Drosophila JIL-1 kinase mediates histone H3 Ser10 phosphorylation, maintains higher order chromatin structure, and is implicated in dosage compensation

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Drosophila JIL-1 kinase mediates histone H3 Ser10 phosphorylation, maintains higher order chromatin structure, and is implicated in dosage compensation

by

Yanming Wang

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
Major professors: Kristen M. Johansen and Jørgen Johansen

Iowa State University
Ames, Iowa
2001
This is to certify that the Doctoral dissertation of

Yanming Wang

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

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For the Major Program

Signature was redacted for privacy.

For the Graduate College
To my wife and my parents, for their love and patience
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Drosophila JIL-1 kinase mediates histone H3 Ser10 phosphorylation, maintains higher order chromatin structure, and is implicated in dosage compensation

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The Drosophila JIL-1 tandem Ser/Thr kinase is associated with chromosomes/chromatin throughout the cell cycle in early embryos and localized to hundreds of sites on the open interband regions on third instar larvae polytene chromosomes. Interestingly, the level of JIL-1 is upregulated on the male X chromosome, which is hypertranscribed for dosage compensation. The distribution of JIL-1 overlaps with that of MSL (male specific lethal) proteins and JIL-1 is associated with the MSL complex.

To further study the function of JIL-1 in vivo, a series of JIL-1 mutants from hypomorphs to null were generated. Analyzing the phenotypes of JIL-1 mutants, we found that JIL-1 is required for the viability of both females and males. Moreover, in the surviving flies of JIL-1 hypomorphic mutants, the number of males is less than that of females, implicating that JIL-1 plays a role in dosage compensation. JIL-1 is also required during early embryonic development and for the maintenance of normal higher order polytene chromosome structure in third instar larvae.
Our studies also showed that JIL-1 is involved in the histone H3 Ser10 phosphorylation signaling pathway. The JIL-1 immunocomplex can phosphorylate the Ser10 site in a synthetic H3 N-terminal peptide *in vitro*. *In vivo*, Ser10 phosphorylation levels in the third instar larvae of JIL-1 mutants are dramatically reduced. In addition, the decreased H3 Ser10 phosphorylation level can be restored with a GFP-JIL-1 transgene. Moreover, the phosphorylated H3 Ser10 is elevated and colocalized with JIL-1 on the male X chromosome.

Thus, our data suggest a model whereby JIL-1 is involved in a signaling pathway that regulates histone H3 Ser10 phosphorylation in *D. melanogaster*, which is required to maintain the appropriate higher order chromatin structure to facilitate chromatin functions, such as gene expression, dosage compensation, and so on.
CHAPTER 1. INTRODUCTION

Organization of this dissertation

This dissertation begins with background information about the JIL-1 project: what is known about the molecular properties and \textit{in vivo} distribution of JIL-1, and the putative involvement of JIL-1 in the dosage compensation pathway. This background information is the important starting point for my study. Following the background, questions raised, tested, and discussed in this dissertation are listed together with a brief description of the research approaches applied to address these questions.

Chapter 2 is a literature review. The abundance of literature related to chromatin structure and function, which was utilized during this study to design experiments to better understand the function of JIL-1, cannot all be included within the confines of this chapter. Therefore, literature related to chromatin structure, the modification of chromatin structure by ATP-dependent chromatin remodeling complexes, and the covalent histone modification enzymes is discussed. In addition, examples of how the above mentioned activities are coordinated with the general transcription machinery are shortly summarized. Last, dosage compensation related studies are reexamined with emphasis given to the \textit{Drosophila} system.

Chapter 3 describes the experimental materials and methods used in this study.

Chapter 4 presents results and discussion emphasizing the work that I have been primarily involved with during my study. If the work was mainly done by another person, the name of that person is referred to. One thing I want to address here is that all of the beautiful confocal imaging of fluorescent labeled samples was done by Dr. Jørgen Johansen, which will not be noted again in the following text.

Chapter 5 is general conclusions of these studies.

References cited in this dissertation are listed together at the end.
Background of JIL-1

The JIL-1 kinase was identified using mAb 2A to screen a λgt11 genomic expression library of *Drosophila*. The mAb 2A stains a dynamically changing nuclear structure during the cell cycle (Johansen, 1996; Johansen et al., 1996). A partial DNA sequence screened from this library was used to screen other libraries to get the full length cDNA. The JIL-1 cDNA encodes a protein of 1207 amino acids including two Ser/Thr kinase domains (Hanks et al., 1988). Whereas the KD1 (kinase domain I) of JIL-1 is highly conserved showing striking homology with members of the MSK/RSK subfamilies, the KDII (kinase domain II) is quite diversified from other kinase domains (Fig 1). Noteworthy is that JIL-1 contains a long N-terminal domain and a long C-terminal domain that are not found in MSKs and RSKs (Johansen et al., 1999).

