2011

Functional analysis of the JIL-1 histone H3 kinase in Drosophila

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Functional analysis of the JIL-1 histone H3 kinase in *Drosophila*

by

Weili Cai

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular and Developmental Biology

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Ames, Iowa
2011

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ABSTRACT

Epigenetic regulations play a crucial role in control of gene expression and development. Histone modification is one of the epigenetic mechanisms that contribute to regulation of chromatin structure and gene expression. In Drosophila melanogaster, JIL-1, the predominant interphase histone H3S10 kinase, localizes specifically to euchromatic interband regions of polytene chromosomes, and is upregulated two-fold on the male X chromosome. Genetic interaction assays with JIL-1 hypomorphic and null allelic combinations demonstrated that JIL-1 can counterbalance the gene-silencing effect of the three major heterochromatin components Su(var)3-9, Su(var)3-7, and HP1a on position-effect variegation. Our data suggested that the epigenetic H3S10ph mark functions to counteract heterochromatic spreading and gene silencing in Drosophila.

JIL-1 can be divided into four main domains including a NH2-terminal domain (NTD), the first kinase domain (KDI), the second kinase domain (KDII), and a COOH-terminal domain (CTD). Transgenic analysis demonstrated that the CTD of JIL-1 is necessary and sufficient for correct chromosome targeting to autosomes, but that both COOH- and NH2-terminal sequences were necessary for upregulation on the male X chromosome. Another construct ΔCTD that lacks the CTD domain but has histone H3S10 kinase activity can be localized to chromatin by the NTD and was able to rescue autosomes as well as partially rescue male X polytene chromosome morphology.

Furthermore, to study the role of histone H3S10 phosphorylation in transcription we examined the distribution of JIL-1 and histone H3S10 phosphorylation under both heat shock and non-heat shock conditions. There was no redistribution or upregulation of JIL-1 or histone H3S10 phosphorylation found at transcriptionally active puffs after heat shock treatments. Also in JIL-1 null mutant backgrounds, heat shock-induced puffs were strongly labeled by the antibody to the elongating form of RNA polymerase II (Pol IIo\textsuperscript{ser2}), indicating
that Pol II^{ser2} is actively involved in heat shock-induced transcription in the absence of histone H3S10 phosphorylation. These results suggested a model where transcriptional defects in the absence of histone H3S10 phosphorylation are a result of structural alterations of chromatin rather than direct effects on Pol II activation.

To further study the interplay between JIL-1 and transcription regulation, a genome-wide analysis of JIL-1 kinase binding sites by ChIP-seq was conducted and combined with an analysis of whole genome transcription level changes by RNA-seq in the absence of JIL-1. We found that most of the identified JIL-1 binding peaks locate around 200 bp upstream of transcription start sites and that in the absence of H3S10 phosphorylation by JIL-1 a number of normally actively expressed genes were repressed, whereas some inactive genes were activated. Moreover, in the absence of JIL-1 the gene expression level changes of two JIL-1 target genes showed alterations in H3K9 dimethylation levels. Taken together, all these observations suggested a model that H3S10 phosphorylation mainly facilitates gene expression of active genes by maintaining an open chromatin structure at promoter regions by counteracting heterochromatization.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

In eukaryotes chromatin plays an important role in regulating gene expression. Although the higher order of chromatin structure is extremely compacted, many biological processes such as, gene transcription, DNA replication and repairs still proceed. To facilitate these processes, chromatin organization needs to be changed dynamically. There are three mechanisms that can alter the chromatin structure: ATP dependent chromatin remodeling, histone covalent modification, histone variants and histone exchange with variants (Baker and Grant, 2007; Henikoff, 2008).

ATP dependent chromatin remodeling is able to catalyze nucleosome sliding along the DNA. Usually the remodeling complex can move the histone octamer to the nucleosome free DNA or open the nucleosome to expose the DNA transiently (Becker and Horz, 2002; Li et al., 2007). During histone exchange, chromatin remodeling complexes can help the eviction and deposition of different histone variants, which have different deposition pathways. Histone can also be covalently modified at many sites and most of the modifications happen in the NH₂-terminal tails. By using specific antibodies and mass spectrometry, several modifications have been characterized: acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, proline isomerization and biotinylation (Kouzarides, 2007; Stanley et al., 2001). Usually there are several functions for the histone modification: disrupting the connection between nucleosomes, changing the nucleosome structure directly, and recruiting other proteins, which may contain enzymatic activities to remodel the chromatin structure (Kouzarides, 2007). The model “histone code” was used to describe the relationship between multiple
modifications (Strahl and Allis, 2000). In this model a specific set of histone modification “codes” are needed to trigger a specific biological process.

Histone H3S10 phosphorylation, one of the histone modifications, was first characterized in mitotic chromosomes. Until now histone H3S10 phosphorylation appears to have two contradictory functions: for example, the high level of H3S10 phosphorylation in mitotic condensed chromosomes implied a function in chromosome condensation and segregation (Hendzel et al., 1997; Wei et al., 1999). On the other hand, it was later found that histone H3S10 phosphorylation can play a role in chromatin remodeling and activate gene transcription (Strelkov et al., 2002; Sassone-Corsi et al., 1999). The mammalian MSK1/2 kinase, RSK and yeast SNF1 have been identified as interphase histone H3S10 kinases (Kouzarides et al., 2007; Sassone-Corsi et al., 1999). Possibly, these apparently contradictory functions can be triggered by combination with different histone modifications such as, H3K14 acetylation and H3S28 phosphorylation (Turner, 2000; Stral and Allis, 2000). But the actual cellular effect of H3S10 phosphorylation during interphase is still not well known.

JIL-1, a tandem kinase of *Drosophila melanogaster*, was identified by screening a *Drosophila* expression library with the monoclonal antibody mAb2A (Johansen, 1996). JIL-1 contains 1,207 amino acids, which can be divided into four domains: NH₂ domain (NTD), kinase domain I (KDI), kinase domain II (KDII) and COOH-domain (CTD). In contrast to RSK and MSK, both JIL-1’s NH₂-domain and COOH-domain are much longer (Jin et al., 1999; Bao et al., 2008). By phylogenetic analysis, JIL-1 shows homology to both *Drosophila* RSK and human MSK (Soloaga et al., 2003) but is much closer to MSK. The KDI shows 63% amino acid identity to MSK, and 47% to RSK. However the KDII does not show very high identity to either MSK or RSK (Jin et al., 1999). Just as MSK does in mammals, JIL-1 also acts as a histone H3S10 kinase both *in vitro* and *in vivo*. *In vitro* kinase assays show
that JIL-1 can phosphorylate histone H3 and also can be autophosphorylated (Jin et al., 1999). In the JIL-1 null mutant, the total level of histone H3S10 phosphorylation decreases dramatically, however the signal of histone H3S10 phosphorylation in the mutant larvae neuroblast cells (mitotic cells) remains the same (Wang et al., 2001), whereas H3S10 phosphorylation levels in non-dividing salivary gland cells is not detectable. Thus JIL-1 is the major histone H3S10 kinase at interphase in Drosophila.

JIL-1 is localized to interband regions of all the polytene chromosomes and upregulated on the male X chromosome as shown by immunostaining (Jin et al., 1999). Co-immunoprecipitation and GST-pull down assays suggest a physical interaction between JIL-1 and the MSL complex (Jin et al., 2000). Another position effect variegation (PEV) assay shows that without normal levels of JIL-1 protein, males are unable to properly dosage compensate the X-linked white gene (Lerach et al., 2005). These data suggest that JIL-1 is involved in the dosage compensation pathway.

JIL-1 is an essential protein for the development of Drosophila melanogaster. Without JIL-1, animals cannot survive to the adult stage. When the level of JIL-1 in the larvae decreases, the polytene chromosome structure is disrupted both in males and females (Wang et al., 2001; Deng et al., 2005), and the male X chromosome is especially affected, with extensive puffing and total loss of its banding pattern (Wang et al., 2001; Deng et al., 2005). Further electron-microscopy analyses of the JIL-1 null mutant polytene chromosomes shows that there are multiple ultrastructural defects including misalignment of interband chromatin fibrils, intermixing of decondensed chromatin regions, and increased ectopic contacts (Deng et al., 2005). Also, decreased JIL-1 levels can result in the enhancement of PEV of the centromeric P-element insertion 118E-10 (Bao et al., 2007; Wang et al., 2011). All these observations indicate that JIL-1 is required for the maintenance of the chromatin structure and is involved in regulation of gene expression.
In hypomorphic and null JIL-1 mutants, histone H3K9 dimethylation and HP1 (Cowell et al., 2002; Schotta et al., 2002; Grewal and Elgin, 2003), heterochromatin markers, spread to ectopic locations on the chromosome arms especially on the X chromosome. By reducing the dose of the SU(VAR)3-9 gene but not HP1, the null JIL-1 mutant autosome phenotype as well as the lethality can be largely rescued. The SU(VAR)3-9 histone methyltransferase, HP1, and SU(VAR)3-7 (Jaquet et al., 2006) are the key components of pericentric heterochromatin. SU(VAR)3-9 catalyzes histone H3K9 dimethylation and helps recruit HP1 and SU(VAR)3-7, which together assemble the pericentric heterochromatin. Reducing the dose of SU(VAR)3-7 also can largely improve the viability of the JIL-1 null mutant as well as SU(VAR)3-9, despite no alteration in the ectopic spreading of histone H3K9 dimethylation (Deng et al., 2010). Further evidence of the counterbalancing between HP1, SU(VAR)3-9, SU(VAR)3-7 and JIL-1 is provided by the PEV assay. In w^m4, DX1 and w^{118E-10}, the three PEV systems tested, HP1, SU(VAR)3-9 and SU(VAR)3-7 loss of function alleles act as strong haplo-suppressors, whereas loss of function JIL-1 alleles act as strong enhancers. However, in the double mutants the variegation effects are neutralized (Wang et al., 2011). These observations suggest a model where JIL-1 mediated histone H3S10 phosphorylation can counteract heterochromatic spreading and gene silencing that is mediated by the key components of pericentric heterochromatin: SU(VAR)3-9, H3K9 dimethylation, HP1 and SU(VAR)3-7 (Deng et al., 2005; Deng et al., 2010; Wang et al., 2011).

In addition, two potential partners of JIL-1 have been identified by yeast two hybrid screens. First is Lola zf5, a novel splice variant from the lola locus, which interacts with KDI of JIL-1 (Zhang et al., 2003). Lola is a BTB-domain protein that contains a zinc finger domain, suggesting a role in transcription regulation (Crowner et al., 2002). Genetic interaction between JIL-1 and lola indicates that JIL-1 might function in the same pathway
with the Lola transcription factor (Zhang et al., 2003). Another partner is lamin Dm0 (Bao et al., 2005). Lamin Dm0 is an essential protein to maintain nuclear envelope structure (Lenz-Bohme et al., 1999; Guillemin et al., 2001). Further study shows that JIL-1 is required to maintain nuclear morphology and integrity of nurse cells during oogenesis (Bao et al., 2005) suggesting that JIL-1 might cooperate with Lamin Dm0 to maintain and modify chromatin structure and nuclear architecture. In this thesis, three questions were addressed by a series of experiments:

**What are the different domain functions of JIL-1?**

Since JIL-1 is a multi-domain protein, it is of interest to examine the different domain functions. By expressing various truncated proteins in a *JIL-1* null mutant background, we found that the COOH-terminal domain of JIL-1 is necessary and sufficient for correct chromosome targeting to the autosomes but that both COOH- and NH$_2$- terminal sequences are necessary for enrichment on the male X chromosome. However, another truncated protein JIL-1 ΔCTD (without the COOH- domain) which retains histone H3S10 kinase activity is able to rescue autosome and partial male X polytene chromosome morphology. All these findings indicate that JIL-1 may participate in regulating chromatin structure by multiple and partially redundant mechanisms.

**Does JIL-1 and H3S10 phosphorylation directly affect RNA polymerase II mediated transcription elongation?**

Recently, an alternative model was proposed that JIL-1 and JIL-1 mediated histone H3S10 phosphorylation are required for transcription elongation in *Drosophila* based on analyses of transcriptionally active puff regions during the heat shock response (Nowak and Corces, 2000; Nowak et al., 2003; Ivaldi et al., 2007). In order to examine the role of
histone H3S10 phosphorylation in transcription under both heat shock and non-heat shock conditions, we used three different commercially available histone H3S10 phosphorylation antibodies, as well as an acid-free polytene chromosome squash protocol that preserves the antigenicity of the histone H3S10 phospho-epitope. We found that JIL-1 and H3S10 phosphorylation are not upregulated on the transcribed active puff regions after heat shock. Furthermore, strong labeling of antibody to the elongating form of RNA polymerase II (Pol II\(^{\text{ser2}}\)) in the \textit{JIL-1} null mutant indicates that heat shock induced transcription elongation was not inhibited in the absence of JIL-1 and histone H3S10 phosphorylation. Taken together, these data suggest that JIL-1 mediated histone H3S10 phosphorylation is not required for Pol II-dependent transcription at active loci and instead support a model in which transcriptional defects in the absence of histone H3S10 phosphorylation might be a result of structural alterations of chromatin.

**Is H3S10 phosphorylation targeted to specific genomic locations?**

In order to understand the correlation between the chromosome structure and gene expression, we conducted a genome-wide analysis of JIL-1 binding sites by using chromatin immunoprecipitation combined with sequencing (ChIP-seq) and whole genome transcription level changes by RNA-seq upon depletion of JIL-1. ChIP-seq identified 1,675 peaks of JIL-1 binding that mapped to 200 bp before the transcription start site (TSS) of the adjacent gene. Also RNA-seq results showed that loss of JIL-1 could trigger either repression or activation of gene expression. The genes that showed significant effects, normally highly expressed genes were mostly suppressed while low expression level and inactive genes were activated. All these observations suggested that JIL-1 might play a role in maintaining an opening chromatin structure of the promoter region of some active genes in order to facilitate their high expression levels.
THESIS ORGANIZATION

Chapter 1 of this dissertation is a general introduction that begins with background about chromatin remodeling as well as the JIL-1 kinase. A list of research questions is given with brief discussion, followed by a literature review of JIL-1’s functions with respect to chromatin structure, chromatin remodeling, histone modification, Pol II related transcription regulation and dosage compensation in *Drosophila melanogaster*.

Chapter 2 to Chapter 4 is composed of three papers. The first paper was published in *Journal of Biological Chemistry* in November of 2008 and describes the functional mapping of JIL-1 domains. Dr. Xiaomin Bao made the ΔCTD transgenic flies, analyzed CTD and ΔCTD domain functions in the *JIL-1* null mutant (Fig. 1C, 2 and 3), and discovered that the CTD can directly interact with histone H3 (Fig. 6 and 7). Robert Krencik made the CFP-CTD transgene. Dr. Huai Deng made and analyzed the NTD/CTD transgene. Weili Cai made and analyzed the NTD and KDI/II transgenes. Weili Cai also performed western blot analysis and stainings to test JIL-1 and H3S10ph levels and localization patterns of each domain (Fig. 1B, 4 and 5). Dr. Weiguo Zhang contributed part of Fig. 2B.

The second paper was published in *Development* in July of 2008. This paper demonstrated that JIL-1 and histone H3S10 phosphorylation are not required for RNA pol II mediated transcription at active loci. Weili Cai made the primary observations and collected most of the data for this paper. Dr. Huai Deng examined the Pol Ilo*sec2* level in the *JIL-1*null and *JIL-1* Δ, *su(var)*3-906 double mutants as shown in Fig. 8B. Dr. Xiaomin Bao did quantitative RT-PCR to test the Hsp70 mRNA level changes in wild type, *JIL-1*null and *JIL-1* Δ, *su(var)*3-906 double mutants as shown in Fig. 8C.

The third paper is a manuscript being prepared for submission and focuses on JIL-1 genome-wide localization and function in the regulation of gene expression. Weili Cai
performed the JIL-1 ChIP experiments and extracted RNA from wild type and null mutants. Dr. Sanzhen Liu made the alignment of RNA-seq data and identified affected genes in JIL-1 null mutants. Weili Cai made the alignment of ChIP-seq data and identified binding sites for JIL-1. Weili Cai, Chao Wang and Lu Shen all contributed to the statistical analysis of JIL-1 ChIP-seq and RNA-seq data. Yeran Li performed H3K9me2 ChIP assays and Weili Cai performed quantitative PCR as shown in Fig. 5.

Chapter 5 of this thesis is a general conclusion that discusses the functions of JIL-1, and proposes potential experiments for future research.
LITERATURE REVIEW

CHROMATIN STRUCTURE AND ORGANIZATION

In eukaryotic cells, the genomic DNA is packed into a highly compact structure called chromatin. Chromatin is very important for many biological processes such as transcription, DNA replication and DNA repair. Chromatin is composed of nucleosomes, which are the fundamental subunits of the chromatin. 146 bp of DNA is wrapped around a histone octamer comprised of two each of histone H2A, H2B, H3 and H4 to form a nucleosome core particle (Oudet et al., 1975; Luger et al., 1997). Histones inside one nucleosome interact with each other through a "histone fold" structural motif, which contains three helices and two loops. An H2A-H2B dimer binds with the H3-H4 tetramer through the interaction between H4 and H2B (Luger et al., 1997). However around 25% of the histone residues extend out from the histone core. They are largely unstructured and called histone tails. Histone tails can be covalently modified by a number of different enzymes. These modifications are thought to be very important in regulating chromatin structure (Strahl and Allis, 2000).

With the help of linker histone H1 (Woodcock and Horowitz, 1995), a chain of nucleosomes can be further packed into a 30 nm fiber (Finch and Klug, 1976). The 30 nm fiber usually is thought to be the first order of folding of a nucleosome array. It is clear that nucleosome arrays are further compacted into higher order structure to form chromosomes. However the higher folding of chromatin is not well understood yet. Several models have been proposed: radial loop model, hierarchical folding/axial glue model, and chromatin network model (Saitoh et al., 1994; Kireeva et al., 2004; Poirier and Marko, 2002). All these folding models of chromatin folding originate from mitotic chromosome studies (Hirano,
At interphase, chromatin folding shows different levels from 10-30nm fibers to 60-130 nm fibers (Belmont and Bruce, 1994), but the chromatin organization and dynamic changes are still not clear (Cremer et al., 2006). Previous studies suggested that different chromosome territories are formed in the interphase nucleus (Cremer et al., 2002).

