ROLE OF THE YEAST HISTONE METHYLTRANSFERASE SET2 AND ITS REGULATORY DOMAINS IN RNA POLYMERASE II TRANSCRIPTION

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ABSTRACT

KELBY O. KIZER: Role of the yeast histone methyltransferase Set2 and its regulatory domains in RNA polymerase II transcription

(Under the direction of Dr. Brian Strahl)

Eukaryotic transcription requires the careful regulation of chromatin structure in order to allow access of RNA polymerase II (RNAPII) to DNA. Histone modifications are well established as regulators of chromatin structure and gene transcription. Although the influences of histone acetylation and phosphorylation have been investigated extensiviely, the role of histone methylation has recently become a topic of intense study in the field of chromatin biology. The newly discovered histone H3 methylase Set2 is the sole enzyme in yeast responsible for H3 lysine 36 (K36) methylation. Although earlier work suggested a role for Set2 and K36 methylation in transcriptional repression, the role of K36 methylation in yeast remained largely unexplored. Through the studies presented here, we have identified and characterized a link between Set2 and actively transcribing RNA polymerase II (RNAPII). Importantly, in Chapter 2 we present data identifying a novel domain in Set2 that is responsible for interaction with RNAPII. Further analysis of this domain revealed its presence in a number of Set2 homologues in other species, thereby stimulating further studies of the role of Set2 and K36 methylation in gene transcription across multiple organisms.

In addition to studies of the downstream roles of Set2 and K36 methylation in transcription, in Chapter 3 we describe our investigation into a network of proteins that are involved in the upstream regulation of Set2. These studies suggest an important link

between the regulation of nucleosome conformation and subsequent K36 methylation, further supported by concurrent studies from other laboratories. Through our studies of Set2 and histone methylation, we also developed and improved several experimental methods that are presented in Chapter 4. Finally, in Chapter 5 we describe the contributions of our work in the larger context of recent studies from other laboratories. We also discuss relevant questions for future work regarding Set2 as well as histone modifications in general.

Sola gratia

Sola fide

Sola scriptura

Solus Christus

Soli Deo gloria

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As a growing scientist, I have learned a great deal in regard to avenues for the improvement of human health. However, at the same time, I have had a mixture of

excitement, anger, jealousy, and sadness while in graduate school. Through the stress of this process, I have thought and acted in anger toward almost everyone at some time. As the years have passed, it has become even clearer to me that I cannot save even myself. The dedication of this dissertation is a glimpse into this truth, a full and clear testimony of which I cannot effectively communicate on these pages.

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LIST OF ABBREVIATIONS AND SYMBOLS

6AU	6-azauracil
α	anti (in regard to an antibody directed against indicated target antigen)
AWS	associated with SET
BLAST	basic local alignment search tool
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
COMPASS	complex proteins associated with Set1
CTD	carboxy-termial domain
D	deleted
DNA	deoxyribonucleic acid
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4
H3K36me1	histone 3 lysine 36 mono-methylation
H3K36me2	histone 3 lysine 36 di-methylation
H3K36me3	histone 3 lysine 36 tri-methylation
H3K4me3	histone 3 lysine 4 tri-methylation
H3K56ac	histone 3 lysine 56 acetylation
H3K79me3	histone 3 lysine 79 tri-methylation
HAT	histone acetyltransferase
HDAC	histone deacetylase
HhH	helix-hairpin-helix
HMT	histone methyltransferase

IP	immunoprecipitation
kDa	kilo Dalton
L	liter
LB	Luria broth
M	molar (mol/L)
ml	milliliter
mM	millimolar
mM	micromolar
PCR	polymerase chain reaction
PHD	plant homeodomain
RNAPII	RNA polymerase II
RT	reverse transcriptase
SC	synthetic complete (media)
SAM	*S*-adenosylmethionine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Ser2	serine 2
Ser5	serine 5
SET	Su(var)3-9, enhancer of zeste, trithorax
SRI	Set2 Rpb1 interacting
WCE	whole cell extract
YPD	yeast peptone dextrose (media)

CHAPTER 1

INTRODUCTION

1.1 Overview of chromatin

The existence of chromatin was predicted over a century ago, however its importance was largely overlooked until the mid 20th century. Over the past century, dramatic evidence was uncovered which implicates chromatin in the control of genome integrity and activity. Here we describe relevant history of predictions, observations, and conclusions regarding chromatin and its role in the regulation of gene transcription.

Discovery of chromatin

In the late 19th century, following the first isolations of nuclear components and the observation of chromosome segregation during mitosis, Walther Flemming hypothesized that a type of scaffold exists which supports the key nuclear materials (Flemming, 1882). The term "chromatin" was coined based on the dye-affinity of this nuclear matrix and in reference to this theoretical scaffold. Following the identification of chromatin and chromosomes, much scientific effort focused on uncovering details of the fundamental carrier of genetic information. A potential influence of chromatin in the 'central dogma' of the genetic code was largely overlooked when Watson and Crick first published the structure of DNA. (Watson and Crick, 1953a; Watson and Crick, 1953b).

Histones, the building block of chromatin

Chromatin composes a balancing act between efficiency of packing and accessibility that is carefully maintained in the nucleus of every eukaryotic cell. In humans, approximately 2 m of DNA is condensed within a 6 μm nucleus (Orphanides and Reinberg, 2000). Using an analogy, this condensation is roughly equivalent to 2 km of 16 gauge wire coiled into a 6 mm diameter sphere. This compaction is accomplished through an association between DNA and specialized proteins known as histones, that ultimately result in the formation of chromosomes. Although histones were discovered just two years after Flemming published his chromatin hypothesis, it was nearly one hundred years before

histones again became the subject of intense study (Kossel, 1884; Ciba Foundation Study Group No.24, 1966; Kornberg and Lorch, 1999).

In the early 1960s, Bonner and Butler found that histones affected the transcription of DNA by RNA polymerase II (Huang and Bonner, 1962; Butler, 1965). This discovery led to many exciting studies to determine the means by which histones and DNA interact to form a chromosome. Through the following two decades five unique types of histones were identified, known as Histone 2A (H2A), Histone 2B (H2B), Histone 3 (H3), Histone 4 (H4), and the linker Histone 1 (H1) (Phillips and Johns, 1965; Kornberg, 1977; Kornberg and Lorch, 1999). Early X-ray structural data indicated that a regular, repeating structure existed along the length of chromatin fibers, while *in vitro* reconstitution experiments indicated that four forms of the histones (H2A, H2B, H3 and H4) associate to form a tetramer (Kornberg and Thomas, 1974). Furthermore, histone tetramers were found to associate into an octamer of histones, known as the nucleosome. Approximately 147 base pairs of DNA wrap around a nucleosome, compacting DNA and ultimately forming the higher-order structure of chromatin as diagrammed in **Figure 1.1** (Kornberg and Thomas, 1974; Oudet et al., 1975; Kornberg, 1977; Felsenfeld, 1978; Hansen, 2002). When the nucleosome composition was first identified, it remained unclear how this structure would impact DNA replication and transcription.

1.2 Transcription through chromatin

A crucial function of chromatin is the packaging of DNA into an environment that is not easily accessible to other protein complexes that require access to the genome. Researchers in the late 1960's discovered that the histone proteins could affect RNA polymerase activity on DNA templates, a clue that implicated histones in the regulation of gene expression (Huang and Bonner, 1962; Butler, 1965). Evidence continued to mount which ultimately demonstrated that the histone proteins are crucial dynamic components in

the regulation of gene transcription (Felsenfeld, 1978; Williamson and Felsenfeld, 1978; Grunstein, 1990; Workman and Kingston, 1998; Orphanides and Reinberg, 2000). Prior to gene transcription, therefore, chromatin must first be unraveled in order for RNA polymerase and other transcription factors to gain access to the DNA template (van Holde et al., 1992; Workman and Kingston, 1998; Kornberg and Lorch, 1999; Sims, III et al., 2004). Continuing with the earlier analogy of DNA as a wire: it is not sufficient to simply compact the wire, but rather mechanisms must exist for the controlled loosening at particular regions such that writing on the wire (that represent the DNA sequence) could actually be visible. This 'balancing act' of chromatin involves the protection of DNA through compaction, while also allowing regulated structural changes necessary for transcription.

RNA polymerase II transcription

Transcription of DNA to mRNA requires recruitment of RNA polymerase II (RNAPII) and a variety of transcription factors. Transcription is accomplished through three general phases: 1) initiation, where transcription factors and chromatin structure allow RNAPII to assemble and the nascent mRNA is capped; 2) elongation, where RNAPII processivity is maintained through the body of a gene by the actions of various transcription factors that assist RNAPII in maintenance of the chromatin environment; and 3) termination, where the DNA sequence and RNAPII-associated proteins signal for the addition of the polyadenylation signal and the dissociation of RNAPII (Lee and Young, 2000; Orphanides and Reinberg, 2000; Orphanides and Reinberg, 2002). Eukaryotic RNAPII is composed of twelve subunits, named Rpb1 to Rpb12 in order of decreasing size (Corden, 1990; Young, 1991). The largest RNAPII subunit, Rpb1, contains a unique sequence at its C-terminus, known as the Carboxy Terminal Domain (CTD). This CTD is composed of a heptad sequence that is repeated in tandem 26 times in the yeast *Saccharomyces cerevisiae* and 52 in mammals (Lee and Greenleaf, 1989). Despite the differences in the number of repeats, and the unstructured nature of this "domain", it is highly conserved across

eukaryotes. Transcription factors, including many involved in mRNA processing, are known to bind to the RNAPII-CTD (Komarnitsky et al., 2000; Morris et al., 1999; Morris and Greenleaf, 2000; Schroeder et al., 2000). Therefore, this domain is thought to be a "binding platform" through which other as-yet-unidentified proteins may bind and influence transcription.

The phosphorylation state of the CTD is significant to our understanding of RNAPII function. The CTD is highly phosphorylated during active transcription, while non-transcribing Rpb1 is unphosphorylated (Cadena and Dahmus, 1987; Weeks et al., 1993). Phosphorylated RNAPII does not exist as a homogenous population, however, but rather is marked with a particular phosphorylation pattern along the CTD corresponding to the phase of transcription. Based on the numbering system assigned to the heptapeptad repeat sequence ($Y_1S_2P_3T_4S_5P_6S_7$), the serines at positions two (Ser2) and five (Ser5) within the CTD are differentially phosphorylated during transcription, with Ser5 phosphorylation found most densely during initiation and early elongation while phosphorylated Ser2 is found preferentially during the elongation and termination phases of transcription (for current review, see (Phatnani and Greenleaf, 2006). The kinases that are responsible for the Ser2 or Ser5 phosphorylation states in yeast have been identified as Ctk1 and Kin28, respectively (Hengartner et al., 1998; Lee and Greenleaf, 1989). A crucial component of RNAPII transcription is the regulation of nucleosome positions along genes. As nucleosomes are known to be inhibitory for RNAPII association and activity along genes, the regulation of chromatin structure is a primary mechanism by which gene activity can be regulated (Butler, 1965; Grunstein, 1990; Huang and Bonner, 1962; Williamson and Felsenfeld, 1978; Workman and Kingston, 1998). This is demonstrated by the two general forms of chromatin, euchromatin and heterochromatin.

Heterochromatin and euchromatin

In general, eukaryotic chromatin can be classified according to two forms, known as heterochromatin and euchromatin. Heterochromatin consists of the most tightly packed form of chromatin, where access to the DNA is limited and transcription is generally repressed, in part due to the increased nucleosome density at those regions (Morse, 1993; Williamson and Felsenfeld, 1978; Workman and Kingston, 1998; Workman, 2006). Studies in *Drosophila melanogaster* ascribed the property of transcription repression to heterochromatin (Eissenberg et al., 1990; Hediger and Gasser, 2006; James and Elgin, 1986; Wallrath and Elgin, 1995). In contrast to heterochromatin, euchromatin consists of lightly packed nucleosomes and allows easier access of RNAPII to DNA, resulting in a region of active transcription. In most organisms, heterochromatin exists near telomeres and the chromosome centromeres. Mammalian heterochromatin occurs in a wide variety of regions, including large portions of the Y chromosome of males and the inactivated X chromosome in females (Heard and Disteche, 2006). In contrast, the active X chromosome in female mammals is characterized as largely euchromatin. A wide array of chromatin-associated proteins exist, some of which bind preferentially to a particular chromatin state (Mellor, 2006a). One example of this specificity is heterochromatin protein 1 (HP1) in humans, which is generally associated with heterochromatin or repressed genes in euchromatic regions (Hediger and Gasser, 2006; Hiragami and Festenstein, 2005). Budding yeast, like higher eukaryotes, contains euchromatic and heterochromatic-like regions. Unlike higher eukaryotes however, almost the entirety of budding yeast's genome exists as euchromatin. The regions in yeast most resembling heterochromatin are the silent mating type loci and sub-telomeric regions (Aparicio et al., 1991; Chien et al., 1993; Loo and Rine, 1994; Rusche et al., 2003; Thompson et al., 1993). Alteration of chromatin structure is known to occur, in part, through the binding of transcription factors to euchromatin regions which disrupts nucleosomes, thereby enhancing transcription initiation at these regions

(Morse, 1993; Workman, 2006). In addition, chromatin structure can be altered by the nucleosome remodeling family of proteins, such as Swi/Snf, which can reposition nucleosomes and overcome the repressive effects of chromatin (Cairns et al., 1994; Kwon et al., 1994; Lorch et al., 1999; Peterson et al., 1994). Given the variety of factors involved in the regulation of heterochromatin and euchromatin, what is the mechanism by which successive generations of a cell 'remember' which regions of the genome should be transcriptionally active or repressed? What proteins or molecular markers are involved in this process? These questions continue to be topics of intense study and, in part, will be discussed in this report.

1.3 Histone Post-translational Modifications

A thorough understanding of the histone proteins is key to elucidating the mechanisms of transcription regulation. While our knowledge in the field of chromatin structure and function has increased greatly since this work began, here we focus on our understanding of the histone post-translational modifications at the time our studies began. In subsequent chapters, our findings are discussed in the context of relevant works from other laboratories that were published concurrently. Finally, in Chapter 5, our most recent understanding of chromatin biology will be addressed.

The first high resolution crystal structures of the nucleosome firmly established that the histone proteins pack with DNA at a region termed the 'globular domain', while the smaller N- and C-terminal 'tail' regions of histones extend away from the core of the chromatin polymer (Arents et al., 1991; Luger et al., 1997). Specific residues along these 'tail' regions and certain sites within the globular domain are targets for various post-translational modifications such as acetylation, phosphorylation, ubiquitination, and methylation (van Holde, 1989; Wu and Grunstein, 2000; Berger, 2002). While most organisms have multiple enzymes capable of catalyzing a particular modification at a

specific site, lower eukaryotes typically possess only one enzyme specific for each modification site. Therefore, analysis of chromatin modifications in yeast provides for a more rapid analysis of the functions of an individual modification (Peterson and Laniel, 2004). Despite the existence of additional histone modifications in mammals as compared to budding yeast, the ease of genetic manipulation in yeast as well as our extensive knowledge of their genome provides for their role as an important model system. Our studies presented here were all performed using the budding yeast model system. Indeed, both human and yeast histone post-translational modifications are known to be contributors in the regulation of a variety of transcription processes, including alterations of chromatin structure, the induction or repression of transcription, and mRNA processing. Our work focuses on particular post-translational modifications of Histone H3 and their downstream functions. Diagrammed in **Figure 1.2** are the histone H3 modifications in yeast and related enzymes known to exist when we began the studies described in this report.

The histone code hypothesis

It was postulated by the Allis laboratory and others that histone post-translational modifications regulate chromatin function through a 'histone code' (Strahl and Allis, 2000; Turner, 2000). This hypothesis postulates that specific factors are recruited to a particular combination of histone modifications which then subsequently signal for various biological outcomes. This 'histone code' hypothesis continues to be tested intensively, but regardless of the extent of the code's complexity it is generally agreed to be a valid theory for a cellular transcriptional control mechanism (Jenuwein and Allis, 2001; Marmorstein, 2001; Zhang and Reinberg, 2001; Berger, 2002; Millar et al., 2004). Additional support for the histone code is manifested through the discovery of unique regulatory pathways for certain modifications such as the *trans*-histone regulatory pathway where ubiquitination of Histone H2B is required for H3 K4 and K79 (but not K36) methylation in yeast (Briggs et al., 2002; Ng et al., 2003a). A key facet of the code is the recognition of uniquely modified (or unmodified)

histones by certain regulatory proteins, that then signal for specific downstream processes. These proteins, when bound, can signal for repression or activation of transcription, replication origin firing, or proper repair of DNA damage, as described below. One example of this process includes the recruitment of HP1 to methylated Histone H3 lysine 9 in higher eukaryotes, subsequently catalyzing the formation of heterochromatin (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). The yeast heterochromatin protein Sir3 has been found to promote gene silencing in yeast when bound to H4 tails, but is inhibited by H4 acetylation at lysine 16 (Brachmann et al., 1995; Carmen et al., 2002; Rusche et al., 2003). Another example is the involvement of acetylated histone H4 in the timing of replication origin firing through the recruitment of replication machinery (Vogelauer et al., 2002). In addition, activation of transcription is signaled by histone acetylation through the recruitment of various transcription factors (Marmorstein and Roth, 2001; Wang et al., 2002). In yeast, phosphorylation of serine 10 was found to be required for lysine 14 acetylation, further demonstrating functional cross-talk between separate modifications (Lo et al., 2000). Experiments focused on histone phosphorylation also support the histone code, as phosphorylation of histone H3 at serine 10 is correlated with transcriptional activation during the heat-shock response in *Drosophila*, and is likely involved with transcriptional activity in other species (Bjorklund et al., 1999; Nowak and Corces, 2000). However, the mechanism for phosphorylation in transcriptional activation was not carefully investigated until later (Nowak and Corces, 2004). The most recent data available, as discussed in Chapter 5, suggests that the 'histone code' consists of a complex cross-communication between nucleosomal structure, DNA sequence, and histone modifications that coordinate to regulate transcription.

In addition to observations of various histone modifications and their influence on biological processes, clues to the mechanisms of these processes were uncovered through the identification of binding domains responsible for recognizing particular histone

modifications. The bromodomain was found to have specificity for binding to acetylated lysine residues (Dhalluin et al., 1999), while the chromodomain (present in HP1) was found to prefer methylated lysine residues (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). Consistent with the theory of the histone code, it was predicted that these and other domains would be identified in new proteins, and would be responsible for recognizing other sites of histone modification that ultimately signal for unique downstream processes (Marmorstein and Berger, 2001; Marmorstein, 2001). As described further in Chapter 5, recent results have supported these predictions.

Implications in Disease

Given the role of histone modifications in normal cellular processes, are there obvious phenotypes when the enzymes that catalyze these modifications are mis-localized or their functions are otherwise disrupted? It is now known that the improper regulation of chromatin modifying factors has consequences for human cancers, possibly due to the contribution of chromatin structural changes in gene regulation. The evidence linking cancer to the enzymes responsible for histone modifications is highlighted in several prominent reviews (Jacobson and Pillus, 1999; Hanahan and Weinberg, 2000; Johnstone, 2002; Schneider et al., 2002; Yang, 2004). In addition to a role for histone modifications in cancer, truncations of the catalytic histone methylase domain in the human Set2 homologue, Nsd1, are linked to Sotos syndrome (Kurotaki et al., 2002). Another putative Set2 homologue in humans, Nsd2, is deleted in Wolf-Hirschhorn syndrome (Stec et al., 1998). The human SET domain-containing protein Ezh2, in addition to being upregulated in various tumor cell lines, interacts with a protein known to be mutated in various inherited disorders including ATR-X syndrome (Cardoso et al., 1998). Interestingly, deletions of any individual histone modifying enzyme do not have an obvious phenotype in yeast. However, it is predicted that this is due to redundant or backup functions of each modification state, preventing a modification's function from being elucidated through cursory observation (Adams and Kamakaka, 1999;

Wittschieben et al., 2000; Zhang and Reinberg, 2001; Morillon et al., 2005; Zhang et al., 1998). Therefore, a more thorough investigation into the roles of particular yeast modifications has been the topic of study for many laboratories. In Chapter 2 we present data from two studies which investigate the function of histone methylation at H3 lysine 36.

1.4 Histone methylation

Histone methylation is a post-translational modification of particular interest that is found on lysine and arginine residues (Wu and Grunstein, 2000; Zhang and Reinberg, 2001; Khan and Hampsey, 2002). At the time our studies began in 2002, lysine methylation had been identified in higher eukaryotes at positions 4, 9, 27, 36, 79 of H3 and 20 of H4, although methylation in budding yeast is only known to exist at positions 4, 36, and 79 of H3. Arginine residues can be mono- or di-methylated, while lysine residues can be mono-, di-, or tri-methylated. Interestingly, studies reveal that each of these modification states may be responsible for unique biological functions, adding another layer of complexity to the histone code (Santos-Rosa et al., 2002; Zhang and Reinberg, 2001). The recent flurry of discoveries into the roles of histone methylation is largely due to the identification of the specific histone methyltransferase enzymes which target the particular sites along histones, as well as the development of tools to study these sites (Kouzarides, 2002; Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001).

Although the existence of histone methylation has been known for some time, only recently has its biological significance emerged. The regulation of chromatin structure is a key component of transcriptional regulation and histone modifications are known to play a pivotal role in this process. Histone lysine methylation is no exception, as highlighted in a number of studies published over the past six years that implicated this modification in the regulation of gene expression (Hampsey and Reinberg, 2003; Kouzarides, 2002; Lachner and Jenuwein, 2002; Rea et al., 2000; Rice and Allis, 2001; Stallcup, 2001), and when mis-

regulated, as a contributor to human diseases such as mixed lineage leukemia (MLL) (Chadwick et al., 2000; Cui et al., 1998; Hanahan and Weinberg, 2000; Huang, 2002; Jacobson and Pillus, 1999; Kurotaki et al., 2002; Peters et al., 2001; Schneider et al., 2002).

In addition to a general role in gene regulation, multiple lines of evidence indicated that methylation can negatively or positively regulate transcription, depending on the site of methylation. Tri-methylation of H3 K4 was shown to correlate with actively transcribing genes (Santos-Rosa et al., 2002), while methylation of H3 K9 in most eukaryotes is correlated with repressed regions of the genome (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). Consistent with the histone code hypothesis, it is predicted that each histone modification can result in the preferential binding (or dissociation) of a regulatory protein that ultimately effects a particular change in gene expression. This pattern has been demonstrated at sites of acetylation, as discussed earlier, yet the binding of HP1 to methylated H3 K9 was the first example of this pattern to be demonstrated at a site of lysine methylation. Do similar mechanisms of methyl-lysine and matching binding protein exist for all sites of methylation? The biochemical mechanisms by which lysine methylation contributes to specific biological consequences is the focus of the experiments presented in Chapter 2 as well as a number of other more recent studies discussed in Chapters 3 and 5.

Although a relatively frequent turnover of histone acetylation (via de-acetylation) occurs (Kurdistani and Grunstein, 2003), initial data suggested that histone methylation is quite stable (Byvoet et al., 1972; Duerre and Lee, 1974; Goll and Bestor, 2002). This observation appears inconsistent with a potential role of methylation in signaling for the positive or negative regulation of transcription, as introduced above. How could an non-removable modification effectively function as a signal along chromatin? A widely considered theory is that of ‘cellular memory’, whereby long-lasting modifications can serve as effective markers for transcription (Turner, 2002). Certain transcription studies also

revealed the phenomenon of histone replacement whereby a cell 'removes' methylation through the exchange of a modified with an unmodified histone (Goll and Bestor, 2002; Briggs et al., 2001; Ahmad and Henikoff, 2002). The most recent studies in the field of histone methylation have revealed a set of enzymes responsible for de-methylation, which will be discussed in Chapter 5, in the context of recent work published since our studies.

Histone lysine methyltransferases in yeast

The focus of our work is histone lysine methylation in budding yeast, and in particular methylation at lysine 36. Three histone methyltransferases (HMTs) have been identified in yeast that are responsible for methylating lysines 4, 36 and 79 of H3. Two of these enzymes, Set1 and Set2, contain a domain first identified in the *Drosophila* proteins Su(var)3-9, Enhancer of zeste, and Trithorax. This region, known as the SET domain, was found capable of regulating gene activities (Tschiersch et al., 1994; Zhang and Reinberg, 2001; Jones and Gelbart, 1993). Structural examples of the SET domain solidified our understanding of the mechanism by which it catalyzes methyl group transfer from *S*-adenosylmethionine (SAM) to a histone substrate (Zhang et al., 2002; Zhang et al., 2003). The SET domain containing HMTs Set1 and Set2 are the sole enzymes in yeast responsible for methylating lysine 4 and 36, respectively (Briggs et al., 2001; Strahl et al., 2002; Zhang et al., 2002; Zhang et al., 2003). A third HMT, Dot1, is specific for methylase activity for lysine 79 but does so through a catalytic domain unique from the SET domain (Dlakic, 2001; Feng et al., 2002; Ng et al., 2002; San Segundo and Roeder, 2000; Singer et al., 1998). Set1 and Set2 share regions of homology adjacent to the SET domain, known as the pre-SET and post-SET domains (Nagy et al., 2002; Rea et al., 2000; Zhang and Reinberg, 2001). These domains contain several invariant cysteines, through which the enzymes recognize their particular substrates and catalyze methyl transfer. While the post-SET domains of Set1 and Set2 are nearly identical, the pre-SET domains are more variable. Structural data of several human SET domains suggests that the post-SET domain, via its

invariant cysteines, forms a zinc cluster that is important in catalysis. In contrast to the post-SET domain, the pre-SET domain also forms a zinc cluster, but is implicated in the substrate recognition (Xiao et al., 2003a; Zhang et al., 2002; Zhang et al., 2003). The pre-SET domain in Set2 is also termed the AWS domain, for Associated With SET, to indicate its distinction from the Set1 post-SET domain and the preference to recognize H3K36 as its substrate. Despite the similarities in the domain architecture between Set1 and Set2, these enzymes are regulated using very diverse mechanisms.

Set1 functions in a complex of seven proteins known as COMPASS (Briggs et al., 2001; Krogan et al., 2002a; Miller et al., 2001; Nagy et al., 2002; Roguev et al., 2001). Although Set1 contains the catalytic SET domain responsible for methyl transfer to H3 lysine 4, deletion of any other COMPASS members results in a moderate to complete abolition of K4 methylation *in vivo* (Miller et al., 2001; Nagy et al., 2002; Roguev et al., 2001). Despite the recent discovery of Set1, significant inroads toward an understanding of COMPASS regulation and downstream function have been completed by several laboratories. These discoveries involve an interesting requirement of ubiquitination on histone H2B for subsequent H3 K4 methylation. This *trans*-histone regulatory pathway involves the mono-ubiquitination of H2B at lysine 123 which is required for subsequent H3 K4 and K79 methylation (**Fig. 1.2**; Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002). This discovery is another example of the cross-talk that exists between separate histone modifications which is possible even across separate histones.

It is interesting to note that the H2B *trans*-tail pathway does not influence H3 K36 methylation, suggesting that another mechanism may exist for the regulation of Set2-mediated K36 methylation. In contrast to Set1, no evidence exists that Set2 functions within a complex. Aside from the discovery of Set2 as the yeast HMT specific for lysine 36 methylation, little data was available regarding the function of Set2 and K36 methylation (Strahl et al., 2002). The unanswered questions regarding this newly identified enzyme led

us to begin detailed studies of its function and regulation. In order to better appreciate the significance of our studies, we will first explore data regarding Set2's function *in vitro* and a more detailed background of Set2's domain architecture.

1.5 Set2 is the H3 lysine 36 methylase in yeast

With the identification of Set2 as the HMT responsible for K36 methylation in yeast, combined with observations that K36 methylation is a modification conserved from yeast to humans, it was anticipated that Set2 would influence gene regulation as do many of the other histone-modifying enzymes (Kouzarides, 2002; Lachner and Jenuwein, 2002; Sims, III et al., 2003; Strahl et al., 2002; Zhang and Reinberg, 2001). Our investigation into Set2's function began with observations of its domain structure. As diagrammed in **Figure 1.3**, Set2 contains several confirmed functional domains: the catalytic SET domain and the cysteine rich regions described earlier. The AWS domain is the version of a pre-SET domain, likely unique to Set2 and its homologues based on its substrate specificity (Nagy et al., 2002; Rea et al., 2000; Zhang et al., 2003; Zhang and Reinberg, 2001).

Although Set2 has been identified as a histone methyltransferase, little is known about this protein's biological function, the function of the methyl modification it catalyzes, or the mechanisms of these activities. Several lines of evidence provided leads for subsequent investigation of these unknown areas, including sequence homology comparisons which revealed that Set2 contains a putative WW domain and coiled-coil motif as illustrated in **Figure 1.3** (Strahl et al., 2002). Coiled-coil motifs consist of α-helices that are often involved in protein-protein interactions. Interestingly, several reports demonstrated that yeast WW domains bind to the phosphorylated CTD of RNAPII (Morris et al., 1999; Morris and Greenleaf, 2000). Could Set2 interact with RNAPII via its WW domain and therefore target Set2 to histone H3? Whether these regions within Set2 were *bona fide* functional domains and contributed to Set2's function was unclear. Initial experiments have

demonstrated that Set2 functions as a repressor of transcription *in vitro*, suggesting K36 methylation could function in a manner analogous (but opposite) to K4 methylation (Strahl et al., 2002; Lutfiyya et al., 1995). Results indicating that Set2 acted as a transcriptional repressor remained unconfirmed *in vivo*. These early lines of evidence laid the groundwork for hypotheses we have tested regarding Set2 and its downstream function.

1.6 Unanswered questions regarding Set2

The complexity of the histone code can seem endless. Even in the yeast model system, much remains to be investigated regarding each histone modification, its regulation, and downstream functions. The fast-paced field of chromatin research requires continual revisions of models for the regulation and function of a histone modification. In the following section I will present some of the unresolved questions regarding Set2 and H3 K36 methylation in yeast that are addressed in subsequent chapters.

Set2 domain structure and the function of K36 methylation

Deletion of *SET2* results in a complete loss of global K36me2 levels as observed by immunoblot analysis (Strahl et al., 2002). It was unknown, however, whether Set2 locates its H3 substrate by simple diffusion, or if it is targeted specifically to chromatin. Do the putative WW and coiled-coil regions in Set2 have functional relevance? Since there is no evidence that Set2 functions in a complex as does Set1, could the WW or coiled-coil regions be responsible for Set2 interaction with an as-yet-unidentified binding partner? Based on the observation that certain WW domain-containing proteins bind to the phosphorylated RNAPII-CTD, does Set2 bind to the CTD as well? What are the downstream functions of Set2-catalyzed methylation? Will Set2, like Set1, be found to contribute to transcriptional regulation? This would seem to be likely, as *SET2* was initially identified as a repressor of the *GAL4* promoter (Lutfiyya et al., 1995), which was later supported by additional evidence that Set2 can act as a repressor via its methylase activity in a plasmid-based transcription

repression assay (Strahl et al., 2002). These early data did not provide clear evidence as to whether Set2 functions to regulate transcription via regulated targeting to specific genes, nor the means by which K36 methylation could accomplish this. These represent the unresolved issues which we began to investigate, the results of which are presented in Chapter 2. Additional studies from our laboratory and others further dissected the downstream roles of this modification, and will be discussed in the context of our work, in Chapter 5.

Regulation of Set2 activity on chromatin

Set2 has been identified as a histone methyltransferase capable of *in vitro* methylation of nucleosomal substrates, but not free histones (Strahl et al., 2002). Does the fact that Set2 prefers nucleosomal substrates *in vitro* have biological relevance *in vivo*? If K36 methylation is responsible for specific biological effects, could the level of K36 methylation be regulated *in vivo*? What are the upstream factors, if any, that are responsible for regulating Set2 activity? Based on new data from our studies and others, presented in Chapter 2, we studied specific transcription factors for a potential influence on the regulation of Set2 activity and describe these results in Chapter 3.

Figure 1.1: Illustration of chromatin fiber condensation.

Shown are the steps involved in the folding of extended nucleosomal arrays into maximally folded chromatin fibers. Reprinted, with permission, from the *Annual Review of Biophysics and Biomolecular Structure*, Volume 31 (c) 2002 by Annual Reviews (www.annualreviews.org).

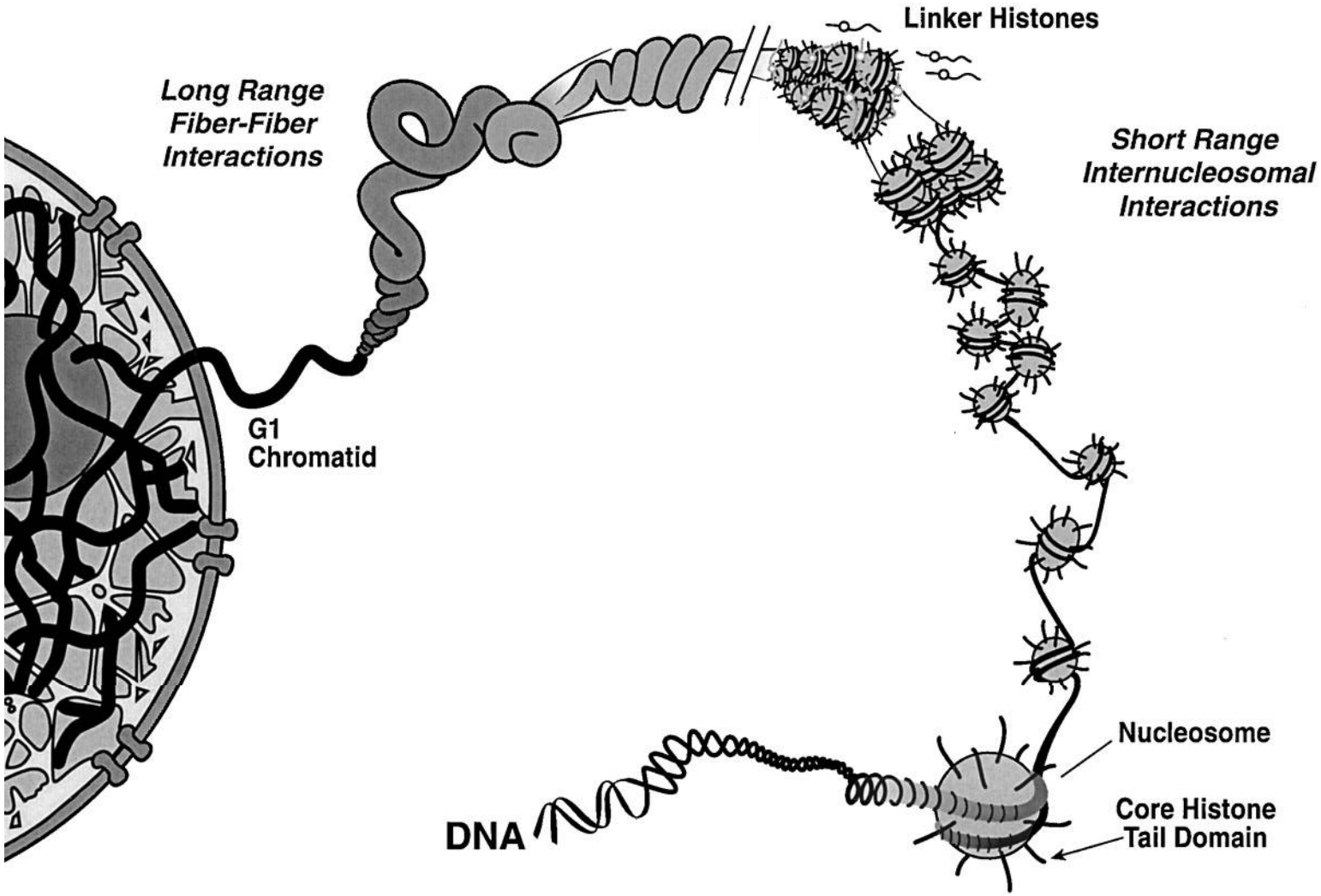
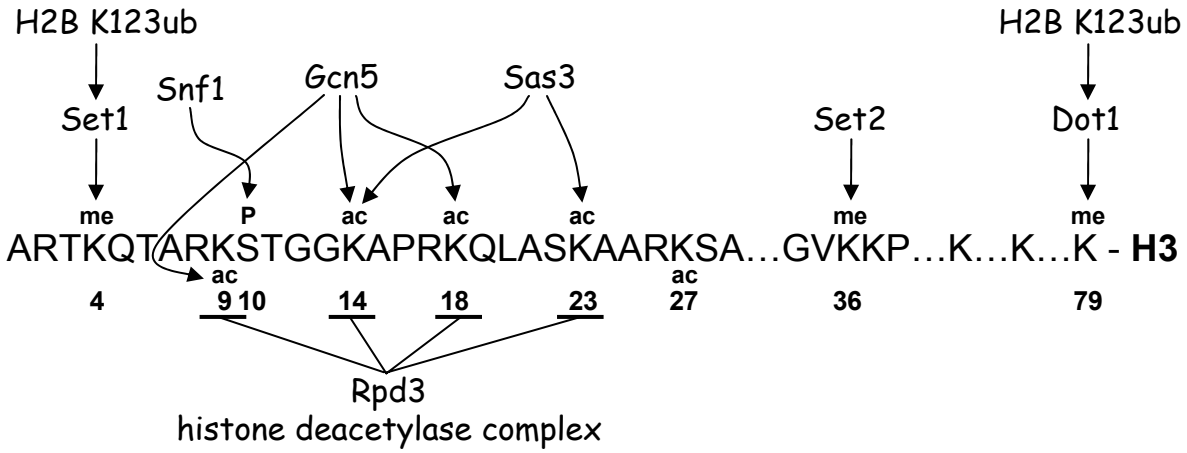

Linker Histones
Long Range
Fiber-Fiber
Interactions
Short Range
Internucleosomal
Interactions
G1
Chromatid
Nucleosome
DNA
Core Histone
Tail Domain

Figure 1.2: Yeast histone H3 post-translational modifications and related enzymes.

(**A**) The histone H3 post-translational modifications known to occur in yeast at the beginning of this study (2002) are indicated, along with the enzymes that catalyze or remove these modifications. (**B**) Indicated here are protiens known to bind to H3 residues in a modification-dependent manner. A current listing of yeast H3 modifications will be presented in Chapter 5. Reprinted and adapted from *TRENDS in Genetics*, Volume 22, Mellor, J., Dynamic nucleosomes and gene transcription, 320-329, Copyright 2006, with permission from Elsevier (Mellor, 2006).

A

Modification catalysis



B

Modification recognition

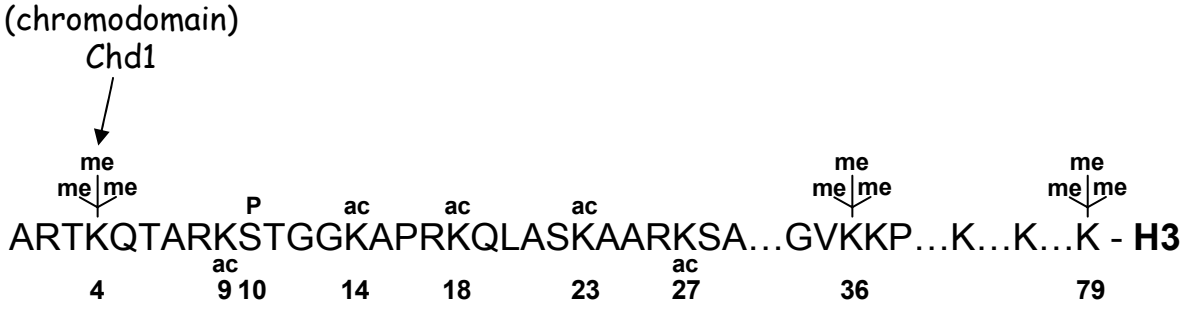


Figure 1.3: Schematic representation of the domain structure of Set2.

The N-terminus of Set2 contains the SET domain, which is flanked by cysteine rich region noted as the post-SET (PS) and AWS domains (AWS is predicted to be present in putative Set2 homologues only). The C-terminus of Set2 contains a putative proline-binding WW domain and a coiled-coil motif, expected to be functionally relevant in protein-protein interactions involving Set2.


Set2
120-243
AWS
SET
PS
476 - 507
WW
548 - 618
CC
733

CHAPTER 2

A NOVEL DOMAIN IN SET2 IS REQUIRED FOR INTERACTION WITH PHOSPHORYLATED RNA POLYMERASE II AND LINKS HISTONE H3 K36 METHYLATION WITH TRANSCRIPTION

Components of this chapter were published as:

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Kizer K.O., Phatnani H.P., Shibata Y., Hall H., Greenleaf A.L., Strahl B.D. (2005) A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Mol. Cell Biol.,* 25, 3305-3316.

Summary

Genetic and biochemical evidence has linked histone methylation to the regulation of chromatin structure and gene transcription. The recently discovered histone H3 methylase Set2 was found to be the sole enzyme in yeast responsible for H3 lysine 36 (K36) methylation. Although earlier work suggested a role for Set2 and K36 methylation in transcriptional repression, the role of K36 methylation in yeast remained largely unexplored. Through our collaborative work, we identified an association between the phosphorylated C-terminal domain (CTD) of RNA polymerase II (RNAPII) and a region of Set2, implying that this enzyme has an important role in the transcription elongation process. Here we show that a novel domain in the C-terminus of Set2 is responsible for interaction between Set2 and RNAPII. This domain, termed the Set2 Rpb1 Interacting (SRI) domain, is encompassed by amino acid residues 619 to 718 in Set2 and is found to occur in a number of putative Set2 homologues from fission yeast to humans. Unexpectedly, BIACORE analysis reveals that the SRI domain binds specifically, and with high affinity, to CTD repeats that are doubly modified (serine 2 and serine 5 phosphorylated), indicating that Set2 association across the body of genes requires a specific pattern of phosphorylated RNAPII. Deletion of the SRI domain not only abolishes Set2-RNAPII interaction, but also abolishes K36 methylation *in vivo*, indicating that this interaction is required for establishing K36 methylation on chromatin. Using 6-azauracil (6AU) as an indicator of transcription elongation defects, we found that deletion of the SRI domain confers a strong resistance to this compound, which was identical to that observed with *set2* deletion mutants. Furthermore, yeast strains carrying *set2* alleles that are catalytically inactive or yeast strains bearing point mutations at K36, were also found to be resistant to 6AU. These data suggest that it is the methylation by Set2 that affects transcription elongation. In agreement with this, we have determined that deletion of *SET2*, its SRI domain, or amino acid substitutions at K36 result in an alteration of RNAPII occupancy levels over transcribing genes. Taken together, these data

indicate K36 methylation, established by the SRI domain-mediated association of Set2 with RNAPII, plays an important role in the transcription elongation process.