The *in vivo* distribution study of JIL-1, which utilized a GFP tagged JIL-1 (GFP-JIL-1) transgene and immunostaining of various fly tissues with JIL-1 antibody, found that JIL-1 is widely distributed in many tissues at different developmental stages and is associated with chromatin/chromosomes (Jin et al., 1999). In early embryos, the GFP-JIL-1 signal was found to be associated with chromatin/chromosomes throughout the cell cycle (Fig 2C). In the developing egg chambers of ovaries, GFP-JIL-1 was found in the diploid nuclei of follicle cells and formed fibrous structures in the polyploid nurse cell nuclei (Fig 2A, B). The nurse cell nucleus is a place where active transcription occurs and all of the maternal mRNAs required for the early embryo development are produced. Taking advantage of the huge larval polytene chromosomes in the salivary gland of third instar larvae, immunostaining with JIL-1 antibodies indicated JIL-1 is distributed along hundreds of bands on all of the female polytene chromosomes, while it is upregulated on the dosage compensated male X chromosome marked by hyperacetylated H4K16 (H4Ac16) (Fig 3; Jin et al, 1999; Turner et al., 1992; for dosage compensation, see literature review). Furthermore, the staining pattern
Fig 1. JIL-1 is a tandem Ser/Thr kinase that belongs to the MSK subfamily. (A) The JIL-1 kinase has two kinase domains with the conserved features of Ser/Thr kinase (Hanks et al., 1988). The KDI (kinase domain I) of JIL-1 shares 63% and 47% identity with human MSK-1 and D. melanogaster RSK, respectively, while the KDII (kinase domain II) of JIL-1 has only 32% and 28% identity with its MSK-1 and RSK counterpart. (B) Phylogenetic analysis to study the relationship of JIL-1 and its homologs. A consensus maximum parsimony tree generated from the sequences of the KDI of JIL-1 and its closely related homologs demonstrated JIL-1 and MSK form a subfamily that is separate from and yet closely related to the RSK kinase subfamily. The yeast PKC was used as an outgroup for constructing the tree (modified from Johansen et al., 1999).
Fig 2. GFP-JIL-1 is associated with chromosomes/chromatin throughout the cell cycle.
(A) GFP-JIL-1 was found in the nuclei of diploid follicle cells and polyploid nurse cells in the developing fly ovary egg chambers. (B) GFP-JIL-1 forms a fibrous network in a nurse cell nucleus. (C) GFP-JIL-1 is associated with the chromosomes throughout the cell cycle in the early embryos (modified from Jin et al., 1999).
Fig 3. Localization of JIL-1 on the larval polytene chromosomes. (A, B, and C) JIL-1 antibody stains hundreds of bands that correspond to the interband regions of all of the female chromosomes. (A) JIL-1 was found to form hundreds of bands on all arms of the female polytene chromosomes. (B) The same polytene chromosome as shown in (A) was stained with Hoechst to visualize the condensed band regions. (C) In this composite figure, there is little overlap between the JIL-1 antibody staining and the Hoechst DNA staining, suggesting JIL-1 is preferentially localized to the open interband regions. (D and E) The level of JIL-1 protein, as indicated by antibody staining, is greatly elevated on the male X chromosome compared with that on the autosomes. (F and G) The bands stained by JIL-1 on the male X chromosome are largely excluded from the bands of Hoechst staining. Noticeable is the weak staining pattern of the male X by Hoechst (F), demonstrating the open chromosome configuration of the male X (modified from Jin et al., 1999).
of JIL-1 is largely excluded from that of the DNA dye Hoechst, which stains the more condensed band regions on polytene chromosomes. This would indicate that JIL-1 is localized to the open interband regions on the polytene chromosomes.

The staining pattern of JIL-1 on the male X is reminiscent of that of MSL (male specific lethal) dosage compensation proteins. Double labeling of polytene chromosomes with both JIL-1 antibody and antibodies against members of the MSL dosage compensation complex showed that JIL-1 colocalizes with the MSL proteins on the male X chromosome (Fig 4; Jin et al., 2000). Furthermore, when MSL complex is ectopically recruited to the female X chromosomes, more JIL-1 is recruited onto the female X chromosome, hence the level of JIL-1 on the MSL bound female X chromosome is also upregulated, suggesting the elevated level of JIL-1 on the male X is due to the function of the MSL complex (Jin et al., 2000).