Some insect cells contain specialized chromosomes called polytene chromosomes. In these cells the DNA undergoes repeated rounds of replication without mitosis (Zhimulev, 1996). In *Drosophila melanogaster*, the salivary glands polytene chromosomes undergo many rounds of endoreplication and 1,024 copies of sister chromatids align in parallel. One of the characteristic features of polytene chromosome is clear banding patterns, which can be used to identify chromosomal rearrangements and deletions. Usually the band regions are corresponding to inactive chromatin while interband regions are thought to be active chromatin with higher transcription levels (Zhimulev et al., 2004). Polytene chromosomes are about 200 micron in length (de Grauw et al., 1998), so they are good models to study chromatin structure remodeling, especially interphase chromatin organization, at the cytological level. Any alteration of interphase chromatin packing can be easily detected by polytene chromosome morphology. For example, reduced levels of JIL-1 result in shortening and coiling of the polytene chromosome arms, misalignment of interband chromatin fibrils, and an intermixing of band/interband regions (Wang et al., 2001; Deng et al., 2005). Similar defects also can be seen in some other chromatin protein mutants such as *Chromator* and *Z4* (Rath et al., 2006; Eggert et al., 2004). Also, similar “puffy” male X chromosome phenotypes have been found in the mutants of a number of genes including *JIL-1* (Wang et al., 2001; Deng et al., 2005), *SU(VAR)3-7* (Spierer et al., 2005), *nurf301* (Badenhorst et al., 2002) and *ISWI* (Deuring et al., 2000). These puffed male X chromosome phenotypes suggested that all these genes might interact directly or indirectly with the dosage compensation complex (Hilfiker et al., 1997).
Interphase chromatin contains two distinct chromatin types in higher eukaryotes that include heterochromatin, which is tighter and generally transcriptionally inactive and euchromatin, which is less compacted and transcriptionally active (Weiler and Wakimoto, 1995). Heterochromatin can be further divided into constitutive heterochromatin and facultative heterochromatin. Constitutive heterochromatin is around the chromosome centromeres and telomeres, while facultative heterochromatin is regulated and not consistent between cell types in one species (Oberdoerffer and Sinclair, 2007). The organization of heterochromatin and euchromatin is important to control the gene expression. When euchromatic genes were translocated close to heterochromatin, clonal gene inactivation will occur and give rise to a phenomenon called position effect varigation (PEV) (Girton and Johansen, 2008). More than 150 mutations which can enhance or surpress PEV have been isolated (Schotta et al., 2003). All these modifiers are called E(var) or SU(var) and found to be associated with regulation of chromatin structure, histone modification and DNA replication.

CHROMATIN REMODELING

HISTONE MODIFICATIONS

It has been shown that the NH₂-terminal tails of core histones can be covalently modified over 60 residues. The modifications include: acetylation (ac), methylation (me), phosphorylation (ph), ubiquitylation (ub), sumoylation (su), ADP ribosylation (ar), biotinylation, deimination, and proline isomerization (Turner, 2005; Kouzarides, 2007). These modifications play important roles in transcription regulation, DNA replication, and chromosome segregation. Some of the histone modifications could directly alter higher
order chromatin structure (Shogren-Knaak et al., 2006), while others function as a binding platform for other factors that drive downstream nuclear processes (Wolffe and Hayes, 1999; Cheung et al., 2000). A “histone code” model has been proposed to explain the relationship and coordination between multiple histone modifications (Strahl and Allis, 2000). In this model, different histone modifications can recruit proteins by specific protein domain recognition. These recruited proteins can act as regulators of chromatin structure and gene expression. For example, the bromodomain (Tamkun et al., 1992), a conserved motif found in many transcription factors, can bind to acetylated lysine residues on histone H3 or H4. Sometimes a set of the histone modifications can form different combinatorial codes and a set of specific regulatory proteins can read these “codes” and initiate specific nuclear processes (Jenuwein and Allis, 2001).

**Histone Acetylation**

Histone acetylation is generally associated with gene activation. There are three families of histone acetyltransferase: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ/YBF2/SAS2/TIP60) and CBP/p300 (Sterner and Berger, 2000). Acetylation of histone H3K14, mediated by the SAGA complex, a GCN5 acetyltransferase complex, is associated with gene activation. Also in Drosophila, histone H4K16 acetylation by MOF, a MYST domain containing protein, is required for 2-fold higher transcription of the male X linked genes (Hilfker et al., 1997; Akhtar et al., 2000). Histone acetylation can be removed by the histone deacetylases (HDAC). HDAC can be classified into four families: HDAC I (HDAC1, 2, 3 and 8), HDAC II (HDAC 4, 5, 6, 7, 9 and 10), HDAC III (sirtuins) and HDAC IV (HDAC 11) (Kouzarides, 2007). HDAC proteins can also deacetylate non-histone lysines (Choudhary C et al., 2009). Histone acetylation turnover is important for correct gene expression, so control of (histone acetyltransferase) HATs and HDACs activities can
directly regulate gene expression. HDAC inhibitors have a long history of use as mood stabilizers and anti-epileptics. Recently more and more HDAC inhibitors are being studied as potential therapeutic drugs for cancer treatment (Dokmanovic, 2005; Dokmanovic et al., 2007; Bolden et al., 2006). The bromodomain, a conserved motif, has been found to specifically bind to acetylated residues (Tamkun et al., 1992; Dhalluin et al., 1999). This motif is composed of about 110 amino acids and has been identified in over 100 chromatin associated proteins from yeast to human, including CBP, GCN5 and TAFII 250 (Jacobson RH et al., 2000; Mujtaba et al., 2007). This recognition is thought to be a major connection for histone-protein association and chromatin remodeling.

Some previous studies indicated that histone acetylation is less frequently associated with gene inactivity. In Drosophila H4K12 acetylation might function in heterochromatin formation by directing HP1 and H3K9 dimethylation recruitment (Swaminathan et al., 2005). However, a recent study in the aging mouse suggested that histone H4K12 acetylation levels are downregulated, which might be associated with less open chromatin structure and decreased gene expression (Peleg et al., 2010). Moreover, a study of H4K12 acetylation in yeast suggested that H4K12 acetylation is involved in telomeric heterochromatin formation with the functions of maintaining partial plasticity of their telomeric heterochromatin (Zhou et al., 2011). Therefore, histone acetylation might play a general role in heterochromatin dynamics in eukaryotes.

**Histone Methylation**

Different residues and the extent of histone methylation (mono- di- or tri-methylation) can be correlated with either transcription activation or repression. Histones can be methylated on lysine and arginine residues. The enzyme that methylates histone lysine is called a histone lysine methyl-transferase (HMT). There are two types of HMTs:
SET domain proteins SU(VAR)3-9, Enhancer of Zeste, Trithorax) and non-SET domain proteins (Dot1) (Martin et al., 2005). Among all the characterized histone lysine methylations, methylation of H3K4, H3K36 and H3K79 are correlated with gene activation while methylations on H3K9, H3K27 and H4K20 are involved in repression of gene expression (Shlatifard, 2006; Kouzarides, 2007).

In *Drosophila* histone H3K9 dimethylation is the major heterochromatin marker. The PEV suppressor SU(VAR)3-9, a SET domain containing HMT, methylates to histone H3K9. SU(VAR)3-9 has been shown to catalyze most of the dimethylations of the histone H3K9 residues which in turn can recruit HP1 (heterochromatin protein 1) by binding a conserved chromodomain on HP1 (Lachner et al., 2001; Schotta et al., 2002; Grewal and Elgin, 2003). SU(VAR)3-9 and HP1a are inherent components of pericentric heterochromatin and are important factors for silencing of reporter genes by heterochromatic spreading in *Drosophila*. Another SET domain protein, Trx, can specifically methylate histone H3K4 and is involved in maintenance of gene expression. Histone H3K27 methylation is mediated by the Polycomb group protein, EZH2, which is associated with HOX gene repression (Kouzarides, 2007). Recently, studies revealed that histone methylation is not a stable mark. LSD1 and JmjC-domain proteins have been identified as histone lysine demethylases (Klose et al., 2006; Klose and Zhang, 2007). More and more studies suggested that histone demethylases are very important in vertebrate development and are also involved in transcription regulation (Webby et al., 2009).

Compared to lysine methylation, arginine methylation is not well characterized. Till now there are five arginine methylation sites that have been reported: H3R2, H3R8, H3R17, H3R26 and H4R3 (Wysocka et al., 2006; Guccione et al., 2007). Arginine methylations are conducted by protein arginine methyltransferases (PRMTs). It has been reported that arginine methylation plays roles in transcription activation, RNA processing, and DNA repair
(Shilatifard, 2006).

**Histone Phosphorylation**

In contrast to histone acetylation and methylation, much less is known about histone phosphorylation. Histone can be phosphorylated on serine and threonine residues, and can be recognized by 14-3-3 protein (Macdonald et al., 2005). High levels of histone H3S10 phosphorylation and H3S28 phosphorylation, mediated by Aurora B kinase, are found on mitotic chromosomes, and are well known mitotic markers (Giet and Glover, 2001; Adams et al., 2001). At first H3S10 phosphorylation was correlated with chromosome condensation and segregation (Hendzel et al., 1997; Wei et al., 1999). H3S10 phosphorylation was also found in mitogenically stimulated cells (Mahadevan et al., 1991; Barratt et al., 1994). MSK1/2 kinase has been found responsible for the phosphorylation of histone H3S10 during transcription activation of immediate early genes such as *C-fos* and *C-jun* (Clayton et al., 2000), suggesting that histone H3S10 phosphorylation is also involved in transcription activation. In *Drosophila*, the JIL-1 kinase has been identified as the major interphase histone H3S10 kinase (Wang et al., 2001). JIL-1 localizes to less dense euchromatic interband regions on polytene chromosomes and plays an important role in maintenance of higher order euchromatin chromatin structure by antagonizing heterochromatization (Wang et al., 2001; Zhang et al., 2006; Deng et al., 2005; Deng et al., 2007; Wang et al., 2011). Recently, ectopic targeting of JIL-1 by using the lacI/lacO tethering system shows that JIL-1 mediated histone H3S10 phosphorylation can cause decondensation of chromatin (Deng et al., 2008). Studies in the Corces group suggested that JIL-1 mediated H3S10 phosphorylation is linked to heat-shock induced puff formation, and is required for releasing transcriptionally paused Pol II (Ivaldi et al., 2007). However, studies in our laboratory argue against a direct role of histone H3S10 phosphorylation in transcription elongation (Deng et
al., 2008; Cai et al., 2008). Other histone phosphorylations also have important functions including transcription, DNA damage repair and apoptosis. Phosphorylation of H4S1 and H2AS1 are also involved in mitotic chromosome condensation (Barber et al., 2004).

Phosphorylation of mammalian H2A.X (S139) is the hallmark of DNA double strand breaks. It is shown to be important in recruiting the repair machinery such as INO80 and the SWR1 complex (van Attikum et al., 2007; Cleaver et al., 2011). H2BS14 phosphorylation in human and H2BS10 phosphorylation in yeast are linked to the apoptotic process (Ahn et al., 2005; Ajiro et al., 2010).

**Other Histone Modifications and Interplay of Different Histone Modifications**

All the other histone modifications are less abundant and not well understood (Kouzarides, 2007). Although polyubiquitylation is usually thought to be a signal for protein degradation, histone H2A and H2B monoubiquitylation has been found to be involved in regulation of transcription initiation, elongation and DNA repair (Weake and Workman, 2008). Monoubiquitylation of mammalian histone H2B K120 promotes the production of intact transcripts in vitro (Pavri et al., 2006). Sumoylation (SUMO) is a large modification. Histone sumoylation is correlated with gene repression through recruitment of histone deacetylase and heterochromatin protein 1 (Shiio et al., 2003). In yeast, sumoylations of histone H2A, H2B and H4 negatively regulate transcription (Nathan et al., 2006). Poly ADP ribosylation, mediated by PARPs (poly-ADP-ribose polymerases), might be related to decondensation of chromatin and repair of DNA double strand breaks (Tulin and Spradling, 2003; Kotova et al., 2011).

Histone modifications usually can affect each other. For example, histone H3K14 acetylation is preferentially added to H3S10 phosphorylated histones rather than non-phosphorylated forms (Cheung et al., 2000). However, H3S10 phosphorylation can inhibit
H3K9 methylation (Rea et al., 2000). Cross talk between H3S10 phosphorylation and H4K16 acetylation promotes transcription elongation in mammalian cells (Zippo et al., 2009). H2B ubiquitylation is required for H3K4 and H3K79 methylation (Dover et al., 2002; Darwonto et al., 2010). All of these evidence suggest that histone modifications have cross talk and might form different “histone codes” to mediate different downstream processes.

ATP DEPENDENT CHROMATIN REMODELING COMPLEXES

As discussed above that the genomic DNA is folded into a highly compact structure. Nucleosome structure needs to be disrupted prior to transcription. ATP dependent remodeling is one of the important and well characterized mechanisms involved in chromatin regulation. All ATP-dependent chromatin-remodeling complexes contain an ATPase subunit that belongs to the SNF2 superfamily. They have been classified into four families: ISWI family, CHD family, INO80 family, and SWI/SNF family (Morrison et al., 2004; Hargreaves et al., 2011). INO80 family also shows deacetylase activity (Kusch et al., 2004). The SWI/SNF family includes yeast SWI/SNF (ySWI/SNF), yeast RSC, the Drosophila Brahma complex, and the human BRM (hBRM) and BRG1 (hBRG1) complexes (Armstrong et al., 2002; Corona and Tamkun, 2004).

In Drosophila, there are three major families of ATP dependent complexes: SWI2/SNF2, ISWI, and CHD (Bouazoun and Brehm, 2006). The brahma complex belongs to SWI2/SNF2 family. It is homologous to the yeast SWI/SNF complex (Tamkun et al., 1992). In this complex, brahma acts as the ATPase (Workman and Kingston, 1998). There are three kinds of ISWI containing ATP dependent chromatin remodeling complexes: NURF (nucleosome remodeling factor), ACF (ATP-utilizing chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex), which all use ISWI as ATPase
(Farkas et al., 2000, Fyodorov et al., 2004). In the mutants of ISWI, a perturbed male X chromosome structure can be seen, which suggests that ISWI functions in building up the chromosome structure (Corona et al., 2002; Sala et al., 2011). The Drosophila CHD family contains four proteins: dCHD1, dCHD3, dMi-2 and Kismet. The CHD family proteins are unique because of the combination of chromo, SNF2-related helicase ATPase, and DNA-binding domains. It has been reported that the CHD family is associated with the regulation of chromatin structure and gene transcription (Bouazoune and Brehm, 2006).

RNA POLYMERASE II-MEDIATED TRANSCRIPTION

Generating the complementary RNA copy from a DNA template is called transcription. Transcription includes several steps: initiation, promoter clearance, elongation and termination (Kornberg, 1999). During transcription DNA double strands need to be unwound and rewound, therefore regulation of chromatin remodeling is important. RNA polymerase is the enzyme that synthesizes this complementary RNA. In eukaryotes, there are several kinds of RNA polymerases classified by the type of RNA they synthesize. Among those, RNA polymerase II is the most studied type, and it synthesizes precursors of all mRNA and most snRNA and microRNA (Kornberg, 1999; Sims 3rd et al., 2004). It is a 550 kDa complex containing 12 subunits. Combined with a subset of general transcription factors, and regulatory proteins, RNA polymerase can form a larger complex called the RNA polymerase II (Pol II) holoenzyme. Pol II contains an extended carboxy-terminal domain (CTD) that distinguishes it from the other two eukaryotic RNA polymerases. The Pol II CTD consists of up to 52 repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Corden, 1990; Meinhart et al., 2004). The CTD is thought to extend from the core polymerase and can be phosphorylated throughout the transcription steps. Different levels
and sites of phosphorylation of the CTD affect its conformation and regulate transcription elongation, RNA processing, and termination. The hallmark of transition from transcription initiation to elongation is Pol II hypophosphorylation to hyperphosphorylation. Phosphorylation of the CTD predominantly occurs at Ser2 and Ser5. Ser5 phosphorylation, mediated by TFII H, occurs at the initiation stage (Phatnani and Greenleaf, 2006). DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) will be recruited to the paused form of Pol II and facilitate Pol II pausing in the promoter-proximal region (Narita et al., 2003). TFIIS also associates with paused Pol II. Positive transcription-elongation factor b (P-TEFb) is composed of CDK9 and cyclin T and is very important to regulate the level of transcription elongation (Price, 2000). P-TEFb phosphorylation of DSIF, NELF and Ser2 of the Pol II CTD stimulate elongation of transcription. Phosphorylated NELF dissociates from the transcription complex and DSIF, and enables the process of productive elongation (Price, 2000). TFIIS also facilitates escape of Pol II from the pausing site (Adelman et al., 2005). With the development of modern biochemical, molecular and genetic methods, hundreds of factors that participate in transcription regulation have been identified. Different combinations of them are involved in different stages of transcription and enable the RNA polymerase II to access DNA from the remodeled nucleosomes to produce a full length RNA transcript (Conaway et al., 2011).

In *Drosophila*, polytene chromosomes can provide a unique view of gene transcription *in vivo*. The activation of transcription is associated with a dramatic chromatin change and can be observed as chromosome puffs (Lis, 2007). Chromosome puffs are diffuse uncoiled regions of the polytene chromosome that are associated with high levels of transcription. Developmental puffs come and go during the different stages of development (Ashburner, 1972). They provide landmarks along each chromosome. Heat shock also can activate the transcription of a set of heat shock genes and induce the formation of
chromosome puffs along the polytene chromosomes. Therefore, studying the transcriptional regulation of genes located in these heat shock puffs is a very common and useful model for investigating mechanisms of gene activation and polymearase II mediated transcription (Lis, 2007).

**DOSAGE COMPENSATION IN DROSOPHILA MELANOGASTER**

Dosage compensation is a regulatory process to equalize the amount of X chromosome gene expression between males and females. Different species utilize diverse mechanisms of dosage compensation. In mammals, dosage compensation for X-linked gene expression between males and females is achieved by inactivating one of the two X chromosomes in female cells through a highly condensed and heterochromatic structure termed the Barr body. In *C. elegans*, the gene products of X chromosomes of hermaphrodites (XX) are reduced by half to equalize transcription levels to those of the single X chromosome in males. In *Drosophila*, equal gene expression has been proposed to be achieved by upregulating gene transcription two-fold on the male X chromosome. All these X chromosome structural and gene expression changes are mediated by dosage compensation machinery that must recognize and associate specifically with the dosage compensated X chromosome(s).

The complex in *Drosophila* is called the male specific lethal (MSL) complex. The MSL complex contains at least five proteins: MSL-1, MSL-2, MSL-3, MLE (maleless) and MOF (male absent on the first genes) and two non coding RNAs: roX1 and roX2 (Meller and Kuroda, 2002; Sass et al., 2003). This protein-RNA complex is about 2M dalton (Gorman and Baker, 1994). Recently a sixth protein, JIL-1, which is upregulated on the male X chromosome, has been shown to coimmunoprecipitate with components of the MSL
complex. Loss-of-function $JIL-1$ mutations suggest a vital role for JIL1 in addition to a role in X chromosome dosage compensation. (Jin et al., 2000; Wang et al., 2001; Zhang et al., 2003)

The MSL complex binds to hundreds of sites on the male X chromosome. Within this complex, MSL1 and MSL2 interact with each other and form the platform for assembly of the MSL complex. Due to the male-specific expression of MSL2, the complex only assembles in males. MSL1 serves as a central scaffold for the complex assembly. The amino-terminal domain of MSL1 binds to MSL2 (Copps et al., 1998; Scott et al., 2000) and the carboxyl-terminal domain of MSL1 binds to both MSL3 and MOF (Scott et al., 2000). It has been shown that formation of the MSL1/MSL3/MOF complex leads to a significant increase in the histone acetyltransferase activity of MOF (Morales, 2004). Both MSL3 and MOF have been shown to play a role in incorporation of roX RNAs into the complex (Akhtar, 2000). Mle is highly homologous to human RNA helicase A. Assembly of MLE into the complex is presumably via interaction with the roX RNA, since MLE contains an RNA binding domain (Copps et al., 1998) and MLE binding to chromosomes is RNase sensitive.