2.1 Background

Successful synthesis of mRNA by RNA polymerase II (RNAPII) requires tight regulation of the initiation, elongation, and termination processes of transcription. The process of transcription elongation is affected in part by the binding of regulatory factors to the phosphorylated C-terminal domain (CTD) of the RNAPII subunit Rpb1. Recent studies have highlighted an important role for histone methylation in the process of transcription. Earlier studies of methylation in yeast revealed an unexpected *trans*-histone regulatory pathway where H2B ubiquitination is required for subsequent H3 K4 and K79 methylation catalyzed by Set1 and Dot1, respectively. Interestingly, H3 K36 methylation is not regulated by this pathway, suggesting an alternate mechanism may exist to signal for the catalysis of this modification. Our early investigations involving the regulation of Set2 revealed that this H3 K36 methylase associates with RNAPII *in vivo* (Xiao et al., 2003b). Other laboratories independently observed similar results, which shed light on a possible mechanism for Set2 localization on genes and a role in transcription (Krogan et al., 2003b; Landry et al., 2003; Li et al., 2003; Li et al., 2002; Schaft et al., 2003). Furthermore, these studies revealed that Set1 and Set2 are associated with RNAPII at different stages of the transcription elongation cycle. While Set1 associates with RNAPII via the Paf1 transcription elongation complex in a manner that is dependent on the serine 5 (Ser5) phosphorylated CTD, Set2 is recruited to RNAPII in a manner that is dependent on the CTD and the Ctk1 kinase (CTDK-I) that effects serine 2 CTD phosphorylation (Gerber and Shilatifard, 2003; Hampsey and Reinberg, 2003; Krogan et al., 2003a; Krogan et al., 2003b; Li et al., 2003; Ng et al., 2003b; Xiao et al., 2003b). Importantly, while studies show that Set1 preferentially associates with the 5' end of genes, Set2 is found throughout the coding regions of genes (Krogan et al., 2003b; Ng et al., 2003b; Schaft et al., 2003; Xiao et al., 2003b). These observations imply that K4 and K36 methylation have differing roles in the transcription elongation process.

Several lines of evidence indicate that Set2 is a phospho-CTD binding protein (Gerber and Shilatifard, 2003; Hampsey and Reinberg, 2003; Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Ng et al., 2003b; Xiao et al., 2003b). For example, truncations of the RNAPII CTD severely reduce global K36 methylation levels *in vivo* (Li et al., 2003; Xiao et al., 2003b). In addition, deletion of CTDK-I results in a total abolition of K36 methylation (Krogan et al., 2003b; Xiao et al., 2003b). These data have led to the model that Set2 preferentially binds to RNAPII that is phosphorylated at the Ser2 position of the CTD, which is supported by findings that Set2 binds to Ser2 phosphorylated CTD repeats *in vitro* (Li et al., 2002; Li et al., 2003). Thus, a function for the CTDK-I generated phospho-CTD in either the recruitment of Set2 and/or the control of K36 methylation activity has been proposed (Shilatifard, 2004; Sims, III et al., 2004).

While the association of Set2 with RNAPII is well established through work from our laboratory and others, the region in Set2 required for this interaction, and the functional significance of this interaction on K36 methylation and transcription elongation remained poorly defined. In this chapter, we present components of two studies which identified the Set2 region responsible for RNAPII interaction. Importantly, work from our first study as presented in this chapter, revealed that the C-terminus of Set2 is responsible for RNAPII interaction (Xiao et al., 2003b). This unexpected result is contradictory to an earlier report (Li et al., 2002), but was first reconciled through studies in our laboratory. This unusual result is then more carefully addressed and finally reconciled through results from our second study, presented here, where we identified the precise boundaries of a novel domain in Set2 that mediates RNAPII interaction (Kizer et al., 2005). We show that deletion of this Set2-Rpb1 interaction (SRI) domain abolishes K36 methylation on chromatin *in vivo* and leads to a transcription elongation defect, as assayed by 6-azauracil (6AU). In support of a direct role for Set2 methylation in the transcription elongation process, we find that *set2* mutants or H3 K36 point mutations that prevent K36 methylation result in 6AU phenotypes

similar to those of a complete *SET2* deletion, which we in turn correlate with altered distribution of RNAPII along genes. These results define a novel domain in Set2 responsible for functional interaction with RNAPII and strongly suggest that the K36 methylation mediated by Set2 influences transcription elongation.

2.2 Materials and Methods

Yeast strains

The p3Flag-KanMX plasmid was used as a PCR template for genomic tagging of Set2 (Gelbart et al., 2001). This provided for the generation of either full-length Set2 (Set2-3Flag) or a form deleted of the SRI domain ($Set2_{(1-618)}$-3Flag) by homologous recombination. Primers used to generate Set2-3Flag in the BY4742 background were constructed previously (Xiao et al., 2003b). The primers for $Set2_{(1-618)}$-3Flag were: 5'-CAAAAGGAAGAGTCCAAAAAACTAGTGGAAGCAAAAGAGGCTAAGCGGTTGAAAAGGGAACAAAAGCTGGAG-3' (forward) and 5'-AAAGAATTTATTCCAGTTGTGCTCTAGTCTTTGGGACTGGGAGACCGTTTTTCTTTACTATAGGGCGAATTGGGT-3' (reverse). Bases which anneal to the p3Flag-KanMX plasmid are underlined, while the remaining sequence corresponds to the *SET2* locus insertion position. The *set2Δ* and wild-type strains of the BY4742 background were obtained from Research Genetics, while the YCB652 strain, carrying an integrated *URA3* gene, was obtained from Dr. Jeff Smith, University of Virginia School of Medicine (Smith et al., 2000). The *SET2* gene was deleted in the W303 and YCB652 backgrounds using a PCR product amplified from genomic DNA obtained from the BY4742 *set2Δ* strain, in which the *SET2* gene had already been replaced by the *KanMX* gene (Research Genetics).

The H3/H4 shuffle strain WZY42 (in the S288C background) was used in 6AU analyses of H3 point mutants, and replacement of wild-type H3 with H3 mutants was accomplished as described (Briggs et al., 2001; Zhang et al., 1998). Plasmids coding for

the H3 S10A and K4R mutants have been described previously (Briggs et al., 2001; Hsu et al., 2000). All other H3 point mutations were prepared by standard PCR-based site-directed mutagenesis using materials and methods previously described (Briggs et al., 2001; Zhang et al., 1998).

Yeast WCE and nuclei preparation

Yeast WCEs were prepared as described previously, but differed in the extraction buffer (Xiao et al., 2003b). The extraction buffer used consisted of 50 mM Tris-HCl at pH 8.0, 300 mM NaCl, 1 mM $Mg(C_2H_3O_2)_2$, 1 mM imidazole, 0.1 % NP40, 0.5 mM EDTA and 10 % glycerol. In addition, this buffer contained 0.5 % phosphatase inhibitor cocktail I (Sigma), PMSF (2 mM), and Leupeptin/Pepstatin/Aprotinin mix each at 2 μg/ml. Nuclear extracts were prepared as previously described from strains grown in 200 ml of YPD medium to an OD_{600} of 1.5 (Edmondson et al., 1996).

Electrophoresis and immunoblotting

Western blotting and SDS-PAGE analyses were performed according to procedures and reagents obtained from Amersham Life Sciences. The ECL Plus Western Blotting Detection Kit (Amersham Pharmacia Biotech) was used for specific antibody detection. The rabbit anti-Me_2(K36) antibody was obtained from Upstate Biotechnology Inc. and used at a dilution of 1:3000. The rabbit anti-Me_3(K36) antibody was obtained from Abcam Inc. (AB9050) and used at a dilution of 1:2500. The antibody targeted against the C-terminus of H3 was obtained from Abcam Inc. (AB1791) and used at a dilution of 1:5000. The anti-phospho-CTD antibodies H5 and H14 were obtained from Covance Inc.

Generation of SET2 expression constructs

The Set2 constructs containing a C-terminal Flag epitope [$Set2_{(1-618)}$, $Set2_{(262-475)}$, $Set2_{(445-538)}$, $Set2_{(528-638)}$, $Set2_{(619-733)}$, $Set2_{(634-733)}$, $Set2_{(619-718)}$, and $Set2_{(619-703)}$] were generated by PCR amplification using Vent DNA polymerase (New England Biolabs) and the Set2-Flag PN823 expression construct as the template. Full-length Set2, $Set2_{(1-261)}$, and

Set2^{R195G} constructs were prepared previously (Strahl et al., 2002; Xiao et al., 2003b). The PCR products were cloned into the PN823 yeast expression plasmid and sequenced for accuracy. Primer sequences are available upon request. For *in vitro* phospho-CTD binding experiments, the Set2$_{(1-618)}$ and Set2$_{(619-733)}$ constructs were subcloned into the pMAL-c2G vector (New England Biolabs) and proteins were purified according to the manufacturer's protocol.

Immunoprecipitations

Co-IP experiments involving the various mutant Set2-Flag constructs or Set2-3Flag strains were performed essentially as previously described (Xiao et al., 2003b). In brief, a *set2Δ* strain (in the BY4742 background) was transformed with the indicated series of Set2 expression constructs, grown to an OD_{600} of ~1.0 in synthetic complete media lacking uracil, and WCEs were prepared using the described extraction buffer above. Co-IPs were performed in a final volume of 0.9 ml, equalized with extraction buffer, containing 1.5 mg of WCE protein (or 2.0 mg for genomically-tagged strains). The extracts were incubated with 12.5 μl of pre-equilibrated αFlag affinity beads (M2; Sigma) for 2 h at 4°C, after which extracts were washed 3x2min in extraction buffer. The beads were eluted in SDS-PAGE loading buffer with incubation at 100°C for 5 min, and bead-bound proteins analyzed by immunoblot analysis using antibodies targeted against the phospho-CTD.

ChIP assays

The chromatin immunoprecipitation assay using the H3 K36 di-methyl antibody was performed as described previously (Xiao et al., 2003b). Primers were used to amplify regions of *SCC2* in the following ranges relative to the ATG start site: -277, -27; 2, 238; 984, 1222; 3044, 3276; 3981, 4222; 4489, 4679. Intergenic chromosome V primers were used as a reference and loading control, as previously reported (Komarnitsky et al., 2000). The previously characterized general RNAPII-CTD antibody (not specific to any CTD modification state) was used in ChIP assays for RNAPII detection (Schroeder et al., 2000).

Set2-3Flag and Set2$_{(1-618)}$-3Flag purification

Set2-3Flag and Set2$_{(1-618)}$-3Flag proteins were purified as previously described (Xiao et al., 2003b). A Coomassie-stained gel was used to visualize the associated proteins and mass spectrometry analysis confirmed the presence or absence of Rpb1 and Rpb2 from excised gel slices.

In vitro HMT assays

The *E. coli* strain BL21 was transformed with pMAL vectors expressing the constructs MBP-Set2 and MPB-Set2$_{(1-618)}$, which also contained the C-terminally tagged Flag epitope. Protein expression was induced in 100 μM IPTG for 3 h at 30°C, cells were lysed by sonication and 20 μl HMT reactions were prepared as described previously (Strahl et al., 2002). In brief, lysate volumes were used that resulted in equal amounts of each MBP-Set2 fusion per reaction, as analyzed by Western blotting with the Flag antibody. HMT reactions contained 1 μCi of ^{3}H-SAM (Amersham Biosciences), with or without 6 μg of chicken nucleosomes. Reactions were incubated at 30°C for 20 min and spotted on Whatman P-81 for liquid scintillation counting, or analyzed by SDS-PAGE followed by fluorography.

Far-Western analysis of Set2 fragments using phospho-CTD probes

Far-Western analysis using a phospho-CTD probe (generated by CTDK-I) was performed essentially as described previously (Morris et al., 1997; Morris et al., 1999). In brief, picomole quantities of recombinant MBP-Set2$_{(1-618)}$ and MBP-Set2$_{(619-733)}$ were resolved on a 4-15% Tris-HCl Criterion Gel (Bio-Rad), and transferred to nitrocellulose (Hybond C Extra, Amersham Pharmacia Biotech). The nitrocellulose was stained with Ponceau S to visually ensure protein transfer from the gel. The membrane was blocked at 4°C for 24 h and probed with 2.5 μg of GST-^{32}PCTD in blocking buffer for 3.5 h at 4°C. The membrane was washed, air dried, and exposed to film. Reverse Far-Western analysis was performed according to published methods by resolving recombinant unphosphorylated or phosphorylated GST-CTD fusions on a 4-15% Tris-HCl gel (Phatnani et al., 2004). The gel

was transferred to a nitrocellulose membrane and probed with MBP-Set2 or MBP-Set2$_{(619-733)}$, followed by detection using an antibody against MBP.

Determination of SRI-CTD affinities using BIACORE

The BIACORE sensor chip carrying 3-repeat CTD peptides (2-phospho, 5-phospho, 2+5 phospho, 6PC scrambled control) was generated as previously recorded (Jones et al., 2004; Phatnani et al., 2004). Purified MBP-Set2$_{(619-733)}$ was interacted with the peptides and association and dissociation monitored. The response curves were normalized to that for the 6PC control peptide.

RT-PCR

Reverse transcription (RT)-PCR analysis was performed as previously described (Xiao et al., 2003b; Xiao et al., 2005). Primer sequences are available upon request.

6AU growth assays

Yeast strains used in this assay, except for YCB652, were transformed with the URA3$^+$ CEN plasmid pRS316 and grown in synthetic defined media lacking uracil (SD-Ura). Overnight cultures were diluted 1:20, grown to an OD_{600} of 1.0, and ten-fold serial dilutions were plated on SD-Ura media with or without 6-azauracil (Aldrich) or mycophenolic acid (Sigma), each at 100 μg/ml. Plates were photographed after 30°C incubation for 2-3 days. Liquid cultures used for RT-PCR analysis were grown with 6AU at 50 μg/ml for 120 min. This time point was selected based on a recent study in which the *IMD2* steady-state mRNA levels of a large-scale 6AU screening of yeast deletion mutants were analyzed (Riles et al., 2004).

2.3 Results

A novel C-terminal domain in Set2 mediates RNAPII interaction.

The initial observations made from our laboratory and others of the RNAPII-Set2 association did not reveal, however, which region(s) of Set2 are required for this interaction.

Using a series of Set2 expression constructs containing a fused C-terminal Flag epitope, we examined various forms of Set2 in a co-immunoprecipitation (Co-IP) study with antibodies generated against RNAPII to determine the region of Set2 responsible for CTD interaction. Either full-length *SET2*, vector only control, or the indicated *SET2* mutant was expressed in a *set2* deletion mutant (*set2Δ*) and whole cell extracts (WCEs) were prepared. We found that a C-terminal portion of Set2 was capable of interacting with RNAPII (**Fig. 2.1**), which formed a component of the collaborative work from our laboratory (Xiao et al., 2003b). In this study, and those from other laboratories, the association between Set2 and the phosphorylated CTD was well established (Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Schaft et al., 2003).

Since our initial analyses demonstrated that specific deletion of the WW domain in Set2 does not disrupt Set2-RNAPII co-precipitation, we hypothesized that the coiled-coil region of Set2 may be responsible (Xiao et al., 2003b). Thus, we generated another series of Set2 mutant yeast expression constructs with a C-terminal Flag epitope tag (**Fig. 2.2A**) and used them in a Co-IP experiment with antibodies generated against RNAPII as in Fig. 2.1. Either full-length *SET2*, vector only control, or the indicated *SET2* mutant was expressed in a *set2* deletion mutant (*set2Δ*) and whole cell extracts (WCEs) were prepared. As expected, immunoprecipitation of full-length Set2-Flag resulted in co-precipitation of RNAPII as detected by immunoblotting with anti-phospho-CTD antibodies (**Fig. 2.2B**). As shown in Fig. **2.2B**, we found through further Set2 truncations that a region at the C-terminus of Set2, encompassing amino acid residues 619-733, is both necessary and sufficient to mediate the interaction of Set2 with RNAPII (compare last two lanes). We therefore termed this region the Set2 Rpb1 Interacting (SRI) domain.

We next sought to determine the precise boundaries of the SRI domain. To accomplish this, we generated additional Set2 constructs containing N- and C-terminal truncations of the SRI region (**Fig. 2.2C**) and used them in co-IP analyses as before (**Fig.**

2.2B). Results revealed that N-terminal truncation of the SRI domain beyond Set2 amino acids 619 abolished RNAPII binding. However, binding was still possible with a C-terminal truncation up to amino acid 718 of Set2 (**Fig. 2.2D**), thereby identifying the boundaries of the SRI domain as amino acids 619-718.

Set2 also contains a putative coiled-coil motif, a region found in other proteins to be a mediator of protein-protein interactions (**Xiao 2003**). As with the WW domain, we tested the coiled-coil motif of Set2 for a potential role in mediating the Set2-RNAPII interaction. To test this possibility, we generated a series of constructs that expressed forms of Set2 which were deleted of precise regions within the coiled-coil motif (**Fig. 2.3A**). As we found in the case of a WW domain deletion of Set2, we found that deletion of the Set2 coiled-coil motif did not disrupt the Set2-RNAPII interaction (**Fig. 2.3B**) Unexpectedly, when we analyzed WCEs of the indicated strains by western blot with an antibody directed against trimethylated H3K36 (H3K36me3), we found that expression of a form of Set2 deleted of the coiled-coil region could not rescue H3K36me3 (**Fig. 2.3C**). This result suggests at least two possibilities for the function of the coiled-coil motif within Set2. First, the coiled-coil motif in Set2 may be required for the proper folding and catalytic function of Set2. We note, however, that a large C-terminal truncation of Set2 (truncated of amino acids 262-733) is still able to methylate *in vitro* (data not shown). A second possibility is that the coiled-coil motif may be responsible for an interaction between Set2 and an as-yet-unidentified factor that, secondary to RNAPII interaction, is required by Set2 for subsequent H3K36 methylation by Set2. Further testing of the coiled-coil motif in Set2 will be required to distinguish between these two possibilities.

Due to the possibility that the observed interaction between the SRI domain and RNAPII shown in **Fig. 2.2** might have been influenced by the high levels of recombinant Set2 protein produced (as these constructs are expressed from a plasmid using the highly active *ADH1* promoter), we genomically tagged Set2 at amino acid 733 or 618 with a triple

Flag sequence and re-examined its association with RNAPII. As shown in **Fig. 2.4A**, full-length Set2 (Set2-3Flag) again co-immunoprecipitated RNAPII as analyzed by immunoblot analysis using the anti-phospho-CTD antibodies. In contrast, a form of Set2 deleted of the SRI domain ($Set2_{(1-618)}$-3Flag) resulted in the abolition of RNAPII interaction. We also confirmed these results by examining the protein associations of Set2 by affinity purification, which revealed that the readily detectable subunits of RNAPII (Rpb1 and Rpb2) were only observed in purifications involving full length Set2 (**Fig. 2.4B**). Additionally, while Rpb1 and Rpb2 were detected by mass spectrometry in gel-excised bands from the full-length Set2 purification, these proteins were not detected in a parallel gel region excised from the SRI domain-deleted form of Set2 (data not shown). Collectively, these data confirm the importance of the SRI domain in mediating Set2-RNAPII interaction.

The SRI domain of Set2 is conserved and interacts with the phosphorylated CTD in vitro.

Previous studies have suggested that Set2 association with RNAPII is dependent, in part, on the Paf1 transcription elongation complex (Henry et al., 2003; Krogan et al., 2003b). Thus, it was a formal possibility that the SRI domain linked Set2 to the polymerase by indirect protein association. To test whether the SRI domain of Set2 is responsible for direct association with the CTD of RNAPII, we generated a variety of maltose-binding-protein (MBP) fusions of Set2 and examined their ability to associate with a recombinant GST-CTD fusion protein that was either unmodified (GST-yCTD) or exhaustively phosphorylated by CTDK-I (GST-yPCTD). Using a "reverse Far-Western" approach (Phatnani et al., 2004), the GST-CTD fusions were resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with MBP fusion proteins carrying full length Set2 (Set2) or only the SRI domain of Set2 ($Set2_{(619-733)}$). Results revealed that both the full length form of Set2 and the SRI domain of Set2 preferentially bound to the phosphorylated CTD (**Fig. 2.5A**). To independently confirm this interaction and further address whether other regions of Set2 may bind to the phosphorylated CTD *in vitro*, we transferred increasing amounts of MBP

fusions of Set2 lacking the SRI or containing only the SRI domain ($Set2_{(1-618)}$ and $Set2_{(619-733)}$, respectively) to nitrocellulose and probed with a CTDK-I phosphorylated GST-^{32}PCTD fusion. As shown in **Figure 2.5B**, this Far-Western approach revealed that while the SRI domain of Set2 bound efficiently to the GST-^{32}PCTD fusion, Set2 lacking the SRI domain did not. While **Figure 2.5B** shows a 3.5 h exposure, it is noteworthy that a 90 h exposure revealed a potential weak interaction of $Set2_{(1-618)}$ to phosphorylated CTD (data not shown), however it is unclear whether such interaction is physiologically relevant (see **Figs. 2.4-2.7** and (Phatnani et al., 2004). In summary, our results show that the SRI domain in Set2 binds directly to the phospho-CTD of RNAPII. Thus the ability of the Paf1 complex to modulate Set2 activity is likely an indirect consequence of the fact that this complex can regulate CTD phosphorylation (Mueller et al., 2004).

Next, we examined the specificity of the SRI domain for binding phospho-eptitopes using BIACORE analysis with three-repeat synthetic CTD peptides that were phosphorylated in each repeat at either Ser2 (2-phospho), Ser5 (5-phospho), or both (2+5-phospho). As a control, a Ser-phosphorylated peptide that mimics the charge state of the 2+5-phospho peptide (6PC) was included. Sensor chips containing these CTD peptides were reacted with the SRI domain of Set2 ($Set2_{(619-733)}$) and binding was monitored. Surprisingly, we found that the Set2 SRI domain bound preferentially to CTD repeats that were doubly-phosphorylated (**Fig. 2.5C**, compare 2+5-phospho curve with 2-phospho and 5-phospho curves). Moreover, because these response curves were obtained after subtracting the contribution of the non-specific control peptide (6PC), this binding depends on the presence of both Ser2P and Ser5P in the context of the CTD heptad repeat sequence. Based on additional BIACORE experiments involving titrated amounts of the Set2 SRI domain (not shown), we determined that the Set2 SRI domain binds to the 2+5-phospho peptide (relative to the control) with an apparent dissociation constant of about 6 μM. It is important to note that the ability of the SRI domain to bind to the non-specific

control peptide was nearly equivalent to that found for binding to the individually phosphorylated peptides (not shown). We take this result to suggest that the SRI domain of Set2 has a specific requirement for Ser2 and Ser5 phosphorylated CTD epitopes. Collectively, these results reveal a novel and selective requirement for a specific CTD phosphorylation pattern in Set2 binding to RNAPII.

Given these findings, we next asked whether the SRI domain of Set2 is a conserved phospho-CTD-binding motif found in other proteins in budding yeast and beyond. By performing a PSI-BLAST search, we determined that the SRI domain of Set2 was unique to this enzyme alone in budding yeast (data not shown). However, the SRI domain showed significant homology to the C-terminal regions of proteins in other species that also displayed domain organizations similar to that of Set2 (AWS, SET, postSET, and WW), suggesting that these proteins may be the functional homologues of budding yeast Set2 and function with RNAPII (**Table 2.1**). Interestingly, the proteins identified in **Table 2.1** represent only a subset of proteins that the SMART database revealed to contain AWS, SET, and postSET domains (>70), suggesting that not every putative histone methyltransferase that contains a AWS, SET, and postSET is by default a functional homolog of Set2. Indeed, recent evidence shows that the *Drosophila* Ash1 protein, which falls into the Set2-family of HMTs (by way of having a AWS domain rather than an archetypal PreSET domain), is a H3 lysine 4 methyltransferase (Byrd and Shearn, 2003; Beisel et al., 2002). These results suggest that the SRI domain is a probable indicator of RNAPII-interacting enzymes that catalyze K36 methylation. To determine whether any of the putative SRI domains we identified by our PSI-BLAST search would actively bind to the phospho-CTD, we expressed and purified from bacteria a GST fusion protein carrying the C-terminal 178 residues of the human Huntington Interacting Protein B (HYPB) that includes the region of homology to Set2's SRI domain (**Table 2.1**). Using the Far-Western approach as described for Fig. 2.5B, we found that similar to Set2, the SRI-containing region in HYPB interacts efficiently with a

CTDK-I-phosphorylated GST-^{32}PCTD fusion (data not shown). Furthermore, additional BIACORE analyses (as described in **Fig. 2.5C**) revealed that the human SRI domain displays binding properties nearly identical to those of the budding yeast domain (H.P.P., A.L.G. and P. Zhou, manuscript in preparation). Taken together, these results suggest that the SRI domain is a highly conserved and novel phospho-CTD interacting domain.

The Set2-RNAPII interaction is required for H3 K36 methylation.

Given the conserved nature and potential importance of the SRI domain to Set2's cellular function, we next investigated the consequences of deleting this domain. Although studies suggest that the CTD and its proper phosphorylation are necessary for K36 methylation, it has not been formally excluded that the phospho-CTD might regulate the enzymatic activity of Set2 (Krogan et al., 2003b; Li et al., 2003; Schaft et al., 2003; Xiao et al., 2003b). To determine if the loss of the SRI domain would result in a loss of genome-wide K36 methylation, we measured the K36 di-methylation levels in strains containing WT Set2 or Set2 genomically deleted for the SRI domain. Nuclei were prepared from these strains and then resolved on an SDS-PAGE gel, followed by Western Blotting with an antibody specific to di-methylated K36. Results revealed that deletion of the SRI domain in Set2 abolishes global H3-K36 di-methylation (**Fig. 2.6A**). As a control, we examined the levels of H3 in parallel gels with an antibody specific to the C-terminus of H3, which revealed that the levels of histones were similar in both nuclei preparations. Importantly, the nuclei of both strains showed the presence of Set2 by Western blot analysis using an anti-Flag antibody (**Fig. 2.6A**). This result indicates that the deletion of the SRI domain does not influence the nuclear localization of Set2 or significantly affect its stability. The requirement of the SRI domain for K36 methylation was independently confirmed in parallel studies in which a different strain background (W303) was genomically tagged either at the C-terminus or at the beginning of the SRI domain at residue 618 (data not shown). In addition, we used the chromatin immunoprecipitation (ChIP) assay to analyze chromatin modifications at a

gene-specific resolution and also observed a loss of K36 methylation at the *SCC2* gene when the SRI domain of Set2 is removed (**Fig 2.6C**). Analysis of the *PMA1*, *ENO1* and *ADH1* genes yielded similar results (data not shown).

To test the possibility that the SRI domain itself might regulate Set2's catalytic activity, we analyzed recombinant full-length Set2 or SRI-deleted MBP-Set2 fusion proteins in HMT assays with chicken oligo-nucleosomes. Results showed that both forms of the enzyme were equally active for K36 methylation, indicating that the SRI domain is not required for the catalytic activity of Set2 *in vitro* (**Fig. 2.6B**). In fact, we find that a region of Set2 encompassing contains the AWS, SET, and postSET domains (amino acid residues 1-260 in Set2) is fully active for histone methylation *in vitro*, indicating that the C-terminus of Set2 does not intrinsically regulate its HMT activity (data not shown).

Set2 methylation influences transcription elongation and RNAPII occupancy on genes.

Growth phenotypes observed in the presence of the drug 6-azauracil (6AU) are frequently used as indicators of defects in transcription elongation (Exinger and Lacroute, 1992; Mandal et al., 2002; Wu et al., 2003; Morillon et al., 2003b). We therefore asked whether deletion of SET2, or prevention of the Set2-RNAPII association by deletion of the SRI domain, would exhibit 6AU-dependent phenotypes. We began by examining the 6AU phenotypes caused by Set2 deletion in several strain backgrounds. Various wild-type (W303, BY4742/SC288C, YCB652) and matched set2D strains were grown on control media (no drug) or media containing 6AU. The parent strain YCB652 contained integrated URA3 gene, which is required for the 6AU assay, while others were transformed with the *URA3* plasmid pRS316 (Smith et al., 2000). The survival and colony sizes of each strain were monitored after several days of growth and compared to control plates. As shown in Figure 2.7A, we found that deletion of *SET2* in these strain backgrounds resulted in a significant resistance phenotype to 6AU. Similar results were also observed when we used media containing mycophenolic acid, another drug that reveals elongation defects but

through a mechanism unique from 6AU (data not shown). Furthermore, we analyzed a *dst1* null strain of the BY4742 background and observed the characteristic 6AU sensitivity known to exist for this mutant (**Fig. 2.7A** and see (Archambault et al., 1992; Orphanides et al., 1999; Wu et al., 2003).

Given previous studies have demonstrated that a proper response to 6AU is the induced expression of the *IMD2* gene, which is a result of the elongation machinery's response to depleted nucleotide pools (Shaw et al., 2001), we sought to verify that the resistance phenotype observed in the *set2Δ* deletion mutant was not due to an aberrant effect on the metabolism of 6AU. Using semi-quantitative RT-PCR, we monitored the expression levels of *IMD2* and *SNR6* (a RNA polymerase III transcribed gene used as a control) in the presence or absence of 6AU. As shown in **Figure 2.7B**, we found that the expression of the *IMD2* gene was increased to equal degrees in both WT and *set2Δ* strains in the presence of the drug, confirming that the loss of Set2 results in a *bona fide* transcription elongation defect. Importantly, the *IMD2* gene was not induced in the absence of 6AU for either the WT or *set2Δ* strains, indicating that Set2 does not act to repress the basal expression of this gene (**Fig. 2.7B**). In addition to our results with Set2, 6AU resistance has also been observed from the deletion or mutation of a variety of other elongation factors including Chd1, Bye1, Isw1, and forkhead factor 1 (Alen et al., 2002; Woodage et al., 1997; Wu et al., 2003).

Since transcription elongation defects are typically correlated with changes in the occupancy and distribution of RNAPII along genes, we therefore asked whether the loss of Set2 would result in an alteration in RNAPII levels on actively transcribed genes. Using an antibody that recognizes the general levels of RNAPII irrespective of its phosphorylation status (Schroeder et al., 2000), we examined RNAPII levels on the promoters and coding regions of active genes in WT and *set2Δ* strains by ChIP. As shown in **Figure 2.7C**, we found that RNAPII levels in the *set2* deletion mutant were significantly increased in the

middle to late coding region of the actively transcribing *SCC2* gene, as compared to the WT control strain. Interestingly, the gene locations that showed an RNAPII increase were also the same locations determined to be highly methylated by Set2 (see **Fig. 2.6C**), suggesting the possibility that a relationship may exist between regions of chromatin highly methylated at K36 and RNAPII occupancy potential. We next examined a variety of other active genes in the *set2* deletion mutant to determine how general this RNAPII defect would be. We examined the promoter and coding regions of *TOM1*, *MDN1*, *PMA1*, *ENO1,* and *FIR1* for the presence of RNAPII as described above and found a similar pattern of RNAPII increase in the *set2* deletion mutant as was observed for *SCC2* (data not shown). We addressed the possibility that the observed increases in RNAPII might be a result of a general increase in transcript formation for these genes in the absence of Set2. Indeed, Set2 has been shown to play a role in the basal repression of *GAL4* (Landry et al., 2003). We therefore examined the expression of the genes indicated above by semi-quantitative RT-PCR and observed that the increased density of RNAPII did not correlate with any change in the steady-state mRNA levels (**Fig. 2.7D** and data not shown). These mRNA results were also confirmed independently by examining the gene expression microarray profiles found in WT and *set2*Δ cells (personal communication, N. Krogan and J. Greenblatt). Furthermore, we also examined TBP levels by ChIP at the promoters of several genes listed above (*SCC2* and *TOM1)* and found no significant increases in TBP occupancy in the *set2* deletion mutant (data not shown). Our data indicate that Set2 does not function as a basal repressor of the genes analyzed, but rather affects the precise levels of RNAPII on genes, further supporting the 6AU results suggesting that Set2 can influence RNAPII elongation.

The above results suggest Set2 is important for transcription elongation, but do not reveal whether this function of Set2 is dependent on its association with RNAPII and/or K36 methylation. To test if loss of the interaction between Set2 and RNAPII is responsible for the elongation defect, we assayed the growth of the SRI deleted ($Set2_{(1\text{-}618)}$-3Flag), *SET2*

deleted, and the Set2-3Flag strains using 6AU. We observed that deletion of the SRI domain resulted in a resistance to 6AU that was similar to the *set2* deletion mutant, indicating that the interaction between Set2 and RNAPII is necessary for the normal transcription elongation functions of Set2 (**Fig. 2.8A**).

We next asked whether K36 methylation *per se* is important for the activity of Set2 in this process. In one case, we transformed *set2Δ* cells with a plasmid coding for either full-length Set2 (*SET2*) or a form of Set2 containing a point mutation (*set2*^{R195G}) that has been shown to abolish K36 methylation activity *in vitro* and *in vivo* (Strahl et al., 2002). We found that expression of *SET2* in the *set2Δ* strain nearly restored WT levels of 6AU sensitivity (**Fig. 2.8B)**. However, *set2Δ* cells expressing *set2*^{R195G} showed resistance to the drug (**Fig. 2.8B**), consistent with a role for K36 methylation in the elongation process. In the second case, we asked whether amino acid substitutions at K36 that prevent methylation (K36A and K36R) would result in resistance to 6AU and RNAPII density increases. As shown in **Fig. 2.8C**, the K36A and K36R strains were significantly resistant to 6AU as compared to the wild-type H3 strain (WT), whereas strains with mutations at other sites of methylation (K4 and K79) or sites of phosphorylation (serine 10) were not. In addition, the 6AU resistance caused by the K36A or K36R mutations were not suppressed by mutation of lysine 4 (**Fig. 2.8C**, K4R/K36R). Significantly, we also found the same pattern of increased RNAPII density in the K36A strain as with the *set2Δ* strain (**Fig. 2.8D**), suggesting that the specific lack of K36 methylation is the primary cause of the 6AU phenotype and RNAPII defect. These data strongly implicate the methylation by Set2 as being functionally important in the elongation process.

2.4 Discussion

Recent work in *S. cerevisiae* has revealed an unexpected role for the Set1 and Set2 histone methyltransferases in transcription elongation. Although several studies found that

Set2 associates with the elongating polymerase, we found that the C-terminus of Set2, independent of the WW domain, was responsible for this interaction (**Fig 2.1**; (Xiao et al., 2003b). Despite the discovery of the Set2-RNAPII interaction, very little is known regarding the influence of Set2 or K36 methylation on transcription elongation. Our results presented in this chapter reveal that (i) a novel region in the C-terminus of Set2, but not including the WW domain as previously reported, is necessary and sufficient for the functional interaction between Set2 and RNAPII; (ii) this domain, which we have named the SRI domain, is within amino acids 619-718 of Set2 and bind with high affinity to doubly-phosphorylated CTD repeats; (iii) the Set2-RNAPII interaction, established through the SRI domain, is required for H3 K36 methylation; and (iv) K36 methylation mediated by Set2, in particular, influences RNAPII density along the 3' end of certain genes and therefore suggests are role in RNAPII elongation.

A novel phospho-CTD binding motif in Set2.

Based on recent observations of other RNAPII CTD-interacting proteins, we expected that the WW and/or coiled-coil regions in Set2 would mediate its association with RNAPII. Surprisingly, neither of these domains were found to be involved in RNAPII binding as demonstrated in **Figs. 2.1-2.5**. Instead, a region from 619-733 in Set2, which we have termed the SRI domain, was found to be both necessary and sufficient for Set2-RNAPII interaction. This is in conflict with an earlier report which stated that the WW domain of Set2 is required for the association of this enzyme with RNAPII (Li et al., 2002). However, the technical approach used in this earlier study to analyze the involvement of the WW domain in RNAPII interaction employed a genomic insertion of the TAP tag in front of the WW domain of Set2, which removed its entire C-terminus including the SRI domain. Even though the WW and/or coiled-coil regions in Set2 are not likely to be involved in its association with RNAPII, they may play important roles in Set2 function by mediating the

association of Set2 with other phosphorylated proteins and/or itself since Set2 is thought to exist as a homodimer in the cell (Schaft et al., 2003; Strahl et al., 2002).

Through further examination of the binding requirements of the SRI domain, we uncovered that this domain binds preferentially to the CTD phosphorylated at both serines 2 and 5, as compared to the singly-phosphorylated form or a charge-control peptide (**Fig. 2.5C**). This discovery strongly suggests that a synergistic relationship between CTD phosphorylation and Set2 binding exists. Interestingly, Ser2 phosphorylation is predominantly found in the body of genes while it has been thought that Ser5 phosphorylation is generally restricted to the promoters and 5' region of these same genes. Recent studies, however, reveal that Ser5 phosphorylation is indeed found at significant levels throughout the transcribed regions of genes (Ahn et al., 2004). Furthermore, a recent paper has noted that CTDK-I preferentially phosphorylates serine 2 of the heptad repeat only when the serine 5 position is already phosphorylated (Jones et al., 2004). Thus, it seems likely that doubly phosphorylated CTD epitopes exist in yeast that may serve to direct the interaction of Set2 (and perhaps other phospho-CTD-interacting proteins).

Based on our blast search analysis, the SRI domain appears to be conserved in other organisms only in proteins that have a domain architecture nearly identical to that of Set2 (those containing AWS or SAC, SET, Post-SET, and WW; see **Table 1**). We take these data to suggest that the SRI domain is a probable indicator of proteins that are *bona fide* functional homologues of Set2. Consistent with this, we have determined that a construct containing the SRI region of HYPB (see **Table 1**) binds directly to the phosphorylated CTD. Furthermore, later studies in our laboratory also reveal the putative *S. pombe* homolog of Set2 (see **Table 1**) to be a robust K36 HMT that also interacts with RNAPII (Morris et al., 2005). Based on these observations, it is likely that Set2 and K36 methylation have a conserved function in transcription elongation.

A role for H3 K36 methylation in transcription elongation.

Previous studies have implicated Set2 in the transcription elongation process. This has been established not only by the numerous biochemical and genetic analyses that have been performed on Set2, but also by the use of 6AU, a drug commonly used as an indicator for elongation defects. The compound 6AU depletes the available nucleotide pool by inhibition of the enzyme IMP dehydrogenase (IMPDH), which is responsible for catalyzing the rate-limiting step in *de novo* synthesis of GTP (Jackson et al., 1975; Exinger and Lacroute, 1992; Hyle et al., 2003). Inhibition of IMPDH results in a challenge to the transcription elongation machinery, which is continually in need of free nucleotides. Thus, strains with altered transcription elongation function display a sensitivity to the drug different from their wild-type parent strain. Our 6AU assays revealed that *SET2* deletion or deletion of the region coding for the SRI domain itself results in resistance to the drug, whether the *URA3* gene is genomically inserted or carried ectopically on a plasmid (**Fig. 2.7A**). This is in contrast to some previous studies that showed that *set2* mutants are modestly sensitive to 6AU (Krogan et al., 2003b; Li et al., 2002; Schaft et al., 2003). While it is unclear why varying 6AU results have been reported, it is noteworthy that others have also seen a 6AU resistance phenotype with *set2* deletion mutants (Li et al., 2003; Xiao et al., 2005) and D. Stillman, personal communication), indicating the differences may lie in context-specific differences in the genetic background used.

In addition to *set2* deletion, resistance to 6AU has also been observed in strains mutated for a number of other factors including Ess1, Bye1, Chd1, Isw1, and Fkh1 (Alen et al., 2002; Wu et al., 2003; Woodage et al., 1997). Many of these factors have been shown to influence transcription elongation via regulation of specific phases in the transcription elongation cycle such as the transition between elongation and initiation or termination (Wu et al., 2003; Morillon et al., 2003b; Morillon et al., 2003a). While 6AU phenotypes alone are not sufficient to allow conclusions regarding the mechanistic details of how a protein affects

the elongation phase of transcription, 6AU resistance can be interpreted as a consequence of increased elongation efficiency resulting from the deletion of a factor which negatively regulates elongation. Indeed, it was found that strains deleted for the Ess1 suppressor, Bye1, display resistance to 6AU, and this protein may negatively regulate transcription elongation by inducing RNAPII pausing at elongation-arrest sites (Wu et al., 2003). In addition, Chd1-null strains were found to exhibit 6AU resistance, which may be due to an indirect effect on elongation by its influence on termination (Alen et al., 2002; Woodage et al., 1997).

In an effort to better understand the nature of the 6AU phenotype of the *set2* deletion mutant, we examined the distribution of RNAPII along genes. Interestingly, we found that RNAPII levels significantly increased at the 3' ends of genes in the absence of Set2 or when methylation is inhibited (**Figs. 2.7C, 2.8D**). Furthermore, this increase generally paralleled K36 methylation levels, indicating the possibility of a direct mechanistic link between the levels of K36 methylation and RNAPII occupancy (**Figs. 2.6C, 2.7C**). While our results strongly implicate K36 methylation as having a direct role in RNAPII elongation, the precise role is currently unclear. We propose three possibilities based on an influence of K36 methylation on any of the three general phases of transcription: 1) K36 methylation may generate a chromatin structure that is less permissive for RNAPII passage. In this case, K36 methylation acts as a negative regulator of RNAPII elongation, and the loss of this 'mark' permits increased RNAPII density and passage along genes. However, since the termination event at the 3' end would be under tight checkpoint control and would be rate limiting, the loss of K36 methylation would result in a buildup or backlog of RNAPII across the gene, while overall transcript production remains consistent. Such a buildup of RNAPII might be viewed as advantageous to cells when presented with 6AU stress. 2) Set2 mediated K36 methylation could influence transcription initiation, allowing additional RNAPII association and initiation, but with a relatively fixed rate of termination the RNAPII could

accumulate at the 3' of a gene. Finally, 3) termination efficiency could be diminished in the absence of K36 methylation. Thus, similar to *chd1* mutants, the reduced efficiency of termination would result in a backlog of RNAPII across the gene. Among these possibilities, we suggest scenario one is most likely, given the 6AU resistance of *set2Δ* strains and the fact that negative regulators of RNAPII elongation have been found to result in 6AU resistance when these genes were deleted. In summary, our results provide strong evidence that K36 methylation, mediated by the SRI-dependent association of Set2 with RNAPII, plays a role in the elongation phase of transcription. This result is further supported by a genome-wide studies completed during the course of our work that revealed H3K36me2 is present solely on RNAPII transcribed genes (Rao et al., 2005). The rise of microarray analysis of histone modifications has become crucial to studies in the field of chromatin biology, and will be discussed further in Chapter 5. Whether Set2's elongation role is mediated through the association of factors that bind to K36 methylation, or through the ability of this modification to control chromatin structure directly by regulating nucleosome-nucleosome or nucleosome-DNA interactions was unknown at the time of this work, has been of keen interest in later studies as will be discussed in Chapter 3-5.