Protein phosphorylation plays a fundamental role in various cellular signal transduction pathways, such as cell growth, cell differentiation, cell division, and gene expression (Hunter, 2000). The finding that JIL-1, a putative Ser/Thr protein kinase, is colocalized with the MSL proteins and upregulated on the male X suggests that protein phosphorylation may also play a role in the dosage compensation pathway.

In addition to its elevated male X distribution, JIL-1 is also distributed on autosomes in males and is evenly distributed on all of the chromosome arms in females, suggesting a more general role for JIL-1 in addition to its possible role in dosage compensation. The association of JIL-1 with chromosomes in the early embryos and in the nuclei of the nurse cells and follicle cells in the developing egg chambers hints that JIL-1 could play some roles in other developmental processes.
Fig 4. The JIL-1 staining pattern overlaps with that of MSL and H4Ac16 staining. (A, B, and C) Double labeling of the same polytene chromosome with anti-JIL-1 antibody and anti-acetylated H4-Lys16 (H4Ac16) antibody. JIL-1 was found to colocalize with the H4Ac16 isoform at most sites, as shown in the composite (A) with predominant yellow signals. (D, E, and F) Double labeling with JIL-1 and MSL1 antibodies showed JIL-1 staining overlaps with that of MSL1 on the male X chromosome. MSL-3 and JIL-1 double labeling also showed overlapping staining patterns (G, H and I). The arrowheads in (A, D, and G) point out that JIL-1 is localized to telomere regions where MSL staining is excluded (modified from Jin et al., 2000).
Research questions

Bearing the previous studies of JIL-1 in mind, I set up a series of experiments to answer the following questions:

1. What is the substrate of the JIL-1 kinase?

To reach this end, I have tried to express and purify JIL-1 in *E. coli*. After finding that the *E. coli* expressed JIL-1 is kinase inactive, I switched to *in vitro* immunocomplex kinase assays to test various putative substrates. After the finding of loss of function JIL-1 mutants, the phosphorylation level of putative substrate was analyzed with phosphorylation and sequence specific antibody developed for the substrate.

2. Is there a physical interaction between JIL-1 and members of the MSL complex?

To address this question, I have used the V5 mAb against the JIL-1-V5 fusion protein expressed in the *Drosophila* Schneider 2 (S2) cells and affinity purified anti-MSL antibodies to carry out co-immunoprecipitation experiments to study protein-protein interactions. In addition, the GST-JIL-1 fusion proteins expressed in *E. coli* were used for *in vitro* pull down experiments to further study these putative interactions and map the interacting region(s) of JIL-1 with the MSL complex.

3. What are the JIL-1 mutant phenotypes and how do these mutant phenotypes reflect the function(s) of JIL-1? This question can be divided into several small questions:
   a. Is JIL-1 important for the viability of males, females or both?

To answer this question, we requested a P-element insertion line, EP(3)3657 or EP3, in which an EP element was inserted into the first exon of JIL-1 locus, upstream of the starting methionine. This line turns out to be a hypomorph and JIL-1 expression is not totally blocked. Starting from this line, a stronger hypomorph Z60 and a null allele Z2 were generated using imprecise P-element excision methods. The excised part of the gene was mapped by PCR. The imprecise excision and PCR work were done by Dr. Kristen M.
Johansen and Weiguo Zhang. The importance of JIL-1 protein for the viability of both males and females was analyzed in these mutant strains. The number of males and the number of females of the surviving JIL-1 mutants were counted and compared to analyze the effect of loss of JIL-1 on the viability of males and females separately.

b. Is JIL-1 important for regulating early embryonic development?

Since JIL-1 is associated with chromosomes in the early embryos, we reasoned it might play a role in early embryo development. To address this question, early embryos produced by the homozygous EP(3)3657 parents were analyzed by Hoechst staining for DNA and tubulin staining for microtubules.

c. Is JIL-1 important for maintaining polytene chromosome structure?

Since JIL-1 can form hundreds of bands on the polytene chromosomes in the third instar larvae, we investigated what is the effect of loss of JIL-1 on polytene chromosomes by staining polytene chromosomes from EP(3)3657, Z60, and Z2 with Hoechst and MSL2 antibody to study the chromosome structure.

