutement aux régions codantes des gènes activement transcrits. Le recrutement de Spt2p est dépendant du chaperon d'histone Spt6p. Spt2p interagit directement avec Spt6p et cette interaction est partiellement inhibée par la phosphorylation de CKII. Ainsi, nos résultats suggèrent que la phosphorylation par CKII module la fonction du facteur d'élongation Spt2p en altérant son interaction avec le chaperon d'histone Spt6p.

2.3 Abstract

Spt2p/Sin1p is a DNA-binding protein with HMG-like domains that has been shown to play a role in chromatin modulation associated with transcription elongation in *S. cerevisiae*. Spt2p controls nucleosome levels in coding regions and is essential for the inhibition of spurious transcription in yeast. In order to better understand the role of this factor in chromatin changes linked to transcription, we used biochemical approaches to identify its interacting partners. We found that Spt2p is associated with all subunits of casein kinase II (CKII). Using different purification methods, we herein show that CKII-Spt2p interaction is stable. Next, we found that CKII phosphorylates Spt2p in vitro as well as in vivo. Moreover, our data indicate that CKII is the sole kinase responsible for Spt2p phosphorylation. Interestingly, we found that CKII phosphorylates Spt2p at serine and tyrosine residues, which confirms the reports that CKII is a dual-specificity kinase. Finally, we identified the phosphorylated residues in two small regions, RI (226-230) and RII (277-281) that each contain four amino acid residues. Mutating the phosphorylation sites in RI and RII to acidic residues leads to a significant loss of Spt2p chromatin function, suggesting that phosphorylation by CKII negatively regulates the function of Spt2p. Importantly, we found that mimicking a permanent phosphorylation state results in the loss of Spt2p recruitment to coding regions of actively transcribed genes. Spt2p recruitment is dependent on the histone chaperone Spt6p. Interestingly, Spt2p interacts directly with Spt6p, and this interaction is partly inhibited by CKII phosphorylation. Thus, our data suggest that phosphorylation by CKII modulates the function of elongation factor Spt2p by disrupting its interaction with histone chaperone Spt6p.

2.4 Introduction

The genome of the eukaryotic cell is packaged in a topologically complex structure known as chromatin. The nucleosome, the basic fundamental unit of chromatin, is composed of a core histone octamer formed by two molecules each of H2A, H2B, H3, and H4, and wrapped around by 146 DNA bp (Luger et al., 1997). Chromatin represents an additional level of regulation for all DNA-mediated processes, including transcription, replication, repair and recombination, by acting as a platform where biological signals are

integrated and molecular responses take place. The packaging of eukaryotic DNA into chromatin helps in accommodating the enormous length of DNA within the confines of a very small nuclear space. Each histone in the nucleosome contains a globular domain and highly dynamic N- and C-terminal extremities rich in basic residues. These extremities not only contribute to the structure of chromatin, but also undergo a wave of post-translational modifications that include acetylation, methylation, and phosphorylation (reviewed in (Albert et al., 2007; Kouzarides, 2007)). These modifications influence individual gene expression as well as the formation of functional chromosomal domains. In addition to histones, several non-histone proteins are important for defining chromatin structure. Among these are DNA-binding proteins and chromatin-associated factors that alter nucleosome structure and function.

Spt2p/Sin1p is a DNA-binding protein with HMG-like domains, was originally identified by mutations that suppress insertion of transposon Ty (hence the name Suppressor of Ty 2) in the promoter of the *HIS4* gene (Winston et al., 1984). Spt2p is also known under the name of Sin1p (Swi-independent1) since mutations in Spt2p suppress the loss of the SWI/SNF chromatin-remodeling complex (Pollard and Peterson, 1997). Mutations in *SPT2* suppress the abnormal phenotypes associated with mutations in the SAGA complex and with partial deletions of the CTD of the largest subunit of RNAPII, Rpb1p (Perez-Martin and Johnson, 1998a; Peterson et al., 1991; Pollard and Peterson, 1997). These studies strongly suggested a negative role for Spt2p in transcription initiation. Recent studies have shown that Spt2p plays an important role in maintaining chromatin structure during transcription elongation via its interaction with several transcription elongation factors such as the Paf1p1p and HIR/HPC complexes (Nourani et al., 2006). Moreover, Spt2p is important for the synthesis of the 3-end of mRNA and also plays a significant role in genome stability and transcription-dependent GCRs (Hershkovits et al., 2006; Nourani et al., 2006; Sikdar et al., 2008)

Sequence analysis of Spt2p has revealed several important structural features. Spt2p possesses two HMG-like domains found in several proteins that are associated with chromatin structure and gene expression, as well as a C-terminal tail rich in polar residues (Kruger and Herskowitz, 1991; Lefebvre and Smith, 1993). Spt2p was shown to bind four-

way junction and crossing DNA, a feature commonly found in HMG proteins (Novoseler et al., 2005). Despite its functional diversity, the exact mechanistic details of Spt2p function and regulation are not well understood. To gain insight into the cellular role of Spt2p and its regulation, we decided to identify the different interacting partners of Spt2p. Tandem affinity purification (TAP) of Spt2p yielded peptides of casein kinase II (CKII), an essential protein kinase in eukaryotes. In yeast, it is composed of two catalytic and two regulatory subunits. Deletion of the two catalytic subunits of CKII is lethal, but deletion of a single catalytic subunit produces no obvious growth defect (Bidwai et al., 1992). CKII is known to be a serine/threonine kinase although several reports show that it might also act as a tyrosine kinase in yeast (Marin et al., 1999; Vilk et al., 2008; Zhu et al., 2000).

CKII subunits have been found to copurify with many chromatin-modifying factors like Spt6p and the FACT complex (Krogan et al., 2002), but the role of CKII in processes related to chromatin structure modulation remains elusive. In this report, we present findings that shed light on the role of CKII in the regulation of chromatin modulation by Spt2p. We show that CKII stably interacts with Spt2p and phosphorylates the latter both in vitro and in vivo. By combining mass spectrometry, deletion and point mutation analyses, we identified two small regions of five amino acids each that are located in Spt2p CTD and are targeted by this kinase. Interestingly, Spt2p CTD was previously shown to play an essential role in the function of Spt2p (Lefebvres and Smith 1993). Interestingly, we next found that CKII phosphorylation inhibits Spt2p function. Moreover, phosphorylation of Spt2p at the CKII sites was found to result in the activation of spurious transcription, indicating a defect in the reassembly of coding regions of the chromatin upon RNAPII passage. Importantly, in the absence of CKII activity, we observed increased Spt2p recruitment in the transcribed regions of various genes. These data clearly indicate that phosphorylation by CKII inhibits recruitment of Spt2p to the chromatin of coding regions. We previously showed that the recruitment of Spt2p to these locations is dependent on the elongation factor Spt6p. In this work, we show that Spt2p interacts directly with Spt6p and provide evidence that this interaction is directly inhibited by CKII-mediated phosphorylation. Taken together, the present findings show for the first time that chromatin modulation associated with transcription elongation involves phosphorylation of the elongation factor Spt2p by CKII, and strongly suggest that CKII regulates chromatin

reassembly by modulating the recruitment of Spt2p by the essential histone chaperone Spt6p.

2.5 Materials and methods

2.5.1 *S. cerevisiae* strains, media and plasmids

All strains are constructed using standard genetic methods, either by crossing or by haploid transformation, and are isogenic to S288C (Table 2.1) (Winston et al., 1995). The *SPT2-TAP, SPT2-FLAG, SPT2-1-FLAG* (amino acid 1-213), *SPT2-179-FLAG* (amino acid 1-179), *SPT2-13MYC, CKB2-TAP, CKA1-13MYC and CKA2-13MYC* alleles were constructed by integrating the DNA encoding the particular epitope at the 3'-end of the corresponding gene (Gelbart et al., 2001; Longtine et al., 1998; Puig et al., 2001). The *cka1Δ::KANMX6* allele was constructed by replacing the open reading frame with a KANMX6 marker (Longtine et al., 1998). The point mutation in the *CKA2* allele (D225N) was introduced as described by Kitazono et al. (Kitazono et al., 2002), using natMX6 (a nourseothricin resistance cassette) as a selection marker.

All yeast media, including YPD, synthetic complete (SC), omission media (SC–) and media containing 5-fluoroorotic acid (5-FOA) were prepared as described previously (Rose et al., 1990). All strains were grown at 30°C unless otherwise stated. All plasmids were constructed using standard molecular biology techniques. GST-fusion and 6xHis fusion plasmids were constructed by insertion of PCR-amplified fragments into the appropriate sites of pGEX-4T3 (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) and pet15b (Novagen, EMD4Biosciences, Gibbstown, NJ). Plasmids pRS415 and pRS416 have been described elsewhere (Sikorski and Hieter, 1989). Spt2p and Spt2p-FLAG along with its promoter (-900 bp upstream of start codon to +500 bp downstream of stop codon) were amplified from genomic DNA and inserted into plasmids pRS415 and pRS416. Mutagenesis was done using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies Canada Inc. Mississauga, ON) according to the manufacturer's instructions.

2.5.2 Protein purification

Recombinant 6xHis-tagged and GST-tagged proteins were expressed in *Escherichia coli* (BL21) and purified with Ni^{2+}-NTA agarose (QIAGEN Inc., Toronto, ON, Canada) or glutathione-Sepharose (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) following the manufacturer's protocol. Tandem affinity purification of Spt2p and Ckb2p was carried out as described previously (Puig et al., 2001). Briefly, two liters of yeast culture were grown to OD_{600} value of 2. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 5 mM β-glycerophosphate, 5 mM sodium butyrate, 5 mM NaF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF and 0.5 mM DTT). Cells were lysed by bead beating, cell lysate was centrifuged and the supernatant collected. The extract (supernatant) was pre-cleared with 200 μl of Sepharose CL-6B beads (Sigma) for 1 h at 4°C. Then, 200 μl of IgG Sepharose beads (GE Healthcare) were added to the pre-cleared extract and incubated with rotation for 3 h at 4°C. The beads were then washed with 20 ml of lysis buffer in the column and then equilibrated with 10 ml tobacco etch virus (TEV) cleavage buffer (10 mM Tris HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM EDTA and 1 mM DTT). Proteins were eluted from beads with 200 units of TEV protease in 1 ml of TEV cleavage buffer for 2.5 h at 16°C. The beads were washed once with 3 ml of calmodulin binding buffer (10 mM Tris HCl pH 8, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM Mg acetate, 2 mM $CaCl_2$, 1 mM imidazole pH 7.0, and10 mM β-mercaptoethanol) and this fraction was pooled with the eluate and incubated with 25 μl of protein A-Sepharose beads (GE Healthcare) for 15 min at 4°C to remove residual IgGs leaking from the IgG Sepharose beads. Then 3 μl of 1 M $CaCl_2$ were added to raise the concentration of $CaCl_2$ to the optimum value for binding to calmodulin beads, and then incubated with 200 μl of calmodulin resin (Stratagene) overnight at 4°C. The calmodulin beads were then washed with 20 ml of calmodulin binding buffer. Proteins were eluted with calmodulin elution buffer (10 mM Tris HCl pH 8, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 1mM Mg acetate, 1 mM imidazole (pH 7.0), 10 mM β-mercaptoethanol and 20 mM EGTA supplemented with protease inhibitors) in five 200-μl fractions and analyzed by SDS PAGE followed by silver staining.

2.5.3 GST pulldown assays and co-immunoprecipitation

Five hundred ng of GST-tagged Spt2p (GST-Spt2p) coupled to beads were incubated with 500 ng of 6xHis-tagged Spt6p (His-Stp6p) in pulldown buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 100 μg/ml bovine serum albumin [BSA], 0.5 mM dithiothreitol [DTT], 0.1% NP-40, and 2 μg/ml leupeptin and pepstatin, 5 μg/ml aprotinin) for 3 hours at 4°C. Beads were washed three times with pulldown buffer and bound proteins were analyzed by western blot against 6xHis-tag. Coimmunoprecipitation was performed as described elsewhere (Nourani et al., 2001). Ten μl of anti-FLAG M2 agar beads were incubated with yeast whole cell-extract (5 mg total protein) in binding buffer A (20 mM Hepes [pH 7.5], 300 mM NaCl, 10% glycerol, 0.1% NP-40, 2 μg/ml of leupeptin and pepstatin, 5 μg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]) overnight at 4°C. Beads were washed three times with binding buffer. Bound proteins were eluted with 3X-FLAG peptide and analyzed by western blot.

2.5.4 In vitro phosphorylation

In vitro phosphorylation of recombinant proteins was performed as described (Sawa et al., 2004; Utley et al., 2005), with slight modifications. One μg of 6xHis-tgged proteins were incubated with CKII purified from yeast in kinase buffer (final concentrations of 80 mM NaCl/KCl, 25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM DTT, 50 μM cold ATP, and 1 μCi of [γ-^{32}P]ATP) for 30 min at 30°C. Samples were run on 12% SDS-PAGE, blotted onto nitrocellulose, dried, and exposed to film. CKII used in the reaction was purified from yeast (Ckb2p-TAP) as described in section 2.5.2. An aliquot of 0.1 μl of fraction 3 was used for each reaction One μg of GST-tagged proteins coupled to beads were incubated with yeast whole-cell extract at 4°C for 3 h in binding buffer B (20 mM Hepes [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% NP-40, 2 μg/ml of leupeptin and pepstatin, 5 μg/ml of aprotinin, 1 mM PMSF). Beads were washed three times with binding buffer B and once with kinase buffer (without γ-^{32}P-labeled ATP), and the phosphorylation reaction was carried out by the addition of kinase buffer containing γ-^{32}P-labeled ATP.

2.5.5 Dephosphorylation and 2D-PAGE

For dephosphorylation with alkaline phosphatase, cells were lysed in 1X New England Biolabs (NEB) buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9 @25°C). Cell lysate was cleared by centrifugation at 15,000 *g* for 30 min. Ten µg of total protein were incubated with 10 units of alkaline phosphatase at 37°C for 1 h (calf intestinal alkaline phosphatase, from NEB; Whitby, ON, Canada). The reaction was stopped by trichloroacetic acid (TCA) precipitation and precipitated proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). For 2D-PAGE, soluble proteins were precipitated by 20% TCA followed by an acetone wash. Precipitated proteins were solubilized in isoelectric focusing (IEF) buffer (8 M urea, 2% CHAPS, 18.5 mM DTT in 2% immobilized pH gradient buffer [pH 7–11; GE Healthcare) and separated in the first dimension using a nonlinear immobilized pH gradient (Immobiline DryStrip NL 7-11, GE Healthcare) at 90 kV/h. After IEF, proteins were resolved in the second dimension by standard SDS–PAGE and analyzed by western blot.

2.5.6 RNA extraction and Northern blot

Total RNA was isolated using the hot phenol method (Schmitt et al., 1990). Northern blot analysis was performed as described previously (Nourani et al., 2006). Forty µg of RNA were separated on a 1% agar-formaldehyde MOPS gel and transferred to a nylon membrane. The *FLO8*, *SCR1* and *SER3* probes were amplified by PCR and radiolabeled by random priming. The sequences of oligonucleotides used are listed in Table 2.2

2.5.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were performed as described previously (Nourani et al., 2006). Ten µl of anti-FLAG beads (Sigma) and 1 µl of anti-Myc antibody (Covance; Princeton, NJ) were used per immunoprecipitation. The oligonucleotides used for qPCR are listed in Table 2.2

Table 0.1: *S. cerevisiae* strains used

Strain	Genotype	Source
YW01	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-FLAG::KANMX2*	This work
YW02	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-1-XFLAG::KANMX6*	This work
YW03	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-179-FLAG::KANMX6*	This work
YW04	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-116-FLAG::KANMX6*	This work
YW06	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-TAP::URA3*	This work
YW13	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-FLAG:: KANMX4 CKA1-13Myc:: KANMX6*	This work
YW22	*MATα ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT2-FLAG::KANMX4 CKA2-13Myc::KANMX6*	This work
YTS07 *(ckII^{ts})*	*MATα ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2 D225N::NATMX4 SPT2-13MYC::KANMX6*	This work
FY2442	*MATaura3Δ0 his3Δ200 leu2Δ1 lys2-128δ*	(Nourani et al., 2006)
FY2427	*MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ SPT2-13MYC::KANMX6*	(Nourani et al., 2006)
FY2432	*MATα ura3-52 his3Δ200 leu2Δ1 lys2-128δ spt2Δ0::KANMX6*	(Nourani et al., 2006)
FY2436	*MATα ura3Δ0 his3Δ200 leu2Δ1 lys2-128δ RPB3-HA1::LEU2 cdc73Δ::KANMX spt2Δ0::KANMX6 FLAG-SPT6*	(Nourani et al., 2006)
FY 2455	*MATa ura3-52 his3Δ200 his4-912δ leu2Δ1 Lys2-128d spt2Δ0::NAT paf1Δ::KANMX SPT6-FLAG/pLL15*	(Nourani et al., 2006)

Table 0.2: Sequences of oligonucleotides used in these studies

Locus	Sequence	Amplicon	Used in
AZR1	FOR : 5' GCAAACTTAATGGGTGG REV : 5' TACCTGCTAACACTACC	+842 to + 1036	ChIP
FKS1	FOR : 5' GAATAGAGTCAT TACGCC REV : 5' GAATACATCATCCCAGAC	+1062 to +1305	ChIP
FKS1	FOR : 5' TTTGACTGAAGGTGAGGA REV : 5' ATCAGATTTACCGTCACC	+3114 to +3252	ChIP
FKS1	FOR : 5' GGACTGGTAAGTGGTATG REV : 5' CAGAGAGTAAATTGGGGG	+5126 to +5361	ChIP
MET10	FOR : 5' ACCACTAACGACGAGTCC REV : 5' AGGCTGCTGAAATGTCTG	+1528 to +1663	ChIP
NO-ORF	FOR : 5' GCTGTCAGAATATGGGGCCGTAGTA REV : 5' CACCCCGAAGCTGCTTTCACAATAC	ChrV : 9716 to 9864	ChIP
PCA1	FOR : 5' AGGTTATAGGTGCCAGAG REV : 5' TTGACGCGGAGATAGTGG	+1571 to +1783	ChIP
PDC1	FOR : 5' CTCTCAAGTCTTATGGGG REV : 5' GGCAACAAGGATAGGTGG	+1221 to + 1499	ChIP
PMA1	FOR : 5' CCTCTTCATCATCCTCTTC REV : 5' CGGCGGCAACTGGACCATCGT	+17 to +208	ChIP
PMA1	FOR : 5' CGCTGGTGGTCAAGGTCATTT REV : 5' CTGGCAAACCGACTGGGACAC	+837 to +1018	ChIP
PMA1	FOR :5' CTATTATTGATGCTTTGAAGACCTCCAG REV : 5' TGCCCAAAATAATAGACATACC CCATAA	+2018 to +2290	ChIP
POL1	FOR : 5' TGATCCCCATAAGCGAG REV : 5' ACTAACATGGCGAGCGG	+2784 to +2909	ChIP
RPL5	FOR : 5' CGGTATTACCCACGGTTT REV : 5' GGCACCAGTGGTGGTTCT TT	+258 to +471	ChIP
RPL10	FOR : 5'TGTGCCGGTGCGGATAGATT REV : 5' CACCAGCTTCTCTCTTCTT	+313 to +565	ChIP
RPL25	FOR : 5' CTAAGGCTACTGCCGCTAA REV : 5' CTTGACGGCCTTCTTGATTT	+428 to +714	ChIP
FLO8	FOR : 5' TGATGCCACTAAGGATGAGAATAA REV : 5' GGTCTTCAACCATACCAATATTCC	+1515 to +2324	*FLO8* probe
SCR1	FOR : 5' ATGATCAACTTAGCCAGGACAT CCATA REV : 5' GTTCAACTAGCGAAGCCGCCAAA TTAA	-246 to +468	*SCR1* probe

2.6 Results

2.6.1 Spt2p is associated with casein kinase II subunits

In order to further understand the cellular function of Spt2p, we decided to identify its interacting partners in vivo. For that, we constructed a yeast strain expressing a TAP-tagged Spt2p hybrid protein. We purified Spt2p-TAP sequentially on IgG and calmodulin columns and analyzed the proteins by SDS-PAGE followed by silver staining (Figure 1.1A). In addition to Spt2p, we observed that three other proteins of 45 kDa, 40 kDa and 30 kDa copurified with this factor. Analysis of the excised gel bands by mass spectrometry identified them as the four subunits of the CKII complex. Twenty-eight, eight, seven and six peptides originating from Cka1p, Ckb1p, Cka2p and Ckb2p, respectively, were identified (Figure 1.1B). These results suggest that the multiprotein kinase CKII interacts in vivo with Spt2p.

CKII has a broad range of targets and interacts with many proteins in yeast. To confirm that the CKII complex is stably and specifically associated with Spt2p, we first constructed yeast strains expressing TAP-tagged Spt2p and Myc-tagged versions of two different CKII subunits. We purified Spt2p using TAP tag epitope labeling and found that both Cka1p-Myc and Cka2p-Myc co-eluted with Spt2p after three purification steps (Figure 2.1C and 2.1D). Importantly, no such co-elution was observed in control strains that do not express TAP-tagged Spt2p. This clearly suggests that Cka1p-Myc and Cka2p-Myc signals observed after TAP purification are specific for Spt2p.

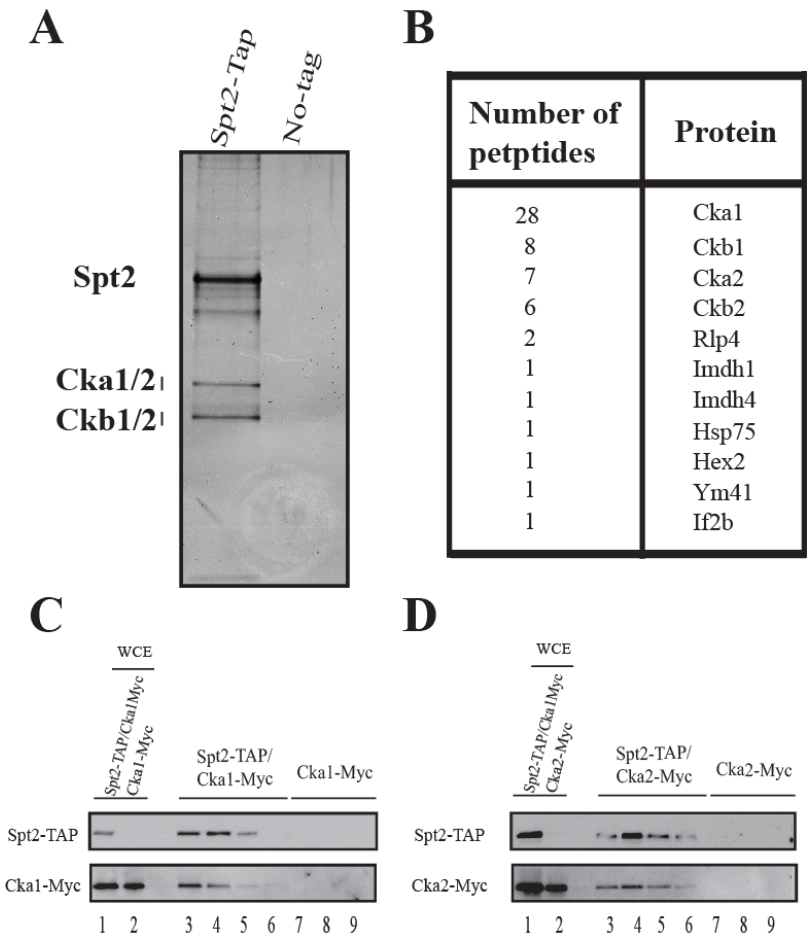

Figure 0.1: Spt2p copurifies with CKII subunits

(A) Silver staining of Spt2p-TAP after 12% SDS-PAGE; the purification control was obtained from an extract of yeast expressing untagged *SPT2*. (B) Various peptides identified by mass spectrometry after TAP. (C & D) TAP of Spt2p from a strain expressing Spt2p-TAP and Cka1p-Myc or Cka2p-Myc. A control for purification was obtained from cells expressing untagged *SPT2* and *CKA1-MYC* or *CKA2-MYC*. Purified fractions were analyzed by western blot using antibodies against the TAP and Myc epitopes.

2.6.2 The CKII/Spt2p interaction is stable

We next wanted to confirm that the CKII complex interacts stably with Spt2p. We used other purification strategies. We first purified FLAG-labelled Spt2p from two different yeast strains that were also expressing either *CKA1-MYC* or *CKA1-MYC* and assessed whether the two CKII subunits might copurify with Spt2p. As shown in Figure

2.2A, Cka1p-Myc and Cka2p-Myc indeed copurified with Spt2p-FLAG. These data show that CKII and Spt2p independently copurify with each other. Finally, we wanted to determine the relative stability of the Spt2p-CKII complex. For that purpose, we used a multistep purification protocol combining four steps of TAP to two other purification steps at a high salt concentration (see Figure 2.2B). First, we tandem affinity-purified Ckb2p from a strain expressing Spt2p-FLAG and then the CKII complex was subjected to FLAG-immunoprecipitation followed by FLAG peptide elution. These last 2 steps were performed at a high salt concentration (300 mM NaCl). As shown in Figure 2.2B, after being submitted to a total of 6 purification steps, Spt2p was found to copurify with the CKII complex, clearly indicating that the association between the elongation factor and the kinase is strong and stable.

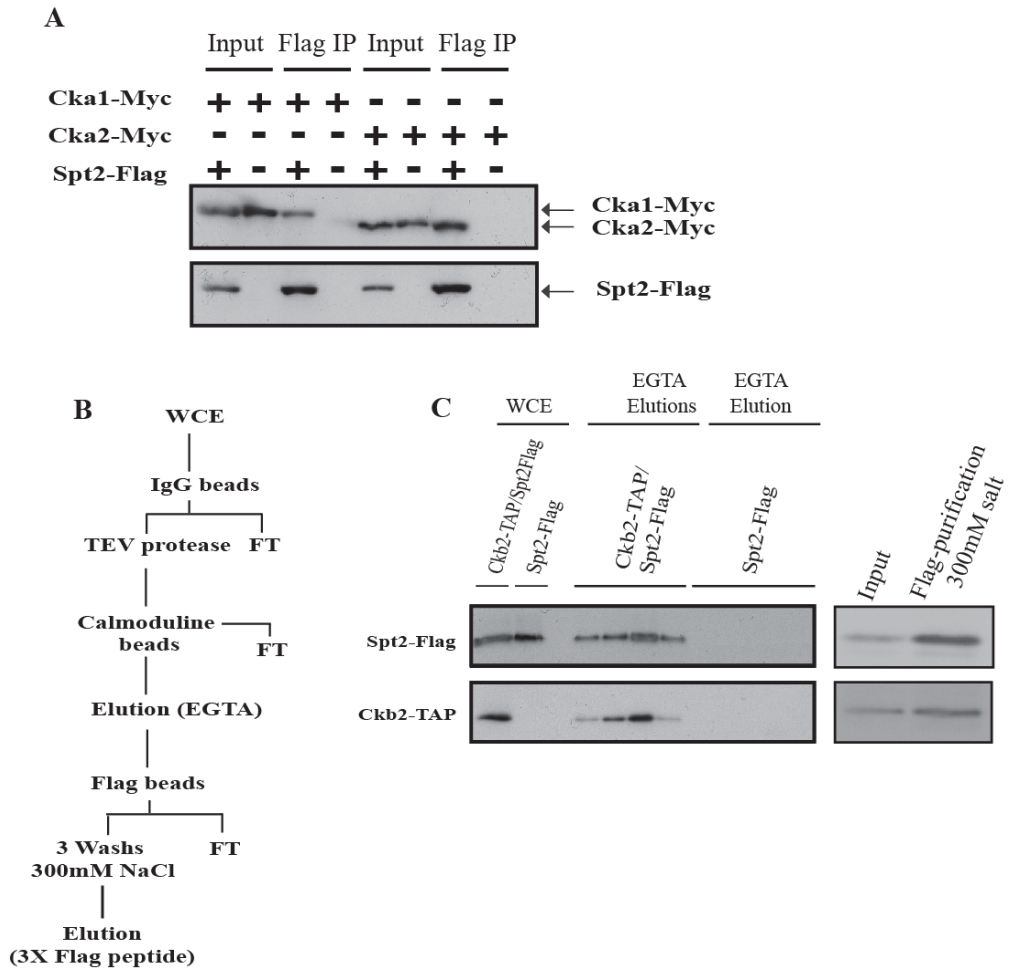

Figure 0.2 : CKII stably interacts with Spt2p

(A) Extracts from yeast cells expressing either *CKA1-MYC* or *CKA2-MYC* and *SPT2-FLAG* were immunoprecipitated with anti-FLAG antibody. After eluting bound proteins from the anti-FLAG beads with FLAG peptide, the eluted fraction was probed with anti-Myc and anti-FLAG antibodies. (B) Schematic representation of the multistep purification strategy used to assess the stability of the Spt2p-CKII interaction. (C) Extracts from yeast cells expressing Ckb2p-TAP and Spt2p-FLAG were subjected to multiple steps of purification as shown in B. Following TAP, samples were analyzed by western blot with antibodies against the FLAG and TAP tags (*left* panels). TAP-purified samples were further subjected to FLAG purification followed by western blot analysis with antibodies against FLAG and TAP tag (*right* panels). IP, immunoprecipitation; FT, flow-through; TEV, tobacco etch virus; WCE,

2.6.3 Spt2p is a phosphoprotein in vivo

The association between Spt2p with CKII led us to think that Spt2p could be a substrate of CKII. Before addressing this issue directly, we first elected to determine if Spt2p is a phosphoprotein in vivo. We always observed that the apparent mass displayed upon Spt2p SDS-PAGE was higher than the expected value, and therefore hypothesized that this altered electrophoresis signature might be associated with post-translational modifications (data not shown). To assess whether this altered migration behavior on SDS-PAGE is the consequence of phosphorylation, we submitted whole-cell extracts from wild-type (WT) cells to alkaline phosphatase treatment followed by SDS-PAGE. As shown in Figure 2.3A, this treatment did not change the migration behavior of Spt2p. This might have arisen from two different possibilities. First, Spt2p is not phosphorylated in vivo and its association with CKII would therefore not result in a significant modification. Alternatively, phosphorylation of Spt2p might not significantly affect its mobility in SDS-PAGE, and therefore, different Spt2p species might not be resolved through this approach. To further examine this possibility, we repeated the experiment except that the Spt2p species were separated by 2D-PAGE, where the first electrophoresis separates proteins according to their electric charge and the second is an SDS-PAGE step that separates proteins according to their molecular weight. Interestingly, we clearly observed two different species of Spt2p, with one of these species being apparently much more acidic than the other, suggesting the possible addition of phospho group(s) (see Figure 2.3B). The acidic form of Spt2p appeared to represent the major species present in vivo. Importantly, alkaline phosphatase treatment had a dramatic effect on the ratio of these two Spt2p species and shifted most of the total Stp2p protein toward the less acidic isoform. These data strongly suggest that Spt2p is a phosphoprotein in vivo.

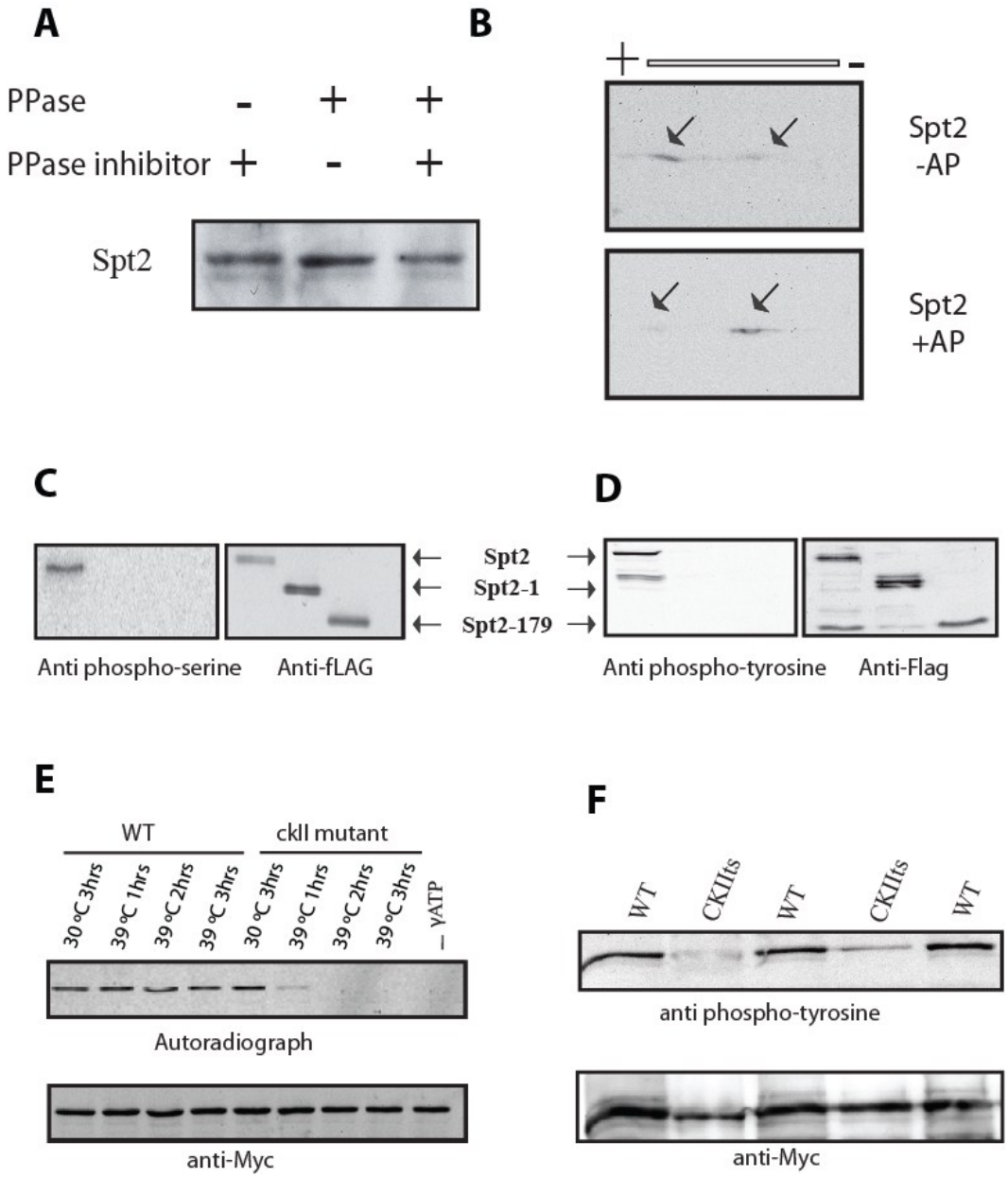

Figure 0.3: CKII is required for the phosphorylation of Spt2p in vivo
(A) A total cell extract from strains expressing Spt2p-FLAG was treated with alkaline phosphatase (AP) ± a phosphatase inhibitor. The samples were resolved by SDS-PAGE and analyzed by western blot against A FLAG-epitope. (B) Different isoforms of Spt2p as identified by 2D-PAGE; total cell lysates from strain expressing Spt2p-FLAG were subjected to 2D–PAGE (isoelectric focusing, followed by SDS–PAGE) and analyzed by western blot with a FLAG antibody. The *top* panel shows different isoforms of Spt2p without phosphatase treatment (-AP). The *bottom* panel shows that the isoelectric point of most acidic form of Spt2p (phosphorylated form) is shifted to a higher value after AP treatment (+AP). (C) Whole-cell extracts were prepared from yeast strains expressing Spt2p-FLAG, Spt2p-1FLAG (amino acids 1-213) and Spt2p-179FLAG (amino acids 1-179). Different versions of Spt2p were immunoprecipitated with anti-FLAG beads followed

by immunoblotting with anti-phosphoserine (*left* panel) or anti-FLAG antibodies (*right* panel) (D) Spt2pFLAG, Spt2p-1FLAG (amino acids 1-213) and Spt2p-179FLAG (amino acids 1-179) were immunoprecipitated with anti-FLAG beads followed by immunoblotting with anti-phosphotyrosine (*left* panel) and anti-FLAG antibodies (*right* panel). (E) Whole-cell extracts were prepared from *WT* or *CKII*^{ts} cells grown at the permissive or restrictive temperature (30ºC or 39ºC as indicated). Spt2p-Myc was immunoprecipitated with anti-Myc antibody, and the pellets were subjected to a kinase assay by the sole addition of radiolabeled ATP. (F) Spt2p-Myc was immunoprecipitated from WT and ckII^{ts} strains and probed with phosphotyrosine (*top* panel) or Myc antibodies (*bottom* panel).

2.6.4 Spt2p is phosphorylated in its CTD and on both serine and tyrosine residues in vivo

Using another approach, we first aimed to confirm that Spt2p is a phosphoprotein in vivo, and secondly, to analyze the type of phosphorylated residue(s) thus formed. Indeed, several reports indicate that CKII phosphorylates serine, threonine as well as tyrosine residues (15-18). Therefore, we determined whether Spt2p is endowed with serine/threonine kinase as well as tyrosine kinase activities. To address these questions, Spt2p was purified by immunoprecipitation from yeast strains and analyzed by SDS-PAGE and western blotting with antibodies raised against phosphoserine or phosphotyrosine residues (Figure 2.3C and 2.3D). Interestingly, a band of the size expected for Spt2p was observed with both antibodies, suggesting that Spt2p is phosphorylated on serine as well as tyrosine residues. We next assessed which Spt2p domain is phosphorylated. For this purpose, we analyzed the in vivo phosphorylation status of two different mutated versions of Spt2p. As shown in Figure 2.3C and 2.3D, deletion of the Spt2p CTD resulted in the loss of the phosphotyrosine and phosphoserine signals. This indicates that Spt2p is phosphorylated on serine and tyrosine residues in vivo, and that these sites are located in the CTD (residues 213 to 333) of this factor. Since we did not test phosphothreonine antibodies, the data by no means exclude the possibility that Spt2p is also phosphorylated on threonine residues by CKII,

2.6.5 CKII kinase activity is essential to Spt2p phosphorylation in vivo

Our data clearly indicate that Spt2p is a phosphoprotein in vivo and is stably associated with the protein kinase CKII. We next addressed the possibility that Spt2p is phosphorylated by CKII in vivo through various approaches. First, we purified Spt2p from a wild-type or CKII thermosensitive mutant ($ckII^{ts}$) grown at the permissive (30°C) or restrictive temperatures (37°C) for various time intervals. These purified Spt2p complexes were incubated with [γ-^{32}P]-ATP and analyzed by SDS-PAGE followed by autoradiography (Figure 2.3E). Spt2p protein purified from wild-type yeast was found to be phosphorylated under permissive or restrictive conditions at all times. In contrast to this, Spt2p extracted from $ckII^{ts}$ cells grown at the restrictive temperature was not phosphorylated. The latter findings indicate that depletion of CKII from yeast cells affects Spt2p phosphorylation. Our observations strongly suggest that Spt2p phosphorylation in vivo requires CKII. Using a second approach, we asked whether the in vivo phosphorylation level of Spt2p is affected by cellular depletion of CKII. For that purpose, we immunopurified Spt2p from wild-type or $ckII^{ts}$ mutant strains. These cells were grown to mid log phase and then submitted to a heat shock at the restrictive temperature for 2 h. We then analyzed the pattern of Spt2p phosphorylation by anti phosphotyrosine western blot (Figure 2.3F). Depletion of CKII activity in the $ckII^{ts}$ mutant was clearly associated with loss of the Spt2p-specific phosphotyrosine signal. We therefore conclude that Spt2p phosphorylation in vivo requires CKII kinase activity.

2.6.6 CKII phosphorylates the CTD of Spt2p in vitro

Our results show that Spt2p phosphorylation level is regulated by CKII. Whether this regulation is the result of a direct phosphorylation by CKII remains to be determined. To directly address this possibility, we first tested the ability of CKII to phosphorylate Spt2p in vitro. Tandem affinity-purified CKII was incubated with or without recombinant His10-Spt2p in presence of [γ-^{32}P]-ATP. Following SDS-PAGE and autoradiography, we observed a clear band corresponding to the size expected for Spt2p that was absent in the control reactions (Figure 2.4A). On the other hand, a band of higher mobility was observed

in the presence or absence of Spt2p, which likely corresponded to autophosphorylated CKII. We thus concluded that Spt2p is a direct target of CKII. Interestingly, as shown above, deletions in the C-terminal region of Spt2p alter the in vivo phosphorylation level of the protein, indicating that this domain might be targeted by CKII. To test this possibility directly, we purified recombinant domains of Spt2p fused to GST and tested the ability of purified yeast CKII to phosphorylate these fusion peptides (Figure 2.4B). As shown in Figure 2.4C, in addition to the GST control, we analyzed full-length Spt2p, an Spt2p fragment resulting from the deletion of the CTD (residues 1-213), and Spt2p CTD itself (residues 200-333). Only full-length Spt2p and Spt2p CTD were found to be phosphorylated by yeast CKII. This result clearly indicates that CKII directly phosphorylates Spt2p CTD. In addition, we analyzed the phosphorylation of the various Spt2p domains by a WT or CKII-depleted yeast extract (Figure 2.4D). We found that the Spt2p CTD is the sole domain found to be phosphorylated in vitro in yeast whole-cell extracts, and that CKII activity is required for such phosphorylation. Therefore, CKII is the sole protein kinase that interacts with Spt2p and directly phosphorylates this factor in vivo.

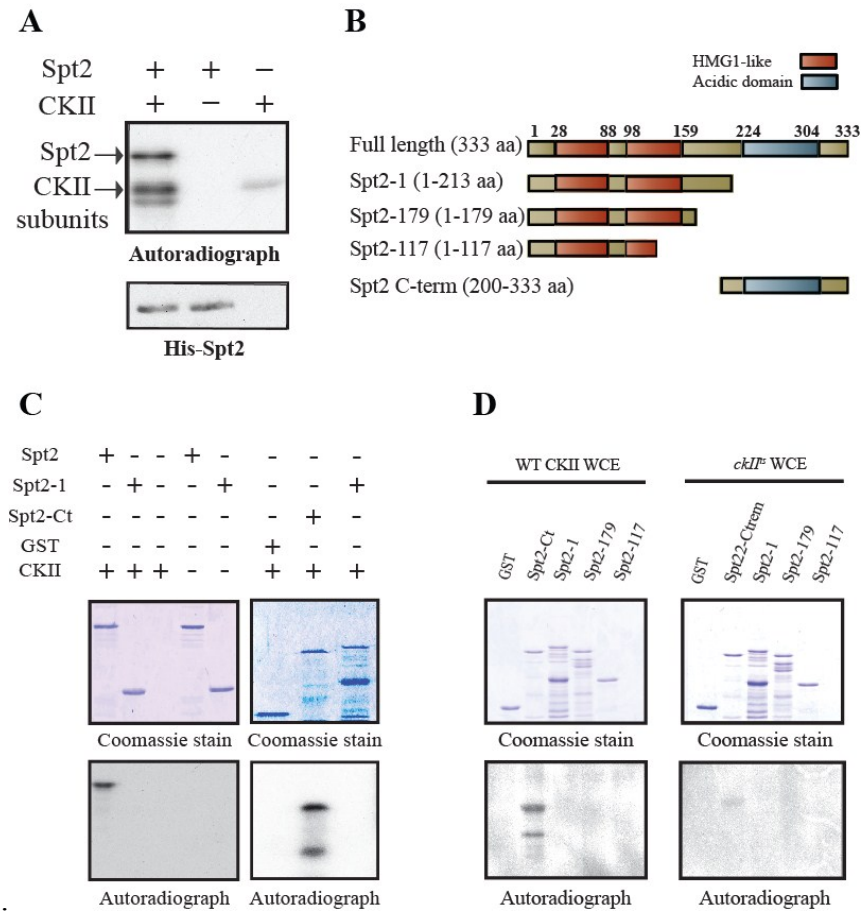

Figure 0.4: CKII directly phosphorylates Spt2p

(A) Recombinant Spt2p-His was incubated with CKII purified from yeast (lane 1) or in a mock reaction (no CKII) (lane 3) in the presence of radiolabeled ATP. Lane 2 shows a control reaction with CKII only. Samples were resolved by SDS-PAGE followed by transfer to a nitrocellulose membrane and autoradiography. The *top* panel shows an auto radiograph, while the *bottom* panel shows an immunoblot with anti-6xHis. (B) Different regions of recombinant Spt2p purified as GST fusion constructs.
(C) Full-length Spt2p and various versions of the protein were incubated with CKII purified from yeast in the presence of radiolabeled ATP. Samples were resolved by SDS-PAGE and analyzed by autoradiography. (D) Different domains of Spt2p fused to GST were incubated with whole-cell extracts from WT or ckII^{ts} strains. After GST pulldown, pellets were directly phosphorylated by adding radiolabeled ATP.

2.6.7 Identification of CKII phosphorylation sites in the Spt2p elongation factor

Our analyses of purified yeast Spt2p with phosphoserine- and phosphotyrosine-specific antibodies and in vitro studies led us to hypothesize that the sites of Spt2p phosphorylation are localized within its CTD (residues 200-333) and involve tyrosine and serine (Figure 2.3 and 2.4), although threonine residues cannot be excluded by any means at this stage. We next wanted to map the phosphorylation sites more precisely. To identify the phosphorylated residues, we subjected recombinant Spt2p to in vitro phosphorylation using yeast CKII, followed by mass spectrometry. Several mass spectrometry attempts failed to detect any phosphorylated residue, despite the fact that each analyzed sample was heavily phosphorylated, as detected by autoradiography. Because of the highly charged nature of Spt2p CTD, only a small part of this domain was recovered by mass spectrometry. Nonetheless, two C-terminal peptides derived from Spt2p were recovered by mass spectrometry after in vitro phosphorylation, but none of these peptides contained a phosphorylated residue. Thus, these C-terminal peptides were excluded from our future analyses (Supplemental Figure 1.12).

To identify the phosphorylated residue(s) in elongation factor Spt2p, we therefore decided to use an indirect approach. First, we looked for the serine and tyrosine residues that are located at CKII consensus sites and could be potentially targets of this kinase. We identified five potential residues (see Figure 2.5A) in Spt2p CTD, namely four tyrosine (Y226, Y230, Y279 and Y281) and one serine (S277) residues. Interestingly, none of these sites is located in the two peptides recovered by mass spectrometry. To directly assess if these residues are phosphorylated by CKII, we thus decided to mutate these residues to alanyl or phenylalanyl residues and examine phosphorylation of the recombinant Spt2p mutant forms by purified yeast CKII. We first tested the phosphorylation status of each mutant individually and found that none of these single mutations affect the overall level of Spt2p phosphorylation (data not shown). Careful examination of the potential phosphorylation sites show that these residues are located within two distinct short stretches of amino acids (see Figure 2.5A). The first region (region I: RI) contains two sites

(Y226 and Y230) whereas the second region (region II: RII) contains three potential sites (S277, Y279 and Y281). Our data show that the Spt2p CTD contains several potential targets for CKII-mediated phosphorylation. In fact, CKII might phosphorylate all sites located within one of the two regions. To test this hypothesis, we combined mutations in each region and determined the effect of these combinations on phosphorylation levels. We therefore substituted potential target residues in RI or RII to non-phosphorylatable amino-acids (i.e. S→A, Y→F) and assessed the ability of CKII to modify the resulting Spt2p mutants. As shown in Figure 2.5B, modification of RI has no detectable effect whereas RII mutations significantly reduced the CKII phosphorylation. We thus concluded that residues present within the RII region are the major targets for CKII phosphorylation. However, mutations of RII did not completely abolish CKII-mediated phosphorylation, suggesting the presence of other sites in the CTD of Spt2p. Another possibility is that RI might still be phosphorylated in presence of intact RII sites, albeit at a low level, and be phosphorylated to a higher level in the absence of RII phosphorylation sites. To address this possibility, we combined mutations in RI and RII and tested the extent of phosphorylation induced by CKII. Importantly, the latter experiments showed that the Spt2p protein, when mutated in both RI and RII regions (Spt2p RI+RII), is no longer phosphorylated by CKII (Figure 2.5B). We thus conclude that in vitro, CKII phosphorylates two regions in Spt2p CTD. These regions respectively contain 2 and 3 residues that are targeted by CKII.

Next, we investigated the actual sites of Spt2p phosphorylation in vivo. To address this question, we used 2D-PAGE to analyze the Spt2p RI+RII mutated protein and compare its migration to that of the WT protein. Whole-cell extracts from *spt2*Δ strains that had been transformed with a plasmid expressing either the Spt2p-FLAG or its RI+RII mutated version fused to the FLAG epitope, were subjected to 2D-PAGE followed by western blotting using anti-FLAG antibodies. As shown in Figure 2.5C, the acidic Spt2p isoform was the major one observed in wild-type cells, while the abundance of these acidic species was strongly diminished in the whole-cell extract from the strain expressing the Spt2p RI+RII mutated protein. This indicates that RI+RII mutations abolish in vivo phosphorylation of Spt2p and show that these sites are the major CKII targets in vivo.

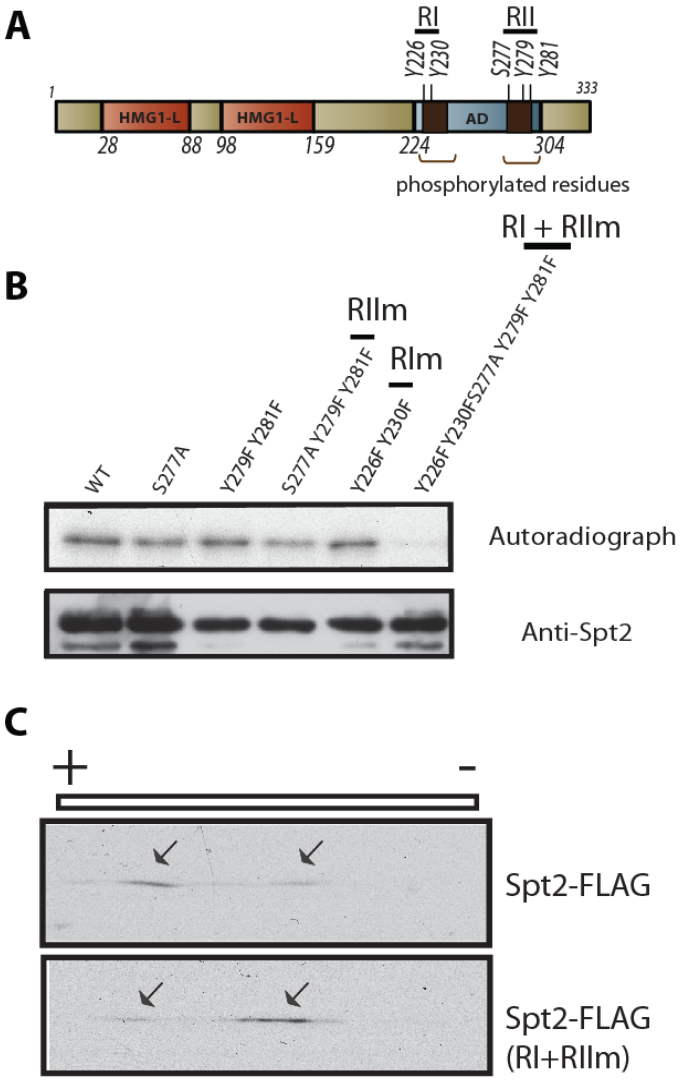

Figure 0.5 : Identification of Spt2p phosphorylated residues
(A) Potential CKII target sites reside in the C-terminal region of Spt2p. (B) Recombinant Spt2p-His and different point mutants of Spt2p, mutated at CKII consensus sequences in the CTD as indicated were phosphorylated by purified yeast CKII. The *top* panel shows an autoradiogram and the *bottom* panel, anti-His immunoblot. (C) 2D-PAGE analysis of Spt2p (WT) and Spt2p (RI+RII with S→A mutations). Total cell lysates from strains expressing either Spt2p-FLAG or Spt2p-FLAG (RI +RII with S→A mutations) were resolved by 2D-PAGE and analyzed by western blot with an anti-FLAG antibody. The *top* panel shows that most of WT Spt2p is represented by the acidic (phosphorylated) isoform. On the other hand, the acidic form of Spt2p is drastically reduced when CKII target sites in RI and RII are mutated to alanine residues (*bottom* panel).

2.6.8 Phosphorylation of RI and RII by CKII modulates Spt2p function

After mapping the Spt2p residues that are phosphorylated in vivo, we sought to determine the functional significance of these post-translational modifications. Although

CKII phosphorylates up to 5 sites in the Spt2p CTD, many of them might be devoid of any functional significance. To test this hypothesis, we constructed different yeast strains expressing an Spt2p protein bearing a substitution at a single phosphorylation site. Each of these mutations replaces a phosphorylatable residue with a non-phosphorylatable amino acid. To analyze the function of these alleles, we looked for specific phenotypes associated with Spt2p function. For instance, *SPT2* deletion suppresses Ty and δ insertion mutations in the *HIS4* and *LYS2* promoters (i.e. the archetypical SPT phenotype). This phenotype is directly associated with the functions of Spt2p in chromatin structure. In the WT strain containing the *lys2-128*δ allele the *LYS2* gene is not expressed, and cells therefore become lysine auxotrophs. The loss of Spt2p function suppresses this phenotype and thus rescues cells containing the *lys2-128*δ allele from lysine auxotrophy. We first exploited this phenotype in cells expressing *spt2* mutant alleles bearing a single substitution to a non-phosphorylatable residue in the RI or RII regions. As shown in Figure 2.6A and 2.6B, loss of a functional Spt2p enabled cells with the *lys2-128*δ allele to grow in media lacking lysine. On the other hand, reintroducing the WT *SPT2* gene or single mutations in region I or II conferred lysine auxotrophy. This shows that loss of a single phosphorylation site in either RI or RII does not affect Spt2p function. We next examined the phenotypic effects of various combinations of mutations in RI or RII, which led to similar results. Moreover, when mutating all five sites to non-phosphorylatable residues (S→A and Y→F), strains containing the *lys2-128*δ allele also became lysine auxotrophs (Figure 2.6C). Taken together, these data show that CKII-mediated phosphorylation of target residues within regions RI or RII has no effect on Spt2p function.