MOF functions as a histone acetyltransferase which specifically acetylates histone H4 K16. The acetylation of histone H4K16 shows a higher level on the male X chromosome (Hilfiker et al., 1997; Akhtar and Becker, 2000; Prestel et al., 2010). It has been shown that this modification can directly influence the decondensation of chromatin structure $in vitro$ (Shogren-Knaak et al., 2006). Histone acetylation is a key step for the activation of gene transcription. So in the male flies, the higher level of histone H4K16 acetylation may play a central role to change the chromatin structure and upregulate gene transcription (Akhtar and Becker, 2000; Kind et al., 2008). Interestingly, in our laboratory, we found that JIL-1 is upregulated and colocalized with the MSL complex on the male X chromosome (Jin et al., 2000). Histone H3S10 phosphorylation, which is mediated by JIL-1, also shows a higher
level on the male X chromosome than on autosomes. In JIL-1 null mutants, polytene chromosomes in both males and females are distorted, the male X chromosome is especially perturbed, showing a total loss of banding pattern and show a shortening and puffing phenotype (Wang et al., 2001; Deng et al., 2005). All these results could suggest that these two modifications may coordinate with each other to remodel the chromatin and activate gene expression.
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CHAPTER 2. THE COOH-TERMINAL DOMAIN OF THE JIL-1 HISTONE H3S10 KINASE INTERACTS WITH HISTONE H3 AND IS REQUIRED FOR CORRECT TARGETING TO CHROMATIN

A paper published in the *Journal of Biological Chemistry*

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**SUMMARY**

The JIL-1 histone H3S10 kinase in Drosophila localizes specifically to euchromatic interband regions of polytene chromosomes and is enriched two-fold on the male X chromosome. JIL-1 can be divided into four main domains including a NH$_2$-terminal domain, two separate kinase domains, and a COOH-terminal domain. Our results demonstrate that the COOH-terminal domain of JIL-1 is necessary and sufficient for correct chromosome targeting to autosomes, but that both COOH- and NH$_2$-terminal sequences are necessary for enrichment on the male X chromosome. We furthermore show that a small 53 amino acid region within the COOH-terminal domain can interact with the tail-region of histone H3 suggesting that this interaction is necessary for the correct chromatin targeting of the JIL-1 kinase. Interestingly, our data indicate that the COOH-terminal domain alone is sufficient to rescue JIL-1 null mutant polytene chromosome defects including those of the male X chromosome. Nonetheless, we also found that a truncated JIL-1 protein, which was without the COOH-terminal domain, but retained histone H3S10 kinase activity, was able to rescue autosome as well as partially rescue male X polytene chromosome morphology. Taken together these findings indicate that JIL-1 may participate in regulating chromatin structure by multiple and partially redundant mechanisms.
INTRODUCTION

The JIL-1 tandem kinase is a multi-domain protein that localizes specifically to euchromatic chromosome regions, phosphorylates histone H3S10 at interphase, and is enriched almost two-fold on the transcriptionally hyperactive male X chromosome (1-3). Furthermore, the JIL-1 kinase has been functionally implicated in counteracting heterochromatization and gene silencing and is required for maintaining proper chromosome morphology (4-8). In polytene autosomes loss of JIL-1 leads to misalignment of interband chromatin fibrils and to increased ectopic contacts between non-homologous regions (7). Furthermore, there is an abnormal coiling of the chromosomes with an intermixing of euchromatic regions and the compacted chromatin characteristic of banded regions. Especially affected by loss of JIL-1 is the male X chromosome where chromatin is dispersed into a diffuse network without any discernable banded regions that leads to a characteristic "puffed" appearance (7).

JIL-1 can be divided into four main domains including a NH$_2$-terminal domain (NTD1), the first kinase domain (KDI), the second kinase domain (KDII), and a COOH-terminal domain (CTD) (1). Interestingly, mutations resulting in truncations of the COOH-terminal domain of JIL-1 lead to chromatin mislocalization of the protein (5) and give rise to some of strongest suppressor-of-variegation (Su(var)) phenotypes yet described of the wm4 allele (4). Thus, in order to determine which sequences function to localize JIL-1 to chromatin and to enrich it on the male X chromosome we have undertaken a domain analysis of the JIL-1 protein by expressing deletion constructs of JIL-1 transgenically in JIL-1 null mutant flies. Our results demonstrate that the CTD of JIL-1 is necessary and sufficient for correct chromosome targeting to autosomes, but that both COOH- and NH$_2$-terminal sequences are necessary for enrichment on the male X chromosome. In addition, the CTD has an unusual organization, being highly acidic in its first half (pI<4) and
highly basic in its second half (pI>11), the latter of which contains a predicted globular tertiary structure (9). We show by in vitro deletion construct analysis that a small 53 amino acid region of the putative CTD globular domain specifically binds to the tail region of histone H3. Thus, our findings indicate that this sequence within the COOH-terminal region of JIL-1 represents a novel histone H3 binding domain that is required for the correct localization of the JIL-1 kinase to chromatin.

MATERIALS AND METHODS

JIL-1 GFP/CFP- and V5-tagged fusion constructs

A full length JIL-1 (1-1207) construct (JIL-1-FL), a NH₂-terminal domain construct of JIL-1 from residue 1-260 (NTD), a KDI/KDII construct containing the two kinase domains (255-888), and a NTD/CTD construct containing most of the NH₂-terminal domain (1-190) and the COOH-terminal domain (927-1207) in tandem were cloned into the pUAST vector with in-frame V5 tags (from Invitrogen's V5-pMT vector) at the COOH-termini using standard methods (10). Similarly, a ΔCTD construct from residue 1-926 with an in-frame GFP-tag at the NH₂-terminus as well as a CTD construct containing sequences from aa 927-1207 with an in-frame CFP-tag were cloned into the pUAST vector. For those constructs not containing the endogenous JIL-1 nuclear localization sequence (NLS) that is situated in the NH₂-terminal domain (1) the NLS from Clontech's NLS-pECFP vector was added to the NH₂-terminus. The fidelity of all constructs was verified by sequencing at the Iowa State University Sequencing facility.

Drosophila melanogaster stocks

Fly stocks were maintained at 23°C according to standard protocols (11). Canton-S was used for wild type preparations. The JIL-1z2 null allele is described in Wang et al. (3)
as well as in Zhang et al. (12). The GFP-JIL-1 line (P[hsp83-GFP-JIL-1, w+]) has been previously characterized in Jin et al. (1) and in Wang et al. (3). JIL-1 construct pUAST lines were generated by standard P-element transformation (BestGene, Inc.) and expression of the transgenes were driven using the hsp70-GAL4 (P[w[mC]=GAL4-hsp70.PB}) driver (obtained from the Bloomington Stock Center) introduced by standard genetic crosses. The hsp83 and hsp70 promoters are leaky (1, 13) and were used without heat shock treatment. Expression levels of each of the JIL-1 constructs were monitored by immunoblot analysis as described below. Balancer chromosomes and markers are described in Lindsley and Zimm (14).

**Immunohistochemistry**

Standard polytene chromosome squash preparations were performed as in Kelley et al. (15) using the 5 min fixation protocol, and acid-free squash preparations were done following the procedure of DiMario et al. (16). Antibody labeling of these preparations was performed as described in Jin et al. (1) and in Wang et al. (3). In most preparations the male X chromosome was identified by double labeling with MSL antibody as previously described (2). Primary antibodies used in this study include rabbit anti-H3S10ph (Cell Signaling), rabbit anti-histone H3 (Cell Signaling), rabbit anti-GFP (Invitrogen), chicken anti-GFP (Aves Labs), anti-Chromator mAb 6H11 (17), rabbit anti-MSL-2 (generous gift of Dr. M. Kuroda, Harvard University, Boston), anti-GST mAb 8C7 (17), anti-V5 mAb (Invitrogen), anti-myc mAb 9E10 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-lamin Dm0 (9), rabbit anti-JIL-1 (1), chicken anti-JIL-1 (2), and anti-JIL-1 mAb 5C9 (2). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype- specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to
visualize primary antibody labeling. The final preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a high resolution Spot CCD camera. Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images non-linear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes. For quantification of the relative levels of antibody staining of X chromosomes and autosomes, images of polytene chromosome squashes were analyzed using the NIH-Image software as previously described (1). In short, the staining intensity of the autosomes from male preparations was averaged and normalized to a value of one and compared to the average pixel intensity for that of the X chromosome.

**Immunoblot analysis**

Protein extracts were prepared from whole third instar larvae or in some experiments from dissected salivary glands homogenized in a buffer containing: 20 mM Tris-HCl pH8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na3VO4, 1 mM PMSF, 1.5 µg/ml aprotinin. Proteins were separated by SDS-PAGE according to standard procedures (10). Electroblot transfer was performed as in Towbin et al. (18) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electrophoretic transfer to 0.2 µm nitrocellulose, and using anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody. Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).
**Pull-down assays**

In pull-down assays GST-fusion proteins containing JIL-1 sequences were used to pull down endogenous nuclear proteins from S2 cell lysates as previously described (9). In addition, pull-down experiments were performed with histone H3 GST-fusion proteins of lysates from an S2 cell line stably transfected with a myc-tagged CTD domain of JIL-1 (927-1207). This construct was subcloned into the pMT/V5-His vector (Invitrogen) with NH$_2$-terminal insertions of a 6-myc tag from the 6-CMYC clone #CD3-128 (Arabidopsis Biological Resource Center) as well as of a NLS. Initially the JIL-1 GST-fusion proteins CTD-A (886-1033), CTD-B (1033-1207), and CTD-G (1065-1196) which have been previously described (9) and a full-length histone H3 (1-135) construct that was RT-PCR amplified from mRNA extractions from S2 cells and cloned into the pGEX4T vector (Amersham Pharmacia Biotech) were expressed in Escherichia coli using standard techniques (10). For subsequent experiments, GST-fusion protein constructs with various truncations of histone H3 and the COOH-terminal domain of JIL-1 were generated by PCR amplification and insertion into the pGEX4T vector. The additional histone H3 GST-fusion proteins were GST-H3-T (1-74) and GST-H3-C (56-135), and the additional JIL-1 GST-fusion proteins were CTD-G1 (1105-1196), CTD-G2 (1105-1144), and CTD-G3 (1144-1196). All GST fusion protein constructs were verified by sequencing. The GST-tag of all constructs were added NH$_2$-terminally. For the in vitro protein-protein interaction assays, approximately 2 µg of the appropriate GST-fusion protein were coupled with glutathione agarose beads and incubated with 300 µl of S2 cell lysate (from 107 cells/ml) at 4°C overnight on a rotating wheel. The beads were washed four times for 10 min each in 1 ml PBS with 0.5% Tween-20, and proteins retained on the glutathione agarose beads were analyzed by SDS-PAGE and immunoblotting as previously described (9).
**Overlay experiments**

The truncated GST-JIL-1 fusion proteins GST-NTD (1-211) and GST-CTD (927-1207) have been previously described (2, 9). The JIL-1 GST-fusion protein CTD-G3 (1144-1196) is described above. The respective GST-fusion proteins were expressed in BL21 cells and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer’s instructions (Amersham Pharmacia Biotech). For the overlay interaction assays either bovine histones (Worthington), individually purified bovine histones (Boehringer Mannheim), or Drosophila histone extractions from S2 cells performed as described below were fractionated by SDS-PAGE and electroblotted to nitrocellulose. The blots were subsequently incubated with approximately 2 µg of either GST-NTD, GST-CTD or CTD-G3 GST-fusion protein overnight at 4°C in PBS with 0.5% Tween-20 and 5% non-fat milk on a rotating wheel. The blots were washed four times for 10 min each in PBS with 0.5% Tween-20 and binding detected by anti-GST mAb 8C7 (17).

Histone extraction from Drosophila S2 cells - 10 ml of S2 cell culture containing ~10^7 cells/ml was pelleted by slow spinning, and washed once with 5 ml ice-cold PBS. The re-pelleted cells were resuspended with 2 ml of lysis buffer (10 mM Tris-HCL pH 8.0, 1 mM KCl, 1.5 mM MgCl2, 0.5% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1.5 µg/ml Aprotinin), and incubated on ice for 30 min. The nuclei were collected by spinning at 5000 rpm for 10 min at 4°C, and resuspended in 0.4 ml 0.4 N H2SO4 and incubated on a rotating wheel at 4°C for 30 min to overnight. After incubation, the supernatant was collected by spinning at 13,200 rpm for 10 min, and histones were precipitated by adding TCA (100%) to a final concentration of 20% and incubating on ice for 30 min. The precipitated histone proteins were harvested by spinning at 13,200 rpm for 10 min, washed twice with cold acetone (-20°C), air dried, and resuspended in the appropriate amount of H2O.
RESULTS

The CTD-domain is required for proper targeting of JIL-1 to chromatin. Recently, Ebert et al. (4) mapped six JIL-1Su(var)3-1 alleles to a small region in the JIL-1 COOH-terminal domain (Fig. 1A). The mutations in these alleles lead to COOH-terminally truncated proteins that mislocalize to ectopic chromatin sites and are not enriched on the male X chromosome (4, 5) implying an important function for the CTD domain in proper chromatin localization of JIL-1. In order to further explore the role of the different JIL-1 domains in chromosome targeting we expressed three GFP- or CFP-tagged JIL-1 UAS P-element insertion constructs transgenically in JIL-1 null mutant animals: a full-length construct (GFP-JIL-1), a construct without the COOH-terminal domain (ΔCTD), and a construct containing only the COOH-terminal region (CTD) (Fig. 1A). In these studies a hsp70-GAL4 driver line was used without heat shock. The hsp70 promoter is leaky (13) and promoted sufficient construct expression at or near wild type levels (Fig. 1B). As previously reported (3) expression of a full-length GFP-JIL-1 construct in a JIL-1 null mutant background rescued all aspects of the mutant polytene chromosome phenotype and like endogenous JIL-1, GFP-JIL-1 was enriched on the male X chromosome (Fig. 1C, Table 1). Interestingly, as illustrated in Fig. 1C we found that expression of the CTD domain alone was sufficient for localization to chromatin and for restoration of polytene chromosome morphology to or near that of wild type preparations. That the CTD localized to the same interband chromatin sites normally occupied by full-length JIL-1 was further indicated by double labeling studies with antibody to Chromator, a chromosomal protein that is specific to interband polytene regions in a pattern identical to that of endogenous JIL-1 (17, 19). Figure 2A shows such a double-labeled polytene squash preparation where CTD localization is indicated in green, Chromator localization in red, and where co-localization is indicated by the predominantly yellow regions in the composite image. At some interband
locations in the composite image the color is more green or orange than pure yellow as a result of slight differences in the relative staining intensity of the two antibodies, which can vary from preparation to preparation. We also expressed the CTD domain in a wild type background. As illustrated in Fig. 2B under these conditions detection of endogenous JIL-1 on the polytene chromosomes was significantly reduced and enrichment was no longer observed on the male X chromosome. However, localization of the MSL complex as detected by MSL-2 antibody labeling was not affected (Fig. 2B, middle panel). These findings suggest that the CTD domain competed with endogenous JIL-1 for the same chromatin binding sites.

For comparison, we next expressed the ΔCTD construct which is without the CTD domain in JIL-1 null mutant larvae. As illustrated in Fig. 1C the ΔCTD was also able to fully rescue autosome morphology in polytene squash preparations. However, in most cases the morphology of the male X chromosome was only partially restored with remnants of "puffed" regions (Fig. 1C). In addition, it should be noted that autosome chromatin morphology in the polytene squash preparations was "better" than normal when the ΔCTD construct was expressed in JIL-1 mutant (Fig. 1C) as well as in wild type (Fig. 3) backgrounds. In such preparations, the chromosomes spread very easily and were wider with a more crisp and well resolved banding pattern as compared to typical wild type preparations. Furthermore, while the antibody labelings demonstrated that the ΔCTD was localized to chromatin (Fig. 1A) its distribution was broader and present at numerous ectopic locations (Fig. 3B) as compared to the CTD. This was especially evident in polytene squash preparations from third instar larvae where the ΔCTD construct was expressed in a wild type background (Fig. 3). In these preparations the distribution of the ΔCTD was determined by labeling with antibody to its GFP-tag and endogenous JIL-1 was determined using JIL-1 antibody (2) to a COOH-terminal epitope not present in the ΔCTD
Furthermore, the antibody labelings indicated that the ΔCTD similarly to the CTD does not show enrichment on the male X chromosome (Figs. 1C and 3A). However, unlike the CTD, ΔCTD did not appear to have any effect on the endogenous JIL-1 chromatin distribution including on the male X chromosome (Fig. 3A). This indicates that the binding sites and/or mechanisms for chromatin association of the CTD and ΔCTD are different.

Both NTD- and CTD-domain sequences are required for JIL-1 enrichment on the male X chromosome. The findings that neither CTD nor ΔCTD was enriched on the male X chromosome suggested that contributions from more than one JIL-1 domain are needed for this enrichment to occur. In order to explore this possibility we generated a series of V5-tagged JIL-1 UAS P-element insertion constructs and expressed them transgenically in JIL-1 mutant backgrounds. These constructs are diagrammed in Fig. 4A and included a full-length JIL-1 construct (JIL-1-FL), a construct containing the two kinase domains (KDI/KDII), a construct containing only the NH₂-terminal domain (NTD), as well as a construct where the NH₂- and COOH terminal domains were fused together (NTD/CTD). The criteria for assessment of chromosome morphology rescue were based on the degree to which autosomes were uncoiled, on the degree to which "puffing" of the male X chromosome was reduced, as well as on the extent of restoration of discrete band/interband regions of the chromosome arms. As illustrated in Fig. 4B JIL-1-FL had the same properties as GFP-JIL-1 and rescued all aspects of the JIL-1 null polytene chromosome phenotype (Table 1). However, KDI/KDII had no detectable localization to chromosomes although partial rescue of autosome coiling and restoration of some band/interband regions were observed (Fig. 4B). Interestingly, in contrast to the autosomes there was no improvement in male X chromosome morphology (Fig. 4B). Furthermore, we found that like the CTD the NTD could associate with chromatin; however, the localization was relatively evenly distributed on the chromosome arms and there was no discernable rescue of either autosome or male...
X chromosome morphology (Fig. 4B). Nonetheless, we found that the construct bringing together the NTD and CTD domains (NTD/CTD) was sufficient both for chromosome localization to interband regions and for enrichment on the male X chromosome (Fig. 4B). In order to quantify the level of this enrichment, we determined the average pixel intensity of NTD/CTD immunostaining on the male X chromosome and compared it to the autosomal staining intensity which was normalized to a value of 1.0. In five polytene chromosome squashes the difference in X chromosome NTD/CTD staining intensity compared to that of autosomes was 1.8±0.3, identical to the value of 1.8 previously determined for male wild type polytene chromosomes by Jin et al. (1). Interestingly, chromosome morphology was only partially restored by the NTD/CTD in contrast to the full rescue by the CTD domain alone (Fig. 4B). This suggests that the addition of the NTD domain without the kinase domains in some way interfered with the rescue of chromosome morphology provided by the CTD domain alone.