4. Is the in vivo phosphorylation of the in vitro identified putative substrate of JIL-1 immunocomplex correlated with the function of JIL-1?

To analyze the distribution of the phosphorylated isoform of the putative JIL-1 substrate, phosphorylation and sequence specific antibody and JIL-1 antibody were used for double labeling in larval polytene nuclei. In the JIL-1 loss of function mutants, the phosphorylation level of the putative substrate of the JIL-1 immunocomplex was studied by western blots with phosphorylation and sequence specific antibodies.

5. Are there any other proteins that interact with JIL-1?

To answer this question, I did two-hybrid screens with different portions of JIL-1 as bait. Two of the JIL-1 interacting candidates found in the two-hybrid screen were further analyzed.
by double immunostaining and/or in vitro protein-protein interaction assays, such as coimmunoprecipitation, and GST-pull down.

Taking together, the study of the questions raised above will help us to better understand the function of JIL-1. The results of these studies support a hypothesis that the JIL-1 kinase works in the histone H3 Ser10 phosphorylation signal transduction pathway and is involved in the regulation of chromatin structure that putatively affects gene expression, such as dosage compensation, and other chromatin functions.
CHAPTER 2. LITERATURE REVIEW

Chromatin structure and organization

The basic unit of chromatin is the nucleosome, which is comprised of a histone octamer made up of an H3-H4 tetramer and two H2A-H2B heterodimers, organizing about 200bp of DNA together with histone H1 (Kornberg, 1974; Kornberg, 1999). Although the model of nucleosome organization was proposed in 1974, the high-resolution structure was not resolved until 23 years later (Luger et al., 1997). The X-ray structure of the nucleosome provides a structural basis to interpret the many processes that involve DNA and chromatin as a template, such as transcription, replication, recombination, condensation and segregation. In this crystal structure, the histone octamer forms the nucleosome core wrapped up by 1.7 turns of a left-handed DNA superhelix. Each histone protein contains a domain with three α helices, which forms the histone fold, and two unstructured tails. The interaction between the histone fold domains forms a handshake-like structure mediating the formation of the H2A-H2B heterodimer and the H3-H4 heterodimer. Two H3-H4 heterodimers are packed into a tetramer that is attached by an H2A-H2B dimer at each side to form the octamer. Approximate 121bp of the 146bp DNA used in the nucleosome crystal are organized by the histone octamer with each heterodimer binding about 30bp of DNA (Luger et al., 1997). A remarkable feature of the nucleosome core structure is the ordered nature of the histone N-terminal tails and the involvement of these tails in nucleosome-nucleosome interaction (Rhodes, 1997). The histone tails protrude out from minor-groove channels of the DNA helix after every 20bp of DNA. A cluster of basic residues on the H4 tail contacts the acidic surface of an H2A-H2B dimer from a neighboring nucleosome, forming multiple hydrogen bonds and salt bridges. Although the N-terminal tails are very long, they do not form any secondary structure and only one-third of them are visible. This reported structure
offers many insights into how transcription is regulated and how protein factors interact with
the nucleosome on the chromatin.

Little is still known, however, about how nucleosomes are folded into higher order
chromatin structure during interphase and further packed to form condensed metaphase
chromosomes. Chromatin is dynamically folded and unfolded to accommodate transcription
and DNA replication, while tightly packed to facilitate the faithful segregation of
chromosomes into daughter cells (Hirano, 2000; Tumbar et al., 1999). Many multiple protein
complexes involved in these processes have been identified, including covalent histone
modification complexes, chromatin remodeling complexes, and chromatin condensation
complexes. However, how these multiple-protein complexes regulate the dynamic folding
and unfolding of chromatin is still poorly understood.