Next, we reasoned that phosphorylation by CKII might in fact negatively regulate the function of Spt2p. If that was the case, mimicking a permanent phosphorylation state might inhibit Spt2p function. To test this hypothesis, we substituted each residue in regions RI or RII with an acidic amino acid, thus mimicking phosphoprotein isoforms of Spt2p in a stable state. Interestingly, while mutations of RI targets to acidic residues had no effect on lysine auxotrophy present in the *lys2-128*δ test strains, test strains expressing Spt2p isoforms where the same type of mutations was introduced within the RII region partly lost the lysine auxotrophy phenotype (Figure 2.6C). Moreover, a strain expressing an Spt2p protein where CKII target sites in both RI and RII were concomitantly changed to acidic

residues completely lost lysine auxotrophy, as seen by direct comparison with the WT control strain transformed with vector only. This indicates that mimicking phosphorylation of CKII target sites within RI and RII completely inhibits Spt2p function. Finally, it is noteworthy that these phenotypes correlate well with CKII preference for sites within the RII region that we had observed in vitro.

We next aimed at determining the possible effect of mutations in the RI and RII regions on the function of Spt2p in chromatin modulation during transcription elongation. The Paf1p1p complex (Paf1p-Cdc73p) is involved in chromatin modulation associated with transcription elongation. Interestingly, Spt2p is known to be functionally associated with the Paf1p complex and *SPT2* deletion leads to a phenotype that is synthetic with concomitant mutations in the Paf1p complex (Nourani et al., 2006). We therefore determined whether phosphorylation of RI and RII target residues might be functionally related to the Paf1p complex component. The Paf1p complex subunits Cdc73p and Paf1p are necessary for the normal growth of cells lacking functional *SPT2*. Moreover, the *spt2*Δ*cdc73*Δ strain is thermosensitive, whereas the double mutation *spt2*Δ*paf1*Δ is lethal. To determine if CKII-mediated phosphorylation is functionally associated with the Paf1p complex and transcription elongation, we assessed whether *spt2* mutants with various substitutions at CKII target sites are able to complement *SPT2 deletion* in the *spt2*Δ*cdc73*Δ and *spt2*Δ*paf1*Δ strains. We first transformed *spt2*Δ*cdc73*Δ cells with empty plasmid or with vectors encoding WT *SPT2* or *SPT2* bearing various mutations at CKII phosphorylation sites. As shown in Figure 2.6D, similar to *spt2*Δ cells, *spt2*Δ*cdc73*Δ strains expressing a phosphomimetic *SPT2* allele in both RI and RII regions do not grow at the higher (non-permissive) temperature. This suggests that the latter mutations lead to a phenotype that is synthetic with the *cdc73*Δ mutation. On the other hand, the double deletion *spt2*Δ*paf1*Δ is lethal in yeast. We addressed the possibility that *SPT2* mutations in RI or RII might rescue the lethal phenotype *spt2*Δ*paf1*Δ. For that purpose, we constructed a *spt2*Δ*paf1*Δ strain rescued with a plasmid carrying the *SPT2* and *URA3* genes. We transformed this strain with another plasmid containing either WT *SPT2* or various *spt2* mutant alleles, as indicated in Figure 2.6E. We next forced the loss the *SPT2*/*URA3* plasmid by growth on 5-FOA. *SPT2* alleles with substitutions to glutamic or aspartic acid residues at CKII target sites in RI and RII could not rescue the 5-FOA-induced loss of *SPT2*. These

data indicate that *SPT2* phosphomimetic mutations in RI and RII are synthetically lethal with the *paf1*Δ mutation. Taken together, our observations strongly suggest that phosphorylation of CKII target sites within RI and RII inhibits the function of Spt2p in the modulation of chromatin structure associated with transcription elongation.

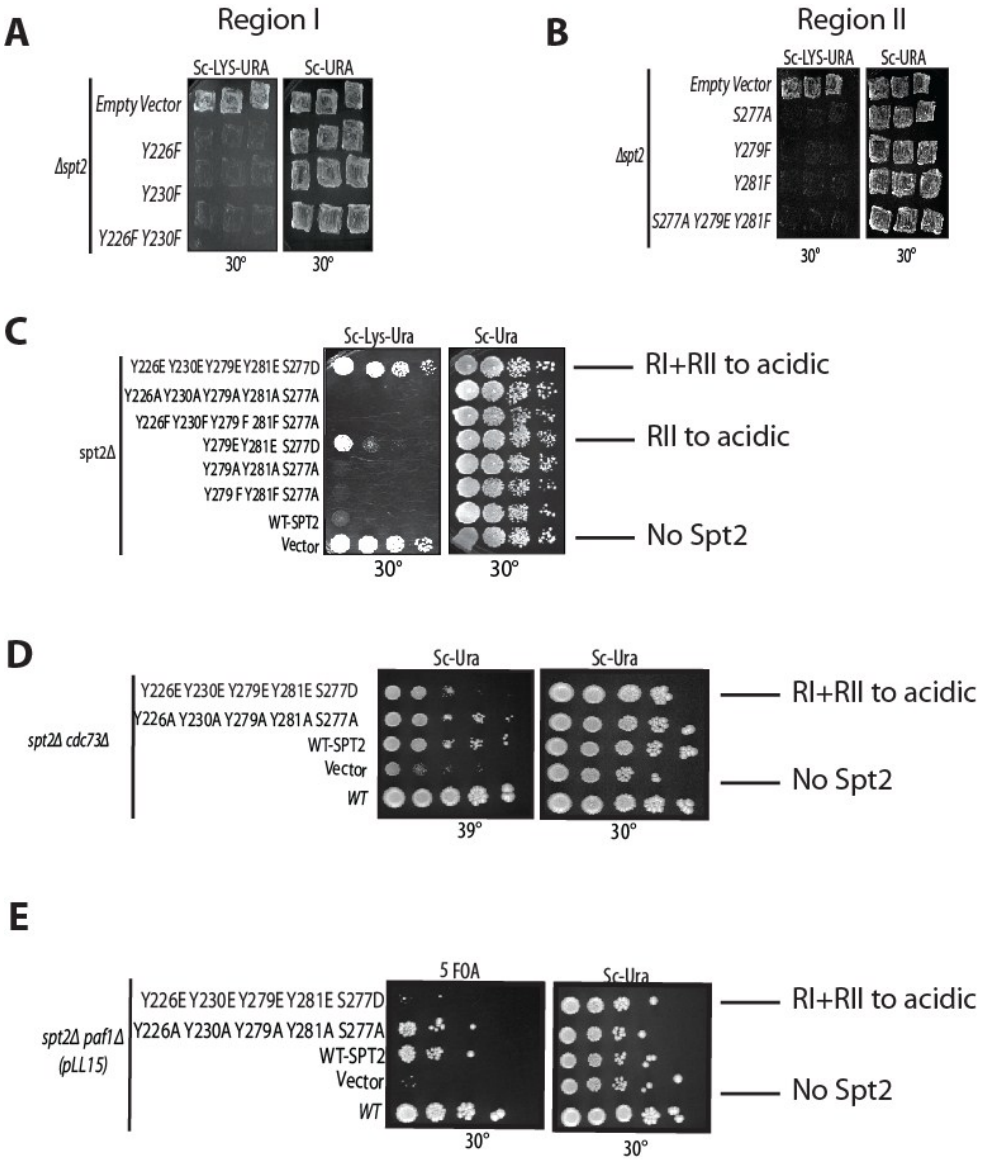

Figure 0.6 : Phosphorylation by CKII negatively regulates the function of Spt2p
(A, B & C) An *spt2*Δ mutant was transformed with pRS416 or a plasmid carrying WT *SPT2* or various *SPT2* point mutants as indicated and spotted on media lacking uracil and

lysine (*SPT* phenotype). (D) The *spt2*Δ*cdc73*Δ strain was transformed with the prs416 plasmid or a plasmid encoding WT *SPT2* or *spt2* mutant versions, as indicated and grown under various conditions. (E) The *spt2*Δ *paf1*Δ strain is non-viable and therefore harbors the *SPT2* gene on a plasmid bearing the *URA* marker (*pLL15*). The *spt2*Δ*paf1*Δ (pLL15) strain was transformed with various plasmids as indicated (with *LEU* markers). After serial dilutions, suspension aliquots of these strains were spotted on medium containing 5- FOA to monitor the loss of the *URA* plasmid.

2.6.9 Mutations of CKII phosphorylation sites affect chromatin refolding and the inhibition of spurious transcription in yeast

Nucleosomes unfolded upon the passage of RNAPII are restored by a machinery involving Spt2p (Thebault et al., 2011). One consequence of a defect in nucleosome refolding is the loss of transcriptional inhibition at TATAA sites, which are located within coding regions (Carrozza et al., 2005; Cheung et al., 2008; Kaplan et al., 2003; Keogh et al., 2005; Nourani et al., 2006). A direct result of this is the occurrence of spurious transcription from these cryptic promoters (Carrozza et al., 2005; Cheung et al., 2008; Kaplan et al., 2003; Keogh et al., 2005; Nourani et al., 2006). The data shown above indicate that CKII phosphorylation could inhibit the function of Spt2p in nucleosome refolding associated with transcription. Interestingly, Spt2p cooperates with the Paf1p and HIR/HPC complexes in the inhibition of transcription initiation from the *FLO8* cryptic promoter and therefore, represses spurious transcription (Nourani et al., 2006). We next aimed to determine if the CKII-mediated phosphorylation of Spt2p RI/RII regions plays any role in the refolding of chromatin in coding regions and in the repression of spurious transcription. For this purpose, we analyzed the role of Spt2p phosphorylation by CKII in cryptic transcription using a reporter for *FLO8* cryptic initiation in which the 3'-coding region of *FLO8* has been replaced with the *HIS3* coding region (Figure 2.7A). In this construction, *HIS3* is only expressed when the *FLO8* cryptic promoter is active, indicating a significant defect in the refolding of nucleosomes after the passage of RNAPII. In addition, the *FLO8* promoter was replaced with the *GAL1* promoter to allow regulation and modulation of transcription levels from the normal *FLO8* initiation site (Nourani et al., 2006). We then tested the expression of this *GAL1-FLO8-HIS3* reporter in various mutants by assaying growth on histidine-free medium, using either glucose or galactose as the sole carbon source. In these tests, growth on galactose rather than glucose is a more permissive

condition to detect cryptic initiation. Our results (Figure 2.7B) show that, as seen with *spt2*Δ, mutation of CKII target sites within RI and RII to acidic residues allows expression from the *FLO8* cryptic promoter when cells are grown on galactose. In the *spt2*Δ *cdc73*Δ double mutants, we observed growth in glucose media lacking histidine, indicating a stronger rate of spurious transcription in this mutant (with phosphomimetic RI/RII sites) when combined with a *CDC73* (encoding one of Paf1p complex subunits) deletion mutant (Figure 2.7C).

To test cryptic initiation at *FLO8* more directly, we also performed Northern hybridization analysis of WT *FLO8* gene expression. These experiments were performed in *spt2*Δ*cdc73*Δ mutants and transformed with either WT *SPT2* or its RI/RII phosphomimetic mutant forms. In these experiments, RNA samples were prepared at the start of and 2 h after a shift to the non-permissive temperature. Our results (Figure 2.7D) show that in strains containing either the WT *SPT2* or its RI/RII non-phosphorylatable mutant alleles, only the full-length *FLO8* transcript was observed. In contrast, in the absence of *SPT2* (empty vector) or in strains expressing the RI/RII phosphomimetic allele, the *FLO8* short transcript was produced at a significant level. These results further support the notion that the CKII-mediated phosphorylation of the Spt2p RI/RII target sites inhibits the ability of Spt2p to regulate chromatin refolding during transcription elongation.

Finally, we examined the effect of mutations of the phosphorylation sites on the regulation of the *SER3* gene encoding 3-phosphoglycerate dehydrogenase, which catalyzes the first step in the serine and glycine biosynthetic pathway. We have recently shown that Spt2p regulates *SER3* gene expression by modulating chromatin structure in the intergenic region encoding *SRG1*, a repressor RNA that inhibits *SER3* expression by competition for transcription (cf. section 1.5.4.1). To address this question, we performed Northern analysis of *SER3* expression in WT and *spt2*Δ strains transformed with plasmids encoding WT *SPT2* or various phosphorylation mutant alleles of that gene. Our results (Figure 2.7E) show that like in the wild-type strain, all phosphorylation mutants inhibit *SER3* transcription. The *spt2* mutant with phosphomimetic CKII target sites complements the *spt2*Δ as well as WT strains in the normal regulation of the *SRG1-SER3* system. This important observation indicates not only that mutating CKII consensus target sites in the RI/RII regions to acidic

residues does not abolish all *SPT2* functions, but also that it specifically inhibits chromatin reassembly in a subset of genes.

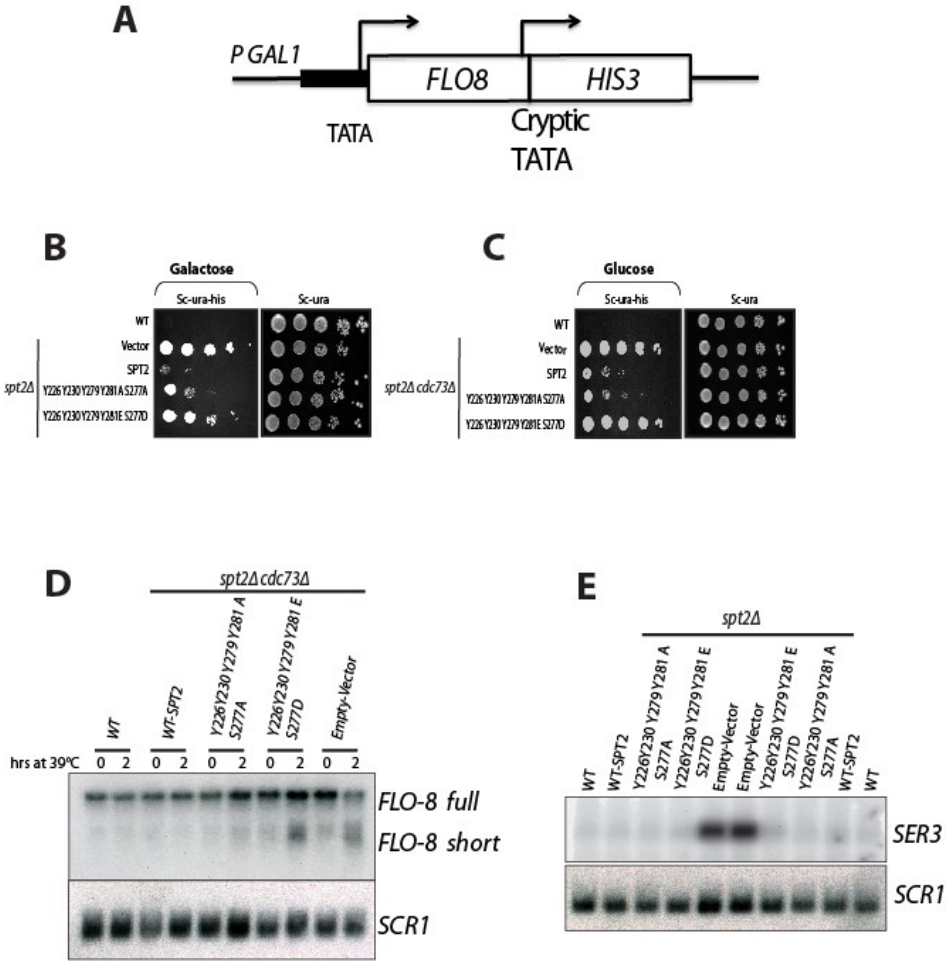

Figure 0.7 : Mutation of Spt2p phosphorylation sites to acidic residues affects chromatin assembly

(A) Schematic representation of the *pGAL1-FLO8-HIS3* reporter. (B & C) *spt2*Δ and *spt2*Δ*cdc73*Δ strains harboring the *pGAL1-FlO8-HIS3* reporter were transformed with plasmids (WT *SPT2* and mutant forms as indicated). *GAL1-FLO8-HIS3* expression was determined by growth on medium lacking histidine, using either glucose or galactose as sole carbon source. (D*) spt2*Δ and *spt2*Δ*cdc73*Δ strains transformed with the indicated plasmids were grown at either 30°C ($T_{t=0}$ = 39°C) or 39°C for 2 h, Total RNA was extracted and analyzed by Northern blot with a probe against *FLO8* 3'-end. (E) Phosphomimetic mutants exhibit normal *SRG1-SER3* regulation: total RNA was isolated from WT and *spt2*Δ strains (transformed with the indicated plasmids) grown at 30°C in YPD and Northern analysis was carried out using an *SER3* probe. *SCR1* (RNA subunit of the signal recognition particle) was used as control for total RNA loading.

2.6.10 Phosphorylation by CKII regulates Spt2p recruitment to the coding region of transcribed genes

We showed above that mutating the CKII phosphorylation sites (RI/RII) of Spt2p to phosphomimetic residues inhibits Spt2p function in chromatin modulation associated with transcription and thereby activates spurious transcription. There are several possible explanations for this observation. First, CKII-mediated phosphorylation might directly inhibit the activity of Spt2p at the sites of transcription. Alternatively, phosphorylation could modulate interaction of Spt2p with partners involved in a common function and therefore, might indirectly inhibit nucleosome refolding. Phosphorylation by CKII could also modulate the recruitment of Spt2p to the sites of transcription. Importantly, we previously showed that Spt2p function is strictly linked to its recruitment to active transcription sites (Nourani et al., 2006). Inactivation of Spt2p by CKII therefore likely results in an inhibition of its recruitment to transcribed regions. To investigate this hypothesis, we analyzed the association of WT Spt2p and its phosphomimetic RI/RII mutants to the transcribed regions of various active genes. As shown in Figure 2.8A, the level of WT Spt2p association with *PMA1, ACT1, RPL5, RPL10 and RPL25* transcribed regions was higher than to the control region *No-ORF* (a non-coding, purely intergenic region on chromosome V), as expected. On the other hand, a drastic reduction in Spt2p recruitment to the transcribed regions of these active genes was observed in the phosphomimetic RI/RII *spt2* mutants. This key observation indicates that the loss of Spt2p function in chromatin regulation that is noted in phosphomimetic RI/RII *spt2* mutants is associated with a significant decrease in the association of the mutant Spt2p with the chromatin sites regulated by this factor. Moreover, it strongly suggests that Spt2p phosphorylation by CKII directly represses its function through an inhibition of its recruitment to sites of active transcription. We also tested the association between Spt2p and moderately transcribed genes. As indicated in Figure 2.8B, Spt2p enrichment in these genes is low. Only a small reduction in the association of the phosphomimetic Spt2p mutant was observed in some of these transcribed regions such as *PCA1* and *PDC1*. These data suggest that regulation by CKII phosphorylation is mostly significant in the transcribed regions of highly active genes. Previous reports have shown that a loss of elongation factors or chromatin marks associated with transcription in coding regions

sometimes reflects changes in the distribution of these elements across these regions rather than a net loss. We therefore asked whether the recruitment of phosphomimetic *spt2* mutants is similar in all regions of the genes affected. We performed *SPT2* ChIP assays at different regions of the highly active *PMA1* gene or the moderately transcribed *FKS1* gene using chromatin from WT *SPT2* or from its phosphomimetic mutant form (Figure 2.8C and 2.8D). We found that the phosphomimetic mutation affected recruitment in all regions of the genes tested and that this effect was markedly stronger for the highly transcribed *PMA1* gene.

We next determined the effect of mutating the RI/RII CKII target sites of Spt2p to non-phosphorylatable residues on the association of the resulting mutant factor with coding regions of differentially transcribed genes (Figure 2.9A and 2.9B). Overall, we did not observe a significant change in Spt2p recruitment at highly transcribed genes (Figure 2.9A). This data indicates that inhibition of CKII-mediated phosphorylation does not stimulate the recruitment of Spt2p to transcribed gene regions, in agreement with the lack of detectable phenotype observed for this type of mutations in Figures 6 and 7. However, one important exception was noted, namely in the moderately transcribed *PDC1* and *FKS1* genes (Figure 2.9B and 2.9D), where we observed an increase in the recruitment of Spt2p in the non-phosphorylatable mutant.

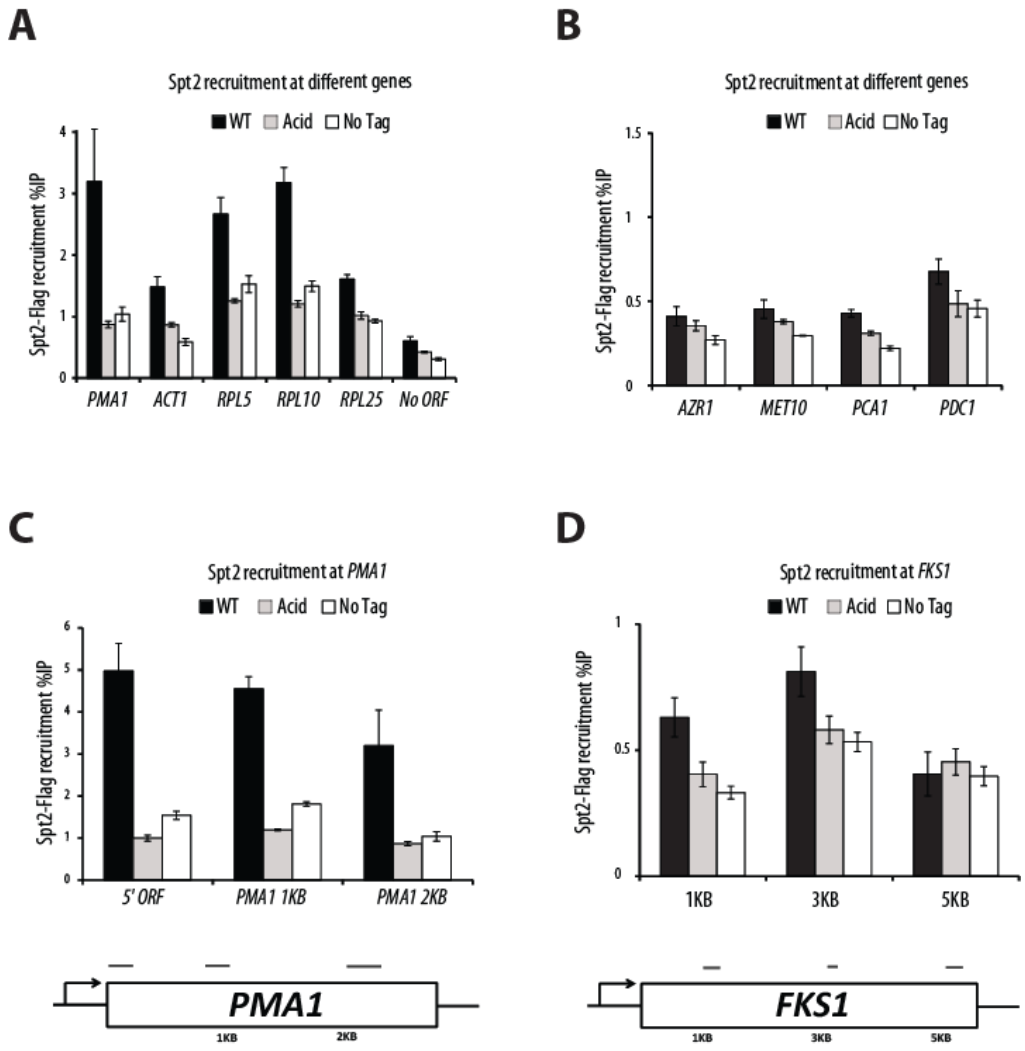

Figure0.8: A phosphomimetic Spt2p mutant is not recruited to the coding region of transcriptionally active genes.

Yeast cells from the untagged strain or a strain expressing *SPT2*-FLAG (WT or phosphomimetic ["acid"] mutant forms) were grown in YPD. Cells were fixed with formaldehyde and chromatin immunoprecipitation was then performed using anti-FLAG beads. The values shown represent the means ± SE from three independent experiments. (A) Association of Spt2p (WT and phosphomimetic ["acid"] mutant) with the coding region of transcriptionally active genes *(PMA1, ACT1, RPL5, RPL10, RPL25* and *No-ORF*. (B) Association of Spt2p (WT and phosphomimetic ["acid"] mutant) with the coding region of moderately transcribed genes (*AZR1, MET10, PCA1* and *PDC1*). (C) & (D) Association of Spt2p with various regions of transcriptionally active genes (*PMA1* and *FKS1*). The *horizontal bars* in the bottom represent the regions analyzed by quantitative PCR.

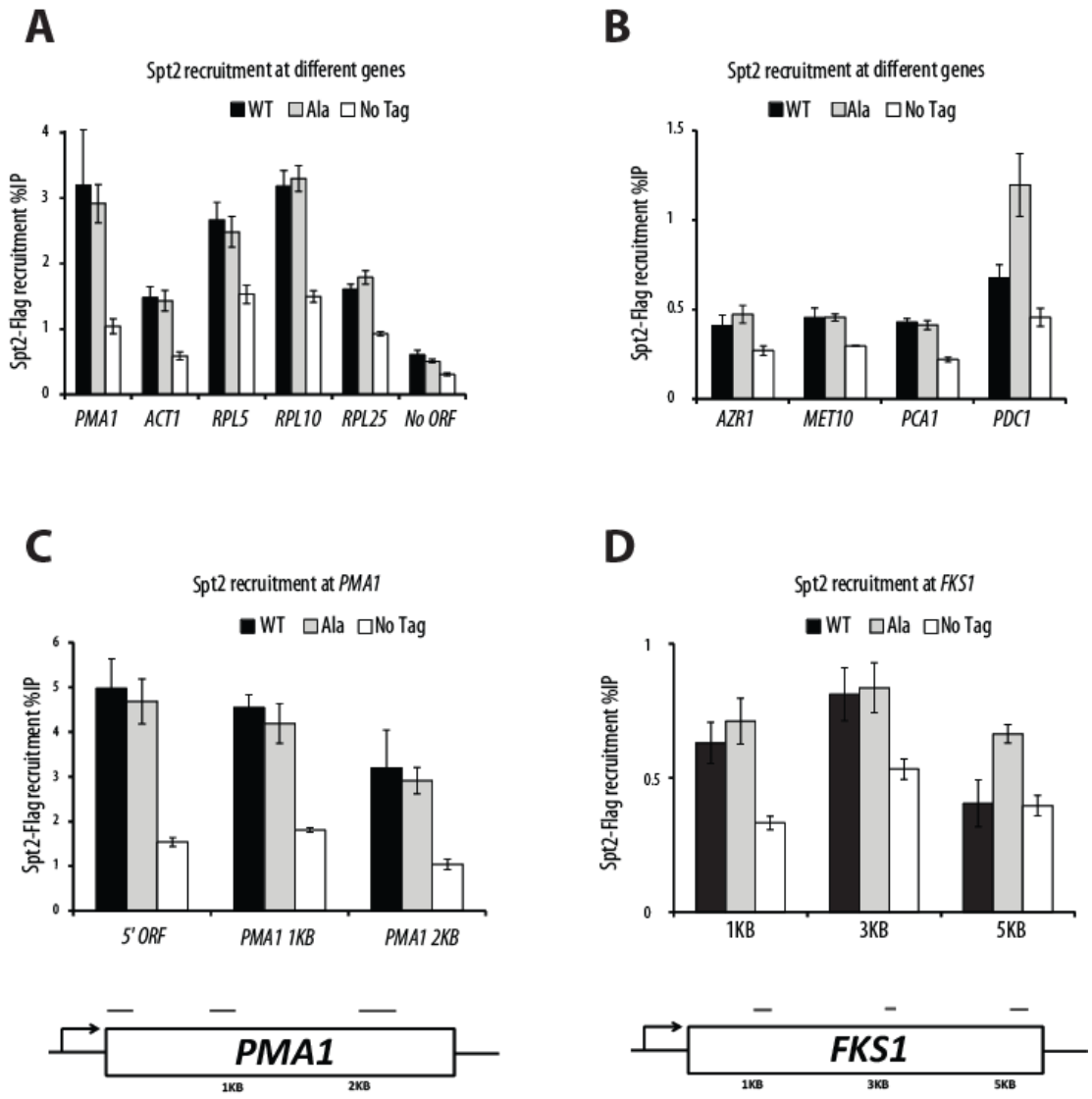

Figure 0.9 : Non-phosphorylatable mutants of Spt2p are recruited to chromatin
Chromatin immunoprecipitation analysis of Spt2p-FLAG (WT) and non-phosphorylatable *spt2* mutant ("Ala") were performed as described in Figure 2.8. The values shown represent the means ± SE from three independent experiments. (A) Association of WT Spt2p and its non-phosphorylatable mutant with the coding region of transcriptionally active genes *(PMA1, ACT1, RPL5,RPL10, RPL25)* and *No-ORF*. (B) Association of WT Spt2p and its non-phosphorylatable mutant with the coding region of moderately transcribed genes (*AZR1, MET10, PCA1, PDC1*). (C) & (D) Association of WT Spt2p and its non-phosphorylatable mutant with various regions of transcriptionally active genes (*PMA1* and *FKS1*). The horizontal bars in the bottom represent the regions analyzed by quantitative PCR.

2.6.11 Phosphorylation of Spt2p by CKII partly inhibits Spt2p-Spt6p interaction

Spt2p function is strictly linked to its recruitment to active transcription sites (Nourani et al., 2006). Importantly, our data strongly suggest that this recruitment is regulated by CKII phosphorylation. Moreover, we previously observed that Spt2p is recruited to the coding region of transcriptionally active genes in an Spt6p-dependent manner (Nourani et al., 2006; Thebault et al., 2011). We reasoned that regulation of Spt2p recruitment by CKII could involve the modulation of the Spt2p-Spt6p relationship. Interestingly, mutations in the *SPT6* gene lead to drastic loss of Spt2p from active regions of transcription and affects the refolding of nucleosomes in the wake of RNAPII transcriptional activity, suggesting that Spt6p is required to the recruitment of Spt2p in vivo (Nourani et al., 2006; Thebault et al., 2011) and Annex 2. There is also genetic evidence for an involvement of both *SPT2* and *SPT6* in the same pathway, i.e. these two genes are epistatic (Nourani et al., 2006) and Thebault et al., 2011). Taken together, these observations suggest a mechanism through which Spt6p directly targets Spt2p, recruiting it to the sites of transcriptional elongation, and indicate that CKII could modulate this mechanism through direct phosphorylation of Spt2p. However, it is unclear whether Spt6p directly recruits Spt2p or stabilizes Spt2p interaction with chromatin after its recruitment. To address this question, we determined if Spt2p and Spt6p might interact directly. For this purpose, recombinant GST, GST-Spt2p and 6xHis-Spt6p were produced, purified and used in a GST pulldown assay (Figure 2.10A). Our results show that GST-Spt2p interacts with Spt6p while no such interaction is observed with the GST control (Figure 2.10A). This data indicate that Spt6p interacts directly with Spt2p and suggest that it recruits Spt2p or stabilizes its association with chromatin in a direct fashion.

Our data indicate that phosphorylation of two regions in Spt2p (RI and RII) located in the CTD of Spt2p plays a crucial role in the regulation and of Spt2p association with chromatin. Therefore, Spt6p and phosphorylation of Spt2p appear to play opposite roles in the recruitment or stabilization of Spt2p at transcription active sites. We reasoned therefore that phosphorylation might inhibit Spt6p interaction with Spt2p. To test if phosphorylation of Spt2p has any role in the Spt6p-Spt2p interaction, we phosphorylated the 6xHis-Spt6p/Gst-Spt2p complex and looked for loss of Spt6p from the complex after

phosphorylation by CKII (Figure 2.10B). Interestingly, after phosphorylation of the complex, we observed the release of a significant amount of Spt6p from the complex. Thus, direct phosphorylation of Spt2p by CKII may partially inhibit its interaction with Spt6p. Altogether; our data strongly suggest that CKII destabilizes the association of Spt2p with sites of active transcription, at least in part, by promoting dissociation of the Spt6p-Spt2p complex from these sites.

Finally, our data suggest the possibility that phosphorylation of Spt2p by CKII results in the disruption of the Spt2p-Spt6p complex. Since Spt6p recruits Spt2p to chromatin, CKII phosphorylation would therefore inhibit the recruitment of Spt2p to chromatin. To test this hypothesis in vivo, we analyzed the association of Spt2p with two different coding regions, namely *PMA1* and *POL1*, in the presence (wild-type) or absence of CKII (*ckII*^{ts}). In the WT strain, we observed a significant association of Spt2p-Myc to both *PMA1* and *POL1* coding regions (Figure 2.10 C). This association was substantially increased in the absence of CKII-mediated phosphorylation. Thus, these data show that phosphorylation of Spt2p by CKII inhibits its recruitment to chromatin in vivo.

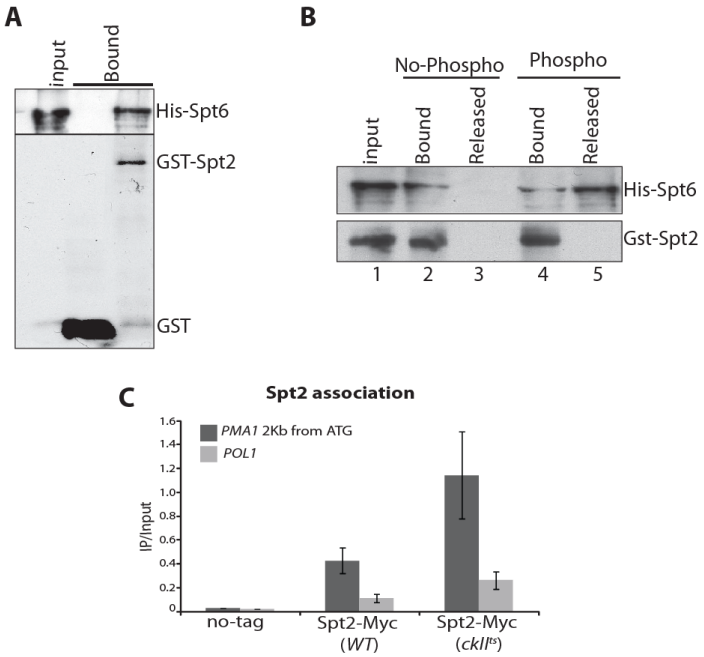

Figure 0.10: Phosphorylation regulates the interaction of Spt2p with Spt6p and chromatin

(A) Spt2p directly interacts with Spt6p. A GST pulldown assay was performed using recombinant proteins 6xHis-Spt6p and GST-Spt2p. GST alone was using as control. Input and bound proteins were resolved by 10% SDS-PAGE and analyzed by western blot with

antibodies against the 6xHis tag. (B) Release of 6xHis-Spt6p from a GST-Spt2p/HisSpt6p complex. GST-Spt2p/His-Spt6p complex was coupled to glutathione beads. Beads were washed with kinase buffer and subjected to in vitro phosphorylation. The supernatant was analyzed using an antibody against the 6xHis tag to monitor the release of 6xHis-Spt6p from the complex. (C) Chromatin immunoprecipitation analysis of Spt2p-Myc. Wild-type (WT) and *ckII*^{ts} yeast cells expressing Spt2p-Myc were grown in YPD. The untagged strain was used as a control. Cells were fixed with formaldehyde and chromatin immunoprecipitation was then performed using an anti-Myc antibody. The values shown represent the mean ± SEM from 3 independent experiments.

2.7 Discussion

Spt2p is a transcription elongation factor involved in the modulation of chromatin structure associated with transcription. It plays important roles in the refolding of chromatin structure and the maintenance of nucleosome structure in the wake of transcription. In the absence of this factor, spurious transcription is activated and genes are not properly regulated. In the present study, we purified Spt2p from yeast and identified its interacting partners. We found a direct and stable association of Spt2p with all CKII subunits (Figure 2.1). Our results demonstrate for the first time that Spt2p is phosphorylated both in vitro and in vivo by CKII (Figure 2.3 and 2.4). Moreover, our data indicate that CKII is the sole kinase responsible for Spt2p phosphorylation in vivo (Figure 2.3). We identified five CKII target sites in Spt2p CTD (Figure 2.5). Mutation of these sites abolishes Spt2p phosphorylation by CKII, indicating that they are the major phosphorylated sites in this factor (Figure 2.5). Interestingly, these sites are located in two pentapeptidic regions, RI and RII, in Spt2p CTD. We mutated RI/RII to either non-phosphorylatable residues or phosphomimetic (acidic, i.e. glutamyl or aspartyl) residues. RI/RII mutations to acidic residues was associated with an *SPT* phenotype and the activation of spurious transcription. In fact, mimicking a permanent phosphorylation state inhibits most, but not all Spt2p chromatin-related functions, suggesting that CKII activity represses this factor. We found that this inhibition is associated with a loss of Spt2p recruitment to sites of active transcription. Importantly, the recruitment of Spt2p to sites of active transcription is directed by the Spt6p histone chaperone. We have shown that Spt2p interacts directly with Spt6p and that phosphorylation of Spt2p by CKII partially inhibits this interaction. Finally, in the absence of CKII, we observed a marked increase in the association of Spt2p to

chromatin. Taken together, our data indicate that CKII phosphorylates and inhibits Spt2p function, at least in part, by repressing the interaction of Spt2p with the Spt6p histone chaperone. Since Spt6p recruits Spt2p to chromatin, inhibition of this interaction results in the release of Spt2p from these sites.

Interestingly, our in vitro experiments showed that RII is the major target of CKII and that RI is a minor site of CKII-mediated phosphorylation. There is a clear correlation between the preferred sites of phosphorylation and the phenotypes corresponding to mutations of these sites. The mutation of both sites is equivalent to a complete loss of CKII-mediated phosphorylation while mimicking phosphorylation of these sites is associated with the strongest loss of Spt2p function.

2.7.1 CKII is the sole kinase that phosphorylates Spt2p

We provide strong evidence that Spt2p interacts with CKII in vivo. First, all subunits of the CKII complex are found to copurify with Spt2p (Figure 2.1 and Figure 2.2). This is an important observation as it rules out the possibility that the association between Spt2p and CKII is an artifact of purification. Indeed, it is difficult to imagine that all four CKII subunits would still coincidentally coelute with Spt2p after four purification steps due to random factors. Second, we performed purification in the reverse order and found that Spt2p still copurifies with the CKII complex. Third, we also independently purified two different subunits of CKII and recovered Spt2p alongside with these subunits after several purification steps. Fourth, although our purification protocols are very stringent, we nonetheless decided to further increase the stringency of our purifications and assess whether the Spt2p-CKII interaction might resist to such treatment. For that purpose, we first purified one CKII subunit from a strain expressing an Spt2p hybrid protein tagged with the FLAG epitope. The purified CKII complex was then subjected to two other purification steps at high ionic force using a FLAG-immobilized column and elution with FLAG peptide. After these six steps of purification at different salt concentrations we still found that Spt2p coeluted with the CKII subunits. The fifth and last line of evidence that Spt2p is stably associated with CKII in vivo came from mutant analysis showing that deletion of the

N-terminal part of Spt2p significantly affects its interaction with CKII (data not shown). Taken together, our data provide strong evidence that CKII is stably associated with Spt2p in vivo.

CKII associates with Spt2p and phosphorylates this factor in its C-terminal region in vivo. Indeed, our results show that Spt2p might be a substrate for this enzyme in vitro. Moreover, Spt2p immunopurified from yeast shows the presence of both phosphoserine and phosphotyrosine in Spt2p molecules in vivo. We wanted to make sure that the phosphotyrosine molecules are specifically generated by CKII in vivo. This is an important question since it is known that CKII primary targets are serine and threonine residues. For that, we immunopurified Spt2p from cells depleted of CKII activity and found that loss of this enzyme is associated with loss of phosphotyrosine Spt2p molecules. Therefore, CKII does indeed phosphorylate Spt2p on tyrosyl residues in vivo. This is consistent with other reports indicating that yeast CKII phosphorylates several targets on tyrosine residues (Marin et al., 1999; Vilk et al., 2008; Wilson et al., 1997; Zhu et al., 2000). Next, we asked if other kinase(s) might phosphorylate Spt2p. After depleting cells from the CKII complex, we found that these extracts cannot phosphorylate Spt2p anymore. Therefore, our data clearly indicate that CKII phosphorylates Spt2p in yeast cells and CKII is the sole kinase that targets this factor.

2.7.2 Identification of CKII phosphorylation sites in the Spt2p protein

After showing that CKII stably interacts with Spt2p and phosphorylates the latter both in vitro and in vivo, we then aimed at identifying the phosphorylation sites. Using a direct approach via mass spectrometry, we analyzed the structure of Spt2p protein purified from yeast. Unfortunately, this approach was unsuccessful because Spt2p is a highly charged protein and very limited yield of the key phosphorylated regions of Spt2p (i.e. the CTD) could be obtained via conventional mass spectrometry approaches. We therefore elected to phosphorylate recombinant Spt2p in vitro and analyze the resulting product by mass spectrometry. These reactions were well controlled and we have obtained strong evidence that recombinant Spt2p is heavily phosphorylated using such a protocol (data not

shown). Surprisingly, even the latter approach failed to identify any phosphorylated peptide. The coverage of Spt2p CTD that is the main target of CKII phosphorylation was strongly underrepresented and very low yield of peptides from this domain were identified by mass spectrometry. Our inability to directly analyze the sites of Spt2p phosphorylation by mass spectrometry led us to use an indirect approach to address that question. From our knowledge that both in vivo and in vitro, deletion of the CTD (213-333) leads to a complete loss of phosphorylation by CKII, careful examination showed that in fact only two very short regions contain potential sites of Ser/Thr phosphorylation by CKII. The first region (RI) corresponds to the 226-230 pentapeptide and the second (RII), to the 277-281 pentapeptide. Importantly, mutating only one out of the five potential RI/RII sites did not affect total phosphorylation, possibly suggesting that Spt2p has multiple potential sites of CKII-mediated phosphorylation. However, mutating all RII sites affected phosphorylation of Spt2p by CKII. Importantly, mutating all RI/RII phosphorylation sites to alanyl or phenylalanyl residues completely abolishes phosphorylation by CKII. This indicates that phosphorylation of RII sites represents the major fraction of total Spt2p phosphorylation, while RI is only a minor site of CKII-mediated phosphorylation. This is consistent with our functional tests of the Spt2p phosphorylation mutant. Indeed, converting the three RII sites to phosphomimetic residues (S277D, Y279E and Y281E) led to defects in Spt2p function, whereas two additional mutations of the same type (Y226E and Y230E) aggravated the resulting *SPT* phenotype (Figure 2.65 and 2.7). Taken together, our results show that phosphorylation of Spt2p by CKII targets two regions in the CTD and negatively regulates Spt2p function.

2.7.3 Spt2p phosphorylation induces the dissociation of the Spt2p/Spt6p complex and inhibits Spt2p recruitment to sites of active transcription

Previous studies have shown that Spt2p associates with the coding regions of transcriptionally active genes and that this association is partly dependent on Spt6p function (Nourani et al., 2006). Whether Spt6p helps in recruiting Spt2p to chromatin or stabilizing its interaction with chromatin post-recruitment is not clear. We did not detect the presence of Spt6p peptides in TAP-Spt2p preparations. However, mass spectrometry was used to analyze only the lower-sized, very abundant protein bands of these preparations.

The Spt2p-Spt6p interaction is also very likely to occur only at the chromatin level and therefore, could not be detected in soluble fractions. Several reports suggest that the histone chaperone FACT requires an HMG box-containing factor, namely Nhp6p, to interact with chromatin and display activity (Biswas et al., 2005; Formosa et al., 2001; Rhoades et al., 2004). Similar to FACT, it is likely that Spt6p needs an HMG box factor to interact efficiently with nucleosomes and initiate the unfolding/refolding reaction during transcription. Indeed, in vitro experiments showed that Spt6p could not interact with nucleosomes without the assistance of the HMG box factor Nhp6p (McDonald et al., 2010). Interestingly, there is no known functional link between Spt6p and Nhp6p. We believe that Spt2p is the HMG box factor that might assist Spt6p during chromatin assembly/disassembly associated with transcription. We already reported that Spt6p recruits Spt2p. Here, we provide evidence that this recruitment likely occurs via a direct interaction with chromatin (see Figure 2.10). The present work also provides evidence that phosphorylation by CKII likely inhibits the recruitment of Spt2p. We further demonstrate that the latter effect of phosphorylation is achieved, at least in part, through a disruption of the Spt2p-Spt6p interaction. The present data allow us to propose a model for the mechanism through which CKII modulates the activity of Spt2p in the chromatin refolding associated with transcription. In this model, Spt6p is recruited by RNAPII CTD to sites of active transcription in order to modulate chromatin structure. However, this function of Spt6p requires the presence of other factors, among which is Spt2p, which is recruited by Spt6p to these sites during elongation. This interaction is important for Spt6p function and allows it to interact with nucleosomes, to start unfolding its structure to facilitate RNAPII transit, and to redeposit nucleosomes in the wake of transcription by RNAPII. The cycle of Spt6p action on chromatin unfolding/refolding is completed by disruption of the Spt2p-Spt6p complex, which, according to our working model, is accomplished in part by the phosphorylation of Spt2p by CKII, which contributes to the release of Spt2p from chromatin. Interestingly, this is consistent with our observations showing that phosphorylated Spt2p binds to native yeast chromatin less efficiently than unphosphorylated Spt2p (data not shown).

In summary, we provide for the first time evidence that an elongation factor is directly regulated by CKII. This regulation may play an important role in the regulation of

chromatin structure in transcribed regions. We also provide evidence that CKII regulates Spt2p via the regulation of its interaction with an important and conserved histone chaperone, namely Spt6p. Our findings provide new insight in the regulation of chromatin modulation associated with transcription, and involve the kinase CKII and the histone chaperone Spt6p. Additional work is needed to understand more precisely the mechanism of regulation of the Spt2p-Spt6p interaction by CKII.

Acknowledgments

We thank Nikita Avvakumov for helpful comments. This work was supported by a CIHR grant to A.N. A.N. holds a Canadian Research Chair and G.B. holds an NSERC PhD fellowship.

2.8 Supplemental Figures

Figure 0.11 Complete sequence of Spt2p
The peptides covered by mass spectrometry are shown in *red*. The CKII target sites are shown in *green*.

Chapter 3

Casein Kinase II regulates the Function of the Histone Chaperone Spt6p

Wajid Bhat[1], Anne Ruffiange[1] and Amine Nourani[1]*

1. Centre de recherche en cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec (CHUQ), 9 McMahon Street, Quebec City, Qc G1R 2J6 Canada

*To whom correspondence should be addressed

E-mail: Amine.Nourani@crhdq.ulaval.ca

3.1 Foreword

This Chapter is in the form of a manuscript. I performed most of the experiments presented in this manuscript. Anne Ruffiange realized one of the GST pulldown assays represented in Figure 3.6 D and the spot tests represented in Figure 3.5 E. I also wrote the manuscript.

3.2 Résumé

CKII est une protéine kinase ubiquitaire chez les eucaryotes et impliquée dans une variété de processus cellulaires. Dans cette étude, nous démontrons que la CKII est directement impliquée dans la modulation de la structure de la chromatine lors de l'élongation de la transcription. Nous avons trouvé que la déplétion de CKII des cellules de *levure Saccharomyces cerevisiae* conduit à la production de transcrits à partir de promoteurs cryptiques ainsi qu'à la réduction des niveaux de triméthylation de l'histone H3K36, un indicateur de la chromatine transcriptionnellement active. Ces observations démontrent un rôle important de la CKII dans la modulation de la chromatine. Nous avons découvert que la CKII interagit avec le facteur d'élongation de la transcription Spt6p et le phosphoryle in vivo et in vitro. Nous avons localisé les sites de phosphorylation à la région N-terminale non structurée et avons démontré que la modification post-traductionnelle de ces résidus est importante pour la fonction de Spt6p. Nous avons par la suite montré que Spt6p interagit directement avec Set2p, la méthyltransférase de H3K36, et que CKII régule cette interaction. De plus, CKII régule directement la formation du complexe Spt6p-Iws1p in vivo et in vitro. Finalement, nous avons trouvé que la CKII est requise pour maintenir les niveaux protéiques de Spt6p dans des conditions spécifiques, suggérant que la formation du complexe Spt6p/Iws1p est requise pour sa stabilité. Dans l'ensemble, nos résultats indiquent que la fonction de Spt6p est régulée par un mécanisme de modifications post-traductionnelles inconnu jusqu'à maintenant impliquant directement sa phosphorylation par CKII et qui module son association en complexe avec Iws1p et Set2p.

3.3 Abstract

CKII is a protein kinase ubiquitously present among eukaryotes and implicated in various cellular processes. In this study, we demonstrate that CKII is involved directly in chromatin structure modulation during transcription elongation. We found that CKII depletion from yeast cells results in spurious transcription from cryptic promoters and in reduced trimethylation of histone H3 K36, a chromatin mark narrowly associated with the elongation of transcription. These observations indicate an important function of CKII in the modulation of chromatin. We found that CKII interacts with the transcription elongation factor Spt6p and phosphorylates it in vivo and in vitro. We mapped the phosphorylation sites to the N-terminal unstructured region and demonstrated that the modification of these residues is important for Spt6p function. Interestingly, we show that Spt6p interacts directly with the H3K36 methyltransferase Set2p and that CKII regulates this interaction. Furthermore, CKII directly regulates the assembly of the Spt6p-Iws1p complex both in vivo and in vitro. Finally, we found that CKII is required for the maintenance of Spt6p protein level in specific conditions, suggesting that formation of the Spt6p/Iws1p complex is required for its stability. Altogether, our data indicate that Spt6p function is regulated by a previously unknown mechanism of post-translational modification involving direct phosphorylation by CKII and that modulates the formation of its complex with Iws1p and Set2p.

3.4 Introduction

All DNA-related processes within the cell such as replication, transcription and repair occur in the context of chromatin. The basic unit of chromatin, the nucleosome, consists of 147 base pairs of DNA wrapped around a histone octamer (Kornberg, 1977). To make DNA accessible to the various macromolecules involved in the processes listed above, cells have developed intricate mechanisms to modulate chromatin structure so that the

machinery responsible for a particular process gets access to DNA. Different factors can alter chromatin structure; these include histone modifiers, ATP-dependent chromatin remodelers, histone chaperones and histone variants. Modulation of chromatin structure during transcription elongation has been the focus of several recent studies [reviewed in (Li et al., 2007a; Selth et al., 2010)]. Diverse sets of factors have been identified to play important role in altering chromatin structure during transcription elongation, e.g. SWI/SNF, Chd1p, Spt6p, the FACT complex (Spt16p-Pob3p), Set2p, Set1p and the Paf1p complex [reviewed in (Selth et al., 2010; Sims et al., 2004)].

One of the important factors for chromatin dynamics during transcription elongation is Spt6p. It was initially identified in a classic genetic screen that was aimed at finding mutations suppressing the phenotypes of *Ty* and *δ* insertion mutations in the promoter of the *HIS4* gene (Winston et al., 1984). This screen led to the identification of genes grouped together as *SPT* (*Suppressor of Ty*). Over time, some gene products of this class were shown to be important for chromatin structure and transcription elongation, such as Spt2p, Spt4p/Spt5p, Spt6p and Spt16p [reviewed in (Yamaguchi et al., 2001)]. Around the same time, mutations in *SPT6* were identified that suppress the loss of the chromatin remodeling complex SWI/SNF (Neigeborn et al., 1986) and bypass the requirement of the transcription factor Adr1p for expression of alcohol dehydrogenase II which is encoded by the *ADH2* gene in yeast (Denis, 1984). These initial screens suggested a role of Spt6p in transcription and laid the foundation for subsequent studies, which ultimately established the important role of Spt6p in regulating chromatin structure. Since then, mounting genetic and biochemical evidence suggested a role for Spt6p as a transcription elongation factor. Spt6p shows functional interaction with transcription elongation factors TFIIS and Spt4p/Spt5p (Hartzog et al., 1998). In addition, Spt6p physically interacts with the elongating form of RNAPII (Yoh et al., 2007). Importantly, Spt6p stimulates transcription elongation in vitro (Endoh et al., 2004) and also enhances the elongation rate of RNAPII in vivo (Ardehali et al., 2009). Spt6p is also important for di-and trimethylation of histone H3K36, which are marks of active transcription located in coding regions.

The exact nature of Spt6p function during transcription elongation is unknown. However, genetic and biochemical evidence suggests that Spt6p regulates chromatin structure as a histone chaperone. Spt6p directly interacts with histones and is capable of inducing nucleosome assembly in vitro (Bortvin and Winston, 1996). In addition, Spt6p is important for nucleosome reassembly along promoter regions during transcriptional repression (Adkins and Tyler, 2006; Davie and Dent, 2006). Furthermore, Spt6p controls histone levels in the coding region of some genes, and is important for the proper refolding of chromatin in the wake of transcription elongation, which otherwise leads to spurious transcription from cryptic promoters (Kaplan et al., 2003). Cryptic transcription is widespread in the *spt6* mutant yeast and ≥1,000 genes have shown to undergo cryptic transcription in this mutant (Cheung et al., 2008). Genome-wide studies in the *spt6-1004* mutant show loss of nucleosomes in highly transcribed genes as compared to the WT strain (Ivanovska et al., 2011). Recently, we and others have shown that Spt6p is involved in the maintenance of chromatin repression in the *SER3* promoter at the *SRG1-SER3* locus (Hainer et al., 2011; Thebault et al., 2011).