Histone H3S10 phosphorylation by JIL-1 deletion proteins. To test whether the histone H3S10 residue could be phosphorylated when the three JIL-1 deletion proteins ΔCTD, CTD and KDI/KDII were expressed we labeled acid-free polytene squash preparations (16, 20) with H3S10ph antibody. As illustrated in Fig. 5A H3S10 phosphorylation was detected by the H3S10ph antibody in an overlapping pattern with that of ΔCTD or JIL-1-FL, but not with KDI/KDII or CTD. These results were further corroborated by immunoblot analysis which showed that JIL-1-FL and ΔCTD were able to phosphorylate H3S10, whereas KDI/KDII, CTD and NTD were not (Figs. 5B and 5D). Figures 5C and 5E show immunoblots of the JIL-1-FL, ΔCTD, KDI/KDII, CTD and NTD proteins assayed for H3S10 phosphorylation activity in Figs. 5B and 5D. While H3S10 phosphorylation by JIL-1-FL was slightly above wild type levels, phosphorylation of H3S10
by the ΔCTD construct was considerably more robust, possibly reflecting additional phosphorylation activity at the ectopic sites.

The CTD-domain of JIL-1 interacts with the tail region of histone H3. The polytene chromosome localization studies of the various JIL-1 domains indicated that both the CTD and the NTD may have the ability to bind to chromatin. Major constituents of chromatin include the four histones H2A, H2B, H3, and H4 that together with DNA form nucleosomes (reviewed in 21). We therefore used overlay assays to test for interactions between these chromatin components and the NTD and CTD domains. For the initial screening we used two GST-fusion proteins with the NTD (GST-NTD) and CTD (GST-CTD) domains of JIL-1, respectively. In the overlay assays purified bovine histones and Drosophila histone extracts from S2 cells were fractionated by SDS-PAGE, transferred to nitrocellulose paper, and incubated with glutathione agarose bead-purified GST-NTD and GST-CTD. Protein interactions were detected with a mAb to GST. As illustrated in Fig. 6A, B we found that the CTD domain, but not the NTD domain, could specifically interact with both bovine and Drosophila histone H3 in these assays. To further verify this interaction and to determine whether tail or core regions of histone H3 were involved we performed additional pull-down assays. For these experiments we generated a myc-tagged CTD construct (myc-CTD) that was stably expressed in Drosophila S2 cells. In addition, we generated GST-fusion proteins of full-length histone H3 (GST-H3), the tail region of histone H3 (GST-H3-T), and the core region (GST-H3-C). In the pull-down experiments the three histone H3 GST-fusion proteins were coupled to glutathione agarose beads, incubated with lysate from myc-CTD-expressing S2 cells, washed, fractionated by SDS-PAGE and analyzed by immunoblot analysis using a myc-specific antibody. Whereas the beads-only controls and GST-H3-C showed no pull-down activity, both GST-H3 and GST-H3-T were able to pull down myc-CTD as detected by myc antibody (Fig. 6C, E). Immunoblot blot analysis of the GST
proteins purified in these experiments and detected with GST-antibody showed that approximately equivalent levels of the three fusion proteins were present in these assays (Fig. 6D, F). Taken together these experiments indicate that the CTD domain of JIL-1 can directly interact with the tail region of histone H3.

The region of JIL-1 that was found to interact with histone H3, the JIL-1 CTD-domain, can be further divided into two distinct regions: an acidic region from residue 887-1033 and a basic region from residue 1034-1207 (Fig. 7A). Furthermore, Bao et al. (9) identified a putative globular domain present within the basic region that spans residues 1065-1196. Thus, in order to better define the sequences of JIL-1 responsible for the molecular interaction between JIL-1 and histone H3, we generated GST fusion proteins comprising these three regions, CTD-A, CTD-B, and CTD-G, respectively (Fig. 7A) and performed pull down experiments of proteins from S2 cell lysate as described above. As shown in Fig. 7B both the CTD-B and CTD-G fusion proteins pulled down a 17 kDa protein detected by histone H3 antibody that was also present in the S2 cell lysate. This band was not present in pull down assays with the CTD-A fusion protein or in beads-only controls (Fig. 7B). Immunoblot analysis of each of the input GST fusion proteins probed with anti-GST antibody showed comparable levels of GST-fusion proteins in each of the pull-down assays (Fig. 7C). To further refine the region of the CTD domain responsible for the interaction with histone H3 we generated three additional GST-fusion proteins spanning different regions of the globular domain, CTD-G1, CTD-G2, and GST-G3 as diagrammed in Fig. 7D. As shown in Fig. 7E the CTD-G, CTD-G1, and CTD-G3 fusion proteins all pulled down histone H3 as a 17 kDa protein detected by H3 antibody. This band was not present in pull-down assays with the CTD-G2 fusion protein or in beads-only controls (Fig. 7E). Immunoblot analysis of each of the input GST fusion proteins probed with anti-GST antibody showed comparable levels of GST-fusion proteins in each of the pull-down assays.
We further verified that the interaction of CTD-G3 was specific to histone H3 in overlay assays as described above, only in this case separately purified bovine histone subtypes were used. As illustrated in Fig. 7G CTD-G3 bound only to histone H3 on immunoblots as detected with GST antibody. These results suggest that a small 53 amino acid region (aa 1144-1196) of the prospective globular domain of the COOH-terminal domain of JIL-1 is sufficient for mediating molecular interactions with the tail region of histone H3.

**DISCUSSION**

In this study we show that the CTD domain of the JIL-1 kinase is required for proper localization to chromatin and that chromosome morphology defects observed in JIL-1 null backgrounds can be fully rescued by expression of this domain. We furthermore provide evidence that a small 53 amino acid region within the CTD domain can interact with the tail-region of histone H3 suggesting that this interaction is necessary for the correct chromatin targeting of the JIL-1 kinase. This is supported by the findings that constructs (ΔCTD, this study) and mutations (JIL-1Su(var)3-1 alleles, 4, 5) that delete this region in the CTD result in mislocalization of the protein to ectopic chromatin sites. Nonetheless, that localization to chromatin still occurs in the absence of the CTD indicates that JIL-1 possesses a second mechanism for chromatin association and we provide evidence that this association is mediated by sequences residing in the NTD. In contrast, KDI/KDII which contained only the two kinase domains without the NTD and CTD sequences did not show any detectable localization to chromatin.

Surprisingly, our data indicate that histone H3S10 phosphorylation is not required for maintaining proper polytene chromosome morphology and band/interband organization and that the CTD alone is sufficient to rescue JIL-1 null mutant polytene chromosome
defects including those of the male X chromosome. One possibility is that the CTD may serve as a binding platform for an unidentified protein or protein complex that has the ability to maintain chromosome morphology but which can function only after proper localization through interactions with the CTD of JIL-1. Another possibility is that binding of the CTD to chromatin by itself is sufficient to stabilize chromatin structure through bridging interactions with surrounding molecules. Nonetheless, we also found that a JIL-1 deletion protein (ΔCTD) which was without the CTD domain but retained histone H3S10 kinase activity and which could be localized to chromatin by sequences in the NTD was able to rescue autosome as well as partially rescue male X polytene chromosome morphology. Taken together these findings indicate that JIL-1 may participate in regulating chromatin structure by multiple and partially redundant mechanisms. That JIL-1 kinase activity is one of these mechanisms is supported by LacI-JIL-1 tethering experiments by Deng et al. (8) which demonstrated that JIL-1 mediated ectopic histone H3S10 phosphorylation is sufficient to induce a change in higher order chromatin structure from a condensed heterochromatin-like state to a more open euchromatic state. This effect was absent when a "kinase dead" LacI-JIL-1 construct with point mutations in each of the two kinase domains of a crucial lysine required for catalytic activity (22) was expressed (8). Interestingly, the expression of KDI/KDII in JIL-1 null mutants was able to partially rescue autosome, but not male X chromosome morphology, despite that it did not localize to chromatin or phosphorylate histone H3S10. We speculate that KDI/KDII either retains or gains the ability to phosphorylate an unknown molecule which in turn influences chromatin structure.

As indicated above several of the expressed constructs had differential effects on the rescue in JIL-1 mutants of chromatin structure of autosomes and of the male X polytene chromosome. However, it is well documented that the male X polytene chromosome structure is unique (23, 24) as also reflected in JIL-1 mutant chromosome morphology.
(Deng et al., 2005). For example, in JIL-1 mutants the coiling and folding of the autosomes is not observed for the male X chromosome (7). Instead the "puffed" appearance of the male X chromosome is caused by dispersal of chromatin into a diffuse network of condensed and euchromatic regions (7). One possibility is that the inherent differences in chromosome structure may be linked to the increased transcriptional activity of the male X chromosome which correlates with a more open chromosome architecture, such that despite the fact that it contains half the DNA content, the normal male X chromosome has the same width as the paired female X chromosome and the autosomes (7, 25). This more open chromatin structure is likely to be maintained by the activity of the MSL dosage compensation complex and the MOF histone acetyltransferase (23, 24). This leads to hyperacetylation of histone H4 and this particular chromatin modification consequently has the potential to modulate the binding and function of JIL-1 on the male X chromosome. We show that contributions from sequences within both the NTD and CTD are required for enrichment of JIL-1 on the male X chromosome. However, while the CTD showed proper localization to interband regions identical to endogenous JIL-1 the localization of the NTD was relatively evenly distributed on the chromosome arms which included ectopic regions. This suggests that the CTD may provide high affinity binding to specific chromatin sites whereas the NTD may mediate a more general and low affinity chromatin association the nature of which remains undetermined. Thus, the special male X polytene chromatin environment could be envisioned to promote higher affinity interactions with the NTD that only occur in the context of prior CTD mediated localization and which in turn result in enhanced JIL-1 binding. Alternatively, it has been shown that JIL-1 associates with the MSL complex and that it can physically interact with MSL complex proteins (2) wherefore it is possible that such interactions may govern the JIL-1 enrichment. In this context it should be noted that the in vitro finding of Jin et al. (2) that the MSL-
complex interaction with JIL-1 may be mediated by the kinase domains is not supported by
the present data. In this study we detected no binding of KDI/KDII to the male X
chromosome in the JIL-1 null mutant background even though the MSL-complex is present
on the mutant X chromosome (2, 3).

In addition to the interaction with histone H3 demonstrated here previous studies
have identified lamin Dm0 (9) as well as the chromodomain containing protein, Chromator
(19), as direct interaction partners with the CTD domain of JIL-1. While lamin Dm0 like
histone H3 binds to the predicted globular domain of the basic region of the CTD,
Chromator binds to sequences in the acidic region. Furthermore, Zhang et al. (26)
showed that JIL-1 physically interacts via its first kinase domain with the zf5 splice variant of
the complex Lola locus, the expression of which is restricted to early embryogenesis.
Thus, JIL-1 is a multi domain protein that functions in a number of developmental contexts
in addition to its general role in regulating chromatin structure. In future experiments it will
be of interest to determine the structural basis for these interactions and specifically how
JIL-1 through its COOH-terminal domain is integrated into nucleosome and chromatin
organization.

REFERENCES


FIGURE LEGENDS

FIGURE 1. Expression of JIL-1 deletion constructs transgenically in a JIL-1 null mutant background. (A) Diagrams of the JIL-1 GFP- or CFP-tagged constructs analyzed. The region in the CTD where JIL-1SU(var)3-1 alleles resulting in COOH-terminally truncated proteins have been mapped (Ebert et al., 2004) is indicated by a bracket. (B) Immunoblot labeled with JIL-1 antibody of protein extracts from wild type (wt) and from JIL-1 null larvae expressing GFP-JIL-1, CTD, and ΔCTD, respectively. Labeling with lamin antibody was used as a loading control. The relative migration of molecular size markers is indicated to the left of the immunoblot in kDa. (C) Polytenes chromosome squash preparations from male JIL-1z2/JIL-1z2 third instar larval salivary glands expressing GFP-JIL-1, ΔCTD, or CTD, respectively. The left panel shows a squash preparation from a JIL-1 null mutant larvae for comparison. Protein localization (in green) was identified using either JIL-1 (anti-JIL-1) or GFP (anti-GFP) antibodies whereas DNA (in blue or gray) was labeled by Hoechst. The male X chromosome is indicated by an X.

FIGURE 2. Expression of the CTD construct in JIL-1 null and in wild type larvae. (A) Double labeling of a female polytene squash preparation from a JIL-1z2/JIL-1z2 third instar larvae expressing the CTD construct. The CTD (in green) was labeled with GFP-antibody and the interband specific protein Chromator (in red) was labeled with mAb 6H11. The composite image (comp) shows the co-localization between the CTD and Chromator as indicated by the predominantly yellow color. (B) Expression of the CTD in wild type larvae dramatically reduced chromosomal levels of endogenous JIL-1 and prevented enrichment on the male X chromosome (X). The upper panel shows the distribution of JIL-1 and the enrichment of JIL-1 on the male X chromosome (X) in a control wild type polytene squash preparation. The labeling of endogenous JIL-1 was with a JIL-1 NH2-
terminal domain specific antibody that does not recognize the CTD domain. The polytene chromosome squash preparations in the upper and middle panels were double labeled with MSL-2 antibody in order to show MSL complex localization and with GFP antibody in the lower panel to indicate the distribution of CTD-CFP. The middle and lower panel are from independent experiments.

**FIGURE 3.** Expression of the ΔCTD construct in wild type larvae. (A) Polytene squash preparation from a wild type larvae expressing the ΔCTD construct. The ΔCTD (in red) was labeled with GFP antibody, endogenous JIL-1 (in green) with JIL-1 antibody to a COOH-terminal epitope not present in the ΔCTD, and the DNA (in gray) was labeled with Hoechst. The male X chromosome is indicated by an X. (B) Higher magnification image of a region of a male X chromosome from a preparation similar to that in (A) showing the extensive ectopic localization of ΔCTD (arrows).

**FIGURE 4.** Expression of JIL-1 deletion constructs transgenically in a JIL-1 null mutant background. (A) Diagrams of the JIL-1 V5-tagged constructs analyzed. (B) Polytene chromosome squash preparations from male JIL-1z2/JIL-1z2 third instar larval salivary glands expressing JIL-1-FL, KDI/KDII, NTD and NTD/CTD, respectively. Protein localization (in green) was identified using V5 antibody whereas DNA (in blue or gray) was labeled by Hoechst. The male X chromosome is indicated by an X. The asterisk indicate labeling of NTD in the nucleolus.

**FIGURE 5.** Immunocytochemical and immunoblot analysis of histone H3S10 phosphorylation in JIL-1 null larvae expressing JIL-1 transgenes. (A) Acid-free polytene chromosome squash preparations from female JIL-1z2/JIL-1z2 third instar larval salivary
glands expressing JIL-1-FL, ΔCTD, KDI/KDII, and CTD respectively. Protein localization (in green) was identified using V5 (anti-V5) or GFP (anti-GFP) antibody, histone H3 phosphorylation (in red) was identified using H3S10ph antibody, and DNA (in blue or gray) was labeled by Hoechst. (B) Immunoblots labeled with H3S10ph antibody of protein extracts from salivary glands from wild type (wt) and JIL-1z2/JIL-1z2 (z2/z2) larvae as well as from JIL-1 null larvae expressing JIL-1-FL, ΔCTD, KDI/KDII, and NTD, respectively. Labeling with histone H3 (H3) antibody was used as a loading control. (C) Immunoblots of the JIL-1-FL, ΔCTD, KDI/KDII, and NTD proteins assayed for H3S10 phosphorylation activity in (B) as detected by V5 and GFP antibody. The relative migration of molecular size markers is indicated to the left of the immunoblots in kDa. (D) Immunoblots labeled with H3S10ph antibody of protein extracts from salivary glands from wild type (wt) and JIL-1z2/JIL-1z2 (z2/z2) larvae as well as from JIL-1 null larvae expressing CTD and ΔCTD, respectively. Labeling with histone H3 (H3) antibody was used as a loading control. (E) Immunoblots of the CTD and ΔCTD proteins assayed for H3S10 phosphorylation activity in (D) as detected by JIL-1 antibody. The relative migration of molecular size markers is indicated to the left of the immunoblots in kDa.

FIGURE 6. The CTD domain of JIL-1 interacts with the tail region of histone H3. In overlay experiments purified bovine histones (A) and Drosophila histone extractions from S2 cells (B) were fractionated by SDS-PAGE, immunoblotted, incubated with JIL-1 NTD or CTD GST-fusion protein, and interactions detected with a GST mAb (right panels). Ponceau S labeling of the fractionated histone proteins are shown in the left panels. In pull-down experiments (C and E) lysate from S2 cells stably expressing a myc-tagged CTD domain of JIL-1 (myc-CTD) and incubated with GST-histone H3 fusion constructs (GST-H3, GST-H3-T, and GST-H3-C) or with beads only was pelleted with glutathione-agarose beads.
and the interacting protein(s) fractionated by SDS-PAGE, immunoblotted, and probed with myc antibody. Unincubated S2 cell lysate was included as a control (right lanes). In these experiments interactions between JIL-1 myc-CTD and GST-H3 as well as GST-H3-T were detected but not with GST-H3-C. (D and F) Immunoblots of the GST-fusion proteins used for the pull-down experiments in (C and E) detected with GST antibody. The relative migration of molecular size markers is indicated to the left of the immunoblots in kDa.

**FIGURE 7.** Mapping of the JIL-1 COOH-terminal interaction domain with histone H3. (A and D) Diagrams of the JIL-1 COOH-terminal domains to which GST-fusion proteins were made for mapping. In pull-down experiments (B and E) lysate from S2 cells incubated with the various GST-fusion constructs (CTD-A, CTD-B, CTD-G, CTD-G1, CTD-G2, and CTD-G3) or with beads- only was pelleted with glutathione-agarose beads and the interacting protein(s) fractionated by SDS-PAGE, immunoblotted, and probed with histone H3 antibody. Unincubated S2 cell lysate was included as a control (right lanes). (C and F) Immunoblots of the GST-fusion proteins used for the pull-down experiments in (B and E) detected with GST antibody. The relative migration of molecular size markers is indicated to the left of the immunoblots in kDa. (G) In overlay experiments individually purified bovine histones were fractionated by SDS-PAGE, immunoblotted, incubated with the JIL-1 CTD-G3 GST-fusion protein, and interactions detected with a GST mAb (left panel). Ponceau S labeling of the fractionated histone proteins is shown in the right panel.

**FOOTNOTES**

We thank members of the laboratory for discussion, advice, and critical reading of the manuscript. We also wish to acknowledge Ms. V. Lephart for maintenance of fly
stocks and Mr. Laurence Woodruff for technical assistance. We especially thank Dr. M. Kuroda for providing the MSL antibody and Dr. Y. Wang for the *Drosophila* histone extraction protocol. This work was supported by National Institutes of Health grant GM62916 to K.M.J.