The interphase chromatin can be divided into two categories, gene rich euchromatin
and gene poor heterochromatin (Weiler and Wakimoto, 1995). Heterochromatin is condensed
even in interphase, replicated later in S phase, and often found around centromeres or
adjacent to telomeres. The DNA in heterochromatic regions is often highly repetitive satellite
DNA and transposable elements with very few genes. There are approximately 40
heterochromatic loci found in D. melanogaster, including the tandemly arrayed 18S and 28S
rDNAs, the rolled/ERK-A MAP kinase gene, and some other genes (Weiler and Wakimoto,
1995 and references therein). Although the heterochromatic environment is important for the
expression of some heterochromatic genes, the rearrangement of euchromatic genes close to
the heterochromatin regions often inhibits their expression. A well-known example of this
phenomenon, called position effect variegation (PEV), occurs in Drosophila. When the white
gene is rearranged closely to the centromeric heterochromatin by chromosome breakage and
rejoining, white gene expression is epigenetically turned on or turned off in fly eyes,
suggesting the condensed heterochromatin is an expandable structure that is generally
repressive for transcription (Wallrath, 1998). Conversely, euchromatin is decondensed in interphase, replicated early in S phase, and contains most genes of the genome. The unfolded feature of euchromatin offers a favorable environment for transcription. However, a recent study found some regions of euchromatin can silence a miniwhite reporter gene in both sexes of *D. melanogaster* (Kelly and Kuroda, 2001). In addition, when *roxl*, a non-coding RNA gene involved in the dosage compensation process, was placed adjacent to the silenced miniwhite reporter gene, the reporter gene expression demonstrated a variegated phenotype (see below for dosage compensation). However, whether these euchromatic silencing sites adopt a mechanism similar to that of heterochromatin, and how they are correlated with the interspersed band and interband regions along the length of chromosomes still need further study.

Chromatin is also organized into compartments of distinct structure and function (Lamond and Earnshaw, 1998). In the interphase nuclei, chromosomes are organized into individual territories that are separated by interchromosomal channels (Huang, 1998). Active genes tend to be found at the surface of the chromosomal territories where transcription occurs, and RNA transcripts are released into the channels, processed, and transported out of the nucleus. This model predicts that regulation of the size of the chromosome surface and the dynamic redistribution of active and inactive genes in the territories are important for transcription regulation. Moreover, it seems that the position of chromatin in the nucleus correlates with the timing of DNA replication (Li et al., 1998; Ma et al., 1998; Wei et al., 1998). In cultured Chinese hamster ovary cells, the early replicating DNA (R band) was found dispersed throughout the nuclear interior, whereas the late replicating DNA (G band) and heterochromatic DNA were found close to the nuclear periphery (Ma et al., 1998).
ATP dependent chromatin remodeling machinery

It is proposed that the ground state of transcription in eukaryotic organisms is restrictive because of the organization of histones and DNA into nucleosomes (Kornberg and Lorch, 1999; Struhl, 1999). Electron cryomicroscopy studies offered direct imaging evidence that the nucleosome is a natural barrier to transcription and causes pausing of the RNA polymerase (Bednar et al., 1999). Additionally, the access of protein factors to their DNA binding sites is spatially prohibited by the nucleosome. For example, nucleosomes were shown to prevent the binding of TBP (TATA binding protein) to the TATA element, therefore, the majority of yeast core promoters are not associated with TBP in the absence of functional activators (Kuras and Struhl, 1999).

The organization of DNA into nucleosomes and chromatin raises the question of how the transcription machinery gains access to the DNA template. Genetic and biochemical studies in yeast, D. melanogaster, and human cells have identified a new class of multiple protein complexes that utilize ATP to remodel nucleosome and chromatin structure (Bjorklund et al., 1999; Trivier et al., 1996; Tyler and Kadonaga, 1999; Workman and Kingston, 1998). In D. melanogaster, four different ATP-dependent chromatin remodeling complexes have been identified. The Brahma complex is the fly counterpart of the yeast SWI/SNF complex, which contains more than 7 subunits including the BRM (Brahma) ATPase. The other three complexes, NuRF, ACF, and CHRAC, contain ISWI (imitation of switch) as the ATPase. The NuRF (nucleosome remodeling factor) complex remodels nucleosomes by promoting histone octamer sliding along the DNA (Hamiche et al., 1999; Mizuguchi et al., 1997). The ACF (ATP-utilizing chromatin assembly and remodeling factor) complex can change the internucleosomal spacing and modulate the nucleosomal configuration at the promoter regions. Working together with CAF-1 (chromatin assembly factor -1), the ACF complex can facilitate chromatin assembly in vitro in the presence of
ATP as well (Ito et al., 1997). The CHRAC (chromatin accessibility complex) was found to induce nucleosome sliding without dissociation from DNA (Langst et al., 1999). In general, these complexes promote the accessibility of DNA sites to protein factors, which would otherwise be blocked by the nucleosome. Members of the ATP-dependent chromatin complexes are evolutionary conserved among yeast, D. melanogaster, and human (Bjorklund et al., 1999; Tyler and Kadonaga, 1999).