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Table 2.1 Putative Set2 homologues identified by PSI-BLAST searching with the SRI domain

species	gene name[i]	gi accession	protein sequence position of putative SRI domain	identity/similarity to SRI domain[ii]
S. cerevisiae	Set2	6322293	619-718	n/a
C. albicans	Ca019.9324	46435920	723-820	35% / 55%
H. sapiens	HYPB	30410779	1956-2056	23% / 37%
M. musculus	XP_135176	38090181	2393-2493	23% / 37%
D. melanogaster	CG1716	24641786	2262-2356	21% / 39%
N. crassa	XP_322355	32403484	569-653	19% / 37%
S. pombe	SPAC29B12	2408044	703-778	17% / 42%

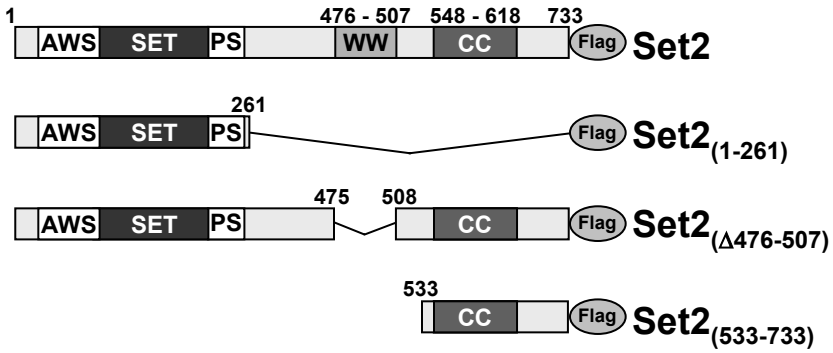
[i] All proteins identified contain a domain architecture similar to yeast Set2, including the AWS, SET, postSET and in some cases WW domains. It is notable that the putative SRI domains, as with Set2, are found in the C-termini of these proteins.

[ii] Identity refers to the percentage of identical amino acids present between the yeast Set2 SRI domain and the putative SRI domain of the indicated homolog, while similarity indicates amino acid replacements that exhibit similar charge or hydrophobicity.

Figure 2.1: The C-terminus of Set2 interacts with the phosphorylated CTD of RNAPII.

(**A**) Schematic representation of the Set2-Flag constructs used to probe for RNAPII interaction. The SET domain along with its flanking Cys-rich domains (AWS and PS), WW domain (WW), and coiled-coil motif (CC) are shown. All constructs contained a C-terminal Flag epitope. (**B**) *set2Δ* cells were transformed with either vector only or the indicated Set2-Flag constructs and WCEs were prepared. These extracts were immunoprecipitated with the α-Flag antibody followed by immunoblot analysis with the (H5; α-Ser2P), (H14; α-Ser5P), or α-Flag antibodies. Sizes of the molecular weight markers are shown and asterisks indicate the location of expected Set2-Flag products. All input extracts showed equivalent levels of hyperphosphorylated RNAPII (data not shown).

A


1
476 - 507
548 - 618
733
AWS
SET
PS
WW
CC
Flag
Set2
261
Set2(1-261)
475
508
Set2(Δ476-507)
533
Set2(533-733)

B

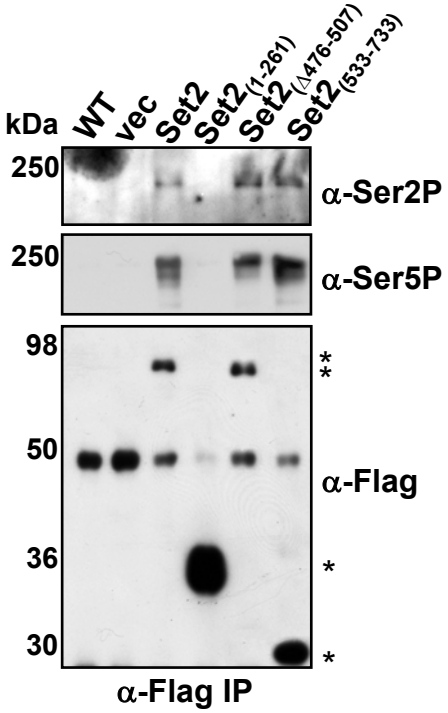
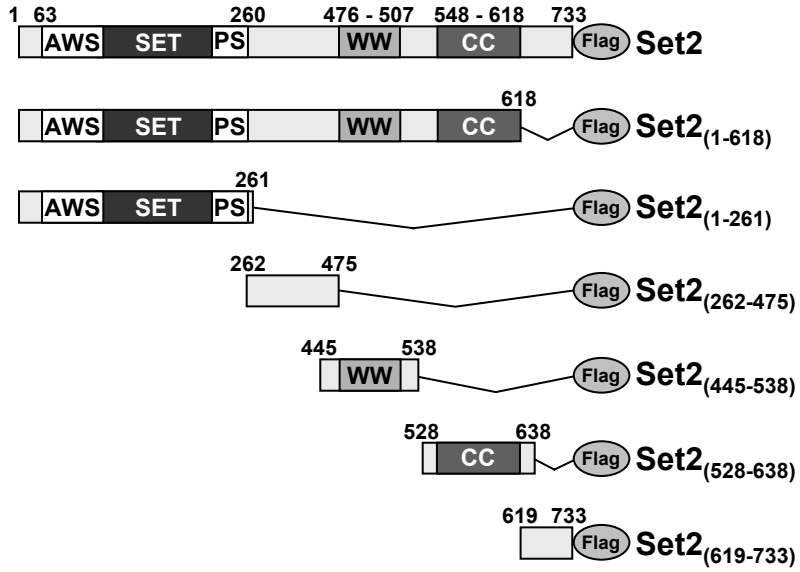

kDa
WT
vec
Set2
Set2(1-261)
Set2(Δ476-507)
Set2(533-733)
250
α-Ser2P
250
α-Ser5P
98
50
36
30
α-Flag
α-Flag IP

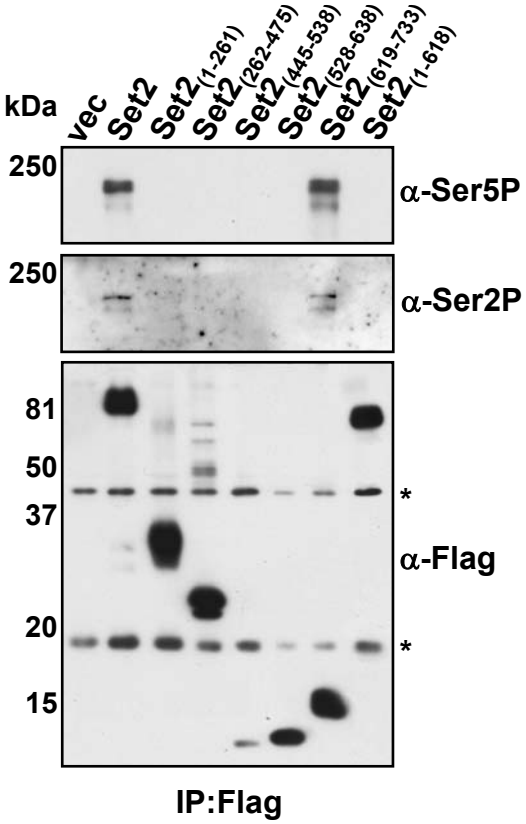
Figure 2.2: Identification of a novel region in Set2 required for RNAPII binding.

(**A**) Schematic representation of the Set2 constructs used to probe for RNAPII interaction as in Fig. 2.1. (**B**) *set2Δ* cells were transformed with either vector only or plasmids coding for the indicated Set2-Flag construct and whole cell extracts (WCE) were prepared. WCEs were immunoprecipitated with a-Flag beads followed by immunoblotting with antibodies directed against serine 5 phosphorylated CTD (H14; α-Ser5P), serine 2 phosphorylated CTD (H5; α-Ser2P), or the Flag epitope. Significant to mention is that the H5 antibody may also recognize serine 5 CTD phosphorylation in addition to serine 2 phosphorylation (Jones et al., 2004). Asterisks indicate the location of non-specific Flag antibody reactive species. (**C**) Schematic representation of the Set2-SRI domain constructs used to determine the boundaries of the functional SRI domain. N- and C-terminal truncations of the SRI domain were made in fifteen amino acid increments as shown. All constructs contained a C-terminal Flag epitope. (**D**) *set2Δ* cells were transformed with the indicated plasmids, WCE were prepared, and co-IPs performed using the antibodies indicated in (B). Sizes of the molecular weight markers are shown.

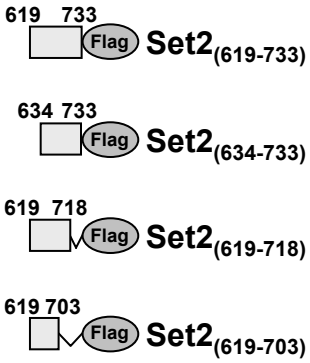
A


1
63
260
476 - 507
548 - 618
733
AWS
SET
PS
WW
CC
Flag
Set2
618
Set2(1-618)
261
Set2(1-261)
262
475
Set2(262-475)
445
538
Set2(445-538)
528
638
Set2(528-638)
619
733
Set2(619-733)

B


kDa
vec
Set2
Set2(1-261)
Set2(262-475)
Set2(445-538)
Set2(528-638)
Set2(619-733)
Set2(1-618)
250
α-Ser5P
250
α-Ser2P
81
50
37
20
15
*
*
α-Flag
IP:Flag

C


619 733
Flag
Set2(619-733)
634 733
Flag
Set2(634-733)
619 718
Flag
Set2(619-718)
619 703
Flag
Set2(619-703)

D

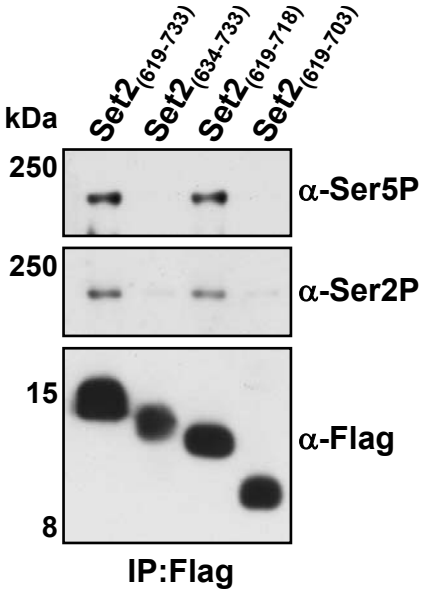

kDa
Set2(619-733)
Set2(634-733)
Set2(619-718)
Set2(619-703)
250
α-Ser5P
250
α-Ser2P
15
8
α-Flag
IP:Flag

Figure 2.3: The coiled-coil region in Set2 is required for H3K36me3, but dispensable for RNAPII interaction.

(**A**) Schematic representation of the Set2 constructs used for examining the coiled-coil (CC) region and potential effects on Set2-RNAPII interaction, as in Fig. 2.1. (**B**) *set2Δ* cells were transformed with either vector only or plasmids coding for the indicated Set2-Flag construct and whole cell extracts (WCE) were prepared. WCEs were immunoprecipitated with anti-Flag beads followed by immunoblotting with antibodies directed against serine 5 phosphorylated CTD (H14; α-Ser5P) or the Flag epitope. Input levels of serine 5 phosphorylated CTD are indicated at the top of the panel. (**C**) Western blot of *set2Δ* cells transformed with the indicated constructs and blotted with antibodies directed against the Flag epitope (α-Flag), tri-methylated H3 lysine 36 (α-H3K36me3), or the C-terminus of H3 (α-H3).

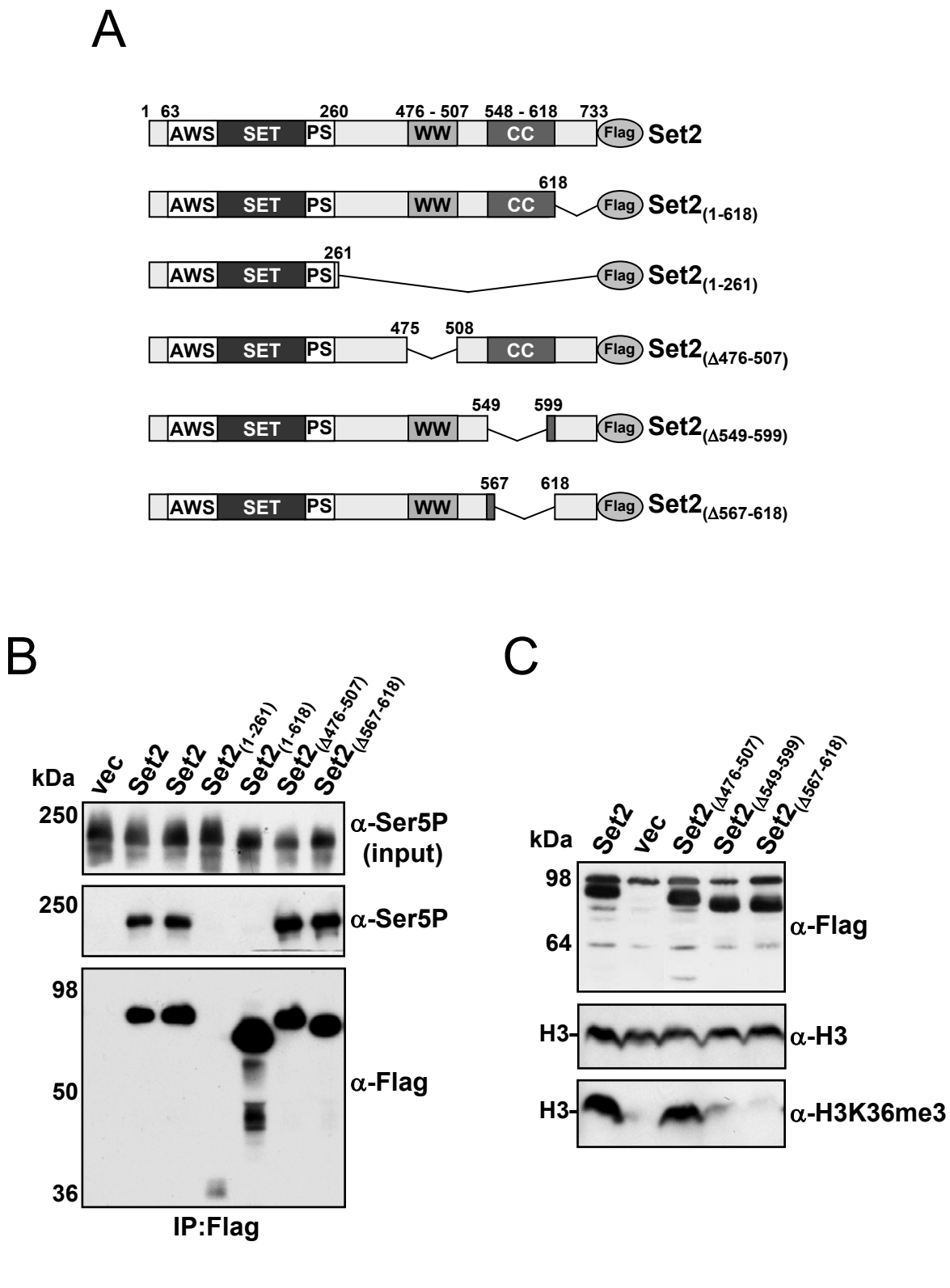
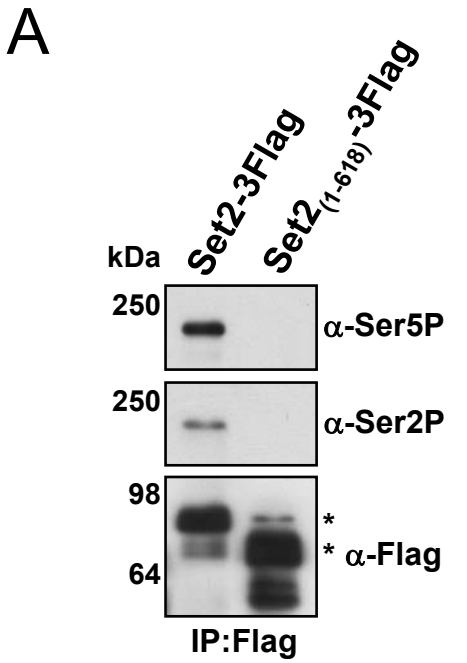

A
1
63
260
476 - 507
548 - 618
733
AWS
SET
PS
WW
CC
Flag
Set2
618
Set2(1-618)
261
Set2(1-261)
475
508
Set2(Δ476-507)
549
599
Set2(Δ549-599)
567
618
Set2(Δ567-618)
B
kDa
vec
Set2
Set2
Set2(1-261)
Set2(1-618)
Set2(Δ476-507)
Set2(Δ567-618)
250
α-Ser5P (input)
250
α-Ser5P
98
50
36
α-Flag
IP:Flag
C
kDa
Set2
vec
Set2(Δ476-507)
Set2(Δ549-599)
Set2(Δ567-618)
98
64
α-Flag
H3
α-H3
H3
α-H3K36me3

Figure 2.4: The SRI domain is required for interaction of Set2 with RNAPII.

(**A**) Yeast strains containing full length Set2 (Set2-3Flag) or a form of Set2 without the SRI domain ($Set2_{(1-618)}$-3Flag) were made via genomic tagging with the 3xFlag epitope. Whole cell extracts (WCEs) of these strains were immunoprecipitated with anti-Flag beads followed by immunoblotting with antibodies directed against serine 2 phosphorylated CTD (H5; α-Ser2P), serine 5 phosphorylated CTD (H14; α-Ser5P) or the Flag epitope. Sizes of the molecular weight markers are shown and asterisks indicate the location of expected Set2-Flag products. (**B**) WCEs from the strains in (A) were incubated with anti-Flag resin and the resulting bound proteins eluted with 3xFlag peptide. Eluted proteins were resolved by a 4-12% NuPAGE gel and examined by Coomassie staining. Arrows indicate the protein identity of bands in the Set2-3Flag lane that were examined by Mass Spectrometry (MS), while analysis of parallel regions in the $Set2_{(1-618)}$-3Flag lane were negative for the presence of Rpb1 or Rpb2. Sizes of the molecular weight markers are shown. This panel was prepared by H. Hall as a component of the published results (Kizer et al. 2005).


A
Set2-3Flag
Set2(1-618)-3Flag
kDa
250
α-Ser5P
250
α-Ser2P
98
64
*
* α-Flag
IP:Flag

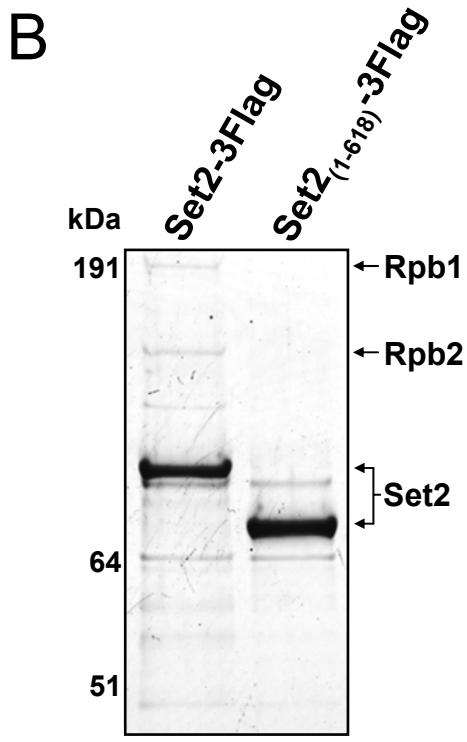
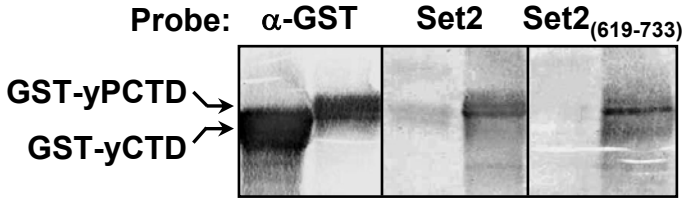

B
Set2-3Flag
Set2(1-618)-3Flag
kDa
191
Rpb1
Rpb2
Set2
64
51

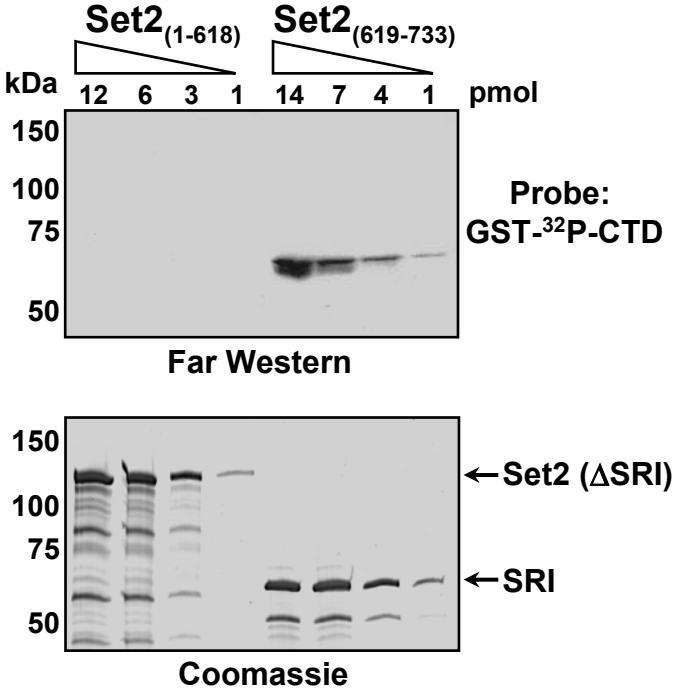
Figure 2.5: The SRI domain of Set2 binds synergistically to the phosphorylated CTD.

(**A**) "Reverse" Far-Western. GST-CTD and CTDK-I-phosphorylated GST-CTD (GST-PCTD) fusion proteins were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were probed individually with purified recombinant full length MBP-Set2 (Set2) or with MBP-SRI ($Set2_{(619-733)}$), and the bound MBP fusions detected with an anti-MBP antibody. As a control, a duplicate membrane was probed with an anti-GST antibody (α-GST) to demonstrate the presence of both GST-CTD fusion proteins. (**B**) Increasing amounts of two MBP fusion proteins ($Set2_{(1-618)}$ and $Set2_{(619-733)}$) were resolved in two SDS polyacrylamide gels; one gel was subjected to Far-Western analysis with GST-^{32}P-CTD as a probe, and the other was stained with Coomassie. (**C**) BIACORE analysis of the SRI domain. The MBP-SRI fusion protein (MBP-$Set2_{(619-733)}$) was analyzed by surface plasmon resonance (BIACORE) for binding to distinct phosphorylated synthetic three-repeat CTD peptides. These peptides were either Ser5 phosphorylated (5-phospho), Ser2 phosphorylated (2-phospho) or both Ser2 and Ser5 (2+5-phospho) in each repeat (see Materials and Methods). Response Units, on the Y axis, represent binding to the peptides. The binding response to a scrambled control peptide carrying six $SerPO_4$ residues (see Chapter 2.2) has been subtracted from each of the three response curves. Only the peptide carrying both $Ser2PO_4$ and $Ser5PO_4$ in each repeat revealed binding above control levels. We estimate the affinity of this interaction, with subtraction of background binding to the control peptide, to be 6 μM. These experiments and panels were prepared by H. Phatnani and A. Greenleaf as a component of our collaborative work (Kizer et al. 2005).

A


Probe:
α-GST
Set2
Set2(619-733)
GST-yPCTD
GST-yCTD

B


Set2(1-618)
Set2(619-733)
kDa
12 6 3 1
14 7 4 1
pmol
150
100
75
50
Probe:
GST-32P-CTD
Far Western
150
100
75
50
←Set2 (ΔSRI)
←SRI
Coomassie

C

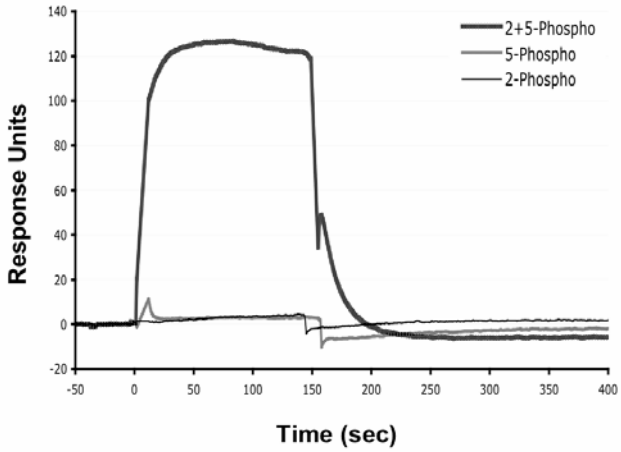

2+5-Phospho
5-Phospho
2-Phospho
Response Units
140
120
100
80
60
40
20
0
-20
-50
0
50
100
150
200
250
300
350
400
Time (sec)

Figure 2.6: Deletion of the SRI domain in Set2 abolishes H3 K36 di-methylation.

(**A**) Yeast nuclear extracts prepared from *set2Δ* cells or the indicated genomically tagged strains in the BY4742 background were probed with antibodies against di-methylated lysine 36 at H3 (α-H3K36me2) to monitor the role of the SRI domain in global K36 methylation levels. An antibody directed against the C-terminus of H3 (α-H3) was used as a loading control. Nuclear levels of Set2 in these strains were monitored using the a-Flag antibody. A similar loss of K36 methylation was observed when the SRI domain was genomically deleted in the W303 background (data not shown). (**B**) Full-length (Set2-Flag) or SRI-domain truncated ($Set2_{(1\text{-}618)}$-Flag) recombinant forms of Set2, which also contained an N-terminal MBP epitope, were prepared and analyzed for their HMT activity *in vitro*. HMT reactions were prepared with bacterial lysates containing the indicated Set2 constructs with or without nucleosomes. Identical samples were analyzed by the filter-binding assay (upper) and fluorography (middle). Immunoblotting with the Flag antibody (lower) was performed to ensure equal amounts of protein were present in each reaction. (**C**) WT or SRI-deleted yeast strains were analyzed by chromatin immunoprecipitation (ChIP) for K36 di-methyl levels on genes. DNA isolated from the K36 methylation IPs was used in PCR reactions with primer pairs for the indicated regions of *SCC2* gene (top). PCR products of the input DNA (Input) and ChIP DNA (H3K36me2) are shown (middle). The asterisks indicate the location of a PCR product pertaining to an intergenic region at chromosome V (ChV), which was used as a loading control in all PCR reactions. The histogram displays the relative enrichment values for K36 di-methylation (bottom). The values were calculated by dividing the ratio of band intensities for IP DNA/ChV with the ratio of intensities for the Input DNA/ChV. Similar results were found for the *PMA1*, *ENO1* and *ADH1* genes (data not shown).

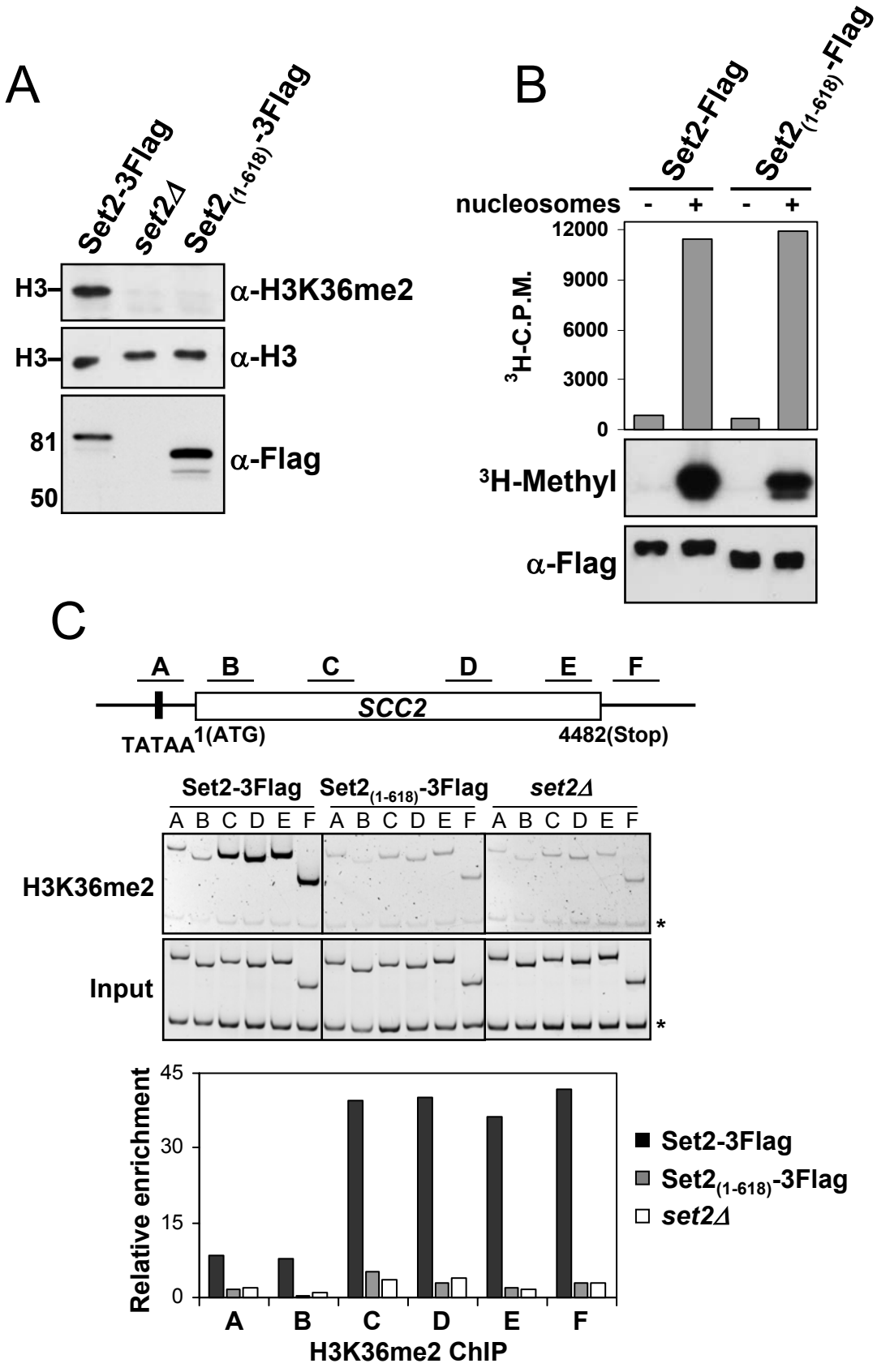

A
Set2-3Flag
set2Δ
Set2(1-618)-3Flag
H3
α-H3K36me2
H3
α-H3
81
50
α-Flag
B
Set2-Flag
Set2(1-618)-Flag
nucleosomes - + - +
12000
9000
6000
3000
0
3H-C.P.M.
3H-Methyl
α-Flag
C
A B C D E F
SCC2
TATAA
1(ATG)
4482(Stop)
Set2-3Flag
Set2(1-618)-3Flag
set2Δ
A B C D E F A B C D E F A B C D E F
H3K36me2
Input
*
*
45
30
15
0
Relative enrichment
A B C D E F
H3K36me2 ChIP
Set2-3Flag
Set2(1-618)-3Flag
set2Δ

Figure 2.7: Deletion of *SET2* results in an elongation phenotype and a disruption of RNAPII occupancy on genes.

(**A**) Various strains containing either wild-type (WT), *set2Δ* or *dst1Δ* alleles were plated on synthetic dextrose-uracil medium with or without 6-azauracil (6AU, 100 μg/ml) and grown at 30°C for 2 to 3 days to monitor for transcription elongation phenotypes. All strains contained the plasmid pRS316 containing the *URA3* gene, except yeast strain YCB652, which contains an integrated *URA3* gene. Results using mycophenolic acid (100 μg/ml) were found to yield identical results (data not shown). (**B**) The loss of Set2 does not aberrantly affect the increased expression of *IMD2* in 6AU. Semi-quantitative RT-PCR was used to monitor the expression of *IMD2* and *SNR6* (a pol III transcribed gene used as a control) in WT or *set2Δ* strains in the absence or presence of 6AU (50 μg/ml). The results of RT-PCR reactions with (PCR amplification cycles indicated above) or without (-RT) reverse transcriptase are shown. The fold change in *IMD2* expression in each condition are indicated, based on averages of the three cycling parameters for each strain, with WT set to 1.0 as reference. This panel was prepared by Y. Shibata as a component of the collaborative project (Kizer et al. 2005). (**C**) WT or *set2Δ* strains were analyzed by ChIP for RNAPII levels. Isolated DNA from the RNAPII IPs were used in PCR reactions with primer pairs for regions of *SCC2* as indicated in the schematic. The data shown represents the average of thirteen individual ChIP assays from separate cell pellets. The standard error of the mean (SEM) is indicated. Asterisks indicate the relative *set2Δ* RNAPII enrichment values that were statistically significant as compared to their WT counterparts ($p<0.01$ for primer set D and $p<0.001$ for primer sets E and F). ChIP analyses using other antibodies against the RNAPII CTD (8GW16, Covance), Rpb3 (NeoClone), or the N-terminus of Rpb1 (y-80, Santa Cruz), revealed a similar pattern of RNAPII distribution as displayed in the figure (not shown). (**D**) *SCC2* expression was unchanged in *set2Δ* despite the increased RNAPII density detected. The expression levels of *SCC2* and *SNR6* (a pol III transcribed gene used as a control) were monitored by semi-quantitative RT-PCR as in (B), with the relative change in *SCC2* expression for the *set2Δ* strain displayed relative to WT. This panel was prepared by Y. Shibata as a component of the collaborative project (Kizer et al. 2005).

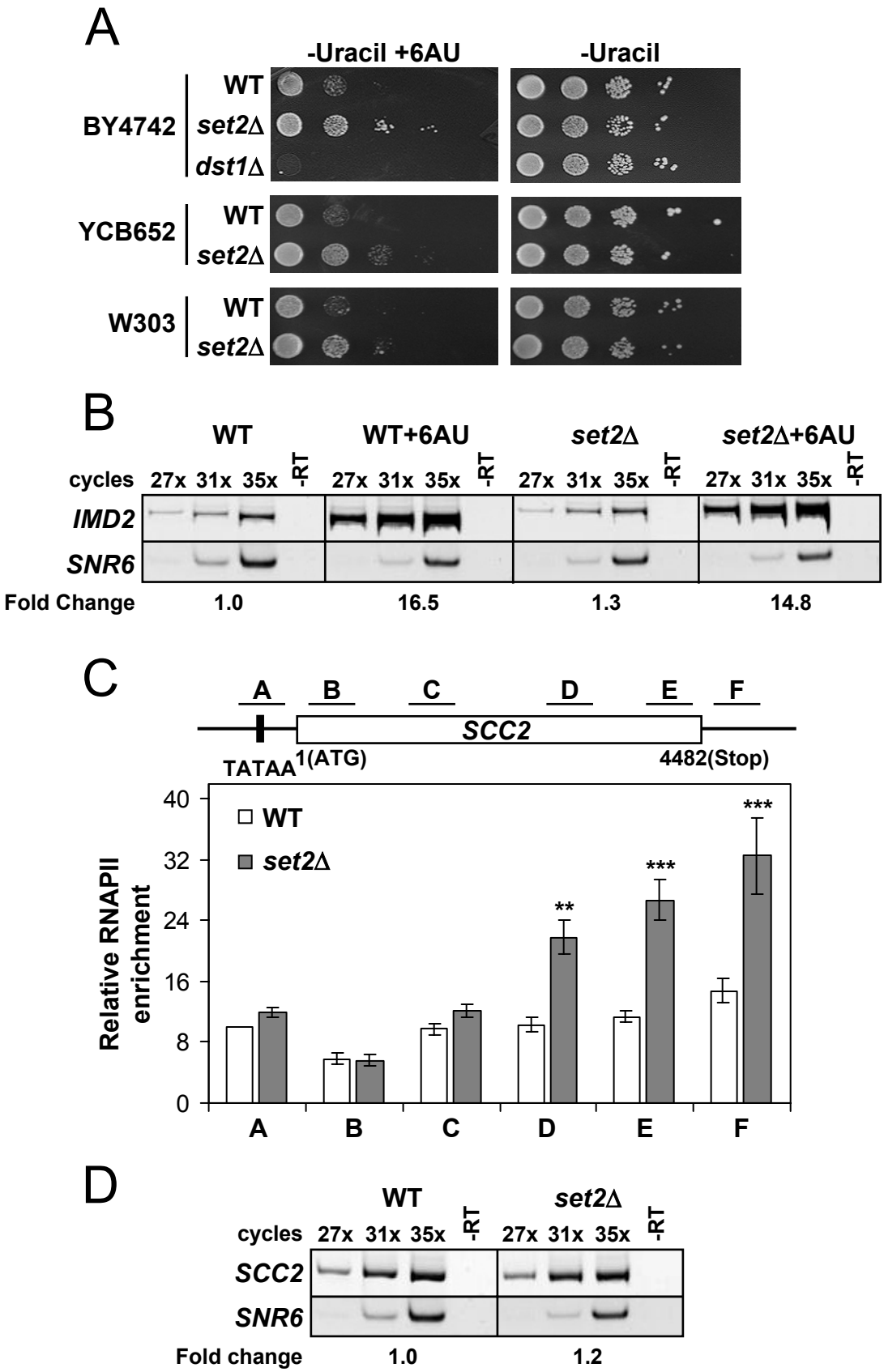
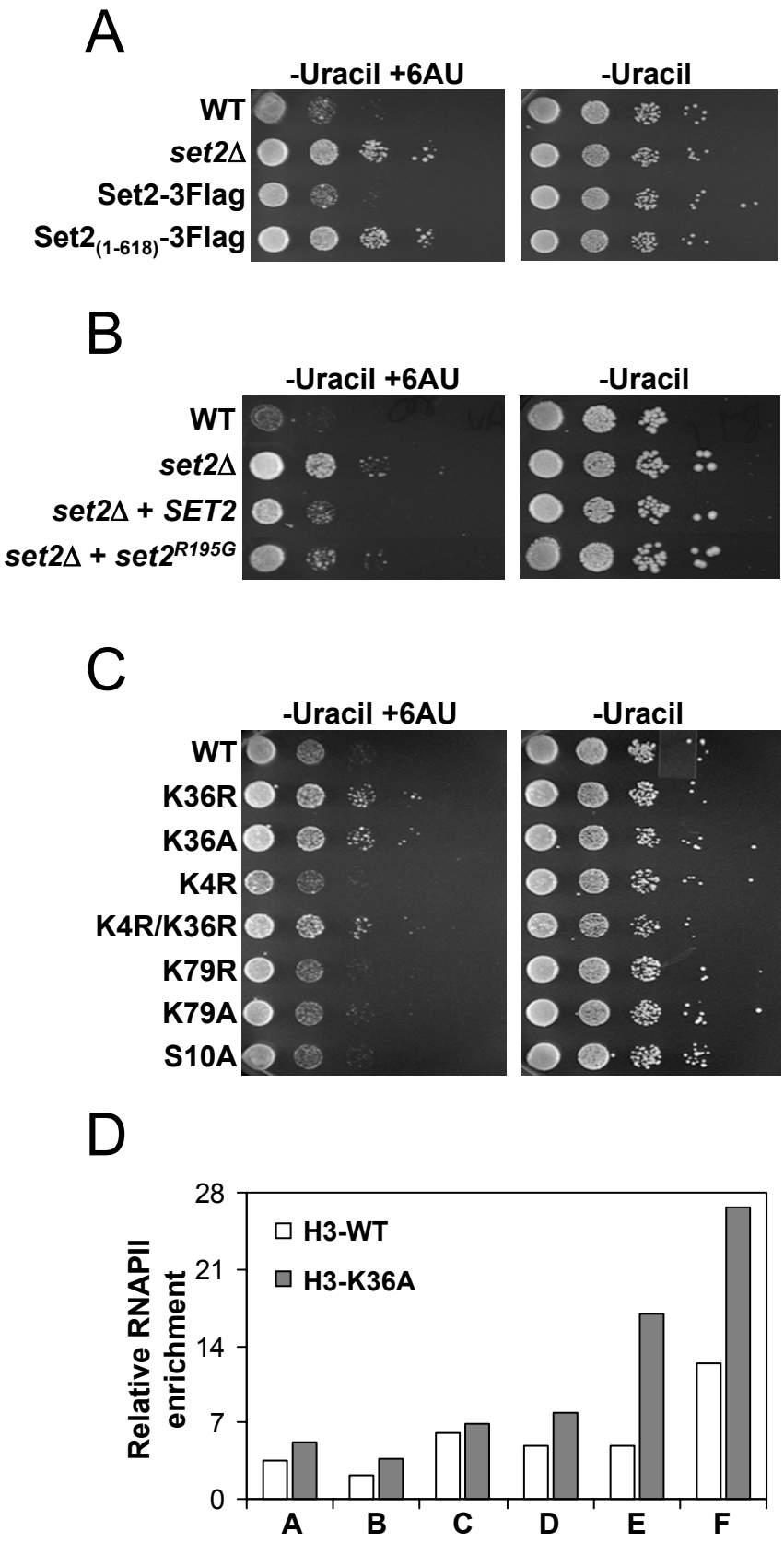

A
-Uracil +6AU
-Uracil
BY4742
WT
set2Δ
dst1Δ
YCB652
WT
set2Δ
W303
WT
set2Δ
B
WT
WT+6AU
set2Δ
set2Δ+6AU
cycles
27x 31x 35x -RT
IMD2
SNR6
Fold Change
1.0
16.5
1.3
14.8
C
A B C D E F
SCC2
TATAA
1(ATG)
4482(Stop)
Relative RNAPII enrichment
WT
set2Δ
40
32
24
16
8
0
**
***
***
A B C D E F
D
WT
set2Δ
cycles
27x 31x 35x -RT
SCC2
SNR6
Fold change
1.0
1.2

Figure 2.8: K36 methylation directly influences transcription elongation.

(**A**) Genomically tagged strains containing either full length Set2 (Set2-3Flag) or Set2 deleted of the SRI domain ($Set2_{(1-618)}$-3Flag) were generated and assayed, as in Fig. 2.7A, for growth on 6AU as compared to WT and *set2Δ* strains. (**B**) *Set2Δ* strains were transformed with a plasmid expressing wild-type Set2 (*SET2*) or a mutant form of Set2 which abolishes its catalytic activity ($set2^{R195G}$) and assayed for growth on 6AU as before. (**C**) Yeast strains (WZY42 derived) bearing various point mutations on histone H3 were assayed for growth on 6AU as in Fig. 2.7A. (**D**) WT or *set2Δ* strains were analyzed by ChIP for RNAPII levels as in Fig. 2.7C. The histogram data is representative of two independent experiments which revealed similar results.