The abbreviations used are: NTD, NH$_2$-terminal domain; CTD, COOH-terminal domain; KDI, kinase domain I, KDII, kinase domain II, Su(var), suppressor-of-variegation, NLS, nuclear localization sequence; MSL, male specific lethal.
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CHAPTER 3. POL II MEDIATED TRANSCRIPTION AT ACTIVE LOCI DOES NOT REQUIRE HISTONE H3S10 PHOSPHORYLATION IN DROSOPHILA

A paper published in Development

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SUMMARY

JIL-1 is the major kinase controlling the phosphorylation state of histone H3S10 at interphase in Drosophila. In this study we used three different commercially available H3S10ph antibodies as well as an acid-free polytene chromosome squash protocol that preserves the antigenicity of the H3S10 phospho-epitope to examine the role of histone H3S10 phosphorylation in transcription under both heat shock and non-heat shock conditions. We show that there is no redistribution or upregulation of JIL-1 or H3S10 phosphorylation at transcriptionally active puffs in such polytene squash preparations after heat shock treatment. Furthermore, we provide evidence that heat shock induced puffs in JIL-1 null mutant backgrounds are strongly labeled by antibody to the elongating form of RNA polymerase II (Pol IIo^ser2) indicating that Pol IIo^ser2 is actively involved in heat shock induced transcription in the absence of H3S10 phosphorylation. This was supported by the finding that there was no change in the levels of Pol IIo^ser2 in JIL-1 null mutant backgrounds compared to wild type. That mRNA from the six genes that encode the major heat shock protein Hsp70 in Drosophila is transcribed at robust levels in JIL-1 null mutants was directly demonstrated by qRT-PCR. Taken together these data are inconsistent with the model that Pol II dependent transcription at active loci requires JIL-1 mediated histone H3S10 phosphorylation and instead support a model where transcriptional defects in the
absence of histone H3S10 phosphorylation are a result of structural alterations of chromatin.

INTRODUCTION

The JIL-1 tandem kinase in Drosophila localizes specifically to euchromatic interband regions of polytene chromosomes and is the predominant kinase controlling the phosphorylation state of histone H3S10 at interphase (Wang et al., 2001). JIL-1 is essential for viability and reduced levels of JIL-1 protein leads to a global disruption of chromosome structure (Jin et al., 2000; Wang et al., 2001; Zhang et al., 2003; Deng et al., 2005) as well as to extensive ectopic spreading of heterochromatic factors (Zhang et al., 2006). These findings suggested a model where maintenance of histone H3S10 phosphorylation levels at euchromatic chromatin regions is necessary to counteract heterochromatization and gene silencing (Wang et al., 2001; Ebert et al., 2004; Zhang et al., 2006; Bao et al., 2007). Recently, based on analyses of transcriptionally active regions during the heat-shock response (Nowak and Corces, 2000; Nowak et al., 2003; Ivaldi et al., 2007) an alternative model was proposed where JIL-1 is required for transcription by the RNA polymerase II (Pol II) machinery (Ivaldi et al., 2007). According to this model, rather than contributing to global chromosome structure, JIL-1 mediated histone H3S10 phosphorylation maintains a local chromatin environment that serves as a platform for the recruitment of the positive transcription elongation factor b (P-TEFb) and the consequent release of Pol II from promoter-proximal pausing (Ivaldi et al., 2007). Ivaldi et al. (2007) further suggested that histone H3S10 phosphorylation by JIL-1 is a hallmark of early transcription elongation in Drosophila and that this histone modification is required for the transcription of the majority, if not all, genes in this organism. However, this model is contradicted by the findings of Deng et al. (2007) that demonstrate that the lethality as well
as some of the chromosome morphology defects associated with the null JIL-1 phenotype to a large degree can be rescued by reducing the dose of the Su(var)3-9 gene. Su(var)3-9 is a histone methyltransferase that is necessary for pericentric heterochromatin formation (Schotta et al., 2002; Elgin and Reuter, 2007) and that plays an important role in silencing of reporter genes by heterochromatic spreading (reviewed in Weiler and Wakimoto, 1995; Girton and Johansen, 2008). If JIL-1 had a critical role in promoting transcription at a majority of genes by regulating transcriptional elongation, it is difficult to envision how lethality can be rescued to near wild type levels in the complete absence of JIL-1 and interphase histone H3S10 phosphorylation (Deng et al., 2007). Furthermore, Deng et al (2008) recently showed that JIL-1 mediated ectopic histone H3S10 phosphorylation is sufficient to induce a change in higher order chromatin structure from a condensed heterochromatin-like state to a more open euchromatic state and that these changes are not associated with enhanced transcriptional activity. Thus, these findings are incompatible with the transcriptional elongation model for JIL-1 function and we therefore attempted to repeat the experiments on which it is based using three different histone H3S10 phosphorylation (H3S10ph) antibodies as well as a newly developed acid-free polytene chromosome squash technique (DiMario et al., 2006) that preserves the antigenicity of the H3S10 phospho-epitope. We show that many of the key findings of Nowak and Corces (2000), Nowak et al. (2003), and Ivaldi et al. (2007) are likely to be artifacts caused by non-specific antibody cross-reactivity and by fixation procedures that are not suitable for reliable antibody detection of interphase phosphorylated histone H3S10. Taken together the results of Deng et al. (2007; 2008) and the findings presented here are inconsistent with the model of Ivaldi et al. (2007) that Pol II dependent transcription at active loci requires JIL-1 mediated histone H3S10 phosphorylation and instead support a model
where transcriptional defects in the absence of histone H3S10 phosphorylation are a result of structural alterations of chromatin.

**MATERIALS AND METHODS**

**Drosophila melanogaster stocks and heat shock induction**

Fly stocks were maintained at 23°C according to standard protocols (Roberts, 1998). Canton-S was used for wild type preparations. The *JIL-1^22* null allele is described in Wang et al. (2001) as well as in Zhang et al. (2003) and the recombined *JIL-1^22 Su(var)3-9^06* chromosome in Deng et al. (2007). The P-element insertion mutant allele *tws^02414* was obtained from the Bloomington Stock Center and the *tws^p* allele (Mayer-Jaekel et al., 1993) was the generous gift of Dr. D.M. Glover (University of Cambridge, Cambridge, England). Balancer chromosomes and markers are described in Lindsley and Zimm (1992). For heat shock experiments wandering third instar larvae were subjected to 25 min of heat shock treatment at 37°C as described previously by Nowak et al. (2003).

**Immunohistochemistry**

Salivary gland nuclei smush preparations were made as described in Wang et al. (2001), standard polytene chromosome squash preparations were performed as in Kelley et al. (1999) using the 5 min fixation protocol, and acid-free squash preparations were done following the procedure of DiMario et al. (2006). Antibody labeling of these preparations was performed as described in Jin et al. (1999) and in Wang et al. (2001). Primary antibodies used in this study include rabbit anti-H3S10ph (Epitomics, Upstate, and Cell Signaling), mouse anti-H3S10ph (Upstate and Cell Signaling), rabbit anti-histone H3 (Cell Signaling), mouse anti-lamin Dm0 (Gruenbaum et al., 1988), rabbit anti-HSF (gift from Dr. C. Wu, National Cancer Institute, Bethesda, MD), mouse anti-Pol IIo^ser2* (H5, Covance),
rabbit anti-JIL-1 (Jin et al., 1999), chicken anti-JIL-1 (Jin et al., 2000), and anti-JIL-1 mAb 5C9 (Jin et al., 2000). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype- specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. The final preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a high resolution Spot CCD camera. Confocal microscopy was performed with a Leica confocal TCS NT microscope system. A separate series of confocal images for each fluorophor of double labeled preparations were obtained simultaneously with z-intervals of typically 0.5 µm using a PL APO 63X/1.40 oil objective. A maximum projection image for each of the image stacks was obtained using the ImageJ software (http://rsb.info.nih.gov/ij/). Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images non-linear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes.

**Immunoblot analysis**

Protein extracts were prepared from salivary glands dissected from third instar larvae (or in some experiments from whole larvae) homogenized in a buffer containing: 20 mM Tris-HCl pH8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na3VO4, 1 mM PMSF, 1.5 µg/ml aprotinin. Proteins were separated by SDS-PAGE according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 µm nitrocellulose, and using anti-mouse or anti-
rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody. A buffer containing: 0.9% NaCl, 100 mM Tris, pH 7.5, 0.1% Tween-20, 5% BSA was used for blocking procedures in experiments using the Pol II<sup>ser2</sup> mAb H5 as per the manufacturer's instructions (Covance). Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).

**Analysis of gene expression by qRT-PCR.**

Total RNA was extracted from 12 pooled whole third instar larvae of each genotype (wild type, JIL-1<sup>122</sup>/JIL-1<sup>122</sup>, and JIL-1<sup>122</sup>/JIL-1<sup>122</sup>, Su(var)3-9<sup>06</sup>) with or without heat shock using the MicroPoly(A)Purist™ Small Scale mRNA Purification Kit (Ambion) following the manufacturer’s instructions. cDNA derived from this RNA using SuperScript™ II Reverse Transcriptase (Invitrogen) was used as template for quantitative real-time PCR performed with the Stratagene Mx4000 real-time cycler. In addition, the PCR mixture contained Brilliant II SYBR Green QPCR Master Mix (Stratagene) as well as the corresponding primers: rp49, 5’-AACGTTTACAAATGTGTATTCCGACC-3’ and 5’-ATGACCATCCGCCAGCATAACCC-3’; hsp70, 5’-GTCATCACAGTTCAGCCTACTTCAAC-3’ and 5’-CTGGGGTGATGGATAGGTTGAGGTTC-3’. Cycling parameters were 10 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 60 sec at 59°C, and 40 sec at 72°C. Fluorescence intensities were plotted against the number of cycles using an algorithm provided by the manufacturer. mRNA levels were quantified using a calibration curve based on dilution of concentrated cDNA. mRNA values from the larvae were normalized to that of ribosomal protein 49 (Rp49).
RESULTS

Histone H3S10ph antibody characterization and acid-free polytene squash labeling

The aim of this study was to examine the role of histone H3S10 phosphorylation in transcriptional regulation in Drosophila. However, many of the commercially available antibodies to this histone modification have been mostly used as a marker for mitotic chromosomes (Hendzel et al., 1997; Wei et al., 1998) and as a result they are poorly characterized with regards to detection of interphase histone H3S10 phosphorylation levels and distribution. Consequently, it is important to verify the specificity and suitability of these antibodies for experimental use at interphase to avoid artifacts. In this study we have therefore adapted the "smush" preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique where nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution with a physiological pH (Wang et al., 2001). The procedure furthermore takes advantage of the finding that JIL-1 kinase is the kinase responsible for interphase histone H3S10 phosphorylation (Wang et al., 2001) and that both JIL-1 and H3S10 phosphorylation are upregulated on the male X chromosome (Wang et al., 2001). Consequently, reliable histone H3S10ph antibodies would be expected to show co-localization with JIL-1, show upregulation on the male X chromosome, and all labeling should be absent in homozygous JIL-1 null nuclei as illustrated in Fig. 1. The upregulation of H3S10ph labeling on the male X chromosome is a particularly useful indicator of proper antibody recognition. Furthermore, on immunoblots of salivary gland protein extracts in JIL-1 null mutant backgrounds histone H3S10 labeling should be absent or greatly reduced as well. It should be noted that whole larval extracts are not suitable for such immunoblots because of the presence of a significant population of mitotic nuclei.
where the H3S10 residue is phosphorylated by the Aurora B kinase (Giet and Glover, 2001). Using these criteria we screened various commercially available mono- and polyclonal antibodies from three different manufacturers. The results are summarized in Table 1. We found that several of the antibodies failed one or more of the above criteria and that different lots of the same antibody in some cases had different properties. Of the suitable antibodies the rabbit mAb from Epitomics (epi), the rabbit pAb (lot 2 and 3) from Cell Signaling (cs), and the rabbit pAb (lot 32219) from Upstate (up) were selected for further use in the present studies.

A limitation of the smush procedure is that the visualization of chromatin structure and bands is inferior to the normal squash technique. However, as previously reported (Wang et al., 2001) the highly acidic fixation conditions of conventional squash protocols (Zink and Paro, 1989; Kelley et al., 1999) prevents reliable antibody labeling of the histone H3S10 phospho-epitope. In such preparations H3S10ph antibody labeling is extremely weak and except for rare cases the upregulation of H3S10 phosphorylation on the male X chromosome (Wang et al., 2001; data not shown) cannot be detected indicating incomplete or defective antibody recognition. In this study, to overcome these difficulties we have adopted the acid-free squash technique of DiMario et al. (2006) originally developed to preserve the fluorescence of GFP-tagged proteins in fixed preparations. As illustrated in Fig. 2A-C this technique also preserves the antigenicity of the H3S10 phospho-epitope as indicated by the robust antibody labeling in both male and female squash preparations by three different H3S10ph antibodies that includes the upregulation on the male X chromosome. The extensive co-localization of H3S10ph with JIL-1 is particularly evident in the confocal images in Fig. 2A. Furthermore, on immunoblots of protein extracts from third instar larval salivary glands H3S10ph labeling was greatly reduced in JIL-1 null mutant backgrounds confirming that the antibodies recognized the H3S10ph epitope. However, it
should be noted that the Epitomics H3S10ph antibody in contrast to the other two antibodies showed strong labeling of the chromocenter (Fig. 2B, asterisks). Although, it was more difficult to properly spread the chromosomes and the chromatin structure as labeled by Hoechst was slightly less well-preserved in acid-free squashes compared to conventional squash preparations our data strongly suggest that the acid-free squash procedure is the method of choice in all antibody labeling studies of histone H3S10 phosphorylation in polytene squash preparations.

**JIL-1 and histone H3S10 phosphorylation are not upregulated at transcriptionally activated loci during heat shock**

To determine the distribution of JIL-1 before and after heat shock we double labeled polytene chromosome squash preparations with the JIL-1 mAb 5C9 and with antibody to the elongating form of RNA polymerase II (Pol IIo\textsuperscript{ser2}), which is phosphorylated at serine 2 in the COOH-terminal domain and which serves as a marker for active transcription (Weeks et al., 1993; Boehm et al., 2003; Ivaldi et al., 2007). In non-heat shock preparations both JIL-1 and Pol IIo\textsuperscript{ser2} were localized to a large number of euchromatic interband regions as illustrated in Fig. 3A (left panel). The composite image in Fig. 3A (left panel) further shows that while there may be some co-localization between JIL-1 and Pol IIo\textsuperscript{ser2} as previously reported (Ivaldi et al., 2007), relatively low levels of JIL-1 were observed at many sites where there were especially high levels of Pol IIo\textsuperscript{ser2} staining such as at developmental puffs. After 25 min of heat shock treatment there was a striking change in the distribution of Pol IIo\textsuperscript{ser2} labeling whereas in contrast there was no appreciable redistribution of JIL-1. The Pol IIo\textsuperscript{ser2} labeling was reduced at most sites while being upregulated at heat shock puffs where transcription of heat shock activated genes was occurring. This was especially prominent at the heat shock loci 87A/C and 93D as illustrated in Fig. 3B.
Notably there were no indications of a concomitant upregulation of JIL-1 at these sites (Fig. 3B). This result was confirmed using two other JIl-1 antibodies, a chicken pAb as well as a rabbit pAb (data not shown).

We next used the acid-free polytene squash technique to determine the distribution of H3S10ph before and after heat shock using three different H3S10ph antibodies (i.e., Epitomics, Cell Signaling, and Upstate). To mark heat shock puffs and other regions of enhanced transcription the preparations were double labeled with antibody to Pol IIo<sup>ser2</sup>. As illustrated in Fig. 4A-D there was no obvious change in H3S10ph distribution before and after heat shock as detected by the Epitomics and Cell Signaling H3S10ph antibodies. Importantly, as also observed for JIL-1 (Fig. 3B) there was no upregulation at the 87A/C heat shock puffs although they were robustly labeled by the Pol IIo<sup>ser2</sup> antibody (Fig. 4B and D). In contrast, we found that the Upstate H3S10ph antibody strongly labeled the 87A/C puffs after heat shock (Fig. 4G, F). However, contrary to the Cell Signaling and Epitomics H3S10ph antibodies the Upstate pAb also labeled heat shock puffs in polytene chromosome squashes from JIL-1 null mutant larvae that are devoid of histone H3S10ph phosphorylation (Fig. 5). Since similar results were obtained with two different lots of the Upstate H3S10ph pAb (Table 1) we conclude that the labeling of heat shock puffs by this antibody is due to non-specific cross-reactivity possibly with proteins involved in the heat shock response. Furthermore, immunoblot analysis with all three H3S10ph antibodies of protein extracts from salivary glands before and after heat shock confirmed that there was no change in the overall level of histone H3S10 phosphorylation (Fig. 4G) as indicated by the polytene squash labelings (Fig. 4A, C, and E). In contrast, there was a clear down regulation in the levels of Pol IIo<sup>ser2</sup> in response to heat shock (Fig. 4H).

Previously, evidence has been presented that protein phosphatase 2A (PP2A) activity may regulate histone H3S10 phosphorylation at interphase (Nowak et al., 2003).
Using a P-element insertion mutation into the regulating subunit of PP2A, \textit{tws}^p, that causes reduced catalytic activity (Mayer-Jaekel et al., 1993), Nowak et al. (2003) showed that on immunoblots of extracts from \textit{tws}^p mutant larvae there is a higher level of H3S10 phosphorylation than in wild type larvae. This difference was attributed to reduced PP2A phosphatase activity indicating that PP2A may function as a H3S10ph phosphatase at interphase (Nowak et al., 2003) in addition to its role as a mitotic H3S10ph phosphatase (Mayer-Jaekel et al., 1993). However, since whole larval extracts were used it remained a possibility that the increased upregulation of H3S10ph levels was due solely to decreased dephosphorylation of mitotic H3S10ph. Using a likely null PP2A regulatory subunit P-element mutation, \textit{tws}^{02414}, we confirmed a higher level of H3S10 phosphorylation in extracts from homozygous \textit{tws}^{02414} mutant larvae as compared to wild type larvae (Fig. 6A). However, when extracts were compared from salivary glands which do not contain mitotic cells there was no difference (Fig. 6B). Furthermore, in extracts from salivary glands of homozygous \textit{tws}^p mutant larvae with or without heat shock there was no difference in H3S10 phosphorylation levels as detected by the Epitomics H3S10ph mAb (Fig. 6C). Taken together these results indicate that the PP2A phosphatase may play a role in H3S10 dephosphorylation only at mitosis and not at interphase.