Mutagenesis studies of D. melanogaster ISWI, the ATPase subunit of the three chromatin-remodeling complexes (NURF, CHRAC, and ACF), revealed ISWI is important for both cell viability and gene expression during development. Mutations in ISWI caused structural changes in the male X chromosome, underscoring the function of ISWI in chromosome remodeling. Colocalization studies indicated the ISWI protein does not colocalize with RNA PolII, suggesting the ISWI protein is not directly involved in transcription (Deuring et al., 2000).

Although chromatin remodeling complexes were originally identified as factors that promote gene activation, more recent studies indicated these complexes are also involved in transcription repression (Tyler and Kadonaga, 1999). A DNA microarray study of global gene expression in yeast revealed the SWI/SNF complex is important for not only gene expression but also gene repression (Holstege et al., 1998). The identification of proteins with chromatin remodeling activity and histone deacetylase in the NuRD (the nucleosome remodeling and deacetylation) complex suggests histone deacetylation, the histone modification related to repression, could be facilitated by nucleosome remodeling as well (Tong et al., 1998). In addition, BRG1 and hBRM, the human homologs of SWI/SNF, can bind to the Retinoblastoma (Rb) protein and inhibit the activity of the transcription activator E2F (Muchardt and Yaniv, 1999). Perhaps whether activation or repression of gene
expression occurs depends on the protein factors that bind to the chromatin when the chromatin remodeling complex opens it up.

**Histone modifications that regulate chromatin structure and function**

It has long been known that certain residues of the histone tails can be modified by phosphorylation, acetylation, methylation, ADP-ribosylation, and/or ubiquitination (Strahl and Allis, 2000). These histone tail modifications were proposed to play a fundamental role in regulating and executing appropriate functions in response to extracellular stimuli (Cheung et al., 2000). Extensive studies in the recent literature demonstrated that the precise regulation of many DNA and chromatin involved processes, such as transcription, replication, recombination, condensation and segregation, depends on the appropriate histone modifications and compatible chromatin environment.

The long and extended N-terminal histone tails are easily accessible to multiple protein factors, making them attractive substrates for modifications and good candidates for mediating protein and chromatin interaction (Luger et al., 1997). Although the function of these histone tails is still elusive, a common view is that histone tails might serve as a signal platform on which diverse enzymes add and erase modifying moieties, and to which multiple protein complexes are recruited and released according to what histone modification or combined histone modifications exist (Strahl and Allis, 2000).

Compared with other kinds of histone modifications, histone acetylation is the most extensively studied (Strahl and Allis, 2000). The finding of HATA1 (histone acetyl transferase A 1) in *Tetrahymena* and its yeast homolog, GCN5, ignited a series of studies of histone acetylation (Brownell et al., 1996). HATA1 was identified using a SDS-polyacrylamide gel electrophoresis based activity assay from *Tetrahymena* macronuclei where hyperacetylated histones are enriched and transcription takes place. *In vivo*, the HAT
activity of GCN5 was required for the expression of various genes in yeast (Kuo et al., 1998). Recent studies found that many components of the transcription complexes, such as the TAF\textsubscript{II}250 of the TFIID and P/CAF (P300/CBP associated factor), possess intrinsic histone acetyl transferase activity, suggesting histone acetylation is closely correlated with transcription (Mizzen et al., 1996; Yang et al., 1996). Using nuclear magnetic resonance (NMR) to study the solution structure of the bromodomain of P/CAF and its binding ligand, including histone tail peptides, the bromodomain was found to bind only to the lysine-acetylated H3 and H4 peptides (Dhalluin et al., 1999). The bromodomain, a conserved sequence of about 100 amino acids, is found in many chromatin associated proteins, including many nuclear histone acetyltransferases (HATs).

The study of histone modification was greatly facilitated by the development of site- and modification-specific antibodies. Using antibodies against histone H4 isoforms acetylated at lysine 5, 8, 12, or 16, a pioneering study in Drosophila showed that acetylated lysine 5 and 8 isoforms are distributed throughout euchromatic arms, whereas the acetyl-lysine 12 isoform is distributed to the \(\beta\)-heterochromatin in the chromocenter. Interestingly, the acetyl-lysine 16 isoform is associated with the transcriptionally hyperactive X chromosomes in male larvae (Turner et al., 1992). This work suggests that different acetylated H4 isoforms have unique and discrete effects on chromatin function. Further studies, applying these acetylation-specific antibodies on mammalian chromosomes, found the inactivated female X is hypoacetylated (Jeppesen and Turner, 1993). Recently, a series of phospho-, acetyl-, and methyl-histone site specific antibodies were developed and have already led to many important discoveries in histone modification studies (see below).