A
-Uracil +6AU
-Uracil
WT
set2Δ
Set2-3Flag
Set2(1-618)-3Flag
B
-Uracil +6AU
-Uracil
WT
set2Δ
set2Δ + SET2
set2Δ + set2R195G
C
-Uracil +6AU
-Uracil
WT
K36R
K36A
K4R
K4R/K36R
K79R
K79A
S10A
D
Relative RNAPII enrichment
28
21
14
7
0
H3-WT
H3-K36A
A
B
C
D
E
F

CHAPTER 3

REGULATION OF SET2-CATALYZED H3 K36 METHYLATION: CTK1 AND THE HhH DOMAIN OF SPT6 REGULATE SET2 AND H3K36me3.

Components of this chapter will be published as:

Youdell, M.*, Kizer, K.O.*, Kisseleva-Romanova, E.*, Duro, E., Strahl, B.D., and Mellor, J. (2007) The transcription elongation factor Spt6 promotes trimethylation of lysine 36 on histone H3. (submitted to *EMBO*)

* These authors made an equal contribution to this work.

Summary

Transcription in eukaryotes by RNA polymerase II requires the regulated disruption and reassembly of nucleosomes. A variety of protein complexes have been identified that mediate nucleosome disassembly/reassembly during transcription including FACT (facilitates chromatin transcription) and Spt6. Mutations in these histone chaperones lead to reassembly defects that result in the unregulated control of internal initiation in gene bodies. Here we show that Spt6 is required for Set2-catalyzed tri-methylation of histone H3 at lysine 36 (H3K36me3). Specifically, we find that the HhH domain of Spt6, from amino acids 931-994, is essential for H3K36me3, but not H3K36me1 or me2. We further show that the loss of H3K36me3 is accompanied by a loss of Set2 at the protein level, indicating an important role for the HhH domain in Set2 stability. Surprisingly, overexpression of Set2 in a Spt6 HhH mutant fails to restore H3K36me3. This data suggests that the HhH domain of Spt6 regulates some aspect of chromatin structure that is important for Set2-catalyzed methylation, which is supported by *in vitro* studies using recombinant Set2 on chromatin from a *spt6* mutant strain. Further investigations reveal that Ctk1 controls Spt6 association to genes, and in agreement with this, the loss of Ctk1 also reduces Set2 proteins levels. While the mechanism for Ctk1 regulation of H3K36me3 is thought to be primarily through Ctk1 phosphorylation of the RNA polymerase II (RNAPII) carboxy-terminal domain, our work demonstrates a new pathway of Ctk1 regulation of H3K36me3. Intriguingly, we find that the Bur1 kinase that stimulates RNAPII elongation also controls H3K36me3 and cryptic initiation, suggesting that Bur1, like Spt6, may be an upstream regulator of H3K36me3 via a similar mechanism of influence upon nucleosome organization.

3.1 Background

Histone lysine methylation is not regulated in a generic fashion, but rather in unique ways, consistent with the theory of the histone code (Briggs et al., 2002; Jenuwein and Allis, 2001; Lachner et al., 2003; Xiao et al., 2003b; Zhang and Reinberg, 2001). Methylation at H3 K4 in yeast is regulated through association of the Paf1 complex with RNAPII, subsequent H2B ubiquitination by PAF complex members, and then methylation by Set1. H3 K4 methylation is dependent on phosphorylation at the Ser5 position along the CTD, whereas Set2 directly interacts with the RNAPII-CTD in a manner dependent on Ctk1 phosphorylation of the Ser2 positions (Krogan et al., 2003b; Krogan et al., 2003a; Li et al., 2003; Li et al., 2002; Ng et al., 2003b; Schaft et al., 2003; Xiao et al., 2003b). Therefore Ctk1, with its role in maintaining RNAPII processivity along genes (Mason and Struhl, 2005), is also one factor involved in the regulation of Set2-catalyzed K36 methylation and its downstream function. After the publication of results from our laboratory and others which suggested a repressive effect of H3 K36 methylation in transcription elongation (Kizer et al., 2005; Li et al., 2003; Xiao et al., 2003b), several studies examined these effects in greater detail and found that H3 K36 methylation is a target for Rpd3C(S) binding through its Eaf3 member (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Through the K36me-Eaf3/Rpd3 association, Set2 can direct deacetylation at H3K14 and H4K12 in the coding regions of genes. Removing acetylation associated with the elongating polymerase is important to repress spurious transcription initiation from cryptic start sites within certain ORFs. These new data, published since the work presented in Chapter 2 (Kizer et al., 2005; Xiao et al., 2003b), promoted our interest in determining the pathway by which Set2 activity is regulated, and will also be discussed further in Chapter 5 where we present an up-to-date picture of the role and regulation of K36 methylation. As Set2 is known to be linked to transcription and RNAPII density along certain genes (Kizer et al., 2005; Li et al., 2003; Schaft et al., 2003; Xiao et al., 2003b), we selected two factors for examination, which at the

time, were known to be involved in the regulation of chromatin structure or certain phases of RNAPII transcription. In this chapter we describe our attempt to better understand the regulation of Set2 through an examination of these factors.

One factor we investigated for involvement in the regulation of Set2 activity is the Bur1 kinase, which was examined in an earlier study for its role in transcription elongation. Through the study by the Buratowski group, Bur1 kinase was examined for its ability to phosphorylate the CTD of Rpb1 (Keogh et al., 2003). Unlike Ctk1 or Kin28, Bur1 was not found to be a significant source of CTD phosphorylation *in vivo*, however it was found to associate with actively transcribing genes and to interact genetically with Ctk1, suggesting that Bur1 is required for efficient transcription elongation. The role of the Bur1/2 kinase in transcription was later expanded by others who showed that Bur1 and Bur2 selectively regulate H2B ubiquitination and H3 K4 tri-methylation (Laribee et al., 2005; Wood et al., 2005a). Through studies of the Rpd3C(S), it was also discovered that deletions of the Rpd3C(S) members, or *SET2* deletion, could rescue the growth of *BUR1* null strains, which are otherwise characterized as inviable (Keogh et al., 2003; Keogh et al., 2005). Based on these data we considered whether Bur1 would also regulate Set2 activity on genes.

In addition, existing data suggests that Spt16-Pob3 (yeast FACT) is involved in transcription elongation through genetic and physical interactions with defined elongation factors (Formosa et al., 2002; Krogan et al., 2003b; Squazzo et al., 2002). Other evidence suggested FACT may influence transcription initiation including data that FACT contributes to the repression of cryptic initiation (Kaplan et al., 2003; Malone et al., 1991). Preliminarily, we tested whether Spt16 and Set2 may functionally interact, however we found no evidence for an interaction *in vivo* or *in vitro* despite several attempts (data not shown). Although we initially considered FACT as a candidate protein in the upstream regulation of Set2, a recent paper from the Stillman laboratory, in collaboration with members of the Strahl lab, tested this hypothesis and found that *SET2* deletion can suppress the growth defects of a *spt16* or

pob3 mutation (Biswas et al., 2006). In their publication, they concluded that Set2 and FACT have opposing roles in transcription, as FACT promotes binding of RNAPII at promoters, while Set2 negatively regulates transcription as suggested by our earlier work (Kizer et al., 2005) and later by others (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005).

Secondly, we considered Spt6 as a possible regulator of Set2 function based in part on evidence that it controls the elongation and termination phases of transcription (Hartzog et al., 1998; Kaplan et al., 2003; Kaplan et al., 2005). Spt6 is capable of assembling nucleosomes *in vitro* and binding to H3 (Bortvin and Winston, 1996). As RNAPII transcribes through nucleosomes, histone removal and deposition are, in general, in equilibrium (Kristjuhan and Svejstrup, 2004; Schwabish and Struhl, 2004). Due to the ability of Spt6 to bind H3, others have postulated that Spt6 contributes to the maintenance of nucleosome integrity during the eviction and deposition occurring during transcription (Formosa et al., 2002; Saunders et al., 2003). Interestingly, it was also shown that certain Spt6 mutations result in faulty nucleosome reformation across genes and cryptic initiation from within certain genes (Kaplan et al., 2003). Based on these data we postulated that Spt6 is an upstream regulator of Set2 function.

Here we present an analysis of these candidate upstream regulators of Set2. However, several publications released while our studies were in progress either validated our preliminary data or resulted in changes to our hypotheses. These will be addressed in the Results and Discussion sections.

3.2 Materials and Methods

Yeast strains, genetic manipulation, and media

Yeast rich media (YPD) was prepared as 1% yeast extract, 1% peptone, and 2% dextrose. Media containing raffinose (YPR) or galactose (YPG) contained 2% raffinose or

2% galactose in place of dextrose. Yeast cultures (100ml) were grown in YPD, YPR, or YPD (as indicated) at 30°C with shaking, or at 39°C for 90min for inactivation of the *spt6-1004* allele. *S. cerevisiae* strains are based on the BY4741 background (Open Biosystems) unless otherwise indicated. The FY2181 "WT" *Mat*a *leu2Δ1 his4-912, lys2-128, SPT6/FLAG*, and FY2180 "*spt6-1004*" *Mat*a *leu2Δ1 his4-912; lys2-128; spt6-1004/FLAG* were a gift of F. Winston. The *bur1* double-deletion strains were a kind gift of S. Buratowski (Keogh et al., 2005). Genetic manipulations in these strains were performed using single step gene replacement with DNA fragments generated by PCR amplification (Gelbart et al., 2001). Genetic manipulations were confirmed by PCR detection of disruption cassettes and then by western blotting.

Generation of an antiserum specific for Set2

Bacterially expressed, recombinant Set2 containing N-terminal amino acids 1-261 was prepared using induction and purification procedures as previously described (Strahl et al., 2002). This N-terminal Set2 fragment was emulsified in Freund's Complete Adjuvant and injected into rabbits following protocol and procedures from Covance, Inc. The resulting bleeds from several injected rabbits were screened for specificity to Set2 via western blot analysis of yeast wild-type and set2Δ whole cell extracts. Antisera were compared to the matched pre-immunune serum for each rabbit as a control, and the bleed of highest antibody avidity was selected.

Preparation of WCE and immunoblotting

WCEs and western blotting protocols were performed as described in our earlier publication and presented in Chapter 2.2. The source and blotting concentrations of the H3K36me3, general H3, and G6PDH antibodies are described in Chapter 4.2 and were previously reported (Kizer et al., 2006). The Set2 antiserum was used at a concentration of 1:11000 except where otherwise indicated.

Co-immunoprecipitation

Co-IP experiments involving the WT and *spt6-1004* strains with the genomic *GAL1-SET2* fusion were performed essentially as described previously (Ch. 2 and (Kizer et al., 2005). In brief, WCEs were prepared from *GAL*-induced cell pellets of the WT and *spt6-1004* strains. Co-IPs contained 0.75 mg of extract brought to a final volume of 0.8 ml with Buffer II. The components of the WCE Buffer II have been described previously (Kizer et al., 2005; Kizer et al., 2006) and Chapter 2.2 and 4.2. Each sample was then incubated with 3 μl of Set2 (or H3 antibody in the case of the *spt6-1004* Histone H3 Co-IP) strain HA antibody for 2 h at 4°C, after which 10 ml of protein A sepharose beads were added to each tube and incubated for an additional 1 h. After incubations, the supernatant in each sample was separated from protein A beads by centrifugation, beads were rinsed 2 x 10 min with Buffer II, eluted using SDS-PAGE loading buffer and boiled 4 min prior to separation by SDS-PAGE.

Histone methyltransferase (HMT) assays

Yeast strains containing *SET2* expressed from *GAL1* promoter were grown in raffinose to prevent Set2 expression. Whole cell extracts were prepared as described (Kizer et al., 2005), except that the extraction buffer contained 150mM NaCl, 50mM Tris pH 9.0, 0.5mM EDTA and 10% glycerol. These extracts were then sonicated using the same procedure as described for the ChIP assay. Preparation and expression of full-length recombinant Set2 fused to calmodulin binding protein (rSet2) was performed essentially as described (Kizer et al., 2005; Strahl et al., 2002), where the resulting cell lysate was used directly for the in vitro histone methyltransferase assay. HMT reactions contained 100μg of sonicated yeast cell extract with or without 2μl of rSet2 lysate, HMT buffer (50 mM Tris-pH 9.0), and 50μM *S*-adenyosylmethionine. Samples were incubated for 45min at 30°C, resolved by SDS-PAGE and analyzed for H3K36 methylation by western blotting.

RT-PCR

Total RNA was prepared using hot acid-phenol, reverse transcribed, and *SET2* mRNA was analyzed by our collaborators using real time PCR with a Corbett Rotorgene and Sybr Green mix (Sensymix, Quantace). Real time PCR was used to amplify regions corresponding to those shown at *STE11*. Data was calculated (IP-NO)/TOT and expressed as a percentage of input. Error bars reflect the standard deviation of the average signal obtained between different experiments (n=2 to 4). The positions of *SET2* primers, of 20 bp, were +1880 (forward primer) and +2019 (reverse primer), relative to the start site.

3.3 Results

Spt6 is required for H3 K36 tri-methylation

Earlier observations of an spt6 mutant revealed that, like *set2Δ*, the *spt6-1004* mutation allows for initiation from cryptic sites within certain genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Kaplan et al., 2003; Keogh et al., 2005). As illustrated in **Figure 3.1A**, the *spt6-1004* mutation produces a form of Spt6 containing a deletion of the helix-hairpin-helix (HhH) domain between residues 931-994 (Doherty et al., 1996; Kaplan et al., 2000; Kaplan et al., 2003). This mutation results in temperature sensitive growth and altered nucleosomal structure at 39°C, (Kaplan et al., 2003). Therefore, we hypothesized that the *spt6-1004* mutant, like *set2Δ*, would have reduced or abolished K36me3. Recently, the Prelich laboratory investigated this possibility and found that H3K36me3 was absent in the presence of the *spt6-1004* mutation at a non-permissive temperature of 37°C (Carrozza et al., 2005; Chu et al., 2006). Interestingly, we found that *spt6-1004* mutation results in an abolition of H3K36me3 at *both* the permissive and non-permissive temperatures (**Fig. 3.1B**).

The Spt6-1004 mutant can still interact with H3 at 30°C

Spt6 has been shown to function similar to a H3 chaperone (Bortvin and Winston, 1996). Through our collaboration with the Mellor laboratory, we asked whether the *spt6-*

1004 mutant is incapable of interaction with H3, which could be related to the loss of H3 K36me3. Our collaborators found that FLAG-tagged Spt6 or *spt6-1004* could equally co-immunoprecipitate H3 in cell extracts prepared at the permissive temperature of 30°C (**Fig. 3.2A**, used with permission). In addition, our collaborators found that chromatin from the WT and Spt6^{1004} strains, when analyzed by micrococcal nuclease digestion, revealed an equivalent ladder of nucleosomes at the *STE11* locus (**Fig 3.2B**, used with permission). Collectively, these data along with previously published results (Kaplan et al., 2003; Kaplan et al., 2005) indicate that the Spt6 HhH domain is not required for H3 interaction or the distribution of nucleosomes at 30°C.

Loss of the Spt6 HhH domain affects Set2 protein levels

We further examined the relationship between Set2 and Spt6 by monitoring the levels of Set2 protein in the *spt6-1004* mutant at 30°C. The FY2181 (WT) and FY2181 (*spt6-1004*) strains were grown at 30°C, WCEs prepared and analyzed by western blotting with antibodies directed against H3 K36me3, H3 or the N-terminus of Set2. In addition, the *set2Δ*, *ctk1Δ*, and WT strains of the BY4741 background were analyzed in parallel. Unexpectedly, we found a near complete loss of Set2 protein in the *ctk1Δ* and *spt6-1004* strains. Although we note differences exist in the levels of Set2 across various WT strain backgrounds (**Fig. 3.3A-B**), the level of H3 K36me3 correlates with the amount of histone H3 in the extracts. We then asked whether this loss of Set2 protein could be due to decreased steady state transcript levels. As shown in **Figure 3.3C**, our collaborators found that *SET2* mRNA levels in the *spt61004* strain are equivalent to those in the WT strain. This suggests that despite subtle variations in Set2 protein levels based on the parent strain background, Ctk1 and Spt6 may regulate H3 K36me3 at least in part, through Set2 protein levels. Given this unexpected result suggests a link between Ctk1 and Spt6 in the regulation of Set2 function, we examined the Spt6 protein levels in a *ctk1Δ* strain. We found that *CTK1* deletion results in a significant reduction of Spt6 protein (**Fig. 3.3D**, used with

permission), indicating that in addition to regulating Set2-RNAPII binding via phosphorylation of the CTD, Ctk1 may also influence Set2 activity indirectly through the reduction of Spt6 protein abundance.

The Spt6 HhH domain is required for H3 K36 methylation by Set2

Given our results in **Fig. 3.3A** suggest that the absence of H3 K36me3 in the *spt6-1004* mutant is due to reduced Set2 protein levels, we then predicted that a restoration of Set2 protein would restore H3 K36me3 in the *spt6-1004* strain. We restored Set2 protein levels in the WT and *spt6-1004* strains by expressing *SET2* from its genomic locus using the *GAL1* promoter and then inducing *SET2* through growth in galactose. Similar levels of Set2 protein were detected in WCE prepared from the WT and *spt6-1004* strains when induced with galactose (**Fig. 3.4A, lanes 7 and 8**), while no Set2 is detectable in these same strains grown in raffinose (**Fig. 3.4A, lanes 3 and 4**). Unexpectedly, restoration of full Set2 protein levels in the *spt6-1004* strain was unable to restore H3 K36me3 (**Fig. 3.4A**). Although Set2 protein levels were restored in the *spt6-1004* strain, we reasoned that the inability of Set2 to methylate H3 K36 could be due to a defect in Set2's ability to bind to RNAPII. Using our N-terminal Set2 antibody, we performed co-IPs in both the WT and *spt6-1004* strains when *SET2* is driven by the *GAL1* promoter and expression is induced. When we blotted for the presence of RNAPII, we found that Set2 from both the WT and *spt6-1004* strains were able to immunoprecipitate phosphorylated RNAPII-CTD equally well (**Fig. 3.4B**). This result demonstrates that the inability of Set2 to methylate chromatin in the *spt6-1004* strain is not due to an inability of Set2 to interact with the polymerase. Collectively, these data support an intriguing possibility that the HhH domain of Spt6 is required for Set2 activity on genes, perhaps by arranging nucleosomes in a manner suitable for recognition by Set2.

Spt6 may configure H3 within nucleosomes for recognition by Set2

To test our hypothesis that Spt6 configures nucleosomes for recognition by Set2, we analyzed chromatin from the WT and *spt6-1004* strains in which *SET2* is not expressed

(**Fig. 3.4A lanes 4 and 5**). Using extracts from these strains as a source of chromatin, we incubated with or without recombinant Set2 and S-adenosylmethionine (SAM) for 30 min at 30°C. We analyzed these reactions by western blotting using antibodies directed against H3 K36me3, H3, or Set2. In support of our hypothesis, we detected a significant reduction in the ability of recombinant Set2 to methylate chromatin from the *spt6-1004* extract as compared to the WT extract (**Fig. 3.5**). For the control reactions, no H3 K36me3 was detectable when the extracts were incubated in the absence of recombinant Set2 (**Fig. 3.5, lanes 3 and 4**) or in the absence of SAM (data not shown). These results suggest that the chromatin in the *spt6-1004* strain is defective as a substrate for Set2.

Bur1 kinase is required for H3 K36 tri-methylation

Based on genetic interaction data (Krogan et al., 2002a) and published results showing that mutations in the BUR kinase can lead to cryptic initiation patters similar to Spt6 mutations, we next examined a potential upstream effect of Bur1 on Set2-catalyzed H3 K36me3. As deletion of *BUR1* is inviable, we could not measure H3 K36me3 in that background, but instead examined a strain deleted of the Bur1 cyclin, Bur2. Indeed, by western blot analysis we found that global H3 K36me3 levels in a *bur2Δ* strain are nearly abolished (**Fig. 3.6A**). These data were in agreement with earlier unpublished results by R. Laribee and T. Xiao.

Given deletion of *SET2* and downstream factors (Rpd3C(S) members) were found to suppress lethality of a *BUR1* deletion, and that *bur1* and *bur2* mutations were shown earlier to result in cryptic initiation within the *FLO8* gene (Kaplan et al., 2003), we next asked whether H3 K36me3 is affected in these strains. The rescued growth of the *BUR1* deletion in these double-deletion strains allowed us to test whether the absence of Bur1 would affect global levels of H3 K36me3. Interestingly, deletion of the genes coding for Rpd3C(S) members Eaf3, in combination with *BUR1* deletion, resulted in a loss of H3 K36me3 (**Fig. 3.6B**). These results are in support of a regulatory role for Bur1 upstream of Set2. In

support of these data, a later publication from the Prelich laboratory independently observed these results and further characterized the Bur1 effect on K36me3 at a gene specific level (Chu et al., 2006).

3.4 Discussion

The proteins Ctk1, Bur1, and Spt6 are already known to be required for proper transcription initiation or elongation (Kaplan et al., 2003; Keogh et al., 2003; Xiao et al., 2003b). We have demonstrated in this work a defect in H3K36me3 in a *bur1*, *bur2*, or *spt6-1004* mutant (**Fig. 3.1 and 3.6**), which during the course of our studies has been confirmed by recent independent observations (Chu et al., 2006). Our work presented here, along with other recent studies (Chu et al., 2006; Laribee et al., 2005), suggests that a likely component of Bur1 and Spt6's influence on transcription initiation and elongation is through their influence on H3K36me3. As discussed below, we propose a model whereby Ctk1, Bur1, and Spt6 influence H3K36me3 either directly or indirectly through proper configuring of nucleosomes prior to and after each round of transcription. To arrive at this model, we will first consider data regarding Spt6, followed by a discussion of Bur1 and then incorporate new and old data regarding Ctk1.

Spt6 as an upstream regulator of Set2

Collectively, the work presented here (and that of others) has demonstrated a link between Spt6, H3 K36 methylation and the repression of cryptic initiation (**Figs 3.1-3.6)** (Chu et al., 2006; Kaplan et al., 2003; Kaplan et al., 2005; Keogh et al., 2005). Prior work has already implicated Spt6, Spt4, and Spt5, in transcription initiation and elongation (Hartzog et al., 1998). Of particular interest is our observation that the *spt6-1004* mutation leads to a reduction of Set2 protein levels. The *spt6-1004* allele is deleted of the HhH domain, a domain which is suggested to recognize and bind to DNA (Doherty et al., 1996). Although this region is absent in *spt6-1004*, this strain has been shown to maintain normal

nucleosome density and positions at the permissive temperature (Kaplan et al., 2003; Kaplan et al., 2005). A disruption of H3 levels and nucleosome density over active genes is detected at the non-permissive growth temperature for *spt6-1004* of 39°C (Kaplan et al., 2003), while the binding of Spt6 to H3 is unaffected in the *spt6-1004* strain at the permissive temperature (**Fig. 3.2B**). As this additional loss of function is only detected during heat-inactivation of *spt6-1004*, it would appear that Spt6 has distinct functions including: 1) a histone chaperone function required for nucleosome positioning (Kaplan et al., 2003) and 2) a DNA binding capability via the HhH domain leading to separate downstream functions such as H3K36 methylation (Bortvin and Winston, 1996; Doherty et al., 1996). The distinct functions of Spt6 is supported by data of other Spt6 mutants which have normal levels of H3K36me3, still contain the HhH domain, yet are defective for chaperone function (Chu et al., 2006).

We propose the following possibilities which could explain the regulation of H3K36me3 by Spt6. 1) Through the ability of the Spt6 HhH domain to bind DNA, we propose that Spt6 regulates the orientation and/or configuration of nucleosomes in a manner conducive for Set2 catalysis of H3K36 methylation, which may first require Spt6 to bind histones via its histone chaperone activity. Our results presented in **Figure 3.5** support this model, as do other published data (Kaplan et al., 2003; Kaplan et al., 2005). We do not rule out a potential effect of Spt6 on any of the recently identified histone demethylases (for recent review, see (Shi and Whetstine, 2007), as will be discussed further in Chapter 5. Yet, based on our model, what is the cause of the reduction in Set2 protein levels? We note that an influence of Spt6 in the proper translation of Set2-coding mRNA cannot yet be ruled out, however we predict that the absence of a properly configured nucleosomal substrate for Set2 releases the protein for targeting by degradation machinery. This is consistent with recent data that revealed an abnormally short half-life for Set2 (Belle et al., 2006). The preference of Set2 for properly configured nucleosomes is suggested by earlier data

revealing Set2's preference for nucleosomes over free histones *in vitro* (Strahl et al., 2002). 2) Spt6 may stabilize Set2, perhaps through a direct or indirect interaction with Set2, dependent in some unknown fashion on the HhH domain of Spt6. We note that our collaborators were unable to detect any interaction between Set2 and Spt6 *in vivo*, however the possibility remains that Spt6 stabilizes Set2 indirectly through Spt6 interaction with an unknown factor. 3) Spt6 may recruit other factors to chromatin via its HhH domain, which in turn affects chromatin structure and/or Set2 in a way that promotes Set2 recognition of nucleosomal substrates. Consistent with this possibility, Spt4 and Spt5 have been found to interact with Spt6 and also influence nucleosomal structure (Krogan et al., 2003b; Lindstrom et al., 2003; Swanson and Winston, 1992). In addition, a recent report reveals a striking similarity between the functions of Spt6 and Spt2, such as Spt2 association with the coding regions of genes, repression of transcription from cryptic promoters, and regulation of the histone H3 levels over transcribed regions (Nourani et al., 2006). When *SPT2* deletion was combined with deletions of either *CDC73* (PAF complex) or *HIR3*, but not *SPT2* deletion alone, cryptic transcription was observed as with *spt6-1004* (Nourani et al., 2006). Strikingly, the recruitment of Spt2, also a histone chaperone, was found to be defective in the *spt6-1004* mutant (Nourani et al., 2006). Spt2 has also been shown to directly recruit polyadenylation machinery (Hershkovits et al., 2006). 4) Finally, the HhH domain itself may adjust the chaperone function of Spt6, allowing it to adjust nucleosomal structure in a way not yet understood. We considered this possibility based on the recently identified acetylation within Hsp90 which allows subsequent regulation of this protein's chaperone function (Scroggins et al., 2007). Based on existing data, the first and third possibilities described above are the most consistent with the established coupling of histone modifications with the regulated disruption and reassembly of nucleosomes during transcription, as chromatin shifts between a transcriptionally permissive or non-permissive state (Mellor, 2006a; Workman, 2006). We therefore have based our model of Spt6 function

on the first explanation described above, which also appears most consistent with data regarding the effects of transcription factors Bur1 and Ctk1 on H3K36 methylation as discussed below.

Bur1 kinase as an upstream regulator of Set2

The Bur1 kinase has already been shown to selectively influence H3K4 tri-methylation through reduction of Paf1 complex-mediated H2B ubiquitination (Laribee et al., 2005; Wood et al., 2005a). We and others have observed a reduction of H3K36 methylation in *bur1* or *bur2* mutants, while Bur1 is also implicated in the pathway of Set2 regulation via genetic links between Bur1, the Rpd3C(S) members (Eaf3 and Rco1), and Rad6 (**Fig. 3.6,** (Chu et al., 2006; Keogh et al., 2005). How is this regulation of Set2-dependent H3K36 methylation accomplished by Bur1? We suggest the following three possibilities to address this question.

One possibility involves an influence of Bur1 on other transcription elongation factors with similar function, such as the yeast DSIF complex, which includes Spt4 and Spt5. Recently it was shown that when Spt4 is absent, a selective reduction of H3K4me3 results (Qiu et al., 2006). In addition, this reduction follows, and is dependent on, a similar reduction of PAF complex recruitment to genes. Based on similarities between the roles of yeast DSIF and Bur1 in the regulation of H3K4me3 and PAF-mediated H2B ubiquitination, we suggest that Bur may influence Spt4 and Spt5 which then regulate chromatin integrity in a manner similar to that which we proposed for Spt6. It is also interesting to note that Spt6, like Spt4 and Bur1, has been shown to affect the recruitment of the PAF complex member Ctr9 to genes, although Spt6's effect does not appear significant enough to affect H3K4me3 levels (Chu et al., 2006; Kaplan et al., 2005). In addition to the similar functions of Spt6 and Spt2 described earlier, we note that Spt2 has been shown to interact genetically with PAF. Most importantly, PAF is required for Spt2 recruitment to transcribing genes. As Bur1 has been shown to affect PAF complex-mediated H2B ubiquitination, it follows that Bur1 may

affect Spt2 recruitment to genes, via the Bur1 effect on PAF (Nourani et al., 2006). As described earlier, deletion of *SPT2* in combination with deletion of *CDC73* (PAF), but neither deletion alone, results in cryptic transcription as seen with mutation of *SET2, BUR1* or *SPT6*. Consistent with these data, we found no defect in H3K36me3 in a *SPT2* deletion strain (data not shown). Based on the observation of a cryptic initiation defect in the *SPT2* and *CDC73* double deletion strain, it remains a possibility that Spt2, with PAF complex, have redundant roles in the regulation of H3K36me3. Mutation of Bur1 is known to cause cryptic transcription (Kaplan et al., 2003), consistent with an effect on H3K36me3, but is likely not due to Bur1's effect on Paf1, since *CDC73* deletion alone does not result in the presence of cryptic transcripts (Nourani et al., 2006). This supports a model that one downstream function of Bur1, possibly through PAF, is to regulate other transcription factors such as Spt2 – a protein similar to function in Spt6 through configuring nucleosomes (Nourani et al., 2006). We recognize that existing data indicates Spt4/5 and Spt6 may have opposing roles, however we also note that dual roles (positive and negative) have been reported for certain transcription factors, such as Spt4/5 (Lindstrom et al., 2003). Further investigation of potential effects of Bur1 on Spt6, Spt2, or Spt4/5 will be crucial in testing this hypotheses.

A second possibility of the mechanism for Bur1 regulation of Set2 involves Bur1-mediated phosphorylation of Set2 (or other factors) which in turn influences Set2 recognition of nucleosomal substrates and subsequent catalysis of H3K36me3. A third possibility is that Bur1 regulation of Set2 does not involve Spt2, Spt4/6 or Spt6, but rather the defect of H3K36me3 in a *BUR1* deletion may be a consequence of general transcription defects in a *BUR1* mutant. This possibility could be consistent with observations that the level of H3K36me3 is correlated with actively transcribing genes, which would likely be affected by mutation of the transcription factor Bur1 (Keogh et al., 2003; Pokholok et al., 2005), as will be described further in Chapter 5. These last two possibilities have been suggested by

others (Chu et al., 2006), however in our opinion, the first possibility is the most consistent with existing data demonstrating similarities of Spt4, Spt5, and Spt6 in the regulation of transcription elongation (Winston, 2001).

An alternate mechanism for Ctk1 regulation of Set2-catalyzed H3 K36 methylation

The enzyme Ctk1 was previously thought to regulate Set2 function solely through phosphorylation of Ser2 repeats along the CTD. Our data are the first to reveal that Set2 protein levels are nearly abolished in the absence of Ctk1. In addition, Set2 protein levels are also reduced in a *spt6-1004* mutant while Spt6 protein is itself in lower abundance when *CTK1* is deleted. Our work suggests a new mechanism for Ctk1 regulation of Set2-catalyzed H3K36me3 - through Ctk1's affect on the Spt6 protein.

A model for nucleosomal integrity and Set2-mediated methylation during transcription

It has been shown that strains containing either the *spt6-1004* allele at 39°C, deletion of *CTK1* or deletion of *BUR2* have reduced histone H3 levels (Chu et al., 2006; Laribee et al., 2005; Xiao et al., 2007). Could it be that *spt6-1004*, *ctk1Δ*, or *bur1Δ* each result in a defect in nucleosome integrity via a conformation change of histone H3, while overall nucleosome density is unaffected, that results in the observed effects on Set2 protein and H3K36me3? We propose a model, illustrated in **Figure 3.7**, whereby nucleosome integrity is influenced either directly as in the case of Spt6, or indirectly through Spt6 by Ctk1, or potentially via Spt2 by Bur1. Defects in nucleosome integrity could restrict recognition by the methyltransferase Set2, and contribute to the downstream effect of increased acetylation and cryptic initiation. We predict that when Set2 is unable to locate and bind to the proper substrate, it is subsequently targeted for degradation.

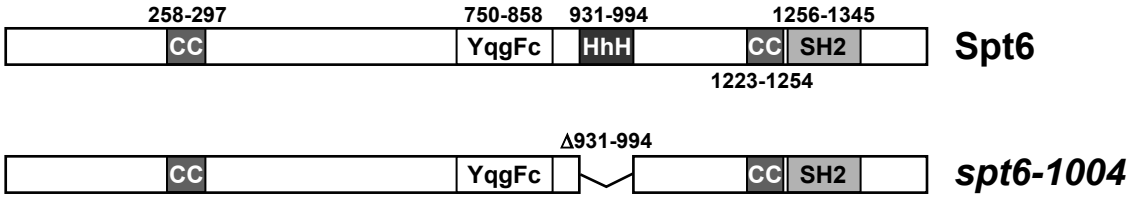
Our model is consistent with suggestions that only certain histone modifications are present on a nucleosome at a particular point in the transcription cycle (reviewed in (Mellor, 2006a). As most studies, including ours, were performed in unsynchronized cells, changes in H3K36me3 could occur at various stages of transcription but would be missed using our

analysis methods. Much work is needed to test this model. We note that our observed results which indicate Set2 has a reduced ability to methylate chromatin in the *spt6-1004* strain (**Figs. 3.1 and 3.4**) only suggest a role that altered nucleosomes are the cause of this effect. Even carefully purified nucleosomes from the *spt6-1004* could potentially have an unknown negative regulator of Set2 present. Ideally, future work will involve the preparation of reconstituted nucleosomes in the presence or absence of Spt6 (and compared to the Spt6 mutant of the *spt6-1004* allele). A thorough test of the hypothesized pathways of Bur1, Spt6, and Ctk1 influence on Set2 (**Fig. 3.7**), regardless of the results, will provide for a greater understanding of the upstream regulation of Set2.

Figure 3.1: Spt6 is required for histone H3 lysine 36 tri-methylation.

(**A**) Schematic representation of the protein products of the *SPT6* or *spt6-1004* strains. The following domains and their known or predicted functions are indicated in the schematic. The YqgFc domain (YqgFc) is likely a non-functional derivative of a Holliday junction resolvase catalytic domain. The Helix-hairpin-helix domain (HhH) is predicted to have generic DNA binding capability based on structural evidence of this domain in other proteins (Doherty et al., 1996). The SH2 domain (SH2) was identified in humans as a phospho-tyrosine recognition domain found to bind proteins at residues near, but not including, a phosphorylated tyrosine. The putative coiled-coil motifs (CC) are also indicated. The positions of the indicated domains and predicted functions were identified using the SMART database and published results (Kaplan et al., 2003). (**B**) The indicated strains were grown at to the reported permissive (30°C) or non-permissive (39°C) temperatures for the *spt6-1004* allele (Kaplan et al., 2003) and WCEs were prepared. Extracts were resolved by SDS-PAGE, and analyzed by western blot using antibodies directed against H3K36me3 (αH3K36me3), the metabolic enzyme glucose-6-phosphate dehydrogenase (αG6PDH; loading control), or the C-terminus of H3 (αH3; loading control).

A


258-297
750-858
931-994
1256-1345
CC
YqgFc
HhH
CC
SH2
Spt6
1223-1254
Δ931-994
CC
YqgFc
CC
SH2
spt6-1004

B

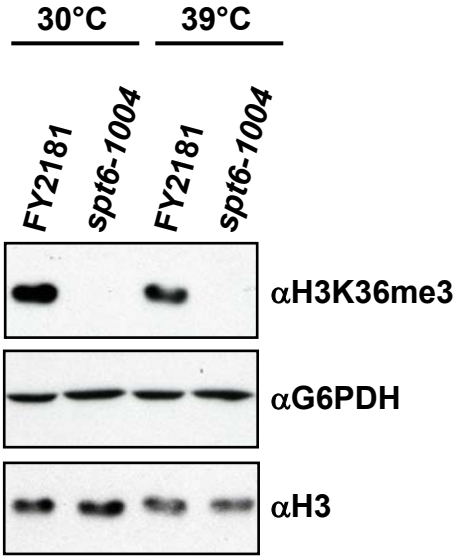
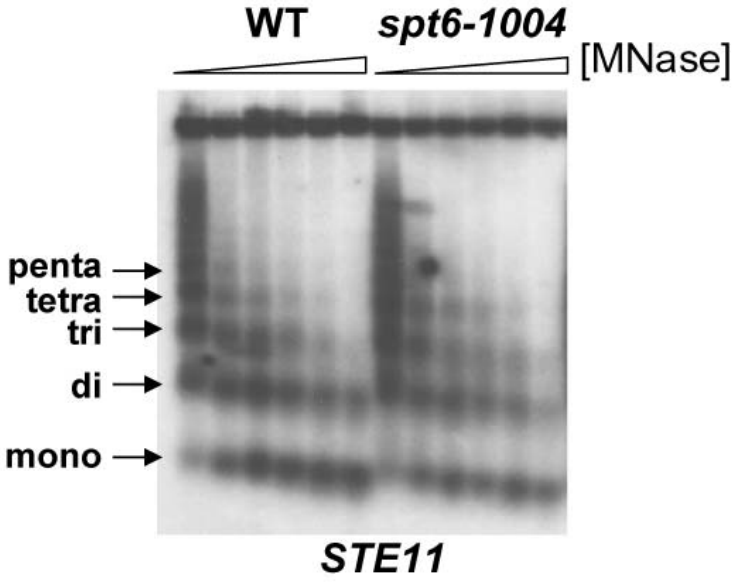

30°C
39°C
FY2181
spt6-1004
FY2181
spt6-1004
αH3K36me3
αG6PDH
αH3

Figure 3.2: Spt6-dependent nucleosome density and H3-binding are unchanged in the *spt6-1004* strain at 30°C.

(**A**) A micrococcal nuclease (MNase) digestion was completed using the indicated strains, cultured at 30°C, permeabilized and incubated with increasing concentrations of micrococcal nuclease (MNase; 15, 40, 75, 150, 300 and 600 units per ml) for 4 minutes at 30°C. DNA was isolated, separated by size and hybridized with a probe from the 3' region of *STE11*. The positions of migration of mono-, di- and tri-, tetra- and penta- nucleosomal sized fragments are indicated. This panel and matching protocol were obtained from our collaborators, the Mellor laboratory, and used with permission from J. Mellor. (**B**) Total cell extracts from strains FY2181 and FY2180 expressing FLAG-tagged derivatives of Spt6 cultured at 30°C were immunoprecipitated with (+) or without (-) anti-FLAG antibodies and precipitates collected on protein A sepharose. Precipitated material, together with aliquots of the input cell extracts (Input, lane 1) and the supernatant remaining after separation of precipitates (Sup) with (+) (lanes 2 and 5) or without (-) (lanes 3 and 7) antibody were separated on a 15% gel and western blotted with an antibody to the C-terminal region of histone H3 (Abcam). Lanes 4 and 6 are empty. This panel and matching protocol were completed by our collaborators, the Mellor laboratory, and used with permission from J. Mellor.

A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spt6-1004
[MNase]
penta
tetra
tri
di
mono
STE11

B

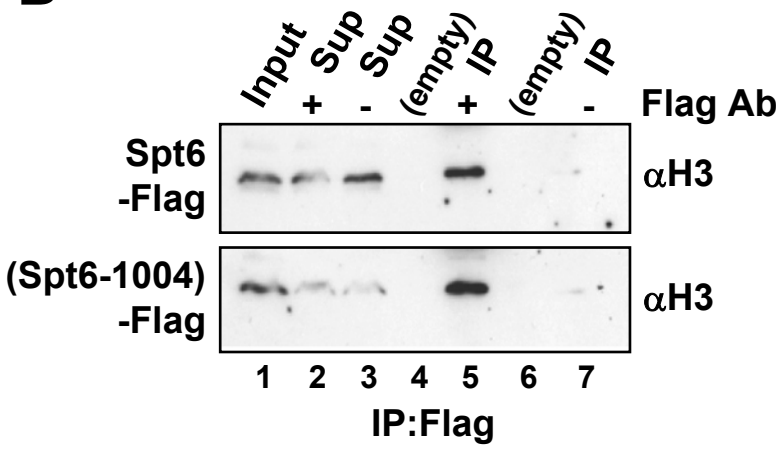

Input
Sup
Sup
(empty)
IP
(empty)
IP
+
-
+
-
Flag Ab
Spt6
-Flag
αH3
(Spt6-1004)
-Flag
αH3
1 2 3 4 5 6 7
IP:Flag

Figure 3.3: Ctk1 and Spt6 control Set2 protein stability.

(**A**) The indicated strains were grown at 30°C and WCEs were prepared. Extracts were analyzed by western blotting using antibodies directed against Set2 (αSet2), H3K36me3 (αH3K36me3), the metabolic enzyme glucose-6-phosphate dehydrogenase (αG6PDH; loading control), or the C-terminus of H3 (αH3; loading control). (**B**) The indicated strains of the BY4741 background were grown as in part (A) and analyzed by western blotting using antibodies directed against Set2 (αSet2), H3K36me3 (αH3K36me3), or the C-terminus of H3 (αH3; loading control). The *cps30Δ* strain (lacking the COMPASS member Cps30) was used as an additional positive control for the presence of Set2. The Set2 band of higher intensity, as compared to the same strain in (A), is due to a longer exposure time and higher antibody concentration (1:10000 vs. 1:11000) used here. (**C**) Steady-state levels of *SET2* mRNA were monitored using PCR amplification of *SET2* and U4 snRNA primers. The data are displayed as a ratio of signals for the *SET2* mRNA over the U4 snRNA loading control. This panel and matching protocol were obtained from our collaborators at the Mellor laboratory and used with permission. (**D**) WCEs were prepared from WT and *ctk1Δ* strains in which the HA-tagged Spt6 was expressed from the genomic locus. HA-Spt6 levels were monitored by western blot using antibodies against HA (α-HA) and tubulin (atubulin) as a loading control. This panel and matching protocol were obtained from our collaborators at the Mellor laboratory and used with permission.