**JIL-1 and H3S10 phosphorylation are not required for transcription at active loci during heat shock**

Deng et al. (2007) have recently provided evidence that the lethality as well as some of the chromosome defects associated with the \textit{JIL-1} null phenotype can be substantially rescued by reducing the dose of the \textit{Su(var)3-9} gene. This suggests that the Pol II transcriptional machinery has the capacity to function more or less normally in the complete absence of JIL-1 mediated interphase histone H3S10 phosphorylation. We therefore
investigated the distribution of Pol II^{ser2} labeling and heat shock induced transcription in both $\text{JIL-1}^{12}/\text{JIL-1}^{12}$ null as well as in $\text{JIL-1}^{12}/\text{JIL-1}^{12} \text{Su(var)3-9}^{06}$ double mutant backgrounds. In $\text{JIL-1}^{12}/\text{JIL-1}^{12} \text{Su(var)3-9}^{06}$ larvae the adult eclosion rate increases to 60\% that of wild type larvae as compared to 0\% for $\text{JIL-1}^{12}/\text{JIL-1}^{12}$ null larvae (Deng et al., 2007). Fig. 7A shows robust antibody labeling of Pol II^{ser2} in polytene chromosome squashes from both genotypes even though the chromatin structure was greatly perturbed. This included the characteristic "puffed" male X chromosome in $\text{JIL-1}$ null larvae (Fig. 7A, upper panel). Furthermore, on immunoblots of extracts from wild type, $\text{JIL-1}^{12}/\text{JIL-1}^{12}$, and $\text{JIL-1}^{12}/\text{JIL-1}^{12} \text{Su(var)3-9}^{06}$ salivary glands there was no detectable difference in Pol II^{ser2} levels (Fig. 7B). This indicates that transcript elongation by the Pol II machinery is likely to be functional in $\text{JIL-1}$ null mutant backgrounds. To further investigate this possibility we double labeled $\text{JIL-1}$ mutant polytene chromosome squashes after they were heat shock treated with Pol II^{ser2} antibody and with antibody to the heat shock transcription factor HSF (Fig. 8A). When inactive, HSF is diffusely distributed at very low levels; however, following heat shock HSF redistributes very prominently to heat shock induced puffs (Westwood et al., 1991; Ivaldi et al., 2007). As shown in Fig. 8A although the chromosome morphology is greatly disrupted, puffed regions can be clearly identified in $\text{JIL-1}$ null mutant backgrounds as defined by the presence of decondensed chromatin and strong HSF antibody labeling. Importantly, these heat shock induced puffs are also strongly labeled by Pol II^{ser2} antibody in an overlapping pattern with that of the HSF antibody (Fig. 8A, arrows). Furthermore, as also confirmed by immunoblot analysis (Fig. 8B), Pol II^{ser2} levels are greatly reduced at non-heat shock sites (Fig. 8A). This suggests that Pol II^{ser2} is actively involved in heat shock induced transcription in $\text{JIL-1}$ null mutants. To test this directly we used quantitative RT-PCR to measure the transcription of the six nearly identical genes that encode Hsp70, the major heat shock protein in $\text{Drosophila}$ (Gong and Golic, 2004) under
heat shock and non-heat shock conditions. Primers were designed that would amplify transcripts from all six \textit{hsp70} genes whereas primers specific to the gene encoding the ribosomal non-heat shock sensitive protein Rp49 was used for normalization. We performed two independent experiments where total RNA was isolated from wild type, \textit{JIL-1^{12}/JIL-1^{12}}, and \textit{JIL-1^{12}/JIL-1^{12} Su(var)3-9^{06}} third instar larvae and where qRT-PCR determination of transcript levels was performed in duplicate. As illustrated in Fig. 8C, very low levels of \textit{hsp70} mRNA transcripts in both wild type and \textit{JIL-1} mutant backgrounds were detected under non-heat shock conditions. However, a robust increase in \textit{hsp70} mRNA transcript levels was detected in response to heat shock treatment in all three genotypes relative to \textit{rp49} transcript levels (Fig. 8C). The increase in \textit{JIL-1^{12}/JIL-1^{12}} null mutant larvae was at least two orders of magnitude larger than under non-heat shock conditions and was about 1/3 that observed in wild type larvae. Interestingly, the heat shock-induced increase in \textit{hsp70} mRNA levels improved considerably to almost 2/3 of wild type levels in larvae where \textit{Su(var)3-9} levels were reduced by half (e.g. \textit{JIL-1^{12}/JIL-1^{12} Su(var)3-9^{06}} larvae).

**DISCUSSION**

A number of studies have suggested that regulation of early stages of transcriptional elongation may be a relatively common phenomenon in higher eukaryotes (reviewed in Hartzog and Tamkun, 2007) and that histone H3S10 phosphorylation may play an important role in specific transcriptional responses to signaling stimuli (Mahadewan et al., 1991; Lo et al., 2001; Ivaldi et al., 2007). In this study we characterized three commercially available histone H3S10ph antibodies and used an acid-free squash protocol to revisit the role of histone H3S10 phosphorylation in transcription in \textit{Drosophila} under both heat shock and non-heat shock conditions. We show that there is no change in the levels of the
elongating form of RNA polymerase II in larvae from *JIL-1* null mutant backgrounds compared to wild type. Furthermore, we provide evidence that heat shock induced puffs in *JIL-1* null mutant backgrounds are strongly labeled by Pol II*ser2* antibody in an overlapping pattern with that of HSF antibody indicating that Pol II*ser2* is actively involved in heat shock induced transcription in the absence of H3S10 phosphorylation. That mRNA from the six genes that encode the major heat shock protein Hsp70 in *Drosophila* is transcribed at robust levels in *JIL-1* null mutants was directly demonstrated by qRT-PCR. Thus these data strongly suggest that histone H3S10 phosphorylation by *JIL-1* is not involved in transcriptional elongation in *Drosophila*. The finding that there is no redistribution of *JIL-1* or H3S10 phosphorylation to transcriptionally active puffs in wild type polytene squash preparations during the heat shock response further supports this conclusion. These results are contrary to those reported by Nowak and Corces (2000), Nowak et al. (2003), and Ivaldi et al. (2007). However, the discrepancies may largely be due to the reliance in these studies on the Upstate H3S10ph pAb which our study indicates is unsuitable for analysis of heat shock induced transcription due to non-specific cross-reactivity at heat shock induced puffs. Nonetheless, it should be noted that the present study confirms the findings of Ivaldi et al. (2007) that the chromatin remodeling associated with heat shock puff formation still occurs in *JIL-1* null mutants despite the disruption of chromatin structure and the absence of H3S10 phosphorylation.

Previous studies indicated that the lethality of *JIL-1* null mutants may be due to ectopic Su(var)3-9 activity and the disruption of chromatin structure (Zhang et al., 2006; Deng et al., 2007). At interphase *JIL-1* phosphorylates the histone H3S10 residue in euchromatic regions of polytene chromosomes (Jin et al., 1999; Wang et al., 2001) suggesting as a plausible model that this phosphorylation during interphase prevents Su(var)3-9 mediated heterochromatization and gene repression at these sites (Zhang et al.,
2006; Deng et al., 2007). However, the present results clearly indicate that such repression is not global and that the Pol II machinery is functional as indicated by the robust transcription of heat shock induced genes in JIL-1 null mutants. Furthermore, while dosage compensation of the white locus in males is impaired in hypomorphic JIL-1 mutant backgrounds, white expression in females is relatively unaffected (Lerach et al., 2005). Thus, the lethality may be caused instead by a severe repression of a few essential genes and/or a more graded decrease in expression of a larger number of genes due to the altered chromatin structure. The latter scenario is supported by the finding that the expression of heat shock induced genes in JIL-1 null mutant backgrounds increases when chromatin structure is improved by reducing the levels of the heterochromatic factor Su(var)3-9 (Deng et al., 2007; this study). That JIL-1 levels can directly affect gene expression was recently demonstrated by experiments that showed that loss-of-function JIL-1 alleles act as enhancers of position-effect-variegation (PEV) whereas the gain-of-function JIL-1\textsuperscript{Su(var)3-1} allele acts as a suppressor of PEV at pericentric sites (Bao et al., 2007). The JIL-1\textsuperscript{Su(var)3-1} allele is one of the strongest suppressors of PEV so far described (Ebert et al., 2004) and it generates truncated proteins with COOH-terminal deletions that mislocalize to ectopic chromosome sites (Ebert et al., 2004; Zhang et al., 2006). Thus, the dominant gain-of-function effect of the JIL-1\textsuperscript{Su(var)3-1} alleles may be attributable to JIL-1 kinase activity at ectopic locations leading to misregulated localization of the phosphorylated histone H3S10 mark counteracting the spreading and gene repression of Su(var)3-9. This is supported by the finding of Deng et al. (2008) that ectopic H3S10 phosphorylation at interphase can function as a causative regulator of higher-order chromatin structure in vivo. Furthermore, studies of PEV of the \textit{w}\textsuperscript{imd} allele have indicated that loss of JIL-1 function also can cause a change in the levels of heterochromatic factors at the chromocenter that indirectly can affect gene expression at nearby loci (Lerach et al.,
2006; reviewed in Girton and Johansen, 2008). In future experiments it will be of interest to further explore a possible mechanistic link between H3S10 phosphorylation and the regulation of chromatin structure and gene expression in *Drosophila*.

**ACKNOWLEDGMENTS**

We thank members of the laboratory for discussion, advice, and critical reading of the manuscript. We also wish to acknowledge Ms. V. Lephart for maintenance of fly stocks and Mr. Laurence Woodruff for technical assistance. We especially thank Dr. C. Wu for providing the HSF antibody and Dr. D.M. Glover for the *tws*<sup>p</sup> allele. This work was supported by National Institutes of Health grant GM62916 to K.M.J.

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**FIGURE LEGENDS**

**Fig. 1.** JIL-1 and histone H3S10ph antibody labeling of salivary gland nuclei smush preparations. JIL-1 (in green) and H3S10ph (in red) co-localizes on chromosomes from both female (upper panel) and male (middle panel) wild type nuclei. The middle panel illustrates the characteristic upregulation of JIL-1 and H3S10ph labeling on the male X chromosome (X). Labeling of both JIL-1 and H3S10ph is absent in *JIL-1^{1z2}/JIL-1^{1z2} (z2)* null mutant nuclei (lower panel). The H3S10ph antibody used was from Cell Signaling.

**Fig. 2.** Immunocytochemistry and immunoblot characterization of three different H3S10ph antibodies. (A-C) Acid-free polytene chromosome squash preparations from male and female third instar larvae double labeled with antibodies to JIL-1 (in green) and H3S10ph (in red). H3S10ph labeling with antibody from Cell Signaling (cs) is shown in (A), from Epitomics (epi) in (B), and from Upstate (up) in (C). Composite images (comp) of the labelings are shown to the left. The labeling of all three H3S10ph antibodies shows co-localization with JIL-1 and upregulation on the male X chromosome (X). The Epitomics H3S10ph antibody in contrast to the two other antibodies showed strong labeling of the chromocenter (B, asterisks). The images in (A) are projection images from confocal sections. (D-F) Immunoblots of protein extracts from salivary glands from wild type (wt), *JIL-1^{1z2}/JIL-1^{1z2} (z2)*, and *JIL-1^{1z2}/JIL-1^{1z2} Su(var)3-9^{06} (z2, 3-9)* larvae labeled with H3S10ph antibody from Cell Signaling (D), Epitomics (E), and Upstate (F). H3S10ph antibody labeling by all three antibodies is greatly reduced in *JIL-1* null mutant backgrounds. Labeling with histone H3 (H3) antibody was used as a loading control.
Fig. 3. JIL-1 is not upregulated at actively transcribed regions during the heat shock response. (A) Polytenes chromosome squash preparations from wild type larvae (wt) triple labeled with Pol IIo\textsuperscript{ser2} antibody (in green), JIL-1 mAb 5C9 (in red), and Hoechst (DNA, grey/blue) with (right panel) and without (left panel) heat shock treatment. At many sites that showed especially high levels of Pol IIo\textsuperscript{ser2} staining such as at developmental puffs, there were relatively low levels of JIL-1 (arrow). After heat shock treatment (A, right panel) Pol IIo\textsuperscript{ser2} labeling was reduced at most sites while being dramatically upregulated at heat shock induced puffs (A, right panel boxed area) while there was no discernable redistribution of JIL-1. (B) Higher magnification of the labeling of heat shock puffs 87A/C and 93D in the area indicated by a white box in (A, upper right panel) showing that there was no upregulation of JIL-1 at these heat shock puffs.

Fig. 4. Polytenes chromosome distribution of H3S10ph in response to heat shock treatment. (A-F) Acid-free polytenes chromosome squash preparations from female third instar larvae triple labeled with antibodies to Pol IIo\textsuperscript{ser2} (in green), H3S10ph (in red), and with Hoechst (DNA, blue/grey). H3S10ph labeling with antibodies from Epitomics is shown in (A), from Cell signaling in (C), and from Upstate in (E) with (+HS) and without (-HS) heat shock treatment. (B, D, and F) Higher magnification images of the heat shock induced puffs 87A/C (boxed regions) labeled by the H3S10ph antibodies from Epitomics (B), Cell Signaling (D), and Upstate (F) respectively. (G) Immunoblots of protein extracts from salivary glands from wild type larvae without heat shock treatment (wt) and with heat shock treatment (wt (HS)) labeled with H3S10ph antibody from Cell Signaling (D), Epitomics (E), and Upstate (F). Labeling with histone H3 (H3) antibody was used as a loading control. (H) Immunoblots of protein extracts from salivary glands from wild type
larvae without heat shock treatment (wt) and with heat shock treatment (wt (HS)) labeled with Pol Illo$^{ser2}$ antibody. Labeling with lamin antibody was used as a loading control.

Fig. 5. Polytene chromosome labeling by three different H3S10ph antibodies in response to heat shock treatment in *JIL-1* null mutants. Acid-free polytene chromosome squash preparations from female *JIL-1^{22}/JIL-1^{22}* third instar larvae triple labeled with antibodies to Pol Illo$^{ser2}$ (in green), H3S10ph (in red), and with Hoechst (DNA, blue/grey) after heat shock treatment. H3S10ph labeling by antibodies from Cell Signaling, Epitomics, and Upstate are shown. Arrows indicate the likely position of the 87A/C heat shock puff regions.

Fig. 6. Immunoblot analysis of histone H3S10 phosphorylation in mutants of the 55 kd regulatory subunit of protein phosphatase 2A. (A) H3S10ph antibody labeling of protein extracts from wild type (wt) and *tws^{02414}/tws^{02414}* (tws$^{02414}$) whole third instar larvae. (B) H3S10ph antibody labeling of protein extracts from wild type (wt) and *tws^{02414}/tws^{02414}* (tws$^{02414}$) salivary glands. (C) H3S10ph antibody labeling of protein extracts from *tws^{P}/tws^{P}* (tws$^{P}$) and *JIL-1^{22}/JIL-1^{22}* (z2) salivary glands with (HS) and without heat shock treatment. The H3S10ph antibody was from Epitomics (epi) and labeling with histone H3 (H3) antibody was used as a loading control.

Fig. 7. Immunocytochemical and immunoblot labeling of Pol Illo$^{ser2}$ in *JIL-1* null mutant backgrounds. (A) Robust Pol Illo$^{ser2}$ antibody labeling (in green) of chromosomes including the male X (X) chromosome in polytene chromosome squash preparations from *JIL-1^{22}/JIL-1^{22}* (z2), and *JIL-1^{22}/JIL-1^{22}* *Su(var)3-9*^{06} (z2, 3-9) third instar larvae. The DNA (blue/grey) was labeled by Hoechst. (B) Immunoblot of protein extracts
from wild type (wt), JIL-1^{z2}/JIL-1^{z2} (z2), and JIL-1^{z2}/JIL-1^{z2} Su(var)3-9^{06} (z2, 3-9) larvae labeled with Pol II^{ser2} antibody. Labeling with lamin antibody was used as a loading control.

Fig. 8. Analysis of Pol II^{ser2} distribution and transcription at active loci during the heat shock response in JIL-1 mutant backgrounds. (A) Polytene chromosome squash preparations from JIL-1^{z2}/JIL-1^{z2} (z2), and JIL-1^{z2}/JIL-1^{z2} Su(var)3-9^{06} (z2, 3-9) larvae triple labeled with Pol II^{ser2} antibody (in green), HSF antibody (in red), and Hoechst (DNA, grey/blue) after heat shock treatment. Arrows point to heat shock puff regions. (B) Immunoblot of protein extracts from salivary glands from JIL-1^{z2}/JIL-1^{z2} (z2) and JIL-1^{z2}/JIL-1^{z2} Su(var)3-9^{06} (z2, 3-9) larvae with (+HS) and without (-HS) heat shock treatment labeled with Pol II^{ser2} antibody. Labeling with lamin antibody was used as a loading control. (C) Transcript levels of hsp70 mRNA in JIL-1 mutant null backgrounds in response to heat shock treatment. hsp70 transcript levels were determined by qRT-PCR and normalized to the mRNA levels of the control non-heat shock protein Rp49 (ribosomal protein 49) both without and after heat shock treatment. The data shown are the average from two independent experiments where total RNA was isolated from wild type (wt), JIL-1^{z2}/JIL-1^{z2} (z2), and JIL-1^{z2}/JIL-1^{z2} Su(var)3-9^{06} (z2, 3-9) larvae and where each determination of transcript levels was performed in duplicate. The error bars indicate the SDM.
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CHAPTER 4. GENOME-WIDE ANALYSIS OF THE BINDING SITES OF THE JIL-1 H3S10 KINASE AND ITS CONTRIBUTION TO MODULATION OF GENE EXPRESSION

A paper to be submitted

Weili Cai, Chao Wang, Lu Shen, Yeran Li, Sanzhen Liu, Changfu Yao, Patrick Schnable, Jack Girton, Jørgen Johansen and Kristen Johansen.

SUMMARY

The major interphase histone H3S10 kinase, JIL-1, localizes specifically to euchromatic interband regions of polytene chromosomes in Drosophila. Genetic interaction assays with JIL-1 hypomorphic and null allelic combinations demonstrated that JIL-1 is able to counterbalance the gene-silencing effect of the heterochromatin. In order to further determine the interplay between epigenetic chromatin modifications and gene expression, we conducted a genome-wide analysis of JIL-1 binding sites by ChIP-seq as well as an RNA-seq analysis in the absence of JIL-1 and JIL-1 mediated H3S10 phosphorylation in salivary gland preparations. We found that most of the 1,675 identified JIL-1 binding peaks locate around 200 bp upstream of transcription start sites (TSS). Furthermore, we compared the transcriptome profiles of salivary glands from wild type and the JIL-1^{22}/JIL-1^{22} null mutants by next generation sequencing. Interestingly, in the absence of H3S10 phosphorylation by JIL-1 the expression of 68% of normally active genes (1,057 out of 1,556) was repressed, whereas the expression of most normally inactive genes (177 out of 181) was activated. Taken together, these observations suggest a model where histone H3S10 phosphorylation may play a dual role in modulating gene expression depending on the state and context of other epigenetic marks, but that H3S10 phosphorylation mainly
facilitates gene expression of active genes by maintaining an open chromatin structure at promoter regions by counteracting heterochromatization.