Spt6p shows physical and functional interaction with different transcriptional elongation factors. Spt6p shows functional interaction with transcription elongation factors Spt4p/Spt5p and TFIIS (Hartzog et al., 1998). Proteomics studies have found Spt6p in complex with other elongation factors like Spt5p, Spt4p, FACT and Iws1p (Krogan et al., 2002; Lindstrom et al., 2003). The only protein that copurifies consistently with Spt6p under different conditions is Iws1p (Interacts with Spt6) (Krogan et al., 2002). Iws1p, also called Spn1p, was also identified in a genetic screen to find suppressors of a post recruitment-defective TBP allele (*spn1* for suppresses post recruitment functions gene number 1) (Fischbeck et al., 2002). Iws1p is an essential protein which is highly conserved among eukaryotes and which plays an important role in the dynamics of chromatin structure. Mutations in Iws1p lead to an Spt$^-$ phenotype and to synthetic lethality with *spt6*, *spt4p*, or *spt5* transcription elongation factor mutants (Lindstrom et al., 2003; McDonald et al., 2010). The interaction of Spt6p with Iws1p is important for chromatin structure and is

required for various steps of transcription and H3K36 methylation (McDonald et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008).

Very little is known on the regulation of Spt6p during transcription elongation. Interestingly, peptides derived from the various subunits of a protein kinase, casein kinase II (CKII), were found in the purified fractions of Spt6p and other transcription elongation factors (Chapter 2; (Krogan et al., 2002). Importantly, Spt6p and two of its interacting partners, namely Iws1p and Spt5p, are phosphorylated at CKII consensus target sequences in vivo (Krogan et al., 2002). Given that CKII interacts with chromatin modulating factors and potentially phosphorylates some of its targets, we wanted to study whether CKII plays a role in chromatin structure by regulating the function of elongating factors. We have shown that CKII phosphorylates and regulates the function of transcription elongation factor Spt2p (Chapter 2). In this study, we have specifically addressed the mechanism underlying the functional interaction between Spt6p and CKII. We found that CKII plays an important role in regulating chromatin structure and suppresses transcription from cryptic promoters. We also found that CKII phosphorylates Spt6p and Iws1p in vivo as well as in vitro. Interestingly our data shows that phosphorylation by CKII regulates the Spt6p-Iws1p interaction as well as the levels of H3K36me3, a marker of active transcription. Finally we show that defects in chromatin structure in a *ckII*^{ts} mutant strain are suppressed by phosphomimetic mutations of Spt6p. Taken together, our data shows that CKII regulates structure and dynamics of chromatin transcribed regions by modulating the function of the Spt6p/Iws1p complex.

3.5 Materials and methods

3.5.1 *S. cerevisiae* strains and plasmids

All strains used in this study are isogenic to S288C (Winston et al., 1995) and are listed in Table 3.1. Strains were constructed by standard methods, either by crosses or by transformation. *SPT6-TAP, CKB2-TAP, SET2-FLAG, CKA1-13MYC, CKA2-13MYC, IWS1-13MYC* alleles were constructed by integrating the DNA encoding the particular

epitope at the 3'-end of the respective gene (Gelbart et al., 2001; Longtine et al., 1998; Puig et al., 2001). The *cka1Δ::KANMX6, set2Δ::KANMX6* alleles were constructed by replacing the open reading frame with *KANMX6* marker (Longtine et al., 1998). The point mutation in the *CKA2* allele (D225N) was introduced as described in (Kitazono et al., 2002) with NatMX6 as a selection marker. The plasmids were constructed using standard molecular biology techniques. GST-fusion and 6-His fusion plasmids were constructed by insertion of PCR amplified fragments into the appropriate sites of the pGEX-4T3 (GE Healthcare) and pet15b vectors (Novagen). The pCC11 (WT SPT6) plasmid is described elsewhere (Clark-Adams and Winston, 1987). Plasmids pCCM1 (Spt6p 3S-A), pCCMA (SPT6 7S-A) and pCCMD (SPT6 7S-D) were generated by mutagenesis of pCC11. Mutagenesis was performed using '*Quik Change Multi* Site-Directed Mutagenesis Kit' from Agilent Technologies following the manufacturer's protocol. All mutations were verified by sequencing. FLAG-SPT6 was amplified from genomic DNA and inserted in the YCp50 plasmid. Plasmid YCp has been described elsewhere (Rose et al., 1987)

3.5.2 Purification of proteins and native chromatin

Recombinant 6His-tagged and GST-tagged proteins were expressed in *E. coli* (BL21). Cells were grown to an optical density of 0.4 at 30°C, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further grown at 18°C overnight. Proteins were purified with Ni^{2+}-NTA agarose (QIAGEN) or glutathione-Sepharose (GE Healthcare) following the manufacturer's protocol. Tandem affinity purification of Spt6p and Ckb2p was carried out as described in section 2.5.2. Yeast native chromatin was prepared as described in Annex 1.

3.5.3 In vitro phosphorylation

In vitro phosphorylation of recombinant proteins was done as described (Utley et al., 2005), with slight modifications. Five hundred ng of recombinant proteins were incubated

with CKII purified from yeast in kinase buffer (final concentrations: 80 mM NaCl/KCl, 25 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 50 µM cold ATP, and 1 µCi of [γ-$^{32}PO_4$]ATP) for 30 min at 30°C. Samples were resolved by 10% SDS-PAGE, and gels were next dried and exposed to film. CKII used in the reaction was purified from yeast (Ckb2p-TAP) as described in section 2.5.2. An aliquot of 0.1 µl of fraction 3 was used for each reaction.

3.5.4 GST-pulldown assays, 6xHis-pulldown assay and co-immunoprecipitation

Three hundred ng of GST-Iws1p coupled to beads were incubated with 300 ng of 6xHis-spt6 in pulldown buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 100 µg/ml BSA, 0.5 mM DTT, 0.1% NP-40, and 2 µg/ml each of leupeptin and pepstatin, 5 µg/ml aprotinin) for 3 h at 4°C. Beads were washed three times with pulldown buffer and bound proteins were analyzed by western blot against a 6xHis-tag antibody. For GST-pulldown with phosphorylated proteins, proteins were first mixed in phosphorylation buffer (final concentrations: 80 mM NaCl/KCl, 25 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 50 µM ATP, and 1 µCi of [γ-$^{32}PO_4$]ATP plus or minus CKII (TAP-Ckb2p) and incubated at 30°C for 30 min. The salt concentration was then raised to 150 mM and the other components of the pulldown buffer were added followed by pulldown as described above. For the His pulldown assay, 300 ng of recombinant 6xHis-Spt6p or 6xHis-Iws1p coupled to Ni-NTA agar beads were incubated with an equal amount of Ckb2p-TAP purified from yeast in pulldown buffer for 3 h at 4°C. Beads were washed three times with pulldown buffer and bound proteins were analyzed by western blot with an antibody against TAP-tag. Co-immunoprecipitation was carried out as described elsewhere (Nourani et al., 2001). Two hundred ml of yeast cultures were grown to an OD_{600} value of 1.0. Cells were harvested and washed with wash buffer (20 mM Hepes (pH 7.5), 150 mM NaCl). Then cells were resuspended in 1 ml of lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 5 mM β-glycerophosphate, 5 mM sodium butyrate, 50 mM NaF, 2 µg each of leupeptin and pepstatin per ml, 5 µg of aprotinin/ml, 1 mM PMSF). Cell lysis was done by bead beating and cell lysate cleared by centrifugation. For

immunoprecipitation of the TAP-tagged protein, cell extract (5 mg of total protein) was incubated with 10 µl of IgG sepharose beads in lysis for 4 h at 4°C. Beads were washed three times with lysis buffer and bound proteins were analyzed by western blot. For immunoprecipitation of FLAG-tagged proteins, 10 µl of anti-FLAG M2 agar beads were incubated with cell extract (5 mg of total protein) in lysis buffer overnight at 4°C. Beads were washed three times with binding buffer. Bound proteins were eluted by 3X-FLAG peptide and analyzed by western blot. For western blotting, samples were boiled in 1X Laemmli buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 0.0625 M Tris HCl (pH 6.8)) for 10 min and separated by SDS PAGE, followed by transfer to nitrocellulose membrane (GE Healthcare). Blots were probed with the following specific antibodies: anti-TAP (CAB1001, Open Biosystems; 1:2500 dilution), anti-FLAG (Sigma F3165; 1:5000 dilution), anti-Myc (MMS-150R, Covance; 1:2500 dilution), anti-6His (631212, Clontech; 1:5000 dilution), anti-H3K36me3 (ab9050, Abcam; 1:2000 dilution) and anti-H4 (ab7311, Abcam; 1:2000 dilution).

3.5.5 RNA extraction and Northern blot

Total RNA was isolated from exponentially growing yeast cultures using the hot phenol method (Schmitt et al., 1990). Northern blot analysis was performed as described previously (Nourani et al., 2006). Forty µg of RNA were dissolved in 16 µl of buffer (25 mM MOPS (pH 7.5), 5 M Na acetate, 1 mM EDTA, 8% formaldehyde and 60% formamide), heated to 70ºC for 10 min, chilled on ice for 5 min and centrifuged briefly before loading on the gel. RNA was separated on denaturing agar gel (1% agar in MOPS containing formaldehyde). The RNA was then transferred to a nylon membrane, crosslinked and hybridized with labeled probe. The *FLO8* and *SCR1* probes were amplified by PCR and radiolabeled by random priming. The sequences of oligonucleotides used for the amplification of these probes are listed in Table 2.2.

3.5.6 Histone methyltransferase assay

Recombinant purified 6His-Set2p (100 ng) was incubated with short oligonucleosomes (500 ng) from yeast along with 50 μM *S*-adenosylmethionine (SAM) in methyltransferase buffer (final concentrations: 50 mM Tris [pH 9.0], 1 mM PMSF, and 0.5 mM DTT) for 30 min at 30°C. Samples were resolved by SDS-PAGE and analyzed for H3K36me2 and H3K36me3 by western blotting.

Table 3.1: *S. cerevisiae* strains used

Strain	***Genotype***	***Source***
YW0031	*MATa ura3-52/ura3D0 his3D200 leu2D1 lys2-128d spt6Δ0::KANMX6/pCC11 (*WT *SPT6 on plasmid)*	*This work*
YW0032	*MATa ura3-52/ura3D0 his3D200 leu2D1 lys2-128d spt6Δ0::KANMX6/pCCMA*	*This work*
YW0033	*MATa ura3-52/ura3D0 his3D200 leu2D1 lys2-128d spt6Δ0::KANMX6/pCCMD*	*This work*
YW0034	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SPT6-TAP::URA3*	*This work*
YW0035	*MATa ura3D0/ura3-52 his3Δ200 leu2Δ1 lys2-128δ SPT6-TAP::URA IWS1-13Myc::KANMX6*	*This work*
YW0036	*MATα ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 SPT6-TAP::URA3*	*This work*
YW0037	*MATα ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N:v :NATMX4 SPT6-TAP::URA3 IWS1-13Myc::KANMX6*	*This work*
YW0038	*MATa ura3D0 his3Δ200 leu2Δ1 lys2-128δ SET2-FLAG::KANMX4*	*This work*
YW0039	*MATa ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 SPT6-TAP::URA3 SET2-*	*This work*

	FLAG::KANMX4	
YW0040	*MATa ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 SPT6-TAP::URA3 SET2-FLAG::KANMX4*	*This work*
YW0041 (set2Δ ckII^{ts})	*MATa ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 set2Δ0::KANMX6*	*This work*
YW0042 (cdc73Δ ckII^{ts})	*MATa ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 cdc73Δ::KANMX6*	*This work*
YW0043 (spt16-179 ckII^{ts})	*MATa ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2D225N::NATMX4 cdc73Δ::KANMX6*	*This work*
YTS07 (ckII^{ts})	*MATα ura3D0/ura3-52 leu2Δ1 his3Δ200 lys2-128δ cka1Δ0::KANMX6 cka2 D225N::NATMX4 spt16-197*	*This work*

3.6 Results

3.6.1 CKII is important for proper chromatin structure

Casein kinase II (CKII) subunits interact with many factors important for chromatin dynamics during transcription elongation such as Spt2p, Spt6p, Spt16p, Spt5p and Chd1p (Chapter 2; (Krogan et al., 2002)), but the exact role of CKII in modulating chromatin structure during transcription elongation has not been studied. Mutations in different factors important for chromatin structure and transcription elongation result in spurious transcription from cryptic promoters (Carrozza et al., 2005; Cheung et al., 2008; Kaplan et al., 2003; Keogh et al., 2005). To investigate whether CKII plays a role in chromatin dynamics, we constructed a conditional mutant of CKII (*ckII^{ts}*) and analyzed the transcripts of the model gene *FLO8* in this mutant. *FLO8* contains cryptic promoters located within its coding region which are activated when chromatin is not properly refolded after RNAPII-mediated transcription. As shown in Figure 3.1A, Northern blot analyses performed on total RNA isolated from *ckII^{ts}* cells show very high levels of short cryptic transcripts in the *FLO8* gene as compared to WT cells. These results show that CKII plays an important role in the maintenance of proper chromatin structure in coding regions.

Di- and trimethylation of histone H3K36 by Set2p is an important mark of active transcription and these methylation states have been shown to be important for the suppression of cryptic transcription (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Lee and Shilatifard, 2007). These methylation states are regulated by various transcription elongation factors like Spt6p, the Paf1p complex and the Bur1p-Bur2p complex (Carrozza et al., 2005; Chu et al., 2007; Chu et al., 2006; Youdell et al., 2008). As CKII subunits were found to copurify with some of these factors, we investigated whether CKII plays a role in H3K36 methylation. Immunoblotting of whole-cell extracts from WT and *ckII*^{ts} with antibody against H3K36me3 and H3K36me2 reveals that CKII activity is required for the maintenance of H3K36me3 but not for H3K36me2 as shown in Figure 3.1B. This observation further suggests that CKII plays an important role in the modulation of chromatin associated with transcription elongation.

To establish the function of CKII in chromatin structure and transcription elongation, we elected to study the genetic interaction of CKII with transcription elongation factors directly involved in chromatin structure dynamics. For this, we generated different double mutants of various transcription factors in combination with *ckII*^{ts} and looked at various phenotypes related to the maintenance of chromatin structure. Interestingly, single- and double-mutant phenotype analysis shows a genetic interaction between *CKII* and both *SPT16* and *CDC73*, but not with *SPT6* and *SET2* (Figure 3.1C). Combining *ckII*^{ts} with the *cdc73*Δ mutation led to strong growth defects at the various temperatures tested, while mutations in the *SPT16* gene partly suppressed the growth defects of *ckII*^{ts} at 33ºC. Combining *ckII*^{ts} and either the *set2*Δ or *spt6-140* mutations did not result in obvious synthetic interactions. Given that *SET2* deletion and mutations in the *SPT6* gene exhibit defects in chromatin structure similar to those found in *ckII*^{ts} mutants and since combining *ckII*^{ts} with either of these mutations does not lead to a synthetic phenotype, we postulated that CKII, Spt6p and Set2p act in the same pathway that modulates chromatin structure during elongation.

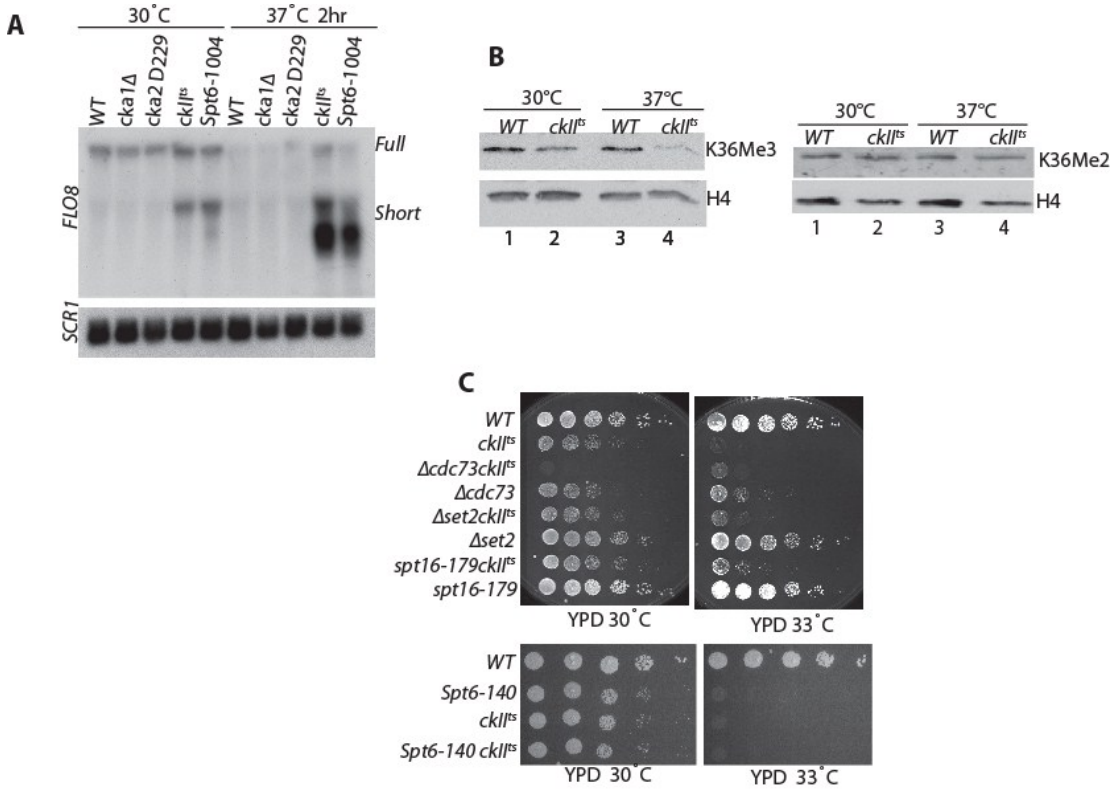

Figure 3.1 : CKII regulates chromatin structure during transcription elongation
(A) CKII is important for the suppression of spurious transcription from the *FLO8* cryptic promoter. WT*, cka1*Δ*, cka2 D225N, ckII^{ts} and spt6-1004* strains were grown at 30°C or at 39°C as indicated. Total RNA was isolated and analyzed by Northern blot with a probe specific to the 3'-end of *FLO8*. *SCR1* served as a loading control. The *FLO8* probe clearly shows full-length transcripts and short transcripts originating from a cryptic promoter within the ORF of *FLO8*. (B) CKII regulates the methylation state of H3K36. Total cell extracts from WT and *ckII^{ts}* cells grown in YPD at 30°C or 39°C for 2 h were probed with antibodies against H3K36me3, H3K36me2 and H4. A clear drop is seen in H3K36me3 levels in *ckII^{ts}* cells at 39°C as compared to WT (compare lanes 4 and 3 in *left* panel). No effect is observed on H3K36me2 levels in *ckII^{ts}* mutants (*right* panel). (C) *CKII* genetically interacts with transcription elongation factors involved in chromatin modulation. Serially diluted aliquots from WT*, ckII^{ts}, set2*Δ, *set2*Δ *ckII^{ts}*, *cdc73*Δ*, cdc73*Δ *ckII^{ts}, spt16-179,* and *spt16-179 ckII^{ts}* cultures were seeded in fresh YPD at the indicated temperatures. The *ckII^{ts}* mutation is synthetically sick with the *cdc73*Δ mutation and the *spt16-179* mutation suppresses the growth defects observed in *ckII^{ts}* cells at 33°C. The *set2*Δ *ckII^{ts}* and *spt6-140 ckII^{ts}* double mutants showed no obvious synthetic phenotypes.

3.6.2 Spt6p interacts with Set2p and this interaction is dependent on CKII

Di-and trimethylation of H3K36 is an important mark associated with transcription elongation. Methylation of H3K36 is catalyzed by the Set2p methyltransferase which is recruited to regions of active transcription through its interaction with the CTD of RNAPII phosphorylated at S2 (Kizer et al., 2005). This methylation mark of H3K36 catalyzed by Set2p plays an important role in the restoration of chromatin structure in the wake of elongating RNAPII (Carrozza et al., 2005; Keogh et al., 2005). Moreover, a drop in H3K36me3 levels is observed in *ckII*^{ts} mutants. In order to investigate if CKII directly regulates Set2p methyltransferase activity, we used the in vitro methyltransferase assay on purified proteins. Yeast native chromatin from *set2*Δ cells was used as a substrate for recombinant Set2p in the presence or absence of CKII and ATP. As shown in Figure 3.10, CKII has no direct effect on Set2p methyltransferase activity in vitro. We next assessed whether the in vivo function of Set2p is affected by CKII. Spt6p plays an important role in regulating the trimethylation of H3K36 in yeast (Youdell et al., 2008). The mechanistic details of how Spt6p regulates methylation of K36 remain unknown. Interestingly, in human cells, Sp6/Iws1p interacts with HYPB/Set2p, and this interaction regulates the trimethylation of H3K36 (Yoh et al., 2008). To determine if such an interaction also occurs in yeast, we generated a strain that coexpresses TAP-tagged Spt6p and FLAG-tagged Set2p. We immunoprecipitated Spt6p-TAP and analyzed the precipitated samples by western blot. As shown in Figure 3.2A, Set2p-FLAG co-precipitates with Spt6p-TAP, and this interaction between Spt6p and Set2p could play an important role in regulating H3K36 trimethylation.

Given that Spt6p interacts with Set2p (Figure 3.2A), we were interested to see whether this interaction is compromised in *ckII*^{ts} mutants, thereby accounting for the drop in trimethylation levels. We immunoprecipitated Spt6p-TAP from WT and *ckII*^{ts} strains and analyzed the samples by western blot for the association of Set2p-FLAG with Spt6p-TAP. As expected, Set2p co-immunoprecipitates with Spt6p in the WT strain, but this interaction

is lost in the *ckII*^{ts} mutant strain (Figure 3.2B). Taken together, our data show that Spt6p interacts with Set2p in vivo and that this interaction is regulated by CKII.

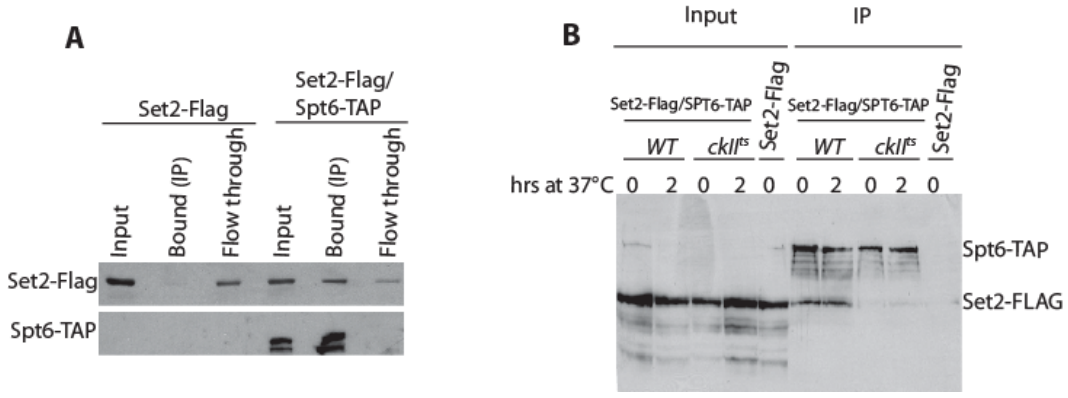

Figure 3.2: The Spt6p-Set2p interaction is dependent on CKII
(A) Spt6p interacts with Set2p. Total cell lysates from strains expressing Spt6p-TAP and Set2p-FLAG were immunoprecipitated using IgG beads. The precipitated proteins were analyzed by western blot with antibodies against the FLAG and TAP tags. A cell lysate from a strain expressing only Set2p-FLAG was used as control. (B) The Spt6p-Set2p interaction is dependent on CKII activity. WT and *ckII*^{ts} strains expressing Spt6p-Tap and Set2p-FLAG were grown at 30°C followed by heat shock at 37°C for 2 h. Cell extracts were prepared, and Spt6p-TAP was immunoprecipitated with IgG beads. Precipitated samples were analyzed by western blot with an antibody against FLAG-Tag. A cell extract from a stain expressing only Set2p-FLAG served as a control.

3.6.3 CKII interacts with and phosphorylates Spt6p in vitro and in vivo

Our data indicate a role of CKII in the modulation of chromatin structure during transcription. We next aimed to address the mechanism responsible for the effect of CKII activity in this process. Several observations initially suggested that CKII might fulfill this function by regulating Spt6p activity. First, we had observed that CKII regulates the Spt6p-Set2p interaction. Second, our genetic analyses indicated that CKII subunit genes and *SPT6* are epistatic and act in the same pathway. Third, peptides from the various subunits of CKII

had been identified by mass spectrometry in tandem affinity-purified Spt6p samples (Gavin et al., 2002; Krogan et al., 2002). Fourth, Spt6p was found to be phosphorylated at three CKII consensus motifs (Krogan et al., 2002). To investigate the functional relationship between CKII and Spt6p, we first assessed whether this kinase stably interacts with Spt6p in vivo. For this purpose, we purified FLAG-Spt6p from yeast strains containing two epitope-tagged CKII subunits. As shown in Figure 3.3A, the CKII subunits Cka1p-Myc and Cka2p-Myc copurified with FLAG-Spt6p. To test if this interaction is direct, we performed a His pulldown assay using purified 6xHis-Spt6p or 6xHis-Iws1p. Ckb2p purified from yeast by TAP was incubated with 6xHis-Spt6p or 6xHis-Iws1p coupled with Ni-NTA agarose. As shown in Figure 3.3B, Ckb2p-TAP interacts with 6xHis-Spt6p and 6xHis-Iws1p. This observation led us to conclude that CKII interacts directly with the Spt6p complex in vivo.

We next determined whether Spt6p is phosphorylated by CKII. To test whether Spt6p is a direct target for CKII phosphorylation, we performed an in vitro phosphorylation assay using recombinant 6xHis-Spt6p and tandem affinity-purified Ckb2p from yeast. CKII was able to incorporate [γ]-$^{32}P_i$ into Spt6p, as shown in Figure 3.3C. This clearly indicates that Spt6p is a direct substrate for CKII in vitro and suggests that this kinase might phosphorylate Spt6p in vivo. Interestingly, it has previously been shown that Spt6p is a phosphoprotein in vivo. In fact, Spt6p is phosphorylated at various CKII consensus motifs (Albuquerque et al., 2008; Krogan et al., 2002). To investigate if Spt6p is phosphorylated by CKII in vivo, we immunopurified FLAG-Spt6p from WT and *ckII*^{ts} yeast strains and analyzed the purified material by western blot with an antibody against phosphoserine/phosphothreonine. As shown in Figure 3.3D, Spt6p was clearly recognized by the anti-phosphoserine/phosphothreonine in WT extracts, further confirming that Spt6p is indeed a phosphoprotein in vivo. This signal was significantly altered in the *ckII*^{ts} mutant at the restrictive temperature, suggesting an important role for CKII in the regulation of the phosphorylation state of Spt6p in vivo. Taken together, our data clearly indicate that CKII interacts directly with Spt6p and phosphorylates this elongation factor both in vitro and in vivo.

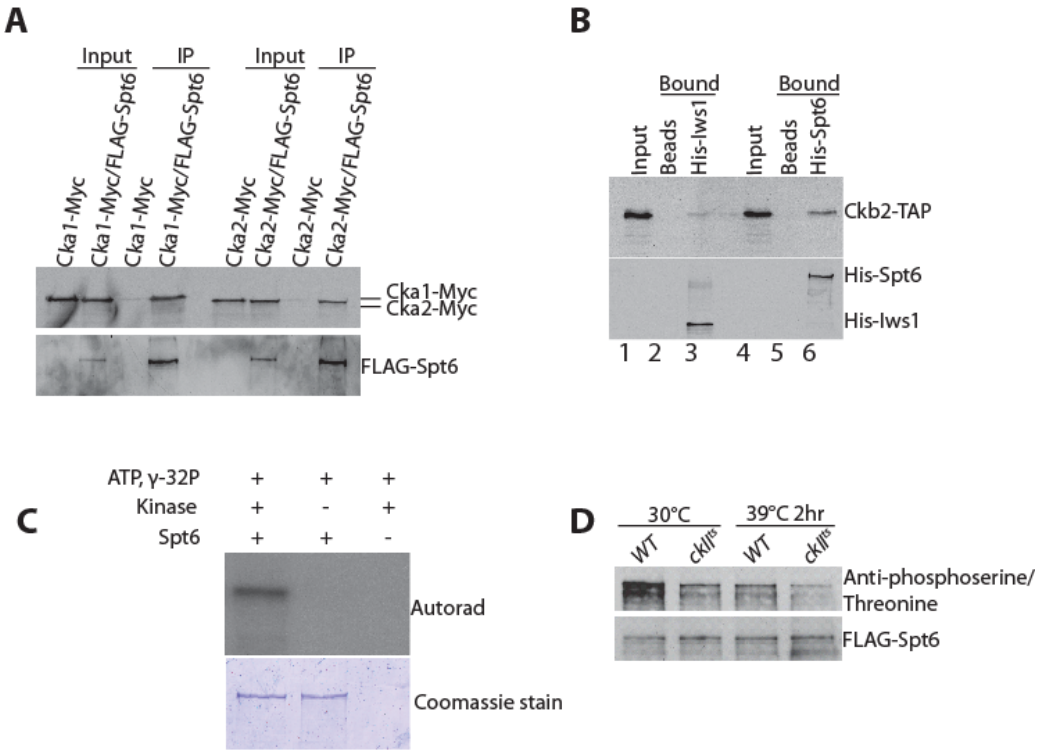

Figure 3.3 : CKII Interacts with Spt6p and phosphorylates it in vivo and in vitro
(A) Spt6p interacts with different subunits of CKII in vivo. FLAG-Spt6p was immunopurified from strains also expressing Cka1p-Myc or Cka2p-Myc and the purified samples were analyzed by western blot with antibodies against the Myc and FLAG epitopes. A purification control was obtained using strains expressing either Myc-tagged Cka1p or Cka2p-Myc but tag-free Spt6p. (B) Spt6p interacts with CKII in vitro. A His pulldown assay was performed using equal amounts of Ckb2p-TAP purified from yeast and recombinant 6xHis-Spt6p (lane 6) or 6xHis-Iws1p (lane3). Ni-NTA agar beads alone served as a control (lanes 2 & 5). (C) CKII phosphorylates Spt6p in vitro. Recombinant 6xHis-Spt6p was incubated with CKII purified from yeast (lane1) or was mock-reacted (no CKII) (lane 2) in the presence of [γ-$^{32}PO_4$] ATP. The proteins were resolved by SDS-PAGE followed by Coomassie staining and autoradiography. The *top* panel shows the autoradiogram and the *bottom* panel illustrates Coomassie staining of the gel. (D) Spt6p is phosphorylated in vivo in a CKII-dependent manner: FLAG-Spt6p was immunopurified from WT and *ckII*^{ts} strains grown at 30°C or following a 2-hour heat shock at 39°C. The purified samples were analyzed by western blot with antibodies against phosphoserine/phosphothreonine or the FLAG epitope.

3.6.4 The Spt6p-Iws1p interaction is dependent on phosphorylation by CKII

Our data indicate that CKII and Spt6p are involved in the modulation of chromatin structure during transcription elongation. We have also shown that *SPT6* and the CKII subunit genes are epistatic with respect to the latter function. Furthermore, we found that CKII interacts directly with the Spt6p complex and controls the phosphorylation state of Spt6p. The next important question we addressed is how phosphorylation by CKII might regulate Spt6p function. Spt6p shows physical and functional interactions with other transcription elongation factors like Spt4p/Spt5p and Iws1p (Krogan et al., 2002; Swanson and Winston, 1992). The physical interaction between Spt6p and Iws1p is quite stable and plays a crucial role in the maintenance of a proper chromatin structure (Krogan et al., 2002; McDonald et al., 2010). Moreover, this interaction is conserved and is also observed in human cells. Recently, the interacting domains of Spt6p and Iws1p have been mapped at high precision and the crystal structure of the Spt6p–Iws1pcomplex determined (McDonald et al., 2010). Mutations at the interface between Spt6p and Iws1p lead to severe growth defects at higher temperatures as well as defects in chromatin structure and the appearance of a Spt$^-$ phenotype. Mutations that completely disrupt the Spt6p-Iws1p interaction are lethal (McDonald et al., 2010). Taken together, these observations suggested that the Spt6p/Iws1p interaction is required for chromatin reassembly. Interestingly, similar to Spt6p, Iws1p is phosphorylated at the CKII consensus sequence in vivo (Albuquerque et al., 2008; Krogan et al., 2002; Li et al., 2007c). We therefore hypothesized that phosphorylation might regulate Spt6p ability to regulate chromatin structure by regulating the Spt6p-Iws1p interaction. To address the latter hypothesis, we tagged Spt6p with TAP epitopes in WT and *ckII*^{ts} strains and purified it from these two strains grown at both permissive and restrictive temperatures. In Figure 3.4A, purified samples were analyzed by SDS-PAGE and proteins were visualized by silver staining. As expected, TAP of the Spt6p-Iws1p complex from the WT strain at permissive temperature produced two clear bands corresponding to Spt6p and Iws1p sizes, respectively. Interestingly, a significant reduction in Iws1p levels is observed in the *ckII*^{ts} strain at the permissive temperature. Moreover, TAP of the Spt6p-Iws1p complex from the *ckII*^{ts} strain at the restrictive

temperature indicated a clear loss of Iws1p protein. We next used an alternative approach to confirm that CKII activity is required for the maintenance of a normal Spt6p-Iws1p interaction. In this experiment, we tagged Spt6p with the FLAG tag in strains expressing Iws1p-Myc in the WT or *ckII*^{ts} strains. We purified FLAG-tagged Spt6p and determined the levels of Iws1p-Myc by western blot in the various strains described. As shown in Fig. 4B, a clear drop in Iws1p protein levels after purification of FLAG-labeled proteins in the *ckII*^{ts} mutant at both the permissive and restrictive temperatures. This approach confirmed that depletion of intracellular CKII activity leads to an important loss of Spt6p/Iws1p complex in vivo.

3.6.5 Direct phosphorylation by CKII is required for the assembly of the Spt6p-Iws1p complex

Although our data suggest that the direct phosphorylation of Spt6p by CKII may have an impact on the formation of the Spt6p/Iws1p complex, we cannot rule out an indirect, non-specific effect of the depletion of an essential kinase with multiple essential roles. To address the hypothesis that direct phosphorylation events control the assembly and/or stability of the Spt6p/Iws1p complex, we used recombinant Spt6p and Iws1p to test if direct phosphorylation of these proteins by purified CKII has a role in stability the Spt6p/Iws1p complex. We performed GST pulldown assays using GST-Iws1p and 6xHis-Spt6p in the presence or absence of a functional CKII. The recombinant proteins were either phosphorylated prior to pulldown or used as without prior in vitro phosphorylation. Following pulldown, the input proteins and the bound fractions were analyzed by western blot. As shown in Figure 3.4C, a clear increase in the level of 6xHis-Spt6p bound to GST-Iws1p was detected when in vitro phosphorylation was carried out prior to the binding reaction, indicating that phosphorylation allowed a more efficient interaction between Spt6p and Iws1p before pulldown. Thus, both in vivo and in vitro results clearly support a role for CKII-mediated phosphorylation in the Spt6p-Iws1p interaction. Taken together, these data clearly suggest that direct phosphorylation by CKII regulates the formation of the Spt6p/Iws1p complex.

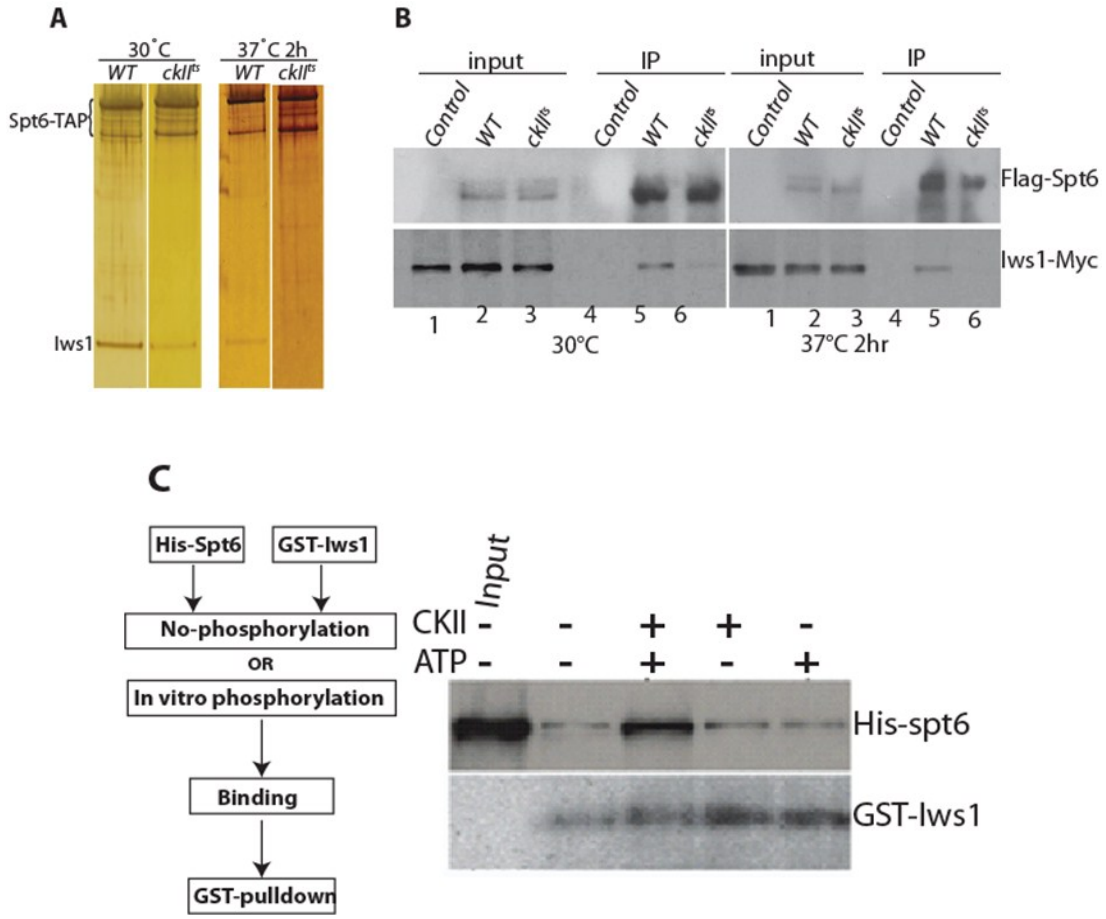

Figure 3.4 : CKII regulates the Spt6p-Iws1p interaction
(A) TAP of Spt6p**:** Spt6p TAP was carried out using *WT* and *ckII*^{ts} strains grown at 30°C or after a 2-hour heat shock at 39°C. Purified proteins were resolved by SDS-PAGE (4-12% gradient gels) and visualized by silver staining**.** Loss of Iws1p is observed in Spt6p-TAP isolated from *ckII*^{ts} cells. (B) FLAG purification of Spt6p**:** Extracts from WT and *ckII*^{ts} cells expressing FLAG-Spt6p and Iws1p-Myc were incubated with anti-FLAG M2 agar beads. Bound proteins were eluted from the anti-FLAG beads with FLAG peptide. The eluted fractions were probed with anti-Myc and anti-FLAG antibodies. Control purification was done using a strain expressing Iws1p-Mys but untagged Spt6p. (C) In vitro interaction GST pulldown assay: GST pulldown was done using recombinant proteins GST-Iws1p and 6xHis-Spt6p. GST itself served as negative control. Pulldown was preceded or not by in vitro phosphorylation by CKII. Input and bound proteins were resolved by 10% SDS-PAGE and analyzed by western blot with antibodies against the 6xHis-tag and GST. Phosphorylated proteins clearly interact more efficiently than non-phosphorylated proteins (compare lane 3 with other lanes).

3.6.6 Mutation of CKII consensus sequences impairs Spt6p function

We have shown that direct phosphorylation of Spt6p and/or Iws1p by CKII is required for the normal assembly of the Spt6p/Iws1p complex both in vivo and in vitro. However, our observations raise a number of questions regarding the exact molecular mechanism underlying this regulation. Various hypotheses could be formulated to account for the mechanism involved. First, Spt6p phosphorylation might trigger the interaction with Iws1p. An equally valid possibility would be that direct modification of Iws1p by CKII is the key event in the regulation of Spt6p/Iws1p interaction. A third hypothesis would be that phosphorylation at different sites on both proteins is in fact required for assembly of the complex.

Importantly, our data clearly indicate that the state of Spt6p phosphorylation is controlled by CKII. We therefore aimed to identify the sites in Spt6p that are phosphorylated by CKII and to then determine whether phosphorylation controls the Spt6p-Iws1p interaction. Previously, 3 potential CKII phosphorylation sites (S94, S134 and S206) in Spt6p were shown to be indeed modified in vivo (Krogan et al., 2002). We purified recombinant Spt6p with all three CKII target sites mutated to alanyl residues and subjected the mutant and WT proteins to in vitro phosphorylation using CKII purified from yeast. As expected, the Spt6p mutant protein in which the three CKII target sites had been eliminated exhibited a reduced level of total phosphorylation as compared to the WT form. However, a residual level of Spt6p phosphorylation was still present in that mutant protein, as shown in Figure 3.5 B. These results indicate that additional CKII phosphorylation sites are present in Spt6p. Indeed, five CKII phosphorylation target sites have been reported for Spt6p in vivo (Albuquerque et al., 2008; Li et al., 2007c).

Interestingly, all the above-mentioned phosphorylation sites (including the 3 sites studied in our mutagenesis experiments described above) are located in the N-terminal region of Spt6p. Furthermore, using the NetPhosK 1.0 server, we predicted a total of 7 potential CKII target sites in the N-terminal region of Spt6p. We therefore elected to mutate all seven

CKII target sites to alanyl residues and to produce and collect the resulting recombinant protein. We then subjected the mutant protein thus generated to in vitro phosphorylation by purified CKII as discussed above. As shown in Figure 3.5 B, no [γ]-$^{32}P_i$ was incorporated from radiolabeled ATP into a mutant Spt6p protein in which all 7 CKII potential target sites had been eliminated, indicating that these sites include all CKII phosphorylation sites actually used in vivo.

Next, to analyze the function of these CKII target sites, we constructed strains expressing Spt6p mutant forms in which the various CKII target sites were selectively eliminated. To get insight on the role of CKII-mediated Spt6p phosphorylation, we probed a set of phenotypes associated with Spt6p function. For instance, one aspect of the *SPT* phenotype is a defect in Spt6p function that suppresses Ty and δ insertion mutations in the promoter of the *HIS4* and *LYS2* genes. Thus, a WT strain with a *lys2-128δ* allele is unable to grow in the absence of lysine. A defect in Spt6p function suppresses this phenotype and the strain is no longer auxotrophic for lysine. To exploit this characteristic, we first evaluated the *SPT6* mutant allele bearing 3 potential CKII sites (S94, S134 and S206) with S→A substitutions. As shown in Figure 3.11, WT cells (*SPT6*) are lysine auxotrophs whereas cells expressing the *spt6* (3S-A) mutation grew to some extent on lysine-free media. This result indicates that Spt6p is partly dysfunctional when 3 out of the 7 potential CKII target sites are mutated to alanyl residues. However, when all 7 CKII target sites were mutated to alanyl residues, cells exhibited a more pronounced loss of lysine auxotrophy, as shown in Fig. 3.5C. The latter observation suggests that Spt6p function is more severely affected upon the loss of all CKII phosphorylation target sites. The observed loss of Spt6p function was also associated with increased thermosensitivity. When all seven CKII target sites had undergone S→A substitutions, cells grew normally at 30°C but showed strong growth defects at 37°C (Figure 3.5 C). This is in agreement with our observation that mutating all 7 identified CKII target sites completely abolishes Spt6p phosphorylation.

At the molecular level, defective Spt6p function is associated with spurious transcription from cryptic promoters within the coding region of transcriptionally active genes. This phenotype is a direct consequence of defective chromatin refolding during transcription

elongation. *FLO8* is a model gene for cryptic transcription studies. To evaluate cryptic transcription in yeast expressing *spt6* mutants resistant to CKII-mediated phosphorylation, we determined *SPT6* mRNA levels by Northern blot analysis. RNA from strains expressing *SPT6* (WT), *spt6* (3S-A) or *spt6* (7S-A) was extracted and following electrophoresis, analyzed with a probe specific for the 3'-end of *FLO8*. We detected significant levels of short cryptic transcripts in yeast expressing *spt6* (7S-A) whereas no such transcripts were observed in yeast expressing either *SPT6* (WT) or *spt6* (3S-A) (Figs. 3.5D and 3.11). This result further supports the notion that CKII target sites found in the N-terminal region of Spt6p are important for the function of that protein, and mutation of all CKII target residues severely impair its function.

We also investigated the effect of constitutive phosphorylation on Spt6p function. For that purpose, we constructed yeast strains expressing an Spt6p mutant protein with all seven CKII target Ser residues changed to phosphomimetic Asp residues (7S-D). Cells were grown at different temperatures and on media lacking lysine to assess thermosensitivity and lysine auxotrophy, respectively as characteristics of the Spt$^-$ phenotype. Our results show that *spt6* (7S-D) cells grow on media lacking lysine (Figure 3.5E) although they were able to grow normally at the temperatures tested (Figure 3.5E). These data therefore indicate that mutations mimicking constitutive phosphorylation also impair Spt6p function to a partial extent. Taken together, these results show that phosphorylation of Spt6p is tightly regulated and that reversible phosphorylation is required for the proper function of Spt6p.

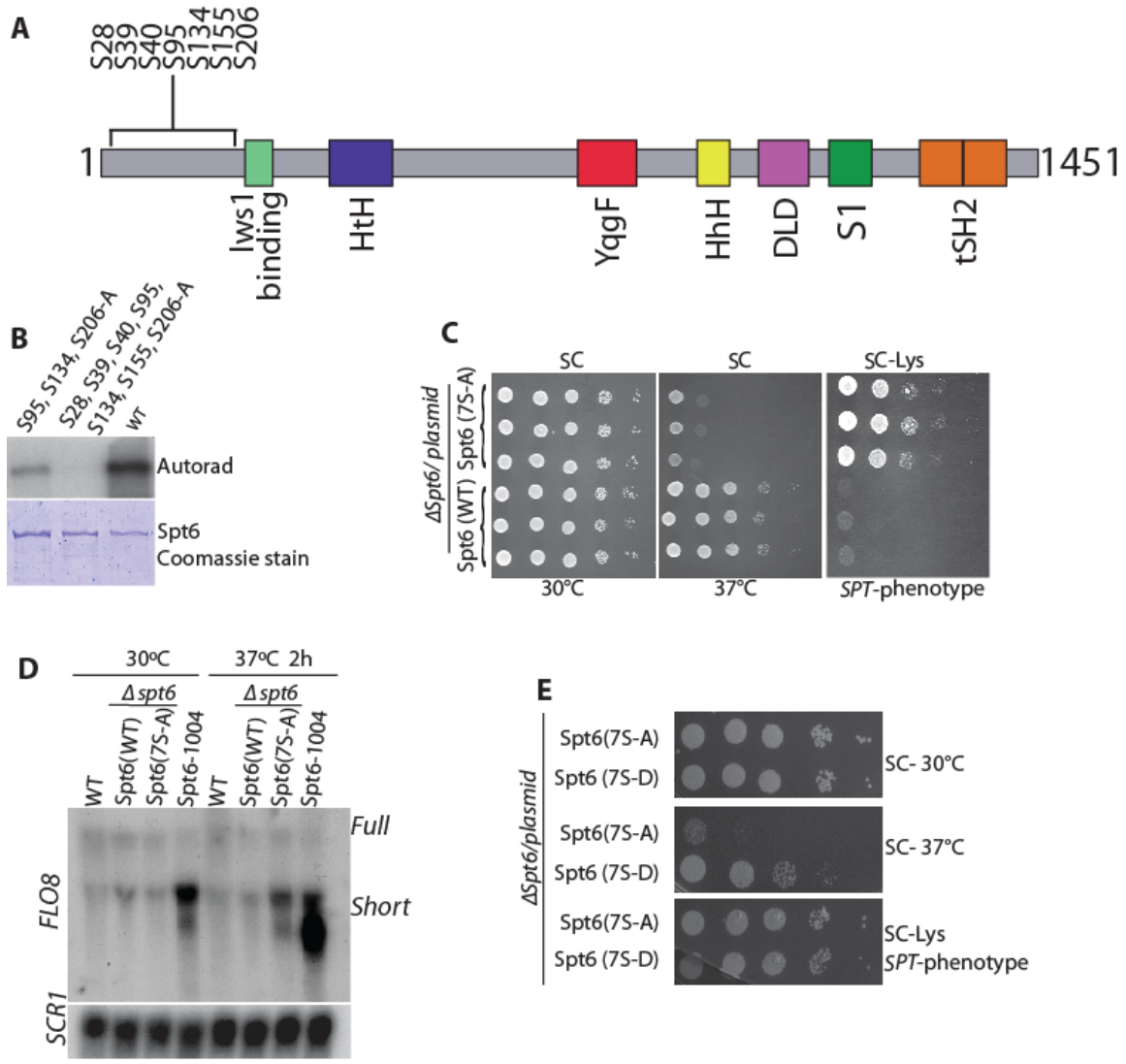

Figure 3.5 : Mutations of the Ser residues within CKII consensus motifs impair Spt6p function

(A) Schematic representation of various domains of Spt6p. The Iws1p-binding domain and phosphorylation sites are all found in the N-terminal region of Spt6p. **(B)** Recombinant 6xHis-Spt6p and the indicated Spt6p mutants were incubated with CKII purified from yeast in the presence of [γ-$^{32}PO_4$] ATP. Proteins were resolved by SDS-PAGE followed by Coomassie staining and autoradiography. *Top panel* shows the autoradiogram and *bottom* panel shows *Coomassie* stain**. (C)** Mutation of CKII consensus residues in Spt6p leads to thermosensitivity and loss of lysine auxotrophy: one SPT6 allele was deleted in WT diploid cells and the mutant was then transformed with plasmids pCC11 *(*WT *SPT6) or* pCCMA (spt6/7S-A) or pCCMD (spt6/7S-D). Diploids were then allowed to sporulate. After dissection, serially diluted aliquots of cultures of representative progenies were spotted on synthetic complete (SC) medium at 30°C or 37°C or SC medium lacking lysine (*SPT* phenotype). **(D)** Mutations of CKII consensus residues in Spt6p result in cryptic

transcription: WT, *spt6Δ/PCC11 and spt6Δ/PCCMA* control cells were grown in YPD at the indicated temperatures. The *spt6-1004* strain was used as positive control. Total RNA was isolated and analyzed by Northern blot with a probe specific for 3'-end of *FLO8*. *SCR1* served as loading control. **(E)** Screening *SPT6* mutants for an SPT phenotype: serially diluted aliquots of *spt6Δ/PCC11 and spt6Δ/PCCMD* cultures were grown on SC medium at 30°C or 37°C or lysine-free SC medium.

3.6.7 CKII-mediated phosphorylation consensus motifs control the Spt6p-Iws1p interaction in vivo.

We established that phosphorylation by CKII *regulates* the interaction between Spt6p and Iws1p and that mutation of CKII consensus residues impairs Spt6p function. Whether Spt6p phosphoserine residues are important for the Spt6p-Iws1p interaction is unclear. To directly demonstrate the importance of these CKII target sites in the Spt6p-Iws1p interaction, we performed a GST pulldown assay using GST-Iws1p and 6xHis-Spt6p (WT or 7S-A). In this case, recombinant proteins were either phosphorylated prior to the pulldown or used in their native form. Our results show that WT Spt6p interacts more strongly with Iws1p when the former is phosphorylated, as shown in Figure 3.6A. In contrast, phosphorylation had almost no effect on the Spt6p-Iws1p interaction when Spt6p target sites for CKII are mutated, clearly showing the importance of these 7 residues in the interaction between Spt6p and Iws1p. To substantiate our in vitro data, we elected to study the Spt6p-Iws1p interaction in strains expressing an Spt6p mutant where all CKII target serine residues had been substituted to alanyl residues. We constructed yeast strains expressing FLAG-*SPT6* (both WT and CKII target site mutants) and *IWS1*-Myc. FLAG Spt6p was purified and proteins were analyzed by western blot using an antibody against the Myc tag. As expected, Iws1p-Myc copurified with WT Spt6p (Figure 3.6B). Interestingly, the amounts of Iws1p co-eluting with the Spt6p CKII site S→A mutants were drastically reduced, as shown in Figure 3.6B. The latter results clearly demonstrate the importance of the CKII target sites for the Spt6p-Iws1p interaction in vivo.