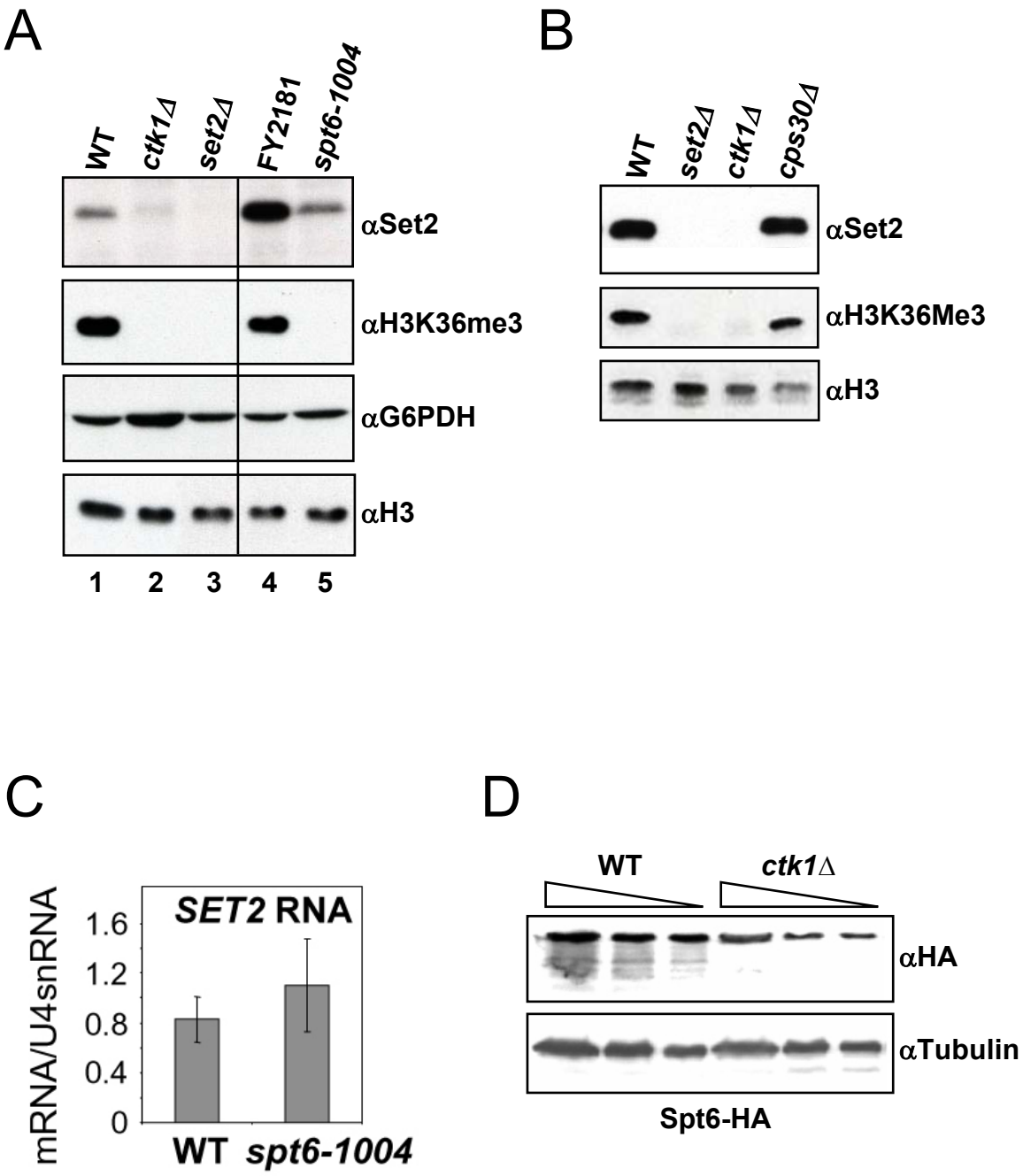
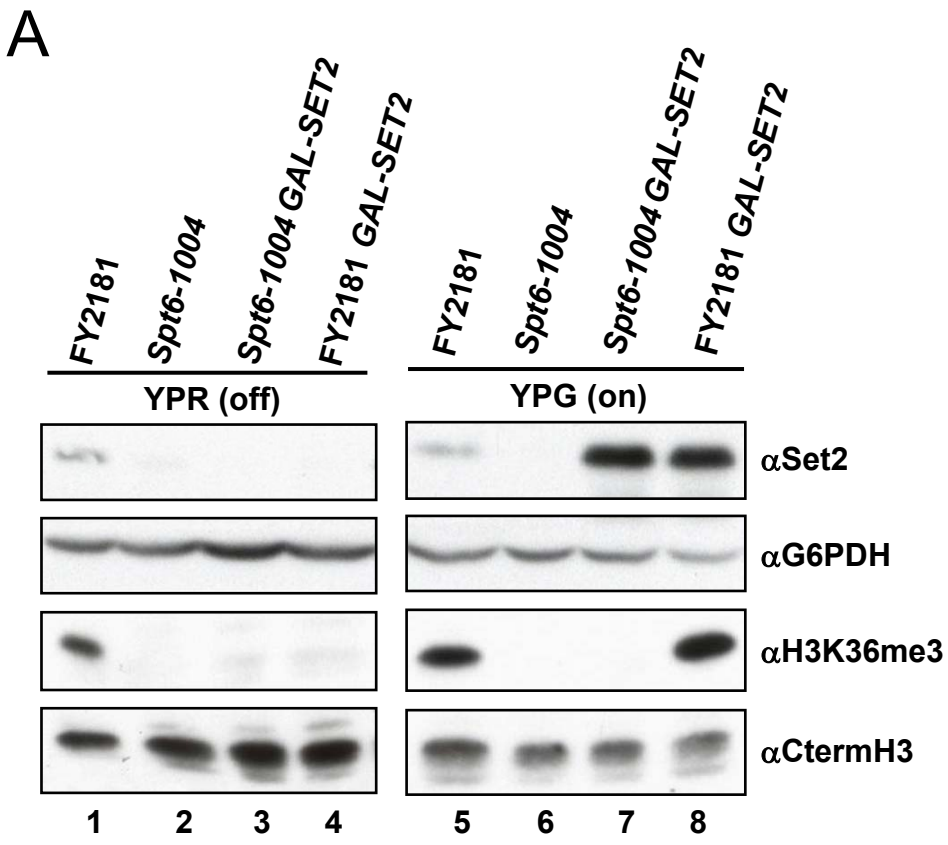

A
WT
ctk1Δ
set2Δ
FY2181
spt6-1004
αSet2
αH3K36me3
αG6PDH
αH3
1 2 3 4 5
B
WT
set2Δ
ctk1Δ
cps30Δ
αSet2
αH3K36Me3
αH3
C
SET2 RNA
mRNA/U4snRNA
1.6
1.2
0.8
0.4
0
WT spt6-1004
D
WT
ctk1Δ
αHA
αTubulin
Spt6-HA

Figure 3.4: Over-expression of *SET2* in the *spt6-1004* strain cannot restore H3K36me3.

(**A**) WCEs were prepared from the indicated strains in which the *GAL1* promoter was fused to the *SET2* genomic locus. Strains were grown at 30°C in raffinose (YPR) for repression of *SET2* (lanes 1-4), or in galactose (YPG) to induce *SET2* expression (lanes 5-8). The extracts were analyzed by western blotting using antibodies against Set2, H3K36me3, histone H3, or G6PDH. (**B**) Set2 from the *spt6-1004* strain was assayed for association with RNAPII by Co-IP. The indicated strains were grown in YPG to induce Set2 expression as in (A), and whole cell extracts were prepared. Co-IPs were performed in a final volume of 0.8 ml, equalized with extraction buffer, containing 1.5 mg of WCE protein. The extracts were incubated with 4μl of Set2 Ab or 4μl of the matched pre-immune serum for 2 h at 4°C, after which each sample was incubated for 1h with 15 ml protein A sepharose beads (GE Healthcare). The beads were then washed 2x10min in extraction buffer, eluted in SDS-PAGE loading buffer, incubated at 100°C for 5 min, and bead-bound proteins analyzed by immunoblot analysis using antibodies targeted against the Ser5 phospho-CTD (H14, Covance Inc.) or the Set2 antibody.


A
FY2181
Spt6-1004
Spt6-1004 GAL-SET2
FY2181 GAL-SET2
YPR (off)
YPG (on)
αSet2
αG6PDH
αH3K36me3
αCtermH3
1 2 3 4 5 6 7 8

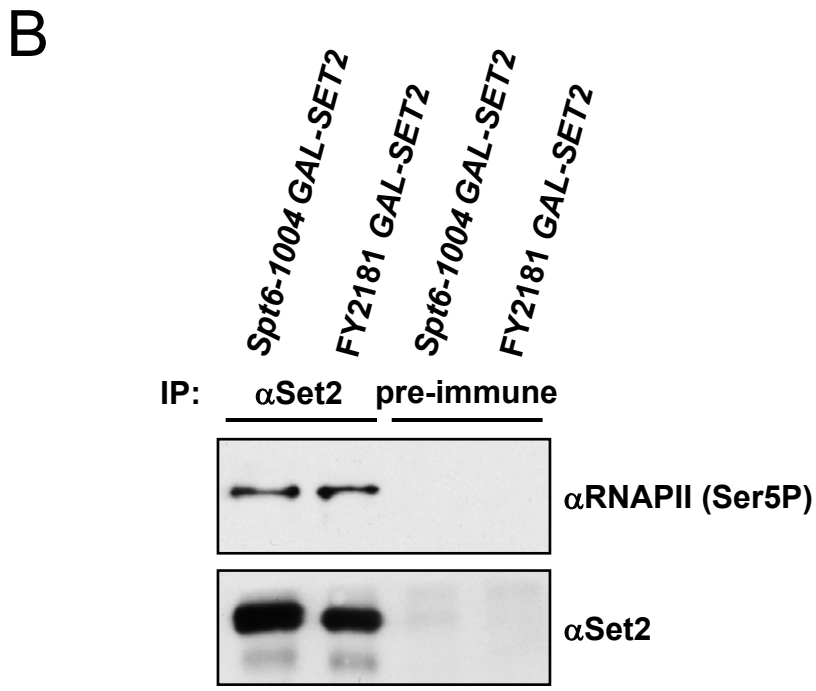

B
Spt6-1004 GAL-SET2
FY2181 GAL-SET2
IP:
αSet2
pre-immune
αRNAPII (Ser5P)
αSet2

Figure 3.5: Chromatin from the *spt6-1004* strain is a poor substrate for Set2.

The indicated strains were grown in YPR (raffinose) to ensure repression of *SET2* under the *GAL1* promoter. Equal amounts of sonicated whole cell extract were used in a histone methyltransferase (HMT) reaction in which the extracts were incubated with 50 μM SAM and with (lanes 1-2) or without (lanes 3-4) an equal amount of purified recombinant Set2 (rSet2) (Kizer et al. 2005) at 30°C for 45 minutes. Aliquots of the reaction were analyzed by western blot using antibodies against Set2, H3K36me3 and histone H3. Asterisks indicate breakdown products of rSet2 and H3, likely due to incubation at 30°C during the HMT reaction.

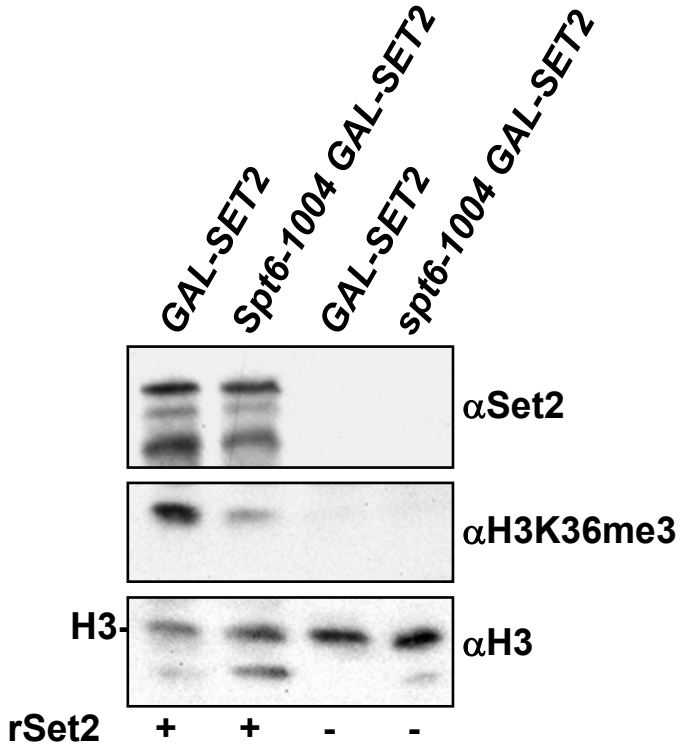
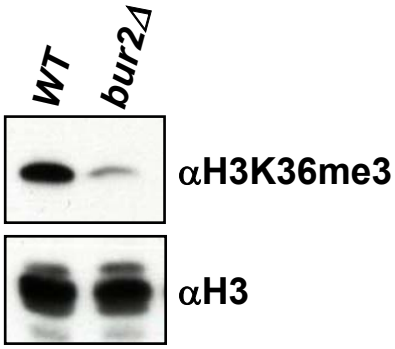

GAL-SET2
Spt6-1004 GAL-SET2
GAL-SET2
spt6-1004 GAL-SET2
αSet2
αH3K36me3
H3-
αH3
rSet2 + + - -

Figure 3.6: Bur1 and Bur2 are required for histone H3 lysine 36 tri-methylation.

(**A**) WCEs of the *BUR2* deletion strain and its matching parent strain (BY4741) were analyzed by western blot using antibodies against H3K36me3 (αH3K36me3) or the C-terminus of H3 (αH3) as a loading control. Approximately two times the extract amount was loaded for the *BUR2* deletion strain (according to total protein concentration) due to the decreased histone levels reported for that strain (Laribee et al., 2005). (**B**) Since *BUR1* deletion is inviable, we tested whether *BUR1* affects H3K36me3 using the indicated double-deletion strains that were reported to rescue *BUR1* lethality (Keogh et al., 2005). WCEs of the indicated strains were analyzed by western blot using antibodies directed against H3K36me3 (αH3K36me3) or the C-terminus of H3 (αH3) as a loading control. The indicated strains were a kind gift from the Buratowski laboratory.

A


WT
bur2Δ
αH3K36me3
αH3

B

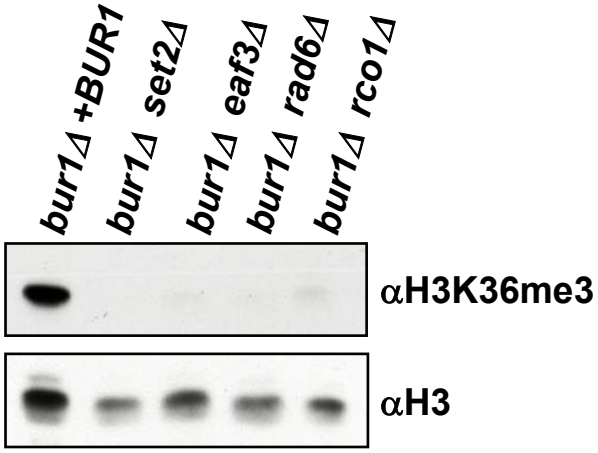
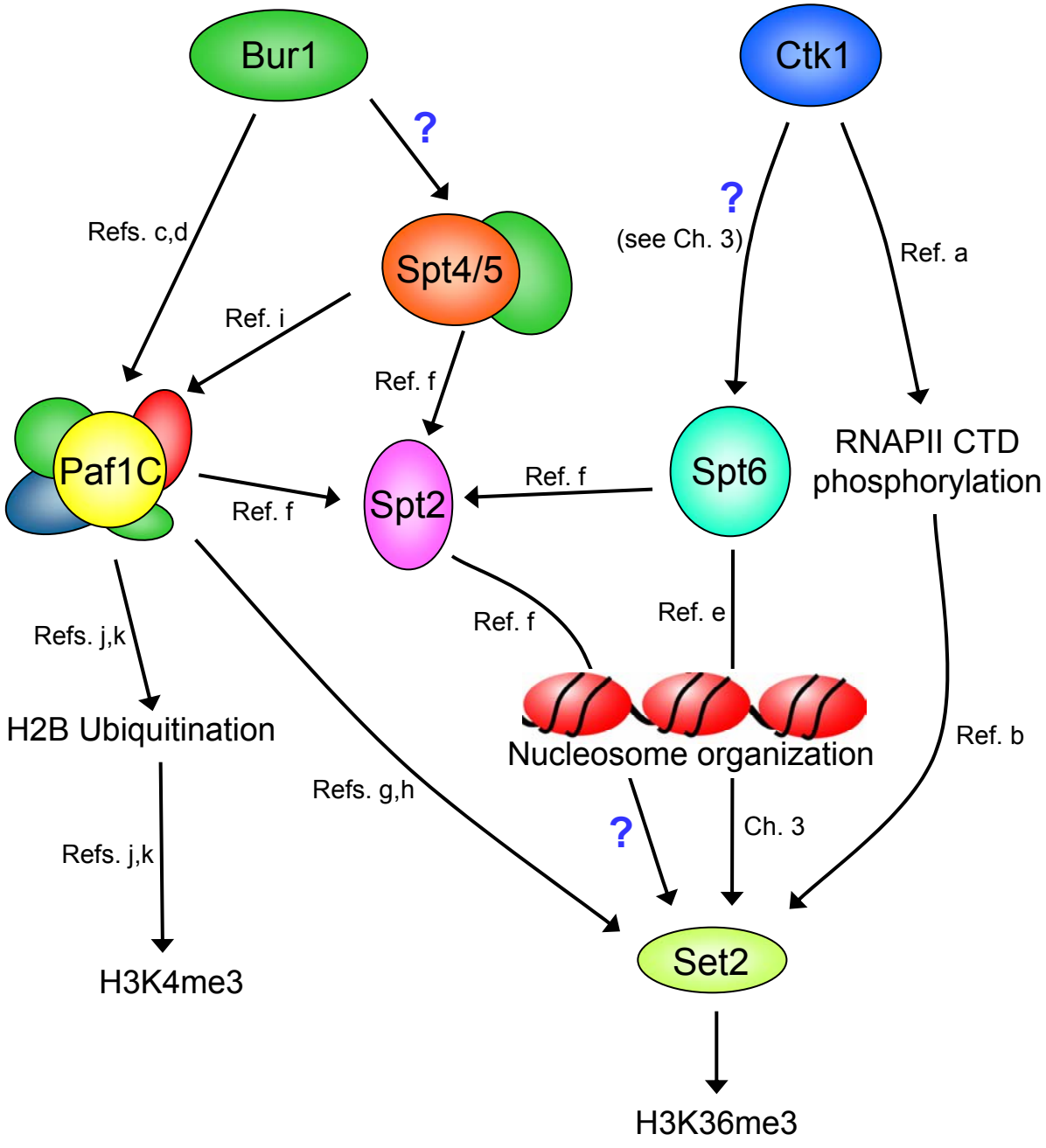

bur1Δ +BUR1
bur1Δ set2Δ
bur1Δ eaf3Δ
bur1Δ rad6Δ
bur1Δ rco1Δ
αH3K36me3
αH3

Figure 3.7: A possible model for the pathway of Ctk1, Bur1, and Spt6 effects on Set2.

Based on results from our laboratory and others, Ctk1, Bur1, and Spt6 are known to control H3K36me3. We have demonstrated that Ctk1 and Spt6 influence Set2 protein levels and therefore may specifically regulate H3K36me3 using similar mechanisms. Alternative possibilities for these effects are discussed in the text. The reference abbreviations indicated in the illustration are as follows: a, (Lee and Greenleaf 1989); b, (Xiao et al. 2003b); c, (Laribee et al. 2005); d, (Wood et al. 2005a); e, (Kaplan et al. 2003); f, (Nourani et al. 2006); g, (Wood et al. 2003); h, (Krogan et al. 2003b); i, (Qiu et al. 2006); j, (Sun et al. 2002); k, (Dover et al. 2002).


Bur1
Ctk1
?
Refs. c,d
Spt4/5
?
(see Ch. 3)
Ref. a
Ref. i
Ref. f
Paf1C
Spt2
Ref. f
Spt6
RNAPII CTD
phosphorylation
Ref. f
Refs. j,k
Ref. f
Ref. e
H2B Ubiquitination
Nucleosome organization
Ref. b
Refs. g,h
?
Ch. 3
Refs. j,k
Set2
H3K4me3
H3K36me3

CHAPTER 4

ACCELERATED NUCLEI PREPARATION AND METHODS FOR ANALYSIS OF HISTONE MODIFICATIONS IN YEAST.

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* These authors made an equal contribution to this work.

Summary

The continuing identification of new histone post-translational modifications and ongoing discovery of their roles in nuclear processes has increased the demand for quick, efficient, and precise methods for their analysis. In the budding yeast *Saccharomyces cerevisiae*, a variety of methods exist for the characterization of histone modifications on a global scale. However, a wide gap in preparation time and histone abundance exists between the most widely used extraction methods, a simple whole cell extraction (WCE) and an intensive histone extraction. In this work we evaluate various published WCE buffers for their relative effectiveness in the detection of histone modifications by western blot analysis. We also present a precise, yet time-efficient method for the detection of subtle changes in histone modification levels. Lastly, we present a protocol for the rapid small-scale purification of nuclei that improves the performance of antibodies that do not work efficiently in WCE, and aids in the detection of histone modifications that are low in abundance. These new methods are ideal for the analysis of histone modifications and could be applied to the analysis and improved detection of other nuclear proteins.

4.1 Background

The investigation of chromatin structure and function in eukaryotes continues to be a fast-paced field. The basic unit of the highly organized chromatin structure is the nucleosome, in which ~147 base pairs of DNA is wrapped around an octamer of the core histone proteins (Kornberg, 1977; McGhee and Felsenfeld, 1980). Chromatin is crucial for protection of the genome from environmental insult, as well as the regulation of all processes in eukaryotes using the DNA template, such as gene transcription, replication, and recombination. The N- and C-terminal "tail" domains of the histone proteins extend away from the nucleosome core particle and, along with the globular domains, are targeted for various post-translational modifications including methylation, acetylation and phosphorylation (Briggs et al., 2002; Feng et al., 2002; Strahl and Allis, 2000). Studies have revealed that these modifications are involved in transcriptional regulation, and likely specify a 'histone code' by which the proper regulation of chromatin structure and gene expression is maintained (Lachner and Jenuwein, 2002; Strahl and Allis, 2000; Turner, 2000).

Histone methylation, in particular, has recently been intensely studied. While lysines can be mono-, di-, or trimethylated, arginines can be either mono- or dimethylated (Briggs et al., 2001; Miranda et al., 2006; Santos-Rosa et al., 2002; Strahl et al., 1999; Strahl et al., 2001; van Holde, 1989; Zhang and Reinberg, 2001). Strikingly, recent studies show that the distinct methyl states of these residues can be independently regulated and are subject to demethylation (Eissenberg and Shilatifard, 2006; Klose et al., 2006; Laribee et al., 2005; Schneider et al., 2005; Shahbazian et al., 2005; Tsukada et al., 2006; Wood et al., 2005a). Modest or dramatic changes (local or genome-wide) in histone methylation can result in significant effects on chromatin organization, the outcome of other histone modification patterns, and gene transcription (Lee et al., 2005; Shilatifard, 2006; Wood et al., 2005b).

Key to our understanding of chromatin function is the detection of even modest changes in the level of histone post-translational modifications. Most often, detection

requires high-quality antibodies specific to the modification of choice and, in many cases, core histones extracted using a time-intensive procedure. Analyses of global changes in histone modifications in yeast commonly begin with a whole cell extraction (WCE), however much variability exists in the components of buffers that are widely used (Briggs et al., 2001; Keogh et al., 2002; Kizer et al., 2005; Xiao et al., 2003b). It is unclear whether the differences between these distinct extraction buffers (i.e. salt type and/or salt concentration) lead to changes in histone yield or purity.

Outside of rapid WCE production, labor-intensive histone acid-extraction protocols are often needed to detect rare or low abundance histone modifications in yeast, which otherwise may be missed by the use of WCE alone (Seligy et al., 1980; Wintersberger et al., 1973). In addition, certain antibodies are difficult to use with WCE due to either low abundance of the antigen or a large relative amount of cytoplasmic proteins in the extract, resulting in detection of a large number of cross-reacting bands. In these cases, the histone acid-extraction method can provide purified histones that are suitable for analysis by western blotting. Although a variety of nuclear isolation/histone acid-extraction protocols over several decades have been described, the vast majority require large scale cultures and multiple time-consuming centrifugation and wash steps (Almer and Horz, 1986; Braunstein et al., 1993; Edmondson et al., 1996; Lowary and Widom, 1989; Santos-Rosa et al., 2003; Seligy et al., 1980; Wintersberger et al., 1973). While a rapid method used to detect the histone ubiquitin moiety has been presented by others, an abbreviated method for the analysis of low-abundance nuclear proteins, histone methyl states, or for detecting small changes in methylation states would be beneficial (Kao and Osley, 2003).

In this chapter, we compare several widely used WCE buffers with the goal of identifying whether a particular salt type or concentration would preferentially enrich for histones. We also describe a titration loading method that maximizes the precision of histone modification analysis via WCE, an approach recently used to demonstrate changes

in various histone modification states (Laribee et al., 2005; Qiu et al., 2006). Finally, we present a protocol for the small-scale and streamlined preparation of crude nuclei suitable for histone modification analyses by western blot procedures. These crude nuclei are enriched in histones, and although the purity is not at the level of most histone purification methods that involve acid extraction, they provide an ideal starting material for the rapid analysis of histone modifications (and presumably other nuclear proteins) that are in low abundance or to which only low avidity antibodies are available.

4.2. Methods

4.2.1 Detection of histone modifications in budding yeast using whole cell extraction.

Whole cell extraction (WCE) by glass bead disruption is the most common method of lysing yeast cells for histone modification analysis. This method is rapid and effective for analyzing most histone modifications from a large number of yeast strains simultaneously. Interestingly, buffers have been used which contain a wide variety of salt types and concentrations, as well as other components (Briggs et al., 2001; Keogh et al., 2002; Kizer et al., 2005; Xiao et al., 2003b). A recent publication thoroughly analyzed sample preparation methods and buffers for their effectiveness in global metabolite extraction, and reported that some buffer components enrich for certain metabolites (Villas-Boas et al., 2005). Therefore, will altering WCE buffer components such as salt type or concentration increase the relative yield of histone proteins extracted, and therefore result in clearer analysis with antibodies directed against histone modifications? We investigated these questions using the following method.

Yeast strains, antibodies, and buffers.

The wild-type (WT), *set2Δ*, *rtf1Δ*, and *bur2Δ* strains in the BY4741 background used in this and following sections were obtained from Open Biosystems. The extraction buffers evaluated for effectiveness in detecting histone modifications are described in **Table 4.1**.

The antibodies used to detect histone modifications were from Upstate Biotechnology and used at the following concentrations for western blotting: H3 lysine 36 di-methyl (H3K36me2, catalog 07-274) used at 1:3000 dilution, general H3 C-terminal (H3, catalog 05-928) for loading controls used at 1:10000 dilution, H3 lysine 4 di-methyl (H3K4Me2, catalog 07-030) used at 1:25000, H3 lysine 79 di-methyl (H3K79me2, catalog 08-835) used at 1:5000 dilution, and H3 lysine 4 tri-methyl (H3K4me3, catalog 07-473) used at 1:5000. Additional antibodies obtained from Abcam were used as follows: H3 lysine 36 tri-methyl (H3K36me3, catalog 9050) used at 1:3000 dilution, and H3 lysine 79 tri-methyl (H3K79me3, catalog 2621) used at 1:3000 dilution. Secondary antibodies used were sheep anti-mouse IgG Horseradish peroxidase and anti-rabbit IgG peroxidase linked (GE Healthcare), each used at 1:5000 dilution.

Comparison of various extraction buffers for effectiveness in detection of histone modifications.

Described below is the method utilized for comparison of various WCE buffers and their effectiveness in histone modification analysis. The approach follows a common WCE preparation using four unique published buffers, differing primarily in their salt type and salt concentration (Briggs et al., 2001; Keogh et al., 2002; Kizer et al., 2005; Xiao et al., 2003b). Complete descriptions of the buffer components are presented in Table 1.

1. Yeast WT, *set2Δ*, and *rtf1Δ* strains were grown overnight and each was inoculated into fresh 100 ml YPD at a starting $O.D._{600}$ of 0.1. Cells were grown to an $O.D._{600}$ of 1.1, then each 100 ml culture was separated into four identical 25 ml portions, pelleted, and stored at -80 °C.
2. The cell pellets were thawed on ice, washed, and then re-suspended in 400 μl extraction buffer. A unique extraction buffer (see **Table 1**) was used in the lysis of one pellet from each of the three strains: WT, *set2Δ*, *rtf1Δ*.

3. WCE was performed for each sample according to a published method, differing in the extraction buffers and loading amounts (Briggs et al., 2001). The extraction method consisted of cell cultures (5 ml) grown overnight, diluted in fresh 100 ml YPD culture to a starting $O.D._{600}$ of 0.1, and grown to a final $O.D._{600}$ of 1.0. Pelleted cells were resuspended in extraction buffer and then disrupted by acid-washed glass beads using a mini-beadbeater (Biospec Products) for 3 x 30 sec pulses with 1 min on ice between each step. Tube bottoms were punctured, and cell extracts were separated by brief centrifugation. Lysates were clarified by centrifugation at 16,000 x g for 15 min, after which the supernatant (WCE) was transferred to a fresh tube for protein analysis.
4. WCE protein concentrations were determined using Coomassie Plus reagent (Pierce Biochem) according to the manufacturer's directions and assayed using the Bradford method.
5. Following addition of 10 μl 2 x Laemmli SDS-PAGE loading buffer to 10 μl of each WCE, the samples were boiled for 5 min.
6. Samples (30 μg) were resolved by SDS-PAGE using a 15% acrylamide gel.
7. Resolved proteins were transferred to PVDF (Millipore Immobilon-P) using a Hoefer TE-77 semi-dry transfer unit at 45 mA per 8 x 7 cm membrane for 90 min total.
8. Membranes were each blocked in 10 ml of a 5% non-fat dry milk solution and TBS (50 mM Tris pH 8.0, 138 mM NaCl, 2.7 mM KCl) at room temperature for 1 h. Histone modifications were detected by incubation of primary antibody overnight at 4 °C. Membranes were then washed 2 x 5 min in TBS-Tween (TBS + 0.1% Tween 20), and secondary antibodies were added to a fresh 10 ml solution of 5% milk and TBS-Tween with incubation at room temperature for 2 h, then washed as before. Membranes were developed using the ECL Plus Western Blotting Detection Kit (GE Healthcare) according to the manufacturer's directions.

The relative effectiveness of each buffer tested is presented in **Fig. 4.1**. Although we initially predicted that changes in salt type or concentration of the WCE buffers would result in a significantly greater elution of proteins and/or histones in the lysate, we found that analysis of total protein concentration revealed that the concentration of all WCEs examined were nearly the same - within 10% (data not shown). In addition, we found that the relative concentration of histones and the background binding of the antibodies to cellular proteins were nearly identical regardless of the buffer components tested (data not shown). These data indicate that changes in the salt types or concentrations (i.e. ionic strength) within the ranges we tested have little to no effect on the global extraction of protein in the WCE method, nor does it affect the purity of histones extracted.

4.2.2. Titration and stripping approach for precise detection of changes in specific histone modifications

To determine whether a gene deletion or mutation affects a particular histone modification, histones from the wild type and deletion strain must be equally loaded for comparison. In most cases, especially when a deletion completely abolishes a histone modification, the histone loading amount may not be critical. However, when a deletion only partially reduces or increases a given histone modification, loading too much WCE (or using too high an antibody concentration) can cause an over or underestimate of the amount of modified histone. In addition, loading too little WCE could appear as though a factor abolishes a particular modification when it may only moderately reduce that modification. To address this issue, we have combined a titration assay and membrane stripping procedure. The membrane stripping approach we present here ensures proper comparison between modification levels and equal histone loading, as variations in sample loading or transfer efficiency are common and can make for difficult interpretations. This method is particularly useful when more than one histone modification is being investigated. In our

experience, a single membrane can be stripped and reused up to four times if the membrane has not dried after each immunoblotting assay. The titration and stripping approach is described below.

1. Prepare cells and WCE using a general method such as that described in section 4.2.1.
2. For each mutant or strain to be analyzed, load at least three different amount of protein in adjacent gel lanes. Suggested ranges should begin near 10 μg of WCE and increase at two or three-fold increments when using the Hoefer Mighty Small gel apparatus (1.0 mm spacers, 10 well combs).
3. Analyze the extracts using a standard western blotting approach for the first antibody of choice.
4. Begin the stripping procedure by placing the membrane in a plastic bag filled with ~10 ml stripping buffer (65 mM Tris-HCl pH 6.8, 2% SDS, 0.75% β-mercaptoethanol).
5. Seal plastic bag and put it in water bath set at 55 °C for 30 min. Invert bag 3 times every 10 min.
6. Decant stripping buffer, and transfer membrane to a box.
7. Wash membrane with 1 x TBS at room temperature for 5 min, repeat 3 times.
8. Verify that the membrane is stripped of the first antibody by re-probing with the matching secondary antibody and ECL.
9. Proceed to standard immunoblotting assay with the next antibody.

The effectiveness of this approach is demonstrated in **Fig. 4.2**. The use of at least three WCE concentrations per strain in adjacent lanes makes it likely that at least one lane will not produce a saturated signal when blotted with the desired antibody. This, in combination with the stripping approach, allows for effective screening of defects in histone modification levels that may otherwise be missed in a cursory screening. We

note that while a stripping approach is recommended, a non-stripping approach that involves examining parallel blots can also be effective, although it may require multiple independent repeats to quantitatively detect a subtle change in a particular histone modification.

4.2.3. Detection of histone modifications when only low-avidity antibodies are available or the modification is in low abundance

For detecting histone modifications in yeast by western analysis, two types of histone preparations are generally used: the WCE method and histone acid-extraction method. The choice depends on the quality of antibody and/or the relative abundance of the particular histone modification of interest. In the first method, all cellular proteins are extracted with one simple buffer, which allows rapid preparation of histones along with other cellular proteins. However, since the histone abundance is generally low as compared to a histone acid-extraction procedure, the WCE method is most useful when antibody avidity is high. The second method is designed to first isolate nuclei from cells followed by detergent washes and acid extraction; therefore, its histone yield is much higher than in the first method. However, existing versions of this method in the literature require significant time and effort (Almer and Horz, 1986; Braunstein et al., 1993; Edmondson et al., 1996; Lowary and Widom, 1989; Santos-Rosa et al., 2003; Wintersberger et al., 1973). For this reason, a histone purification method is only used when antibody avidity is low, or if the modification of interest is rare.

Small-scale accelerated nuclei preparation

Here we present a modified nuclear extraction method to detect histone modifications. Typical large-scale histone purification methods require approximately seven hours of preparation time once the cell pellet is obtained. This small-scale method requires less than half the time of a histone purification method (3 h versus 7 h), yet is superior to WCE for the detection of low-abundance histone modifications or when an antibody of low avidity is

utilized. In addition, the common nuclei cushion step of most histone purification methods is omitted. The required culture volume is also decreased to 200 ml, from the 1 L volume commonly used in histone purifications. The protocol for this approach is described below.

1. Yeast strains are grown overnight and inoculated into fresh 200 ml YPD at a starting $O.D._{600}$ of 0.1. Inoculated cultures are grown at 30°C with shaking to an $O.D._{600}$ of 1.1.
2. Centrifuge cells at 4000 x g for 15 min at 4 °C, then wash once with 40 ml ice-cold H_2O, and spin as before. For convenience, pellets can be frozen at -80 °C at this step.
3. Suspend each pellet in 3 ml of spheroplasting buffer (1 M Sorbitol, 50 mM K_2HPO_4 pH 6.5 buffer, 0.018% β-mercaptoethanol). Cells pellets should be mixed gently in this and subsequent steps.
4. Centrifuge cells 3500 x g for 10 min at 4 °C.
5. Suspend each pellet in 3 ml of spheroplasting buffer containing 40 units/ml Zymolase 100T (MP Biomedicals).
6. Incubate mixture at 30 °C for 30 min (when ~90 % of the cells should be spheroplasted). Complete spheroplasting can be analyzed by measuring the $O.D._{600}$ in a 1:100 dilution of spheroplasts with 1% SDS. The reaction should not proceed longer than 45 min due to the risk of shearing nuclei.
7. Centrifuge the spheroplasted cells at 4000 x g for 5 min.
8. Wash spheroplasted pellets in 3 ml of spheroplasting buffer (without Zymolase).
9. Gently pellet again as in step 7.
10. Suspend each pellet in 8 ml lysis buffer (18 % Ficoll 400, 20 mM $K_2(HPO_4)$ pH 6.8 buffer, 1 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin.

11. Lyse cells with 20 strokes of a small Dounce homogenizer with pestle A (we find that pestle A is easier to use yet just as effective as pestle B). This step is performed on ice. The homogenizer should be rinsed as follows:
    a. Pour lysed cells into a sterile tube and wipe the homogenizer handle along the tube interior.
    b. Pour 4 ml of fresh lysis buffer over the handle into homogenizer.
    c. Use a pipette to rinse inside of homogenizer with the remaining lysis buffer.
    d. Pipette the buffer rinse into the conical tube, resulting in 12 ml final volume of homogenized solution.
12. Spin samples at 3500 x g for 10 min to remove cell debris (nuclei in supernatant, debris is in pellet).
13. Transfer supernatant into (16 x 76 mm) tubes and pellet nuclei in Type 50 Ti rotor at 50,000 x g for 35 min
14. Suspend nuclei in 200 μl NP buffer (0.34 M sucrose, 20 mM Tris-HCl pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, with protease inhibitors added as in step 10. Vigorous pipetting may be required to suspend the nuclei pellet. Aliquot and store nuclei at -80 °C.

Using the above protocol, nuclei were prepared from the WT, *set2Δ* and *rtf1Δ* strains and western blot analysis was performed as in section 4.2.1. As shown in **Fig. 4.3**, this method is effective for a variety of difficult antibodies such as the H3K36me2 or H3K79me3. However, when comparing WCE versus nuclei, it is evident that nuclei provide no additional benefit when using antibodies that perform well in WCE (**Fig. 4.3**, compare H3K4me3 and H3K36me3 blots). While one might predict that increasing the WCE load could allow for an increased signal for difficult antibodies (see **Fig. 4.3** H3K79me3 and H3K36me2 blots), we note that the maximum amount of WCE that can be loaded is near 100 μg when using our gel apparatus (see section 4.2.2). Beyond this WCE maximum load, the extract lodges in the well and the high protein concentration results in significant smearing and insufficient

resolution of bands. In our experience, dramatic increases in WCE loading do not significantly improve western results with poor performing antibodies. Importantly, equivalent protein concentrations were used between WCE and nuclei (WCE: 60 μg and nuclei: 15, 30, and 60 μg), thus confirming that the nuclei preparation method enriches for the histone proteins and is superior to WCE for the detection of histone modifications. In addition to the analysis of histone modifications, the purified nuclei could be useful for the analysis of any nuclear protein that may otherwise be undetectable in WCE due to low antibody avidity or a low relative abundance of the protein in total cellular extract.

4.3 Concluding Remarks

The methods and approaches outlined in this article provide for time-saving and precise global analysis of even modest changes in histone modifications. We have provided three key points concerning the analysis of histone modifications in yeast: (i) altering the salt type or salt concentration (ionic strength), within the ranges we tested, results in little to no improvement in the ability to detect histone modifications; (ii) a titration and membrane stripping approach is superior in gauging quantitative changes in histone modification levels; and (iii) our abbreviated nuclei preparation method is beneficial in the detection of rare histone modifications or if an antibody's avidity is low.

Interestingly, changes in the ionic strength or salt concentration of WCE buffers do not lead to an appreciable change in the total protein concentration extracted or enrichment of histones (**Fig. 4.1**). Empirically, there is greater freedom in buffer components than may have been predicted. Also of importance is the observation that modest changes in histone modification levels could be missed in a cursory screen of WCEs. The results shown in **Fig. 4.2** demonstrate that a careful, yet rapid analysis of yeast extracts using a titration and stripping approach aids in identifying genes, that when deleted, may have a minor, yet biologically relevant change in specific histone modification levels. This approach has been

utilized in several recent reports to demonstrate selective changes in specific histone modification states (Laribee et al., 2005; Qiu et al., 2006). Without the use of this titration approach, a factor with genuine effects on a specific histone modification could appear to have no effect (**Fig. 4.2A,B** compare lanes 1,4,7 and 3,6,9). Also presented here is an abbreviated nuclei preparation procedure, which provides a midpoint in quality and time expenditure between the rapid WCE and a time-consuming histone acid-extraction (**Fig. 4.3**). Previously, use of antibodies with low avidity often resulted in low-quality blots and required large-scale preparation of core-histones or nuclei for clear detection (Laribee et al., 2005; Shahbazian et al., 2005). The enriched histone procedure provided through the abbreviated nuclei preparation presented in this report is also applicable for use with antibodies that have low avidity or when there is a low abundance of the target modification/protein in the cell that would be difficult to analyze using WCE (see **Fig. 4.3** WCE vs. nuclei). In a similar manner, the enriched nuclei could also be of use for the analysis of low-abundance nuclear proteins. Collectively, the applications discussed in this article allow for clear and reproducible detection of nuclear proteins and/or global changes in histone modification levels.

Acknowledgements

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Table 4.1: Extraction buffers evaluated for effectiveness in detecting histone modifications

	Buffer I[a]	Buffer II[b]	Buffer III[c]	Buffer IV [d]
Salt type and concentration	320 mM $(NH_4)_2SO_4$	300 mM NaCl	600 mM NaCl	200 mM $KC_2H_3O_2$
Other components[e]	200 mM TRIS-HCl pH 8.0	50 mM TRIS-HCl pH 8.0	10 mM TRIS-HCl pH 7.4	20 mM HEPES 7.6
	20 mM EDTA pH 8.0	1 mM EDTA	5 mM EDTA	1 mM EDTA
		0.1% NP40	300 mM sorbitol	10% glycerol
	10 mM EGTA pH 8.0	1 mM $Mg(C_2H_3O_2)_2$	5 mM $MgCl_2$	protease
	5 mM $MgCl_2$	1 mM imidazole	10% glycerol	inhibitors
	1 mM DTT	10% glycerol	protease	
	10% glycerol	protease inhibitors	inhibitors	
	protease inhibitors			

[a] See Xiao et al. 2003
[b] See Kizer et al. 2005
[c] See Briggs et al. 2001
[d] See Keogh et al. 2002
[e] Protease inhibitors at 2 μg/ml each of pepstatin, leupeptin, aprotinin, 2 mM PMSF and 10% glycerol were prepared consistently for all buffers, regardless of original published recipe.