The reversible protein phosphorylation controlled by protein kinases and phosphatases plays a fundamental role in many cascades of signal transduction (Hunter, 2000). Not surprisingly, the phosphorylation of histone H3 at its basic N-terminal tail was
rapidly increased in response to growth factors, phorbol esters, okadiac acid, and protein synthesis inhibitors. The dynamics of histone phosphorylation are correlated with the transcriptional induction of proto-oncogenes, *c-fos* and *c-jun*, suggesting histone phosphorylation might play a role in gene expression as well (Mahadevan et al., 1991; Thomson et al., 1999b). The identification of RSK-2 (ribosomal S6 kinase -2) and MSK1 (mitogen and stress activated kinase 1) and the discovery that these protein kinases are involved in histone H3 Ser10 phosphorylation greatly forwarded the understanding of the histone H3 Ser10 phosphorylation signal transduction pathways that lead to gene expression (Deak et al., 1998; New et al., 1999; Sassone-Corsi et al., 1999). That the JIL-1 kinase is the closest homolog of human MSK1 in *D. melanogaster* makes it intriguing to investigate whether JIL-1 could be involved in the Ser10 phosphorylation pathway. Noteworthy is that RSK-2 mutations are associated with human Coffin-Lowry syndrome (Trivier et al., 1996). The fibroblasts from the Coffin-Lowry syndrome patient or RSK-2 knockout mice failed to phosphorylate H3 Ser10 in response to EGF stimulus as indicated by staining with an antibody that specifically recognizes the phosphorylated H3 at Ser10. The lack of Ser10 phosphorylation in these fibroblasts could be restored by an RSK-2 transgene (Sassone-Corsi et al., 1999), suggesting RSK-2 is required for the EGF induced H3 Ser10 phosphorylation *in vivo*. Moreover, MSK1 can be activated by both mitogen and stress signals and was proposed to mediate H3 Ser10 phosphorylation and hence lead to the expression of immediate early genes *c-Jun* and *c-fos* (Thomson et al., 1999a; Thomson et al., 1999b).

Is there any crosstalk or coordination between the phosphorylation pathway and acetylation pathway if both of them are involved in the regulation of chromatin structure and transcription? Several studies indicated that phosphorylation and acetylation are synergistically regulated during the induction of *c-fos* and *c-jun* gene expression by EGF (Cheung et al., 2000b; Clayton et al., 2000). Using an antibody that is specific to H3
phosphorylated at Ser-10 and acetylated at Lys-14, ChIP (chromatin immunoprecipitation) experiments showed that the phophoacetyl-H3 isoform was associated with the c-jun and c-fos promoters in an EGF stimulation dependent manner. In addition, the H3 N-terminal peptide phosphorylated at Ser10 is a better substrate for the histone acetyltransferase GCN5 than the unphosphorylated peptide, suggesting the synergistic coupling of phosphorylation and acetylation during transcription (Cheung et al., 2000b).

However, phosphorylation of Ser10 was also correlated with mitotic chromosome condensation (Wei et al., 1998). An S10A mutation of the H3 gene in Tetrahymena caused segregation defects during mitosis, demonstrating the importance of Ser10 phosphorylation for appropriate mitotic chromosome behavior (Wei et al., 1999). Recent studies identified Ipl1/aurora kinase as the mitotic histone H3 Ser10 kinase and Glc7/PP1 as the phosphatase catalyzing the reverse reaction to maintain the balance of Ser10 phosphorylation (Hsu et al., 2000). Analysis of the D. melanogaster Aurora B kinase by a RNAi approach in Schneider 2 (S2) cells demonstrated that mitotic Ser10 phosphorylation is controlled by the Aurora B kinase as well (Giet and Glover, 2001).

How could Ser10 phosphorylation have multiple personalities, i.e., how could the same modification be involved in both gene transcription and mitotic condensation? One hypothesis is that H3 phosphorylation actually opens up chromatin to enable the access of condensation machinery during mitosis, and transcription machinery during gene expression (Cheung et al., 2000a), therefore the pathway in action is determined by the protein factors that bind to the modified histones. Another attractive theory is the combined "histone code" hypothesis (Strahl and Allis, 2000). For example, the combination of Ser10 phosphorylation and acetylation at adjacent lysine residues (K9 or K14) might serve as a signal for transcription, while phosphorylation at Ser10 and Ser28 together might dictate condensation (Goto et al., 1999). Both of these proposed models need to be tested in future studies.
Nevertheless, the extensive study of H3 Ser10 phosphorylation has supported the fundamental role of this modification in the regulation of chromatin structure and function. Intrigued by this basic concept, we investigated the possible role of JIL-1 in the Ser10 phosphorylation pathway. Our data support that JIL-1 regulates Ser10 phosphorylation at interphase in *Drosophila* (Wang et al., 2001).