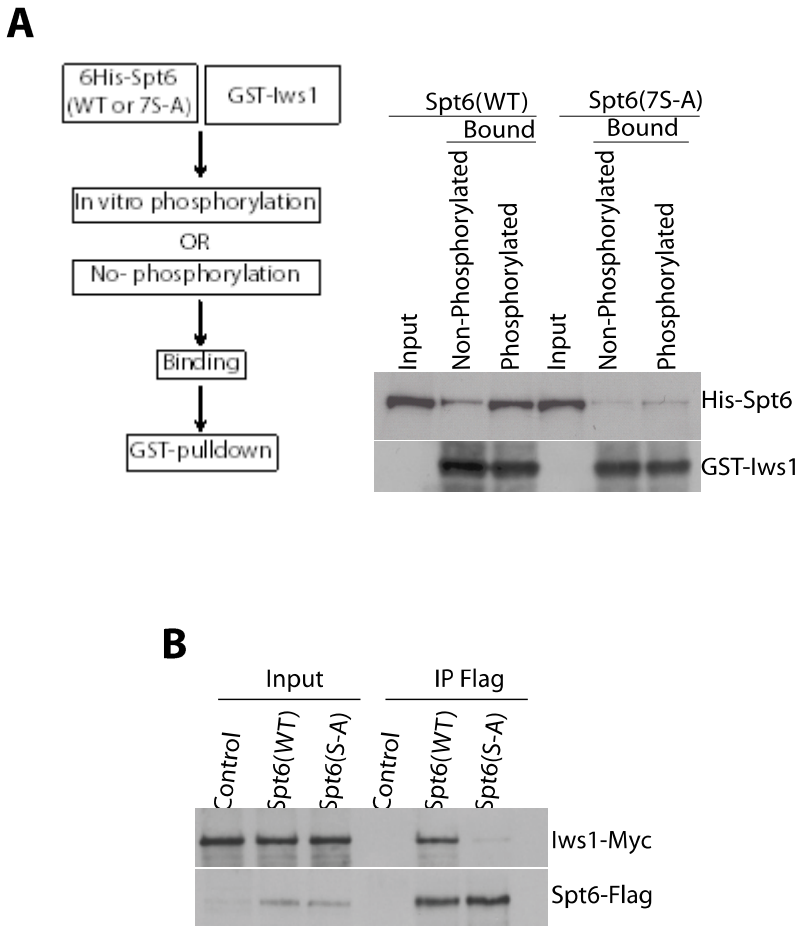

Figure 3.6: A non-phosphorylatable Spt6p mutant (7S-A) interacts less strongly with Iws1p

(A) A GST pulldown assay was performed using recombinant GST-Iws1p and 6xHis-Spt6p proteins (WT or with a 7S→A substitution). The pulldown was preceded or not by in vitro phosphorylation. Input and bound proteins were resolved by 10% SDS-PAGE and analyzed by western blot with antibodies against 6xHis-tag and GST. (B) FLAG-Spt6p (WT) or FLAG-Spt6p (7S-A) were immunoprecipitated from strains also expressing Iws1p-Myc using anti-FLAG beads. Bound proteins were eluted by FLAG peptide and analyzed by western blot with antibodies against the FLAG and Myc epitopes. The S→A mutation of all CKII consensus residues result in a decreased association between Iws1p and Spt6p.

3.6.8 The phosphomimetic mutant of Spt6p rescues the loss of Spt6p-Iws1p complex in *ckII*^{ts} mutants

The data presented thus far show that direct phosphorylation of Spt6p by CKII is critical for the formation of the Spt6p-Iws1p complex. This would suggest that a phosphomimetic Spt6p mutant might interact more strongly with Iws1p than WT Spt6p. To assess this hypothesis, we carried out a GST pulldown assay using GST-Iws1p and 6xHis-Spt6p (wild type or 7S→D). As shown in Figure 3.7A, a Spt6p mutant in which all CKII target Ser residues had been mutated to Asp (7S-D) interacts more strongly with GST-Iws1p than WT Spt6p. We have shown earlier that the Spt6p-Iws1p interaction is lost in the *ckII*^{ts} mutant strain. If such a defect is a direct consequence of the loss of Spt6p phosphorylation, we should be able to restore formation of complex by expressing a phosphomimetic mutant of Spt6p in *ckII*^{ts} mutant strain. To test this, we expressed FLAG-Spt6p (both wild type and 7S-D mutant) in a *ckII*^{ts} strain also expressing Iws1p-Myc. We performed a FLAG affinity purification of Spt6p and analyzed the purified proteins by western blot. As shown in Figure 3.7B, we clearly observe an increase in the amount of Iws1p that co-purifies with Spt6p (7S-D) as compared to WT Spt6p. This further supports the idea that phosphorylation of Spt6p by CKII plays an important role in Spt6p-Iws1p interaction.

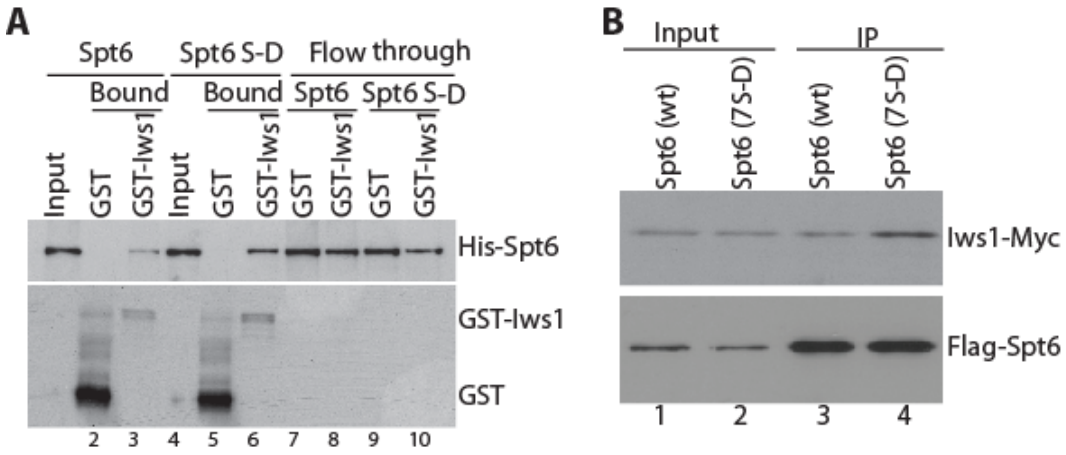

Figure 3.7 : A phosphomimetic mutant of Spt6p (7S-D) interacts efficiently with Iws1p.
(A) A GST pulldown assay was performed using recombinant GST-Iws1p and 6xHis-Spt6p proteins (wild type or 7S→D mutant) with GST as a control. Input, bound and unbound proteins were analyzed by western blot with antibodies against 6xHis and GST. The Spt6p S→D mutant protein (phosphomimetic) interacts more with Iws1p than WT Spt6p (compare lanes 6 and 3). (B) FLAG-Spt6p (WT) or FLAG-Spt6p (7S-D) were purified from the *ckII*^{ts} strain also expressing Iws1p-Myc. Purified proteins were analyzed by western blot with antibodies against the FLAG and Myc epitopes. Iws1p interacts more strongly with a phosphomimetic Spt6p mutant.

3.6.9 Phosphorylation and Spt6p stability under nutritional stress

CKII activity is required for normal re-entry into the cell cycle from stationary phase after cell cycle arrest (Hanna et al., 1995). CKII also phosphorylates Cdc28p, and this phosphorylation is important for the regulation of total protein content and cell size during stationary phase (Russo et al., 2000). Furthermore, CKII plays an important role in the tight regulation of RNA polymerase III activity, which is important for cell cycle arrest in stationary phase and for normal growth once conditions become favorable again (Ghavidel and Schultz, 1997).

Our data thus far suggested that Spt6p/Iws1p complex assembly is dependent on CKII. Because of the important role of CKII in re-entry of quiescent cells into the cell cycle, we examined the dynamics of the Spt6p-Iws1p complex under these conditions. We thus

immunoprecipitated Spt6p-TAP from WT and *ckII*^{ts} (also expressing Iws1p-Myc) strains in stationary phase and at different time points after addition of fresh medium and ensuing de novo progression into the cell cycle (Figure 3.8A). As expected, Iws1p co-immunoprecipitated with Spt6p in WT, but not in *ckII*^{ts} cells (Figure 3.8B). Surprisingly, we did not detect Spt6p-TAP in these fractions. The latter finding led us to hypothesize that Spt6p might be degraded in *ckII*^{ts} cells during stationary phase. Spt6p purified from WT cells generally contains substoichiometric amounts of Iws1p. However, during stationary phase, the Spt6:Iws1p ratio is lower than in exponentially growing cells (Figure 3.8B). These results suggest that the fraction of total Spt6p that interacts with Iws1p might be protected while "free" Spt6p (i.e. not involved in a complex with Iws1p) is degraded during stationary phase. Since the Spt6p-Iws1p interaction is lost in the *ckII*^{ts} strain, such protection afforded by the latter interaction might account for the net loss in Spt6p detected in stationary-phase *ckII*^{ts} cells.

From the above observations, we next assessed whether the loss in Spt6p might be rescued in stationary-phase *ckII*^{ts} cells. We transformed *ckII*^{ts} with plasmids expressing the FLAG-Spt6p (both as WT and the 7S-D mutant forms). We prepared cell extracts in log phase, stationary phase and at different time intervals after adding fresh medium to stationary-phase cells and analyzed total cell extracts by western blot with the antibodies against the FLAG tag. We did not detect Spt6p in stationary phase, but in *ckII*^{ts} strain, levels of Spt6p (7S-D) mutant protein were restored earlier than for the WT protein upon the addition of fresh medium (Figure 3.8C). The restoration of Spt6p (7S-D) mutant protein was similar in the *ckII*^{ts} and WT strains, which clearly shows the existence of a correlation between Spt6p phosphorylation and the amount of Spt6p protein level present upon re-entry into the cell cycle.

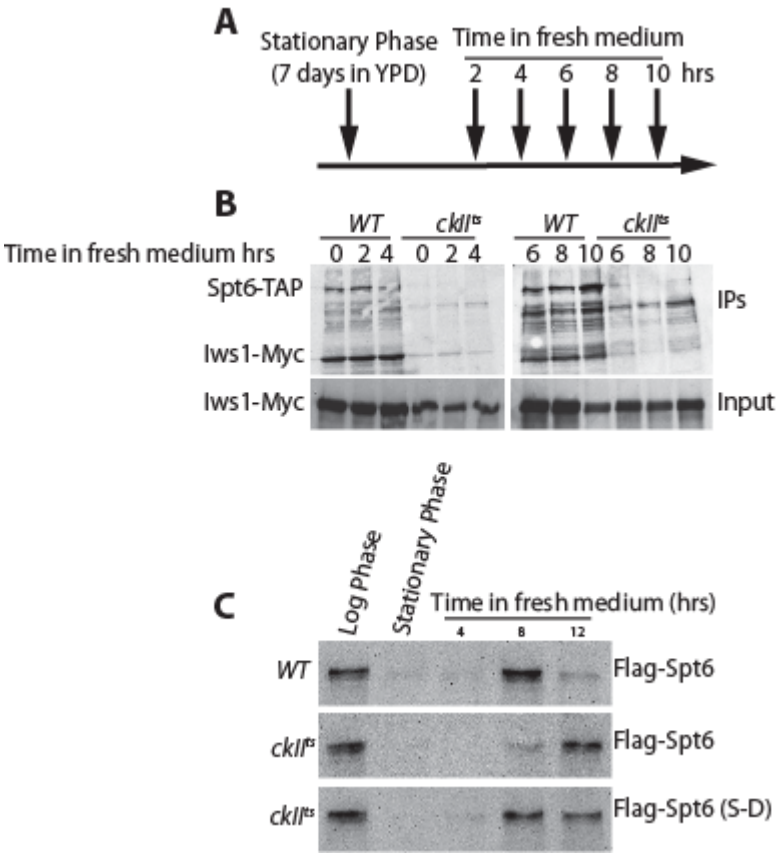

Figure 3.8 : Spt6p-Iws1p interaction in stationary phase is dependent on phosphorylation by CKII

(A) Diagram showing the experimental procedure. Samples were collected at the time points indicated by vertical *arrow*s. Stationary phase is represented by *t*=0 in fresh medium. (B) WT and *ckII*^{ts} strains expressing Spt6p-TAP and Iws1p-Myc were grown to stationary phase, followed by the addition of fresh medium at *t*=0. Samples were collected at different time intervals as indicated and Spt6p-TAP was immunoprecipitated with IgG beads. Immunoprecipitated samples were analyzed by western blot with an antibody against the Myc tag to detect Iws1p-Myc associated with Spt6p. (C) Total cell lysates from WT and ckII^{ts} strains expressing Spt6p-FLAG (in its WT and 7S-D mutant forms) grown under the conditions indicated were probed with an antibody against the FLAG epitope.

3.6.10 Phosphomimetic Spt6p mutations rescue the chromatin defects in *ckII*^{ts} cells

Our data have thus far demonstrated the importance of CKII in the refolding of chromatin. We have also shown that CKII phosphorylates Spt6p and regulates its interaction with Iws1p. This phosphorylation plays an important role in the modulation of chromatin structure and in the inhibition of spurious transcription. However, CKII targets other elongation factors and the phenotypic manifestations of spurious transcription might also result from the action of CKII towards such other factors. To evaluate if the chromatin defects associated with CKII depletion result specifically from defective phosphorylation of Spt6p, we determined whether a phosphomimetic Spt6p mutant form might suppress the chromatin defects found in a *ckII*^{ts} strain. For that purpose, we constructed a *ckII*^{ts} strain expressing a phosphomimetic version of Spt6p (i.e. in which all seven potential CKII phosphorylation sites had undergone an S→D substitution, or 7S-A mutant) in WT and *ckII*^{ts} strains. Using a *FLO8* 3'-end probe, we were able to detect full-length as well as short cryptic transcripts in *ckII*^{ts} strains transformed with the empty vector (Fig. 3.9). However, the level of short cryptic transcripts was significantly decreased when a phosphomimetic mutant form of Spt6p was expressed in the *ckII*^{ts} strain. In contrast, there was no effect on the amount of short transcripts detected in *ckII*^{ts} cells submitted to heat shock and expressing a non-phosphorylatable Spt6p mutant protein (Fig. 3.9). This clearly demonstrates that the chromatin defects found in the *ckII*^{ts} strain are directly correlated with defective Spt6p phosphorylation.

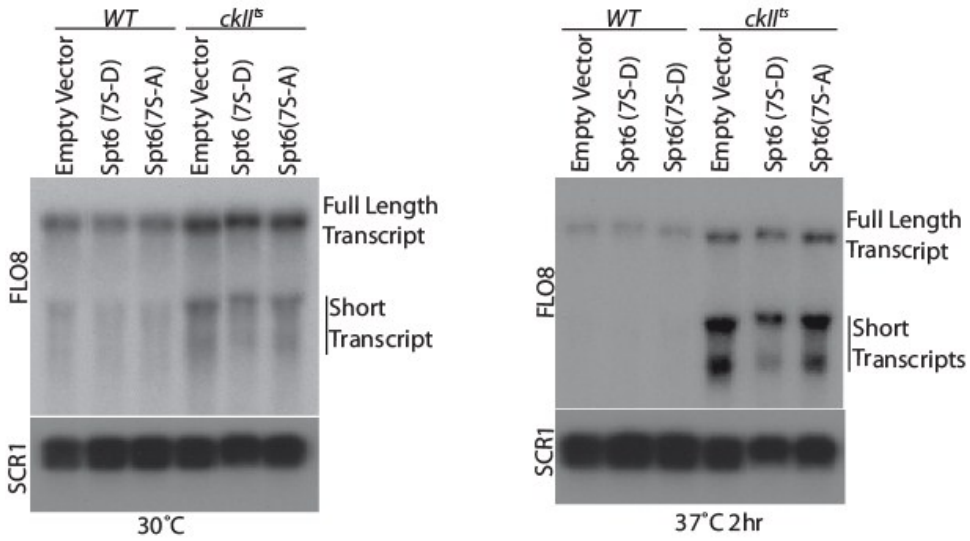

Figure 3.9 : S→D mutations of the whole set of CKII target sites in Spt6p suppress chromatin defects in *ckII*^{ts} cells
WT and *ckII*^{ts} strains were transformed with plasmids encoding CKII-phosphomimetic (*spt6* (7S-D)) or non-phosphorylatable *spt6* (7S-A) (relative to CKII), or with empty vector. The strains were grown in uracil-free SC medium (SC-URA) at 30°C and temperature was then shifted to 37°C for 2 h. Total RNA was extracted and analyzed by Northern blot using a probe for *FLO8* 3'-end. *SCR1* served as a loading control. The *FLO8* probe identifies both full-length *FLO8* mRNA and the short transcript, which has been previously shown to initiate from a cryptic promoter within the coding region of *FLO8*.

3.7 Discussion

The proper refolding of chromatin during transcription elongation is tightly regulated and multiple mechanisms act in concert to fold the chromatin properly in the wake of RNAPII activity. Defective refolding of chromatin after RNAPII-mediated transcription results in transcription initiation and elongation from intragenic cryptic promoters (Carrozza et al., 2005; Cheung et al., 2008; Kaplan et al., 2003). Cryptic promoters are widespread and ≥1,000 genes (17% of all genes) exhibit spurious transcription in the *spt6-1004* mutant (Cheung et al., 2008). The set of defectively transcribed regions is conserved among eukaryotes. Indeed, a recent report has shown the occurrence of spurious transcripts resulting from defects in chromatin structure during transcription elongation in mammalian cells (Lin and Workman, 2011; Xie et al., 2011). It has been previously reported that CKII

subunits associate with various factors that are important for chromatin structure dynamics and suppression of cryptic transcription (Gavin et al., 2002; Krogan et al., 2002). However, the role of CKII in regulating chromatin structure remains largely unknown. Herein we demonstrate for the first time that CKII is of key importance for the restoration of chromatin structure during transcription elongation. Our data shows a clear role of CKII in chromatin structure as exemplified by cryptic transcription observed in the *ckII*^{ts} mutant strain. Importantly, we have observed that the role of CKII is mediated through the regulation of the function of histone chaperone Spt6p. We provide evidence that Spt6p is phosphorylated by CKII both in vivo and in vitro. Next, we have mapped the phosphorylated residues to the N-terminal region of Spt6p and shown that these residues are crucial for Spt6p function. Moreover, we show that phosphorylation of Spt6p by CKII regulates the interaction between Spt6p and Iws1p. This interaction plays a pivotal role in the regulation of chromatin structure during transcription. Finally, we were able to rescue chromatin structure defects of *ckII*^{ts} mutants with a Spt6p phosphomimetic mutant. Altogether, these observations indicate that CKII controls the modulation of chromatin structure associated with transcription via its regulation of the Spt6p/Iws1p complex

Yeast CKII consists of two catalytic and two regulatory subunits. Deletion of both catalytic subunits is lethal whereas deletion of a single catalytic subunit entails no oblivious growth defects, an indication of a certain degree of functional redundancy of the catalytic subunits (Padmanabha et al., 1990). Deletion of catalytic *CKA1* gene and point mutation in *CKA2* result in a conditional mutant exhibiting strong defects in chromatin structure at the restrictive temperature (cf. Fig. 3.1). The fact that different subunits of CKII copurify with various chromatin modulating factors led us to hypothesize that CKII could act via one or more of these factors and that defective chromatin structure in *ckII*^{ts} results from the mis-regulation of one or more of these factors. One of the potential targets of CKII identified through our studies is the histone chaperone Spt6p. It is phosphorylated in vivo at CKII consensus motifs and our genetic analyses indicate that CKII and Spt6p are epistatic and act in the same pathway in the regulation of chromatin structure. Our results confirm the interaction of Spt6p with the various subunits of CKII and show that Spt6p is indeed a

direct target of CKII. Our proteomic studies have shown that Spt6p is phosphorylated at three CKII consensus sites (S94, S134 and S206) in vivo (Krogan et al., 2002). Our in vitro data shows that CKII phosphorylates Spt6p even if these 3 sites are mutated, raising the possibility of additional sites of Spt6p phosphorylation by CKII. Search for additional potential CKII target sites in adjacent regions led to the identification of four additional consensus sites for phosphorylation by CKII in the N-terminal region of Spt6p. In addition, there are reports that five out of these seven potential CKII targets are phosphorylated in vivo (Albuquerque et al., 2008; Krogan et al., 2002; Li et al., 2007c). We mutated all seven CKII target sites and found that CKII cannot phosphorylate Spt6p anymore in the resulting mutants. This data suggests that Spt6p is phosphorylated by CKII and that these post-translational modifications may be of utmost importance in the regulation of Spt6p function.

Spt6p interacts with Iws1p in a relatively stable manner. Both proteins are phosphorylated in vivo at CKII consensus sites (Krogan et al., 2002). The interaction of Spt6p with Iws1p is important for transcription and chromatin structure (McDonald et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Since both proteins are phosphorylated at CKII target sites in vivo, phosphorylation might potentially regulate this interaction. Indeed, we found that the Spt6–Iws1p interaction is partially lost in *ckII*^{ts} mutants under permissive conditions and that this loss is aggravated under restrictive conditions (Fig. 3.4). GST pulldown assays using recombinant proteins which were phosphorylated in vitro by CKII clearly demonstrate a direct role of phosphorylation in the Spt6p-Iws1p interaction (Figure 3.4). Whether phosphorylation of either protein or of both proteins is required for their interaction was not clear. Spt6p had previously been shown to be phosphorylated at three CKII consensus sites (S95, S135 and S207) (Krogan et al., 2002). Mutation of these residues results in partial loss of Spt6p phosphorylation and function as evidenced by the resulting weak Spt$^-$ phenotype. However, this phenotype is far less pronounced than that associated with *ckII*^{ts} mutants which exhibit high levels of cryptic transcription and decreased H3K36 trimethylation. Phosphorylation of additional residues is thus likely important for the regulation of Spt6p function. Mutation of all 7 residues in

the N-terminal region of Spt6p entailed a complete loss of phosphorylation in vitro and further loss of Spt6p function in vivo, as seen by the extent of cryptic transcription, the relative severity of Spt$^-$ phenotype and the strong growth defects observed at higher temperature. Furthermore, the Spt6p-Iws1p interaction was lost in this mutant. More importantly, we were able to restore an Spt6p/Iws1p complex in *ckII*^{ts} mutants by provision of Spt6p bearing phosphomimetic mutations at CKII target sites. Our observations therefore clearly indicate that phosphorylation of Spt6p by CKII regulates the Spt6p/Iws1p complex. However, a functional role of Iws1p phosphorylation cannot be ruled out at this stage. No evidence of CKII consensus residue phosphorylation other than S89 has yet been reported for Iws1p. We found that mutation of this residue did not result in any detectable phenotype associated with Iws1p function (data not shown). However, using NetPhosK 1.0, six other potential CKII target sites were predicted for Iws1p, although none of these residues were reported to be phosphorylated in vivo. We do not rule out the possibility that phosphorylation of these residues by CKII might be important for Iws1p function. Interestingly, we have observed a small increase in Spt6p-Iws1p interaction after in vitro phosphorylation by CKII even upon mutation of the 7 CKII target sites present in Spt6p (Figure 3.6A). Phosphorylation of Iws1p might be responsible for this small increase in Spt6p-Iws1p interaction. Notwithstanding these uncertainties, our data strongly suggest that CKII phosphorylates Spt6p and controls its function through its regulation of the Spt6p-Iws1p interaction.

Intriguingly, all identified phosphoserine residues lie in the N-terminal domain of Spt6p and are adjacent to the region that interacts with Iws1p. Recently, McDonald et al. have mapped precisely the regions in Spt6p and Iws1p that are sufficient for their mutual interaction. Using structural and biochemical approaches, these authors have shown that the Spt6p region interacting with Iws1p spans residues 239 to 268 (McDonald et al., 2010). It is important to note that all of these studies were carried out using Spt6p polypeptides lacking a complete N-terminal region. Nonetheless, in connection with that problem, our data clearly shows that CKII target sites (lying in a region adjacent to the domain interacting with Iws1p) are of key importance in Spt6p function and its interaction with

Iws1p. Therefore, the model proposed by McDonald et al. should be refined to account for the important role played by the N-terminal region through its CKII-mediated phosphorylation. Two hypotheses can be put forward regarding the possible function of the phosphorylation of the N-terminus by CKII. A *first* possibility is that the N-terminal extremity inhibits the Spt6p-Iwsi interaction and this inhibition is relieved by CKII-mediated phosphorylation. Indeed, there are reports showing that the binding properties of a particular domain are inhibited by an immediately adjacent region (Close et al., 2011). In fact, the central core region of Spt6p is able to bind DNA when examined in isolation (i.e. without the two extremities of the polypeptide chain, but this binding ability is lost in the full protein because of the unstructured N-terminal region which contains the sites of phosphorylation by CKII (Close et al., 2011). An *alternative* hypothesis is that phosphorylation acts as a regulatory switch that changes the binding affinity of Spt6p towards different substrates. In the recent study by McDonald et al., Spt6p was shown to bind nucleosomes and Iws1p via the same domain (McDonald et al., 2010) and it was proposed that interaction of Spt6p with Iws1p favors its release from chromatin after histones are loaded onto chromatin. How the preferred interaction partner of Spt6p is switched from chromatin to Iws1p is not clear. Phosphorylation by CKII could in fact act as a regulatory switch, with phosphorylation favoring the interaction of Spt6p with Iws1p and the release of Spt6p after the reassembly of chromatin. Further studies are required in order to evaluate the two above-mentioned hypotheses on the possible role of phosphorylation in this process.

Spt6p plays a vital role in regulating the trimethylation of H3K36. How Spt6p plays its role is still unclear. We have herein shown for the first time that Spt6p interacts with Set2p in vivo in yeast. This interaction is highly dynamic and is lost at high salt concentrations. The Spt6p-Set2p interaction could be important for H3K36 trimethylation. Interestingly, we have found that this interaction (Spt6p-Set2p) is in fact indirect (data not shown). Importantly, the Spt6p-Set2p as well as Spt6p-Iws1p interactions are lost in *ckII*^{ts} mutants, suggesting that Iws1p could be the link between Spt6p and Set2p. In human cells, it is known that Spt6 interacts with HYPB/Set2 and that the interaction is mediated via Iws1

(Yoh et al., 2008). Depletion of Iws1 in human cells results in the disruption of the Spt6-HYPB/Set2 interaction and the loss of H3K36 trimethylation (Yoh et al., 2008). Our data in yeast are in keeping with the mechanisms described in human cells. The possibility that Iws1p is the link between Spt6p and Set2p could account for the loss of the Spt6p-Set2p interaction in *ckII*^{ts} mutants. Regardless of the actual mechanism involved, our observations show that CKII might regulate H3K36 trimethylation by modulating the ternary Spt6p/Iws1p-Set2p interaction.

Another interesting result from this study is the almost complete loss of Spt6p in *ckII*^{ts} mutants in stationary phase. CKII is important for normal re-entry into the cell cycle after stationary phase arrest (Hanna et al., 1995). CKII also phosphorylates Cdc28p, an event which is important for the regulation of total protein content and cell size in stationary phase cells (Russo et al., 2000). The important role of CKII in stationary phase led us to study the Spt6p-Iws1p interaction in this phase. As expected, in WT cells, Spt6p levels were reduced in stationary phase compared to exponentially growing cells. Surprisingly, in the *ckII*^{ts} mutant, Spt6p was almost completely lost in stationary phase, although Iws1p levels were comparable. Generally, Spt6p purified from WT cells contains sub-stoichiometric amounts of Iws1p, but in stationary phase, the Spt6:Iws1p ratio was lower than in exponentially growing cells (Figure 3.8A). Co-immunoprecipitation experiments in stationary phase and at different time intervals after adding fresh medium indicated that in the WT strain, the fraction of Spt6p associated with Iws1p is maintained in stationary phase. Since phosphorylation by CKII is important for the Spt6p-Iws1p interaction, such stabilization of Spt6p-Iws1p complex formation would explain the almost complete loss of Spt6p observed in *ckII*^{ts} mutants. After addition of fresh medium to stationary phase cells, Spt6p protein levels are restored much earlier in WT cells than in *ckII*^{ts} cells. Importantly, in *ckII*^{ts} cells expressing a phosphomimetic Spt6p mutant form (7S-D), protein levels are restored similar to a WT strain. This clearly demonstrates a role for CKII in the stabilization of a pool of Spt6p protein during stationary phase (starvation) and the restoration of normal levels of Spt6p when conditions become favorable again (e.g. upon the addition of fresh media).

Finally, our data suggest a previously unknown and important role of CKII in the modulation of chromatin structure. We provide evidence that CKII plays its role via the phosphorylation of Spt6p and the regulation of the Spt6p-Iws1p interaction. We were able to partially suppress the chromatin defects in *ckII*^{ts} mutants by transformation with a vector expressing *SPT6* bearing phosphomimetic mutations of the protein CKII target sites. This clearly demonstrates a direct link between the chromatin defects observed in *ckII*^{ts} mutations and Spt6p function. Spt6p-Iws1p interaction is critical for proper chromatin structure and this interaction is lost in *ckII*^{ts} mutants. Phosphomimetic mutant forms of Spt6p are able to restore Spt6p/Iws1p complex formation in *ckII*^{ts} mutants, which could explain the suppression of chromatin defects detected in this mutant. Our observations open a novel avenue of research on the modulation of chromatin structure and the regulation of spurious transcription, where post-translational modification of elongation factors indirectly controls this process.

3.8 Supplementary Figures

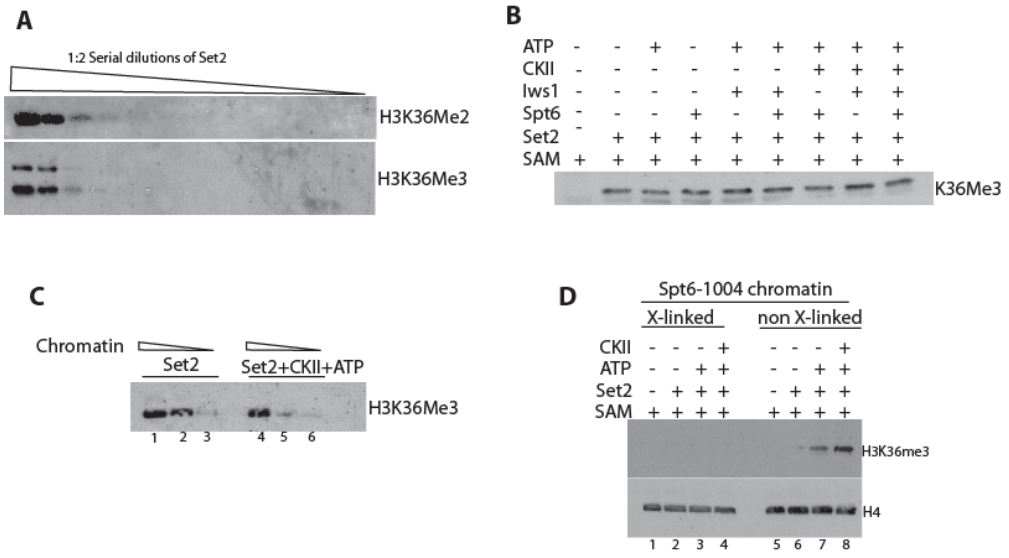

Figure 3.10: Supplementary Figure 1
(A) Serial dilutions (1:1 in methyltransferase buffer) of recombinant 6His-Set2p solutions were incubated with yeast native chromatin (from an *set2*Δ strain) in the presence of SAM (50 µM) for 30 min at 30°C. Samples were resolved by 15% SDS PAGE and analyzed by western blot with antibodies against H3K36me2 and H3K36me3. (B) Yeast native

chromatin from a *set2*Δ strain was incubated with SAM (50 μM) and recombinant Set2p in the presence or absence of various factors as indicated. Samples were analyzed by western blot with an antibody against H3K36me3. (C) Yeast chromatin was incubated with Set2p and SAM in the presence or absence of CKII and ATP followed by western blot analysis with an antibody against H3K36me3. (D) The *spt6*-1004 strain was grown in YPD at 30°C. Cells were then either subjected or not to formaldehyde fixation followed by sonication. Extracts from the two fixation conditions were next incubated with recombinant Set2p and SAM (50 μM) in the presence or absence of ATP and CKII as indicated. Samples from each reaction type were then analyzed by western blot against H3K36me3 and H4 antibodies.

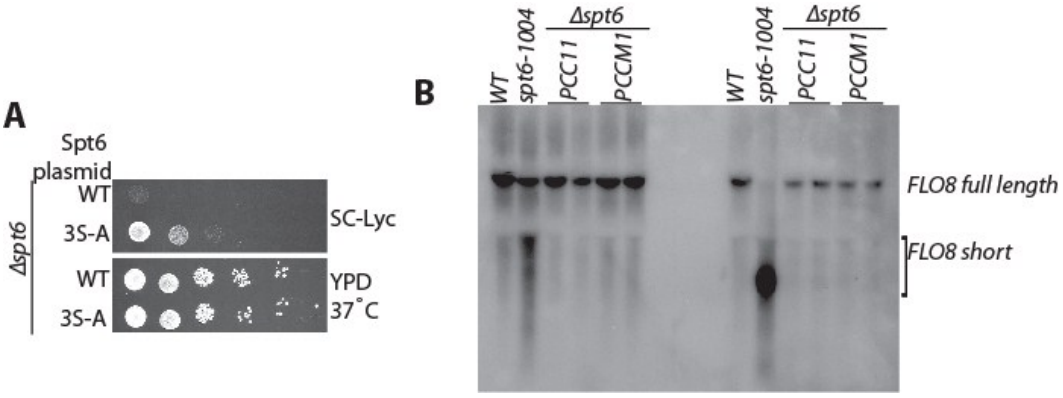

Figure 3.11: Supplementary Figure 2
Mutation of three CKII consensus residues of Spt6p results in a weak *SPT* phenotype.
(A) One SPT6 allele was deleted in WT diploid cells and transformed with plasmids pCC11 *(*WT *SPT6)* or pCCM1 (*spt6*/3S-A*)*. Diploids were then allowed to sporulate. After dissection, serially diluted aliquots of representative progeny cultures were spotted on selective medium (lysine-free SC, SC-Lys) and YPD.
(B) WT*, spt6-1006*, *spt6*Δ*/PCC11* and *spt6*Δ*/PCCM1* strains were grown at 30°C and temperature was then shifted to 37°C for 2 h. Total RNA was isolated and analyzed by Northern blot with a probe specific for the 3'-end of the *FLO8* gene.

Chapter 4

Discussion and Conclusion

4.1 CKII is required for the proper refolding of chromatin structure after the action of elongating RNAPII

Proper refolding of chromatin during the course of transcription is tightly regulated. Defects in chromatin refolding have been linked to spurious transcription from cryptic promoters within the coding regions of different genes (Carrozza et al., 2005; Cheung et al., 2008; Kaplan et al., 2003). Cryptic transcription is a widespread phenomenon in yeast, with nearly 1000 genes displaying such aberrant transcription. In a recent report, cryptic transcription resulting from defective refolding has been reported in mammals (Lin and Workman, 2011; Xie et al., 2011)

Different factors can suppress cryptic transcription, including histones, regulators of histone genes, chromatin remodeling factors, transcription elongation factors and histone chaperones (Cheung et al., 2008). CKII subunits have been associated with some of these factors. Indeed, subunits of CKII have been found to be associated with different factors that modulate chromatin structure during transcription elongation (Gavin et al., 2002; Krogan et al., 2002). In this work, our data further support a physical link between CKII and elongation factors, suggesting a possible role of CKII in chromatin structure dynamics and suppression of cryptic transcription.

CKII is a ubiquitous serine/threonine kinase which has been highly conserved among eukaryotes. In yeast, CKII is made of two catalytic (α and α') and two regulatory (β and β') subunits. Deletion of both catalytic subunits is lethal whereas deletion of a single catalytic subunit entails no obvious growth defects, which suggests some level of functional redundancy (Padmanabha et al., 1990). We constructed a conditional mutant (*ckII*^{ts}) to study the role of CKII in chromatin structure dynamics. Our results show for the first time that CKII plays a role in chromatin refolding during transcription elongation. In the *ckII*^{ts} mutant, we observed strong phenotypes linked with defects in chromatin structure. One of the hallmarks of defective chromatin structure is spurious transcription from intragenic cryptic promoters. Cryptic transcription in the *FLO8* gene has been studied and the cryptic promoter has been precisely mapped within this gene. This gene is widely used as a model to study defects in chromatin refolding. In the *ckII*^{ts} mutant strain, Northern analysis with a probe specific for the 3'-end of *FLO8* gene has shown the presence of short transcripts

originating from the cryptic promoter located within the coding region (Figure 3.1A).The role of CKII in chromatin refolding was further supported by its functional interaction with other factors important for chromatin structure dynamics during transcription elongation. CKII shows functional interaction with the Paf1p complex and Spt16p (Figure 3.1B). These factors are important for the restoration of chromatin structure after completion of transcription elongation by RNAPII. In addition, we also found a decrease in the trimethylation of H3K36 in the *ckII*^{ts} strain. Trimethylation of H3K36 is a mark of active transcription and plays an important role in the restoration of chromatin structure during transcription elongation. Taken together, our data clearly demonstrate a role for CKII in chromatin refolding during transcription elongation.

How CKII plays its role in chromatin modulation associated with elongation is an important question. The CKII subunits are associated with different transcription elongation factors such as Spt16p, Spt6p/Iws1p, Spt4p/Spt5p, and Spt2p (Chapters 2 and 3) (Gavin et al., 2002; Krogan et al., 2002). All of these factors are involved in chromatin dynamics during transcription elongation and suppress transcription from cryptic promoters. The defects in chromatin refolding observed in *ckII*^{ts} mutants might result from the defective properties of one or more of these factors. A schematic representation of the various hypotheses is depicted in Figure 4.1 and will be discussed in the following sections.

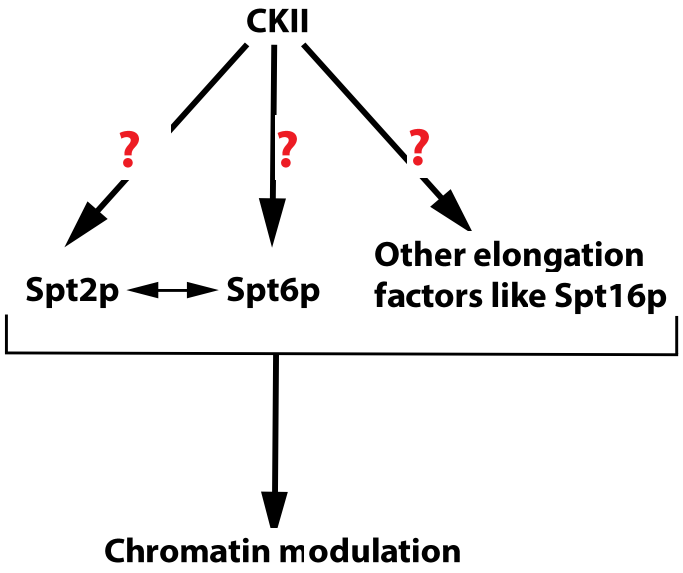

Figure 4.1: Schematic representation of the possible roles of CKII

4.2 CKII interacts with the chromatin component Spt2p and catalyzes its phosphorylation

Spt2p is a non-histone chromatin component with HMG-like domains. It is involved in a wide range of processes. Spt2p plays role in transcription initiation, transcription elongation and in the formation of the 3'-end of mRNA. It maintains nucleosome levels in the coding region of transcriptionally active genes and inhibits spurious transcription from cryptic promoters (Nourani et al., 2006). Recently, we have shown that Spt2p is important to maintain the repressed state of chromatin in the *SER3* promoter at the *SRG1-SER3* locus (Thebault et al., 2011). Despite such diverse roles, the mechanistic details of Spt2p function and its regulation are largely unknown. In order to understand the function of Spt2p in detail, we have aimed to identify the proteins interacting with Spt2p. Spt2p binding partners could help us to understand the function of Spt2p in detail. We conducted tandem affinity purification of Spt2p and analyzed the associated proteins by mass spectrometry. Peptides derived from all four CKII subunits were detected in purified fractions. Copurification of all four CKII subunits with Spt2p after multiple steps of purification suggests that the interaction between Spt2p and CKII is specific and stable. We used stringent conditions and different multistep purification strategies to verify the stability of this interaction. We also performed reverse purification and protein ion exchange chromatography (increasing ionic strength up to 300 mM) and found that Spt2p coelutes and copurifies with the CKII complex. Altogether, our data provide strong evidence that CKII is stably associated with Spt2p in vivo.

A strong association of CKII with Spt2p could suggest that Spt2p is phosphorylated by CKII. Indeed, we found that Spt2p is a direct substrate of CKII in vitro. Moreover, we detected various forms of Spt2p by 2D-PAGE, and phosphatase treatment shifted the isoelectric point of the most acidic Spt2p isoforms, thereby clearly demonstrating that Spt2p is a phosphoprotein in vivo. Importantly, Spt2p immunopurified from yeast is phosphorylated at serine and tyrosine residues in vivo, and phosphorylation of serine as well as tyrosine is strongly reduced in the *ckII*^{ts} strain. This is an important observation, as

although CKII is primarily a serine/threonine kinase, there are reports showing that CKII is a dual kinase and can phosphorylate tyrosine residues as well (Marin et al., 1999; Vilk et al., 2008; Wilson et al., 1997; Zhu et al., 2000). Tyrosine phosphorylation by CKII might be of higher significance for yeast, which, as in higher plants, does not encode tyrosine kinase genes such as those found in animal cells.

Using various mutant versions of Spt2p, we were able to locate CKII target sites to the Spt2p CTD. Spt2p CTD is highly polar, containing two subdomains (226−249 and 277−303) rich in acidic amino acids. A close examination of Spt2p led to the finding of five potential CKII target sites localized to very short regions within the two acidic subdomains (Fig. 4.2). The first region (RI) contains Y226 and Y230 and second (RII) has S277, Y279 and Y281. Mutation of CKII target sites in RI did not affect phosphorylation while mutation of all RII sites affected CKII-mediated phosphorylation to some extent. Importantly, mutating all five potential CKII targets in RI and RII completely abolished phosphorylation by CKII. This indicates that phosphorylation of RII sites represents the majority of Spt2p phosphorylation while RI represents only a fraction of total CKII–mediated phosphorylation, in agreement with our in vivo data. Indeed, replacing the three RII sites with corresponding acidic residues (S277D, Y279E and Y281E) led to defects in Spt2p function, and mutation of two additional tyrosine residues (Y226, Y230) to glutamyl groups aggravated the observed defects in Spt2p function. Altogether, our results show that CKII phosphorylates Spt2p and negatively regulates its function. Importantly, our data indicate that phosphorylation of Spt2p by CKII does not contribute to the reassembly of chromatin. First, Spt2p alone cannot account for the chromatin defects observed in *ckII*^{ts} mutants and secondly, phosphorylation by CKII negatively regulates Spt2p function. In fact, constitutive phosphorylation is the factor that renders Spt2p non-functional. Spt2p possesses HMG-like domains and, as for other HMG proteins, can bind to chromatin non-specifically. Binding of HMG proteins induces changes in chromatin structure and if not controlled properly, may result in GCRs (Reeves, 2010; Sikdar et al., 2008). Indeed overexpression of Spt2p has been linked to stalled replication forks and GCRs (Sikdar et al., 2008). We suggest that phosphorylation could prevent the non-specific binding of Spt2p to chromatin. Otherwise, the abundant Spt2p might create GCRs by stalling replication forks. Therefore, the action of CKII on Spt2p cannot account for the role of CKII in

chromatin refolding and may constitute a more general effect, i.e. in the prevention of non-specific Spt2p binding.

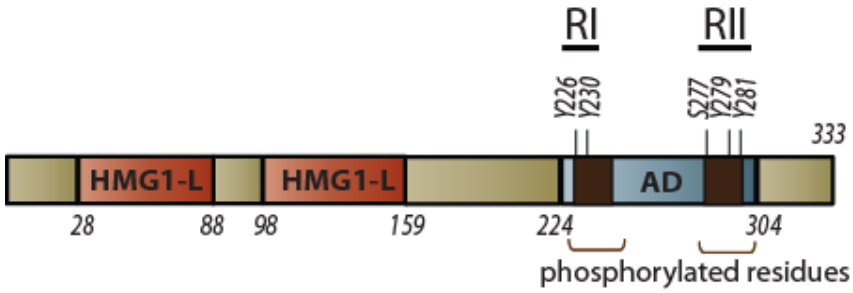

Figure 4.2 : Phosphorylation sites are in the C-terminal region of Spt2p

4.3 CKII phosphorylates and regulates the function of transcription elongation factor Spt6p

CKII regulates chromatin structure, as shown in Chapter 3, but how CKII plays this role constitutes an important question. We have already shown that CKII regulates Spt2p function, but the chromatin defects in *ckII*^{ts} are severe and cannot be explained solely via its action on Spt2p. This suggests that CKII plays role(s) that are additional to the regulation of Spt2p function. Among other possible targets of CKII, Spt6p has been shown to be phosphorylated in vivo at CKII consensus motifs. Furthermore, our genetic analysis indicates that Spt6p and CKII act in the same pathway. Importantly, Spt2p shows functional interaction with Spt6p. These observations led us to hypothesize that CKII might regulate chromatin structure via the modulation of Spt6p function. We have demonstrated that Spt6p interacts with CKII in vivo as well as in vitro and shown that this interaction is direct. This is in accordance with earlier proteomic studies showing that CKII subunits copurify with Spt6p (Krogan et al., 2002). A crucial question, however, was whether Spt6p is directly phosphorylated by CKII. We used purified proteins in in vitro kinase assay and found that CKII purified from yeast was able to incorporate γ-$^{32}P_i$ into recombinant Spt6p from radiolabeled ATP. In addition, probing whole-cell yeast extracts with an antibody against phosphoserine/phosphothreonine showed that Spt6p is a phosphoprotein in vivo.

Phosphorylation levels are severely reduced in *ckII*^{ts} mutants, clearly pointing to an important role of CKII in Spt6p phosphorylation in vivo.

To study the functional significance of Spt6p phosphorylation, we mutated the CKII target sites and looked for phenotypes associated with Spt6p function. Three CKII target sites in Spt6p (S94, S134, S206) were previously shown to be phosphorylated in vivo (Krogan et al., 2002). Mutation of these three residues results in the partial loss of Spt6p function, which is seen as a weak Spt$^-$ phenotype. However, this phenotype was much milder than that associated with *ckII*^{ts} mutants, thus raising the possibility that additional residues of Spt6p are phosphorylated. The latter observation was supported by our finding that Spt6p mutated at these three residues can still be phosphorylated in vitro by CKII, although to a lower extent than for the WT protein. We next used the NetPhosK 1.0 server to identify additional potential CKII targets in the adjacent region. Seven potential CKII target sites were thus identified in the N-terminal region of Spt6p (S28, S39, S40, S94, S134, S155, S206) (Fig. 4.3). Indeed, there are reports that five of these CKII potential targets (S28, S94, S134, S155, and S206) are phosphorylated in vivo (Albuquerque et al., 2008; Krogan et al., 2002; Li et al., 2007c). Instead of tediously mutating each individual residue, we instead mutated either 3 or all 7 CKII target seryl residues to study the effect of phosphorylation on Spt6p function. However, we could not rule out the possibility that phosphorylation of only an intermediary number (4, 5 or 6) of phosphorylated residues are necessary and sufficient to account for the full regulation of Spt6p function by CKII-mediated phosphorylation. Despite these limitations, mutation of all seven CKII potential targets in the N-terminal region of Spt6p was found to completely abolish in vitro phosphorylation of Spt6p and to severely impair its function in vivo, as seen by the strong Spt$^-$ phenotype and temperature sensitivity observed in the resulting phosphorylation mutant. Moreover, we detected transcription from the cryptic promoter in the coding region of *FLO8* in this mutant, which is indicative of defective chromatin structure. Our results thus clearly demonstrate that CKII phosphorylates the transcription elongation factor Spt6p and regulates its function.

Figure 4.3 : Phosphorylation sites reside in the N-terminal region of Spt6p
The N-terminal region of Spt6p is unstructured and contains the domain interacting with Iws1p. Phosphorylated serines are also found in the N-terminal region. The central core of the protein contains various domains. Residues 336–442 encompass a DNA-binding helix–turn-helix (HtH), residues 735–887 (YqgF) present a homology with members of the YqgFc protein superfamily, residues 933–1002 form two consecutive helix–hairpin–helix (HhH) motifs, residues 1019–1104 present a similarity to members of the death domain superfamily (death-like domain or DLD), and the S1 domain (residues 1129–1219) encompass a region of similarity to the RNA-binding domain of ribosomal protein S1. The central core region (HtH, YqgF, HhH, DLD and S1) can bind to DNA when studied in isolation. The C-terminal part contains tandem SH2 domains that bind to the RNAPII isoform phosphorylated at the Ser-2 position of the heptad repeats in the CTD. The figure was adapted from Close et al. (Close et al., 2011).

Given the important role of Spt6p in the reassembly of chromatin in the wake of transcription, our observations may suggest that chromatin defects found in the *ckII*^{ts} mutant are directly associated with the misregulation of Spt6p. If this holds true, we can make the prediction that mutants mimicking the phosphorylation of Spt6p by CKII would bypass the requirement for CKII activity. Thus, this rose the question of whether chromatin defects in *ckII*^{ts} mutant yeast might be reversed by supplementing cells with Spt6p bearing phosphomimetic mutations at CKII target sites. Interestingly, we found that expression of a phosphomimetic mutant of Sp6 (7S-D) in the *ckII*^{ts} strain partially suppressed cryptic transcription, whereas such transcription could not be suppressed by expression of a non-phosphorylatable form of Spt6p in *ckII*^{ts} mutants (7S-A) (Figure 3.9). This led us to conclude that the regulation of Spt6p by CKII is central to the CKII chromatin reassembly function. However, since cryptic transcription in the *ckII*^{ts} mutant is partially repressed by an Spt6p phosphomimetic mutant (7S-D), it is possible that the residual action of CKII is mediated via the phosphorylation of other factors such as Iws1p or Spt16 (Figure 4.4).

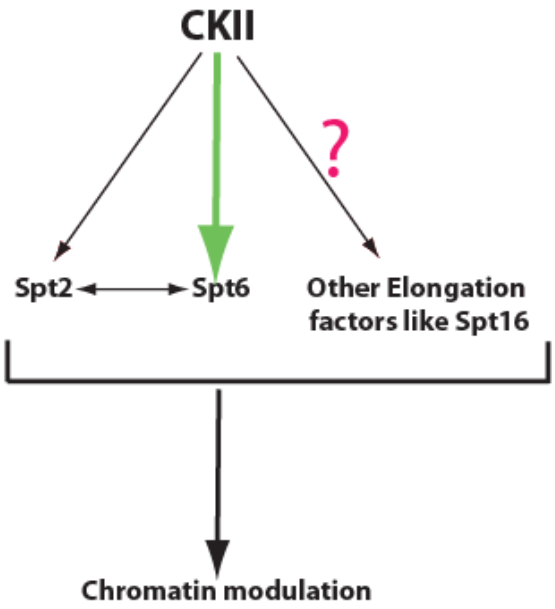

Figure 4.4 : CKII controls chromatin structure by regulating Spt6p function

4.4 Phosphorylation by CKII regulates the Spt6p-Iws1p interaction

Spt6p interacts with Iws1p, a phenomenon that is important for transcription and chromatin structure dynamics. In yeast, mutations in the Spt6p-Iws1p interface lead to severe growth defects at higher temperatures and defects in chromatin structure detected as an Spt$^-$ phenotype (McDonald et al., 2010). Furthermore, mutations that completely disrupt the Spt6p-Iws1p interaction are lethal in yeast (McDonald et al., 2010). This interaction is conserved and is also observed in human cells, where it is involved at various steps of transcription (McDonald et al., 2010; Yoh et al., 2008; Zhang et al., 2008). Both Spt6p and Iws1p are phosphorylated in vivo at CKII consensus sites. We hypothesized that phosphorylation could control Spt6p function by regulating the Spt6p-Iws1p interaction. We observed a drop in Spt6p-Iws1p complex abundance in the *ckII*^{ts} strain, suggesting that CKII may play an important role in the Spt6p-Iws1p interaction. In order to demonstrate that the latter effects directly result from deficient phosphorylation by CKII and not arise indirectly from the lack of a functional CKII, we studied the interaction of Spt6p-Iws1p in vitro using recombinant proteins (GST-Iws1p and 6xHis-*spt6*) in the presence or absence of

phosphorylation. Our data indicate that phosphorylation of at least one of the two proteins could be important for Spt6p-Iws1p interaction.

To study the role of Spt6p phosphorylation in the Spt6p-Iws1p interaction, we purified WT FLAG-Spt6p and mutant FLAG-Spt6p (7S-A) from yeast that were also expressing Iws1p-Myc. We found that the in vivo association of Iws1p with Spt6p is strongly reduced when all Spt6p CKII target sites are removed (by substitution to alanyl residues). This suggests that Spt6p phosphorylation plays a major role in the Spt6p-Iws1p interaction. The importance of Spt6p phosphorylation in the Spt6p-Iws1p interaction was further supported by our in vitro data. Recombinant Spt6p with phosphomimetic mutations (7S-D) interacts more strongly with Iws1p than WT Spt6p. Furthermore, while phosphorylation increased the Spt6p-Iws1p interaction in vitro, this effect was prevented upon substitution of all CKII target sites in Spt6p to alanine. This clearly indicates that Spt6p phosphorylation by CKII regulates the Spt6p-Iws1p interaction. We used the NetPhosK 1.0 server to predict potential CKII phosphorylation motifs in Iws1p. There are a total of seven potential CKII target sites (T54, S72, T86, S89, S123, T253, and T346) in Iws1p. Only one of these CKII target site (S89) has been reported to be phosphorylated in vivo (Krogan et al., 2002). Mutation of the latter residue produces no obvious phenotype. However, we cannot rule out the possibility that other residues might be phosphorylated and important for Iws1p function. Notwithstanding these uncertainties, we conclude that phosphorylation of Spt6p plays a pivotal role in the Spt6p-Iws1p interaction.