Figure 4.1: Evaluation of WCE buffer components reveals equal effectiveness in the detection of histone methyl modifications.

A 100 ml culture of the indicated strains was separated into four identical pellets, and WCE were prepared for each pellet using one of four distinct buffers (see Table 4.1 for a complete list of buffer components). WCEs (30 μg) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 di-methyl lysine 4 (αH3K4me2), tri-methyl lysine 4 (αH3K4me3), tri-methyl lysine 36 (αH3K36me3), and di-methyl lysine 79 (αH3K79me2). An antibody directed against the C-terminus of H3 (αH3) was used as a loading control. Cell pellets, buffers, and extracts prepared by K. Kizer, western transfer and blots prepared T. Xiao.

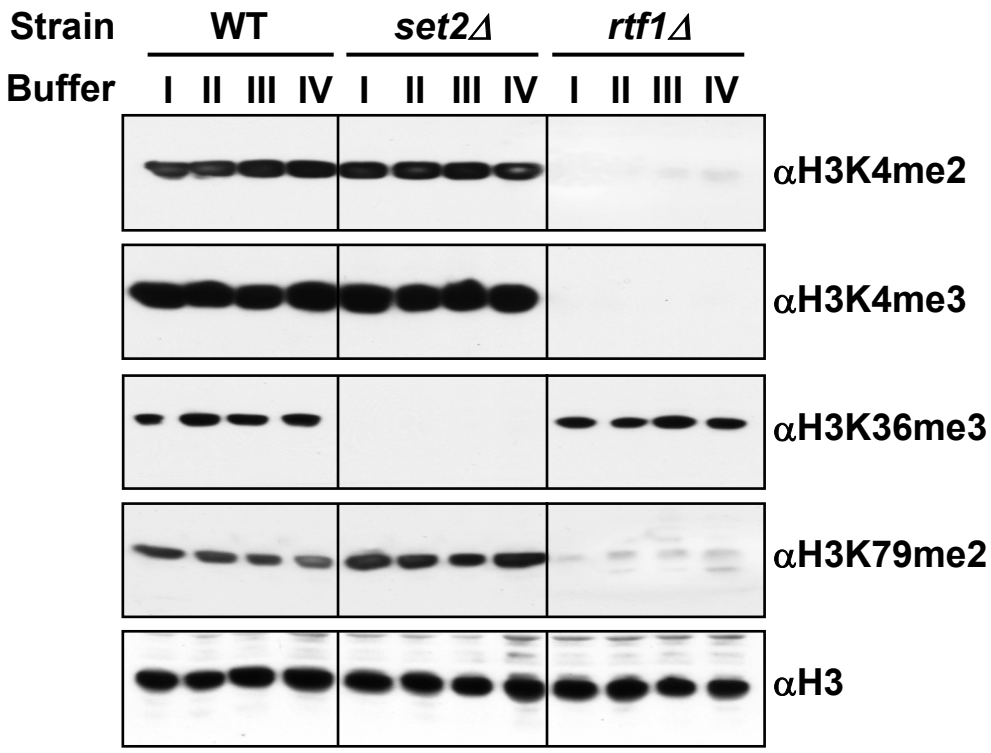
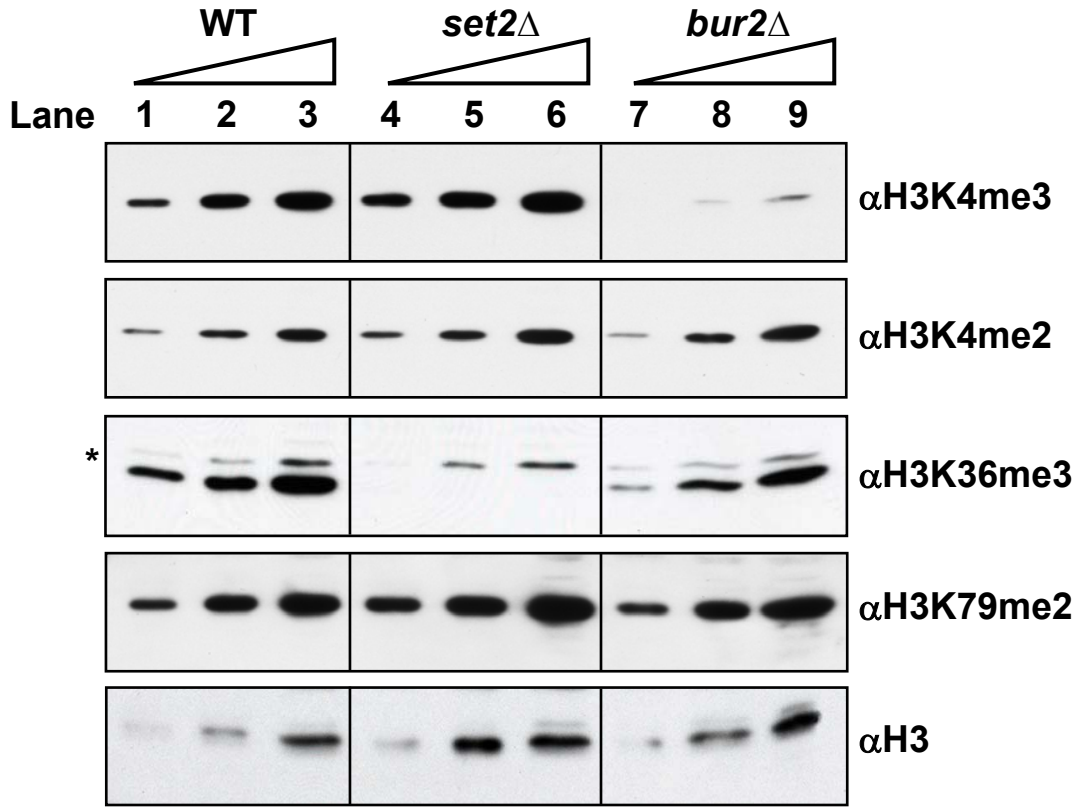

Strain
WT
set2Δ
rtf1Δ
Buffer
I II III IV I II III IV I II III IV
αH3K4me2
αH3K4me3
αH3K36me3
αH3K79me2
αH3

Figure 4.2: Titration and stripping approach is helpful for the precise detection of changes in histone modification levels.

(**A**) Following the standard WCE protocol (see Section 4.2.1.), extracts were prepared from WT, *set2Δ* and *bur2Δ* strains using Buffer II described in Table 4.1. The titration approach consisted of three concentrations of extract from each strain resolved by SDS-PAGE and transferred to PVDF (generally a range between 10 and 90 μg). Each membrane probed for a histone modification was stripped and re-probed for the H3 loading control. We note that the use of the same membrane to blot for the loading control and modification of interest avoids the complications that arise from variations in gel loading and transfer efficiency which occur between gels. However, we also find that comparisons between two independent membranes (one probed for the modification of interest and one probed for the histone loading control) generally results in a similar conclusion, but is best confirmed with multiple independent repeats. Asterisks denote non-specific bands. Cell pellets, buffers, and extracts prepared by K. Kizer, western transfer and blots prepared T. Xiao. (**B**) Quantification of band intensities for the H3K36me3 blot is displayed as a ratio of H3K36me3 band intensity to the corresponding H3 loading control. Band intensities are plotted relative to the WT level (set to 100%) in each lane. Important to note is that at the highest concentration loaded, *bur2Δ* appears to effect a negligible change in H3K36me3 (compare lanes 3 and 9 of the H3K36me3 blot), yet the lowest concentration (lanes 1 and 7) reveals the decreased H3K36me3 found to occur in the absence of Bur2 (Chu et al., 2006). This observation is consistent for these blots regardless of exposure time.

A


WT
set2Δ
bur2Δ
Lane 1 2 3 4 5 6 7 8 9
αH3K4me3
αH3K4me2
*
αH3K36me3
αH3K79me2
αH3

B

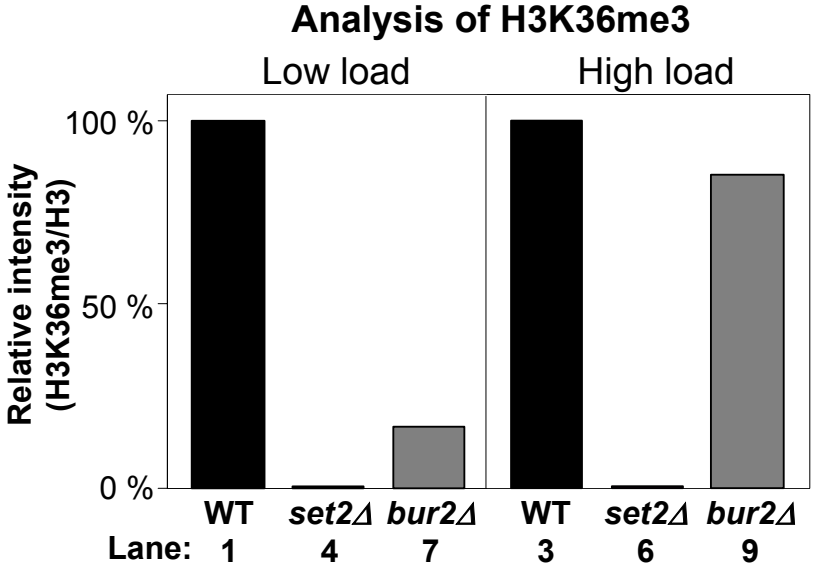
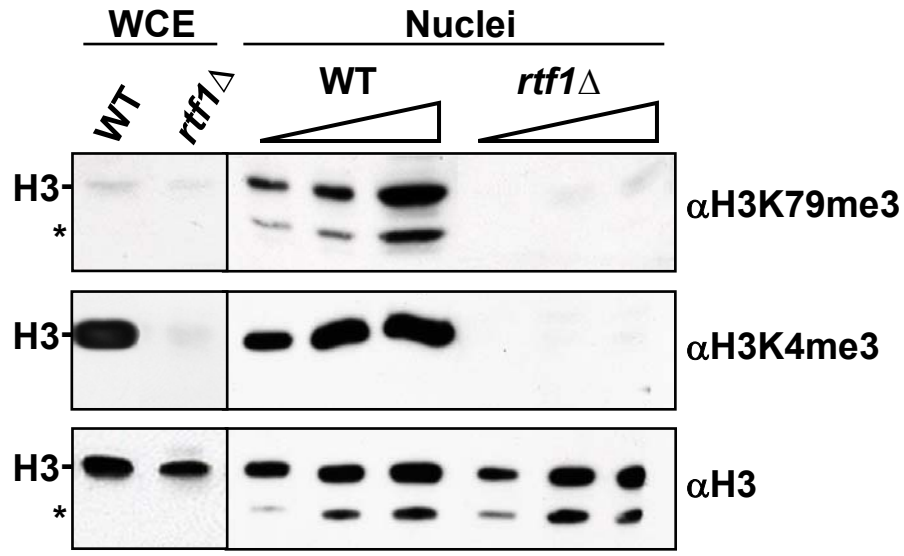

Analysis of H3K36me3
Low load
High load
100 %
50 %
0 %
Relative intensity (H3K36me3/H3)
WT set2Δ bur2Δ WT set2Δ bur2Δ
Lane: 1 4 7 3 6 9

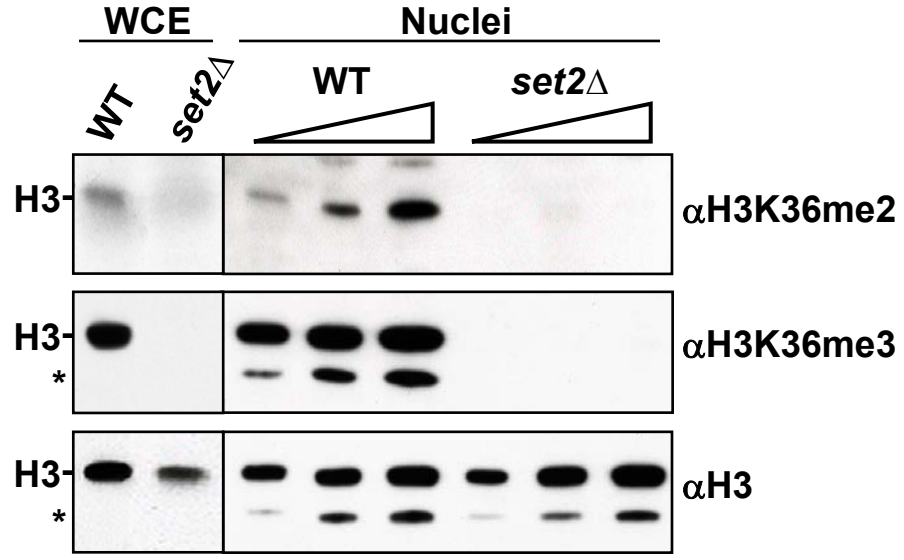
Figure 4.3: The abbreviated nuclei preparation method improves detection of histone modifications with low modification abundance or antibody avidity.

In each panel, nuclei were isolated from the indicated strains using the abbreviated nuclei method and compared to WCEs of identical strains. For comparison of antibody effectiveness, 60 μg WCE was compared to a range of nuclear extract at 15, 30 or 60 μg. The location of H3 is indicated and asterisks indicate a partial N-terminal H3 breakdown products detectable in the nuclear extract. Important to note is that more protease cleavage (breakdown product) of H3 is typically observed in nuclei preparations as compared to WCE preparations. For both panels, cell pellets and buffers prepared by K. Kizer, Nuclei prepared and blotted by T. Xiao. *(***A**) WCEs (left) and nuclei (right) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 tri-methyl lysine 79 (αH3K79me3) and tri-methyl lysine 4 (αH3K4me3) while an antibody directed against the C-terminus of H3 (αH3) was used as a loading control. (**B**) WCEs (left) and nuclei (right) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 di-methyl lysine 36 (αH3K36me2) and tri-methyl lysine 36 (αH3K36me3), with an antibody directed against the C-terminus of H3 (αH3) used as a loading control. Although the abbreviated nuclei extraction method allows for better detection when using a poorly performing antibody, it does not appear to provide an advantage when the antibody performs sufficiently well in WCE (compare WCE vs. nuclei with the H3K36me3 and H3K4me3 antibodies).

A


WCE
Nuclei
WT
rtf1Δ
WT
rtf1Δ
H3
*
αH3K79me3
H3
αH3K4me3
H3
*
αH3

B


WCE
Nuclei
WT
set2Δ
WT
set2Δ
H3
αH3K36me2
H3
*
αH3K36me3
H3
*
αH3

CHAPTER 5

CONCLUSIONS, CURRENT QUESTIONS, AND FUTURE WORK

5.1 Advances in our understanding of the function of H3K36 methylation

The studies presented here, which focused on the yeast methylase Set2, have advanced our knowledge of the functions and regulation of histone lysine methylation. We have reconciled a false assumption from an earlier study (Li et al., 2002) regarding the region of Set2 responsible for RNAPII interaction. As described in Chapter 2, our detailed mapping of the Set2 C-terminus revealed the presence of the SRI domain and allowed for a more careful investigation of the role of Set2-RNAPII interaction on Set2 function. We have advanced our understanding of the repressive effect of H3K36 methylation in transcription through observations of RNAPII density changes in the absence of Set2. Our data presented in Chapter 3 have provided insight into the regulation of Set2 function through observations of Set2 dependence on Ctk1, Bur1, and Spt6. Finally, as described in Chapter 4, we have developed improved methods for the future analysis of histone modifications.

Concurrent studies from other laboratories

As with all scientific endeavors, these studies were not completed in a vacuum. Simultaneous discoveries and ideas from other laboratories were crucial in the direction of our work. Collectively, our work along with studies from other groups over the past six years has matured investigators' abilities to examine and understand the roles of histone modifications in the regulation of the genetic code. Following our identification of the SRI domain in Set2, two independent publications described the structure of the SRI domain (Li et al., 2005b; Vojnic et al., 2006). These studies confirmed our observations that the SRI domain binds to doubly phosphorylated CTD repeats (see **Fig. 2.4**) and our prediction of an SRI domain within the human Set2 homologue (see **Table 2.1**). In addition, a separate study from our laboratory found that the Set2-RNAPII interaction is functionally conserved in the *Schizosaccharomyces pombe* Set2 homologue (Morris et al., 2005). The publication of large-scale yeast genetic analyses during the course of our work has provided much insight into the inter-relationships between various histone modifying enzymes, transcription

factors, and other proteins (Krogan et al., 2002b; Krogan et al., 2003b; Schneider et al., 2004; Wood et al., 2005a; Wood et al., 2005b). These studies provided the basis for follow-up investigations to better understand the related functions of these genetically-linked proteins including Set2 (Keogh et al., 2005; Laribee et al., 2005; Wood et al., 2005a; Wood et al., 2007; Xiao et al., 2005; Xiao et al., 2007; Laribee et al., 2007).

One of many observations resulting from these screens was the discovery that the histone deacetylase (HDAC) Rpd3, and Set2, are required to repress the formation of incomplete transcripts at certain loci. As introduced in Chapter 3.1, these incomplete transcripts are the result of transcription initiation from cryptic start sites within the body of genes. Further study revealed that acetylation at H3 lysine 14 (H3K14ac) and H3 lysine 16 (H3K16ac) allows this defective initiation, which is normally repressed by deacetylation by the HDAC Rpd3 (Kadosh and Struhl, 1998). Rpd3 was found to be a component of two distinct complexes, the Rpd3C(S) and Rpd3C(L) complex (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). The Eaf3 and Rco1 proteins are distinct to the Rpd3C(S) complex, although Rpd3, Sin3 and Ume1 are found within both complexes. These studies revealed that the Eaf3 subunit binds to nucleosomes methylated at H3K36, via its chromodomain, and recruits Rpd3C(S). Through an early phenotypic screen, shown in **Figure 5.1A**, I observed a similarity in function between Set2 and Eaf3 based on a similar characteristic 6AU resistance seen in these deletion strains. My preliminary results were confirmed through the thorough studies published by our laboratory and others (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). In addition, I found that a *RAD6* deletion strain displays sensitivity to 6AU, in contrast to *SET2* deletion (**Fig. 5.1B**), a result that was supported by work from our colleagues that further examined the role of Rad6-mediated histone H2B ubiquitination in transcription (Xiao et al., 2005). This study and others demonstrated the complexity of the upstream regulation of H3K4 and H3K79

methylation, and is a parallel example of the emerging work identifying the regulation of H3K36 methylation.

5.2 Deciphering the pathway of regulation for histone methylation

Links between methylation and RNAPII transcription

Proper transcriptional regulation by RNAPII is accomplished, in part, through the regulated association of a number of factors and complexes. Key to the proper function of RNAPII is the role of the CTD in the association of these regulatory proteins. Through the course of our studies, our laboratory and others have made significant advances in the understanding of RNAPII transcription as it relates to histone modifications in yeast. Subsequent to our discovery of the SRI domain in Set2, two aforementioned studies further characterized the requirement of CTD phosphorylated at *both* Ser2 and Ser5 of the repeat sequence for direct SRI domain binding (Kizer et al., 2005; Li et al., 2005b; Vojnic et al., 2006). As illustrated in **Figure 5.2**, these findings promoted the possibility of Ser5 and Ser2 phospho co-existence along CTD repeats in a transitory stage between transcription initiation and elongation where H3K36 methylation is predominant (Kizer et al., 2005; Krogan et al., 2003b; Li et al., 2003; Xiao et al., 2003b). This doubly-phosphorylated RNAPII-CTD state was not expected to be of significant functional relevance based on the apparent predominance of either Ser5 or Ser2 phosphorylation alone, at either the promoter or 3' end of most genes, respectively (Gerber and Shilatifard, 2003; Hampsey and Reinberg, 2003). Over the past five years, a great deal of evidence has emerged that emphasizes the importance of CTD phosphorylation in recruiting proteins involved in the regulation of transcription and chromatin structure (for review, see (Phatnani and Greenleaf, 2006). Binding to the phosphorylated CTD is crucial for the proper function of a variety of histone-modifying enzymes such as the H2B ubiquitinating Paf1 complex, Set1-containing COMPASS complex and Set2 (Krogan et al., 2003a; Ng et al., 2003a; Shi et al., 1996;

Wood et al., 2005b; Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Schaft et al., 2003; Xiao et al., 2003b; Ng et al., 2003b).

The role of histone methylation in RNAPII transcription must be considered in the context of the complex relationship between the maintenance of chromatin structure, control of the initiation, elongation and termination phases of transcription, mRNA processing, and histone modifications that likely signal for a variety of these processes in ways not yet understood. Examples of this complexity include early observations that suggested the passage of transcribing polymerase results in alternations of chromatin structure, dependent in part on Spt16 and Pob3 (FACT) (Formosa et al., 2002). Later it was found that FACT removes H2A/H2B dimers in preparation for RNAPII passage, and functions cooperatively with H2B ubiquitination via the Paf1 complex to facilitate elongation in a reconstituted human chromatin system (Belotserkovskaya et al., 2003; Pavri et al., 2006). In addition, the ATP-dependent chromatin remodeling protein Isw1 was found to coordinate with histone methylation by Set1 and Set2 to regulate the release of initiated RNAPII into productive elongation (Morillon et al., 2005). The histone chaperones Spt2 and Spt6, along with the elongation factors Spt4 and Spt5 (also known as DSIF) were found to regulate elongation through changes in chromatin structure or by regulating RNA-processing events (Hartzog et al., 1998; Kaplan et al., 2003; Lindstrom et al., 2003; Nourani et al., 2006; Proudfoot et al., 2002; Qiu et al., 2006; Wada et al., 1998). In summary, recent genetic and biochemical studies of the CTD and chromatin-modifying enzymes have revealed a functional interplay in the preparation of chromatin for RNAPII passage, proper RNAPII initiation and elongation, mRNA formation, and then the proper re-establishment of certain histone modifications post-transcription. In regard to these processes, a current model of the dynamic role of nucleosomes in transcription will be discussed in section 5.3.

Upstream regulation of Set2

Observations from our lab and others that deletion of *SET2*, or members of the Rpd3C(S) complex, bypasses lethality of a *BUR1* deletion has revealed clues as to the interconnectedness of elongation factors and Set2 (**Fig. 3.1**, (Chu et al., 2006; Keogh et al., 2005). Other connections have also revealed a requirement of Spt6 in H3K36 methylation (**Figs. 3.2-3.5**, (Chu et al., 2006). Most importantly, these new data give a clearer picture of the function and regulation of Set2-catalyzed H3K36 methylation as well as reveal a potentially complex network of proteins in the upstream regulation of Set2. These publications, in combination, have led to a new model of the function of Set2 and H3K36 methylation along genes (**Fig. 5.2**). In this model, Set2 binds to the Ser2 and Ser5 doubly-phosphorylated CTD via the SRI domain and is recruited to active genes. Set2 is then able to methylate nucleosomes that are properly configured by Spt6 and/or other factors may be regulated by Bur1 such as Spt2 or Spt4 (see **Fig. 3.8** and **Fig. 5.2**). Subsequent methylation recruits Eaf3 and the Rpd3C(S) for deacetylation and restricts RNAPII from initiating at cryptic start sites.

A greater understanding of the configuration of the RNAPII-CTD has also contributed to an updated model of Set2 function along genes. A recent study demonstrated a mode of regulation for H3K36 methylation through the proline isomerase Fpr4. Fpr4 exhibits control over Set2-catalyzed methylation of H3K36 through the isomerization of H3 proline 38 (Nelson et al., 2006). This isomerization results in an H3 configuration that is non-permissive for H3K36me3. This antagonism, however, was only detected at basally transcribed genes, and could be overcome with a high RNAPII density (bringing along Set2) that is present at highly active genes. An additional enzymatic antagonistic relationship with Set2 was demonstrated by the identification of acetylation at H3K36 *in vivo*, catalyzed by Gcn5 (Morris et al., 2006).

Enzymatic demethylation

Histone methylation has long been seen as a stable modification, based in part on early data that suggested that methylation was essentially irreversible (Byvoet et al., 1972; Duerre and Lee, 1974). Subsequent analyses of the mammalian H3K9 methylase G9a supported this earlier view, based on observations that methylation persists for several rounds of cell division even after inactivation of the methylase (Su and Tarakhovsky, 2006). Is methylation simply a static modification? It would seem unlikely that an effective signaling mark could exist with only an "on" state? This question persisted during the course of our work, although the mechanism of histone replacement accounted for the positions of histone variants during transcription and as a mechanism of essentially "demethylating" a nucleosome (Ahmad and Henikoff, 2002; Briggs et al., 2001; Goll and Bestor, 2002; Turner, 2002). Concurrent with our studies, a variety of discoveries have been made in the area of histone demethylation. Specifically, multiple demethylase enzymes have been identified from yeast to human. These enzymes appear specific to the demethylation of particular methylated residues using functional domains and enzymatic mechanisms. The human enzyme LSD1 is specific for demethylation of mono- or dimethyl H3K4 by a amine oxidation mechanism, but is unable to demethylate H3K4me3 (Shi et al., 2004). A separate class of demethylases containing the Jumonji C (JmjC) domain were later identified through which demethylation is accomplished through a hydroxylation mechanism (Schneider and Shilatifard, 2006; Shi and Whetstine, 2007). The demethylase JHDM1, and its homologue in budding yeast, specifically demethylate H3K36me2 (Tsukada et al., 2006). While JHDM2A demethylates H3K9me2 in humans, the demethylase JHDM3A/JMJD2A can remove the trimethyl moiety from H3K9 or H3K36 (Klose et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Although these enzymes have been confirmed as capable demethylases *in vivo*, examinations of the deletion or over-expression of these enzymes revealed only a modest affect on global levels of methylation (Shi and Whetstine, 2007;

Tsukada et al., 2006). This is due at least in part to the specificity of the majority of these enzymes for a mono- or di-methylated substrate, and can account for prior difficulty in their detection. As described in Chapter 3, the upstream regulation of methyltransferases is a key regulatory pathway for the positioning of the methyl modifications. As illustrated in **Figure 5.2**, enzymatic demethylation and histone replacement demonstrate additional mechanisms for the regulation of H3K36 methylation. Based on the improvement of techniques to detect demethylases *in vitro*, it is anticipated that additional demethylases, such as those responsible for demethylation at H3K79, will be discovered in the near future (Schneider and Shilatifard, 2006; Shi and Whetstine, 2007; Trojer and Reinberg, 2006). However, we note that the kinetics of H3K79 methylation and its loss in heterochromatin formation display marked differences from that of H3K4 methylation. A study of heterochromatin formation in yeast found a more rapid loss of H3K4 methylation from that of H3K79 methylation, which more closely represented the rate of cell division (Katan-Khaykovich and Struhl, 2005). Therefore, H3K79 may more closely represent the heritable epigenetic mark initially attributed to all histone methyl modifications due to their predicted stability.

5.3 Distribution of specific histone modification states

Genome-wide analyses of histones and histone modifications

The genome-wide detection of histone modifications has provided significant insight as to the distribution of particular modifications, data that is crucial for future work in understanding the histone code. The past five years have seen a dramatic rise in the use and effectiveness of microarray in the analysis of histone modifications. The first genome-wide analysis of histone modifications in yeast focused on histone deacetylases and the genomic regions they target (Robyr et al., 2002). The principle of microarray analyses centers around the presentation of the entire genome (or representation thereof) prepared

by the spotting of DNA oligos onto microscopic arrays that are available for binding to complementary sequence. Using a traditional ChIP approach, DNA corresponding to a particular protein bound or modified region is amplified by PCR and labeled with fluorophors. The enrichment of DNA corresponding to these protein bound or modified states is compared to DNA prepared from an appropriate control sample, such as ChIP DNA using an unmodified histone antibody, that is labeled with an alternate fluorophore. These DNAs are then hybridized to the microarray chip and enrichment of a particular region along the array is measured by the relative intensities of fluorescence at the wavelengths of each fluorophore. This "ChIP-on-chip" approach allows for the genome-wide detection of regions where a particular protein binds or where particular histone modifications exist.

One of many important genome-wide observations using the ChIP-on-chip approach was the reduction in nucleosome density at active promoters. Prior results revealed a decreased nucleosomal density at the *PHO5* and *GAL* promoters upon gene activation in yeast (Boeger et al., 2003; Fedor and Kornberg, 1989; Lohr, 1997; Svaren and Horz, 1997). These studies focused on specific genes, however, and could not effectively test for nucleosomal removal (or shifting) at a genome-wide scale. Using the ChIP-on-chip approach, various investigators found that nucleosomal occupancy at promoters decreases in proportion to the rate of transcription initiation (Bernstein et al., 2004; Lee et al., 2004). Consistent with this, FACT was shown to remove histone H2A/H2B dimers in the path of transcribing RNAPII (Belotserkovskaya et al., 2003). Further study using high-resolution arrays demonstrated that the majority of nucleosomes are positioned in a well organized fashion along transcribing genes, while depleted in promoters (Yuan et al., 2005).

In regard to H3K36 methylation, several studies have advanced our knowledge of the localization of this modification across the genome. Analysis of the H3K36me2 modification found this mark to be predominant in the body of genes transcribed by RNAPII. This data provided support to existing data that Set2 activity is directed through its

association with RNAPII *in vivo* (Kizer et al., 2005; Rao et al., 2005; Xiao et al., 2005), however the genome-wide results revealed that H3K36me2 did not correlate with the rate of transcription by RNAPII. Interestingly, this study revealed that nucleosomes carrying the H3K36me2 modification were predominantly retained in highly transcribed genes. As nucleosomes are inhibitory for transcription, this result was consistent with later observations of a role for H3K36 methylation in preventing RNAPII initiation at cryptic promoters (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005).

The ChIP-on-chip approach in yeast continues to be optimized by the preparation of higher resolution arrays. A multitude of other studies were published over the past few years using these improved arrays to analyze regions in the genome where specific proteins were bound or where certain histone modifications were present. A later analysis of H3K36me3 revealed that this modification, in contrast to H3K36me2, was positively correlated with transcription rates (Pokholok et al., 2005). These results, in combination with other studies of H3K36 and H3K4 methylation, demonstrate the existence of distinct mechanisms by which modification states are regulated and function in transcription (Chu et al., 2006; Wood et al., 2005a; Wood et al., 2005b; Wood et al., 2007).

Taken together, genome-wide studies of histones and histone modifications in yeast have supported gene-specific analyses by ChIP and global analyses by western blot that demonstrate a correlation between histone modifications and transcription. A variety of recent genome-wide studies in yeast have solidified three key points: 1) histone acetylation and methylation are correlated with active transcription (Kurdistani et al., 2004; Liu et al., 2005; Pokholok et al., 2005; Rao et al., 2005; Xu et al., 2005), 2) histone deacetylases and histones themselves are often negatively correlated with transcription (Kurdistani et al., 2002; Lee et al., 2004; Robyr et al., 2002), while 3) histone variants (i.e. Htz1) are usually correlated with active transcription (Millar and Grunstein, 2006; Zhang et al., 2005). Interestingly, as in the case of H3K36me3 functioning to repress cryptic initiation, a positive

correlation between a particular modification and transcription does not necessarily indicate a modification positively influences transcription. Indeed, the role of histones and histone modifications in transcriptional regulation is a complex 'code' that will only be understood by high resolution genome-wide studies, in combination with biochemical and genetic analyses. Genome-wide studies of histone modifications, however, demonstrate that the location and density of histone modifications only correlate, and not exactly predict, the transcriptional state of a particular gene. It is most likely that histone modifications are only one component of a complex cross-communication between nucleosomal structure, DNA sequence, and histone modifications that allow for transcriptional regulation as a collective 'code'.

Distribution of specific histone modification states at a typical gene

Recent insight into the interaction between RNAPII and chromatin modifying machinery has revealed one mechanism by which specific histone modification states are regulated. The specific modification states at a particular histone residue (e.g. H3K4 mono-, di-, and tri-methylation) were predicted to be independently regulated and to signal for specific downstream functions (Schotta et al., 2004; Turner, 2003). This hypothesis continues to be supported by recent studies, including observations of the selective regulation of H3K4me3 by Bur1 and the Ccr4/Not complex (Laribee et al., 2005; Laribee et al., 2007) and H3K36me3 by Bur1 (Chu et al., 2006). The complexity of transcriptional regulation and histone modifications is further emphasized by a recent study of the CTD kinase, Ctk1 (Wood et al., 2007; Xiao et al., 2007). Deletion of *CTK1* results in a near abolition of H3K4me1, with a corresponding increase in H3K4me2 and H3K4me3 into the body of genes. Results from this study suggested that Ctk1 serves to maintain the configuration of suppressive chromatin by regulating H3K36 methylation (a mark associated with the repression of initiation) and preventing the spread of H3K4me3 into the body of genes (a modification linked to transcription initiation). Over the past five years, our

knowledge of the locations of chromatin modifications has grown from a general knowledge, to a more genome-wide and gene-specific scale. In summary, the distributions of specific histone modification states along a typical gene suggest a direct link to the various stages of RNAPII transcription, as illustrated in **Figure 5.3**. However, a crucial question is illustrated well in a recent review that notes the complexity of histone modifications and the apparent dilemma from observations that histone modifications respond to changes in gene function, rather than predict them, and could potentially mask a more predictable, but as-yet, undetected epigenetic code (Nightingale et al., 2006).

Recently identified histone modifications and binding proteins in yeast

As we return our focus to considering histone modifications on a broader scope, it is relevant to consider the newly identified histone modifications in yeast, as well as discoveries in the binding domains that link functionality to these marks. Several new post-translational modifications in yeast have been identified since our work began. Perhaps the most relevant to our work was the identification of H3K36 acetylation by Gcn5, which may antagonize Set2-catalyzed H3K36 methylation (Morris et al., 2006). The increasing use of mass spectrometry as a tool for the analysis of post-translational modifications on specific proteins has allowed the identification new histone modifications such as acetylation at H3K56 in yeast (Hyland et al., 2005; Masumoto et al., 2005; Xu et al., 2005). Acetylation at H3K56 represents another post-translational histone modification, like H3K79 methylation, that is found within the globular domain rather than the N- or C-terminal tail. Initial results indicate that this modification, as with H3K79me3, H3K4me3, and many chromatin remodeling enzymes, is involved in the DNA damage response (Feng et al., 2002; Masumoto et al., 2005; Ng et al., 2002; Osley and Shen, 2006). Additional work revealed Rtt109 as the HAT responsible for catalyzing H3K56 acetylation (Driscoll et al., 2007), although additional studies also found this HAT activity to be largely dependent on the histone chaperone Asf1 (Adkins et al., 2004; Adkins et al., 2007; Natsume et al., 2007;

Prado et al., 2004; Schwabish and Struhl, 2006). It is interesting to consider a potential pattern illustrated between Asf1-Rtt109 and Spt6-Set2, that reveals a requirement for proper nucleosomal configuration by a specific histone chaperone (Asf1 or Spt6) prior to the addition of a particular histone modification (Rtt109 and H3K56ac or Set2 and H3K36me). Furthermore, the application of mass spectrometry allows the identification of low-abundance modifications that may have a functional role *in vivo*, such as the detection of the monomethylated species at H3K9 and H4K20 in budding yeast (Garcia et al., 2006). These modifications were previously not considered to be conserved in budding yeast as compared to multi-cellular eukaryotes. Based on these and other recent reports, a revised illustration of the histone H3 modifications currently known to exist in yeast are presented in **Figure 5.4A**.

The recent identification of new binding proteins that target specific histone modifications, illustrated in **Figure 5.4B**, allows for a better understanding of the downstream functions of these post-translational marks. Experiments in mammalian systems had already identified bromodomains and chromodomains as binding to acetylated or methylated lysine residues, respectively (Bannister et al., 2001; Dhalluin et al., 1999; Lachner et al., 2001; Nakayama et al., 2001). However, it remained to be established whether chromodomain or bromodomain-containing proteins in yeast would demonstrate the same binding specificity. This question was addressed in part through studies discussed earlier, that identified the Rpd3C(S) member, Eaf3, as recognizing H3K36me3 and binding via its chromodomain (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Therefore, specific downstream functions in transcription are communicated through the recognition of modified histone residues, as predicted by the histone code theory. Studies over the past two years of the mammalian lysine demethylase JMJD2A revealed it to contain a double Tudor domain, which was capable of recognizing H3K4me2 for subsequent demethylation (Huang et al., 2006). Later, the plant homeodomain (PHD) region was found

to recognize modified histones, resulting in the recognition of methylated histone residues, such as H3K4 methylation by the PHD domain of mammalian Ing2 (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006). Examination of all the PHD-containing proteins in yeast later revealed this domain to recognize either H3K4me3 or H3K36me3 residues (Shi et al., 2007). The cross-talk functions of various histone modifications is illustrated by the PHD domain-functionality in the yeast proteins Yng1 and Nto1 (reviewed in (Mellor, 2006b). As components of the NuA3 acetyltransferase complex, Yng1 and Nto1 bind to H3K4me3 and H3K36me3 (weakly) via their PHD domains, which allows subsequent NuA3 association with chromatin (Martin et al., 2006b; Martin et al., 2006a; Shi et al., 2006).

Analysis of *SET2* null cells and point mutations at H3K36 revealed that Set2 is required to prevent the spreading of silenced chromatin from the mating locus *HMR*a and telomeric regions (Tompa and Madhani, 2007). Unexpectedly, deletion of Rpd3C(S) members did not display this effect, indicating that the role of H3K36 methylation in preventing the spread of silenced chromatin is independent of Eaf3 binding and subsequent effects by Rpd3-mediated histone deacetylation (Tompa and Madhani, 2007). Most importantly, recent work has further demonstrated the wide variety of functional consequences for other sites of histone methylation. The modifications within the histone H3 globular domain, H3K56 acetylation and H3K79 methylation, are linked to cell-cycle control through regulation of the DNA damage response (Adkins et al., 2007; Feng et al., 2002; Green et al., 2005; Masumoto et al., 2005; Natsume et al., 2007; Schwabish and Struhl, 2006). In summary, recent studies of yeast histone modifications and their downstream functions has advanced our understanding of the mechanisms through which the histone code allows signaling for downstream processes in transcriptional regulation and the maintenance of DNA integrity. However, turning our focus from studies of specific histone modifications to the level of nucleosomes will best illustrate the current directions in the study of chromatin structure and function.

5.4 Nucleosome dynamics and consequences in transcription

Mechanisms effecting nucleosome dynamics

Histone-DNA interactions compose one of the most stable protein-DNA complexes, yet alterations in these interactions result in nucleosome displacement, re-positioning, or conformational changes. These nucleosome dynamics are a crucial component of transcriptional control (Kouzarides, 2007; Li et al., 2007; Mellor, 2006a; Workman, 2006). To describe the regulation of nucleosome structure and positioning in all eukaryotes, we consider four key components that are responsible for the control of these processes.

The first component is the ATP-dependent nucleosome remodeling family of proteins, such as Swi/Snf, RSC, Chd1, and Isw1 (Lorch et al., 2006). The chromatin remodeling proteins allow the displacement of nucleosomes from DNA, and in particular, from promoter DNA (Kouzarides, 2007; Li et al., 2007; Mellor, 2006a; Workman, 2006). The mechanism for this displacement is thought to be by forcing bulges of DNA into the nucleosome that, through cooperation with various transcription factors, results in a destabilizing effect on the nucleosome-DNA interaction (Cairns, 2005; Lorch et al., 2006).

Nucleosome displacement, or destabilization of nucleosomes through removal of H2A-H2B dimmers, appears to occur primarily at the promoter of active genes and upstream of the transcribing polymerase (Mellor, 2006a; Workman, 2006). This process is facilitated by the second key component in the control of nucleosome dynamics, the histone chaperone proteins. Histone chaperone proteins, such as Asf1, Spt2, Spt6, and FACT, function as histone sinks by which a new or displaced nucleosome is held nearby for reassembly post-RNAPII passage (Kouzarides, 2007; Li et al., 2007; Lorch et al., 2006; Mellor, 2006a; Workman, 2006). Histone chaperones also appear to reconfigure the newly re-assembled nucleosome in a fashion compatible for subsequent histone modification. Examples of this process include the aforementioned Spt6-Set2 and Asf1-Rtt109 relationships (see Chapter 3) through which the histone chaperone (in these examples: Spt6

or Asf1) work in concert with a histone modifier (Set2 or Rtt109) to allow a specific subsequent modification (Adkins et al., 2007; Natsume et al., 2007).

The configuration of nucleosomes also involves incorporation of the histone variant H2A.Z (Htz1), the third component in the control of nucleosome positioning. Recent genome-wide studies of H2A.Z localization found that this variant is enriched in silent promoters, while it is depleted in the promoters of active genes (Guillemette et al., 2005; Li et al., 2005a; Raisner et al., 2005; Workman, 2006; Zhang et al., 2005). Although unmodified H2A.Z is displaced at promoters upon gene activation, H2A.Z that is acetylated at lysine 14 is retained (Millar et al., 2006). Additional studies revealed that acetylation at H4K16 is required for H2A.Z incorporation near silent chromatin at telomeres, contributing to the mechanism by which heterochromatin spreading is controlled (Shia et al., 2006).

The influence of acetylation on the incorporation of H2A.Z within nucleosomes demonstrates the importance of the fourth mode of nucleosomal regulation: histone modification. As described extensively in this report, histone modifications can signal for the recruitment of a variety of transcription factors or other histone modifying enzymes. As discussed in recent reviews (Kouzarides, 2007; Li et al., 2007; Mellor, 2006a; Workman, 2006), histone modifications, in coordination with ATP-dependent remodeling activities, may delineate a transcriptional clock through which progression through the cycles of transcription is directed. This process may also be cell-cycle regulated, as multiple histone modifications are implicated in the regulation of DNA repair activities and inhibition of transcription to allow proper repair (Osley and Shen, 2006). Rather than repeating the many influences of histone modifications in transcriptional regulation, it is important to highlight the fact that histone modifications are only one component influencing nucleosome dynamics.

While nucleosome depletion is generally observed only at active promoters, specific studies have revealed that nucleosomes are depleted within highly active genes, such as the induced *GAL* or heat-shock genes (Lee et al., 2004; Zhao et al., 2005). In addition,

observations of multiple promoters and genes across several species have revealed significant differences in the order in which histone modifications, chaperones, and remodeling factors appear (Mellor, 2006a). These results demonstrate a current principle girding the field of chromatin structure and function: nucleosomal changes are likely specific to any given gene, therefore we cannot make a generalized set of rules that will predict when certain combinations of histone modifications or nucleosomal changes will occur for any gene. The complexity of gene regulation is clearly a topic with room for further investigation and discovery. Most importantly, studies in this area continue to yield results applicable to the treatment of human disease.