**INTRODUCTION**

In eukaryotes, the genomic DNA is highly compacted into a structure called chromatin. The remodeling and organization of higher order chromatin structure is critical for many biological processes, such as transcription, DNA replication and DNA repair (Wolffe and Hayes, 1999). Histone modification is a well known mechanism to alter the chromatin structure and regulate gene expression. In *Drosophila*, the novel tandem kinase, JIL-1, localizes specifically to euchromatic interband regions on polytene chromosomes and predominantly phosphorylates histone H3S10 at interphase (Jin et al., 1999; Wang et al., 2001). JIL-1 is essential for the viability of *Drosophila* (Wang et al., 2001; Zhang et al., 2003) and loss of JIL-1 causes global disruption of polytene chromosome morphology (Wang et al., 2001; Deng et al., 2005). Also reducing JIL-1 level results in the enhancement of PEV of the centromeric P-element insertion 118E-10 (Bao et al., 2007; Wang et al., 2011). Three major heterochromatin markers, histone H3K9 dimethylation, HP1(Schotta et al., 2002) and Su(var)3-7 (Delattre et al., 2004; Jaquet et al., 2006), spread to ectopic locations on the chromosome arms in hypomorphic and null JIL-1 mutants (Zhang et al., 2006; Deng et al., 2010; Wang et al., 2011). Reducing the dose of *Su(var)3-9* and *Su(var)3-7* is able to largely rescue the lethality of JIL-1 null and hypomorphic alleles (Deng et al., 2008; Deng et al., 2010). Moreover, by expressing various truncated JIL-1 proteins in a JIL-1 null mutant and testing the PEV effects on 118E-10 and *wm*4 (Wallrath and Elgin, 1995; Cryderman et al., 1998; Wang et al., 2010), it has been shown that the JIL-1 mediated epigenetic H3S10 phosphorylation marker itself is necessary to prevent heterochromatin spreading independently (Wang et al., 2011). These results indicate that JIL-1 is required
for chromatin structure maintenance and is involved in transcription regulation by counteracting heterochromatin spreading. Recently, Ivaldi et al (2007) proposed that JIL-1 and JIL-1 mediated histone H3S10 phosphorylation are able to help recruitment of positive transcription elongation factor b (P-TEFb) which facilitates transcription elongation by releasing RNA polymerase II (Pol II) from pausing. However, we found that JIL-1 and H3S10 phosphorylation do not accumulate at transcriptionally active puffs upon heat shock treatments. Furthermore, the elongating form of RNA polymerase II (Pol IIoSer2) is present abundantly and heat shock protein Hsp70 is robustly transcribed in the absence of JIL-1 and histone H3S10 phosphorylation (Cai et al., 2008). Our data are inconsistent with Ivaldi’s model that JIL-1 mediated histone H3S10 phosphorylation is required for Pol II-dependent transcription at active loci. In this study, we describe a multilayered analysis of ChIP-seq and RNA-seq datasets to elucidate the correlation between epigenetic chromatin modifications and gene expression. ChIP-seq data analyses show that JIL-1 binds to the chromatin near transcription start sites (TSS) of active genes. In JIL-1^{+/+}/JIL-1^{++} null mutants, the expression of normally active genes (1,057 out of 1,556) is repressed, whereas normally inactive genes (177 out of 181) are activated. In summary, all these observations suggest that JIL-1 and JIL-1 mediated histone H3S10 phosphorylation may play a global role in modulating gene expression depending on the state and context of chromatin.
MATERIAL AND METHODS

*Drosophila melanogaster* stocks

Fly stocks were maintained according to standard protocols (Roberts 1998). Canton S. was used for wild type preparations. The *JIL-1* allele is described in Wang et al. (2001) and in Zhang et al. (2003).

ChIP and ChIP-seq

ChIP experiments were performed as previously described (Wang et al., 2011). 50 pairs of salivary gland were used. Mouse anti-JIL-1 mAb 5C9 (Jin et al., 2000), or anti-GST mAb 8C7 (Rath et al. 2004) was used for immunoprecipitation. DNA from the immunoprecipitated chromatin fragments (500 bp average) and input chromatin was purified by a Wizard SV DNA purification kit (Promega). ChIPed DNA was sequenced on an Illumina Genome Analyzer at the Iowa State University DNA facility. 41 bp sequenced tags were aligned to the *Drosophila melanogaster* genome with Bowtie (Langmead et al., 2009). Binding sites were identified using MACS 1.4.1 (Zhang et al. 2008). The peaks with a false discovery rate of 5% or below were considered as valid binding sites of JIL-1. All the peaks can be shown by integrated genome viewer (IGV 1.5). Chromator and histone H3K9 dimethylation ChIP on Chip data were downloaded from ModENCODE (www.modencode.org).

Quantitative-PCR

Quantitative PCR was carried out using Brilliant® II SYBR Green QPCR Master Mix (STRATAGENE) in conjunction with an Mx4000 (STRATAGENE) PCR machine. Cycling parameters were 10 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. Fluorescence intensities were plotted against the number of cycles.
using an algorithm provided by Stratagene. Template levels were quantified using a calibration curve based on dilution of concentrated DNA. For each experimental condition the relative enrichment was normalized to the corresponding control immunoprecipitation from the same chromatin lysate.

**RNA-seq and analysis**

Total RNA of third instar salivary glands was isolated by UltraClean Tissue & Cells RNA isolation Kit (Mo Bio) and DNA was removed by DNase I kit (Mo Bio). Wild type and *JIL-1*<sup>122</sup>/*JIL-1*<sup>122</sup> null mutant RNA were amplified and sequenced on an Illumina Genome Analyzer at the Iowa State University DNA facility. Raw reads were trimmed to remove low-quality nucleotides via the Data2Bio trimming script. GSNAP (Genomic Short-read Nucleotide Alignment Program, version 2010-03-09) (Wu and Nacu 2010), which allows for gapped alignments, including intron-spanning alignments, was then used to map trimmed reads to the reference genomes. Reads with one unique best match in the reference genome with ≤2 mismatches every 75 bp were used for all subsequent analyses. The read depth of each gene was computed based on the coordinates of mapped reads and annotated locations of genes in the reference genome.

**Identification of differentially expressed genes via Fisher's exact test:**

The trimmed mean of M-values normalization method was used to normalize wild type and mutant RNA-seq reads (Robinson and Oshlack, 2010). The normalized read counts were used to calculate fold-changes (FC) and statistical significance. Fisher's exact test was used to test the null hypothesis that expression of a given gene is not different between the two samples. Only genes having at least 50 mapped reads from the two combined genotypes were tested. Genes identified as candidates for differential expression
were further filtered by correcting for multiple testing using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). To obtain a relatively confident set of differentially expressed genes, we required that differentially expressed genes exhibit >2 fold change and p-value smaller than $2.2 \times 10^{-16}$ in expression.

**Immunohistochemistry**

Polytene chromosome squash preparations were performed as in Cai et al. (2010) using a 5 minute fixation protocol and labeled with chicken anti-JIL-1 (Jin et al., 2000), anti-Chromator mAb 6H11 (Rath et al., 2006), Rabbit anti histone H3K9me2 (Millipore) or anti-JIL-1 mAb 5C9 (Jin et al., 2000). DNA was counterstained by Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype- specific TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution). The final preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a cooled Spot CCD camera. Images were imported into Photoshop where they were pseudocolored, image processed, and merged.

**RESULTS**

**JIL-1 binds chromatin near TSS.**

We have recently demonstrated that the JIL-1 tandem kinase is responsible for phosphorylation of histone H3S10 at interphase (Wang et al., 2001). JIL-1 localizes specifically to euchromatic interband regions of chromosomes and is required for maintenance of euchromatin structure by counteracting the spreading of heterochromatic factors (Wang et al., 2001; Jin et al., 1999; Zhang et al., 2003; Deng et al., 2005; Wang et
al., 2011). Thus elucidation of the higher resolution distribution of JIL-1 within the genome can greatly contribute to understanding the biological functions of JIL-1 and JIL-1 mediated histone H3S10 phosphorylation. JIL-1 chromatin binding sites across the *Drosophila melanogaster* genome were identified by chromatin immunoprecipitation combined with next generation sequencing in salivary glands of third instar larvae. Salivary gland chromatin was immunoprecipitated using the affinity purified monoclonal anti-JIL-1 antibody 5C9 (Jin et al., 2000) and followed by next generation sequencing. More than 10 million reads were obtained from both input and immunoprecipitation samples. We were able to identify 1,679 JIL-1-binding sites by using the MACS algorithm with a 5% false discovery rate (FDR) threshold (Zhang et al., 2008). All these sites were mapped to 1,527 unique gene loci and 81 intergenic regions with average peak length of 2,074 bp. In order to further characterize how JIL-1 localizes in and around genes, peak summits of JIL-1 binding sites were plotted relative to the transcription start site (TSS) of the closest genes. Interestingly, we found that most of the peak centers locate around 200 bp upstream of the proximity of TSS (Fig. 1A, 1B). These ChIP-seq data were validated by salivary gland chromatin immunoprecipitated with the JIL-1 monoclonal antibody 5C9 (Jin et al., 2000), and analysed by quantitative PCR using specific primers for the JIL-1-enriched regions identified by MACS (Sup. 1). Our data indicated that JIL-1 might regulate gene expression by maintaining chromatin structure near the proximity of TSS.

**JIL-1 binds to active gene regions and colocalizes with Chromator.**

Previous studies demonstrated that JIL-1 localizes to euchromatic interband regions which are thought to comprise actively transcribed regions. Therefore, our hypothesis is that JIL-1 is generally associated with active genes. To test this hypothesis, we combined JIL-1 ChIP-seq profiles with wild type transcriptome profiles to examine the locations of
JIL-1 on polytene chromosomes. Among 1,527 JIL-1 target genes, 1,167 genes are actively transcribed in wild type third instar salivary glands (Fig. 2C), which indicates that the majority of JIL-1 protein distributes to active gene regions. It is known that Chromator colocalizes with JIL-1 on polytene chromosomes (Fig. 2B). Genome-wide Chromator distribution is consistent with JIL-1 ChIP-seq profiles according to the Chromator ChIP tilling array data (ModENCODE) (Celniker et al., 2009). Chromator peaks largely overlap with those of JIL-1 at multiple loci including autosomes and the X chromosome (Fig. 2A, Sup. 3). Also specifically for Chromator distribution across genes, the plot of peak centers shows that Chromator locates predominantly near TSS close to or overlapping with JIL-1 binding sites (Sup. 2). Histone H3K9 dimethylation is enriched at heterochromatin domains and correlates with gene silencing (Shilatifard, 2006). JIL-1 and histone H3K9 dimethylation ChIP on Chip binding profiles do not overlap with each other (Fig. 2A). These results suggested that JIL-1 colocalizes with Chromator at the proximity of TSS on euchromatin.

Furthermore, a venn diagram of the JIL-1 and Chromator target genes demonstrated they have 51% overlap. However, histone H3K9 dimethylation only has 10% overlapped target genes with that of JIL-1(Fig. 2D, 2E). All these data further illustrated that JIL-1 is generally associated with the proximity of the TSS region of active genes.

**Loss of JIL-1 results in activation of some genes and repression of others.**

Reduction of JIL-1 levels results in global disruption of polytene chromosome structure. Also, hypomorphic loss-of-function alleles of the JIL-1 histone H3S10 kinase are strong suppressors of position effect variegation (PEV) of the w\textsuperscript{nd} allele (Lerach et al., 2006) and enhancers of PEV of the centromeric P-element insertion 118E-10 (Bao et al., 2007; Wang et al., 2011). Thus, it has been suggested that JIL-1 is required for maintenance of chromatin structure and regulation of gene expression. To determine the
global role of JIL-1 in transcriptional regulation and the relationship between chromatin structure and transcription regulation, we mapped genome-wide transcriptome of third instar larvae salivary glands of wild type and $JIL-1^{z2}/JIL-1^{z2}$ null mutants using RNA-seq analysis. Of nearly 15,000 analyzed genes, 1,737 genes changed at least two-fold ($p < 2.2 \times 10^{-16}$) in $JIL-1^{z2}/JIL-1^{z2}$ null mutants compared to wild type (Fig. 3A). Of all genes, 61% were downregulated while 39% showed an increased expression level in $JIL-1^{z2}/JIL-1^{z2}$ null mutants compared to wild type (Table 1). Consistent with a major role for JIL-1 in transcriptional activation, a box plot of all significantly changed genes shows a global reduction of expression levels (Fig. 3B) These affected genes belong to many functionally diverse gene ontology groups with no obvious common properties involved in the regulation of chromatin structure.

To examine whether loss of JIL-1 has different affects on different chromosomes, affected genes were plotted and summarized relative to their chromosome positions. Genes on the X chromosome do not have significant differences from the autosomes (Fig. 3C). Thus loss of JIL-1 does not affect the X chromosome differently than autosomes. Furthermore, to assess the relationship between chromosome structure and transcription, we selected genes with at least four-fold changes and plotted them along the X chromosome and autosomes (Fig. 4). These highly changed genes appear to distribute randomly and broadly between the X chromosome and autosomes. Therefore, the changes in gene expression in $JIL-1$ mutants could be a consequence of global alteration of chromosome organization.

In order to gain further insight into JIL-1 functions in transcription regulation, we first divided all changed genes into two categories, active and inactive, and the global changes were examined between these two groups. A transcript of one RPKM (reads per kilobase per million reads) corresponds to approximately one transcript per cell after calculation
The genes with greater than one RPKM were classified as active genes in salivary gland cells, otherwise they are considered inactive genes. Different effects on expression after loss of JIL-1 were observed between these two groups. Interestingly, in the absence of JIL-1 the expression of 68% of normally active genes (1,057 out of 1,556) was repressed, whereas the expression of most normally inactive genes (177 out of 181) was activated (Fig. 3D, Table 2). We further classified the active genes into three separate groups comprised of high, moderate or low expression levels that reflected an RPKM from 1-5, 5-30 or above 30. In the absence of JIL-1, 91% of highly expressed genes and 74% of moderately expressed level genes were down-regulated compared to wild type. However, only 25% of low expressed genes decreased (Fig. 3E, Table 3). Moreover, the plot of all changed active genes in JIL-1<sup>+/−</sup>/JIL-1<sup>−/−</sup> null mutants shifts and condenses to the middle compared to the wild type (Fig. 3F). Taken together, our analyses suggested that JIL-1 regulates gene expression differently in different classes of genes.

**Loss of JIL-1 could directly affect gene expression at many loci.**

ChIP-seq mapping and RNA-seq analysis showed that JIL-1 binds near TSS and affects transcription regulation of different classes of genes in different ways. In order to assess the direct effect on gene expression of JIL-1 binding, we combined the results of JIL-1 ChIP-seq and RNA-seq. 273 genes (18% of total target genes) with at least two-fold changes were found to be in common between these two datasets. Also pattern of expression level changes of these 273 genes is similar to that of the whole transcriptome. 184 out of 273 genes were down-regulated and the other 89 genes were up-regulated. In this gene set, 261 genes were normally active and only 12 genes were normally inactive. Specifically, these 12 genes expression levels were all up-regulated in the JIL-1 null mutant. However, the other 261 normally active genes had variant expression level changes
between mutant and wild type. Around 94% of those genes with normally high expression levels and 73% of moderately expressed level genes decreased in the absence of JIL-1, whereas only 27% of low expression level genes are down-regulated (Table 4). However, 1,253 of JIL-1 target genes (82% of the total target genes) do not have significant expression level changes in a JIL-1 null mutant, indicating that JIL-1 might not be active at these binding sites and thus might not show direct effects on gene expression upon loss of JIL-1. All these observations suggested that loss of JIL-1 directly results in gene expression level changes at some loci but not all.

The H3K9 dimethylation levels of two JIL-1 target genes is altered in a JIL-1 mutant.

In a JIL-1 null mutant, the chromatin structure of polytene chromosomes is totally disturbed and the heterochromatin marker H3K9me2 spreads to all chromosome arms especially on the X chromosome (Fig. 5A). Therefore, the transcriptome alteration in a JIL-1 null mutant might be due to heterochromatic marker spreading. In order to test this hypothesis, we quantified the H3K9 dimethylation levels on two JIL-1 target genes in a JIL-1 null mutant by ChIP assay. CG15824 is a normally inactive gene associated with a high level of H3K9 dimethylation. In a JIL-1 null mutant CG15824 expression level is upregulated and simultaneously H3K9 dimethylation level decreases dramatically. On the contrary, mnb, a normally active gene, is repressed in the absence of JIL-1 and is accompanied by an increase of H3K9 dimethylation levels. These results suggested that in the absence of JIL-1, expression level changes of these two JIL-1 target genes, CG15824 and mnb, show a correlation with H3K9 dimethylation level changes (Fig. 5B). Thus, JIL-1 might regulate gene expression by counteracting the spreading of heterochromatin marker H3K9me2.
DISCUSSION

Previous studies demonstrated that JIL-1 and JIL-1 mediated histone H3S10 phosphorylation are required for maintenance of polytene chromosome structure and facilitating transcription by counteracting heterochromatic marker spreading. Furthermore, PEV assays suggested that JIL-1 or JIL-1 mediated H3S10 phosphorylation is able to regulate gene expression at several loci (Lerach et al., 2005; Lerach et al., 2006; Bao et al., 2007). However, it is not known whether this is a global effect across the genome or whether the absence of JIL-1 and H3S10 phosphorylation is important for only certain classes of genes.

In this study, we identified 1,675 loci that contain JIL-1 binding sites from a genome-wide analysis of JIL-1 kinase binding profiles. JIL-1 binding peaks are centered approximately 200 bp upstream of transcription start sites. These 1,675 targets have been mapped to 1,527 unique genes. 1,167 out of 1,527 JIL-1 target genes are actively transcribed, which is consistent with our previous findings that JIL-1 predominantly localizes to interband regions on polytene chromosomes. It is further supported by comparing JIL-1 ChIP-seq profiles with ModENCODE tiling array ChIP data of Chromator and histone H3K9 dimethylation. The results show that JIL-1 peaks substantially overlap with Chromator peaks but not those of H3K9 dimethylation. We previously demonstrated that Chromator also binds to interband regions and colocalizes with JIL-1 on polytene chromosomes (Rath et al., 2006) whereas H3K9 dimethylation enriched regions usually correlate with inactive gene regions (Shlatifard, 2006; Kouzarides, 2007). However, only 50% of JIL-1 target genes overlaps with that of Chromator. There are two reasons to explain this result. One is that immunostaining has lower resolution than ChIP-seq, thus colocalization of proteins by immunostaining does not mean that they target to the same gene loci. The other reason is that Chromator profiles from ModENCODE are generated from 16 hour embryos, whereas
our data use salivary glands as detection sample. The benefits using salivary gland cells from third instar larvae in our experiments are that we can specifically map and correlate the locations of JIL-1 binding sites with the locations of the epigenetic histone H3S10 phosphorylation mark. Salivary gland nuclei are all at interphase excluding contributions from mitotic histone H3S10 phosphorylation. Referring to histone H3K9 dimethylation ChIP on Chip profiles from ModENCODE, the 10% target gene overlaps observed between JIL-1 and H3K9 dimethylation profiles strongly supports that JIL-1 predominantly localizes to active gene regions.

Moreover, we performed an RNA-seq experiment to determine transcriptome changes of third instar larave salivary glands in JIL-1$^{22}$/JIL-1$^{22}$ null mutants. In the absence of JIL-1 and JIL-1 mediated H3S10 phosphorylation, 61% of the total affected genes is repressed while 39% is activated. The broad distribution on all of the chromosome arms and the functional diversity of all the changed genes indicate that transcriptome alteration upon loss of JIL-1 is not caused by one or two specific pathways but instead is likely to be due to overall alterations in chromatin structure. Furthermore, the expression level changes of JIL-1 target genes were analysed. However only 18% (273 out of 1,527) of JIL-1 target genes shows expression level changes in the absence of JIL-1 (Table. 4). ChIP-seq results show the genome-wide JIL-1 binding profiles, while RNA-seq results represent the overall transcription changes including direct and indirect effects by loss of JIL-1. Therefore these two datasets do not have to overlap. It is known that in JIL-1 null mutant polytene chromosome structure is largely disturbed and H3K9 dimethylation spreads to ectopic loci (Zhang et al., 2006; Wang et al., 2011). Further H3K9 dimethylation ChIP assay suggests that in the absence of JIL-1 two JIL-1 target genes, CG15824 and mnb, that show changes in expression levels, also show alterations of H3K9 dimethylation level. These alterations might be a consequence of H3K9 dimethylation redistribution in JIL-1 null mutant. In the
future, it will be of interest to map the genome-wide histone H3K9 dimethylation redistribution sites upon loss of JIL-1. In summary, all these findings suggest a model that JIL-1 and JIL-1 mediated histone H3S10 phosphorylation can facilitate transcription by maintaining an open chromatin structure at the promoter region and counteracting heterochromatin spreading.