Intriguingly, all the phosphorylated residues of Spt6p are concentrated in the N-terminal region, which is predicted to be unstructured. Recent structural and biochemical studies have shown that Spt6p has the following three important structural features (Close et al., 2011).

1. An N-terminal region which is predicted to be unstructured and to contain regions responsible for interacting with Iws1p and nucleosomes (McDonald et al., 2010).
2. A central core region (residues 298–1117) with multiple recognizable domains that are important for nucleic acid binding (Close et al., 2011).

3. A C-terminal flexible region with a tandem SH2 domain. This C-terminal domain displays an affinity for phosphopeptides and binds the RNAPII isoform phosphorylated on Ser-2 of the heptad repeats in the CTD (Sun et al., 2010; Yoh et al., 2007).

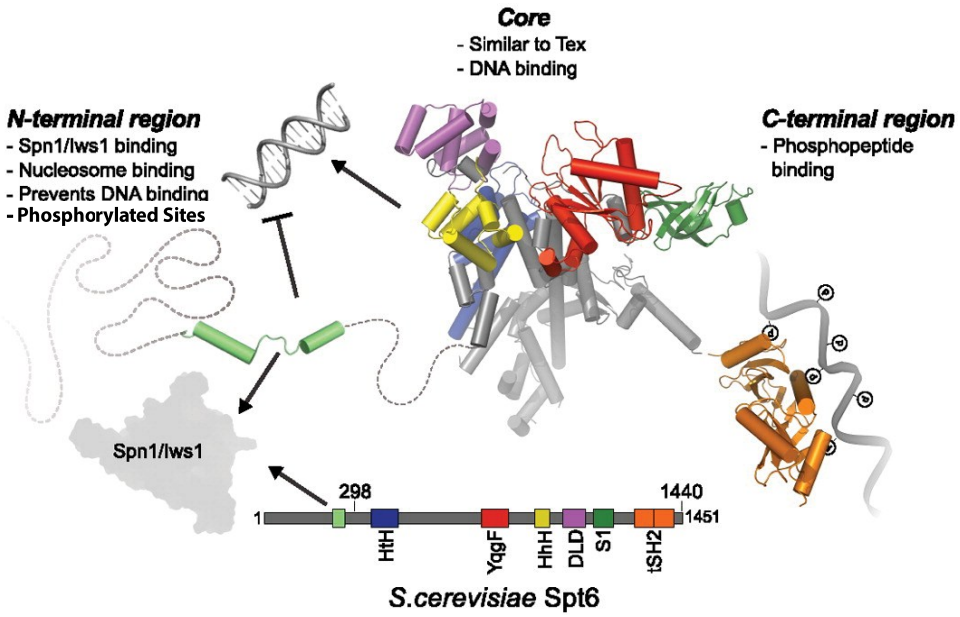

Figure 4.5 : Schematic representation of Spt6p domains
The various regions of Spt6p are shown together with their putative functions. The N-terminal region is unstructured. It contains the region (*light* green) interacting with Iws1p and the nucleosome. The N-terminal region also encompasses the different CKII phosphorylation motifs, all sites being serine residues. The central core contains various distinctive protein domains (HtH, YqgF, HhH, DLD, and S1). This region is able to bind to DNA when studied in isolation. The CTD contains tandem SH2 domains that bind to the RNAPII isoform phosphorylated on Ser-2 of the YSPTSPS heptad repeats in the CTD. Figure adapted from Close et al. (Close et al., 2011).

The N-terminal region, although predicted to be unstructured, is important for Spt6p function. This region harbors all phosphorylated sites as well as the region of interaction with Iws1p. Recently, the region of Spt6p that interacts with Iws1p was precisely mapped and spans residues 239 to 268 (McDonald et al., 2010). Importantly, the proteins used in this study lacked the N-terminal region along with all CKII target sites. Our data clearly demonstrate the importance of the CKII target sites in the Spt6p-Iws1p interaction, an aspect that was obviously overlooked in the studies by McDonald et al. We outlined two

hypotheses that may explain how the N-terminal domain of Spt6p might regulate this interaction. A first possibility is that the extremity of the N-terminal region might inhibit the Spt6p-Iws1p interaction, an inhibition which could be relieved by phosphorylation. There are reported cases where the binding properties of a particular domain is inhibited by an adjacent region. In fact, the central core domain of Spt6p is able to bind DNA, a feature that is lost in the complete protein due to steric hindrance by the N-terminal unstructured region (Close et al., 2011). This observation provides support for a model where the N-terminal domain of Spt6p regulates a structural switch that determines the ability of the protein to bind DNA. Thus, phosphorylation of Spt6p by CKII might simply make its domain of interaction with Iws1p accessible. Alternatively, phosphorylation might act as a regulatory switch changing the binding affinity of Spt6p towards different substrates. The recent study of McDonald et al. has shown that Spt6p binds to Iws1p and the nucleosome via the same domain (McDonald et al., 2010). Using a gel-shift assay, these authors have shown that Spt6p is able to bind recombinant nucleosomes in the presence of the HMG box protein Nhp6Ap. Increasing concentrations of Iws1p compete with nucleosomes for binding to Spt6p and releasing recombinant nucleosomes. The authors have proposed that the interaction of Spt6p with Iws1p favors release of the former from chromatin after nucleosome deposition. How the interaction is switched from chromatin to Iws1p is not clear. Phosphorylation by CKII might act as a regulatory switch where phosphorylation would favor the interaction of Spt6p with Iws1p, thereby promoting the release of Spt6p after the assembly of chromatin. Based on work by McDonald et al. (McDonald et al., 2010) and our own observations, we can propose a model for the role of CKII in chromatin structure dynamics (Figure 4.6).

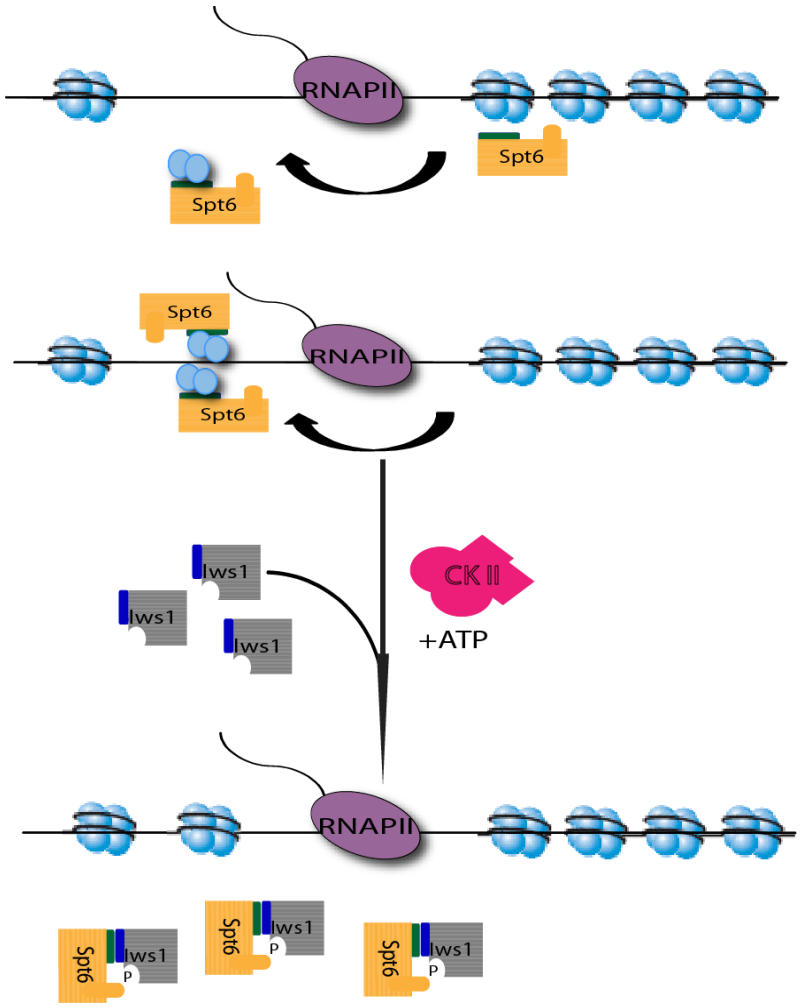

Figure 4.6 : Current model for the role of CKII in chromatin structure dynamics
Spt6p is required for the unfolding and refolding of chromatin during transcription elongation. After reassembly of chromatin, the Spt6p-nucleosome interaction has to be disrupted. Spt6p interacts with the nucleosome and Iws1p via the same domain. CKII phosphorylates Spt6p, thereby favoring the Spt6p-Iws1p interaction and release of the Spt6p-Iws1p complex.

4.5 CKII regulates the interaction of Spt6p with Set2p

One of the important marks of active transcription is the di-and trimethylation of H3K36. Methylation of H3K36 is catalyzed by Set2p methyltransferase. Set2p is recruited to regions of active transcription through its interaction with the RNAPII isoform that is phosphorylated on the Ser-2 residue of the heptad repeats in the CTD (Kizer et al., 2005).

Methylation by Set2p plays an important role in the restoration of chromatin structure after transcription by elongating RNAPII. This chromatin mark (methylation of H3K36) is recognized by the Rpd3S complex which deacetylates H3 and H4, resulting in the "closed" chromatin conformation and inhibition of transcription from cryptic sites after the passage of RNAPII. The methylation states of H3K36 are regulated by different factors such as the Paf1p complex, the Bur1p-Bur2p kinase and Spt6p (Chu et al., 2007; Youdell et al., 2008). Spt6p controls both the dimethylation and trimethylation of H3K36. In the *spt6-1004* mutant, Set2p protein levels are drastically reduced (Youdell et al., 2008). Dimethylation of H3K36 was resumed in the *spt6-1004* mutant when intracellular and chromatin levels of Set2p were restored, but trimethylation of H3K36 could not be reinstated (Youdell et al., 2008). The mechanistic details of the regulation of K36 methylation by Spt6p are as yet unknown. We report for the first time in yeast that Spt6p interacts with Set2p in vivo. This interaction is unstable and is easily lost at higher ionic strength. This interaction of Spt6p with Set2p might be important for the regulation of Set2p function and the methylation state of H3K36. Importantly, recombinant GST-Set2p and His Spt6p did not interact in vitro, suggesting that the Spt6p-Set2p interaction is indirect and that Iws1p might act as the link between Spt6p and Set2p in vivo. The situation is similar to human cells, where it was shown that Spt6p interacts with HYPB/Set2p and Iws1p is the link between the two proteins (Yoh et al., 2008).

Interestingly, in the *ckII*^{ts} mutant, we observed a specific drop in H3K36 trimethylation while dimethylation remained unaffected (Figure 3.1B). In vitro treatment with CKII does not change the activity of recombinant Set2p towards chromatin (supplemental Figure 3.10), indicating that CKII might act indirectly. Importantly, in the *ckII*^{ts} mutant, the interaction of Spt6p with Set2p and Iws1p is lost. A study in human cells has shown that depletion of Iws1p results in the loss of Spt6p-HYPB/Set2p interaction and H3K36 trimethylation (Yoh et al., 2008). The latter effects are similar to the phenotypic characteristics noted in the *ckII*^{ts} yeast mutant. In the *ckII*^{ts} mutant, Spt6p-Iws1p and Spt6p-Set2p interactions are lost. The hypothesis that Iws1p acts as the link between Spt6p and Set2p would account for the loss of the Spt6p-Set2p interaction in *ckII*^{ts} mutants. Further experiments will be needed to determine more precisely the role of Spt6p and CKII in the regulation of H3K36 trimethylation.

4.6 Phosphorylation by CKII protects Spt6p from complete degradation during stationary phase

In response to starvation, microorganisms like *S. cerevisiae* stop growing and enter into a non-proliferating state referred to as *stationary phase*, *quiescence* or G0. Quiescent yeast cells are similar to quiescent mammalian cells and share many characteristics such as lack of genome replication, condensed chromosomes, increased rates of autophagy and reduced rates of translation (Gray et al., 2004). In yeast, CKII is important for normal re-entry into the cell cycle upon exit from stationary phase (Hanna et al., 1995). CKII also phosphorylates Cdc28p, and this phosphorylation is important for the control of total protein content and cell size in stationary phase (Russo et al., 2000). Since CKII regulates Spt6p/Iws1p complex, we were interested to study Spt6p/Iws1p complex in stationary phase. While investigating the Spt6p-Iws1p interaction in stationary-phase cells, we came across an interesting situation. In WT cells, Spt6p and Iws1p levels are reduced during stationary phase when compared to exponentially growing cells, as expected. Surprisingly, in stationary-phase cells, we could not detect Spt6p protein in the *ckII*^{ts} mutant although Iws1p levels were comparable to those found in the WT strain. This suggests a possible role of CKII in stabilizing Spt6p against protein turnover during stationary phase. Under normal conditions in a WT strain, Spt6p levels are higher than for Iws1p, and only a fraction of total Spt6p is associated with Iws1p [Chapter 3, (Krogan et al., 2002)]. Analysis of Spt6p immunopurified from cells in stationary phase and at different time intervals after fresh medium addition shows that in WT strain, a small fraction of Spt6p is protected from degradation in stationary phase. This small pool of Spt6p that is protected from degradation in WT cells is in the form of a complex with Iws1p. This indicates that formation of the Spt6p-Iws1p complex might normally protect Spt6p from complete degradation during stationary phase. Since phosphorylation by CKII is important for the Spt6p-Iws1p interaction, the normal role of the complex in counteracting Spt6p turnover would explain the almost complete loss of Spt6p in stationary-phase *ckII*^{ts} cells.

4.7 Phosphorylation disrupts the Spt2p/Spt6p complex and inhibits the recruitment of Spt2p to sites of active transcription

Our studies of Spt2p and Spt6p regulation by CKII present a rather complex situation. In addition to regulation of the Spt6p-Iws1p complex, we also found that phosphorylation by CKII regulates the interaction between Spt2p and Spt6p. These findings raise several important questions about the basis and implications of such complex levels of regulation. Clearly, Spt2p and Spt6p are functionally linked. It has previously been shown that Spt2p is associated with coding regions of transcriptionally active genes and that this association is dependent on Spt6p (Nourani et al., 2006). Whether Spt6p helps in the recruitment of Spt2p to chromatin or stabilizes its interaction with chromatin post-recruitment is not clear. In vitro binding assays strongly suggest that Spt6p interacts directly with Spt2p. The association of Spt2p with the coding region of transcriptionally active genes is most likely dependent on a direct interaction with both Spt6p and chromatin. Interestingly, phosphomimetic mutants of Spt2p are not recruited to coding regions of transcriptionally active genes, suggesting that CKII-mediated phosphorylation likely inhibits the recruitment of Spt2p. We hypothesized that phosphorylation disrupts the Spt2p-Spt6p interaction on chromatin, resulting in the loss of Spt2p from chromatin. Indeed, we found that phosphorylation by CKII partially disrupts Spt6p-Spt2p in vitro.

Spt2p possesses two HMG1-like domains. HMG proteins such as Nhp6p induce specific changes in chromatin structure that allow different factors to bind chromatin (Stillman, 2010). The histone chaperone yFACT requires the HMG protein Nhp6p to recognize and alter the nucleosome structure (Ruone et al., 2003; Stillman, 2010). It is likely that histone chaperone Spt6p also requires an HMG box factor to interact with nucleosomes and initiate the unfolding/refolding reaction during transcription. Indeed, a recent study has shown that Spt6p could not interact with nucleosomes in vitro in the absence of HMG box factor Nhp6p (McDonald et al., 2010). Importantly, Spt2p has two HMG-like domains and shows physical and functional interactions with Spt6p. We believe that Spt2p is the HMG box protein that might assist Spt6p in vivo during the reaction of chromatin assembly/disassembly associated with transcription.

Our data shows that in vitro phosphorylation by CKII disrupts the Spt6p-Spt2p complex. Importantly, we have also shown that phosphorylation by CKII is required for the formation of the Spt6p-Iws1p complex, which has a potential role in the release of the Spt6p/Iws1p complex from chromatin. Taken together, these results led us to propose a model for the mechanism by which CKII modulates chromatin refolding activity (Figure 4.7). In this model, Spt6p is recruited to sites of active transcription by the CTD of RNAPII. However, Spt6p requires an HMG box protein, which in this case is Spt2p, to perform some of its functions. In the coding region of actively transcribed genes, Spt6p interacts with Spt2p and stabilizes its interaction with chromatin. This interaction is important for Spt6p function and allows the latter protein to interact with nucleosomes, start unfolding its structure to facilitate RNAPII passage, and redeposit nucleosomes after transcription. Once nucleosomes are reassembled properly, the interaction of Spt6p with nucleosomes and Spt2p has to be disrupted and the latter proteins are released from chromatin. We believe that this process is orchestrated via CKII-mediated phosphorylation. Phosphorylation disrupts the Spt6p-Spt2p and Spt6p-nucleosome interactions, and favors the interaction of Spt6p with Iws1p, which helps in turn to release Spt6p/Iws1p and Spt2p from chromatin.

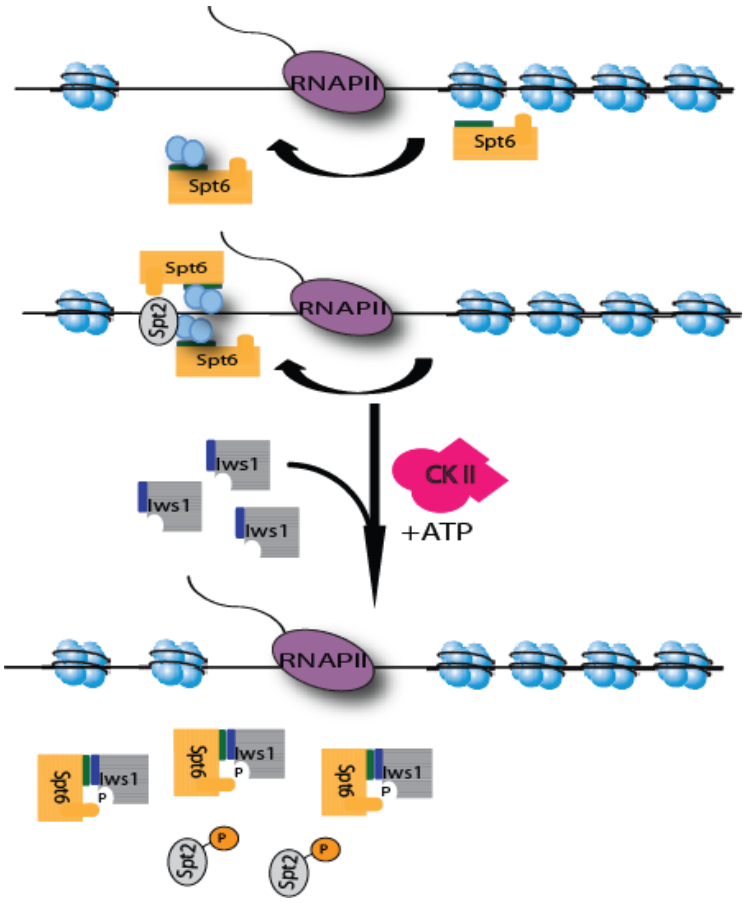

Figure 4.7 : Model for the regulation of the function of transcription elongation factors Spt2p and Spt6 by CKII
In Step 1, Spt6p requires an HMG protein to bind nucleosomes, which is needed for Spt6p function. The two proteins Spt6p and Spt2p interact on the chromatin. After proper folding of the nucleosome, CKII phosphorylates both Spt6p and Spt2p, thus disrupting the Spt6p-Spt2p interaction. Phosphorylation favors the Spt6p-Iws1p interaction, resulting in the release of phosphorylated Spt2p and phosphorylated Spt6p-Iws1p complex from chromatin.

4.8 Conclusion and perspective

The work presented in this dissertation was focused on the study of the regulation of transcription elongation factors. We began with the objective to understand the function and regulation of Spt2p, a chromatin-associated transcription elongation factor. The final picture that emerged from our studies is quite complex. We found that CKII phosphorylates Spt2p and negatively regulates its function. Our results indicate that phosphorylation prevents Spt2p from interacting with chromatin, thus preventing the non-specific

interaction of Spt2p with chromatin. In order to be recruited to the coding region of transcriptionally active genes, Spt2p should be dephosphorylated. It will be interesting to characterize the phosphatase associated with Spt2p dephosphorylation.

The data presented here identify for the first time a role of the protein kinase CKII in the modulation of chromatin during transcription elongation. We used a series of genetic and biochemical methods to study the mechanistic details of this function. Our results show that CKII plays its role via the phosphorylation and regulation of the function of transcription elongation factor Spt6p. Our data shows that CKII regulates the interaction of Spt6p with other proteins that are important for chromatin structure dynamics. We demonstrate that Spt6p phosphorylation favors the Spt6p-Iws1p interaction and propose that phosphorylation favors the release of the Spt6p/Iws1p complex from chromatin once the latter is properly refolded after transcription by elongating RNAPII. For these reasons, the Spt6p/Iws1p complex is released less efficiently from chromatin in a *ckII* mutant strain. This can be tested by ChIP on genes under a *GAL1* promoter. If Spt6p is not released from chromatin after transcription is shut off, we should observe a delay in release of Spt6. In vitro nucleosome binding assays using recombinant proteins (WT and Spt6p phosphomimetic mutant) could be performed to compare the binding of WT Spt6p and phosphomimetic Spt6p mutants to nucleosomes.

In the course of this study, we found that mutation of CKII leads to strong defects in chromatin structure. A non-phosphorylatable mutant of Spt6p presents chromatin defects, although these defects are not as severe as those found in a *ckII* mutant. Furthermore, we were able to partially suppress chromatin defects in *ckII* mutants using phosphomimetic mutants of Spt6p. This suggests that CKII plays roles that are additional to the regulation of Spt6p function. CKII subunits are also associated with other factors important for chromatin modulation such as FACT, Spt4p/5p and Chd1p (Krogan et al., 2002). CKII could be at the helm of the pathways that regulate all these factors (Figure 4.8). It will be highly interesting to decipher the additional pathways of chromatin structure regulation by CKII in future studies.

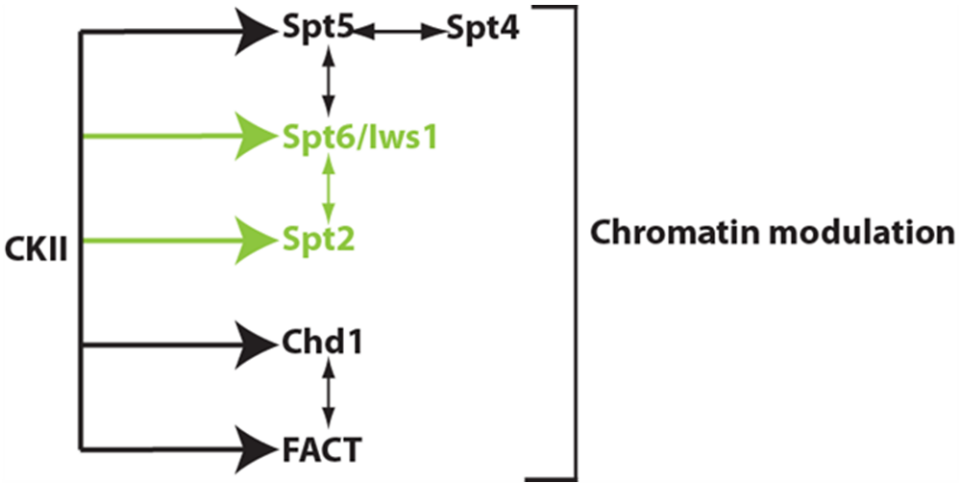

Figure 4.8 : CKII possibly regulates various transcription elongation factors

Spt6p interacts with Iws1p and phosphorylation of Spt6p regulates this interaction. We found that in vitro phosphorylation slightly increases the Spt6p-Iws1p interaction even when the CKII target sites of Spt6p cannot be phosphorylated. Phosphorylation of Iws1p might be responsible for the latter small increase (Figure 3.6). It will be interesting to check chromatin defects when phosphorylation sites of both Spt6p and Iws1p are mutated. This might be done by constructing a yeast strain bearing conditional mutations of these two essential proteins. Previously, Spt6p has been shown to interact physically and functionally with Spt5p (Krogan et al., 2002; Swanson and Winston, 1992). Interestingly, Spt5p and Iws1p are both phosphorylated at CKII consensus motifs in a region with sequence homology, and their phosphorylated serine residues align perfectly (Krogan et al., 2002). It is will be tempting to study whether CKII plays a role in the regulation of the Spt6p-Spt5p interaction. CKII also interacts with elongation factors Chd1p and FACT (Krogan et al., 2002). Importantly, Chd1p and FACT show physical and functional interactions (Costa and Arndt, 2000; Krogan et al., 2002). It could be interesting to study the role of CKII in the regulation of the interaction between FACT and Chd1p as well as their function.

The data presented in this dissertation provide valuable information regarding a novel pathway of chromatin structure regulation via the post-translational modification of transcription elongation factors. The ideas and concepts put forward here, when further developed in future studies, should further enrich our knowledge of the complex regulation of chromatin structure during transcription elongation.

Bibliography

Adkins, M.W., and Tyler, J.K. (2006). Transcriptional activators are dispensable for transcription in the absence of Spt6p-mediated chromatin reassembly of promoter regions. Mol Cell *21*, 405-416.

Ahmad, K., and Henikoff, S. (2002). Histone H3 variants specify modes of chromatin assembly. Proc Natl Acad Sci U S A *99 Suppl 4*, 16477-16484.

Albert, I., Mavrich, T.N., Tomsho, L.P., Qi, J., Zanton, S.J., Schuster, S.C., and Pugh, B.F. (2007). Translational and rotational settings of H2A.Z nucleosomes across the *Saccharomyces cerevisiae* genome. Nature *446*, 572-576.

Albuquerque, C.P., Smolka, M.B., Payne, S.H., Bafna, V., Eng, J., and Zhou, H. (2008). A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol Cell Proteomics *7*, 1389-1396.

Antos, C.L., McKinsey, T.A., Dreitz, M., Hollingsworth, L.M., Zhang, C.L., Schreiber, K., Rindt, H., Gorczynski, R.J., and Olson, E.N. (2003). Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors. J Biol Chem *278*, 28930-28937.

Ardehali, M.B., Yao, J., Adelman, K., Fuda, N.J., Petesch, S.J., Webb, W.W., and Lis, J.T. (2009). Spt6p enhances the elongation rate of RNA polymerase II in vivo. EMBO J *28*, 1067-1077.

Ausio, J., Abbott, D.W., Wang, X., and Moore, S.C. (2001). Histone variants and histone modifications: a structural perspective. Biochem Cell Biol *79*, 693-708.

Avvakumov, N., and Cote, J. (2007). The MYST family of histone acetyltransferases and their intimate links to cancer. Oncogene *26*, 5395-5407.

Bao, Y., and Shen, X. (2007). INO80 subfamily of chromatin remodeling complexes. Mutat Res *618*, 18-29.

Bassett, A., Cooper, S., Wu, C., and Travers, A. (2009). The folding and unfolding of eukaryotic chromatin. Curr Opin Genet Dev *19*, 159-165.

Becker, P.B., and Horz, W. (2002). ATP-dependent nucleosome remodeling. Annu Rev Biochem *71*, 247-273.

Belotserkovskaya, R., Oh, S., Bondarenko, V.A., Orphanides, G., Studitsky, V.M., and Reinberg, D. (2003). FACT facilitates transcription-dependent nucleosome alteration. Science *301*, 1090-1093.

Bernstein, B.E., Liu, C.L., Humphrey, E.L., Perlstein, E.O., and Schreiber, S.L. (2004). Global nucleosome occupancy in yeast. Genome Biol *5*, R62.

Bidwai, A.P., Hanna, D.E., and Glover, C.V. (1992). Purification and characterization of casein kinase II (CKII) from delta cka1 delta cka2 *Saccharomyces cerevisiae* rescued by Drosophila CKII subunits. The free catalytic subunit of casein kinase II is not toxic in vivo. J Biol Chem *267*, 18790-18796.

Biswas, D., Yu, Y., Prall, M., Formosa, T., and Stillman, D.J. (2005). The yeast FACT complex has a role in transcriptional initiation. Mol Cell Biol *25*, 5812-5822.

Bonifer, C. (1999). Long-distance chromatin mechanisms controlling tissue-specific gene locus activation. Gene *238*, 277-289.

Bortvin, A., and Winston, F. (1996). Evidence that Spt6p controls chromatin structure by a direct interaction with histones. Science *272*, 1473-1476.

Brewster, N.K., Johnston, G.C., and Singer, R.A. (2001). A bipartite yeast SSRP1 analog comprised of Pob3 and Nhp6 proteins modulates transcription. Mol Cell Biol *21*, 3491-3502.

Brown, C.E., Lechner, T., Howe, L., and Workman, J.L. (2000). The many HATs of transcription coactivators. Trends Biochem Sci *25*, 15-19.

Bucceri, A., Kapitza, K., and Thoma, F. (2006). Rapid accessibility of nucleosomal DNA in yeast on a second time scale. EMBO J *25*, 3123-3132.

Burnett, G., and Kennedy, E.P. (1954). The enzymatic phosphorylation of proteins. J Biol Chem *211*, 969-980.

Cairns, B.R. (2001). Emerging roles for chromatin remodeling in cancer biology. Trends Cell Biol *11*, S15-21.

Cairns, B.R., Kim, Y.J., Sayre, M.H., Laurent, B.C., and Kornberg, R.D. (1994). A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. Proc Natl Acad Sci U S A *91*, 1950-1954.

Cairns, B.R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B., and Kornberg, R.D. (1996). RSC, an essential, abundant chromatin-remodeling complex. Cell *87*, 1249-1260.

Cairns, B.R., Schlichter, A., Erdjument-Bromage, H., Tempst, P., Kornberg, R.D., and Winston, F. (1999). Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. Mol Cell *4*, 715-723.

Calvert, M.E., Keck, K.M., Ptak, C., Shabanowitz, J., Hunt, D.F., and Pemberton, L.F. (2008). Phosphorylation by casein kinase 2 regulates Nap1 localization and function. Mol Cell Biol *28*, 1313-1325.

Carey, M., Li, B., and Workman, J.L. (2006). RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Mol Cell *24*, 481-487.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.J., Anderson, S., Yates, J., Washburn, M.P.*, et al.* (2005). Histone H3 methylation by Set2p directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell *123*, 581-592.

Chakravarthy, S., Park, Y.J., Chodaparambil, J., Edayathumangalam, R.S., and Luger, K. (2005). Structure and dynamic properties of nucleosome core particles. FEBS Lett *579*, 895-898.

Chester, N., Yu, I.J., and Marshak, D.R. (1995). Identification and characterization of protein kinase CKII isoforms in HeLa cells. Isoform-specific differences in rates of assembly from catalytic and regulatory subunits. J Biol Chem *270*, 7501-7514.

Cheung, V., Chua, G., Batada, N.N., Landry, C.R., Michnick, S.W., Hughes, T.R., and Winston, F. (2008). Chromatin- and transcription-related factors repress transcription from within coding regions throughout the *Saccharomyces cerevisiae* genome. PLoS Biol *6*, e277.

Chi, P., Allis, C.D., and Wang, G.G. (2010). Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer *10*, 457-469.

Chu, Y., Simic, R., Warner, M.H., Arndt, K.M., and Prelich, G. (2007). Regulation of histone modification and cryptic transcription by the Bur1 and Paf1 complexes. EMBO J *26*, 4646-4656.

Chu, Y., Sutton, A., Sternglanz, R., and Prelich, G. (2006). The BUR1 cyclin-dependent protein kinase is required for the normal pattern of histone methylation by SET2. Mol Cell Biol *26*, 3029-3038.

Clapier, C.R., and Cairns, B.R. (2009). The biology of chromatin remodeling complexes. Annu Rev Biochem *78*, 273-304.

Clark-Adams, C.D., and Winston, F. (1987). The SPT6 gene is essential for growth and is required for delta-mediated transcription in *Saccharomyces cerevisiae*. Molecular and cellular biology *7*, 679-686.

Close, D., Johnson, S.J., Sdano, M.A., McDonald, S.M., Robinson, H., Formosa, T., and Hill, C.P. (2011). Crystal structures of the *Saccharomyces cerevisiae* Spt6 core and C-terminal tandem SH2 domain. J Mol Biol *408*, 697-713.

Conaway, R.C., and Conaway, J.W. (2009). The INO80 chromatin remodeling complex in transcription, replication and repair. Trends Biochem Sci *34*, 71-77.

Corona, D.F., and Tamkun, J.W. (2004). Multiple roles for ISWI in transcription, chromosome organization and DNA replication. Biochim Biophys Acta *1677*, 113-119.

Costa, P.J., and Arndt, K.M. (2000). Synthetic lethal interactions suggest a role for the *Saccharomyces cerevisiae* Rtf1 protein in transcription elongation. Genetics *156*, 535-547.

Cote, J., Quinn, J., Workman, J.L., and Peterson, C.L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast Swi/Snf complex. Science *265*, 53-60.

Das, C., and Tyler, J.K. (2012). Histone exchange and histone modifications during transcription and aging. Biochimica et biophysica acta *1819*, 332-342.

Davie, J.K., and Dent, S.Y. (2006). No Spt6p, no nucleosomes, no activator required. Mol Cell *21*, 452-453.

De Koning, L., Corpet, A., Haber, J.E., and Almouzni, G. (2007). Histone chaperones: an escort network regulating histone traffic. Nat Struct Mol Biol *14*, 997-1007.

Denis, C.L. (1984). Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. Genetics *108*, 833-844.

Diebold, M.L., Koch, M., Loeliger, E., Cura, V., Winston, F., Cavarelli, J., and Romier, C. (2010). The structure of an Iws1/Spt6p complex reveals an interaction domain conserved in TFIIS, Elongin A and Med26. EMBO J *29*, 3979-3991.

Dion, M.F., Kaplan, T., Kim, M., Buratowski, S., Friedman, N., and Rando, O.J. (2007). Dynamics of replication-independent histone turnover in budding yeast. Science *315*, 1405-1408.

Dror, V., and Winston, F. (2004). The Swi/Snf chromatin remodeling complex is required for ribosomal DNA and telomeric silencing in *Saccharomyces cerevisiae*. Mol Cell Biol *24*, 8227-8235.

Endoh, M., Zhu, W., Hasegawa, J., Watanabe, H., Kim, D.K., Aida, M., Inukai, N., Narita, T., Yamada, T., Furuya, A., *et al.* (2004). Human Spt6p stimulates transcription elongation by RNA polymerase II in vitro. Mol Cell Biol *24*, 3324-3336.

Erdel, F., Krug, J., Langst, G., and Rippe, K. Targeting chromatin remodelers: signals and search mechanisms. Biochim Biophys Acta *1809*, 497-508.

Fischbeck, J.A., Kraemer, S.M., and Stargell, L.A. (2002). SPN1, a conserved gene identified by suppression of a postrecruitment-defective yeast TATA-binding protein mutant. Genetics *162*, 1605-1616.

Fleming, A.B., Kao, C.F., Hillyer, C., Pikaart, M., and Osley, M.A. (2008). H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. Mol Cell *31*, 57-66.

Formosa, T., Eriksson, P., Wittmeyer, J., Ginn, J., Yu, Y., and Stillman, D.J. (2001). Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. EMBO J *20*, 3506-3517.

Fuchs, T.M., Deppisch, H., Scarlato, V., and Gross, R. (1996). A new gene locus of Bordetella pertussis defines a novel family of prokaryotic transcriptional accessory proteins. J Bacteriol *178*, 4445-4452.

Fuda, N.J., Ardehali, M.B., and Lis, J.T. (2009). Defining mechanisms that regulate RNA polymerase II transcription in vivo. Nature *461*, 186-192.

Gangaraju, V.K., and Bartholomew, B. (2007). Mechanisms of ATP-dependent chromatin remodeling. Mutat Res *618*, 3-17.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., *et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141-147.

Gelato, K.A., and Fischle, W. (2008). Role of histone modifications in defining chromatin structure and function. Biol Chem *389*, 353-363.

Gelbart, M.E., Rechsteiner, T., Richmond, T.J., and Tsukiyama, T. (2001). Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. Mol Cell Biol *21*, 2098-2106.

Ghavidel, A., and Schultz, M.C. (1997). Casein kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein. Genes Dev *11*, 2780-2789.

Ginsburg, D.S., Govind, C.K., and Hinnebusch, A.G. (2009). NuA4 lysine acetyltransferase Esa1 is targeted to coding regions and stimulates transcription elongation with Gcn5. Mol Cell Biol *29*, 6473-6487.

Glover, C.V., 3rd (1998). On the physiological role of casein kinase II in *Saccharomyces cerevisiae*. Prog Nucleic Acid Res Mol Biol *59*, 95-133.

Goldmark, J.P., Fazzio, T.G., Estep, P.W., Church, G.M., and Tsukiyama, T. (2000). The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. Cell *103*, 423-433.

Govind, C.K., Zhang, F., Qiu, H., Hofmeyer, K., and Hinnebusch, A.G. (2007). Gcn5 promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding regions. Mol Cell *25*, 31-42.

Gray, J.V., Petsko, G.A., Johnston, G.C., Ringe, D., Singer, R.A., and Werner-Washburne, M. (2004). "Sleeping beauty": quiescence in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev *68*, 187-206.

Guillemette, B., Bataille, A.R., Gevry, N., Adam, M., Blanchette, M., Robert, F., and Gaudreau, L. (2005). Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. PLoS Biol *3*, e384.

Hainer, S.J., Pruneski, J.A., Mitchell, R.D., Monteverde, R.M., and Martens, J.A. (2011). Intergenic transcription causes repression by directing nucleosome assembly. Genes Dev *25*, 29-40.

Han, P., Hang, C.T., Yang, J., and Chang, C.P. (2011). Chromatin remodeling in cardiovascular development and physiology. Circ Res *108*, 378-396.

Hanna, D.E., Rethinaswamy, A., and Glover, C.V. (1995). Casein kinase II is required for cell cycle progression during G1 and G2/M in *Saccharomyces cerevisiae*. J Biol Chem *270*, 25905-25914.

Hartzog, G.A., Wada, T., Handa, H., and Winston, F. (1998). Evidence that Spt4, Spt5, and Spt6p control transcription elongation by RNA polymerase II in *Saccharomyces cerevisiae*. Genes Dev *12*, 357-369.

Henikoff, S., and Ahmad, K. (2005). Assembly of variant histones into chromatin. Annu Rev Cell Dev Biol *21*, 133-153.

Hershkovits, G., Bangio, H., Cohen, R., and Katcoff, D.J. (2006). Recruitment of mRNA cleavage/polyadenylation machinery by the yeast chromatin protein Sin1p/Spt2p. Proc Natl Acad Sci U S A *103*, 9808-9813.

Hicke, L. (2001). Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol *2*, 195-201.

Hong, L., Schroth, G.P., Matthews, H.R., Yau, P., and Bradbury, E.M. (1993). Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 "tail" to DNA. J Biol Chem *268*, 305-314.

Huber, L.C., Brock, M., Hemmatazad, H., Giger, O.T., Moritz, F., Trenkmann, M., Distler, J.H., Gay, R.E., Kolling, C., Moch, H.*, et al.* (2007). Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. Arthritis Rheum *56*, 1087-1093.

Imbeault, D., Gamar, L., Rufiange, A., Paquet, E., and Nourani, A. (2008). The Rtt106 histone chaperone is functionally linked to transcription elongation and is involved in the regulation of spurious transcription from cryptic promoters in yeast. J Biol Chem *283*, 27350-27354.

Ivanovska, I., Jacques, P.E., Rando, O.J., Robert, F., and Winston, F. (2011). Control of chromatin structure by Spt6: different consequences in coding and regulatory regions. Mol Cell Biol *31*, 531-541.

Jiang, C., and Pugh, B.F. (2009). Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet *10*, 161-172.

Jonsson, Z.O., Jha, S., Wohlschlegel, J.A., and Dutta, A. (2004). Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. Mol Cell *16*, 465-477.

Joshi, A.A., and Struhl, K. (2005). Eaf3 chromodomain interaction with methylated H3-K36 links histone deacetylation to Pol II elongation. Mol Cell *20*, 971-978.

Kamakaka, R.T., and Biggins, S. (2005). Histone variants: deviants? Genes Dev *19*, 295-310.

Kaplan, C.D., Laprade, L., and Winston, F. (2003). Transcription elongation factors repress transcription initiation from cryptic sites. Science *301*, 1096-1099.

Kappes, F., Waldmann, T., Mathew, V., Yu, J., Zhang, L., Khodadoust, M.S., Chinnaiyan, A.M., Luger, K., Erhardt, S., Schneider, R.*, et al.* (2011). The DEK oncoprotein is a Su(var) that is essential to heterochromatin integrity. Genes Dev *25*, 673-678.

Keogh, M.C., Kurdistani, S.K., Morris, S.A., Ahn, S.H., Podolny, V., Collins, S.R., Schuldiner, M., Chin, K., Punna, T., Thompson, N.J.*, et al.* (2005). Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. Cell *123*, 593-605.

Kim, J.H., Lane, W.S., and Reinberg, D. (2002). Human Elongator facilitates RNA polymerase II transcription through chromatin. Proc Natl Acad Sci U S A *99*, 1241-1246.

Kim, T., and Buratowski, S. (2009). Dimethylation of H3K4 by Set1 recruits the Set3 histone deacetylase complex to 5' transcribed regions. Cell *137*, 259-272.

Kingston, R.E., and Narlikar, G.J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev *13*, 2339-2352.

Kireeva, M.L., Walter, W., Tchernajenko, V., Bondarenko, V., Kashlev, M., and Studitsky, V.M. (2002). Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. Mol Cell *9*, 541-552.

Kitazono, A.A., Tobe, B.T., Kalton, H., Diamant, N., and Kron, S.J. (2002). Marker-fusion PCR for one-step mutagenesis of essential genes in yeast. Yeast *19*, 141-149.

Kizer, K.O., Phatnani, H.P., Shibata, Y., Hall, H., Greenleaf, A.L., and Strahl, B.D. (2005). A novel domain in Set2p mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol *25*, 3305-3316.

Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., and Rine, J. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol *2*, E131.

Kornberg, R.D. (1977). Structure of chromatin. Annu Rev Biochem *46*, 931-954.

Kouskouti, A., and Talianidis, I. (2005). Histone modifications defining active genes persist after transcriptional and mitotic inactivation. EMBO J *24*, 347-357.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Krebs, J.E. (2007). Moving marks: dynamic histone modifications in yeast. Mol Biosyst *3*, 590-597.

Kristjuhan, A., and Svejstrup, J.Q. (2004). Evidence for distinct mechanisms facilitating transcript elongation through chromatin in vivo. EMBO J *23*, 4243-4252.

Kristjuhan, A., Walker, J., Suka, N., Grunstein, M., Roberts, D., Cairns, B.R., and Svejstrup, J.Q. (2002). Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region. Mol Cell *10*, 925-933.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M.*, et al.* (2003a). The Paf1p1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell *11*, 721-729.

Krogan, N.J., Kim, M., Ahn, S.H., Zhong, G., Kobor, M.S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J.F. (2002). RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. Mol Cell Biol *22*, 6979-6992.

Krogan, N.J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D.P., Beattie, B.K., Emili, A., Boone, C.*, et al.* (2003b). Methylation of histone H3 by Set2p in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. Mol Cell Biol *23*, 4207-4218.

Krohn, N.M., Stemmer, C., Fojan, P., Grimm, R., and Grasser, K.D. (2003). Protein kinase CK2 phosphorylates the high mobility group domain protein SSRP1, inducing the recognition of UV-damaged DNA. J Biol Chem *278*, 12710-12715.

Kruger, W., and Herskowitz, I. (1991). A negative regulator of HO transcription, SIN1 (SPT2), is a nonspecific DNA-binding protein related to HMG1. Mol Cell Biol *11*, 4135-4146.

Kruger, W., Peterson, C.L., Sil, A., Coburn, C., Arents, G., Moudrianakis, E.N., and Herskowitz, I. (1995). Amino acid substitutions in the structured domains of histones H3 and H4 partially relieve the requirement of the yeast Swi/Snf complex for transcription. Genes Dev *9*, 2770-2779.

Kulaeva, O.I., Hsieh, F.K., and Studitsky, V.M. (2010). RNA polymerase complexes cooperate to relieve the nucleosomal barrier and evict histones. Proc Natl Acad Sci U S A *107*, 11325-11330.

Kuo, M.H., Zhou, J., Jambeck, P., Churchill, M.E., and Allis, C.D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. Genes Dev *12*, 627-639.

Laribee, R.N., Fuchs, S.M., and Strahl, B.D. (2007). H2B ubiquitylation in transcriptional control: a FACT-finding mission. Genes Dev *21*, 737-743.

Larschan, E., and Winston, F. (2001). The *Saccharomyces cerevisiae* SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4. Genes Dev *15*, 1946-1956.

Laskey, R.A., Honda, B.M., Mills, A.D., and Finch, J.T. (1978). Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature *275*, 416-420.

Laurent, B.C., Yang, X., and Carlson, M. (1992). An essential *Saccharomyces cerevisiae* gene homologous to SNF2 encodes a helicase-related protein in a new family. Mol Cell Biol *12*, 1893-1902.

Lawless, M.W., O'Byrne, K.J., and Gray, S.G. (2009). Histone deacetylase inhibitors target diabetes via chromatin remodeling or as chemical chaperones? Curr Diabetes Rev *5*, 201-209.

Lee, C.K., Shibata, Y., Rao, B., Strahl, B.D., and Lieb, J.D. (2004). Evidence for nucleosome depletion at active regulatory regions genome-wide. Nat Genet *36*, 900-905.

Lee, J.S., and Shilatifard, A. (2007). A site to remember: H3K36 methylation a mark for histone deacetylation. Mutat Res *618*, 130-134.

Lee, K.K., and Workman, J.L. (2007). Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol *8*, 284-295.

Lee, S.K., Fletcher, A.G., Zhang, L., Chen, X., Fischbeck, J.A., and Stargell, L.A. Activation of a poised RNAPII-dependent promoter requires both SAGA and Mediator. Genetics *184*, 659-672.

Lee, S.K., Fletcher, A.G., Zhang, L., Chen, X., Fischbeck, J.A., and Stargell, L.A. (2010). Activation of a poised RNAPII-dependent promoter requires both SAGA and Mediator. Genetics *184*, 659-672.

Lefebvre, L., and Smith, M. (1993). Mutational and functional analysis of dominant SPT2 (SIN1) suppressor alleles in *Saccharomyces cerevisiae*. Mol Cell Biol *13*, 5393-5407.

LeRoy, G., Orphanides, G., Lane, W.S., and Reinberg, D. (1998). Requirement of RSF and FACT for transcription of chromatin templates in vitro. Science *282*, 1900-1904.

Li, B., Carey, M., and Workman, J.L. (2007a). The role of chromatin during transcription. Cell *128*, 707-719.

Li, B., Gogol, M., Carey, M., Lee, D., Seidel, C., and Workman, J.L. (2007b). Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. Science *316*, 1050-1054.

Li, B., Howe, L., Anderson, S., Yates, J.R., 3rd, and Workman, J.L. (2003). The Set2p histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem *278*, 8897-8903.

Li, B., Pattenden, S.G., Lee, D., Gutierrez, J., Chen, J., Seidel, C., Gerton, J., and Workman, J.L. (2005a). Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. Proc Natl Acad Sci U S A *102*, 18385-18390.

Li, G., and Reinberg, D. (2011). Chromatin higher-order structures and gene regulation. Curr Opin Genet Dev *21*, 175-186.

Li, X., Gerber, S.A., Rudner, A.D., Beausoleil, S.A., Haas, W., Villen, J., Elias, J.E., and Gygi, S.P. (2007c). Large-scale phosphorylation analysis of alpha-factor-arrested *Saccharomyces cerevisiae*. J Proteome Res *6*, 1190-1197.

Li, Y., Keller, D.M., Scott, J.D., and Lu, H. (2005b). CK2 phosphorylates SSRP1 and inhibits its DNA-binding activity. J Biol Chem *280*, 11869-11875.

Liew, C.C., and Dzau, V.J. (2004). Molecular genetics and genomics of heart failure. Nat Rev Genet *5*, 811-825.

Lin, C.H., and Workman, J.L. (2011). Suppression of cryptic intragenic transcripts is required for embryonic stem cell self-renewal. EMBO J *30*, 1420-1421.

Lindstrom, D.L., Squazzo, S.L., Muster, N., Burckin, T.A., Wachter, K.C., Emigh, C.A., McCleery, J.A., Yates, J.R., 3rd, and Hartzog, G.A. (2003). Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol *23*, 1368-1378.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953-961.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Malik, H.S., and Henikoff, S. (2003). Phylogenomics of the nucleosome. Nat Struct Biol *10*, 882-891.

Marin, O., Meggio, F., Sarno, S., Cesaro, L., Pagano, M.A., and Pinna, L.A. (1999). Tyrosine versus serine/threonine phosphorylation by protein kinase casein kinase-2. A study with peptide substrates derived from immunophilin Fpr3. J Biol Chem *274*, 29260-29265.

Marino-Ramirez, L., Jordan, I.K., and Landsman, D. (2006). Multiple independent evolutionary solutions to core histone gene regulation. Genome Biol *7*, R122.

Martens, C., Krett, B., and Laybourn, P.J. (2001). RNA polymerase II and TBP occupy the repressed CYC1 promoter. Mol Microbiol *40*, 1009-1019.

Martens, J.A., and Winston, F. (2002). Evidence that Swi/Snf directly represses transcription in *Saccharomyces cerevisiae*. Genes Dev *16*, 2231-2236.

Martens, J.A., and Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Curr Opin Genet Dev *13*, 136-142.

Mas, G., de Nadal, E., Dechant, R., Rodriguez de la Concepcion, M.L., Logie, C., Jimeno-Gonzalez, S., Chavez, S., Ammerer, G., and Posas, F. (2009). Recruitment of a chromatin remodelling complex by the Hog1 MAP kinase to stress genes. EMBO J *28*, 326-336.

Matallana, E., Franco, L., and Perez-Ortin, J.E. (1992). Chromatin structure of the yeast SUC2 promoter in regulatory mutants. Mol Gen Genet *231*, 395-400.

McDonald, S.M., Close, D., Xin, H., Formosa, T., and Hill, C.P. (2010). Structure and biological importance of the Spn1-Spt6p interaction, and its regulatory role in nucleosome binding. Mol Cell *40*, 725-735.

Meggio, F., and Pinna, L.A. (2003). One-thousand-and-one substrates of protein kinase CK2? FASEB J *17*, 349-368.

Mello, J.A., and Almouzni, G. (2001). The ins and outs of nucleosome assembly. Curr Opin Genet Dev *11*, 136-141.

Milgrom, E., West, R.W., Jr., Gao, C., and Shen, W.C. (2005). TFIID and Spt-Ada-Gcn5-acetyltransferase functions probed by genome-wide synthetic genetic array analysis using a *Saccharomyces cerevisiae* taf9-ts allele. Genetics *171*, 959-973.

Miller, T., Krogan, N.J., Dover, J., Erdjument-Bromage, H., Tempst, P., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2001). COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc Natl Acad Sci U S A *98*, 12902-12907.

Mito, Y., Henikoff, J.G., and Henikoff, S. (2005). Genome-scale profiling of histone H3.3 replacement patterns. Nat Genet *37*, 1090-1097.

Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science *303*, 343-348.

Morillon, A., Karabetsou, N., O'Sullivan, J., Kent, N., Proudfoot, N., and Mellor, J. (2003). Isw1 chromatin remodeling ATPase coordinates transcription elongation and termination by RNA polymerase II. Cell *115*, 425-435.

Morrison, A.J., and Shen, X. (2009). Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol *10*, 373-384.

Muchardt, C., and Yaniv, M. (1999). ATP-dependent chromatin remodelling: Swi/Snf and Co. are on the job. J Mol Biol *293*, 187-198.

Nechaev, S., and Adelman, K. (2011). Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. Biochim Biophys Acta *1809*, 34-45.

Neigeborn, L., and Carlson, M. (1984). Genes affecting the regulation of SUC2 gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics *108*, 845-858.

Neigeborn, L., Rubin, K., and Carlson, M. (1986). Suppressors of SNF2 mutations restore invertase derepression and cause temperature-sensitive lethality in yeast. Genetics *112*, 741-753.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell *11*, 709-719.

Ni, Z., Saunders, A., Fuda, N.J., Yao, J., Suarez, J.R., Webb, W.W., and Lis, J.T. (2008). P-TEFb is critical for the maturation of RNA polymerase II into productive elongation in vivo. Mol Cell Biol *28*, 1161-1170.

Nourani, A., Doyon, Y., Utley, R.T., Allard, S., Lane, W.S., and Cote, J. (2001). Role of an ING1 growth regulator in transcriptional activation and targeted histone acetylation by the NuA4 complex. Mol Cell Biol *21*, 7629-7640.

Nourani, A., Robert, F., and Winston, F. (2006). Evidence that Spt2/Sin1, an HMG-like factor, plays roles in transcription elongation, chromatin structure, and genome stability in *Saccharomyces cerevisiae*. Mol Cell Biol *26*, 1496-1509.