Newly discovered links between chromatin function and human disease

Extensive evidence regarding the mechanism of carcinogenesis indicate that malignant cells selectively manipulate histone modifying and chromatin remodeling enzymes, thereby altering normal nucleosomal structure or positioning, and result in increased cell proliferation, altered differentiation, and the evasion of apoptosis (Dey, 2006). Histone acetylation, in particular, has been implicated in carcinogenesis although the mechanism for this process is only now becoming understood. HDAC inhibitors are known to inhibit the growth of tumor cells, in part through their ability to reactivate the expression of tumor suppressor genes (Johnstone, 2002). The HDAC inhibitor compounds display a remarkable tumor specificity, although the reason for this specificity are not well understood (Marks et al., 2004). Interestingly, in a study examining the mechanism by which HDAC inhibitors act in the treatment of solid malignancies, investigators found that HDAC inhibitors diminish the demethylase activities at H3K4. This interesting observation links histone demethylase and deacetyase enzymes in the potential treatment of solid malignancies. (Lee et al., 2006). In addition, a newly discovered link between histone acetylation and phosphorylation in yeast revealed a series of histone modification reactions that promote a cellular switch from proliferation to cell death. Histone phosphorylation and subsequent

chromatin condensation has been linked to apoptosis in human cells. A recent report demonstrated that deacetylation of H2B lysine 11 in turn mediates H2B serine 10 phosphorylation, thereby promoting cell death (Ahn et al., 2006).

A study into the mechanism of pathogenesis for Huntington's disease revealed that the H3K9 methylase ESET is highly expressed in patients or transgenic mice with the disease, with a corresponding increase in the level of H3K9me3 (Ryu et al., 2006). This study also revealed that transcriptional activators responsible for ESET expression were elevated in neuronal cells. Examination of the anti-tumor antibiotic mithramycin revealed that this drug interferes with the transcriptional activation of ESET, resulting in a corresponding decrease of H3K9me3 in neuronal cells and a 40% increase in survival of transgenic mice (Ryu et al., 2006). These data further established the role of mis-regulated histone modifications in cancer and other human disease (see Chapter 1.3). The recent data linking histone modifications to human disease demonstrate the functional interplay between histone modifications such as acetylation, methylation, and phosphorylation. The pathways regulating histone modifications must be better understood in order to more directly treat specific human cancers.

5.5 Unanswered questions and future work

Specific questions remaining regarding Set2 regulation

Studies from our laboratory (presented in Chapters 1-4) and others (highlighted in Chapter 5) have significantly advanced our understanding of histone methylation in yeast, specifically methylation at H3K36. This advancement has also revealed a series of new questions regarding Set2 and H3K36 methylation that remain to be answered through future work. In Chapter 2, we described our study of the Set2 domain structure and identification of regions which were shown (SRI domain) or suggested (coiled-coil motif) to have important roles in Set2 function. Further study of the potential role of the coiled-coil or other regions in

Set2 is necessary to better understand the regulation and function(s) of this methyltransferase. Future work in this area could include: 1) testing whether the coiled-coil motif is responsible for protein-protein interactions between Set2 and other factors, relevant to Set2 function, and 2) further study of the domain structure of Set2 to determine mechanisms that may influence the stability of the protein. A careful analysis of the stability of Set2 and mechanisms which control this stability are important avenues of future study based on evidence that mutation or deletion of certain transcription factors, such as Spt6 and Ctk1, influences the protein level of Set2.

In addition to further work focusing on the domain structure of Set2, a more in-depth investigation into the roles of Bur1, Ctk1, and Spt6 control on Set2 methylation may reveal novel mechanisms through which Set2 and other methyltransferases are regulated. Specifically, future work in this area should first involve a careful test of our model of Bur1, Spt6, and Ctk1 influence on Set2 (see Chapter 3). Either support or a rebuke of our model will ultimately increase our understanding of how transcription factors coordinate with Set2 to influence the transcription process. Our model should be further tested through a genetic and biochemical approach in order to determine the pathway though which Set2 protein and subsequent H3K36 methylation is regulated. For example, will further work support the pathway in which Ctk1 regulates Spt6 recruitment to genes, while Spt6 in turn targets nucleosomes for proper configuration for recognition by Set2? In regard to the role of Bur1 in the regulation of histone modifications, the candidate protein Spt2, which has similar functions to Spt6, should be examined to determine whether Bur1 may regulate Spt2, and in turn influence nucleosome conformation in a manner similar to Spt6.

A second example of future work relevant to advancing our understanding of the regulation of Set2 involves studying the dynamics of Set2 association with RNAPII and chromatin. As has been explored in the case of the enzyme responsible for H2B ubiquitination, Rad6 (Xiao et al., 2005), knowing the kinectics of a transcription factor's

association with RNAPII provides for a clearer understanding of the factor's role in transcription, as well as the regulation of this factor's influence. In regard to Set2 and the duration of its interaction with RNAPII, is Set2 always associated with the transcribing polymerase during each round of transcription? Alternatively, is Set2 associated only with the pioneering round of transcription, as apparently seen with Rad6 (Xiao et al., 2005)? To address this new question, we began preliminary studies in which we monitored the association of Set2 at the *FMP27* gene by ChIP, at various time points after induction or after induction was terminated. For this experiment, we used a strain containing a *GAL* promoter flanking the *FMP27* gene, which was a kind gift from K. Struhl and prepared according to their published method (Mason and Struhl, 2003). Our preliminary results presented in **Figure 5.5** indicate that Set2 is associated with the active *FMP27* throughout the gene's transcription, and not simply when the gene is first induced, as with Rad6. A further study of the kinetics of Set2 recruitment and H3K36 methylation on genes could allow for a greater prediction of what proteins may be involved in proper targeting of Set2 to genes. For example, is H3K36me3 removed by demethylases when a gene is turned off, or do methylation levels decrease primarily due to effects of histone exchange? In the absence of H3K36me3, what are the effects of cryptic transcription at the yeast homologues of oncogenes? Ultimately, these specific questions regarding Set2 can lead to broader applications such as the development of new treatment options in humans – especially those linked to defects in histone methylation as described in Chapter 1.3 and 5.4.

The Big Picture: broad questions and applications regarding histone modifications

Although our work has focused largely on a single methyltransferase in yeast, the collective studies of histone modifications and gene regulation in yeast are important to understanding the recently completed human genome project, as well as applications in treating human disease. Importantly, significant links have been established between various histone modification activities and the cell cycle (Millar and Grunstein, 2006).

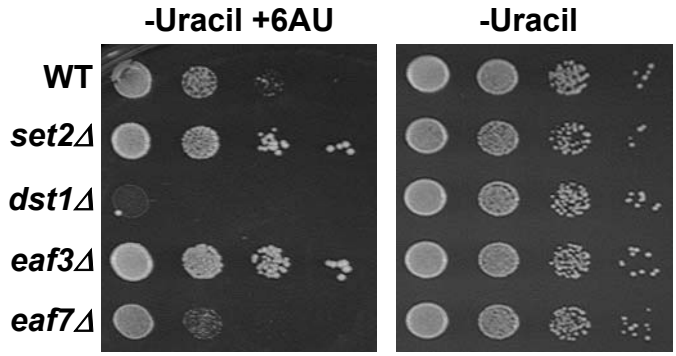
Histone modifications, the DNA damage response, and certain growth-regulating genes are influenced, or controlled by various stages of the cell cycle. How will the improper regulation of histone modifications affect cells that are already carrying damaged DNA? It is possible that a clearer understanding of the interplay between histone modifications and cell cycle control in cancer cells would allow for the development of drugs to re-order these modifications and control tumor progression. Significant headway in this area has already been made in the case of HDAC inhibitors, as described in Section 5.4.

Taken together, existing studies in yeast and humans emphasize that the maintenance of nucleosomal positioning and conformation is largely responsible for downstream effects in histone modification and gene expression or repression. Increasing evidence suggests the coordination of chromatin remodeling activities, such as FACT, Spt6, Spt2, and Asf1, with histone modifications such as H3K4, H3K36 methylation, and H3K56 acetylation (Adkins et al., 2007; Belotserkovskaya et al., 2003; Biswas et al., 2006; Chu et al., 2006; Nourani et al., 2006; Pavri et al., 2006) is a component of the regulation of nucleosomal structure. Further study in the interplay between nucleosome conformation and histone modification activities may allow for accurate predictions of the extent and type of histone modifications present on a given gene, as well as their effects. Ultimately, significant advancement in our understanding of these areas could allow for the monitoring of the proper nucleosomal structure and gene expression in particular cancer cell lines, resulting in the development of drugs or techniques to realign these mechanisms. Most scientific investigations are interesting at the individual experiment level, but the greatest excitement comes when research leads to improvements in human health and our stewardship of it.

Figure 5.1: Deletion of *RAD6* results in sensitivity to 6AU, while *EAF3* or *SET2* deletion display resistance.

(**A**) Based on existing genetic data (Krogan et al., 2002b; Krogan et al., 2003b), several gene deletions in the BY4741 background were tested for similar 6AU phenotypes as *SET2* deletion. Deletion strains were plated on synthetic dextrose-uracil medium with or without 6-azauracil (6AU, 100 μg//ml) and grown at 30°C for 2 to 3 days to monitor for transcription elongation phenotypes as in Figure 2.6. The *DST1* deletion strain is a positive control for 6AU sensitivity. (**B**) A *RAD6* deletion in an H2B-Flag tagged strain was tested for growth on 6AU as in panel A. All strains contained the plasmid pRS316 expressing the *URA3* gene, except yeast strain YCB647, which contains an integrated *URA3* gene.

A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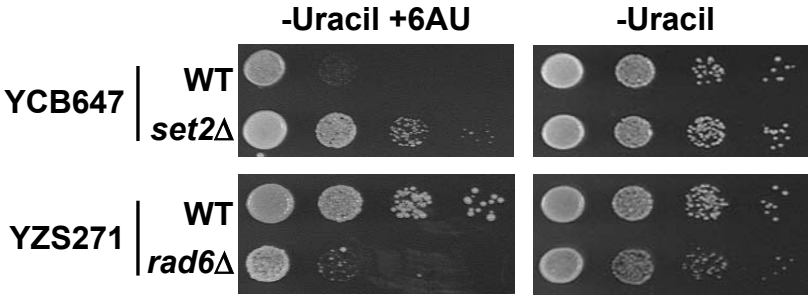
-Uracil
WT
set2Δ
dst1Δ
eaf3Δ
eaf7Δ

B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-Uracil
YCB647
WT
set2Δ
YZS271
WT
rad6Δ

Figure 5.2: Current model of Set2 function along genes.

The binding of Set2 via the SRI domain to the hyperphosphorylated CTD (at Ser2 and Ser5 positions) is shown in this current model of Set2 function. A downstream function of H3K36me3 in recruiting Eaf3 and the Rpd3C(S) is illustrated in the body of the example ORF. The upstream regulatory proteins required for Set2 function along genes, Ctk1 and Spt6, are indicated at the top. Also displayed is the possible demethylation of H3K36me3 by JMJD1 and isomerization of H3P38 by Fpr4, which function to limit H3K36me3 as described in the text. Image designed by Brian Strahl and used with permission.

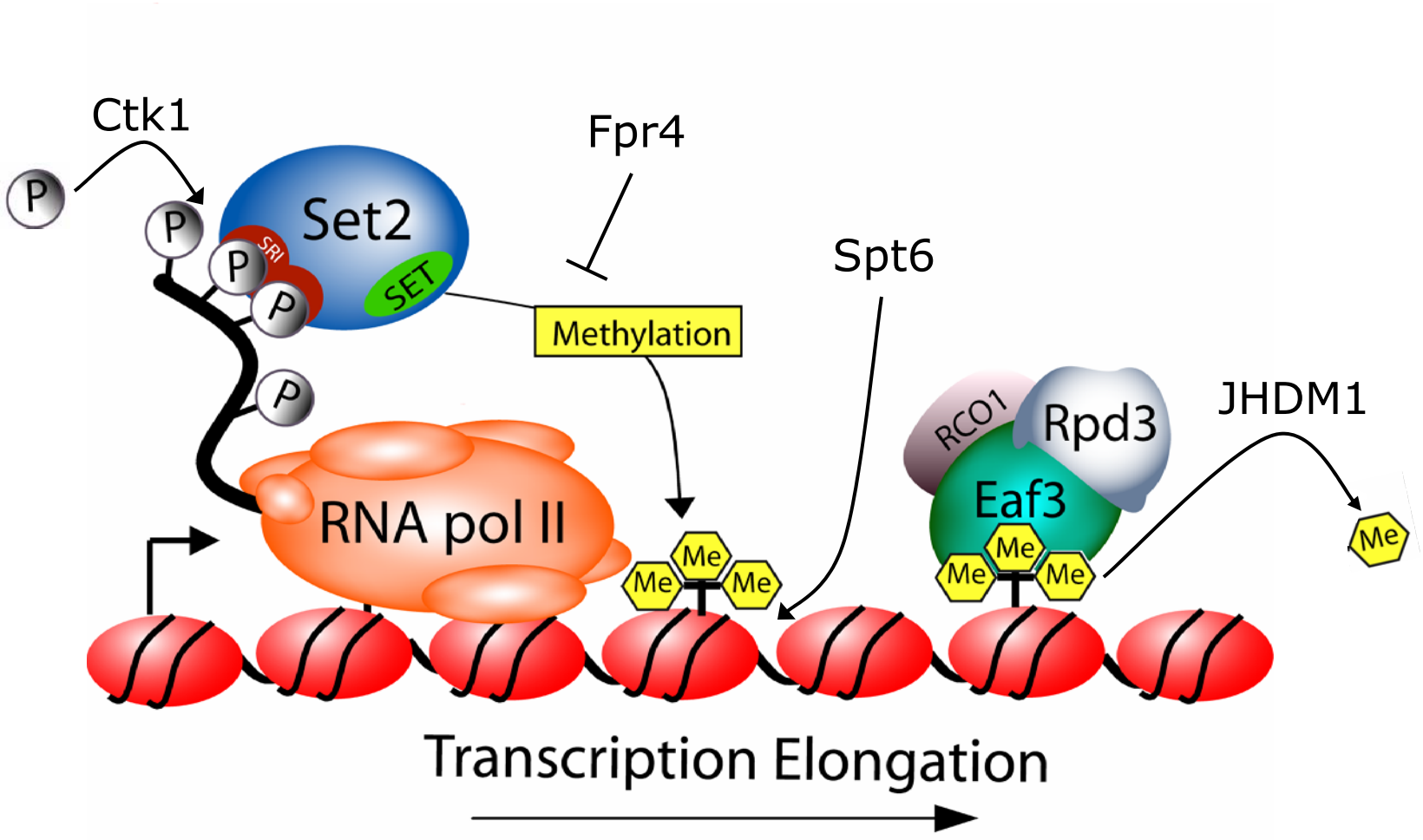

Ctk1
P
P
P
P
P
SRI
Set2
SET
Fpr4
Methylation
Spt6
RNA pol II
RCO1
Rpd3
Eaf3
JHDM1
Me
Me
Me
Me
Me
Me
Me
Transcription Elongation

Figure 5.3: Relative distributions of histone methylation and acetylation at active genes.

A schematic representation of an a typical actively transcribing gene is shown, with the promoter, coding, and intergenic regions indicated. The extent of modification at H3K4, H3K36, H3K79, H3K9, and H3K14 are shown. The level of modification is depicted by the height of the colored shape. For example, H3K4me3 is most enriched at the 5' end of active genes, whereas H3K36me3 is found in increasing amounts at the 3' end of the coding region. Data used to compile this figure was obtained from various gene-specific and genome-wide studies (Millar and Grunstein, 2006; Pokholok et al., 2005; Rao et al., 2005; Wood et al., 2007; Xiao et al., 2007). Adapted by permission from Macmillan Publishers Ltd: *Nature Reviews in Molecular Cell Biology*, Millar, C., and Grunstein M., Genome-wide patterns of histone modifications in yeast., Volume 7, Copyright 2006

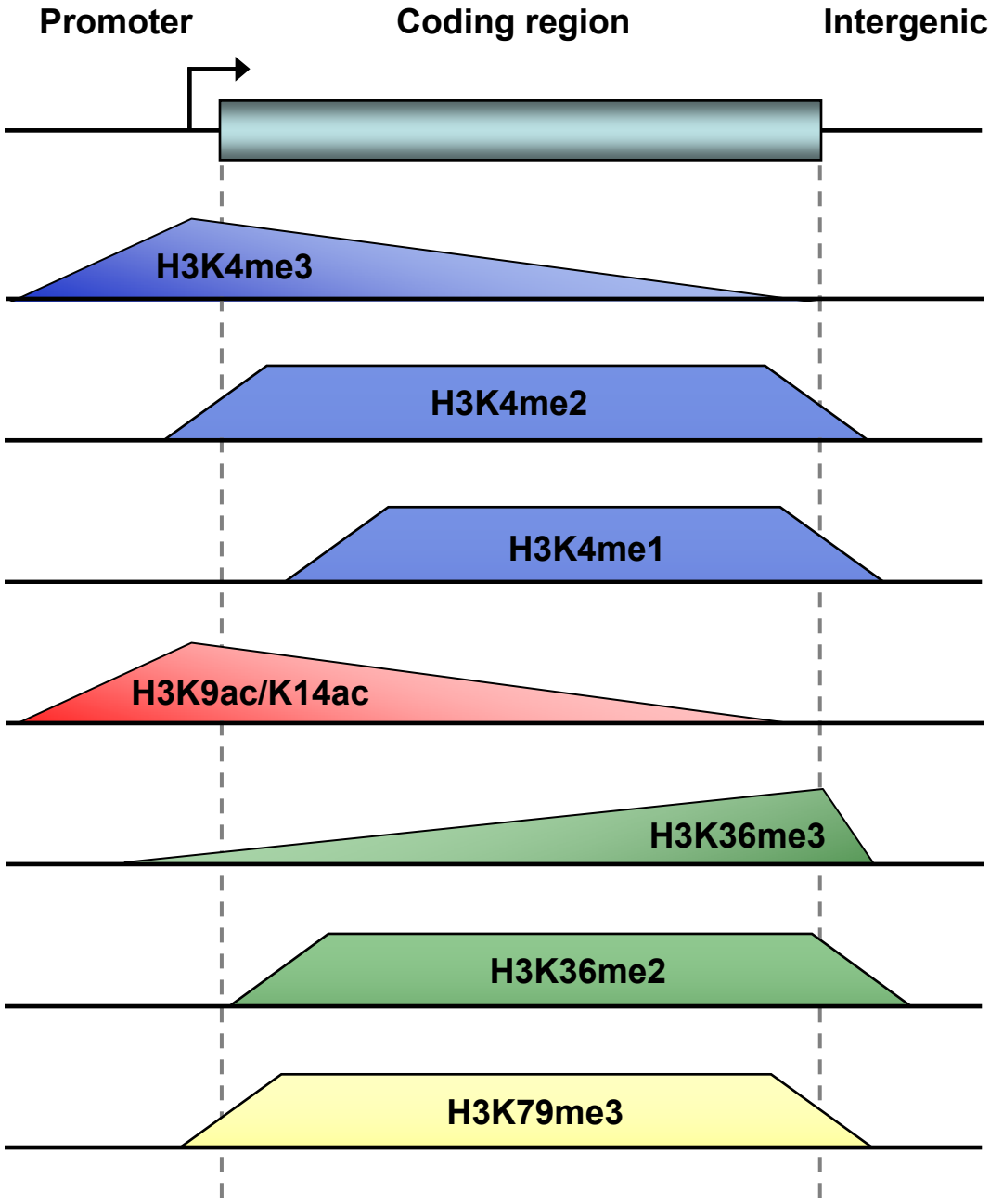
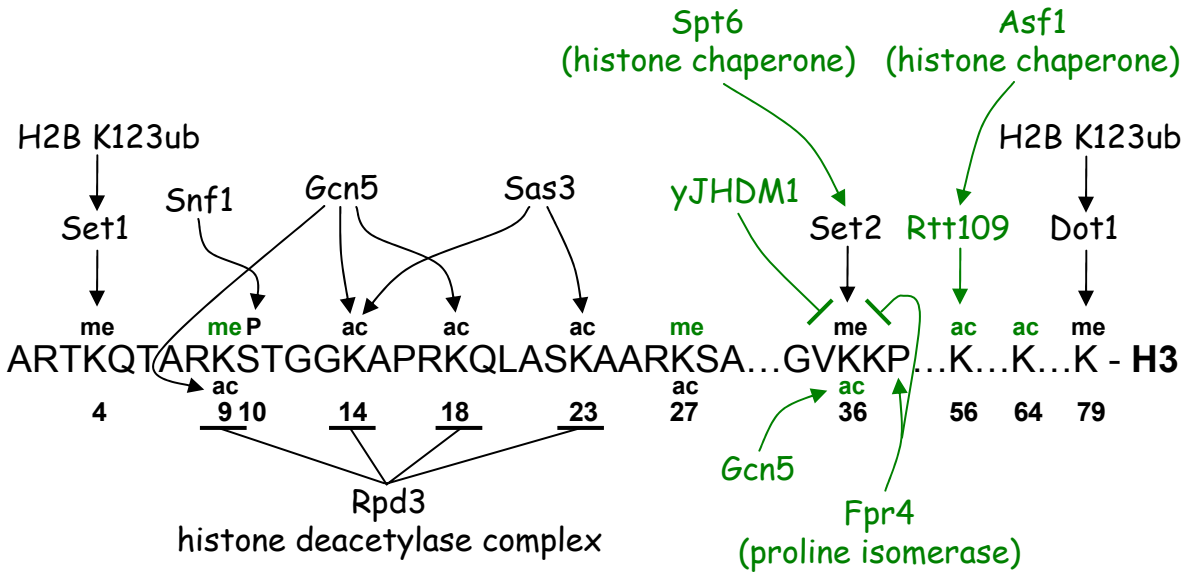

Promoter
Coding region
Intergenic
H3K4me3
H3K4me2
H3K4me1
H3K9ac/K14ac
H3K36me3
H3K36me2
H3K79me3

Figure 5.4: Current depiction of yeast histone H3 post-translational modifications, binding proteins, and related enzymes.

(**A**) Illustration of currently identified histone H3 post-translational modifications found in yeast, along with the enzymes that catalyze or antagonize these modifications. Newly identified modifications are indicated in green and are complied from recent reports (Carrozza et al., 2005; Garcia et al., 2006; Hyland et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Martin et al., 2006; Morris et al., 2006; Natsume et al., 2007; Nelson et al., 2006; Shi et al., 2006). (**B**) Indicated here are the current proteins known to bind to H3 residues in a modification-dependent manner. Adapted from *TRENDS in Genetics*, Volume 22, Mellor, J., Dynamic nucleosomes and gene transcription, 320-329, Copyright 2006, with permission from Elsevier (Mellor, 2006).

A

Modification catalysis, removal, and antagonism



B

Modification recognition

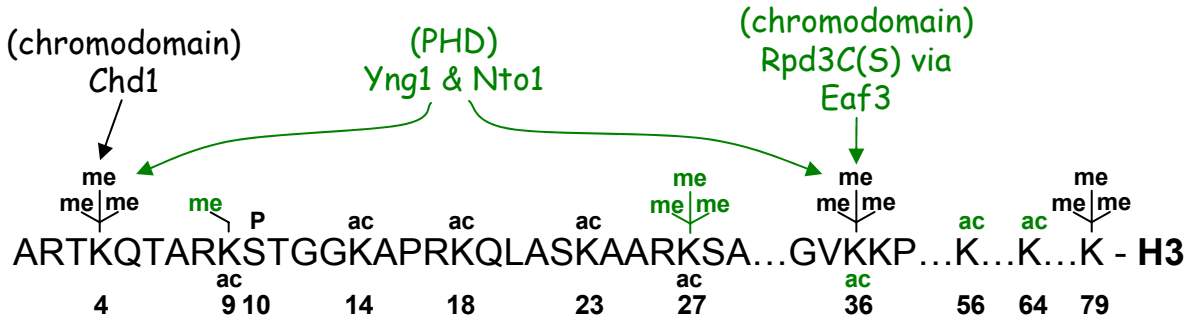
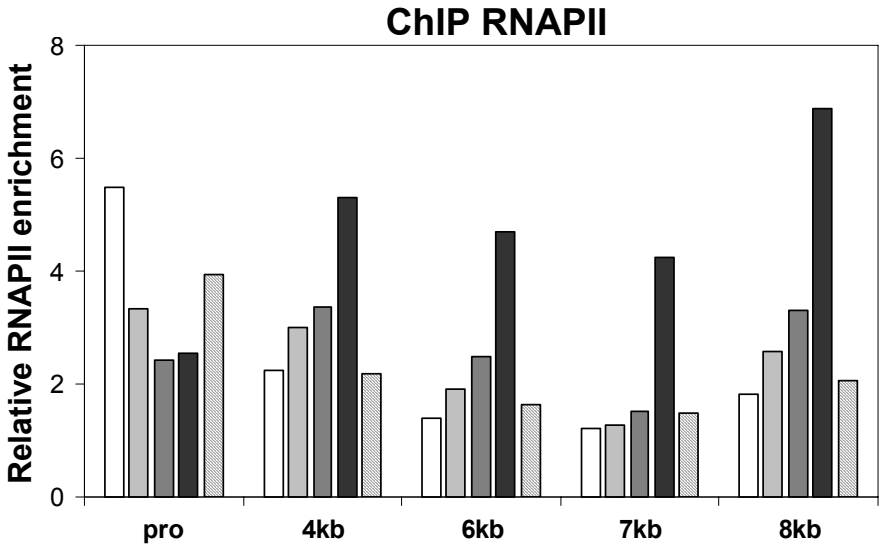


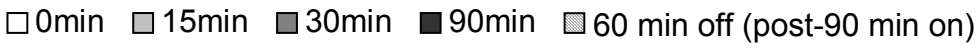
Figure 5.5: Set2 is associated with an active gene through each round of transcription.

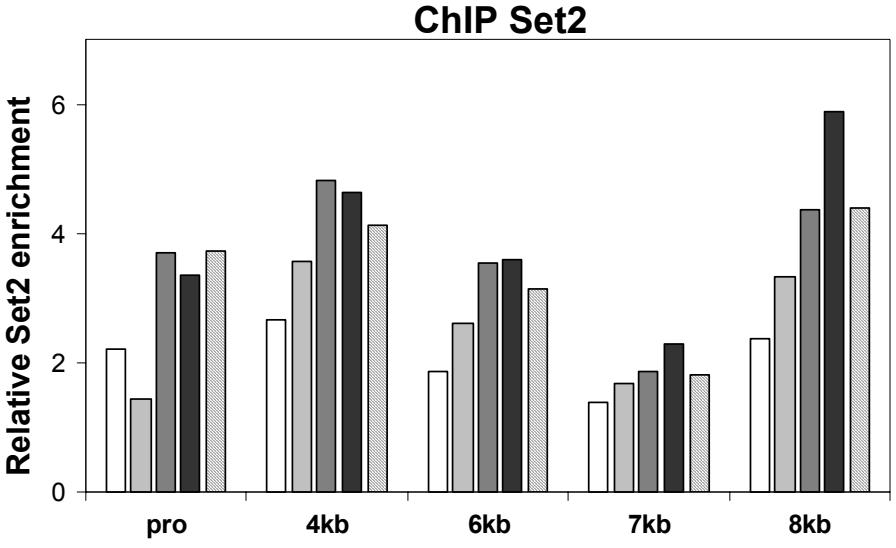
(**A**) The GAL-FMP27 yeast strain was analyzed by chromatin immunoprecipitation (ChIP) to determine the relative enrichment of RNAPII or Set2 on chromatin at the *FMP27* gene during induction with galactose, using the 8WG16 antibody (RNAPII) or the Set2 antibody (described in Section 3.2). Individual cell pellets were collected before or after galactose induction at the indicated timepoints, and ChIP was performed as for Fig. 2.6C. The DNA isolated from each IP was analyzed in PCR reactions using primer pairs for the indicated regions of the *FMP27* gene or an intergenic region at chromosome V (ChV), used as a loading control in all PCR reactions. The histogram displays the relative enrichment values of RNAPII or Set2, calculated by dividing the ratio of band intensities for IP DNA/ChV with the ratio of intensities for the Input DNA/ChV. (**B**) The unaltered GAL-FMP27 yeast strain (F-WT) or one in which *SET2* was deleted (F-*set2Δ*) was analyzed as in (A), but with *GAL* induction terminated through addition of dextrose. The relative enrichment of RNAPII or Set2 at the *FMP27* gene was determined as in (A), using individual cell pellets collected after induction shut-off for the indicated durations. The enrichment of Set2 displayed at the promoter and 4kb region in the case of *SET2* deletion is likely an error in the particular PCR reaction, as these initial data were obtained from only one experiment. The GAL-FMP27 strain was a kind gift from K. Struhl and prepared according to their published method (Mason and Struhl, 2003).

A

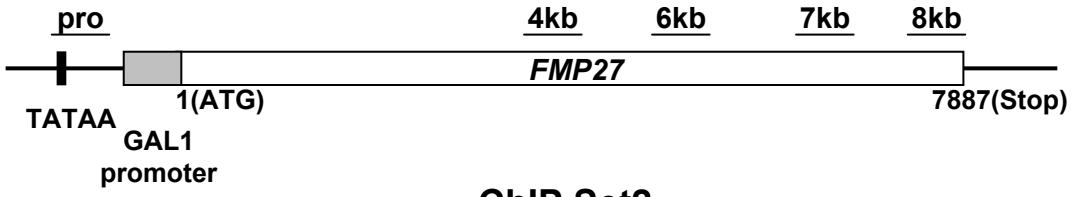

pro
4kb
6kb
7kb
8kb
FMP27
TATAA
1(ATG)
7887(Stop)
GAL1
promoter


ChIP RNAPII
Relative RNAPII enrichment
8
6
4
2
0
pro
4kb
6kb
7kb
8kb


0min
15min
30min
90min
60 min off (post-90 min on)


ChIP Set2
Relative Set2 enrichment
6
4
2
0
pro
4kb
6kb
7kb
8kb

B


pro
4kb
6kb
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8kb
FMP27
TATAA
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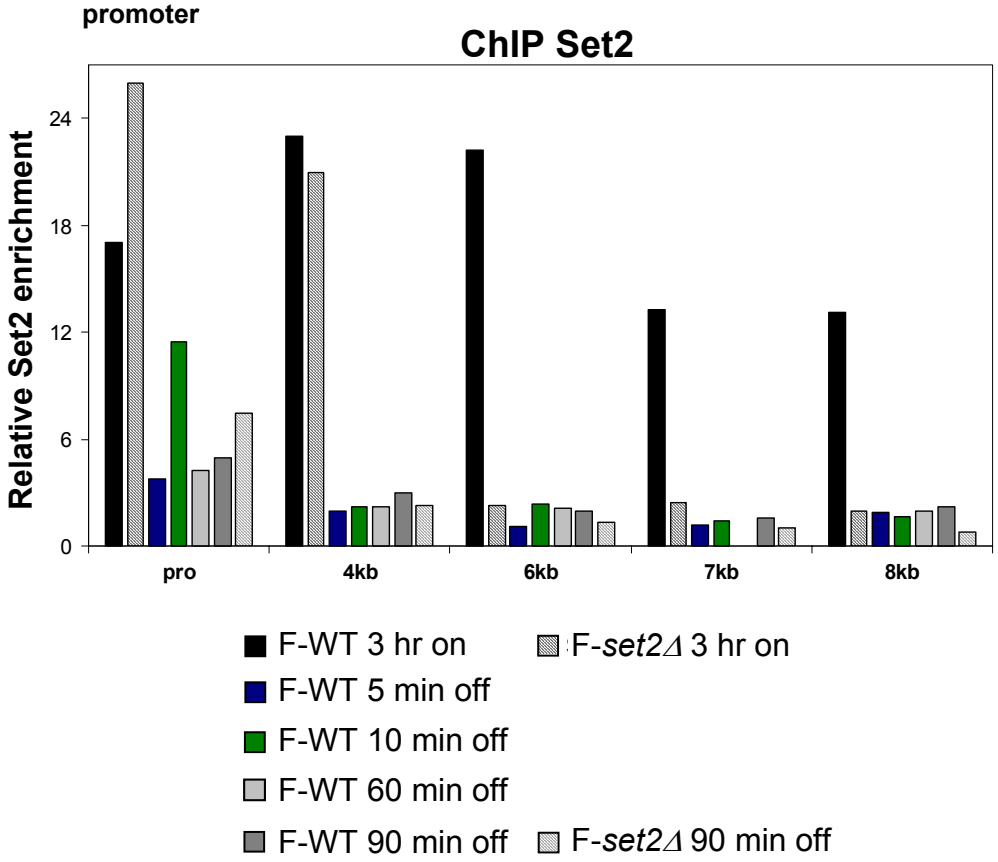
7887(Stop)
GAL1
promoter


ChIP Set2
Relative Set2 enrichment
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12
18
24
pro
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6kb
7kb
8kb
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F-set2Δ 3 hr on
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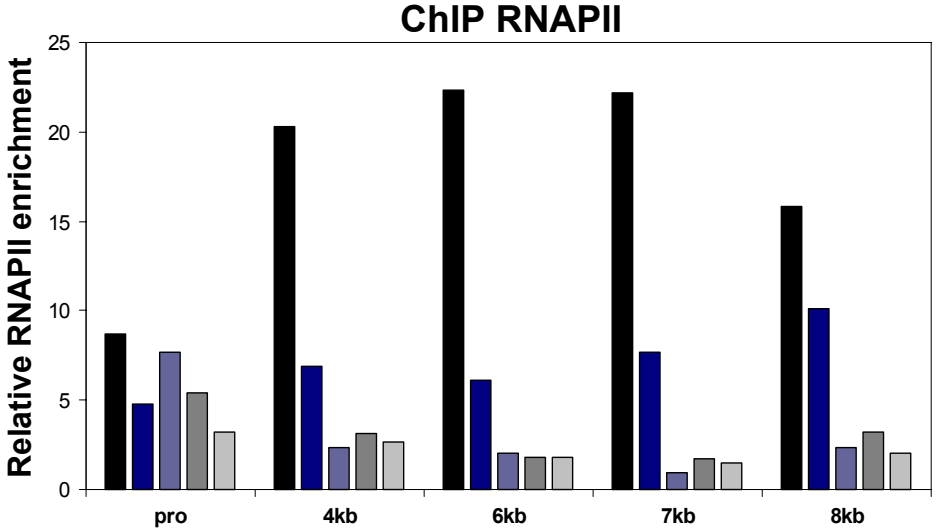
F-set2Δ 90 min off


ChIP RNAPII
Relative RNAPII enrichment
0
5
10
15
20
25
pro
4kb
6kb
7kb
8kb

References

1. Adams,C.R. and Kamakaka,R.T. (1999). Chromatin assembly: biochemical identities and genetic redundancy. Curr. Opin. Genet. Dev. *9*, 185-190.

2. Adkins,M.W., Carson,J.J., English,C.M., Ramey,C.J., and Tyler,J.K. (2007). The histone chaperone anti-silencing function 1 stimulates the acetylation of newly synthesized histone H3 in S-phase. J. Biol. Chem. *282*, 1334-1340.

3. 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.

4. Ahmad,K. and Henikoff,S. (2002). Epigenetic consequences of nucleosome dynamics. Cell *111*, 281-284.

5. Ahn,S.H., Diaz,R.L., Grunstein,M., and Allis,C.D. (2006). Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10. Mol. Cell *24*, 211-220.

6. Ahn,S.H., Kim,M., and Buratowski,S. (2004). Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Mol. Cell *13*, 67-76.

7. Alen,C., Kent,N.A., Jones,H.S., O'Sullivan,J., Aranda,A., and Proudfoot,N.J. (2002). A role for chromatin remodeling in transcriptional termination by RNA polymerase II. Mol. Cell *10*, 1441-1452.

8. Almer,A. and Horz,W. (1986). Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast. EMBO J. *5*, 2681-2687.

9. Aparicio,O.M., Billington,B.L., and Gottschling,D.E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279-1287.

10. Archambault,J., Lacroute,F., Ruet,A., and Friesen,J.D. (1992). Genetic interaction between transcription elongation factor TFIIS and RNA polymerase II. Mol. Cell Biol. *12*, 4142-4152.

11. Arents,G., Burlingame,R.W., Wang,B.C., Love,W.E., and Moudrianakis,E.N. (1991). The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. Proc. Natl. Acad. Sci. U. S. A *88*, 10148-10152.

12. Bannister,A.J., Zegerman,P., Partridge,J.F., Miska,E.A., Thomas,J.O., Allshire,R.C., and Kouzarides,T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120-124.

13. Beisel,C., Imhof,A., Greene,J., Kremmer,E., and Sauer,F. (2002). Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. Nature *419*, 857-862.

14. Belle,A., Tanay,A., Bitincka,L., Shamir,R., and O'Shea,E.K. (2006). Quantification of protein half-lives in the budding yeast proteome. Proc. Natl. Acad. Sci. U. S. A *103*, 13004-13009.

15. 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.

16. Berger,S.L. (2002). Histone modifications in transcriptional regulation. Curr. Opin. Genet. Dev. *12*, 142-148.

17. 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.

18. Biswas,D., Dutta-Biswas,R., Mitra,D., Shibata,Y., Strahl,B.D., Formosa,T., and Stillman,D.J. (2006). Opposing roles for Set2 and yFACT in regulating TBP binding at promoters. EMBO J. *25*, 4479-4489.

19. Bjorklund,S., Almouzni,G., Davidson,I., Nightingale,K.P., and Weiss,K. (1999). Global transcription regulators of eukaryotes. Cell *96*, 759-767.

20. Boeger,H., Griesenbeck,J., Strattan,J.S., and Kornberg,R.D. (2003). Nucleosomes unfold completely at a transcriptionally active promoter. Mol. Cell *11*, 1587-1598.

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

22. Brachmann,C.B., Sherman,J.M., Devine,S.E., Cameron,E.E., Pillus,L., and Boeke,J.D. (1995). The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. *9*, 2888-2902.

23. Braunstein,M., Rose,A.B., Holmes,S.G., Allis,C.D., and Broach,J.R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. *7*, 592-604.

24. Briggs,S.D., Bryk,M., Strahl,B.D., Cheung,W.L., Davie,J.K., Dent,S.Y., Winston,F., and Allis,C.D. (2001). Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. Genes Dev. *15*, 3286-3295.

25. Briggs,S.D., Xiao,T., Sun,Z.W., Caldwell,J.A., Shabanowitz,J., Hunt,D.F., Allis,C.D., and Strahl,B.D. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. Nature *418*, 498.

26. Butler,J.A. (1965). Role of histones and other proteins in gene control. Nature *207*, 1041-1042.

27. Byrd,K.N. and Shearn,A. (2003). ASH1, a Drosophila trithorax group protein, is required for methylation of lysine 4 residues on histone H3. Proc. Natl. Acad. Sci. U. S. A *100*, 11535-11540.

28. Byvoet,P., Shepherd,G.R., Hardin,J.M., and Noland,B.J. (1972). The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. Arch. Biochem. Biophys. *148*, 558-567.

29. Cadena,D.L. and Dahmus,M.E. (1987). Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. J. Biol. Chem. *262*, 12468-12474.

30. Cairns,B.R. (2005). Chromatin remodeling complexes: strength in diversity, precision through specialization. Curr. Opin. Genet. Dev. *15*, 185-190.

31. 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.

32. Cardoso,C., Timsit,S., Villard,L., Khrestchatisky,M., Fontes,M., and Colleaux,L. (1998). Specific interaction between the XNP/ATR-X gene product and the SET domain of the human EZH2 protein. Hum. Mol. Genet. *7*, 679-684.

33. Carmen,A.A., Milne,L., and Grunstein,M. (2002). Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J. Biol. Chem. *277*, 4778-4781.

34. 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., and Workman,J.L. (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell *123*, 581-592.

35. Chadwick,R.B., Jiang,G.L., Bennington,G.A., Yuan,B., Johnson,C.K., Stevens,M.W., Niemann,T.H., Peltomaki,P., Huang,S., and de la,C.A. (2000). Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. Proc. Natl. Acad. Sci. U. S. A *97*, 2662-2667.

36. Chien,C.T., Buck,S., Sternglanz,R., and Shore,D. (1993). Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. Cell *75*, 531-541.

37. 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.

38. Ciba Foundation Study Group No.24 (1966). Histones: Their Role in the Transfer of Genetic Information., A.V.S.de Reuck and J.Knight, eds. (Boston: Little, Brown and Company).

39. Corden,J.L. (1990). Tails of RNA polymerase II. Trends Biochem. Sci. *15*, 383-387.

40. Cui,X., De,V., I, Slany,R., Miyamoto,A., Firestein,R., and Cleary,M.L. (1998). Association of SET domain and myotubularin-related proteins modulates growth control. Nat. Genet. *18*, 331-337.

41. Dey,P. (2006). Chromatin remodeling, cancer and chemotherapy. Curr. Med. Chem. *13*, 2909-2919.

42. Dhalluin,C., Carlson,J.E., Zeng,L., He,C., Aggarwal,A.K., and Zhou,M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491-496.

43. Dlakic,M. (2001). Chromatin silencing protein and pachytene checkpoint regulator Dot1p has a methyltransferase fold. Trends Biochem. Sci. *26*, 405-407.

44. Doherty,A.J., Serpell,L.C., and Ponting,C.P. (1996). The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. Nucleic Acids Res. *24*, 2488-2497.

45. Dover,J., Schneider,J., Tawiah-Boateng,M.A., Wood,A., Dean,K., Johnston,M., and Shilatifard,A. (2002). Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J. Biol. Chem. *277*, 28368-28371.

46. 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.

47. Duerre,J.A. and Lee,C.T. (1974). In vivo methylation and turnover of rat brain histones. J. Neurochem. *23*, 541-547.

48. Edmondson,D.G., Smith,M.M., and Roth,S.Y. (1996). Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. *10*, 1247-1259.

49. Eissenberg,J.C., James,T.C., Foster-Hartnett,D.M., Hartnett,T., Ngan,V., and Elgin,S.C. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A *87*, 9923-9927.

50. Eissenberg,J.C. and Shilatifard,A. (2006). Leaving a mark: the many footprints of the elongating RNA polymerase II. Curr. Opin. Genet. Dev.

51. Exinger,F. and Lacroute,F. (1992). 6-Azauracil inhibition of GTP biosynthesis in Saccharomyces cerevisiae. Curr. Genet. *22*, 9-11.

52. Fedor,M.J. and Kornberg,R.D. (1989). Upstream activation sequence-dependent alteration of chromatin structure and transcription activation of the yeast GAL1-GAL10 genes. Mol. Cell Biol. *9*, 1721-1732.

53. Felsenfeld,G. (1978). Chromatin. Nature *271*, 115-122.

54. Feng,Q., Wang,H., Ng,H.H., Erdjument-Bromage,H., Tempst,P., Struhl,K., and Zhang,Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr. Biol. *12*, 1052-1058.