ACKNOWLEDGEMENTS

We thank members of the laboratory for discussion, advice, and critical reading of the manuscript. We also wish to acknowledge Mr. Atrez Norwood for technical assistance. We especially thank all colleagues for providing their data to the ModENCODE data source. This work was supported by NIH Grant GM062916 (KMJ/JJ).

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FIGURE LEGENDS

**Figure 1.** Genome-wide chromatin binding sites of JIL-1 are enriched upstream of the transcription start site (TSS). (A) The density of JIL-1 binding sequences is plotted relative to the distance from the TSS of the closest gene. Blue dashed line represents the TSS position. (B) Representative examples of JIL-1 binding sites near the promoter regions. These two panels are screen shots from IGV 1.5 (integrated genome viewer). Chromosome length is shown in scale. The red thick bars highlight JIL-1 peaks identified by MACS (FDR<5%). The lower two blue graphs are ChIP-seq profiles of JIL-1 immunoprecipitations and input DNA of salivary gland cells of third instar larvae. Both of these two charts are from chromosome arm 3L. The bottom bars show all the genes annotated in these regions. The repeated small blue arrows in the intron segments show the transcription directions of genes.

**Figure 2.** JIL-1 is associated with active gene regions and colocalizes with Chromator at many but not all positions. (A) Three representative examples of JIL-1 ChIP-seq profiles compared with Chromator and histone H3K9me2 tiling array ChIP data (ModENCODE) from chromosome X, 3L and 4. Green bars represent Chromator peaks; blue bars represent histone H3K9 dimethylation peaks; red bars represent JIL-1 peaks. (B) JIL-1 colocalizes with Chromator on polytene chromosomes. The polytene squash preparations were labeled with antibody to JIL-1 (in green), Chromator (in red) and with Hoechst (DNA, in blue/gray). (C) Venn diagram showing the relationships between JIL-1 associated genes and active genes. (D) Venn diagram showing the relationships between JIL-1 associated genes with histone H3K9 dimethylation associated genes. (E) Venn
Figure 3. JIL-1 increases expression of many genes and represses expression of other genes. (A) A scatter plot for the comparison of gene expression patterns between the two samples. Negative log_{10} p-values from Fisher’s exact test were plotted against the negative log_{2} (Wild type/mutant) fold change for each gene with at least 2 fold change and 2.2x10^{-16} p-values cutoff. Each dot represents a separate gene. Differentially expressed genes with >2-fold increase or decrease in expression are highlighted in red(down-regulated in the mutant) or light blue (up-regulated in the mutant). (B) Box plot of negative log_{2} (Wild type/mutant) fold changes of all genes showing significant changes in expression. (C) Box plot of negative log_{2} (Wild type/mutant) fold changes of all genes showing significant changes in expression classified by chromosome positions. (D) Box plot of negative log_{2} (Wild type/mutant) fold change in gene expression classified by different expression levels: active genes (RPKM≥1) and inactive genes (RPKM<1). (E) Box plot of negative log_{2} (Wild type/mutant) fold change of active gene expression classified by different expression levels: high (RPKM≥30), moderate (30>RPKM≥5) and low (RPKM<5). (F) Histogram plot of all significantly changed active genes in wild type and JIL-1^{+/2}/JIL-1^{t^{22}} null mutants. Total transcription levels are quantified as RPKM.

Figure 4. Genes that exhibit significantly changed expression levels in a JIL-1 null mutant are broadly distributed throughout the genome. (A) (B) (C) (D) (E) Scatter plots show the position distributions of euchromatic genes that have at least a 4-fold change in gene expression in the salivary glands of a JIL-1 null mutant third instar larvae compared to wild type. The x-axis represents the relative cytological positions of genes on the left or right
arms of the second (Chr2L and Chr2R) and third (Chr3L and Chr3R) and the X chromosome. The y-axis corresponds to negative log₂ (Wild type/Mutant) fold change. Each blue dot represents a gene. The chromosomes are drawn to scale based on the length of each arm. Tel and Cen represents the telomere and centromere regions of each arm.

**Figure 5.** H3K9 dimethylation spreads to all chromosomes especially X chromosomes in a *JIL-1^{z2}/JIL-1^{z2}* null mutant. (A) Wild type (WT) and JIL-1 mutant (Z2) polytene squash preparations were labeled with antibody to JIL-1 (in green), Histone H3K9 dimethylation (in red) and with Hoechst (DNA, in blue/gray). (B) ChIP assay of the H3K9 dimethylation level change between wild type and a JIL-1 null mutant. Histograms of the relative enrichment of chromatin immunoprecipitated by anti-H3K9me2 monoclonal antibody from salivary glands from wild type (WT) and JIL-1z2/JIL-1z2 null (Z2) third instar larvae. For each experimental condition the relative enrichment was normalized to the corresponding control immunoprecipitation with anti-GST monoclonal antibody (8C7).

*CG15824* is an inactive gene in wild type and upregulated in JIL-1z2/JIL-1z2 null. *Cdc2* is an inactive gene both in wild type and JIL-1z2/JIL-1z2 null. *Rp49* is an active gene both in wild type and JIL-1z2/JIL-1z2 null. *Mnb* is an active gene in wild type and downregulated in JIL-1z2/JIL-1z2 null.
### Table 1
Whole transcriptome changes in JIL-1<sup>Z2</sup>/JIL-1<sup>Z2</sup> mutants

<table>
<thead>
<tr>
<th></th>
<th>Upregulated gene numbers (% total)</th>
<th>Downregulated gene numbers (% total)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changed genes</td>
<td>676 (39%)</td>
<td>1061 (61%)</td>
<td>1737</td>
</tr>
</tbody>
</table>

### Table 2
Whole transcriptome changes in JIL-1<sup>Z2</sup>/JIL-1<sup>Z2</sup> mutants

<table>
<thead>
<tr>
<th></th>
<th>Upregulated gene numbers (% total)</th>
<th>Downregulated gene numbers (% total)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive genes (RPKM&lt;1)</td>
<td>177 (97.8%)</td>
<td>4 (2.2%)</td>
<td>181</td>
</tr>
<tr>
<td>Active genes (RPKM&gt;1)</td>
<td>499 (32.1%)</td>
<td>1057 (67.9%)</td>
<td>1556</td>
</tr>
</tbody>
</table>
### Table 3
Whole transcriptome changes of active genes in JIL-1<sup>ZZ</sup>/JIL-1<sup>ZZ</sup> mutants

<table>
<thead>
<tr>
<th></th>
<th>Upregulated gene numbers (% total)</th>
<th>Downregulated gene numbers (% total)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low expression level</td>
<td>279 (74.8%)</td>
<td>94 (25.2%)</td>
<td>373</td>
</tr>
<tr>
<td>(RPKM1-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate expression</td>
<td>177 (25.9%)</td>
<td>507 (74.1%)</td>
<td>684</td>
</tr>
<tr>
<td>level (RPKM5-30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High expression level</td>
<td>42 (8.4%)</td>
<td>457 (91.6%)</td>
<td>499</td>
</tr>
<tr>
<td>(RPKM&gt;30)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4
Transcription level changes of JIL-1 target genes in JIL-1<sup>ZZ</sup>/JIL-1<sup>ZZ</sup> mutants

<table>
<thead>
<tr>
<th></th>
<th>Upregulated gene numbers (% total)</th>
<th>Downregulated gene numbers (% total)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low expression level</td>
<td>39 (73.1%)</td>
<td>14 (26.9%)</td>
<td>53</td>
</tr>
<tr>
<td>(RPKM1-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate expression</td>
<td>34 (26.6%)</td>
<td>94 (73.4%)</td>
<td>128</td>
</tr>
<tr>
<td>level (RPKM5-30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High expression level</td>
<td>5 (6.2%)</td>
<td>76 (93.8%)</td>
<td>81</td>
</tr>
<tr>
<td>(RPKM&gt;30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactive (RPKM&lt;1)</td>
<td>11 (100%)</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 2
Fig. 3
Fig. 3
Fig. 4
Fig. 5
Supplementary Data

Supp. 1 Validation of ChIP-seq result by quantitative PCR. Histograms of the relative enrichment of chromatin immunoprecipitated by anti-JIL-1 monoclonal antibody (5C9) from salivary glands of wild type third instar. The relative enrichment was normalized to the corresponding control immunoprecipitation with anti-GST monoclonal antibody (8C7). Caf1-180, CG15824, gkt, phl and mnb are identified peaks from the ChIP-seq experiment. Cdc2 is a negative control with no JIL-1 enrichment at this locus. The graph shows the average result from three independent experiments. Error bars indicate the s.e.m.
Sup. 2 Chromator chromatin binding sites are enriched at the transcription start site (TSS). The density of Chromator binding sequences is plotted relative to the distance from the TSS of the closest genes. Chromator tends to bind with a peak at the TSS. Blue dashed line shows the TSS position.
Sup. 3
Sup. 3 JIL-1 is associated with active gene regions and colocalizes with many but not all Chromator binding sites. Six more examples of JIL-1 ChIP-seq profiles compared with Chromator and histone H3K9me2 tiling array ChIP data (ModENCODE) from chromosome 2L, 2R and 3L. Green and blue bars represent Chromator peaks and histone H3K9 dimethylation peaks. Red bars are JIL-1 peaks.
\[ C = \frac{X \cdot L}{T} \]
\[ N = \frac{m_{\text{totalRNA}} \cdot p \cdot L}{M_{\text{nt}} \cdot 1.66 \times 10^{-24} \cdot L \cdot n_{\text{tiss}} \cdot n_{\text{cell}}} \]
\[ R = 10^9 \frac{X}{T} \]

\( C \), number of reads mapping to transcript 
\( N \), total number of sequenced reads 
\( X \), copies per cell of transcript 
\( T \), total length of transcriptome 
\( L \), transcript length 
\( R \), RPKM (reads per kilobase and million mappable reads) 
\( m_{\text{total}} \), weight of total RNA from sample 
\( p \), proportion of mRNA in Total RNA 
\( M_{\text{nt}} \), average molecular weight of nucleotide 
\( n_{\text{tiss}} \), number of salivary glands in sample 
\( n_{\text{cell}} \), average number per salivary gland

Sup. 4

Sup. 4 The formula to calculate transcripts in each cell in salivary glands (Modified from Mortazavi et al., 2008).
Quantitative PCR Primers

\( phl-F: \) ACAGAAGTGGATGTGGGT

\( phl-R: \) TCGGCCAAAGGATCGTAT

\( mnbF: \) ATCATCGCGAGTGGCTAACCC

\( mnbR: \) GCACACTCAGCCAGCAACAC

\( Caf1-180 \ F: \) AGCCGTATGCAGGATCACTCT

\( Caf1-180 \ R: \) TCTTAACAACGCCAGCGTGCA

\( CG15824 \ F: \) GAACCGAAGTGTGCGCCACCCT

\( CG15824 \ R: \) CATTCTTGGCGACGCGACCAT

\( gkt \ F: \) CCAATCGATTAATTGCCATC

\( gkt \ R: \) CCCGATTCATTTCCCTTCGCA

\( cdc2 \ F: \) GTAGCTAGCTTAGCATCGTT

\( cdc2 \ R: \) CCATATGTGCCCTCGCCAAT

\( Rp49 \ F: \) GGCAAGTATGTCGCTGATT

\( Rp49 \ R: \) CCCATCACAACAGAAGCCA
CHAPTER 5: GENERAL CONCLUSIONS

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Functional Mapping of JIL-1 domains

JIL-1 contains 1,207 amino acids, which can be divided into four main domains including a NH$_2$-terminal domain (NTD), the first kinase domain (KDI), the second kinase domain (KDII) and a COOH-terminal domain (CTD) (Jin et al., 1999; Wang et al., 2001). Previous studies suggested that the JIL-1 C-terminal domain is important for correct chromosomal targeting of the protein. The $JIL-1^{Su(var)3-1}$ allele, which has a truncation of the C-terminal domain of JIL-1, results in chromatin mislocalization of the proteins (Zhang et al., 2006) and gives rise to a strong suppressor effect of PEV for the $w^{md}$ allele (Bao et al., 2007; Elbert et al., 2004). In this study, the results demonstrate that the CTD domain of the JIL-1 kinase is necessary and sufficient for proper localization to chromatin, but that both C- and N-terminal sequences are necessary for upregulation on the male X chromosome. Expression of the CTD domain in a $JIL-1$ null mutant background is able to rescue the chromosome morphology defects. Mislocalization of the truncated JIL-1 $\Delta$CTD proteins further supports that the CTD domain is required for the correct localization of JIL-1 to chromatin. Furthermore, a small 53 amino acid region within the CTD domain has been shown to interact with the tail-region of histone H3 suggesting that this interaction is necessary for the correct chromosomal targeting of the JIL-1 kinase. However, expression of the $\Delta$CTD construct in $JIL-1$ null mutant background, which lacks the C-terminal domain but retains histone H3S10 kinase activity, is able to target to the chromosome arms and partially rescue the polytene chromosome morphology. This result implies that JIL-1 has
another mechanism for chromosomal targeting. The ΔCTD construct includes the NTD and KDI/KDII. We found that the NTD is able to be associated with chromosome while KDI/KDII does not show any detectable localization to chromatin suggesting that NTD is responsible for mediating the chromosomal targeting of the ΔCTD construct. In summary, these findings indicate that JIL-1 may participate in regulating polytene chromosome morphology by multiple and partially redundant mechanisms.

For future study, one interesting direction is to determine the crystal structure of JIL-1 or each domain of JIL-1, which will help us to determine the interactions between JIL-1 and histone H3, especially how JIL-1 through its C-terminal domain is integrated into nucleosome and chromatin organization.

**RNA Pol II-mediated transcription at active loci does not require histone H3S10 phosphorylation in *Drosophila.***

Recently, an alternative model has been proposed based on analysis of transcription activation at active loci by heat shock treatments in *Drosophila* (Ivaldi et al., 2007). In this model, JIL-1 and JIL-1 mediated H3S10 phosphorylation have been linked to the recruitment of positive transcription elongation factor b (P-TEFb), which results in release of Pol II from promoter-proximal pausing (Ivaldi et al., 2007). However, our results are contrary to their model. No redistribution of JIL-1 or H3S10 phosphorylation to transcriptionally active puffs was found in wild type polytene chromosome squashes after heat shock treatments. Protein levels of the elongation form of RNA polymerase II do not change in a *JIL-1* null mutant. Upon heat shock, the heat shock induced puffs are strongly labeled with Pol II<sup>ser2</sup> antibody in the absence of JIL-1 and JIL-1 mediated H3S10 phosphorylation. mRNAs of heat shock protein Hsp70 in *Drosophila* are transcribed at robust levels in *JIL-1* null mutants as quantified by qRT-PCR. All these observations
suggest that gene repression in JIL-1 null mutants is not global and Pol II machinery is functional. Furthermore, ectopic targeting of JIL-1 resulting in high levels of H3S10 phosphorylation and decondensation at tethering sites, is not associated with high levels of Pol II\textsuperscript{ser2} RNA polymerase II, which suggests that JIL-1 and JIL-1 mediated H3S10 phosphorylation are able to regulate higher chromatin structure \textit{in vivo} without inducing a high level of transcription (Deng et al., 2008). Therefore, JIL-1 and interphase H3S10ph might facilitate transcription by generating a less condensed chromatin state and counteracting heterochromatin spreading. In the future, exploring the mechanism of how H3S10 phosphorylation regulates gene expression through maintenance of chromatin structure will greatly contribute to understanding functions of interphase H3S10 phosphorylation in epigenetic processes.

\textbf{Genome-wide analysis of JIL-1 and H3S10ph localization and regulation of gene expression.}

Previous studies already suggested that JIL-1 and JIL-1 mediated H3S10 phosphorylation are able to regulate gene expression at certain loci (Bao et al., 2007; Deng et al., 2008; Deng et al., 2010; Wang et al., 2011). However, it is not known whether this is a global effect or restricted to specific loci. To address this issue, we performed a genome-wide analysis of JIL-1 targeting sites by ChIP-seq, combined with a RNA-seq analysis in the absence of JIL-1 and JIL-1 mediated H3S10 phosphorylation. ChIP-seq data analyses demonstrate that JIL-1 binds to the chromatin upstream of transcription start sites (TSS) of active genes. Loss of JIL-1 and H3S10 phosphorylation results in changes of gene expressions. Consistent with a role of JIL-1 in transcriptional activation, the box plot of all significantly changed genes shows a global reduction of expression levels. However, there are still 39% of all affected genes that are up-regulated in a JIL-1 null mutant suggesting
loss of JIL-1 also can result in activation of gene expression. In the absence of JIL-1 the expression of 68% of normally active genes (1,057 out of 1,556) were repressed, whereas the expression of 98% of most normally inactive genes was activated. Furthermore, the percentage of down-regulated genes increases to 91%, when we consider only the higher expression level genes that have at least 30 RPKM in a wild type background. Since gene expression levels usually are correlated with chromatin state (Weiler et al., 1995; Schotta et al., 2004), this data suggests that JIL-1 might regulate gene expression differently depending on the state and context of chromatin. Interestingly, expression level changes of two JIL-1 target genes in the absence of JIL-1 appear to be correlated with alteration of H3K9 dimethylation. Therefore, in the future, we will be able to determine the effect of ectopic H3K9 dimethylation in global gene silencing by mapping the precise genomic redistribution of repressive heterochromatin marks such as H3K9me2 in a JIL-1 null mutant background.

In summary, all our results suggest a model where the JIL-1 mediated epigenetic histone H3S10 phosphorylation marker may play a global role in regulation of gene expression depending on the state and context of other epigenetic marks, but that JIL-1 mediated H3S10 phosphorylation mainly facilitates gene expression of active genes by maintaining an open chromatin structure and counteracting heterochromatization.

REFERENCES


ACKNOWLEDGEMENT

First, I deeply thank my major professors, Dr. Kristen M. Johansen and Dr. Jørgen Johansen, for their enthusiastic supervision, encouragement and support during all the time of research. I was able to develop many good experimental and critical thinking skills under their excellent guidance. I would also like to thank Dr. Jørgen Johansen for his help in confocal imaging, and thank Dr. Kristen M. Johansen for her critical reading and comments on my dissertation.

I thank Dr. Jack Girton, Dr. Michael Shogren-Knaak, and Dr. Clark Coffman for serving in my POS committee, and for their helpful advice on my research and dissertation. In particular, I thank Dr. Jack Girton for his direct contribution and suggestions to many of my genetic experiments on Drosophila.

I would like to express my gratitude to all those who have been involved in the JIL-1 project. I especially thank Dr. Xiaomin Bao and Dr. Huai Deng for their pioneering works as well as kind help and mentoring at the beginning of my research. I also thank Chao Wang and Yeran Li for their help and discussion on the JIL-1 project. I wish to thank all the present and past members in this laboratory, including Changfu Yao, Saheli Sengupta, Dr. Yun Ding, Dr. Hongying Qi and Mr. Laurence Woodruff for their generous help, suggestion, valuable hints and friendship.

I thank Dr. C. Wu for providing the HSF antibody, Dr. M. Kuroda for providing the MSL antibody and Dr. D.M. Glover for the tws^P allele. I especially thank Dr. Sanzhen Liu for helping me on RNA-seq and ChIP-seq raw data analyses and thank Lu Shen for providing me with statistical support on my RNA-seq and ChIP-seq data analysis. I thank all the colleagues for providing their data to the ModENCODE data source.