Novoseler, M., Hershkovits, G., and Katcoff, D.J. (2005). Functional domains of the yeast chromatin protein Sin1p/Spt2p can bind four-way junction and crossing DNA structures. J Biol Chem *280*, 5169-5177.

Ohkuma, Y. (1997). Multiple functions of general transcription factors TFIIE and TFIIH in transcription: possible points of regulation by trans-acting factors. J Biochem *122*, 481-489.

Olson, E.N. (2006). Gene regulatory networks in the evolution and development of the heart. Science *313*, 1922-1927.

Orphanides, G., LeRoy, G., Chang, C.H., Luse, D.S., and Reinberg, D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell *92*, 105-116.

Orphanides, G., Wu, W.H., Lane, W.S., Hampsey, M., and Reinberg, D. (1999). The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. Nature *400*, 284-288.

Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A.M., Gustafsson, C.M., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J.Q. (1999). Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. Mol Cell *3*, 109-118.

Padmanabha, R., Chen-Wu, J.L., Hanna, D.E., and Glover, C.V. (1990). Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. Mol Cell Biol *10*, 4089-4099.

Park, Y.J., and Luger, K. (2006). Structure and function of nucleosome assembly proteins. Biochem Cell Biol *84*, 549-558.

Pavri, R., Zhu, B., Li, G., Trojer, P., Mandal, S., Shilatifard, A., and Reinberg, D. (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell *125*, 703-717.

Perez-Martin, J., and Johnson, A.D. (1998a). The C-terminal domain of Sin1 interacts with the SWI-SNF complex in yeast. Mol Cell Biol *18*, 4157-4164.

Perez-Martin, J., and Johnson, A.D. (1998b). Mutations in chromatin components suppress a defect of Gcn5 protein in *Saccharomyces cerevisiae*. Mol Cell Biol *18*, 1049-1054.

Peterson, C.L., Dingwall, A., and Scott, M.P. (1994). Five Swi/Snf gene products are components of a large multisubunit complex required for transcriptional enhancement. Proc Natl Acad Sci U S A *91*, 2905-2908.

Peterson, C.L., Kruger, W., and Herskowitz, I. (1991). A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. Cell *64*, 1135-1143.

Phatnani, H.P., and Greenleaf, A.L. (2006). Phosphorylation and functions of the RNA polymerase II CTD. Genes Dev *20*, 2922-2936.

Pilyugin, M., Demmers, J., Verrijzer, C.P., Karch, F., and Moshkin, Y.M. (2009). Phosphorylation-mediated control of histone chaperone ASF1 levels by Tousled-like kinases. Plos One *4*, e8328.

Pollard, K.J., and Peterson, C.L. (1997). Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression. Mol Cell Biol *17*, 6212-6222.

Ponting, C.P. (2002). Novel domains and orthologues of eukaryotic transcription elongation factors. Nucleic Acids Res *30*, 3643-3652.

Poole, A., Poore, T., Bandhakavi, S., McCann, R.O., Hanna, D.E., and Glover, C.V. (2005). A global view of CK2 function and regulation. Mol Cell Biochem *274*, 163-170.

Pray-Grant, M.G., Daniel, J.A., Schieltz, D., Yates, J.R., 3rd, and Grant, P.A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature *433*, 434-438.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218-229.

Rando, O.J., and Chang, H.Y. (2009). Genome-wide views of chromatin structure. Annu Rev Biochem *78*, 245-271.

Recht, J., and Osley, M.A. (1999). Mutations in both the structured domain and N-terminus of histone H2B bypass the requirement for Swi-Snf in yeast. EMBO J *18*, 229-240.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev *12*, 162-169.

Reeves, R. (2010). Nuclear functions of the HMG proteins. Biochimica et biophysica acta *1799*, 3-14.

Rhoades, A.R., Ruone, S., and Formosa, T. (2004). Structural features of nucleosomes reorganized by yeast FACT and its HMG box component, Nhp6. Mol Cell Biol *24*, 3907-3917.

Rocha, W., and Verreault, A. (2008). Clothing up DNA for all seasons: Histone chaperones and nucleosome assembly pathways. FEBS Lett *582*, 1938-1949.

Rodriguez-Paredes, M., Ceballos-Chavez, M., Esteller, M., Garcia-Dominguez, M., and Reyes, J.C. (2009). The chromatin remodeling factor CHD8 interacts with elongating RNA polymerase II and controls expression of the cyclin E2 gene. Nucleic Acids Res *37*, 2449-2460.

Rose, M., Winston, F., and Hieter, P. (1990). Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rose, M.D., Novick, P., Thomas, J.H., Botstein, D., and Fink, G.R. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene *60*, 237-243.

Roth, S.Y., Denu, J.M., and Allis, C.D. (2001). Histone acetyltransferases. Annu Rev Biochem *70*, 81-120.

Rufiange, A., Jacques, P.E., Bhat, W., Robert, F., and Nourani, A. (2007). Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. Mol Cell *27*, 393-405.

Ruone, S., Rhoades, A.R., and Formosa, T. (2003). Multiple Nhp6 molecules are required to recruit Spt16-Pob3 to form yFACT complexes and to reorganize nucleosomes. J Biol Chem *278*, 45288-45295.

Russo, G.L., van den Bos, C., Sutton, A., Coccetti, P., Baroni, M.D., Alberghina, L., and Marshak, D.R. (2000). Phosphorylation of Cdc28 and regulation of cell size by the protein kinase CKII in *Saccharomyces cerevisiae*. Biochem J *351*, 143-150.

Saha, A., Wittmeyer, J., and Cairns, B.R. (2006). Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol *7*, 437-447.

Sarokin, L., and Carlson, M. (1985). Comparison of two yeast invertase genes: conservation of the upstream regulatory region. Nucleic Acids Res *13*, 6089-6103.

Saunders, A., Core, L.J., and Lis, J.T. (2006). Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol *7*, 557-567.

Sawa, C., Nedea, E., Krogan, N., Wada, T., Handa, H., Greenblatt, J., and Buratowski, S. (2004). Bromodomain factor 1 (Bdf1) is phosphorylated by protein kinase CK2. Mol Cell Biol *24*, 4734-4742.

Sawatsubashi, S., Murata, T., Lim, J., Fujiki, R., Ito, S., Suzuki, E., Tanabe, M., Zhao, Y., Kimura, S., Fujiyama, S.*, et al.* (2010). A histone chaperone, DEK, transcriptionally coactivates a nuclear receptor. Genes Dev *24*, 159-170.

Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res *18*, 3091-3092.

Schneider, J., Wood, A., Lee, J.S., Schuster, R., Dueker, J., Maguire, C., Swanson, S.K., Florens, L., Washburn, M.P., and Shilatifard, A. (2005). Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. Mol Cell *19*, 849-856.

Schwabish, M.A., and Struhl, K. (2004). Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol Cell Biol *24*, 10111-10117.

Sekinger, E.A., Moqtaderi, Z., and Struhl, K. (2005). Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. Mol Cell *18*, 735-748.

Selth, L.A., Sigurdsson, S., and Svejstrup, J.Q. Transcript Elongation by RNA Polymerase II. Annu Rev Biochem *79*, 271-293.

Selth, L.A., Sigurdsson, S., and Svejstrup, J.Q. (2010). Transcript Elongation by RNA Polymerase II. Annu Rev Biochem *79*, 271-293.

Shandilya, J., and Roberts, S.G. (2012). The transcription cycle in eukaryotes: From productive initiation to RNA polymerase II recycling. Biochim Biophys Acta *1819*, 391-400.

Shen, X., Mizuguchi, G., Hamiche, A., and Wu, C. (2000). A chromatin remodelling complex involved in transcription and DNA processing. Nature *406*, 541-544.

Shi, X., Potvin, B., Huang, T., Hilgard, P., Spray, D.C., Suadicani, S.O., Wolkoff, A.W., Stanley, P., and Stockert, R.J. (2001). A novel casein kinase 2 alpha-subunit regulates membrane protein traffic in the human hepatoma cell line HuH-7. J Biol Chem *276*, 2075-2082.

Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R., and Peterson, C.L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science *311*, 844-847.

Sikdar, N., Banerjee, S., Zhang, H., Smith, S., and Myung, K. (2008). Spt2p defines a new transcription-dependent gross chromosomal rearrangement pathway. PLoS Genet *4*, e1000290.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19-27.

Simic, R., Lindstrom, D.L., Tran, H.G., Roinick, K.L., Costa, P.J., Johnson, A.D., Hartzog, G.A., and Arndt, K.M. (2003). Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. EMBO J *22*, 1846-1856.

Sims, R.J., 3rd, Belotserkovskaya, R., and Reinberg, D. (2004). Elongation by RNA polymerase II: the short and long of it. Genes Dev *18*, 2437-2468.

Smolle, M., Venkatesh, S., Gogol, M.M., Li, H., Zhang, Y., Florens, L., Washburn, M.P., and Workman, J.L. (2012). Chromatin remodelers Isw1 and Chd1 maintain chromatin structure during transcription by preventing histone exchange. Nature Structural & Molecular Biology *19*, 884-892.

Srinivasan, S., Armstrong, J.A., Deuring, R., Dahlsveen, I.K., McNeill, H., and Tamkun, J.W. (2005). The Drosophila trithorax group protein Kismet facilitates an early step in transcriptional elongation by RNA Polymerase II. Development *132*, 1623-1635.

Stern, M., Jensen, R., and Herskowitz, I. (1984). Five SWI genes are required for expression of the HO gene in yeast. J Mol Biol *178*, 853-868.

Stillman, D.J. (2010). Nhp6: a small but powerful effector of chromatin structure in *Saccharomyces cerevisiae*. Biochim Biophys Acta *1799*, 175-180.

Stoler, S., Keith, K.C., Curnick, K.E., and Fitzgerald-Hayes, M. (1995). A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes Dev *9*, 573-586.

Sudarsanam, P., and Winston, F. (2000). The Swi/Snf family nucleosome-remodeling complexes and transcriptional control. Trends Genet *16*, 345-351.

Sun, M., Lariviere, L., Dengl, S., Mayer, A., and Cramer, P. (2010). A tandem SH2 domain in transcription elongation factor Spt6p binds the phosphorylated RNA polymerase II C-terminal repeat domain (CTD). J Biol Chem *285*, 41597-41603.

Swanson, M.S., and Winston, F. (1992). SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in *Saccharomyces cerevisiae*. Genetics *132*, 325-336.

Tanny, J.C., Erdjument-Bromage, H., Tempst, P., and Allis, C.D. (2007). Ubiquitylation of histone H2B controls RNA polymerase II transcription elongation independently of histone H3 methylation. Genes Dev *21*, 835-847.

Thebault, P., Boutin, G., Bhat, W., Rufiange, A., Martens, J., and Nourani, A. (2011). Transcription regulation by the noncoding RNA SRG1 requires Spt2-dependent chromatin deposition in the wake of RNA polymerase II. Mol Cell Biol *31*, 1288-1300.

Tsukiyama, T. (2002). The in vivo functions of ATP-dependent chromatin-remodelling factors. Nat Rev Mol Cell Biol *3*, 422-429.

Tsukiyama, T., Palmer, J., Landel, C.C., Shiloach, J., and Wu, C. (1999). Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in *Saccharomyces cerevisiae*. Genes Dev *13*, 686-697.

Turner, B.M. (2005). Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol *12*, 110-112.

Utley, R.T., Lacoste, N., Jobin-Robitaille, O., Allard, S., and Cote, J. (2005). Regulation of NuA4 histone acetyltransferase activity in transcription and DNA repair by phosphorylation of histone H4. Mol Cell Biol *25*, 8179-8190.

Van Hooser, A.A., Ouspenski, II, Gregson, H.C., Starr, D.A., Yen, T.J., Goldberg, M.L., Yokomori, K., Earnshaw, W.C., Sullivan, K.F., and Brinkley, B.R. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. J Cell Sci *114*, 3529-3542.

van Vugt, J.J., Ranes, M., Campsteijn, C., and Logie, C. (2007). The ins and outs of ATP-dependent chromatin remodeling in budding yeast: biophysical and proteomic perspectives. Biochim Biophys Acta *1769*, 153-171.

VanDemark, A.P., Blanksma, M., Ferris, E., Heroux, A., Hill, C.P., and Formosa, T. (2006). The structure of the yFACT Pob3-M domain, its interaction with the DNA replication factor RPA, and a potential role in nucleosome deposition. Mol Cell *22*, 363-374.

Varshavsky, A. (2005). Regulated protein degradation. Trends Biochem Sci *30*, 283-286.

Varv, S., Kristjuhan, K., and Kristjuhan, A. (2007). RNA polymerase II determines the area of nucleosome loss in transcribed gene loci. Biochem Biophys Res Commun *358*, 666-671.

Venkatesh, S., Smolle, M., Li, H., Gogol, M.M., Saint, M., Kumar, S., Natarajan, K., and Workman, J.L. (2012). Set2p methylation of histone H3 lysine 36 suppresses histone exchange on transcribed genes. Nature *489*, 452-455.

Verdone, L., Agricola, E., Caserta, M., and Di Mauro, E. (2006). Histone acetylation in gene regulation. Brief Funct Genomic Proteomic *5*, 209-221.

Vilk, G., Weber, J.E., Turowec, J.P., Duncan, J.S., Wu, C., Derksen, D.R., Zien, P., Sarno, S., Donella-Deana, A., Lajoie, G.*, et al.* (2008). Protein kinase CK2 catalyzes tyrosine phosphorylation in mammalian cells. Cell Signal *20*, 1942-1951.

Wang, G.G., Allis, C.D., and Chi, P. (2007). Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling. Trends Mol Med *13*, 373-380.

Wang, W. (2003). The Swi/Snf family of ATP-dependent chromatin remodelers: similar mechanisms for diverse functions. Curr Top Microbiol Immunol *274*, 143-169.

Weake, V.M., and Workman, J.L. (2008). Histone ubiquitination: triggering gene activity. Mol Cell *29*, 653-663.

Wery, M., Shematorova, E., Van Driessche, B., Vandenhaute, J., Thuriaux, P., and Van Mullem, V. (2004). Members of the SAGA and Mediator complexes are partners of the transcription elongation factor TFIIS. EMBO J *23*, 4232-4242.

Whitehouse, I., Rando, O.J., Delrow, J., and Tsukiyama, T. (2007). Chromatin remodelling at promoters suppresses antisense transcription. Nature *450*, 1031-1035.

Widom, J. (1989). Toward a unified model of chromatin folding. Annu Rev Biophys Biophys Chem *18*, 365-395.

Wilson, L.K., Dhillon, N., Thorner, J., and Martin, G.S. (1997). Casein kinase II catalyzes tyrosine phosphorylation of the yeast nucleolar immunophilin Fpr3. J Biol Chem *272*, 12961-12967.

Winston, F., and Carlson, M. (1992). Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. Trends Genet *8*, 387-391.

Winston, F., Chaleff, D.T., Valent, B., and Fink, G.R. (1984). Mutations affecting Ty-mediated expression of the HIS4 gene of *Saccharomyces cerevisiae*. Genetics *107*, 179-197.

Winston, F., Dollard, C., and Ricupero-Hovasse, S.L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast *11*, 53-55.

Wood, A., Schneider, J., Dover, J., Johnston, M., and Shilatifard, A. (2003). The Paf1p1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. J Biol Chem *278*, 34739-34742.

Woodage, T., Basrai, M.A., Baxevanis, A.D., Hieter, P., and Collins, F.S. (1997). Characterization of the CHD family of proteins. Proc Natl Acad Sci U S A *94*, 11472-11477.

Woodcock, C.L., and Dimitrov, S. (2001). Higher-order structure of chromatin and chromosomes. Curr Opin Genet Dev *11*, 130-135.

Woodcock, C.L., and Ghosh, R.P. (2010). Chromatin higher-order structure and dynamics. Cold Spring Harb Perspect Biol *2*, a000596.

Wu, L., and Winston, F. (1997). Evidence that Snf-Swi controls chromatin structure over both the TATA and UAS regions of the SUC2 promoter in *Saccharomyces cerevisiae*. Nucleic Acids Res *25*, 4230-4234.

Xie, L., Pelz, C., Wang, W., Bashar, A., Varlamova, O., Shadle, S., and Impey, S. (2011). KDM5B regulates embryonic stem cell self-renewal and represses cryptic intragenic transcription. EMBO J *30*, 1473-1484.

Yamaguchi, Y., Narita, T., Inukai, N., Wada, T., and Handa, H. (2001). SPT genes: key players in the regulation of transcription, chromatin structure and other cellular processes. J Biochem *129*, 185-191.

Yearling, M.N., Radebaugh, C.A., and Stargell, L.A. (2011). The Transition of Poised RNA Polymerase II to an Actively Elongating State Is a Complex Affair. Genetics Genetics Research International, 7.

Yoh, S.M., Cho, H., Pickle, L., Evans, R.M., and Jones, K.A. (2007). The Spt6p SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export. Genes Dev *21*, 160-174.

Yoh, S.M., Lucas, J.S., and Jones, K.A. (2008). The Iws1:Spt6:CTD complex controls cotranscriptional mRNA biosynthesis and HYPB/Setd2-mediated histone H3K36 methylation. Genes Dev *22*, 3422-3434.

Youdell, M.L., Kizer, K.O., Kisseleva-Romanova, E., Fuchs, S.M., Duro, E., Strahl, B.D., and Mellor, J. (2008). Roles for Ctk1 and Spt6p in regulating the different methylation states of histone H3 lysine 36. Mol Cell Biol *28*, 4915-4926.

Zhang, H., Roberts, D.N., and Cairns, B.R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell *123*, 219-231.

Zhang, L., Fletcher, A.G., Cheung, V., Winston, F., and Stargell, L.A. (2008). Spn1 regulates the recruitment of Spt6p and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol *28*, 1393-1403.

Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev *15*, 2343-2360.

Zhu, H., Klemic, J.F., Chang, S., Bertone, P., Casamayor, A., Klemic, K.G., Smith, D., Gerstein, M., Reed, M.A., and Snyder, M. (2000). Analysis of yeast protein kinases using protein chips. Nat Genet *26*, 283-289.

Zlatanova, J., and van Holde, K. (1998). Binding to four-way junction DNA: a common property of architectural proteins? FASEB J *12*, 421-431.

Annex1

Molecular Cell
Article

Genome-Wide Replication-Independent Histone H3 Exchange Occurs Predominantly at Promoters and Implicates H3 K56 Acetylation and Asf1

Anne Rufiange,[1,4] Pierre-Étienne Jacques,[2,4] Wajid Bhat,[1] François Robert,[2,3] and Amine Nourani[1,*]
[1]Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec (CHUQ), Québec, QC G1R 2J6, Canada
[2]Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, QC H2W 1R7, Canada
[3]Département de Médecine, Faculté de Médecine, Université de Montréal, Montréal, QC H3T 1J4, Canada
[4]These authors contributed equally to this work.
*Correspondence: amine.nourani@crhdq.ulaval.ca
DOI 10.1016/j.molcel.2007.07.011

SUMMARY

In yeast, histone H3/H4 exchange independent of replication is poorly understood. Here, we analyzed the deposition of histone H3 molecules, synthesized during G1, using a high-density microarray histone exchange assay. While we found that H3 exchange in coding regions requires high levels of transcription, promoters exchange H3 molecules in the absence of transcription. In inactive promoters, H3 is deposited predominantly in well-positioned nucleosomes surrounding nucleosome-free regions, indicating that some nucleosomes in promoters are dynamic. This could facilitate induction of repressed genes. Importantly, we show that histone H3 K56 acetylation, a replication-associated mark, is also present in replication-independent newly assembled nucleosomes and correlates perfectly with the deposition of new H3. Finally, we found that transcription-dependent incorporation of H3 at promoters is highly dependent on Asf1. Taken together, our data underline the dynamic nature of replication-independent nucleosome assembly/disassembly, specify a link to transcription, and implicate Asf1 and H3 K56 acetylation.

INTRODUCTION

Major advances were made during the past decade in the understanding of chromatin structure modulation. Most transcriptional regulation involves chromatin remodeling by ATP-dependent activities and posttranslational modifications of histones. In addition to these modulations, recent observations have linked nucleosomal assembly, disassembly, and histone exchange to transcription, suggesting the existence of more drastic means of gene regulation (Workman, 2006). Most inactive genes possess stable positioned nucleosomes in their promoters that inhibit the binding of the transcription complex. Presumably, these nucleosomes are modified, remodeled, and displaced during activation (Workman, 2006). At promoters, histone exchange could be a way to add or remove certain histone modifications that play a role during activation. Interestingly, recent observations have shown that replication-independent replacement of histone H2A by the variant histone Htz1 at promoters of repressed genes could facilitate their activation (Guillemette et al., 2005; Raisner et al., 2005; Zhang et al., 2005). Therefore, replication-independent chromatin assembly-disassembly and histone exchange are potential mechanisms by which genes are regulated.

Higher eukaryotes such as vertebrates or flies contain a variant histone H3.3 that is deposited independently of replication (Mito et al., 2005). Histone H3.3 is incorporated genome-wide in transcribed regions in *Drosophila* or at promoters of active mammalian genes (Chow et al., 2005; Janicki et al., 2004; Mito et al., 2005). However, in chicken hematopoietic cells, the transcription-dependent deposition of H3.3 is less clearly associated with transcription, since it occurs mainly at upstream regions of genes independently of their transcription status (Jin and Felsenfeld, 2006). Recent studies have shown that transcription is associated with eviction of histones, while the repression of galactose-controlled genes is correlated with a rapid redeposition of histones (Schwabish and Struhl, 2004, 2006). This reassembly of chromatin is not consistent with a mechanism coupled to replication during S phase but rather suggests a process independent of replication and coupled to transcription (Schwabish and Struhl, 2004). This process may recycle histones that were displaced by RNA polymerase II (RNAP II) or could use a new source of histones (Workman, 2006).

Histone exchange and deposition independent of replication are important processes that emerge as possible mechanisms of gene regulation (Williams and Tyler, 2007). Using a high-resolution, microarray-based, histone H3 replacement assay performed in G1-arrested yeast cells, we studied the replication-independent histone H3 deposition. We found basal and transcription-coupled

H3 deposition both mainly targeting promoter regions. Importantly, despite strict genome-wide correlation with transcription at open reading frames, we observed an important transcription-independent exchange in promoters of inactive genes. Using high-resolution MNase-ChIP assays, we mapped this exchange to nucleosomes surrounding the nucleosome-free region (NFR) of inactive genes. Detailed analyses on *PHO5* confirmed that positioned nucleosomes of a repressed promoter exchange histone H3 in absence of replication or transcription. This indicates that nucleosomes of inactive genes are in a dynamic state that could poise promoters for activation. Therefore, our data provide evidence of the existence of transcription-dependent and -independent mechanisms of H3 exchange outside of S phase. We finally show that Asf1 contributes largely to the transcription-dependent deposition of new histone H3 and that it is mainly involved at promoters of inducible genes. Interestingly, in contrast to previous reports, our data show that Asf1-dependent acetylation of H3 K56 is associated with DNA replication-independent histone H3 deposition. Our analyses show global strong correlation between H3 exchange and acetylation of H3 K56. We therefore suggest that H3 K56 acetylation is also a mark of replication-independent newly assembled nucleosomes.

RESULTS

To analyze histone H3 dynamics, we used an experimental system based on the method developed to study chromatin reassembly at the *PHO5* promoter upon repression (Schermer et al., 2005) and coupled it to high-resolution microarrays. In this system there are two different sources of histone H3 in the cell, the endogenous histone tagged with the Myc epitope and a galactose-inducible form fused to the Flag tag coexpressed with histone H4. In order to eliminate the contribution of DNA replication-dependent histone deposition, exponentially growing cells containing the double-tag system are blocked in G1 with α factor. After incubation with α factor, cells are either fixed or induced to express Flag-H3 prior to formaldehyde treatment. Next, the levels of Myc-H3, Flag-H3, and RNAP II are assayed either by standard ChIP-QPCR or by ChIP-chip using tiling-path DNA microarrays (Guillemette et al., 2005; Pokholok et al., 2005). Preliminary analyses, performed on selected loci, showed that new Flag-H3 histones were incorporated into various genomic loci with a preferential incorporation at transcribed genes as shown for *PMA1* and *GAL1* coding regions (Figure 1A). We also observed some level of transcription-independent histone H3 incorporation (Figure 1A and see Figure S1 in the Supplemental Data available with this article online).

Newly Synthesized Histone H3 Is Incorporated into Chromatin

Although our preliminary data suggest that the new H3 is incorporated into nucleosomes, it was possible that Flag-H3 crosslinks nonspecifically to DNA of different genomic regions after formaldehyde treatment. To rule out this possibility, we purified chromatin and asked if the new H3 is incorporated into nucleosomes. For that, nuclei from cells expressing new Flag-H3 were isolated, digested with a high concentration of MNase producing mainly DNA fragments ranging from 0 to 300 bp. The product was fractionated on a Superose 6 column as previously described (Utley et al., 2005), and the fractions were analyzed on agarose gels by SDS-PAGE or western blotting. Figure 1B showed that fractions 17–23 contained dinucleosmes or oligonucleosomes, that fractions 25–27 contained monoucleosomes, and that in fractions 29–37, the DNA molecules were smaller than the expected size of nucleosomes. The DNA of mononucleosomes coeluted with all four core histones, whereas fractions 31–35 contained only H2A/H2B dimers as shown by Coomassie-stained SDS-PAGE. Interestingly, the Flag-H3 as well as the constitutive Myc-H3 coeluted with the mononucleosomal DNA and the three other core histones. These observations show that Flag-H3 is incorporated into nucleosomes. Importantly, very little Myc-H3 or Flag-H3 eluted in fractions 31–35. According to their size, free H3 monomers, H3/H4 dimers, or H3/H4 tetramers would elute in fraction 31–37. Therefore, we conclude that the majority of Flag-H3 is associated with chromatin and is incorporated into nucleosomes.

High incorporation of Flag-H3 into nucleosomes should be associated, at least partially, with the replacement of the endogenous Myc-H3. To test this, we conducted a time course experiment in G1-arrested cells and monitored simultaneously the levels of Flag-H3 and Myc-H3 at transcribed genes (Figure 1C). Our data showed that incorporation of the Flag-H3 in transcribed genes was accompanied by a loss of the previously chromatin-associated histone H3 molecules (Myc-H3). This is indicative of a specific chromatin assembly process that uses histones from the free pool to replace existing histone molecules. We refer to this phenomenon as H3 exchange.

Histone H3 Synthesized outside of Replication Is Preferentially Targeted to Transcribed Loci

As suggested by our preliminary analyses (Figures 1A and 1C and data not shown), our genome-wide data showed that the incorporation of new H3 was well correlated with transcription. Figure 1D indeed shows a clear correlation between transcription (as measured by RNAPII occupancy by ChIP-chip) and H3 exchange (as measured by the Flag-H3/Myc-H3 ratio by ChIP-chip) on protein-coding genes. This observation establishes that, outside of replication, deposition of H3 is associated with transcription. However, gene-by-gene analyses by QPCR and genome-wide analyses by ChIP-chip both clearly showed that a basal incorporation is observed at nontranscribed loci, suggesting two different replication-independent chromatin assembly mechanisms (see below and Figure S1). We therefore asked, for a given gene or location, if transcription is the only determinant of new histone incorporation. For that, we analyzed by ChIP assays, in

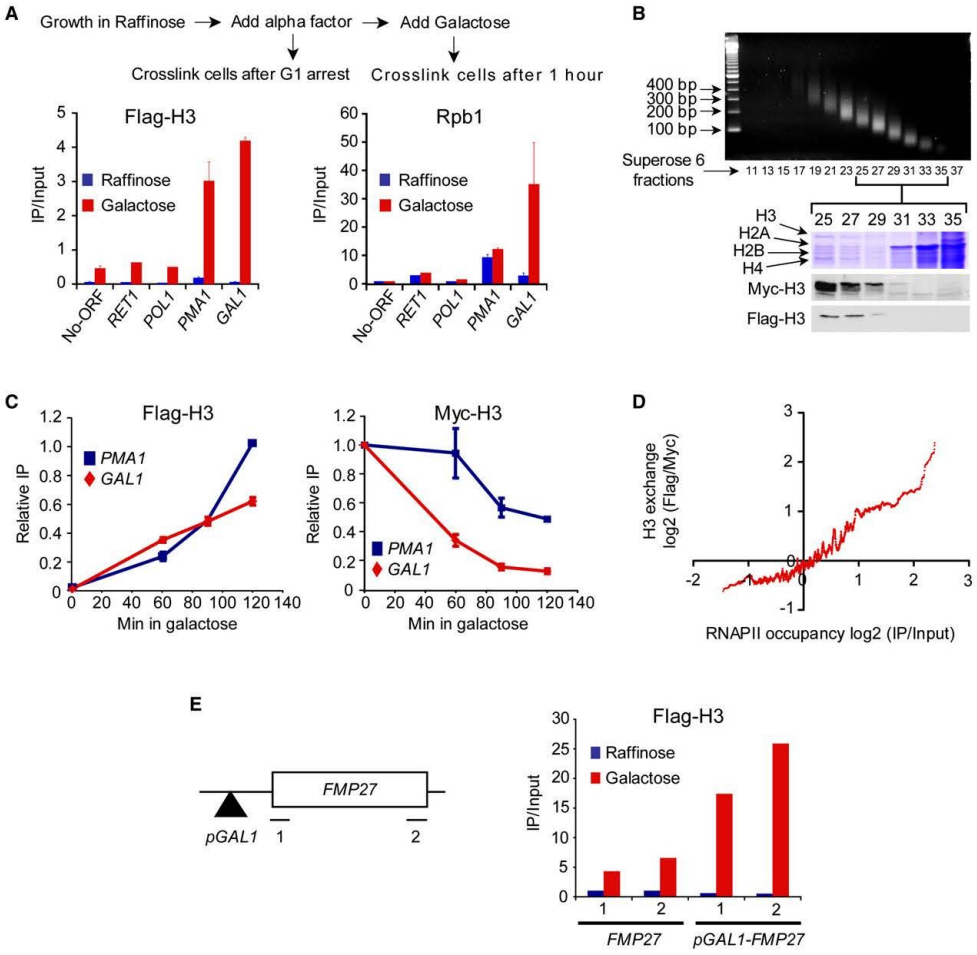

Figure 1. Incorporation of New Histone H3 Is Dependent on Transcription

(A) Preferential incorporation of new Flag-H3 at the coding sequences of active genes *PMA1* and *GAL1* in G1-arrested cells. Yeast cells from the strain (YAN1001) containing the histone double-tag system were grown in raffinose medium to mid-log phase. After G1 arrest by α factor, the cells were subsequently formaldehyde fixed or shifted to galactose medium for 60 min prior to formaldehyde treatment. ChIPs were then performed using the anti-Flag and the anti-Rpb1 antibodies. The values shown (IP/Input) represent the average and standard error of three independent experiments.

(B) Newly synthesized Flag-H3 is incorporated into chromatin. Nuclei were isolated from a strain expressing new Flag-H3 (YAN1002) for 2 hr, treated with MNase, and fractionated on a Superose 6 column. Fractions were run on agarose gels or SDS-PAGE or analyzed by western blots for the presence of Myc-H3 and Flag-H3.

(C) Incorporation of newly synthesized Flag-H3 is correlated with displacement of the endogenous Myc-H3. Cells were treated as described in (A) except that a time course was conducted after galactose addition. Flag-H3 and Myc-H3 association with *GAL1* and *PMA1* coding sequences was analyzed by ChIP. The percent of IP at *PMA1* after 2 hr of induction was set to 1 for Flag-H3 occupancy. The percent of IP before induction at each location was set to 1 for Myc-H3 occupancy. The values shown represent the average and standard error of three independent experiments.

(D) The level of histone H3 exchange correlates with RNAPII occupancy. The chromatin used for Myc-H3, Flag-H3, and Rpb1 ChIP was prepared from WT cells (YAN 1001) treated as described in (A). For each protein-coding gene, the levels of H3 exchange were calculated by averaging the ratio for all the probes covering that gene (see the Experimental Procedures).

(E) Transcription is an important determinant of new Flag-H3 deposition. Yeast cells from strains (YAN1001) or (YAN1002) were treated as described in (A). In YAN1002, *FMP27* is under the control of the *GAL1* promoter.

G1-arrested cells, the association of Flag-H3 with the 5′ or 3′ coding sequences of the long *FMP27* gene in two different strains (Figure 1E). In the first strain, *FMP27* is under the control of its own promoter while in the second strain the *GAL1* promoter drives it. In raffinose medium, only marginal levels of Flag-H3 crosslinking were observed. Galactose induction resulted in a significant Flag-H3 incorporation when *FMP27* was under the control of its own promoter. The new H3 deposition occurred despite low RNAPII occupancy at this gene, suggesting a basal replication and transcription-independent chromatin assembly (Figure 1E and data not shown). Interestingly, the deposition of new H3 at the *GAL1*-driven *FMP27* gene was higher and corresponded to higher RNAPII association (Figure 1E and data not shown). This provides evidence that transcription is an important process driving additional incorporation at open reading frames of a given gene. As a second way to test the role of transcription in the incorporation of new H3, we looked at Flag-H3 occupancy at the heat-shock-inducible gene *HSP104* in different conditions (Figure S2). Again, in absence of high transcription, we observed a basal incorporation after induction of new H3. Activation of transcription following the synthesis of new H3 resulted in a high level of incorporation at *HSP104* coding sequence. Taken together, our data show that, in absence of transcription, there is a basal level of incorporation of new H3. In addition to this basal incorporation, we clearly observed an important additional deposition of new H3 at transcribed regions of active genes. This additional incorporation, intimately associated with transcription, provides direct evidence of chromatin assembly process coupled to transcription in yeast that uses a source in *trans*.

Replication-Independent Histone H3 Exchange Occurs Predominantly in Promoter Regions

We then mapped H3 exchange relative to the 5′ and 3′ ends of genes. To identify possible differences in the distribution of exchange with regard to transcription level, protein-coding genes were divided into six groups according to their level of transcription (based on RNAPII occupancy [Experimental Procedures]). As shown in Figure 2A (left panel), for all groups, exchange occurred predominantly in the 5′ and 3′ regions. Importantly, high H3 exchange in the coding regions occurs only in the most highly transcribed genes (red curve). Furthermore, our data show that an appreciable exchange can be observed at promoters of genes where very low RNAPII association has been detected (violet, blue, and green curves). Therefore, exchange of H3 in open reading frames strictly requires transcription, whereas an important transcription-independent level of exchange is observed in promoters. Specific examples of H3 exchange can be seen in Figure 2B showing exchange over a portion of chromosome II. As indicated by the vertical dotted lines in Figure 2B, H3 exchange was high in every intergenic region. Interestingly, this exchange does not strictly require the presence of a promoter, since it can be observed in intergenic regions abutting two genes transcribed toward each other (region between *YBR094W* and *RXT2* in Figure 2B). However, one should note that, on average, the presence of promoter in intergenic regions is associated with higher exchange (Figure S3). Although the presence of a promoter is not strictly required for H3 exchange in intergenic regions, the promoterless intergenic regions with a high level of exchange tend to have higher levels of RNAPII, suggesting that the observed exchange is transcription dependent rather than basal (data not shown).

We therefore conclude that replication-independent H3 exchange is brought about by two mechanisms: a basal level of exchange occurring mainly in promoters, and a transcription-dependent exchange targeting both intergenic and coding regions.

Histone H3 Exchange at Promoters of Inactive Genes

As mentioned above, despite a clear relation between exchange and transcription rate, appreciable exchange can be detected in the promoter of genes for which no evidence for transcription can be found (violet, blue, and green curves in Figure 2A). More examples can be seen in Figure 3A, which shows both exchange (red) and RNAPII occupancy (green) along a region of chromosome VII. Although most genes in this region are poorly transcribed, a high level of histone H3 exchange was detected at specific locations. For example, the intergenic regions containing the promoters for *TPN1*, *YGL185C*, and *STR3* all showed clear evidence for exchange (dotted vertical lines) despite very low levels of RNAPII. This important observation indicates that promoters could exchange histones H3 in absence of transcription and replication.

H3 exchange in the absence of replication or transcription at promoters of repressed genes could have a very important function in the regulation of these genes. To further our analyses on the H3 exchange at repressed promoters, we studied the promoters of the *PHO5* gene. This gene, extensively used in chromatin studies, has, in repressive conditions, four stably positioned nucleosomes that are lost upon activation (reviewed in Svaren and Horz [1997]). The nucleosomal structure of *PHO5* promoter is remodeled only by transcription activity or presumably by the passage of the replication fork. First we analyzed, in G1-arrested cells, the incorporation of Flag-H3 and Rpb1 at the *PHO5* promoter and in *GAL1* or *POL1* coding sequences (Figure S4). In contrast to *GAL1*, we observed a high level of exchange at the *PHO5* promoter in the absence of RNAPII. We next analyzed in more detail histone exchange at the *PHO5* promoter (Figures 3B and 3C and data not shown). Using ChIP assays, we measured Flag-H3, Myc-H3, or Rpb1 levels at the *PHO5* promoter and ORF. Galactose induction led to an important enrichment of the new H3 at the level of the *PHO5* promoter (Figure 3B, probe C). Interestingly, we saw less enrichment in the *PHO5* ORF (probe E) or in the adjacent upstream region (probe B). The high enrichment in new H3 is associated with a higher eviction of Myc-H3 (Figure 3C) and indicates that the promoter of

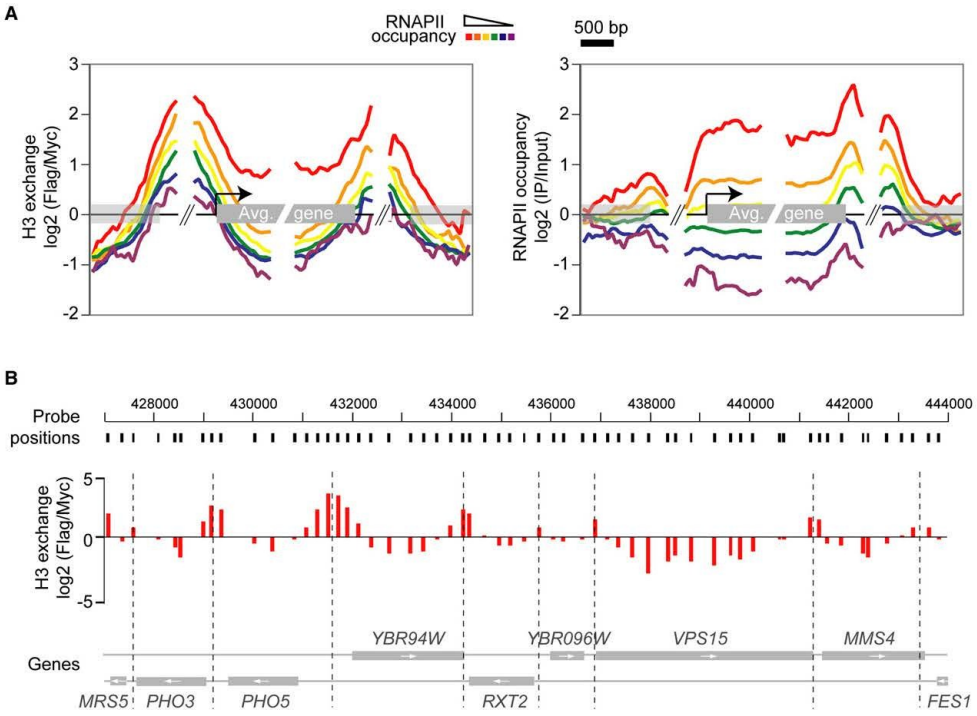

Figure 2. Replication-Independent Histone H3 Incorporation Occurs Mainly in Promoters

(A) Map of histone H3 exchange along genes. Average H3 exchange (left) was mapped relative to the 5′ and 3′ genes boundaries of six groups of genes based on RNAPII occupancy (right) (see the Experimental Procedures).

(B) Histone H3 exchange along a 17 kb region on chromosome II. Exchange is shown in red for all the probes that map within this chromosomal region. The probes are shown as black bars, and the genes (according to the UCSC Genome Browser) are shown in gray.

PHO5 exchanges histones H3/H4 with the free pool in the absence of transcription and replication.

Chromatin structure at inactive promoters was previously shown to contain a NFR flanked by a few well-positioned nucleosomes (Yuan et al., 2005). We therefore mapped exchange on NFRs. As shown in Figure 3D, exchange is very high over the NFR and the surrounding nucleosomes, suggesting that these nucleosomes exchange histone H3 with the free pool more than most other nucleosomes. This is consistent with our observation that promoters, which are the privileged regions for NFRs and positioned nucleosomes, have high levels of exchange. However, these data could also mean that the new H3 molecules bind free DNA in NFRs (as opposed to the surrounding nucleosomes). In order to investigate that possibility, we modified our protocol by replacing sonication with MNaseI treatment followed by immunoprecipitation (MNase-ChIP, Figure S5). Mononucleosomes (Figure S6) were immunoprecipitated with anti-Myc and anti-Flag antibodies. MNase-ChIP samples of Flag-H3 and Myc-H3 immunoprecipitations, performed with chromatin of G1-arrested cells, were then hybridized on a microarray containing 244K overlapping probes covering the whole genome. Although this does not have enough resolution to perfectly distinguish nucleosomes, such an approach can distinguish the NFRs from the neighboring nucleosomes. Consistent with the standard ChIP-chip result shown in Figure 3D, Figure 3E shows that exchange in this assay was higher on the few nucleosomes surrounding the NFR than at more distant nucleosomes. More importantly, it clearly shows that the Flag-H3/Myc-H3 ratio is the lowest in the NFR. This indicates that H3 replacement occurs around, but not on, the NFRs.

We next conducted MNase-ChIP and analyzed the *PHO5* gene by QPCR. Figure 3F shows that nucleosomes −2 and −1 (relative to the ATG) were subject to exchange in these growth conditions. Indeed, galactose induction led to a major enrichment of the new H3 at the level of the nucleosome −2 (probe C) and to a lesser extent to nucleosome −1 (probe D). Interestingly, we saw less

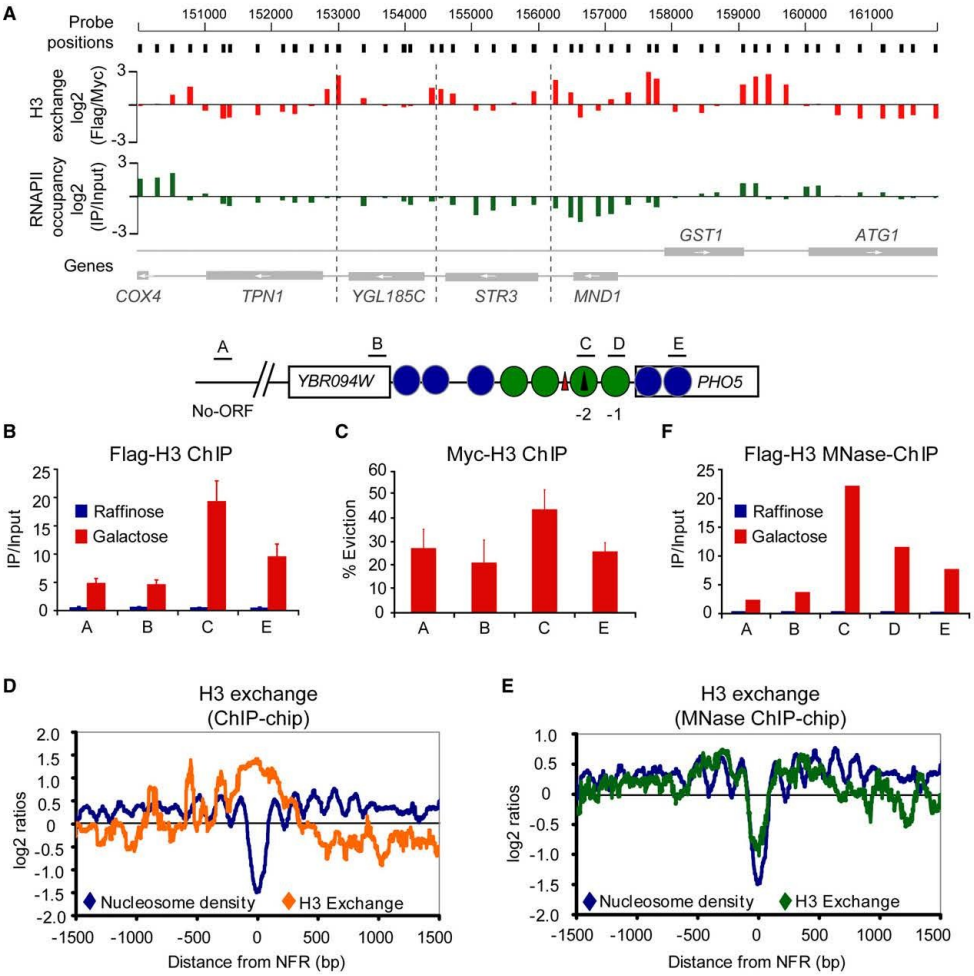

Figure 3. Promoters of Inactive Genes Exchange Histone H3 Independently of Transcription or Replication

(A) Histone H3 exchange (red) and RNAPII occupancy (green) along a 12 kb region containing repressed genes located in chromosome VII.

(B) The promoter of the *PHO5* gene incorporates high level of Flag-H3. In G1-arrested cells, Flag-H3 was induced for 2 hr and its occupancy measured at several positions of the *PHO5* locus. The nucleosomes remodeled during activation are in green, and the triangles represent the Pho4 binding sites. The values shown (IP/Input) represent the average and standard error of three independent experiments.

(C) Pre-existing Myc-H3 molecules are displaced by the incorporation of new Flag-H3. The Myc-H3 association was analyzed by ChIP assays as in (A). The percent eviction quantifying the amount of Myc-H3 displaced was calculated relative to the Myc-H3 occupancy before galactose induction. The values shown represent the average and standard error of three independent experiments.

(D) Histone H3 exchange is high around NFRs. H3 exchange (Flag-H3/Myc-H3), as measured by ChIP-chip, is plotted (orange) relative to the middle of the NFRs. Nucleosome density profile from Yuan et al. (2005) is shown in blue.

(E) Histone H3 exchange is restricted to few nucleosomes surrounding the NFRs. H3 exchange (Flag-H3/Myc-H3), as measured by MNase-ChIP coupled with high-density tiling microarray, is plotted (green). The NFRs and nucleosomes are the same as those used in (D). The chromatin used was extracted from WT cells (YAN1002) treated as in (B).

(F) High incorporation of Flag-H3 is mainly associated with the positioned nucleosome −2 of the *PHO5* promoter. ChIP assays using MNase treated chromatin as described in E. The *PHO5* regions tested are indicated. The values shown for IP/Input represent the average of two independent experiments.

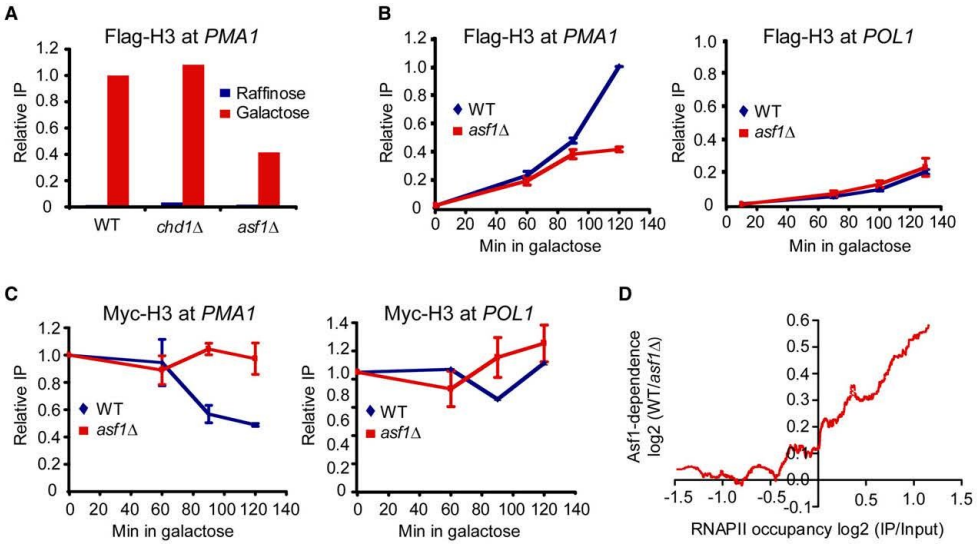

Figure 4. Transcription-Dependent Exchange of Histone H3 Involves Asf1

(A) Incorporation of newly synthesized H3 at *PMA1* is reduced in an *asf1Δ* strain and not in a *chd1Δ* strain. Flag-H3 association was analyzed prior to or following galactose induction of WT (YAN1001), *asf1Δ* (YAN1003), and *chd1Δ* (YAN1004) cells. The enrichment at *PMA1* in WT cells after 2 hr in galactose was set to 1.

(B) Asf1 plays an important role in transcription-dependent histone H3 exchange outside of S phase. Results of a time course experiment showing incorporation of Flag-H3 at the active *PMA1* (5′ coding) gene or the *POL1* (5′ coding) inactive gene are shown. The enrichment at *PMA1* in WT cells after 2 hr in galactose was set to 1. Average and standard error of three independent experiments are shown.

(C) Asf1 is important for displacement of the pre-existing Myc-H3. The time course experiment shows Myc-H3 association with *PMA1* and *POL1*. The values shown (percent IP at given time point relative to percent IP in raffinose) represent the average and standard error of three independent experiments.

(D) Asf1 dependence for histone H3 exchange on promoters correlates with RNAPII occupancy. The average level of Asf1 dependence within the promoter was calculated similarly as in Figure 1D, except that only probes covering the promoter region were used.

enrichment at the *PHO5* ORF (probe E) or the adjacent upstream ORF (probe B). The high enrichment in new H3 is associated with a higher eviction of Myc-H3 (Figure 3C) and shows that positioned nucleosomes in the promoter of repressed genes exchange histones with the free pool in absence of transcription and replication. We obtained similar results with the *CHA1* repressed promoter (Figure S7). This confirms our genome-wide data and shows that some nucleosomes within promoter regions are highly dynamic regardless of transcription. At inactive genes, this dynamic state could poise the promoter for transcription by facilitating the remodeling during induction.

Transcription-Dependent Histone Replacement outside of S Phase Implicates Asf1

Efficient nucleosome assembly requires histone chaperone and ATP-dependent nucleosome remodeling activities (Robinson and Schultz, 2003). Interestingly, yeast extracts lacking either Asf1 or ATP-dependent nucleosome remodeling factor Chd1 are severely defective in replication-independent nucleosome assembly (Robinson and Schultz, 2003). Moreover, Asf1 and Chd1 were previously shown to associate with transcribed regions (Schwabish and Struhl, 2006; Simic et al., 2003). To obtain further insight into replication-independent chromatin assembly in yeast, we asked if Asf1 and Chd1 play an important role in new H3 deposition in vivo. Thus, we measured the Flag-H3 incorporation at *PMA1* coding sequence in wild-type (WT), *asf1Δ*, or *chd1Δ* cells arrested in G1 (Figure 4A). The deletion of *CHD1* gene had no effect on Flag-H3 incorporation. However, we observed a substantial reduction of new H3 incorporation in *asf1Δ* mutant. Importantly, the protein level of Flag-H3 was not affected in *asf1Δ* mutant (data not shown). We therefore analyzed the role of Asf1 in the replication-independent histone deposition in greater detail by looking at the levels of Flag-H3 and Myc-H3 in G1-arrested cells after various times of galactose incubation (Figures 4B and 4C). At the transcriptionnally inactive gene *POL1*, absence of Asf1 had no effect on Flag-H3 deposition. Interestingly, the incorporation of new H3 was significantly decreased at *PMA1*

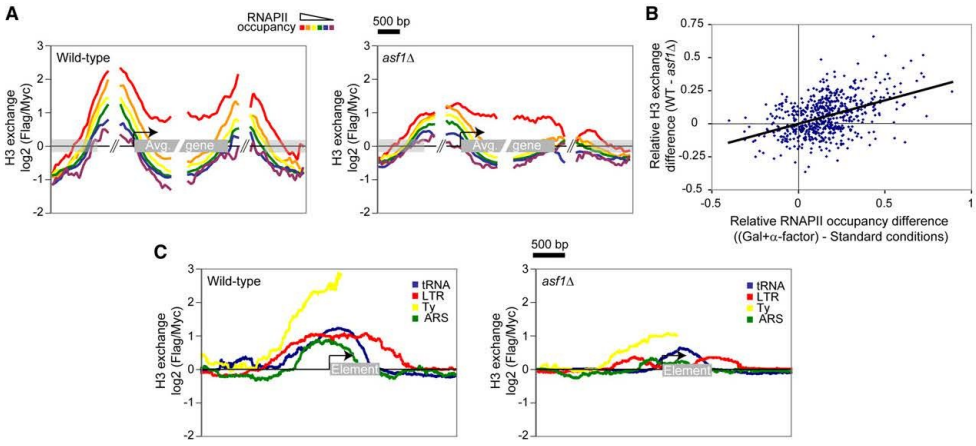

Figure 5. The Effect of the Deletion of *ASF1* on the Replication-Independent Histone H3 Incorporation

(A) Asf1 is mainly responsible for H3 exchange in active promoters. A map of histone H3 exchange along genes in the WT (left, YAN1001) and *asf1Δ* (right, YAN1003) is shown. The mapping was done as in Figure 2A.

(B) The contribution of Asf1 globally correlates with the level of transcriptional induction. The difference in H3 exchange (WT versus *asf1Δ*) measured in our conditions (galactose + α factor) is plotted against the difference in RNAPII occupancy between two conditions (galactose + α factor versus standard conditions) for the highly transcribed yeast genes (see the Experimental Procedures).