55. Flemming,W. (1882). Zellsubstanz, Kern und Zelltheilung., F.C.W.Vogel, ed. (Leipzig.

56. Formosa,T., Ruone,S., Adams,M.D., Olsen,A.E., Eriksson,P., Yu,Y., Rhoades,A.R., Kaufman,P.D., and Stillman,D.J. (2002). Defects in SPT16 or POB3 (yFACT) in Saccharomyces cerevisiae cause dependence on the Hir/Hpc pathway: polymerase passage may degrade chromatin structure. Genetics *162*, 1557-1571.

57. Garcia,B.A., Hake,S.B., Diaz,R.L., Kauer,M., Morris,S.A., Recht,J., Shabanowitz,J., Mishra,N., Strahl,B.D., Allis,C.D., and Hunt,D.F. (2006). Organismal differences in post-translational modifications in histones H3 and H4. J. Biol. Chem.

58. 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.

59. Gerber,M. and Shilatifard,A. (2003). Transcriptional elongation by RNA polymerase II and histone methylation. J. Biol. Chem. *278*, 26303-26306.

60. Goll,M.G. and Bestor,T.H. (2002). Histone modification and replacement in chromatin activation. Genes Dev. *16*, 1739-1742.

61. Green,E.M., Antczak,A.J., Bailey,A.O., Franco,A.A., Wu,K.J., Yates,J.R., III, and Kaufman,P.D. (2005). Replication-independent histone deposition by the HIR complex and Asf1. Curr. Biol. *15*, 2044-2049.

62. Grunstein,M. (1990). Histone function in transcription. Annu. Rev. Cell Biol. *6*, 643-678.

63. 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.

64. Hampsey,M. and Reinberg,D. (2003). Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. Cell *113*, 429-432.

65. Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

66. Hansen,J.C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. Annu. Rev. Biophys. Biomol. Struct. *31*, 361-392.

67. Hartzog,G.A., Wada,T., Handa,H., and Winston,F. (1998). Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae. Genes Dev. *12*, 357-369.

68. Heard,E. and Disteche,C.M. (2006). Dosage compensation in mammals: fine-tuning the expression of the X chromosome. Genes Dev. *20*, 1848-1867.

69. Hediger,F. and Gasser,S.M. (2006). Heterochromatin protein 1: don't judge the book by its cover! Curr. Opin. Genet. Dev. *16*, 143-150.

70. Hengartner,C.J., Myer,V.E., Liao,S.M., Wilson,C.J., Koh,S.S., and Young,R.A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol. Cell *2*, 43-53.

71. Henry,K.W., Wyce,A., Lo,W.S., Duggan,L.J., Emre,N.C., Kao,C.F., Pillus,L., Shilatifard,A., Osley,M.A., and Berger,S.L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev. *17*, 2648-2663.

72. 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.

73. Hiragami,K. and Festenstein,R. (2005). Heterochromatin protein 1: a pervasive controlling influence. Cell Mol. Life Sci. *62*, 2711-2726.

74. Hsu,J.Y., Sun,Z.W., Li,X., Reuben,M., Tatchell,K., Bishop,D.K., Grushcow,J.M., Brame,C.J., Caldwell,J.A., Hunt,D.F., Lin,R., Smith,M.M., and Allis,C.D. (2000). Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell *102*, 279-291.

75. Huang,R.C. and Bonner,J. (1962). Histone, a suppressor of chromosomal RNA synthesis. Proc. Natl. Acad. Sci. U. S. A *48*, 1216-1222.

76. Huang,S. (2002). Histone methyltransferases, diet nutrients and tumour suppressors. Nat. Rev. Cancer *2*, 469-476.

77. Huang,Y., Fang,J., Bedford,M.T., Zhang,Y., and Xu,R.M. (2006). Recognition of histone H3 lysine-4 methylation by the double tudor domain of JMJD2A. Science *312*, 748-751.

78. Hyland,E.M., Cosgrove,M.S., Molina,H., Wang,D., Pandey,A., Cottee,R.J., and Boeke,J.D. (2005). Insights into the role of histone H3 and histone H4 core modifiable residues in Saccharomyces cerevisiae. Mol. Cell Biol. *25*, 10060-10070.

79. Hyle,J.W., Shaw,R.J., and Reines,D. (2003). Functional distinctions between IMP dehydrogenase genes in providing mycophenolate resistance and guanine prototrophy to yeast. J. Biol. Chem. *278*, 28470-28478.

80. Jackson,R.C., Weber,G., and Morris,H.P. (1975). IMP dehydrogenase, an enzyme linked with proliferation and malignancy. Nature *256*, 331-333.

81. Jacobson,S. and Pillus,L. (1999). Modifying chromatin and concepts of cancer. Curr. Opin. Genet. Dev. *9*, 175-184.

82. James,T.C. and Elgin,S.C. (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Mol. Cell Biol. *6*, 3862-3872.

83. Jenuwein,T. and Allis,C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

84. Johnstone,R.W. (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat. Rev. Drug Discov. *1*, 287-299.

85. Jones,J.C., Phatnani,H.P., Haystead,T.A., MacDonald,J.A., Alam,S.M., and Greenleaf,A.L. (2004). C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. J. Biol. Chem. *279*, 24957-24964.

86. Jones,R.S. and Gelbart,W.M. (1993). The Drosophila Polycomb-group gene Enhancer of zeste contains a region with sequence similarity to trithorax. Mol. Cell Biol. *13*, 6357-6366.

87. 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.

88. Kadosh,D. and Struhl,K. (1998). Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. Genes Dev. *12*, 797-805.

89. Kao,C.F. and Osley,M.A. (2003). In vivo assays to study histone ubiquitylation. Methods *31*, 59-66.

90. Kaplan,C.D., Holland,M.J., and Winston,F. (2005). Interaction between transcription elongation factors and mRNA 3'-end formation at the Saccharomyces cerevisiae GAL10-GAL7 locus. J. Biol. Chem. *280*, 913-922.

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

92. Kaplan,C.D., Morris,J.R., Wu,C., and Winston,F. (2000). Spt5 and spt6 are associated with active transcription and have characteristics of general elongation factors in D. melanogaster. Genes Dev. *14*, 2623-2634.

93. Katan-Khaykovich,Y. and Struhl,K. (2005). Heterochromatin formation involves changes in histone modifications over multiple cell generations. EMBO J. *24*, 2138-2149.

94. Keogh,M.C., Cho,E.J., Podolny,V., and Buratowski,S. (2002). Kin28 is found within TFIIH and a Kin28-Ccl1-Tfb3 trimer complex with differential sensitivities to T-loop phosphorylation. Mol. Cell Biol. *22*, 1288-1297.

95. 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., Boone,C., Emili,A., Weissman,J.S., Hughes,T.R., Strahl,B.D., Grunstein,M., Greenblatt,J.F., Buratowski,S., and Krogan,N.J. (2005). Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. Cell *123*, 593-605.

96. Keogh,M.C., Podolny,V., and Buratowski,S. (2003). Bur1 kinase is required for efficient transcription elongation by RNA polymerase II. Mol. Cell Biol. *23*, 7005-7018.

97. Khan,A.U. and Hampsey,M. (2002). Connecting the DOTs: covalent histone modifications and the formation of silent chromatin. Trends Genet. *18*, 387-389.

98. Kizer,K.O., Phatnani,H.P., Shibata,Y., Hall,H., Greenleaf,A.L., and Strahl,B.D. (2005). A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol. Cell Biol. *25*, 3305-3316.

99. Kizer,K.O., Xiao,T., and Strahl,B.D. (2006). Accelerated nuclei preparation and methods for analysis of histone modifications in yeast. Methods *40*, 296-302.

100. Klose,R.J., Yamane,K., Bae,Y., Zhang,D., Erdjument-Bromage,H., Tempst,P., Wong,J., and Zhang,Y. (2006). The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature.

101. Komarnitsky,P., Cho,E.J., and Buratowski,S. (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. *14*, 2452-2460.

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

103. Kornberg,R.D. and Lorch,Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell *98*, 285-294.

104. Kornberg,R.D. and Thomas,J.O. (1974). Chromatin structure; oligomers of the histones. Science *184*, 865-868.

105. Kossel,A. (1884). Ueber einen peptoartigen bestandheil des zellkerns. Z. Physiol. Chem. *8*, 511-515.

106. Kouzarides,T. (2002). Histone methylation in transcriptional control. Curr. Opin. Genet. Dev. *12*, 198-209.

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

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

109. Krogan,N.J., Dover,J., Khorrami,S., Greenblatt,J.F., Schneider,J., Johnston,M., and Shilatifard,A. (2002a). COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression. J. Biol. Chem. *277*, 10753-10755.

110. Krogan,N.J., Dover,J., Wood,A., Schneider,J., Heidt,J., Boateng,M.A., Dean,K., Ryan,O.W., Golshani,A., Johnston,M., Greenblatt,J.F., and Shilatifard,A. (2003a). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol. Cell *11*, 721-729.

111. 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. (2002b). RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol. Cell Biol. *22*, 6979-6992.

112. Krogan,N.J., Kim,M., Tong,A., Golshani,A., Cagney,G., Canadien,V., Richards,D.P., Beattie,B.K., Emili,A., Boone,C., Shilatifard,A., Buratowski,S., and Greenblatt,J.

(2003b). Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol. Cell Biol. *23*, 4207-4218.

113. Kurdistani,S.K. and Grunstein,M. (2003). Histone acetylation and deacetylation in yeast. Nat. Rev. Mol. Cell Biol. *4*, 276-284.

114. Kurdistani,S.K., Robyr,D., Tavazoie,S., and Grunstein,M. (2002). Genome-wide binding map of the histone deacetylase Rpd3 in yeast. Nat. Genet. *31*, 248-254.

115. Kurdistani,S.K., Tavazoie,S., and Grunstein,M. (2004). Mapping global histone acetylation patterns to gene expression. Cell *117*, 721-733.

116. Kurotaki,N., Imaizumi,K., Harada,N., Masuno,M., Kondoh,T., Nagai,T., Ohashi,H., Naritomi,K., Tsukahara,M., Makita,Y., Sugimoto,T., Sonoda,T., Hasegawa,T., Chinen,Y., Tomita Ha,H.A., Kinoshita,A., Mizuguchi,T., Yoshiura,K.K., Ohta,T., Kishino,T., Fukushima,Y., Niikawa,N., and Matsumoto,N. (2002). Haploinsufficiency of NSD1 causes Sotos syndrome. Nat. Genet. *30*, 365-366.

117. Kwon,H., Imbalzano,A.N., Khavari,P.A., Kingston,R.E., and Green,M.R. (1994). Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex. Nature *370*, 477-481.

118. Lachner,M. and Jenuwein,T. (2002). The many faces of histone lysine methylation. Curr. Opin. Cell Biol. *14*, 286-298.

119. Lachner,M., O'Carroll,D., Rea,S., Mechtler,K., and Jenuwein,T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

120. Lachner,M., O'Sullivan,R.J., and Jenuwein,T. (2003). An epigenetic road map for histone lysine methylation. J. Cell Sci. *116*, 2117-2124.

121. Landry,J., Sutton,A., Hesman,T., Min,J., Xu,R.M., Johnston,M., and Sternglanz,R. (2003). Set2-catalyzed methylation of histone H3 represses basal expression of GAL4 in Saccharomyces cerevisiae. Mol. Cell Biol. *23*, 5972-5978.

122. Laribee,R.N., Krogan,N.J., Xiao,T., Shibata,Y., Hughes,T.R., Greenblatt,J.F., and Strahl,B.D. (2005). BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. Curr. Biol. *15*, 1487-1493.

123. Laribee, R. N., Shibata, Y., Mersman, D. P., Collins, S. R., Kemmeren, P., Weissman, J. S., Briggs, S. D., Krogan, N. J., and Strahl, B. D. The CCR4/NOT complex associates with the proteasome and regulates histone methylation. Proc.Natl.Acad.Sci.U.S.A . 2007.
Ref Type: In Press

124. 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.

125. Lee,D.Y., Teyssier,C., Strahl,B.D., and Stallcup,M.R. (2005). Role of protein methylation in regulation of transcription. Endocr. Rev. *26*, 147-170.

126. Lee,J.M. and Greenleaf,A.L. (1989). A protein kinase that phosphorylates the C-terminal repeat domain of the largest subunit of RNA polymerase II. Proc. Natl. Acad. Sci. U. S. A *86*, 3624-3628.

127. Lee,M.G., Wynder,C., Bochar,D.A., Hakimi,M.A., Cooch,N., and Shiekhattar,R. (2006). Functional interplay between histone demethylase and deacetylase enzymes. Mol. Cell Biol. *26*, 6395-6402.

128. Lee,T.I. and Young,R.A. (2000). Transcription of eukaryotic protein-coding genes. Annu. Rev. Genet. *34*, 77-137.

129. Li,B., Carey,M., and Workman,J.L. (2007). The Role of Chromatin during Transcription. Cell *128*, 707-719.

130. Li,B., Howe,L., Anderson,S., Yates,J.R., III, and Workman,J.L. (2003). The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J. Biol. Chem. *278*, 8897-8903.

131. 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.

132. Li,H., Ilin,S., Wang,W., Duncan,E.M., Wysocka,J., Allis,C.D., and Patel,D.J. (2006). Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature *442*, 91-95.

133. Li,J., Moazed,D., and Gygi,S.P. (2002). Association of the histone methyltransferase Set2 with RNA polymerase II plays a role in transcription elongation. J. Biol. Chem. *277*, 49383-49388.

134. Li,M., Phatnani,H.P., Guan,Z., Sage,H., Greenleaf,A.L., and Zhou,P. (2005b). Solution structure of the Set2-Rpb1 interacting domain of human Set2 and its interaction with the hyperphosphorylated C-terminal domain of Rpb1. Proc. Natl. Acad. Sci. U. S. A *102*, 17636-17641.

135. Lindstrom,D.L., Squazzo,S.L., Muster,N., Burckin,T.A., Wachter,K.C., Emigh,C.A., McCleery,J.A., Yates,J.R., III, 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.

136. Liu,C.L., Kaplan,T., Kim,M., Buratowski,S., Schreiber,S.L., Friedman,N., and Rando,O.J. (2005). Single-nucleosome mapping of histone modifications in S. cerevisiae. PLoS. Biol. *3*, e328.

137. Lo,W.S., Trievel,R.C., Rojas,J.R., Duggan,L., Hsu,J.Y., Allis,C.D., Marmorstein,R., and Berger,S.L. (2000). Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. Mol. Cell *5*, 917-926.

138. Lohr,D. (1997). Nucleosome transactions on the promoters of the yeast GAL and PHO genes. J. Biol. Chem. *272*, 26795-26798.

139. Loo,S. and Rine,J. (1994). Silencers and domains of generalized repression. Science *264*, 1768-1771.

140. Lorch,Y., Maier-Davis,B., and Kornberg,R.D. (2006). Chromatin remodeling by nucleosome disassembly in vitro. Proc. Natl. Acad. Sci. U. S. A *103*, 3090-3093.

141. Lorch,Y., Zhang,M., and Kornberg,R.D. (1999). Histone octamer transfer by a chromatin-remodeling complex. Cell *96*, 389-392.

142. Lowary,P.T. and Widom,J. (1989). Higher-order structure of Saccharomyces cerevisiae chromatin. Proc. Natl. Acad. Sci. U. S. A *86*, 8266-8270.

143. 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.

144. Lutfiyya,L., Hesman,T., and Johnston,M. (1995). What activates expression of a transcriptional activator? Analysis of the weak GAL4 promoter. Yeast *11*, S205-S256.

145. Malone,E.A., Clark,C.D., Chiang,A., and Winston,F. (1991). Mutations in SPT16/CDC68 suppress cis- and trans-acting mutations that affect promoter function in Saccharomyces cerevisiae. Mol. Cell Biol. *11*, 5710-5717.

146. Mandal,S.S., Cho,H., Kim,S., Cabane,K., and Reinberg,D. (2002). FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation. Mol. Cell Biol. *22*, 7543-7552.

147. Marks,P.A., Richon,V.M., Miller,T., and Kelly,W.K. (2004). Histone deacetylase inhibitors. Adv. Cancer Res. *91*, 137-168.

148. Marmorstein,R. (2001). Protein modules that manipulate histone tails for chromatin regulation. Nat. Rev. Mol. Cell Biol. *2*, 422-432.

149. Marmorstein,R. and Berger,S.L. (2001). Structure and function of bromodomains in chromatin-regulating complexes. Gene *272*, 1-9.

150. Marmorstein,R. and Roth,S.Y. (2001). Histone acetyltransferases: function, structure, and catalysis. Curr. Opin. Genet. Dev. *11*, 155-161.

151. Martin,D.G., Baetz,K., Shi,X., Walter,K.L., MacDonald,V.E., Wlodarski,M.J., Gozani,O., Hieter,P., and Howe,L. (2006a). The Yng1p plant homeodomain finger is a methyl-histone binding module that recognizes lysine 4-methylated histone H3. Mol. Cell Biol. *26*, 7871-7879.

152. Martin,D.G., Grimes,D.E., Baetz,K., and Howe,L. (2006b). Methylation of histone H3 mediates the association of the NuA3 histone acetyltransferase with chromatin. Mol. Cell Biol. *26*, 3018-3028.

153. Mason,P.B. and Struhl,K. (2003). The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation in vivo. Mol. Cell Biol. *23*, 8323-8333.

154. Mason,P.B. and Struhl,K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. Mol. Cell *17*, 831-840.

155. 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.

156. McGhee,J.D. and Felsenfeld,G. (1980). Nucleosome structure. Annu. Rev. Biochem. *49*, 1115-1156.

157. Mellor,J. (2006a). Dynamic nucleosomes and gene transcription. Trends Genet. *22*, 320-329.

158. Mellor,J. (2006b). It takes a PHD to read the histone code. Cell *126*, 22-24.

159. Millar,C.B. and Grunstein,M. (2006). Genome-wide patterns of histone modifications in yeast. Nat. Rev. Mol. Cell Biol. *7*, 657-666.

160. Millar,C.B., Kurdistani,S.K., and Grunstein,M. (2004). Acetylation of yeast histone H4 lysine 16: a switch for protein interactions in heterochromatin and euchromatin. Cold Spring Harb. Symp. Quant. Biol. *69*, 193-200.

161. Millar,C.B., Xu,F., Zhang,K., and Grunstein,M. (2006). Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. Genes Dev. *20*, 711-722.

162. 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.

163. Miranda,T.B., Sayegh,J., Frankel,A., Katz,J.E., Miranda,M., and Clarke,S. (2006). Yeast Hsl7 (histone synthetic lethal 7) catalyses the in vitro formation of omega-N(G)-monomethylarginine in calf thymus histone H2A. Biochem. J. *395*, 563-570.

164. Morillon,A., Karabetsou,N., Nair,A., and Mellor,J. (2005). Dynamic lysine methylation on histone H3 defines the regulatory phase of gene transcription. Mol. Cell *18*, 723-734.

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

166. Morillon,A., O'Sullivan,J., Azad,A., Proudfoot,N., and Mellor,J. (2003b). Regulation of elongating RNA polymerase II by forkhead transcription factors in yeast. Science *300*, 492-495.

167. Morris,D.P. and Greenleaf,A.L. (2000). The splicing factor, Prp40, binds the phosphorylated carboxyl-terminal domain of RNA polymerase II. J. Biol. Chem. *275*, 39935-39943.

168. Morris,D.P., Lee,J.M., Sterner,D.E., Brickey,W.J., and Greenleaf,A.L. (1997). Assaying CTD kinases in vitro and phosphorylation-modulated properties of RNA polymerase II in vivo. Methods *12*, 264-275.

169. Morris,D.P., Phatnani,H.P., and Greenleaf,A.L. (1999). Phospho-carboxyl-terminal domain binding and the role of a prolyl isomerase in pre-mRNA 3'-End formation. J. Biol. Chem. *274*, 31583-31587.

170. Morris,S.A., Rao,B., Garcia,B.A., Hake,S.B., Diaz,R.L., Shabanowitz,J., Hunt,D.F., Allis,C.D., Lieb,J.D., and Strahl,B.D. (2006). Identification of histone H3 lysine 36 acetylation as a highly conserved histone modification. J. Biol. Chem.

171. Morris,S.A., Shibata,Y., Noma,K., Tsukamoto,Y., Warren,E., Temple,B., Grewal,S.I., and Strahl,B.D. (2005). Histone H3 K36 methylation is associated with transcription elongation in Schizosaccharomyces pombe. Eukaryot. Cell *4*, 1446-1454.

172. Morse,R.H. (1993). Nucleosome disruption by transcription factor binding in yeast. Science *262*, 1563-1566.

173. Mueller,C.L., Porter,S.E., Hoffman,M.G., and Jaehning,J.A. (2004). The Paf1 complex has functions independent of actively transcribing RNA polymerase II. Mol. Cell *14*, 447-456.

174. Nagy,P.L., Griesenbeck,J., Kornberg,R.D., and Cleary,M.L. (2002). A trithorax-group complex purified from Saccharomyces cerevisiae is required for methylation of histone H3. Proc. Natl. Acad. Sci. U. S. A *99*, 90-94.

175. Nakayama,J., Rice,J.C., Strahl,B.D., Allis,C.D., and Grewal,S.I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

176. Natsume,R., Eitoku,M., Akai,Y., Sano,N., Horikoshi,M., and Senda,T. (2007). Structure and function of the histone chaperone CIA/ASF1 complexed with histones H3 and H4. Nature.

177. Nelson,C.J., Santos-Rosa,H., and Kouzarides,T. (2006). Proline isomerization of histone H3 regulates lysine methylation and gene expression. Cell *126*, 905-916.

178. Ng,H.H., Dole,S., and Struhl,K. (2003a). The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J. Biol. Chem. *278*, 33625-33628.

179. Ng,H.H., Feng,Q., Wang,H., Erdjument-Bromage,H., Tempst,P., Zhang,Y., and Struhl,K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev. *16*, 1518-1527.

180. Ng,H.H., Robert,F., Young,R.A., and Struhl,K. (2003b). 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.

181. Nightingale,K.P., O'Neill,L.P., and Turner,B.M. (2006). Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr. Opin. Genet. Dev. *16*, 125-136.

182. 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.

183. Nowak,S.J. and Corces,V.G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. Genes Dev. *14*, 3003-3013.

184. Nowak,S.J. and Corces,V.G. (2004). Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. Trends Genet. *20*, 214-220.

185. Orphanides,G. and Reinberg,D. (2000). RNA polymerase II elongation through chromatin. Nature *407*, 471-475.

186. Orphanides,G. and Reinberg,D. (2002). A unified theory of gene expression. Cell *108*, 439-451.

187. 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.

188. Osley,M.A. and Shen,X. (2006). Altering nucleosomes during DNA double-strand break repair in yeast. Trends Genet. *22*, 671-677.

189. Oudet,P., Gross-Bellard,M., and Chambon,P. (1975). Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. Cell *4*, 281-300.

190. 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.

191. Pena,P.V., Davrazou,F., Shi,X., Walter,K.L., Verkhusha,V.V., Gozani,O., Zhao,R., and Kutateladze,T.G. (2006). Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature *442*, 100-103.

192. Peters,A.H., O'Carroll,D., Scherthan,H., Mechtler,K., Sauer,S., Schofer,C., Weipoltshammer,K., Pagani,M., Lachner,M., Kohlmaier,A., Opravil,S., Doyle,M., Sibilia,M., and Jenuwein,T. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell *107*, 323-337.

193. 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.

194. Peterson,C.L. and Laniel,M.A. (2004). Histones and histone modifications. Curr. Biol. *14*, R546-R551.

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

196. Phatnani,H.P., Jones,J.C., and Greenleaf,A.L. (2004). Expanding the Functional Repertoire of CTD Kinase I and RNA Polymerase II: Novel PhosphoCTD-Associating Proteins in the Yeast Proteome. Biochemistry *43*, 15702-15719.

197. Phillips,D.M. and Johns,E.W. (1965). A FRACTIONATION OF THE HISTONES OF GROUP F2A FROM CALF THYMUS. Biochem. J. *94*, 127-130.

198. 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., Zeitlinger,J., Lewitter,F., Gifford,D.K., and Young,R.A. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. Cell *122*, 517-527.

199. Prado,F., Cortes-Ledesma,F., and Aguilera,A. (2004). The absence of the yeast chromatin assembly factor Asf1 increases genomic instability and sister chromatid exchange. EMBO Rep. *5*, 497-502.

200. Proudfoot,N.J., Furger,A., and Dye,M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

201. Qiu,H., Hu,C., Wong,C.M., and Hinnebusch,A.G. (2006). The Spt4p subunit of yeast DSIF stimulates association of the Paf1 complex with elongating RNA polymerase II. Mol. Cell Biol. *26*, 3135-3148.

202. 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.

203. Rao,B., Shibata,Y., Strahl,B.D., and Lieb,J.D. (2005). Dimethylation of histone H3 at lysine 36 demarcates regulatory and nonregulatory chromatin genome-wide. Mol. Cell Biol. *25*, 9447-9459.

204. Rea,S., Eisenhaber,F., O'Carroll,D., Strahl,B.D., Sun,Z.W., Schmid,M., Opravil,S., Mechtler,K., Ponting,C.P., Allis,C.D., and Jenuwein,T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

205. Rice,J.C. and Allis,C.D. (2001). Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr. Opin. Cell Biol. *13*, 263-273.

206. Riles,L., Shaw,R.J., Johnston,M., and Reines,D. (2004). Large-scale screening of yeast mutants for sensitivity to the IMP dehydrogenase inhibitor 6-azauracil. Yeast *21*, 241-248.

207. Robyr,D., Suka,Y., Xenarios,I., Kurdistani,S.K., Wang,A., Suka,N., and Grunstein,M. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell *109*, 437-446.

208. Roguev,A., Schaft,D., Shevchenko,A., Pijnappel,W.W., Wilm,M., Aasland,R., and Stewart,A.F. (2001). The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. EMBO J. *20*, 7137-7148.

209. Rusche,L.N., Kirchmaier,A.L., and Rine,J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. *72*, 481-516.

210. Ryu,H., Lee,J., Hagerty,S.W., Soh,B.Y., McAlpin,S.E., Cormier,K.A., Smith,K.M., and Ferrante,R.J. (2006). ESET/SETDB1 gene expression and histone H3 (K9) trimethylation in Huntington's disease. Proc. Natl. Acad. Sci. U. S. A *103*, 19176-19181.

211. San Segundo,P.A. and Roeder,G.S. (2000). Role for the silencing protein Dot1 in meiotic checkpoint control. Mol. Biol. Cell *11*, 3601-3615.

212. Santos-Rosa,H., Schneider,R., Bannister,A.J., Sherriff,J., Bernstein,B.E., Emre,N.C., Schreiber,S.L., Mellor,J., and Kouzarides,T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature *419*, 407-411.

213. Santos-Rosa,H., Schneider,R., Bernstein,B.E., Karabetsou,N., Morillon,A., Weise,C., Schreiber,S.L., Mellor,J., and Kouzarides,T. (2003). Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. Mol. Cell *12*, 1325-1332.

214. Saunders,A., Werner,J., Andrulis,E.D., Nakayama,T., Hirose,S., Reinberg,D., and Lis,J.T. (2003). Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo. Science *301*, 1094-1096.

215. Schaft,D., Roguev,A., Kotovic,K.M., Shevchenko,A., Sarov,M., Shevchenko,A., Neugebauer,K.M., and Stewart,A.F. (2003). The histone 3 lysine 36 methyltransferase, SET2, is involved in transcriptional elongation. Nucleic Acids Res. *31*, 2475-2482.

216. Schneider,J., Dover,J., Johnston,M., and Shilatifard,A. (2004). Global proteomic analysis of S. cerevisiae (GPS) to identify proteins required for histone modifications. Methods Enzymol. *377*, 227-234.

217. Schneider,J. and Shilatifard,A. (2006). Histone demethylation by hydroxylation: chemistry in action. ACS Chem. Biol. *1*, 75-81.

218. 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.

219. Schneider,R., Bannister,A.J., and Kouzarides,T. (2002). Unsafe SETs: histone lysine methyltransferases and cancer. Trends Biochem. Sci. *27*, 396-402.

220. Schotta,G., Lachner,M., Peters,A.H., and Jenuwein,T. (2004). The indexing potential of histone lysine methylation. Novartis. Found. Symp. *259*, 22-37.

221. Schroeder,S.C., Schwer,B., Shuman,S., and Bentley,D. (2000). Dynamic association of capping enzymes with transcribing RNA polymerase II. Genes Dev. *14*, 2435-2440.

222. 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.

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

224. Scroggins,B.T., Robzyk,K., Wang,D., Marcu,M.G., Tsutsumi,S., Beebe,K., Cotter,R.J., Felts,S., Toft,D., Karnitz,L., Rosen,N., and Neckers,L. (2007). An acetylation site in the middle domain of Hsp90 regulates chaperone function. Mol. Cell *25*, 151-159.

225. Seligy,V.L., Thomas,D.Y., and Miki,B.L. (1980). Saccharomyces cerevisiae plasmid, Scp or 2 mum: intracellular distribution, stability and nucleosomal-like packaging. Nucleic Acids Res. *8*, 3371-3391.

226. Shahbazian,M.D., Zhang,K., and Grunstein,M. (2005). Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. Mol. Cell *19*, 271-277.

227. Shaw,R.J., Wilson,J.L., Smith,K.T., and Reines,D. (2001). Regulation of an IMP dehydrogenase gene and its overexpression in drug-sensitive transcription elongation mutants of yeast. J. Biol. Chem. *276*, 32905-32916.

228. Shi,X., Finkelstein,A., Wolf,A.J., Wade,P.A., Burton,Z.F., and Jaehning,J.A. (1996). Paf1p, an RNA polymerase II-associated factor in Saccharomyces cerevisiae, may have both positive and negative roles in transcription. Mol. Cell Biol. *16*, 669-676.

229. Shi,X., Hong,T., Walter,K.L., Ewalt,M., Michishita,E., Hung,T., Carney,D., Pena,P., Lan,F., Kaadige,M.R., Lacoste,N., Cayrou,C., Davrazou,F., Saha,A., Cairns,B.R., Ayer,D.E., Kutateladze,T.G., Shi,Y., Cote,J., Chua,K.F., and Gozani,O. (2006). ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature *442*, 96-99.

230. Shi,X., Kachirskaia,I., Walter,K.L., Kuo,J.H., Lake,A., Davrazou,F., Chan,S.M., Martin,D.G., Fingerman,I.M., Briggs,S.D., Howe,L., Utz,P.J., Kutateladze,T.G., Lugovskoy,A.A., Bedford,M.T., and Gozani,O. (2007). Proteome-wide analysis in Saccharomyces cerevisiae identifies several PHD fingers as novel direct and selective binding modules of histone H3 methylated at either lysine 4 or lysine 36. J. Biol. Chem. *282*, 2450-2455.

231. Shi,Y., Lan,F., Matson,C., Mulligan,P., Whetstine,J.R., Cole,P.A., Casero,R.A., and Shi,Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

232. Shi,Y. and Whetstine,J.R. (2007). Dynamic regulation of histone lysine methylation by demethylases. Mol. Cell *25*, 1-14.

233. Shia,W.J., Pattenden,S.G., and Workman,J.L. (2006). Histone H4 lysine 16 acetylation breaks the genome's silence. Genome Biol. *7*, 217.

234. Shilatifard,A. (2004). Transcriptional elongation control by RNA polymerase II: a new frontier. Biochim. Biophys. Acta *1677*, 79-86.

235. Shilatifard,A. (2006). Chromatin Modifications by Methylation and Ubiquitination: Implications in the Regulation of Gene Expression. Annu. Rev. Biochem.

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

237. Sims,R.J., III, Nishioka,K., and Reinberg,D. (2003). Histone lysine methylation: a signature for chromatin function. Trends Genet. *19*, 629-639.

238. Singer,M.S., Kahana,A., Wolf,A.J., Meisinger,L.L., Peterson,S.E., Goggin,C., Mahowald,M., and Gottschling,D.E. (1998). Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae. Genetics *150*, 613-632.

239. Smith,J.S., Brachmann,C.B., Celic,I., Kenna,M.A., Muhammad,S., Starai,V.J., Avalos,J.L., Escalante-Semerena,J.C., Grubmeyer,C., Wolberger,C., and Boeke,J.D. (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. U. S. A *97*, 6658-6663.

240. Squazzo,S.L., Costa,P.J., Lindstrom,D.L., Kumer,K.E., Simic,R., Jennings,J.L., Link,A.J., Arndt,K.M., and Hartzog,G.A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. EMBO J. *21*, 1764-1774.

241. Stallcup,M.R. (2001). Role of protein methylation in chromatin remodeling and transcriptional regulation. Oncogene *20*, 3014-3020.

242. Stec,I., Wright,T.J., van Ommen,G.J., de Boer,P.A., van Haeringen,A., Moorman,A.F., Altherr,M.R., and den Dunnen,J.T. (1998). WHSC1, a 90 kb SET domain-containing gene, expressed in early development and homologous to a Drosophila dysmorphy gene maps in the Wolf-Hirschhorn syndrome critical region and is fused to IgH in t(4;14) multiple myeloma. Hum. Mol. Genet. *7*, 1071-1082.

243. Strahl,B.D. and Allis,C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

244. Strahl,B.D., Briggs,S.D., Brame,C.J., Caldwell,J.A., Koh,S.S., Ma,H., Cook,R.G., Shabanowitz,J., Hunt,D.F., Stallcup,M.R., and Allis,C.D. (2001). Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr. Biol. *11*, 996-1000.

245. Strahl,B.D., Grant,P.A., Briggs,S.D., Sun,Z.W., Bone,J.R., Caldwell,J.A., Mollah,S., Cook,R.G., Shabanowitz,J., Hunt,D.F., and Allis,C.D. (2002). Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol. Cell Biol. *22*, 1298-1306.

246. Strahl,B.D., Ohba,R., Cook,R.G., and Allis,C.D. (1999). Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. Proc. Natl. Acad. Sci. U. S. A *96*, 14967-14972.

247. Su,I.H. and Tarakhovsky,A. (2006). Lysine methylation and 'signaling memory'. Curr. Opin. Immunol. *18*, 152-157.

248. Sun,Z.W. and Allis,C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104-108.

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

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

251. Thompson,J.S., Hecht,A., and Grunstein,M. (1993). Histones and the regulation of heterochromatin in yeast. Cold Spring Harb. Symp. Quant. Biol. *58*, 247-256.

252. Tompa,R. and Madhani,H.D. (2007). Histone H3 Lysine 36 Methylation Antagonizes Silencing in Saccharomyces cerevisiae Independently of the Rpd3S Histone Deacetylase Complex. Genetics *175*, 585-593.

253. Trojer,P. and Reinberg,D. (2006). Histone lysine demethylases and their impact on epigenetics. Cell *125*, 213-217.

254. Tschiersch,B., Hofmann,A., Krauss,V., Dorn,R., Korge,G., and Reuter,G. (1994). The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. EMBO J. *13*, 3822-3831.

255. Tsukada,Y., Fang,J., Erdjument-Bromage,H., Warren,M.E., Borchers,C.H., Tempst,P., and Zhang,Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

256. Turner,B.M. (2000). Histone acetylation and an epigenetic code. Bioessays *22*, 836-845.

257. Turner,B.M. (2002). Cellular memory and the histone code. Cell *111*, 285-291.

258. Turner,B.M. (2003). Memorable transcription. Nat. Cell Biol. *5*, 390-393.

259. van Holde,K.E. (1989). Histone modifications. (New York: Springer), pp. 111-148.

260. van Holde,K.E., Lohr,D.E., and Robert,C. (1992). What happens to nucleosomes during transcription? J. Biol. Chem. *267*, 2837-2840.

261. Villas-Boas,S.G., Hojer-Pedersen,J., Akesson,M., Smedsgaard,J., and Nielsen,J. (2005). Global metabolite analysis of yeast: evaluation of sample preparation methods. Yeast *22*, 1155-1169.

262. Vogelauer,M., Rubbi,L., Lucas,I., Brewer,B.J., and Grunstein,M. (2002). Histone acetylation regulates the time of replication origin firing. Mol. Cell *10*, 1223-1233.

263. Vojnic,E., Simon,B., Strahl,B.D., Sattler,M., and Cramer,P. (2006). Structure and carboxyl-terminal domain (CTD) binding of the Set2 SRI domain that couples histone H3 Lys36 methylation to transcription. J. Biol. Chem. *281*, 13-15.

264. Wada,T., Takagi,T., Yamaguchi,Y., Ferdous,A., Imai,T., Hirose,S., Sugimoto,S., Yano,K., Hartzog,G.A., Winston,F., Buratowski,S., and Handa,H. (1998). DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. Genes Dev. *12*, 343-356.

265. Wallrath,L.L. and Elgin,S.C. (1995). Position effect variegation in Drosophila is associated with an altered chromatin structure. Genes Dev. *9*, 1263-1277.

266. Wang,A., Kurdistani,S.K., and Grunstein,M. (2002). Requirement of Hos2 histone deacetylase for gene activity in yeast. Science *298*, 1412-1414.

267. Watson,J.D. and Crick,F.H. (1953a). Genetical implications of the structure of deoxyribonucleic acid. Nature *171*, 964-967.

268. Watson,J.D. and Crick,F.H. (1953b). Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature *171*, 737-738.

269. Weeks,J.R., Hardin,S.E., Shen,J., Lee,J.M., and Greenleaf,A.L. (1993). Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: correlations with gene activity and transcript processing. Genes Dev. *7*, 2329-2344.

270. Whetstine,J.R., Nottke,A., Lan,F., Huarte,M., Smolikov,S., Chen,Z., Spooner,E., Li,E., Zhang,G., Colaiacovo,M., and Shi,Y. (2006). Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell *125*, 467-481.

271. Williamson,P. and Felsenfeld,G. (1978). Transcription of histone-covered T7 DNA by Escherichia coli RNA polymerase. Biochemistry *17*, 5695-5705.

272. Winston,F. (2001). Control of eukaryotic transcription elongation. Genome Biol. *2*, REVIEWS1006.

273. Wintersberger,U., Smith,P., and Letnansky,K. (1973). Yeast chromatin. Preparation from isolated nuclei, histone composition and transcription capacity. Eur. J. Biochem. *33*, 123-130.

274. Wittschieben,B.O., Fellows,J., Du,W., Stillman,D.J., and Svejstrup,J.Q. (2000). Overlapping roles for the histone acetyltransferase activities of SAGA and elongator in vivo. EMBO J. *19*, 3060-3068.

275. Wood,A., Schneider,J., Dover,J., Johnston,M., and Shilatifard,A. (2005a). The Bur1/Bur2 complex is required for histone H2B monoubiquitination by Rad6/Bre1 and histone methylation by COMPASS. Mol. Cell *20*, 589-599.

276. Wood,A., Schneider,J., and Shilatifard,A. (2005b). Cross-talking histones: implications for the regulation of gene expression and DNA repair. Biochem. Cell Biol. *83*, 460-467.

277. Wood,A., Shukla,A., Schneider,J., Lee,J.S., Stanton,J.D., Dzuiba,T., Swanson,S.K., Florens,L., Washburn,M.P., Wyrick,J., Bhaumik,S.R., and Shilatifard,A. (2007). Ctk complex-mediated regulation of histone methylation by COMPASS. Mol. Cell Biol. *27*, 709-720.

278. 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.

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

280. Workman,J.L. and Kingston,R.E. (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. *67*, 545-579.

281. Wu,J. and Grunstein,M. (2000). 25 years after the nucleosome model: chromatin modifications. Trends Biochem. Sci. *25*, 619-623.

282. Wu,X., Rossettini,A., and Hanes,S.D. (2003). The ESS1 prolyl isomerase and its suppressor BYE1 interact with RNA pol II to inhibit transcription elongation in Saccharomyces cerevisiae. Genetics *165*, 1687-1702.

283. Wysocka,J., Swigut,T., Xiao,H., Milne,T.A., Kwon,S.Y., Landry,J., Kauer,M., Tackett,A.J., Chait,B.T., Badenhorst,P., Wu,C., and Allis,C.D. (2006). A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature *442*, 86-90.

284. Xiao,B., Jing,C., Wilson,J.R., Walker,P.A., Vasisht,N., Kelly,G., Howell,S., Taylor,I.A., Blackburn,G.M., and Gamblin,S.J. (2003a). Structure and catalytic mechanism of the human histone methyltransferase SET7/9. Nature *421*, 652-656.

285. Xiao,T., Hall,H., Kizer,K.O., Shibata,Y., Hall,M.C., Borchers,C.H., and Strahl,B.D. (2003b). Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. Genes Dev. *17*, 654-663.

286. Xiao,T., Kao,C.F., Krogan,N.J., Sun,Z.W., Greenblatt,J., Osley,M.A., and Strahl,B.D. (2005). Histone H2B ubiquitylation is associated with elongating RNA polymerase II. Mol. Cell Biol. *25*, 637-651.

287. Xiao,T., Shibata,Y., Rao,B., Laribee,R.N., O'Rourke,R., Buck,M.J., Greenblatt,J.F., Krogan,N.J., Lieb,J.D., and Strahl,B.D. (2007). The RNA polymerase II kinase Ctk1 regulates positioning of a 5' histone methylation boundary along genes. Mol. Cell Biol. *27*, 721-731.

288. Xu,F., Zhang,K., and Grunstein,M. (2005). Acetylation in histone H3 globular domain regulates gene expression in yeast. Cell *121*, 375-385.

289. Yamane,K., Toumazou,C., Tsukada,Y., Erdjument-Bromage,H., Tempst,P., Wong,J., and Zhang,Y. (2006). JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell *125*, 483-495.

290. Yang,X.J. (2004). The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res. *32*, 959-976.

291. Young,R.A. (1991). RNA polymerase II. Annu. Rev. Biochem. *60*, 689-715.

292. 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.

293. 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.

294. Zhang,W., Bone,J.R., Edmondson,D.G., Turner,B.M., and Roth,S.Y. (1998). Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase. EMBO J. *17*, 3155-3167.

295. Zhang,X., Tamaru,H., Khan,S.I., Horton,J.R., Keefe,L.J., Selker,E.U., and Cheng,X. (2002). Structure of the Neurospora SET domain protein DIM-5, a histone H3 lysine methyltransferase. Cell *111*, 117-127.

296. Zhang,X., Yang,Z., Khan,S.I., Horton,J.R., Tamaru,H., Selker,E.U., and Cheng,X. (2003). Structural basis for the product specificity of histone lysine methyltransferases. Mol. Cell *12*, 177-185.

297. 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.

298. Zhao,J., Herrera-Diaz,J., and Gross,D.S. (2005). Domain-wide displacement of histones by activated heat shock factor occurs independently of Swi/Snf and is not correlated with RNA polymerase II density. Mol. Cell Biol. *25*, 8985-8999.