(C) Asf1 affects H3 exchange in various genetic elements. The average H3 exchange was mapped on the 5′ of tRNA, LTR, and Ty elements and on the middle of ARS in the WT (left) and in *asf1Δ* (right).

after 2 hr of induction ($p = 0.02$). Furthermore, the transcription-dependent eviction of Myc-H3 is also significantly reduced in *asf1Δ* mutant at *PMA1*. These observations indicate that Asf1 plays an important role in transcription-dependent histone H3 exchange outside of S phase at the *PMA1* coding region.

We next asked if Asf1 plays a general role in the transcription-dependent histone H3 deposition in G1-arrested cells. For that, we analyzed, at the genome-wide level, the impact of *asf1* deletion on Flag-H3 deposition. Flag-H3 was immunoprecipitated from crosslinked chromatin extracted from WT and *asf1Δ* cells arrested in G1. The precipitated DNAs were labeled and hybridized on tiling microarray. In Figure 4D, the WT/*asf1Δ* ratio at promoters, which constitutes a measure of the Asf1 dependence, was plotted against RNAPII occupancy. The figure clearly shows a positive correlation between transcription level and the dependence on Asf1. The same was observed at 3′ regions (data not shown). Interestingly, the figure also indicates that Asf1 contributes less to exchange at inactive genes (genes with negative RNAPII occupancy values). This argues for a global role of Asf1 on transcription-dependent (but not on the basal) H3 deposition outside of the replication period.

We next performed a ChIP-chip experiment in *asf1Δ* cells to map precisely the H3 exchange dependent on Asf1. In Figure 5A, we report the average H3 exchange in WT (left panel) and *asf1Δ* (right panel) cells on the same groups of protein-coding genes used in Figure 2A. It shows that Asf1 contributes mainly to the transcription-dependent exchange at promoters and to some extent at terminators, while exchange on the ORFs, as well as the basal exchange at promoters, is not significantly affected. This suggests that alternative activities are required for H3 exchange. Interestingly, a gene ontology search revealed that the most affected genes are involved in the response to pheromone. These genes are activated by our experimental conditions (α factor treatment) suggesting that Asf1 may be more important for exchange at inducible genes than at highly transcribed constitutive genes. In order to address that possibility, we plotted the difference in exchange between the WT and *asf1Δ* versus the difference in RNAPII occupancy measured prior and after the switch to galactose + α factor (Figure 5B). This shows high correlation ($r = 0.45$), suggesting that for a given expression level, a gene is more dependent on Asf1 for H3 exchange at its promoter when it is being induced (see also Figure S8). We conclude that genes are highly dependent on Asf1 for H3 exchange during induction but less so during steady-state high transcription.

Finally, Figure 5C shows that Asf1 is also a major contributor to H3 exchange observed at other genetic elements such as tRNA, Ty, and ARS. This important latter observation suggests a possible direct role of Asf1 in the regulation of chromatin structure of replication origins (see also Figure S9).

Replication-Independent Newly Deposited H3 Is Acetylated on K56

Asf1 is essential for the acetylation of histone H3 on K56 (Recht et al., 2006). Interestingly, recent reports show that this epigenetic mark is cell cycle regulated, is important for DNA damage response, and plays a role in genome stability (Driscoll et al., 2007; Han et al., 2007; Masumoto et al., 2005). These observations clearly indicated that K56 is associated with replication-dependent chromatin assembly and therefore suggest a function for Asf1 in DNA synthesis. However, K56 acetylation and the HAT (Rtt109) responsible for this modification were also found at active genes (Schneider et al., 2006). This observation suggests that K56 acetylation could also be involved in transcription-dependent chromatin assembly. However, this association was observed in nonsynchronized cells, making the evaluation of the relative contribution of replication versus transcription difficult to assess. To distinguish between these two processes, we asked whether K56 acetylation is associated with the transcription-dependent chromatin assembly pathway in G1-arrested cells. To this end, we analyzed K56 acetylation on Flag-H3 immunoprecipitated from WT and *asf1Δ* whole-cell extracts (Figure 6A). The cells used were arrested in G1 and Flag-H3 induced by galactose treatment during 0, 1, or 2 hr. We clearly observed that new Flag-H3 is acetylated on K56 in WT cells arrested in G1. This acetylation is absent from *asf1Δ* cells subjected to the same treatment. This previously unreported observation indicates that K56 histone acetyltransferase activity is present in G1 and that newly synthesized H3 is modified during this step of the cell cycle. We next asked if K56-acetylated H3 is deposited on chromatin at transcribed genes in G1-arrested cells. We analyzed K56 acetylation of H3 by ChIP assays at the *GAL1* coding region in WT or *asf1Δ* mutant cells. When *GAL1* was induced in G1-arrested cells, we observed an increase of K56 acetylation at this gene only in WT cells (Figure 5B). This shows that K56 acetylation is also a mark of transcription-coupled newly assembled nucleosomes outside of S phase.

To have a global view of H3 K56 acetylation, we analyzed by ChIP-chip the incorporation of this mark in G1-arrested cells containing newly synthesized histone. Figure 6D shows that the acetylation of H3 K56 is globally higher in the promoter of transcribed genes, a pattern that is highly similar to the pattern of H3 exchange (compare with Figure 2). Interestingly, we also observed a significant incorporation in the promoters of inactive genes (Figure 6D, blue and violet curves; also see Figure 6C), indicating that H3K56 acetylation is not restricted to transcribed regions. This observation suggests that H3 K56 acetylation correlates with new H3 incorporation. To confirm this observation, we plotted H3 K56 acetylation against H3 exchange (Figure 6E) and observed a very strong correlation ($r = 0.865$). Therefore, we conclude that acetylation of K56 is a general mark of replication-independent, newly assembled nucleosomes in G1 cells.

DISCUSSION

Basal and Transcription-Dependent Histone H3 Exchange outside of Replication

The cell assembles chromatin during S phase in the wake of replication. In addition to replication, a number of other processes involving DNA metabolism are associated with histone deposition and nucleosomal assembly, including transcription and DNA repair. The present work is focused on the understanding of replication-independent histone H3 dynamics in the yeast *Saccharomyces cerevisiae*. The data presented in this study show clear association between transcription and histone H3 incorporation. In addition, we observed promoter-associated genome-wide basal exchange in absence of transcription or replication.

After this work was completed, it was reported that in G1-blocked yeast cells, new histone H3 is incorporated into nucleosomes of the transcribed genes and not into those of some repressed genes, suggesting that, outside of replication, histone H3 exchange is only associated with transcription (Jamai et al., 2007). A careful analysis of our data on genes extensively studied by Jamai et al. shows similar patterns of H3 exchange, namely no exchange at the inactive *STE3* promoter (Figure S11). Because genes showing no or low basal incorporation are rather infrequent in our study, we think that our conclusions are different primarily because we analyzed the exchange globally.

Also, we cannot exclude the fact that some differences lie in the systems used in both studies. Indeed, Jamai and colleagues used a C-terminal HA-tagged histone H3 expressed from a plasmid, while we used an integrated construction that expresses both histone H3 and H4. The co-expression of histone H4 together with histone H3 may be important for the basal exchange pathway. However, this is unlikely, since deletion of the histone H4 allele under the control of *GAL1/10* does not affect the new H3 incorporation at the promoter of the inactive *PHO5* gene (see Figure S12). Finally, there are other differences in the two experimental systems that could explain some differences including the type and the position of the epitope or the origin and the nature of the endogenous histone H3.

In any case, we observe a basal genome-wide exchange at promoters that is independent of transcription and replication. In addition to this basal exchange, there is a specific chromatin-assembly pathway associated with transcription. Finally, while this manuscript was in review, another related study in yeast reported the existence of H3 exchange that is independent of transcription and replication (Dion et al., 2007). Importantly, two other studies in *Drosophila* published at the same time show that histone H3 exchange occurs in promoters of genes in absence of transcription and RNAP II (Mito et al., 2007; Nakayama et al., 2007). This clearly indicates that replication and transcription-independent histone H3 exchange in promoters is a conserved phenomenon that could play an important role in transcription regulation.

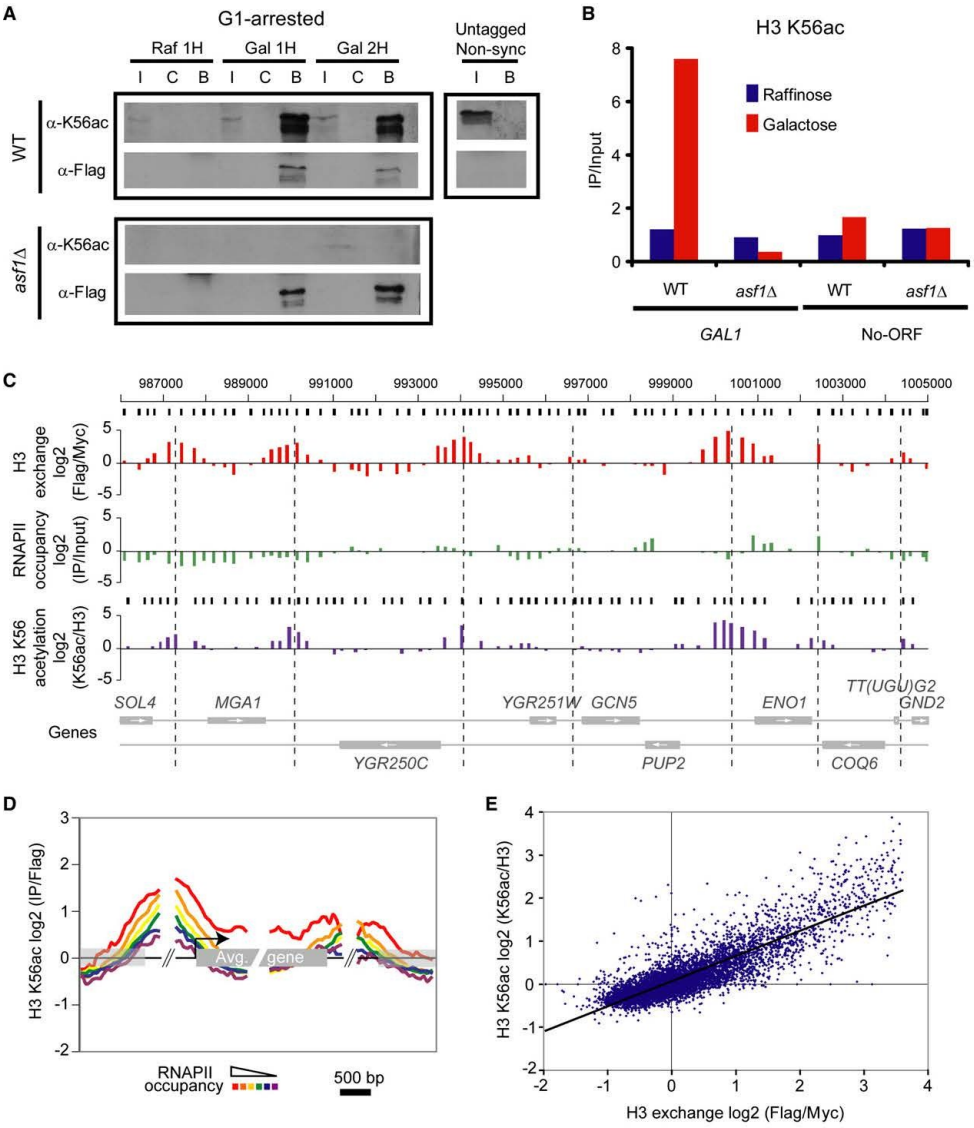

Figure 6. Acetylation of H3 Lysine K56 Is Involved in Transcription-Dependent Chromatin Assembly

(A) Newly synthesized Flag-H3 is acetylated on K56 in G1-arrested cells. Proteins from whole-cell extract ("I") of WT (YAN 1002) or *asf1Δ* (YAN1003) were immunoprecipitated using anti-Flag beads ("B") or control beads ("C") and analyzed for H3 K56 acetylation by western blots. Whole-cell extract of nonsynchronized exponentially growing cells were also tested (right).

(B) Newly synthesized H3 is acetylated on K56 and deposited at the active *GAL1* gene. ChIP assays showing association of K56 acetylation with *GAL1* or No-ORF (intergenic region of chromosome V), before and after galactose induction in WT (YAN 1002) or *asf1Δ* (YAN1003).

(C) Histone H3 exchange along a 19 kb region on chromosome VII as in Figure 2B. Exchange is shown in red, RNAPII in green, and H3 K56ac in purple.

(D) Histone H3 K56 acetylation is enriched in the promoter of active genes. A map of histone H3 K56 acetylation is shown as in Figure 2A.

(E) The level of histone H3 K56 acetylation globally correlates with the level of H3 exchange. H3 K56 acetylation is plotted against H3 exchange. Each dot represents the average value over a gene.

Transcription-Dependent Histone H3 Exchange outside of Replication Occurs Mainly in the 5′ and 3′ Ends of Active Genes

The exchange mapping shows new surprising results that could help to better understand chromatin modulation. Transcription-dependent exchange shows two different peaks of incorporation, located at the promoters and 3′UTR, respectively. The exchange we observed in 3′ regions is probably not due to the presence of a neighboring promoter since it also occurs in intergenic regions located between two genes with convergent transcription. However, we note that, on average, the genome-wide H3 exchange is higher in intergenic regions containing at least one promoter (Figure S3).

The intriguing pattern of H3 exchange reflects less exchange in the body of the active genes. It is possible that initiation and termination of transcription are more disruptive for nucleosomes than elongation. Alternatively, transcription elongation could be, at least partially, associated with a mechanism that uses more histone in *cis* while initiation and termination would favor *trans*-exchange. The use of histone in *cis* would have important consequences. It would protect epigenetic marks such as methylation and acetylation that have been shown to play a major role in the repressive function of chromatin structure in coding regions (reviewed in Workman [2006]). In contrast, *trans*-exchange would scramble any epigenetic mark so that each round of elongation would reset chromatin to a generic form.

H3/H4 Exchange in Intergenic Regions May Poise Repressed Promoters

In promoters of repressed genes, the appreciable exchange has most likely very important biological implications, specifically for the transcriptional regulation. Considering that H2A/H2B dimers are highly dynamic, our data on H3 suggest that nucleosomes of inactive genes could be in equilibrium between association and dissociation. Alternatively, the high incorporation of new H3 could reflect an exchange of H3/H4 dimers, suggesting only partial disassembly of positioned nucleosomes. During the activation process, this equilibrium could be displaced toward partial or total dissociation by the recruitment of chromatin remodelers, histone-modifying activities, or histone chaperones.

Alternatively, it is also possible that specific histone modifications, or incorporation of a variant histone, result in higher "dissociation exchange" of promoter nucleosomes. Recent studies indicate that Htz1, a histone H2A variant, is localized near NFRs of repressed genes and may poise promoters for activation (Guillemette et al., 2005; Raisner et al., 2005; Zhang et al., 2005). Therefore, it is possible that the high exchange rate in promoters of inactive genes is driven by the presence of Htz1 in some of their nucleosomes. Alternatively, the presence of NFRs in intergenic regions (regardless of the presence of Htz1) may play a role in the targeting of basal histone exchange. Finally, it is possible that the binding of regulators in repressing conditions drives the exchange at the promoters of repressed genes. We tested this possibility for *PHO5* and found that Pho2 and Pho4 activators play some role in the kinetic and final level of H3 exchange at the promoter (Figures S13 and S14). Therefore, it is possible that activators binding to promoters will contribute to this basal exchange in promoters of inactive genes.

The important H3/H4 dynamics in promoters could be a way to incorporate or eject a specific chromatin mark that would be important for transcriptional regulation. In any case, high nucleosome dynamics in promoters is likely to have profound biological consequences regarding the regulation of transcriptional induction. After this work was submitted, two studies in *Drosophila* showed that H3.1 is exchanged by H3.3 at regulatory regions of genes independently of the presence of RNAP II (Mito et al., 2007; Nakayama et al., 2007). Similar to what we reported in yeast, this exchange occurs in nucleosomes that surround nucleosomal depleted regions. These observations, together with our report, clearly indicate that transcription- and replication-independent H3 exchange occurs in regulatory regions and is conserved in eukaryotic organisms.

Asf1 and H3 K56 Acetylation Play a Role in Transcription-Dependent Chromatin Assembly outside of S Phase

Asf1 travels with RNAPII and plays a role in histone eviction and deposition upon repression of transcription (Schwabish and Struhl, 2006). Our study provides, to our knowledge, the first global and direct evidence that Asf1 is implicated in replication-independent histone H3 deposition in yeast. We show that Asf1 is important for the cotranscriptional deposition of new H3. Moreover, we find clear genome-wide correlation between Asf1 and transcription-coupled histone H3 exchange. Therefore, we conclude that Asf1 contributes not only to the redeposition of nucleosomes after repression of transcription but also to the genome-wide cotranscriptional deposition of new nucleosomes. Interestingly, our mapping of Asf1 dependency shows that Asf1 plays a much more important role at promoters of active genes than at their coding regions. This observation suggests that Asf1 is a predominant chaperone during initiation, while its role during elongation is less important or redundant with other activities. Finally, we found that, among the active genes, the inducible ones are the most dependent on Asf1 for H3 exchange. This could suggest that Asf1 is crucial for the rapid induction and repression of inducible genes. This is consistent with previous observations regarding galactose-inducible genes or *PHO5* (Adkins et al., 2004; Schwabish and Struhl, 2006).

Asf1 also has important roles during replication. It controls specifically the acetylation of H3 K56, a chromatin mark that was thought to be restricted to S phase and to associate with the replication-dependent chromatin assembly machinery (Masumoto et al., 2005). Recent observations show that expression of Rtt109, the H3 K56 HAT,

is also cell cycle regulated and overlaps largely with the induction of K56 acetylation during S phase (Driscoll et al., 2007). Our data provide new intriguing results, showing that K56 acetylation activity is present in G1. There are different ways to interpret this observation. First, it is possible that the Rtt109 activity is present throughout the cell cycle but cannot be observed during G1 because of the lower level of histone synthesis. Alternatively, induction of new H3 could trigger a signal that would lead to expression of the Rtt109. Interestingly, K56 acetylation was recently linked to transcription elongation (Schneider et al., 2006). However, that study was conducted on nonsynchronized cultures, making the evaluation of transcription-dependent and S phase chromatin assembly difficult. Our experimental system allowed us to observe induction of K56 acetylation in the absence of replication. We found that H3 K56 acetylation is mostly predominant in promoter regions of active genes. Our mapping analyses show that H3 K56 acetylation is also present in these regions of inactive genes. In summary, this epigenetic mark is strongly correlated to H3 exchange. Therefore, we conclude that replication-independent newly assembled nucleosomes are marked by acetylation at H3 K56. Importantly, the K56 acetylation plays a crucial role during replication-coupled chromatin assembly, and the absence of this epigenetic mark has deleterious effects on the repair of DNA damage and genome stability (Driscoll et al., 2007; Masumoto et al., 2005). Our data bring about a possible role for K56 acetylation in transcription regulation of either active or inactive genes, a possibility that will be addressed in future work.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

All *S. cerevisiae* strains used in this study are listed in Table S1. They all originated from crosses between USY6 (generous gift from Dr. Philip Korber) and different strains bearing the desired mutations. The deletions or promoter replacement of genes was constructed by homologous recombination and standard yeast genetics methods. Efficient G1 arrest (at least 95% of cells) was achieved by adding 500 ng/ml of α factor for 3 hr.

Purification of Native Chromatin, Chromatin Immunoprecipitation, and MNase-ChIP Assays

Yeast native chromatin was purified as described elsewhere (Utley et al., 2005). The protocol for Mnase-ChIP assays is generally similar to the standard ChIP procedure. The modifications were done according to Schermer et al. (2005).

Labeling and Hybridization

In both ChIP-chip and MNase-ChIP-chip, DNA was amplified and labeled using ligation-mediated PCR and hybridized as described previously (Guillemette et al., 2005). Yeast full genome tiling arrays containing ~44K (ChIP-chip) or ~244K (MNase ChIP-chip) probes were purchased from Agilent Technologies. The 44K arrays were the same as we used previously (Guillemette et al., 2005) and contain an average of four 60-mer probes (Tm-adjusted) per kilobase across all the nonrepetitive portion of the yeast genome (except for a few experiments that were done on a new generation of microarray (the 4 × 44K) carrying similar characteristics). The 244K array, also made of Tm-adjusted 60-mer probes, contains an average of about 20 probes per kilobase. In this case, most probes are overlapping with the next by approximately ten bases.

More details of experimental procedures including microarray data analysis are described in the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, 17 figures, 2 tables, 8 files, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/27/3/393/DC1/.

ACKNOWLEDGMENTS

We thank Alan Anderson, Jacques Côté, and Alain Verreault for helpful comments and advice on the manuscript. We are grateful to Philip Korber and Michel Strubin for sending reagents. This work was supported by CIHR grants (to A.N. and F.R.). F.R. holds a new investigator award from the Canadian Institutes of Health Research (CIHR). A.N. holds a Canadian Chair Research.

Received: March 3, 2007
Revised: May 28, 2007
Accepted: July 10, 2007
Published: August 2, 2007

REFERENCES

Adkins, M.W., Howar, S.R., and Tyler, J.K. (2004). Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. Mol. Cell *14*, 657–666.

Chow, C.M., Georgiou, A., Szutorisz, H., Maia e Silva, A., Pombo, A., Barahona, I., Dargelos, E., Canzonetta, C., and Dillon, N. (2005). Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division. EMBO Rep. *6*, 354–360.

Dion, M.F., Kaplan, T., Kim, M., Buratowski, S., Friedman, N., and Rando, O.J. (2007). Dynamics of replication-independent histone turnover in budding yeast. Science *315*, 1405–1408.

Driscoll, R., Hudson, A., and Jackson, S.P. (2007). Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56. Science *315*, 649–652.

Guillemette, B., Bataille, A.R., Gevry, N., Adam, M., Blanchette, M., Robert, F., and Gaudreau, L. (2005). Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. PLoS Biol. *3*, e384. 10.1371/journal.pbio.0030384.

Han, J., Zhou, H., Horazdovsky, B., Zhang, K., Xu, R.M., and Zhang, Z. (2007). Rtt109 acetylates histone H3 lysine 56 and functions in DNA replication. Science *315*, 653–655.

Jamai, A., Imoberdorf, R.M., and Strubin, M. (2007). Continuous histone H2B and transcription-dependent histone H3 exchange in yeast cells outside of replication. Mol. Cell *25*, 345–355.

Janicki, S.M., Tsukamoto, T., Salghetti, S.E., Tansey, W.P., Sachidanandam, R., Prasanth, K.V., Ried, T., Shav-Tal, Y., Bertrand, E., Singer, R.H., and Spector, D.L. (2004). From silencing to gene expression: real-time analysis in single cells. Cell *116*, 683–698.

Jin, C., and Felsenfeld, G. (2006). Distribution of histone H3.3 in hematopoietic cell lineages. Proc. Natl. Acad. Sci. USA *103*, 574–579.

Masumoto, H., Hawke, D., Kobayashi, R., and Verreault, A. (2005). A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nature *436*, 294–298.

Mito, Y., Henikoff, J.G., and Henikoff, S. (2005). Genome-scale profiling of histone H3.3 replacement patterns. Nat. Genet. *37*, 1090–1097.

Mito, Y., Henikoff, J.G., and Henikoff, S. (2007). Histone replacement marks the boundaries of cis-regulatory domains. Science *315*, 1408–1411.

Nakayama, T., Nishioka, K., Dong, Y.X., Shimojima, T., and Hirose, S. (2007). Drosophila GAGA factor directs histone H3.3 replacement that prevents the heterochromatin spreading. Genes Dev. *21*, 552–561.

Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell, G.W., Walker, K., Rolfe, P.A., Herbolsheimer, E., et al. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. Cell *122*, 517–527.

Raisner, R.M., Hartley, P.D., Meneghini, M.D., Bao, M.Z., Liu, C.L., Schreiber, S.L., Rando, O.J., and Madhani, H.D. (2005). Histone variant H2A.Z marks the 5′ ends of both active and inactive genes in euchromatin. Cell *123*, 233–248.

Recht, J., Tsubota, T., Tanny, J.C., Diaz, R.L., Berger, J.M., Zhang, X., Garcia, B.A., Shabanowitz, J., Burlingame, A.L., Hunt, D.F., et al. (2006). Histone chaperone Asf1 is required for histone H3 lysine 56 acetylation, a modification associated with S phase in mitosis and meiosis. Proc. Natl. Acad. Sci. USA *103*, 6988–6993.

Robinson, K.M., and Schultz, M.C. (2003). Replication-independent assembly of nucleosome arrays in a novel yeast chromatin reconstitution system involves antisilencing factor Asf1p and chromodomain protein Chd1p. Mol. Cell. Biol. *23*, 7937–7946.

Schermer, U.J., Korber, P., and Horz, W. (2005). Histones are incorporated in trans during reassembly of the yeast PHO5 promoter. Mol. Cell *19*, 279–285.

Schneider, J., Bajwa, P., Johnson, F.C., Bhaumik, S.R., and Shilatifard, A. (2006). Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with the elongating RNA polymerase II. J. Biol. Chem. *281*, 37270–37274.

Schwabish, M.A., and Struhl, K. (2004). Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol. Cell. Biol. *24*, 10111–10117.

Schwabish, M.A., and Struhl, K. (2006). Asf1 mediates histone eviction and deposition during elongation by RNA polymerase II. Mol. Cell *22*, 415–422.

Simic, R., Lindstrom, D.L., Tran, H.G., Roinick, K.L., Costa, P.J., Johnson, A.D., Hartzog, G.A., and Arndt, K.M. (2003). Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. EMBO J. *22*, 1846–1856.

Svaren, J., and Horz, W. (1997). Transcription factors vs nucleosomes: regulation of the PHO5 promoter in yeast. Trends Biochem. Sci. *22*, 93–97.

Utley, R.T., Lacoste, N., Jobin-Robitaille, O., Allard, S., and Cote, J. (2005). Regulation of NuA4 histone acetyltransferase activity in transcription and DNA repair by phosphorylation of histone H4. Mol. Cell. Biol. *25*, 8179–8190.

Williams, S.K., and Tyler, J.K. (2007). Transcriptional regulation by chromatin disassembly and reassembly. Curr. Opin. Genet. Dev. *17*, 88–93.

Workman, J.L. (2006). Nucleosome displacement in transcription. Genes Dev. *20*, 2009–2017.

Yuan, G.C., Liu, Y.J., Dion, M.F., Slack, M.D., Wu, L.F., Altschuler, S.J., and Rando, O.J. (2005). Genome-scale identification of nucleosome positions in S. cerevisiae. Science *309*, 626–630.

Zhang, H., Roberts, D.N., and Cairns, B.R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell *123*, 219–231.

Accession Numbers

The ChIP-chip data in this paper have been deposited in NCBIs Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE8299.

Annex 2

MOLECULAR AND CELLULAR BIOLOGY, Mar. 2011, p. 1288–1300 Vol. 31, No. 6
0270-7306/11/$12.00 doi:10.1128/MCB.01083-10

Transcription Regulation by the Noncoding RNA *SRG1* Requires Spt2-Dependent Chromatin Deposition in the Wake of RNA Polymerase II[▽]

Philippe Thebault,[1] Geneviève Boutin,[1] Wajid Bhat,[1] Anne Rufiange,[1] Joseph Martens,[2] and Amine Nourani[1]*

Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec (CHUQ), Québec, Québec G1R2J6, Canada,[1] and Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania[2]

Received 15 September 2010/Returned for modification 11 October 2010/Accepted 21 December 2010

Spt2 is a chromatin component with roles in transcription and posttranscriptional regulation. Recently, we found that Spt2 travels with RNA polymerase II (RNAP II), is involved in elongation, and plays important roles in chromatin modulations associated with this process. In this work, we dissect the function of Spt2 in the repression of *SER3*. This gene is repressed by a transcription interference mechanism involving the transcription of an adjacent intergenic region, *SRG1*, that leads to the production of a noncoding RNA (ncRNA). We find that Spt2 and Spt6 are required for the repression of *SER3* by *SRG1* transcription. Intriguingly, we demonstrate that these effects are not mediated through modulations of the *SRG1* transcription rate. Instead, we show that the *SRG1* region overlapping the *SER3* promoter is occluded by randomly positioned nucleosomes that are deposited behind RNAP II transcribing *SRG1* and that their deposition is dependent on the presence of Spt2. Our data indicate that Spt2 is required for the major chromatin deposition pathway that uses old histones to refold nucleosomes in the wake of RNAP II at the *SRG1-SER3* locus. Altogether, these observations suggest a new mechanism of repression by ncRNA transcription involving a repressive nucleosomal structure produced by an Spt2-dependent pathway following RNAP II passage.

In eukaryotes, modulation of the chromatin structure is a key aspect of important processes involving DNA. The basic unit of chromatin structure is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of histones (20). In addition to histones, many nonhistone proteins play important roles in regulating chromatin structure and chromatin-related processes (41). In *Saccharomyces cerevisiae*, one such nonhistone chromatin component is the HMG-like protein Spt2/Sin1, which was first identified genetically, by analysis of secondary mutations that suppress Ty and δ insertion mutations (*spt2* for "suppressor of Ty 2"), in the *HIS4* promoter (43).The sequence identity of Spt2 to HMG proteins is weak. However, like these proteins, it binds DNA nonspecifically and has significant affinity to four-way junction DNA, a structure similar to that found at the entrance/exit point of DNA from a nucleosome (25, 46). Several studies indicated that this factor could play a negative role in transcription initiation. Indeed, Spt2 was identified in a second screen for mutations that suppress the loss of the Swi/Snf chromatin remodeling complex and was called *SIN1* (39). In addition, mutations in *SPT2* suppress defects caused by mutations of SAGA histone acetyltransferase complex components (27, 31) and by deletion mutations in the RNA polymerase II (RNAP II) largest subunit gene (*RPB1*) (28). Finally, *spt2* mutations have been shown to derepress the heat shock *SSA3* gene (2).

While these mutant phenotypes suggested a negative role for Spt2 in transcription initiation, our recent work showed that it has an important function in transcription elongation (24). We demonstrated that Spt2 plays a significant role in the maintenance of proper chromatin structure over transcribed regions of the two active genes *PMA1* and *GAL1*. Similarly to other elongation factors, it is required for the repression of transcription from the *FLO8* cryptic promoter within its coding region (24). This factor is also involved in 3′-end processing of RNA and has been shown to specifically affect the polyadenylation of mRNA (11). Finally, in addition to its role in transcription, the *spt2*Δ mutation enhances recombination where transcription is active and causes defects in chromosome segregation and stability (16, 24, 38). Collectively, these observations suggest that Spt2p protects genome integrity at transcriptionally active regions, presumably by maintaining chromatin structure.

Global analyses of Spt2 localization showed that it is generally associated with coding regions of actively transcribed genes (24). However, a few exceptions have been observed, and among these infrequent Spt2 localizations is the *SRG1-SER3* intergenic region. Interestingly, the *SER3* gene is regulated by a transcription interference mechanism involving the transcription of a noncoding RNA (ncRNA) (21), produced from the intergenic region (*SRG1*) where Spt2 is localized. Noncoding RNAs were shown to be major players in gene expression regulation. They are produced by transcription across entire genomes, including intergenic regions, and regulate gene expression by different mechanisms, including RNA interference (RNAi)-mediated pathways of gene repression (10). However, production of some ncRNAs, as is the case for the ncRNA *SRG1*, regulates gene expression in *cis*. Indeed, recent observations indicated that, rather than the ncRNA

* Corresponding author. Mailing address: Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec (CHUQ), Québec, Québec G1R2J6, Canada. Phone: (418) 525-4444. Fax: (418) 691-5439. E-mail: Amine.Nourani@crhdq.ulaval.ca.

▽ Published ahead of print on 10 January 2011.

product itself, it was the act of transcription and its associated processes that were important for the regulation of adjacent genes. Moreover, a few studies showed that the chromatin modulations associated with transcription of ncDNA play a major regulatory role. The activation of the *Schizosaccharomyces pombe fbp1*$^+$ gene requires displacement by ncRNA transcription of key nucleosomes at the *fbp1*$^+$ promoter (12). Similarly, antisense transcription enhances *PHO5* activation by facilitating nucleosome displacement from the *PHO5* promoter (40). Other studies have highlighted the roles of specific chromatin marks induced by the transcription of ncRNAs in the regulation of adjacent genes (reviewed in reference 10). Specifically, the histone posttranslational modifications directed to *GAL1* and *GAL10* regulatory regions by transcription of the ucut Gal ncRNA were shown to directly affect the expression of these genes (13, 30).

In the *SRG1-SER3* system, the act of transcribing *SRG1*, rather than the ncRNA product, mediates regulation of the *SER3* gene (21). The current model posits that in serine-rich medium *SRG1* transcription interferes with the downstream *SER3* promoter, thereby blocking *SER3* expression and unnecessary serine biosynthesis (see Fig. 1A) (23, 37). Although the specific molecular mechanism of transcription interference is unknown, several models were proposed, including promoter occlusion by the transcription machinery, collisions between RNAP II complexes, and promoter competition (37). In the case of the *SRG1-SER3* regulatory system, the current preferred model postulates that the passage of RNA polymerase II transcribing *SRG1* ncDNA through the *SER3* promoter inhibits the assembly of the preinitiation complex and represses *SER3* transcription (21). Importantly, in the absence of *SRG1* ncRNA, Spt2 is completely delocalized from the intergenic region, suggesting a tight association between the regulation by *SRG1* ncRNA and Spt2 targeting to the intergenic DNA (24). Moreover, deletion of the *SPT2* gene affects dramatically the transcription of *SER3*, apparently by disturbing the regulation by transcription interference associated with *SRG1* ncRNA production (24).

In this work, we analyzed the mechanism by which the elongation factor Spt2 modulates expression of the *SER3* gene by ncRNA *SRG1* transcription. We found that the change in ncRNA *SRG1* transcription in the *spt2*Δ background cannot explain the loss of *SER3* repression. Importantly, we show that Spt2 is involved in the shaping of a particular nucleosomal structure at the *SRG1-SER3* locus. Our data indicate that nucleosomes are deposited specifically within the ncDNA *SRG1* region, corresponding to the *SER3* promoter regulatory elements, following the passage of RNAP II. Interestingly, this deposition is severely impaired by the *spt2*Δ mutation, suggesting that the loss of nucleosomes at this region could be the consequence of disrupted nucleosome deposition in the wake of transcription. Therefore, in contrast to previous observations and models involving nucleosome displacement, histone posttranslational modifications, and promoter occlusion by RNAP II, our data suggest a model in which specific nucleosomes, deposited by a transcription-dependent mechanism involving Spt2, play a central role in the repression by ncRNA transcription.

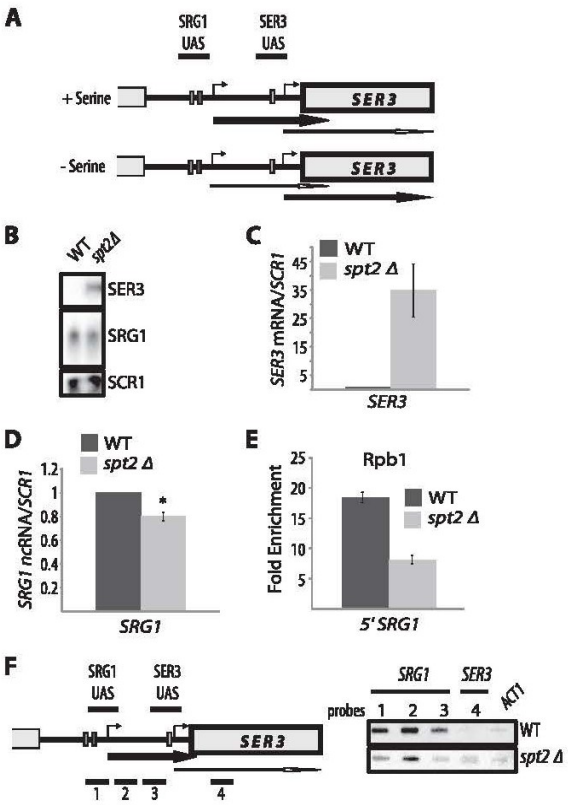

FIG. 1. Deletion of the *SPT2* gene affects *SER3* regulation and reduces ncRNA *SRG1* levels. (A) Diagram explaining the complex regulation of *SER3* by the intergenic transcription of the ncDNA *SRG1*. In the presence of high levels of serine in the medium, ncDNA *SRG1* is actively transcribed and SER3 is inhibited by transcription interference. In the absence of serine, *SRG1* ncDNA transcription activity is reduced and SER3 is then induced. (B) *spt2*Δ mutation affects the transcript levels of *SER3* and ncDNA *SRG1*. Wild-type (WT) (YAN1034) and *spt2*Δ (YAN1035) strains were grown in YPD at 30°C. Total RNA was extracted and analyzed by Northern blotting with a probe against *SER3* and *SRG1*. *SCR1* served as a loading control. (C and D) Quantification of the *SRG1* ncRNA and *SER3* mRNA by RT-qPCR. Total RNA analyzed by Northern blotting as shown in panel B was used to produce cDNA and quantified by qPCR. The relative level is the ratio of the indicated RNA to the *SCR1* transcript level. All values shown are the average results with standard errors from three independent experiments. *, $P < 0.05$. (E) Association of RNAP II with ncDNA *SRG1* is partially dependent on Spt2. Yeast cells from the wild-type (YAN1034) or *spt2*Δ (YAN1035) strains were grown in YPD medium to mid-log phase and then cross-linked with 1% formaldehyde. Chromatin immunoprecipitations were performed using the 8WG16 antibody against Rpb1. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). (F) Run-on assay showing that deletion of *SPT2* results in a decrease of transcription activity at intergenic ncDNA *SRG1*. Diagram showing the different probes used in the run-on assay conducted on the *SRG1-SER3* locus. Radiolabeled RNA extracted from wild-type (YAN1034) or *spt2*Δ (YAN1035) strains was hybridized on membranes containing immobilized probes spanning the *SRG1-SER3* locus. The *ACT1* probe was used as a control.

MATERIALS AND METHODS

***S. cerevisiae* strains, media, and genetic methods.** All *S. cerevisiae* strains (see Table ST1 at http://www.crc.ulaval.ca/nourani/supplemental_data) are isogenic to a *GAL2* derivative of S288C. Strains (44) were constructed by standard methods, either by crosses or by transformation. The *HIS3-pGAL1::SRG1* and *HIS3-pGAL1::FMP27* alleles were constructed by replacing the promoter of the corresponding gene with the *HIS3-pGAL1* cassette (1, 19). *SPT15-3HA*, marked with *HIS3*, was generated by integrating DNA encoding three copies of the HA epitope at the 3′ end of the gene (19). For experiments involving galactose induction, cells were grown to an optical density at 600 nm (OD_{600}) of 0.5 in YP (1% yeast extract, 2% peptone) supplemented with 2% raffinose (YPraf). The cells were then centrifuged, resuspended in YP medium containing 2% galactose (YPgal), and grown for 2 h before being harvested. In the experiments involving glucose repression, cells were grown to an OD_{600} of 0.5 in YPgal; glucose was then added to reach a concentration of 4% in the medium. Efficient G_1 arrest (at least 95%) of cells was achieved by adding 500 ng/ml of α-factor for 2 to 3 h. Sequences of all oligonucleotides used in strain constructions, Northern blotting, reverse transcription (RT)-PCR, run-on transcription, nucleosome scanning assays, and chromatin immunoprecipitation (ChIP) assays are available upon request.

Chromatin immunoprecipitation experiments. Chromatin immunoprecipitation experiments were performed as previously described (22). For the immunoprecipitation of Spt2-13Myc, TBP-3HA, and Gal4, we used, respectively, the antibodies 9E10 anti-Myc (1 μl per immunoprecipitation; Covance), HA.11 anti-HA (1.5 μl per immunoprecipitation; Covance), and Gal4 (DBD):sc-577 (1 μl per immunoprecipitation; Santa Cruz Biotechnology). Immunoprecipitation of Rpb1 was performed using the 8WG16 anti-CTD antibody (2 μl per immunoprecipitation; Covance). The histone H3 immunoprecipitation was done using rabbit anti-H3 antibody (0.2 μl per immunoprecipitation; Abcam). The PCR amplification was performed with 1% of the precipitated material and 0.05% of the input DNA using the LightCycler 480 Sybr green I master kit from Roche.

RNA analyses. Total RNA was isolated using the hot-phenol method (35). In Northern blot analyses, 20 to 40 μg of RNA were separated on a 1% agarose formaldehyde-MOPS gel and transferred to a nylon membrane. The *SRG1*, *SER3*, and *SCR1* probes were amplified by PCR and radiolabeled by random priming. The *SRG1*, *SER3*, and *SCR1* probes were also quantified by RT-quantitative PCR (qPCR). For that process, cDNAs were generated using the Invitrogene M-MLV reverse transcriptase kit and their levels were measured by real-time PCR using LightCycler 480 Sybr green I master kit purchased from Roche. The run-on experiments were conducted as described in reference 21.

Nucleosome scanning assay. Nucleosome scanning experiments were performed using a method adapted from those previously described (3, 17, 42). Cells were grown to 2×10^7 to 3×10^7 cells/ml and treated with formaldehyde (2% final concentration) for 30 min at 30°C and then glycine (125 mM final concentration) for 10 min at room temperature. Formaldehyde-treated cells (1.2×10^9) were harvested by centrifugation, washed with Tris-buffered saline, and then incubated in ZDB buffer (50 mM Tris Cl, pH 7.5, 1 M sorbitol, 10 mM β-mercaptoethanol) containing 1.5 mg Zymolase 20T at 30°C for 30 min on a rocker platform. Spheroplasts were pelleted by low-speed centrifugation, gently washed with NP buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris Cl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.075% NP-40, 1 mM β-mercaptoethanol, and 500 μM spermidine), and resuspended in 1.8 ml NP buffer. Samples were divided into six 300-μl aliquots that were then digested with 0, 1, 2.5, 5, 10, and 20 units of micrococcal nuclease (MNase) (Nuclease S7; Roche) for 45 min at 37°C. Digestions were stopped with 75 μl Stop buffer (5% SDS, 50 mM EDTA) and treated with 100 μg proteinase K for 12 to 16 h at 65°C. DNA was extracted by phenol-chloroform using PLG-H tubes (5 Prime) and incubated with 50 μg RNase A for 1 h at 37°C. DNA was reextracted with phenol-chloroform, precipitated with an equal volume of isopropanol, washed with 100% ethanol, and resuspended in 100 μl TE. MNase digestions were evaluated by two methods. First, one-fifth of digested DNA was separated by gel electrophoresis. Second, previously characterized *GAL1* promoter sequences (3, 8, 18), one within a positioned nucleosome (*GAL1* NB) and a second in an adjacent region (*GAL1* NUB) that is rapidly digested by MNase, were amplified by qPCR from MNase-treated and untreated samples. The MNase concentration that resulted in mostly mononucleosome-sized DNA with a *GAL1* NUB/NB ratio of <15% was subjected to further qPCR using tiled *SER3* primer pairs that amplify 38 unique *SER3* sequences that range from 90 to 114 bp in size, with an average overlap of 69 bp between sequences. For each *SER3* primer set, the amount of protected template was calculated as a ratio between amounts of MNase-digested and undigested samples and then normalized to the amount of protected *GAL1* NB template. All nucleosome scanning assays were done in triplicate using at least two independent strains as indicated in the figure legends. The qPCR data were obtained using an ABI 7300 real-time PCR system with Sybr green reagents (Fermentas). All calculations were performed using Pfaffl methodology for relative quantitation of real-time PCR (29).

RESULTS

Spt2 is required for normal *SER3* repression and affects ncRNA *SRG1* production. The *SER3* gene is regulated by a complex mechanism involving noncoding RNA *SRG1* (ncRNA *SRG1*) transcription (21). As indicated in Fig. 1A, in the presence of serine in the medium, the *SER3* gene is inhibited by the active transcription of the *SRG1* gene. This gene produces a noncoding RNA and its transcription interferes with that of *SER3*. In the absence of serine from the medium, the *SRG1* gene is repressed and *SER3* is in turn rapidly activated (23). Spt2 is recruited to the ncDNA *SRG1*, and its association to this location is tightly linked to the presence of the ncRNA *SRG1* (24). We decided to further study the role of the elongation factor Spt2 in the complex regulation of *SER3*. Since *SER3* regulation is tightly associated with the transcription of the ncRNA *SRG1*, we wanted to know the effect of *SPT2* deletion on the levels of *SER3* mRNA and *SRG1* ncRNA. For that, we extracted total RNA from a wild-type or *spt2*Δ strain and performed Northern blot analyses. We observed a high level of *SER3* transcript in the *spt2*Δ strain, while no detectable amount of such transcript was observed in the wild-type cells (Fig. 1B). This is consistent with our previous observations (24). Interestingly, although the *SER3* mRNA level is dramatically increased in *spt2*Δ cells, the amount of ncRNA *SRG1* appears only slightly reduced in this mutant (Fig. 1B). We decided to quantify precisely the level of both *SER3* and *SRG1* transcripts by RT-qPCR. As indicated in Fig. 1C and D, the deletion of *SPT2* resulted in a dramatic increase of *SER3* mRNA (30- to 40-fold induction) but only a 20% reduction of the noncoding *SRG1* RNA steady-state level.

Recent observations showed that Spt2 plays a significant role in the posttranscriptional events and that it is necessary for the polyadenylation of RNAP II transcripts (11). Therefore, it is possible that the slight reduction of ncRNA *SRG1* is the consequence of a posttranscriptional defect linked to the *SPT2* deletion. To explore the possible role of Spt2 in the stability of the noncoding *SRG1* RNA transcripts, we conducted an experiment to measure the half-life of the ncRNA *SRG1* in wild-type or *spt2*Δ strains (see Fig. S1 at http://www.crc.ulaval.ca/nourani/supplemental_data). Our data indicate that *SRG1* ncRNA stability is not affected by the loss of Spt2.

Our results show that *SRG1* ncRNA levels are slightly affected by the loss of Spt2. Importantly, this effect is not associated with the posttranscriptional function of Spt2, since no defect in the stability of this transcript was observed (see Fig. S1 at the URL above). This finding suggests that a *SRG1* ncRNA drop in the *spt2*Δ strain could be the consequence of a decrease in transcription activity. We wanted to analyze, using different methods, the transcription activity at the *SRG1* region in wild-type and *spt2*Δ strains. For that, we first measured the transcription activity in different regions of the *SRG1-SER3* locus using run-on assays. Radiolabeled RNA was extracted from the wild-type strain or the *spt2*Δ mutant and blotted on membranes containing four probes covering the *SRG1-SER3*

locus and a control probe located in the *ACT1* gene (Fig. 1F). Not surprisingly, as a consequence of the *SER3* derepression in the *spt2*Δ strain, we detected a higher signal at the *SER3* open reading frame (probe 4, Fig. 1F) in this mutant. Importantly, deletion of the *SPT2* gene is associated with a reduction of probe 1, 2, and 3 run-on signals, indicating a lower transcription activity at the intergenic region (*SRG1*) in the absence of Spt2 (Fig. 1F).

In order to confirm our data, we extended our analysis and asked whether the reduction of transcription activity in the absence of Spt2 is associated with the reduction of the RNAP II level at *SRG1*. For that we measured the RNAP II occupancy in the wild-type or *spt2*Δ strain by Rpb1 chromatin immunoprecipitation assays (Fig. 1E). The deletion of *SPT2* led to a decrease of RNAP II association at the 5′ region of *SRG1*, indicating a correlation between the transcriptional activity and the level of RNAP II at this location. Therefore, we concluded that Spt2 is necessary for normal association of the transcription machinery at this location.

A different hypothesis could explain the reduction in transcription activity at this location. Spt2 could be required for the first steps of initiation or during early elongation of transcription at the intergenic region *SRG1*. One of the most important events during initiation is the recruitment of TBP to the TATA box (32). We analyzed the recruitment of TBP-HA to both *SRG1* TATA and *SER3* TATA in the wild-type and *spt2*Δ strains (see Fig. S2 at http://www.crc.ulaval.ca/nourani/supplemental_data). Our experiment shows clearly that TBP-HA association at *SRG1* TATA is similar in both strains, indicating that Spt2 loss affects transcription of ncDNA *SRG1* at a post-TBP recruitment step.

Spt2 role on *SRG1* transcription cannot explain the loss of *SER3* repression. The *SER3* gene is repressed presumably by transcription interference mechanism, whereby passages of RNAP II transcribing the *SRG1* intergenic region obstruct the *SER3* promoter, inhibiting the interaction of activators and initiation factors with their respective binding sites (21). If the transcription interference, in this case, was achieved merely by maintaining a certain transcription rate, then it is possible that the small reduction in transcriptional activity observed at the intergenic region in the *spt2*Δ mutant can disturb the transcription interference and consequently compromise *SER3* repression. We reasoned that, if this hypothesis was true, increasing the *SRG1* ncRNA transcription in the *spt2*Δ mutant to wild-type level would suppress the phenotype associated with this mutation and restore *SER3* repression. We therefore asked whether a higher level of transcriptional activity at the *SRG1* intergenic region is capable of restoring *SER3* repression in *spt2*Δ. To address this, we used an experimental system (Fig. 2A) in which we replaced the *SRG1* promoter by the strong inducible *GAL1* promoter in the wild type and the *spt2*Δ mutant (*pGAL1-SRG1*) and induced transcription of *SRG1*. Our goal was to increase the transcription activity at the intergenic region in the *spt2*Δ mutant in order to reach levels equal to or higher than those found in wild-type cells. We first compared the level of transcription activity under inducing conditions in wild-type, *spt2*Δ, and *spt2*Δ *pGAL1-SRG1* strains by analyzing RNAP II occupancy at the *SRG1* intergenic region via Rpb1 ChIP assays (Fig. 2B). RNAP II occupancy at the *SRG1* intergenic region in the *spt2*Δ *pGAL1-SRG1* strain was significantly higher than that in wild-type or *spt2*Δ cells, where *SRG1* is transcribed from its own promoter. This indicates that replacing the *SRG1* promoter by that of *GAL1*, in *spt2*Δ cells, results in higher transcription activity at the *SRG1* intergenic region. We next wanted to know whether this higher transcription activity correlated with higher ncRNA levels. Total RNA was extracted from wild-type, *spt2*Δ, and *spt2*Δ *pGAL1-SRG1* cells grown under galactose-inducing conditions, and the ncRNA *SRG1* levels were analyzed by Northern blotting (Fig. 2C). As expected, in the *spt2*Δ mutant, the *GAL1* promoter produced a higher level of the ncRNA *SRG1* transcript than the *SRG1* promoter in both wild-type and *spt2*Δ mutant strains (compare lanes 1 and 2 to 4 in Fig. 2C). This observation was confirmed by RT-qPCR (see Fig. S4 at http://www.crc.ulaval.ca/nourani/supplemental_data). We conclude that the *GAL1* promoter induces a higher transcription level at *SRG1* in *spt2*Δ cells. Surprisingly, analyses of the *SER3* transcript level by Northern blotting or RT-qPCR (Fig. 2C and D; also see Fig. S3 at the URL above) indicated that this higher level of transcription activity observed in *spt2*Δ *pGAL1-SRG1* was not associated with repression of the *SER3* gene. We observed instead that higher transcription activity at ncDNA *SRG1* resulted in further derepression of *SER3*. Therefore, suppression of the *SRG1* transcription defect associated with the *spt2*Δ mutation does not restore *SER3* repression. We conclude that the reduction of transcription activity observed in *spt2*Δ cells cannot explain the failure of *SER3* repression by the transcription interference mechanism. Interestingly, higher transcription activity in wild-type cells was also associated with *SER3* activation (Fig. 2C, compare lanes 1 to 3). This indicates that high *SRG1* transcription induced by the *GAL1* promoter is not sufficient by itself for the repression of *SER3*.

Remarkable changes in intergenic *SRG1* transcription are observed when different serine levels are present in the growth media (23). Therefore, we analyzed the *SRG1* and *SER3* transcript levels during a time course with different serine concentrations (see Fig. S5 at http://www.crc.ulaval.ca/nourani/supplemental_data). Our data clearly show that the absence of serine results in a decreased level of *SRG1* and induction of *SER3* transcription in the wild-type strain. When serine was added back, we observed a dramatic increase of *SRG1* level that correlated with *SER3* repression (see Fig. S5). Interestingly, in the *spt2*Δ mutant, the serine regulation of ncRNA *SRG1* is similar to that in the wild-type strain. However, in this mutant *SER3* is never completely repressed, even after a dramatic increase of the ncRNA level in the presence of serine (see Fig. S5). This observation is highlighted in Fig. 2E, where we compare conditions in which the level of ncRNA *SRG1* is significantly higher in the *spt2*Δ strain than in the wild-type strain. Our Northern blot (Fig. 2E) and RT-qPCR (data not shown) analyses demonstrate that the levels of the *SRG1* ncRNA are lower in wild-type cells in the presence of low levels of serine (YPD medium) than those in the *spt2*Δ mutant in the presence of high levels of serine (SD medium with added serine). However, in contrast to the level in the wild type, the higher level of the *SRG1* ncRNA in *spt2*Δ cells is not associated with better repression of *SER3*. This indicates a disconnection between intergenic *SRG1* transcription levels and *SER3* regulation in the *spt2*Δ mutant and confirms that the small reduction of ncRNA transcription observed in these cells cannot

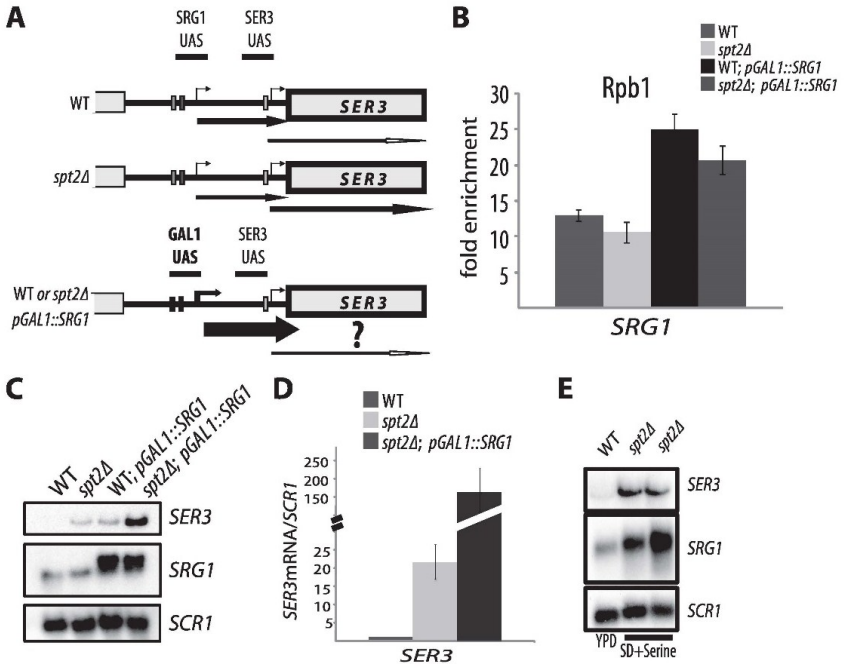

FIG. 2. Increasing the intergenic transcription of ncDNA *SRG1* does not restore *SER3* repression in the *spt2*Δ mutant. (A) Diagram showing the p*GAL1-SRG1* construct. WT, wild type. (B) *GAL1* promoter drives a higher level of transcription at ncDNA *SRG1*. Yeast cells from the wild-type strain (YAN1034), the *spt2*Δ mutant (YAN1035), wild-type *pGAL1-SRG1* (YAN 1040), or the *spt2*Δ strain containing the *pGAL1-SRG1* construct (YAN1039) were grown in YP galactose medium to mid-log phase and then cross-linked with 1% formaldehyde. Chromatin immunoprecipitations were performed using the 8WG16 antibody against Rpb1. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (C) Overproduction of the ncRNA *SRG1* in the *spt2*Δ strain containing the *pGAL1-SRG1* construct is not associated with repression of *SER3*. Total RNA was extracted from cells of wild-type (YAN1034), *spt2*Δ (YAN1035), wild-type *pGAL1-SRG1* (YAN 1040), and *spt2*Δ *pGAL1-SRG1* (YAN1039) strains grown as indicated above. The RNA was analyzed by Northern blotting with probes against *SER3* and *SRG1*. *SCR1* served as a loading control. (D) Quantification of the SER3 mRNA by RT-qPCR. Total RNA analyzed by Northern blotting as shown in panel C was used to produce cDNA that was quantified by qPCR. The relative level is the ratio of the indicated *SER3* mRNA to the *SCR1* transcript level. The values shown represent the average results with standard errors from three independent experiments. (E) High ncRNA *SRG1* levels in serine-rich media are not associated with *SER3* repression in the *spt2*Δ mutant. Yeast cells from the wild-type (YAN1034) or *spt2*Δ (YAN1035) strains were grown, respectively, in YPD or SD medium supplemented with serine. Total RNA was extracted and analyzed by Northern blotting with probes against *SER3* and *SRG1*. *SCR1* served as a loading control. Lanes 2 and 3 are duplicates that represent results of two independent experiments.

explain by itself the loss of *SER3* repression. The transcription of the *SRG1* ncDNA alone is not able to prevent expression of *SER3* when *SPT2* is mutated. Conversely, the presence of Spt2 in wild-type cells is not by itself sufficient for repression of *SER3* when the intergenic *SRG1* region is not transcribed (21). Together, these observations indicate that both intergenic transcription and Spt2 are necessary for repression of *SER3*. Importantly, it also follows that the mere passage of RNAP II and production of ncRNA are not sufficient for silencing of *SER3*. Other transcription-associated events involving Spt2 must be required to achieve full repression of the downstream gene.

Mutation of the histone chaperone *SPT6* has a similar defect in the repression of *SER3*. Our present data show that *SER3* regulation in *spt2*Δ cells is not linked to the modulation of ncRNA *SRG1* transcription. Therefore, the mechanism used by the elongation factor Spt2 to repress *SER3* remains unclear. Interestingly, we previously showed a functional link between the histone chaperone Spt6 and Spt2 (24). Moreover, we found that Spt6 is required for the recruitment of Spt2 to the transcribed regions of some active genes (24). To further understand how Spt2 regulates the *SER3* gene, we asked if the recruitment of this factor to the *SRG1* intergenic region is under the control of Spt6. To address this question, we analyzed the association of Spt2-13Myc with SRG1 by ChIP assays in the wild-type strain, the *spt6-1004* strain, and a control untagged strain (Fig. 3A). In the wild-type strain, the Spt2-13Myc signal at the *SRG1* intergenic region was 10-fold higher than that of the untagged control, confirming a strong specific recruitment to *SRG1*. In contrast, the association of Spt2-13Myc with *SRG1* in *spt6-1004* was very weak, indicating that Spt6 plays an important role in the recruitment of Spt2 to the *SRG1* intergenic region. We next asked if Spt6 recruitment to *SRG1* is dependent on the Spt2 protein. As shown in Fig. 3B, deletion of *SPT2* is associated with partial loss of Spt6-Flag occupancy at *SRG1*. We therefore conclude that Spt2 and Spt6 are dependent on each other for their recruitment to *SRG1*.

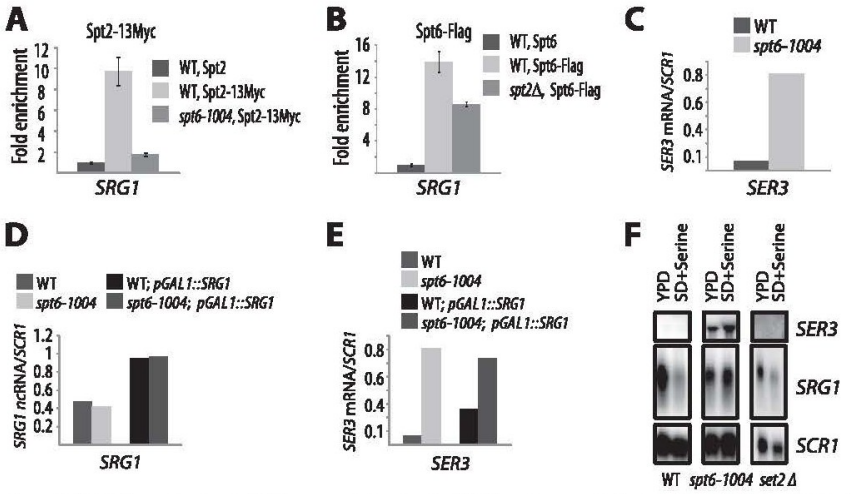

FIG. 3. Spt6 regulates *SER3* independently of *SRG1* transcription level. (A) Recruitment of Spt2 to the ncDNA *SRG1* is dependent on Spt6. ChIP assays of Spt2-13Myc were performed with chromatin extracted from wild-type (WT) (YAN1040), *spt6-1004* (YAN1041), and untagged (YAN1042) strains. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (B) Recruitment of Spt6 to the ncDNA *SRG1* is partially dependent on Spt2. ChIP assays of Spt6-Flag were performed with chromatin extracted from wild-type (YAN1057), *spt2*Δ (YAN1058), and untagged (YAN1042) strains. The fold enrichment is the ratio of %IP of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (C) *SER3* is derepressed in the *spt6-1004* mutant. Total RNA extracted from the wild-type (YAN1034) or the *spt6-1004* (YAN1043) strain was used to produce cDNA that was subsequently quantified by qPCR. The *SER3* mRNA relative level is the ratio of the *SER3* mRNA to the *SCR1* transcript level. The values shown represent the average results of two independent experiments. (D) *GAL1* promoter increases the level of the ncDNA *SRG1* transcripts in the *spt6-1004* mutant. Total RNAs extracted from the wild-type (YAN1034), *spt6-1004* (YAN1043), wild-type *pGAL1-SRG1* (YAN 1040), and *spt6-1004 pGAL1-SRG1* (YAN1044) strains were used in reverse transcription reactions to produce cDNA that was quantified by qPCR. The relative level is the ratio of *SRG1* ncRNA to the *SCR1* transcript level. The values represent the average results of two independent experiments. (E) Overproduction of the ncRNA *SRG1* in the *spt6-1004* strain does not lead to repression of *SER3*. The *SER3* mRNA was quantified using the cDNA obtained in the experiment shown in panel D. The *SER3* relative level is the ratio of *SER3* mRNA to the *SCR1* transcript level. (F) Deletion of the *SET2* gene has no effect on *SER3* repression. Yeast cells from the wild-type (YAN1034), *spt2*Δ (YAN1035), and *set2*Δ (YAN1051) strains were grown, respectively, in YPD or SD medium supplemented with serine. Total RNA was extracted and analyzed by Northern blotting with probes against *SER3* and *SRG1*. *SCR1* served as a loading control. All experiments using *spt6-1004* strains and described in this figure were performed with cells grown at 30°C.

Given the role of Spt2 in the regulation of *SER3* and the fact that its function depends on Spt6 integrity, it became tempting to speculate that similarly to Spt2, Spt6 could be involved in the regulation of *SER3* by transcription of ncDNA *SRG1*. To test this, we first analyzed the *SER3* transcript levels in wild-type and *spt6-1004* strains by RT-qPCR and found a dramatic increase of the *SER3* transcript in *spt6-1004* cells (Fig. 3C). Under these conditions, the derepression of *SER3* was also associated with a small reduction in intergenic *SRG1* transcription, as evidenced by a decreased ncRNA level and a reduced RNAP II occupancy (data not shown). Interestingly, as observed for Spt2, artificially increasing *SRG1* intergenic transcription in *spt6-1004* cells by replacing the *SRG1* promoter with that of *GAL1* resulted in a higher transcription activity without restoring the expected repression of *SER3* (Fig. 3D and E). Thus, similarly to Spt2, Spt6 plays a major role in the regulation of *SER3* by ncRNA *SRG1*. The transcription of *SRG1* alone is not able to prevent expression of *SER3* when *SPT2* or *SPT6* is mutated. In addition, the presence of Spt2 or Spt6 in wild-type cells is not by itself sufficient for repression of *SER3* when intergenic *SRG1* region is not actively transcribed (21). Collectively, these observations indicate that in addition to RNAP II passages and production of ncRNA, other transcription-associated events involving Spt2 and the histone chaperone Spt6 must be required to achieve full repression of the downstream gene.

Histone H3 methylation is not involved in the regulation of *SER3* by transcription of ncDNA *SRG1*. Spt6 is a transcription elongation factor, mainly known to be involved in chromatin modulation associated with elongation (15). In addition to functioning together with Spt2, Spt6 controls the histone H3-lysine 36 methylation by the Set2 enzyme (4, 5, 45). Interestingly, a number of recent studies have reported a link between regulation by transcription of ncRNA and chromatin modifications, specifically histone H3 methylation (reviewed in reference 10). Indeed, regulation of *GAL1* and *GAL10* by the ucut Gal1-10 ncRNA requires methylation of histone H3-K36 and H3-K4 (13). Therefore, the phenotype observed in *spt6-1004* could be associated with the known defects in histone H3-K36 methylation in this mutant (4, 5, 45). To address this possibility directly, we analyzed by Northern blotting the level of *SER3* and *SRG1* in wild-type, *spt6-1004*, and *set2*Δ strains in media containing low or high concentrations of serine (Fig. 3F). In both conditions, the *SER3* transcript was not detected

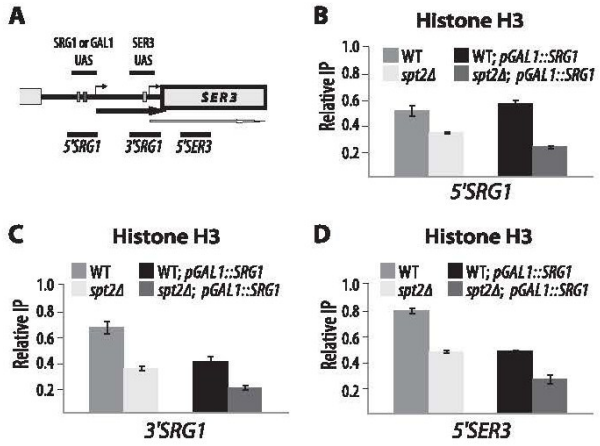

FIG. 4. Spt2 controls the histone H3 occupancy at the *SRG1-SER3* locus. (A) Diagram showing the *SRG1-SER3* regions analyzed by histone H3 ChIP. (B, C, and D) Spt2 is required to maintain normal histone H3 level at the *SRG1* 3′-end region corresponding to the *SER3* promoter. Histone H3 ChIP assays were conducted using chromatin extracted from wild-type (WT) (YAN1034) and *spt2*Δ (YAN1035) strains with or without the *pGAL1-SRG1* construct. For each strain, the value shown represents the ratio of the percent immunoprecipitation (%IP) at the indicated region to the %IP at NoORF. The values shown represent the average results with standard errors from three to six independent experiments.

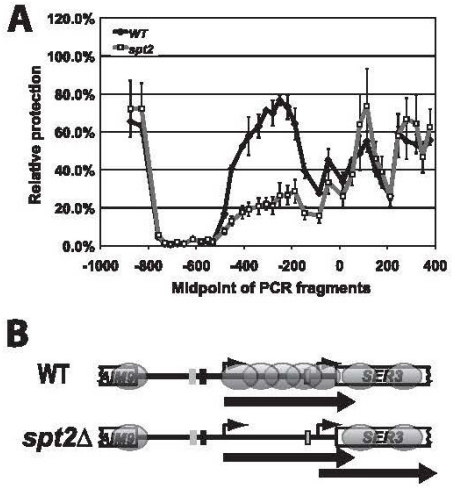

FIG. 5. Spt2 controls the nucleosomal structure at the ncDNA *SRG1*. (A) *spt2*Δ mutation is associated with loss of specific nucleosomes at the ncDNA *SRG1* region corresponding to the *SER3* promoter. A nucleosome scanning assay was performed on wild-type (WT) (OY8 or KY766) and *spt2*Δ (YAN16 or OY8*spt2*) cells that were grown in YPD (*SER3* repressed) at 30°C. Using qPCR, the relative MNase protection of each *SER3* template was calculated as a ratio to that of a *GAL1* promoter template (*GAL1* NB) found within a well-positioned nucleosome in the *GAL1-10* promoter. Each point on the graph shows the mean ± standard error of the mean result from three independent experiments that are plotted at the midpoint of each PCR product. (B) Diagram of the *SER3* locus showing nucleosome positions (ovals) extrapolated from the nucleosome scanning experiments.

in wild-type strains, whereas appreciable amounts were visible in *spt6-1004* cells. Importantly, no *SER3* transcripts were observed in cells bearing the deletion of the *SET2* gene. Moreover, we observed a similar regulation of *SER3* and *SRG1* in wild-type and *set2*Δ strains, while the *SPT6* mutation resulted in constitutive transcription of *SER3* (see Fig. S6 at http://www.crc.ulaval.ca/nourani/supplemental_data). Thus, the Spt6 function in regulating *SER3* cannot be attributed to its role in methylation of H3-K36. In addition, we also analyzed the role of histone H3-lysine 4 methylation in *SER3* regulation by measuring the *SER3* mRNA levels by RT-qPCR in wild-type cells and *spt2*Δ and *set1*Δ mutants (see Fig. S7 at the URL above). This experiment showed that loss of H3-K4 methylation had no significant impact on *SER3* repression. Our observations clearly indicate that in contrast to regulation of *GAL1* and *GAL10* by ncRNA, histone H3-K4 and H3-K36 methylation by Set1 and Set2, respectively, are not required for normal *SER3* repression by ncRNA *SRG1*.

Spt2 is required for maintaining proper nucleosomal structure at the *SRG1* intergenic region. Mutations in *SPT2* or *SPT6* result in spurious transcription from cryptic promoters located within coding regions (15, 24). Presumably, in the absence of normal Spt2 activity, the chromatin structure modified by the passage of transcription machinery is not properly restored, leading to the derepression of cryptic promoters. Importantly, Spt2 was shown to control the level of histone H3 at transcribed regions of *GAL1* and *PMA1* genes (24). This led us to imagine that the role of Spt2 in regulation of *SER3* could have a link with its function in modulating chromatin structure at transcribed regions. To address this question, we first determined whether the deletion of *SPT2* gene affects the histone H3 levels at the *SRG1-SER3* locus (Fig. 4A). To this end, we conducted histone H3 ChIP assays at three different locations around *SRG1-SER3* in wild-type and *spt2*Δ strains. As shown in Fig. 4B, C, and D, H3 occupancy is significantly reduced at this locus in Spt2-deficient cells. This result indicates that chromatin structure within the *SRG1* transcribed region corresponding to the *SER3* promoter could be significantly affected by the loss of Spt2. Interestingly, increasing transcription activity at *SRG1* by inserting the strong *GAL1* promoter does not suppress the loss of histone H3 associated with mutation of *SPT2* at the *SRG1* transcribed region (see Fig. 4B, C, and D). Therefore, the significant histone H3 occupancy change observed in *spt2*Δ cells is independent of the transcription rate. This important observation indicates clearly that *spt2*Δ mutation effect on *SRG1-SER3* chromatin structure is not mediated through the modulation of the *SRG1* transcription activity. It should be noted that inserting the *GAL1* promoter at *SRG1* in wild-type cells resulted in the reduction of histone H3 level in the 3′*SRG1* and 5′*SER3* regions (see Fig. 4C and D). This effect on nucleosome occupancy at *SRG1* could explain the *SER3* derepression observed in wild-type cells and reported in Fig. 2C.

To further study the role of Spt2 in maintaining the nucleosomal structure of *SRG1-SER3*, we determined precise nucleosome positions at this locus by a nucleosome scanning assay similar to those previously described (3, 17, 42). Using this approach first in wild-type cells, we identified two sharp peaks of MNAse protection within the 5′ end of the *SER3* open reading frame, indicating the presence of two well-positioned nucleosomes (Fig. 5A, black line, and Fig. 5B). In addition, we

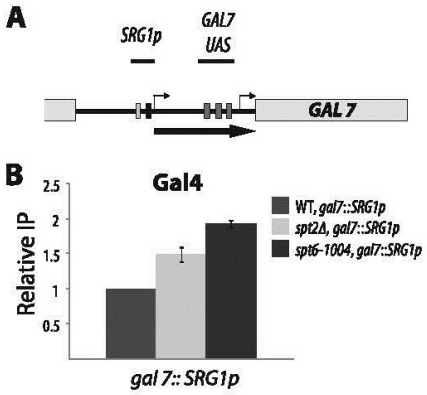

FIG. 6. Mutations of *SPT2* or *SPT6* enhance the accessibility of p*SRG1-GAL7* UAS to the Gal4 activator. (A) Diagram showing the p*SRG1-GAL7* construct used to analyze Gal4 binding in the presence of *SRG1* transcription. (B) Gal4 binding to the p*SRG1-GAL7* UAS is increased in the *spt2*Δ and *spt6-1004* mutants. ChIP assays using anti-Gal4 antibody were conducted on chromatin extracted from wild-type (WT) (FY2257), *spt2*Δ (YAN1045), and *spt6-1004* (YAN1046) strains. For each strain, the fold enrichment shown is relative to the fold enrichment calculated for the wild-type strain and arbitrarily set at 1.0.

observed a broad region of MNase protection that overlaps the *SRG1* transcribed region, which is consistent with the presence of nucleosomes that are randomly positioned. Importantly, this broad region of MNase protection is greatly reduced in *spt2*Δ cells (Fig. 5A, gray line), indicating a loss of nucleosomes over the *SRG1* transcribed region. Taken together with our histone H3 ChIP results, our data suggest that there is a significant disruption of nucleosomal structure at *SRG1* in *spt2*Δ cells. Moreover, our data also show that this effect is independent of the level of *SRG1* transcription.

Mutations in *SPT2* and *SPT6* compromise *SRG1*-mediated inhibition of activator binding. Spt2 is required for normal positioning of nucleosomes in the *SRG1* transcribed region that corresponds to the *SER3* promoter (here called SRG1 3′). In the absence of these nucleosomes, it is possible that transcription activators governing the induction of *SER3* can freely bind their target sites. Because the *SER3* regulators are not known, we decided to use a strain (see Fig. 6A) in which the *SRG1* promoter was inserted in front of the *GAL7* promoter region (21). It was previously shown using this construction that *SRG1* transcription reduces Gal4 binding to the *GAL7* upstream activation sequence (UAS) (21). We asked if *SPT2* or *SPT6* mutations impaired the inhibition of Gal4 binding by *SRG1* transcription. Therefore, we performed Gal4 ChIP assays in wild-type, *spt6-1004*, and *spt2*Δ strains containing the *SRG1* promoter in front of the *GAL7* UAS region. As shown in Fig. 6B, Gal4 binding is significantly increased in the *spt2*Δ and *spt6-1004* mutants. Thus, *SPT2* or *SPT6* mutations compromise the Gal4 inhibition achieved by *SRG1* transcription at the *GAL7* UAS and enhance significantly the accessibility of this region to activators. Importantly, in both *spt2*Δ and *spt6-1004* mutants, Gal4 binding does not increase at the normal *GAL7* promoter (see Fig. S8 at http://www.crc.ulaval.ca/nourani/supplemental_data). We next asked whether these mutations alter the chromatin structure at the *SRG1*::*GAL7* promoter. As shown in Fig. S9 at the URL above, both mutations are associated with a significant loss of histone H3 occupancy at *SRG1*::*GAL7*. Collectively, these observations suggest that a loss of nucleosomes at the *SRG1* region observed in Spt2-deficient cells compromise the *SER3* promoter occlusion to activators.

Nucleosome deposition in the wake of RNAP II passage at the *SRG1* intergenic region is significantly affected in *spt2*Δ cells. We established that Spt2 has an important impact on the chromatin structure of the *SRG1* intergenic region. However, how this elongation factor participates in the maintenance of this chromatin structure remained an open question. Interestingly, this genomic region is robustly transcribed and thus RNAP II activity could lead to displacement of key nucleosomes in this region that must be subsequently reassembled. One could imagine that Spt2 plays a role in nucleosome redeposition in the wake of transcription machinery and that the absence of this factor could lead to a deficient restoration of nucleosomes. To directly test this hypothesis we first used an experimental system described in a number of studies (7, 36). Briefly, through histone H3 ChIP assays, we measured the kinetics of histone H3 redeposition in the transcribed region of the large (8-kb) model gene *FMP27* that is driven by the *GAL1* promoter (36). The rapid repression of *GAL1-FMP27* obtained by adding glucose to the growth medium allowed us to study the rate with which histone H3 is deposited following the last wave of transcription (Fig. 7A). In wild-type cells, upon the addition of glucose, histone H3 levels increased rapidly and reached their maximum at the middle region of *GAL1-FMP27* within 4 min (Fig. 7B). This is consistent with previous studies (36). Deletion of *SPT2* was associated with a significant defect in the H3 redeposition at the *GAL1-FMP27* region after glucose repression. As shown in Fig. 7B, although glucose repression was associated with a small increase of histone H3 occupancy in *spt2*Δ cells, the recovery of nucleosomes in this strain is slower and did not reach the wild-type level even after 20 min of repression. We therefore conclude that the loss of Spt2 has a substantial effect on the nucleosome deposition associated with transcription elongation at *GAL1-FMP27*.

Altogether, our findings suggest the possibility that Spt2 is required for nucleosome redeposition in the wake of transcription at the *SRG1* ncDNA. To address this possibility, we used the same approach described in the legend to Fig. 7A by replacing the *SRG1* promoter with that of *GAL1* and following the histone H3 deposition after the last wave of transcription upon glucose addition (Fig. 7C). We analyzed these kinetics at the *SRG1* transcribed region corresponding to the *SER3* promoter, and the results are reported in Fig. 7D. In wild-type cells, we observed a very rapid increase in histone H3 occupancy that reached its maximum level after 4 to 6 min of repression. Surprisingly, in contrast to the results for *GAL1-FMP27*, the level of histone H3 started to drop and returned to a low level quickly, indicating that nucleosomes that reassembled following RNAP II passage at the ncDNA *SRG1* are very unstable compared to those at *FMP27*. Interestingly, the histone H3 deposition associated with the last wave of transcription was severely impaired in *spt2*Δ cells, indicating an important role of Spt2 in transcription-dependent nucleosome deposition at the region corresponding to the *SER3* promoter.

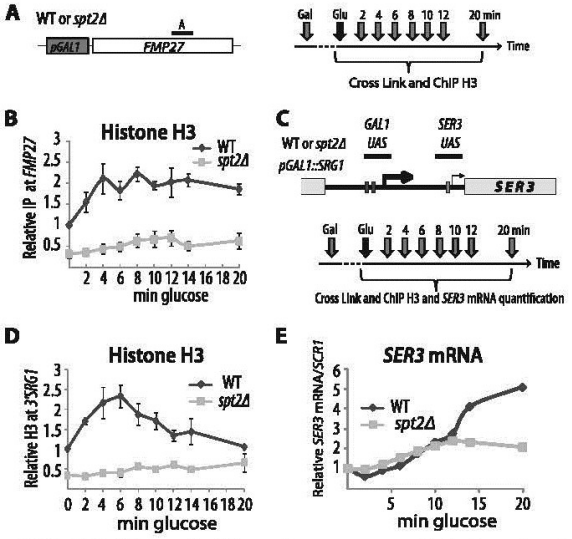

FIG. 7. Spt2 is required for nucleosome reassembly in the wake of RNAP II at the *SRG1* intergenic region. (A) Diagram explaining the experimental procedure designed to analyze histone H3 recovery upon repression of *GAL1-FMP27*. WT, wild type. (B) *spt2*Δ mutation affects significantly histone H3 redeposition associated with the last wave of transcription at the *GAL1-FMP27* transcribed region. Yeast cells from wild-type (YAN1047) or *spt2*Δ (YAN1048) strains were grown in galactose medium to mid-log phase. Glucose was then added to the medium, and the cells were cross-linked and harvested at the indicated time points. Histone H3 level was analyzed by ChIP assays using chromatin extracted from wild-type or *spt2*Δ strains. For each strain, the value of the ratio IP/input calculated for the time zero min was arbitrarily set at 1. (C) Diagram explaining the experimental procedure designed to analyze histone H3 recovery at the *SRG1* 3′ end upon repression of *pGAL1-SRG1*. (D) *spt2*Δ mutation affects significantly histone H3 redeposition associated with the last wave of transcription at the *SRG1* region corresponding to the *SER3* promoter. The experiment was conducted as described for panel B with chromatin extracted from wild-type (YAN1040) or *spt2*Δ cells (YAN1039). The ratio IP/input calculated for time zero in the wild type was arbitrarily set at 1. (E) Spt2 delays maximal *SER3* induction. The *SER3* mRNA and *SCR1* levels were analyzed by RT-qPCR using total RNA extracted from cells treated as described for panel B. For each strain, the value of the ratio *SER3* mRNA/*SCR1* calculated for time zero was arbitrarily set at 1. The values represent the average of two to three independent experiments. Note that at time zero, as expected, the absolute levels in the *spt2*Δ strain are higher than those observed in wild-type cells.

Importantly, the histone H3 steady-state level in the *pGAL1-SRG1 spt2*Δ strain grown in glucose almost reaches the wild-type level (see Fig. S10 at http://www.crc.ulaval.ca/nourani/supplemental_data).

In addition to monitoring histone H3, we analyzed *SER3* transcript levels upon glucose repression of *GAL1-SRG1* (Fig. 7E). In this experiment, we focused on the degree of induction in each strain and not on the absolute levels, which are higher in *spt2*Δ cells. Upon glucose addition, we observed a rapid induction of *SER3* transcripts in wild-type and *spt2*Δ cells. This induction quickly reached a plateau in the *spt2*Δ mutant, while it continued to increase in wild-type cells. The graph in Fig. 7E shows that maximum induction of *SER3* was reached in cells lacking Spt2 after only 10 min. In contrast to this, *SER3* induction continued for at least 10 more minutes in wild-type cells and did not reach a plateau even after 20 min. This delay could be due to the deposition of nucleosomes in the wild-type cells during the first 4 to 6 minutes after *GAL1* promoter shutdown. Later, these nucleosomes are displaced, allowing a further induction of *SER3*. Therefore, our observation suggests that nucleosome deposition associated with the last wave of transcription delays *SER3* induction in wild-type cells. Since the *spt2*Δ mutant is deficient in nucleosome deposition, we did not observe any delay in maximal induction of *SER3* and a plateau was reached after 10 min of glucose repression. Altogether, our data strongly suggest that Spt2 is required for the assembly of nucleosomes associated with transcription of ncDNA *SRG1* and that these nucleosomes play a crucial role in the normal repression of *SER3*.

Our data strongly suggest that Spt2, together with *SRG1* transcription, maintains higher nucleosome occupancy over the *SER3* promoter and therefore represses its transcription. To confirm this, we analyzed histone H3 occupancy in the *srg1-1* mutant. In this strain (Fig. 8A), mutation of the *SRG1* TATA box abolishes its transcription (21). As shown in Fig. 8B, in the absence of *SRG1* transcription, the levels of histone H3 at the *SRG1* region corresponding to the *SER3* promoter dropped significantly, indicating that chromatin structure in this region is dependent on transcription. Interestingly, deletion of the *SPT2* gene did not result in a further decrease in histone H3 occupancy (Fig. 8B). This observation shows that the role of Spt2 in maintaining chromatin structure at the *SER3* promoter is dependent on *SRG1* transcription. Moreover, we found that deletion of *SPT2* did not affect significantly the *SER3* transcript level in the *srg1-1* mutant (Fig. 8C), suggesting therefore that the role of Spt2 in the regulation of *SER3* is associated with *SRG1* transcription.

Spt2 contributes to redeposition of nucleosomes displaced by *SRG1* ncRNA transcription. As shown earlier, there are nucleosomes randomly positioned at the *SRG1* ncDNA region corresponding to the *SER3* promoter. These nucleosomes are displaced and reassembled by continuous passages of RNAP II. We first wanted to know whether this chromatin modulation involves a mechanism that recycles the histones of these nucleosomes or uses new histones. As our data show that Spt2 participates in the deposition of these nucleosomes, we asked specifically whether Spt2 favors the recycling or the use of new histones. To answer these questions, we used an experimental system previously described by us and others (6, 33, 34). In this system, there are two different sources of histone H3 in the cell: the endogenous untagged histone H3 and a galactose-inducible form fused to the Flag tag that is coexpressed with histone H4 (Fig. 9A). In order to eliminate the contribution of DNA replication-dependent histone deposition, exponentially growing cells containing the described construction (Fig. 9B) are blocked in G_1 with α-factor. After incubation with α-factor, cells are either left untreated or induced to express Flag-H3 prior to formaldehyde treatment to cross-link chromatin. Next, the levels of Flag-H3 are assayed by standard ChIP-qPCR at the *SRG1* ncDNA region corresponding to the *SER3* promoter and at a control nontranscribed intergenic region of chromosome V (NoORF) (Fig. 9C and D). As shown in Fig. 9C and D, after induction of the new histone H3 in wild-type cells, we

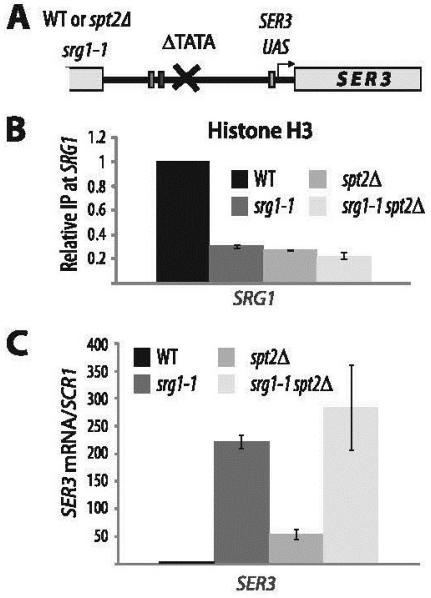

FIG. 8. Chromatin structure at SRG1 depends on active transcription. (A) Diagram showing the *srg1-1* construct. WT, wild type. (B) Mutation of *SRG1* TATA is associated with a significant loss of histone H3 at the *SRG1* 3′-end region corresponding to the *SER3* promoter. Histone H3 ChIP assays were conducted using chromatin extracted from wild-type (YAN1053), *srg1-1* (YAN1054), *spt2*Δ (YAN1055), and *srg1-1 spt2*Δ (YAN1056) strains. For each strain, the value shown represents the ratio IP/input relative to the same ratio calculated for the corresponding wild-type strain and arbitrarily set at 1.0. The values shown are the averages and standard errors of results from three independent experiments. (C) The *spt2*Δ mutation in the *srg1-1* strain does not increase significantly *SER3* derepression. Total RNA extracted from wild-type (YAN1053), *srg1-1* (YAN1054), *spt2*Δ (YAN1055), and *srg1-1 spt2*Δ (YAN1056) strains was used to produce cDNA that was subsequently quantified by qPCR. The *SER3* mRNA relative level is the ratio of the *SER3* mRNA to the *SCR1* transcript level. The values shown are the averages and standard errors of results from three independent experiments.

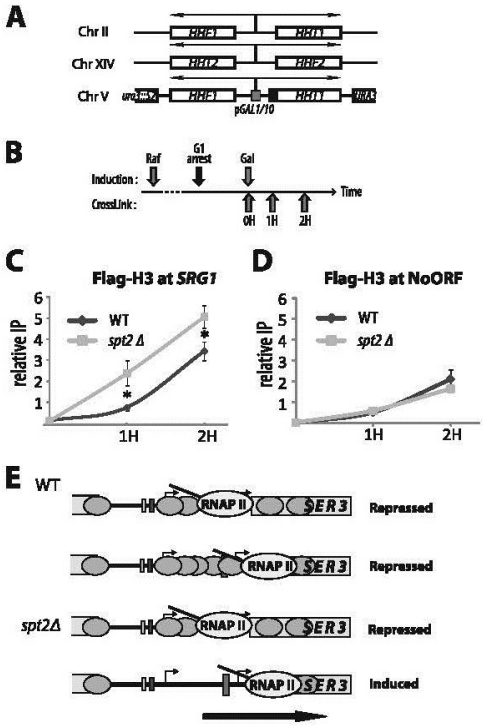

FIG. 9. Spt2 is involved in a nucleosome reassembly pathway that uses old histones at *SRG1*. (A) Diagram representing the different sources of histone H3 and H4 in the yeast strains used in the experiment. (B) Diagram representing the experimental procedure. (C and D) Spt2 inhibits the incorporation of new histone H3 at the *SRG1* region corresponding to the *SER3* promoter. Yeast cells from the wild-type (WT) (YAN1049) or *spt2*Δ strains (YAN1050) containing the construction encoding histone Flag-H3 under the control of the *GAL1* promoter were grown in raffinose-containing medium to mid-log phase. After G_1 arrest by α-factor, the cells were formaldehyde fixed or shifted to galactose medium for 60 or 120 min prior to formaldehyde treatment. ChIP assays were then performed using anti-Flag antibody. The values shown (relative IP/input) represent the averages and standard errors of results from three independent experiments. *, $P < 0.05$. (E) Model of *SER3* regulation by ncRNA *SRG1* transcription.

detected higher levels of its incorporation at *SRG1* than in the control region (NoORF). Thus, *SRG1* ncDNA is associated with a significant replication-independent histone H3 turnover, indicating the existence of a transcription-dependent chromatin assembly mechanism that deposits new nucleosomes. Surprisingly, deletion of *SPT2* resulted in higher deposition of new histone H3 at ncDNA *SRG1*. Moreover, the low level of total H3 in Spt2-deficient cells suggests that, in the *spt2*Δ mutant, the vast majority of nucleosomes at *SRG1* are refolded using newly synthesized histones. This indicates that Spt2 inhibits nucleosome assembly that uses newly synthesized histones. Because the overall nucleosome deposition is reduced in *spt2*Δ cells (Fig. 7), our data suggest that, similarly to yFACT at the transcribed regions of some genes (14), Spt2 favors a dominant nucleosome assembly pathway that uses old histones displaced by elongating RNAP II. In Spt2-deficient cells, the induction of another chromatin assembly pathway that uses new histones is not able to overcome this loss. Thus, Spt2 participates in the major mechanism that refolds chromatin structure in the wake of RNAP II at *SRG1*. This mechanism recycles histones of nucleosomes previously displaced by the transcription machinery and shapes specific chromatin structure that is crucial for *SER3* repression.

DISCUSSION

The modulation of gene expression by ncRNA is an emerging field that has focused mostly on transregulatory mechanisms, including repression by RNAi pathways. However, a growing amount of evidence points toward the existence of various *cis*-directed mechanisms where the process of ncRNA production itself, and not the product, contributes to the regulation of a gene. Interestingly, several observations introduced the notion that the chromatin environment created by the transcription of ncRNA may have a regulatory role (10).

These studies involved different mechanisms where ncRNA transcription displaces nucleosomes or modifies histones to regulate a nearby gene (12, 13, 30). Here we show that *SRG1* ncRNA transcription deposits nucleosomes that participate in the repression of the adjacent *SER3* gene. Our study also brings new insights into the molecular mechanism of transcription interference. Different models of transcription interference have been proposed, including promoter competition, occlusion by elongating RNAP II, or collision between transcription machineries (reviewed in reference 37). We provide evidence that transcription interference in the *SRG1-SER3* system involves deposition of specific nucleosomes in the wake of an elongating RNAP II. Importantly, and in contrast to previous observations, our work shows that histone H3-K4 methylation and H3-K36 methylation are not required for the normal *SER3* regulation by ncRNA transcription. This suggests that the most important factor in this repression is the deposition of nucleosomes and not their posttranslational modifications that could alter their dynamic properties. Finally, we show that the elongation factor Spt2 and the histone chaperone Spt6 play central roles in this regulatory system. Our data indicate that Spt2 participates in the shaping of the transcription-dependent chromatin structure over the *SRG1* transcribed region that controls the activity of *SER3* promoter.

Before this work was completed, the working model of *SER3* regulation had been based on the canonical mechanism of transcription interference, whereby successive passages of RNAP II transcribing the *SRG1* intergenic region obstruct the *SER3* promoter, inhibiting the interaction of activators and initiation factors with their respective binding sites. In the *spt2*Δ or *spt6-1004* mutant strains grown in standard rich medium, we observed a small but consistent reduction of transcription activity at the *SRG1* intergenic region that produces the ncRNA. It was easy to imagine that if regulation by transcription interference was achieved merely by maintaining a certain transcription rate involving the passage of a specific number of RNAP II complexes, a small reduction in the number of these complexes traversing the *SER3* promoter could compromise the interference. However, direct and indirect experimental evidence quickly challenged this simple view. First, our work showed that under conditions where *SRG1* transcription in the *spt2*Δ mutant is higher than that in wild-type cells, the *SER3* gene remained active (Fig. 2E; also see Fig. S5 at http://www.crc.ulaval.ca/nourani/supplemental_data). Second, increasing artificially the transcription rate of the *SRG1* ncRNA in the *spt2*Δ mutant did not restore normal repression (Fig. 2C and D). Third, in *spt6-1004* cells, we observed similar defects in *SER3* repression. This phenotype cannot be explained by variations in *SRG1* transcription (Fig. 3). Fourth, changing the transcription in the wild type from a high level (*SRG1* promoter) to a very high level (*GAL1* promoter) did not further repress *SER3* transcription. In fact, we observed the opposite result, since insertion of the *GAL1* promoter was associated with *SER3* derepression (Fig. 2C, 2D, and 3E; also see Fig. S3 at the URL above). These data suggest a more complicated mechanism of transcriptional interference in which the passages of RNA polymerase II alone are not sufficient to repress *SER3* transcription. Thus, the presence or absence of normal Spt2 and Spt6 function could slightly affect the production of the ncRNA under some conditions, but this marginal role could not explain the loss of *SER3* repression. Finally, taken together, these observations indicate that *SER3* repression is not only mediated by the production of the *SRG1* ncRNA or the frequency of transcription machinery passages over the *SER3* promoter. Instead, it is the entire process of transcription and its associated events such as chromatin modulation that are involved in the interference with the *SER3* promoter. Our observations support an important role of Spt2 in the chromatin modulations associated with *SRG1* transcription that contributes directly to the repression of *SER3*.

Perhaps the most significant observation in this work is the one related to the chromatin structure in the *SRG1* intergenic region (Fig. 4 and 5). Nucleosome scanning assays conducted in wild-type cells indicate that this region contains a large peak representing the presence of nucleosomes randomly positioned within this short DNA segment (Fig. 5). Importantly, our data indicate clearly that nucleosomes are assembled in the wake of transcription at this region (Fig. 7). However, shortly after their deposition, these nucleosomes appear to be displaced. Therefore, our findings suggest that continuous transcription of intergenic *SRG1* is required for the maintenance of nucleosomes at this region. Interestingly, mutation of the *SRG1* TATA box abolishes its transcription and results in a clear depletion of histone H3 at this region (Fig. 8). This is consistent with a recent study showing that nucleosomes over the *SER3* UAS (upstream activation sequence) are present when *SRG1* is transcribed, while inhibition of the intergenic transcription in the absence of serine is associated with a loss of nucleosomes at this position (9a).

The precise mechanism by which Spt2 contributes to the shaping of the nucleosomal structure at the *SRG1* ncDNA remains an interesting question. Several hypotheses could be proposed. It is possible that Spt2 acts after chromatin synthesis and stabilizes the nucleosomes deposited in the wake of RNAP II. Alternatively, Spt2 could be directly involved in transcription-dependent chromatin assembly associated with *SRG1* transcription. The latter possibility is likely to be the case for several reasons. First, Spt2 has two HMG-like domains and could, similarly to other HMG-box proteins, assist histone chaperones in nucleosomal assembly (9, 26). Second, it has functional genetic and physical interactions with the histone chaperone Spt6 which are consistent with such a role (reference 24 and unpublished data). Third, it binds four-way junction DNA, a structure similar to that found at the entrance/exit point of DNA from a nucleosome (25, 46). Consistently, we found that it interacts with mononucleosomes *in vitro* (unpublished data). Future studies focused on Spt2 should help to determine its precise role in chromatin remodeling in the wake of RNAP II.

Our analyses of the replication-independent histone H3 incorporation indicate the existence of at least two different mechanisms involved in the control of transcription-dependent nucleosome deposition at the ncDNA *SRG1*. First, we showed that *SRG1* ncDNA incorporates a high level of new histones in G_1-arrested cells, indicating that transcription at this region is coupled to a replication-independent chromatin assembly pathway that uses new histones. Second, our analyses of histone H3 deposition outside S-phase show that the absence of Spt2 is associated with both a preferential deposition of newly

synthesized H3 and an overall loss of histone H3 (Fig. 4 and 9) at the *SRG1* ncDNA. This indicates that the loss of Spt2 affects an additional mechanism(s) that recycles old histone H3 to reassemble nucleosomes displaced by RNAP II at *SRG1*. This observation is similar to the data recently reported on the yFACT histone chaperone (14). Indeed, the Strubin group clearly showed that newly synthesized histone H3 deposition is induced on a number of genes in the *spt16-197* mutant, while nucleosomal occupancy decreases overall (14). Without excluding other possibilities, one simple explanation of such observations is that two different chromatin assembly pathways coexist at *SRG1*. One uses old histones and recycles them to redeposit nucleosomes displaced by RNAP II and requires Spt2. The other pathway uses newly synthesized histones and is not dependent on Spt2. At *SRG1*, one possibility is that the recycling pathway is dominant, and the induction of another pathway using newly synthesized histone H3 cannot overcome the loss of Spt2. Whether this effect is more general and could be applied to other genomic loci would be interesting to assess. It is likely that this mechanism also functions outside the *SRG1-SER3* locus. Indeed, analyses at other transcribed regions showed that *spt2*Δ mutation is also associated with the induction of newly synthesized H3 deposition (P. Thebault, unpublished data). Finally, given the fact that Spt2 and Spt16 defects have similar phenotypes on histone H3 dynamics at different transcribed regions, it will be interesting to test whether yFACT depletion has any role on *SRG1* histone H3 turnover or chromatin structure and therefore on *SER3* regulation.

Finally, our data suggest a new model of regulation by ncRNA transcription that involves nucleosomes deposited in the wake of the transcription machinery (Fig. 9E). We propose that ncRNA *SRG1* transcription achieves *SER3* repression mainly by creating a repressive chromatin structure. Therefore, in contrast to what has been imagined, transcription interference within the *SRG1-SER3* system is not only achieved by RNAP II passing through the *SER3* promoter but is also mediated by the nucleosomes inserted behind RNAP II complexes as they leave this regulatory region. These nucleosomes are important for the repression of *SER3* transcription that could possibly be initiated in the time frame between two successive rounds of ncRNA *SRG1* transcription. In the *spt2*Δ mutant, these nucleosomes are not assembled correctly, creating an open chromatin context for the *SER3* activator(s). Importantly, and in contrast to previous observations, our work shows that histone H3-K4 and H3-K36 methylation is not required for the normal *SER3* regulation by ncRNA transcription, suggesting that the most important factor is the deposition of nucleosomes and not modifications that play a role in the dynamic properties of these nucleosomes. Overall, we provide strong evidence that Spt2 participates in a regulation by transcription interference via a novel mechanism involving a complex interplay between RNAP II and chromatin dynamics associated with its passage.

ACKNOWLEDGMENTS

We thank Nikita Avvakumov and Jacques Côté for helpful comments and advice on the manuscript.

This work was supported by a CIHR grant to A.N. A.N. holds a Canadian Research Chair, and G.B. holds an NSERC Ph.D. fellowship.

REFERENCES

1. **Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin.** 1993. A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acids Res. **21:**3329–3330.
2. **Baxter, B. K., and E. A. Craig.** 1998. Suppression of an Hsp70 mutant phenotype in Saccharomyces cerevisiae through loss of function of the chromatin component Sin1p/Spt2p. J. Bacteriol. **180:**6484–6492.
3. **Brickner, D. G., et al.** 2007. H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. PLoS Biol. **5:**e81.
4. **Carrozza, M. J., et al.** 2005. Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell **123:**581–592.
5. **Cheung, V., et al.** 2008. Chromatin- and transcription-related factors repress transcription from within coding regions throughout the Saccharomyces cerevisiae genome. PLoS Biol. **6:**e277.
6. **Dion, M. F., et al.** 2007. Dynamics of replication-independent histone turnover in budding yeast. Science **315:**1405–1408.
7. **Fleming, A. B., C. F. Kao, C. Hillyer, M. Pikaart, and M. A. Osley.** 2008. H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. Mol. Cell **31:**57–66.
8. **Floer, M., et al.** 2010. A RSC/nucleosome complex determines chromatin architecture and facilitates activator binding. Cell **141:**407–418.
9. **Formosa, T., et al.** 2001. Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. EMBO J. **20:**3506–3517.

9a. **Hainer, S. J., J. A. Pruneski, R. M. Monteverde, and J. A. Martens.** 2011. Intergenic transcription causes repression by directing nucleosome assembly. Genes Dev. **25:**29–40.

10. **Hartzog, G. A., and J. A. Martens.** 2009. ncRNA transcription makes its mark. EMBO J. **28:**1679–1680.
11. **Hershkovits, G., H. Bangio, R. Cohen, and D. J. Katcoff.** 2006. Recruitment of mRNA cleavage/polyadenylation machinery by the yeast chromatin protein Sin1p/Spt2p. Proc. Natl. Acad. Sci. U. S. A. **103:**9808–9813.
12. **Hirota, K., et al.** 2008. Stepwise chromatin remodelling by a cascade of transcription initiation of non-coding RNAs. Nature **456:**130–134.
13. **Houseley, J., L. Rubbi, M. Grunstein, D. Tollervey, and M. Vogelauer.** 2008. A ncRNA modulates histone modification and mRNA induction in the yeast GAL gene cluster. Mol. Cell **32:**685–695.
14. **Jamai, A., A. Puglisi, and M. Strubin.** 2009. Histone chaperone spt16 promotes redeposition of the original h3–h4 histones evicted by elongating RNA polymerase. Mol. Cell **35:**377–383.
15. **Kaplan, C. D., L. Laprade, and F. Winston.** 2003. Transcription elongation factors repress transcription initiation from cryptic sites. Science **301:**1096–1099.
16. **Kruger, W., and I. Herskowitz.** 1991. A negative regulator of HO transcription, SIN1 (SPT2), is a nonspecific DNA-binding protein related to HMG1. Mol. Cell. Biol. **11:**4135–4146.
17. **Lee, W., et al.** 2007. A high-resolution atlas of nucleosome occupancy in yeast. Nat. Genet. **39:**1235–1244.
18. **Lohr, D.** 1984. Organization of the GAL1-GAL10 intergenic control region chromatin. Nucleic Acids Res. **12:**8457–8474.
19. **Longtine, M. S., et al.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953–961.
20. **Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond.** 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:**251–260.
21. **Martens, J. A., L. Laprade, and F. Winston.** 2004. Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene. Nature **429:**571–574.
22. **Martens, J. A., and F. Winston.** 2002. Evidence that Swi/Snf directly represses transcription in S. cerevisiae. Genes Dev. **16:**2231–2236.
23. **Martens, J. A., P.-Y. J. Wu, and F. Winston.** 2005. Regulation of an intergenic transcript controls adjacent gene transcription in Saccharomyces cerevisiae. Genes Dev. **19**(22)**:**2695–2704.
24. **Nourani, A., F. Robert, and F. Winston.** 2006. Evidence that Spt2/Sin1, an HMG-like factor, plays roles in transcription elongation, chromatin structure, and genome stability in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **26:**1496–1509.
25. **Novoseler, M., G. Hershkovits, and D. J. Katcoff.** 2005. Functional domains of the yeast chromatin protein Sin1p/Spt2p can bind four-way junction and crossing DNA structures. J. Biol. Chem. **280:**5169–5177.
26. **Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey, and D. Reinberg.** 1999. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. Nature **400:**284–288.
27. **Perez-Martin, J., and A. D. Johnson.** 1998. Mutations in chromatin components suppress a defect of Gcn5 protein in Saccharomyces cerevisiae. Mol. Cell. Biol. **18:**1049–1054.

28. **Peterson, C. L., W. Kruger, and I. Herskowitz.** 1991. A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. Cell **64:**1135–1143.
29. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
30. **Pinskaya, M., S. Gourvennec, and A. Morillon.** 2009. H3 lysine 4 di- and tri-methylation deposited by cryptic transcription attenuates promoter activation. EMBO J. **28:**1697–1707.
31. **Pollard, K. J., and C. L. Peterson.** 1997. Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression. Mol. Cell. Biol. **17:**6212–6222.
32. **Pugh, B. F.** 2000. Control of gene expression through regulation of the TATA-binding protein. Gene **255:**1–14.
33. **Rufiange, A., P. E. Jacques, W. Bhat, F. Robert, and A. Nourani.** 2007. Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. Mol. Cell **27:**393–405.
34. **Schermer, U. J., P. Korber, and W. Horz.** 2005. Histones are incorporated in trans during reassembly of the yeast PHO5 promoter. Mol. Cell **19:**279–285.
35. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res. **18:**3091–3092.
36. **Schwabish, M. A., and K. Struhl.** 2004. Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol. Cell. Biol. **24:**10111–10117.
37. **Shearwin, K. E., B. P. Callen, and J. B. Egan.** 2005. Transcriptional interference—a crash course. Trends Genet. **21:**339–345.
38. **Sikdar, N., S. Banerjee, H. Zhang, S. Smith, and K. Myung.** 2008. Spt2p defines a new transcription-dependent gross chromosomal rearrangement pathway. PLoS Genet. **4:**e1000290.
39. **Sternberg, P. W., M. J. Stern, I. Clark, and I. Herskowitz.** 1987. Activation of the yeast HO gene by release from multiple negative controls. Cell **48:**567–577.
40. **Uhler, J. P., C. Hertel, and J. Q. Svejstrup.** 2007. A role for noncoding transcription in activation of the yeast PHO5 gene. Proc. Natl. Acad. Sci. U. S. A. **104:**8011–8016.
41. **West, K. L.** 2004. HMGN proteins play roles in DNA repair and gene expression in mammalian cells. Biochem. Soc. Trans. **32:**918–919.
42. **Whitehouse, I., and T. Tsukiyama.** 2006. Antagonistic forces that position nucleosomes in vivo. Nat. Struct. Mol. Biol. **13:**633–640.
43. **Winston, F., D. T. Chaleff, B. Valent, and G. R. Fink.** 1984. Mutations affecting Ty-mediated expression of the HIS4 gene of Saccharomyces cerevisiae. Genetics **107:**179–197.
44. **Winston, F., C. Dollard, and S. L. Ricupero-Hovasse.** 1995. Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C. Yeast **11:**53–55.
45. **Youdell, M. L., et al.** 2008. Roles for Ctk1 and Spt6 in regulating the different methylation states of histone H3 lysine 36. Mol. Cell. Biol. **28:**4915–4926.
46. **Zlatanova, J., and K. van Holde.** 1998. Binding to four-way junction DNA: a common property of architectural proteins? FASEB J. **12:**421–431.