As mentioned above, in addition to acetylation and phosphorylation, histone tails can also be modified by methylation, ubiquitination, and ADP-ribosylation. A recent study reported the SET domain containing proteins, human Suv39H1 and mouse Suv39h1, possess histone methyltransferase (HMT) activity and preferentially methylate lys9 of histone H3 (Rea et al., 2000). Suv39H1 and Suv39h1 are homologs of *D. melanogaster* Sur(var)3-9 and *S. pombe* clr4. The catalytic region of these proteins was mapped to the SET domain and adjacent cysteine rich regions. To better understand the relationship of phosphorylation, acetylation, and methylation, *in-vitro* peptide kinase assays with Ipl1/aurora were carried out (Rea et al., 2000). These experiments showed that Lys9-acetylated peptide and Lys14-acetylated peptide can serve as better substrates than unmodified peptide for the Ipl1/aurora kinase, while Lys9 dimethylated peptide was poorly phosphorylated. Conversely, the Ser10 phosphorylated peptide cannot be methylated by Suv39h1. These data suggest that Lys9 methylation and Ser10 phosphorylation are antagonistically regulated, while acetylation and phosphorylation are synergistically regulated. *In vivo*, *Suv39h1* null cells exhibit a defect in chromosome condensation and segregation, which was also observed in the *Su(var)3-9* mutant in *D. melanogaster* and the *clr4* mutant in *S. pombe*, suggesting that the HMT function is important for mitotic chromosome behavior. In addition, Ser10 phosphorylation level in *Suv39h1* null fibroblast cells is elevated, supporting the hypothesis that Lys9 methylation impairs Ser10 phosphorylation *in vivo* (Rea et al., 2000). Another study found that HP1 (heterochromatin protein 1), an important protein involved in the maintenance of
heterochromatin structure and PEV regulation, specifically binds to H3 methylated at Lys9 (Lachner et al., 2001). The domain that recognizes methylated K9 was mapped to the chromodomain of HP1. In vivo, heterochromatin HP1 binding is lost in the Suv39h1 mutant fibroblast cells. The notion that Suv39h1 functions as an HMT (histone methyltransferase) and creates binding sites for HP1 in heterochromatic regions was confirmed by the study of clr4 and swi6, the homologs of Suv39h1 and HP1 respectively, in *S. pombe* (Nakayama et al., 2001).

With the importance of histone modification in chromatin structure and function being more and more appreciated, we can expect the coming of more thorough studies of how histone modifications are involved in development, signal transduction, and human disease.

**The role of chromatin modifying activities in signal transduction pathways**

As discussed above, the involvement of RSK-2 and MSK1 in H3 Ser10 phosphorylation plays an important role in *c-fos* and *c-jun* gene expression. The fact that these two enzymes can be activated by the MAP kinase pathway suggests a pathway whereby an extracellular stimulus, such as EGF, activates the MAP kinase pathway, leading to the activation of RSK-2 and MSK1, which in turn leads to histone modification and the elicitation of a specific cellular response (Deak, et al., 1998; Pierrat et al., 1998).

Another piece of evidence linking histone modification to signal transduction comes from the study of how Retinoblastoma (Rb) protein inhibits E2F mediated gene expression. The Rb protein regulates cell cycle progression by binding the E2F transcriptional activator. This silences specific genes that require E2F as an activator and are required for entering the S phase of the cell cycle. Until recently, the mechanism of how Rb silences E2F dependent genes was poorly understood. The finding that HDAC1 (histone deacetylase 1) and Rb
interact with each other provided a breakthrough in the field (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). As the Rb protein is recruited to the promoters of E2F dependent genes by interacting with the transcriptional activation domain of E2F, it brings HDAC1 with it, resulting in deacetylation of histones and producing a chromatin environment unfavorable for gene expression. Furthermore, if histone deacetylation is inhibited by the histone deacetylase inhibitor TSA (trichostatin A), Rb mediated transcription repression is abolished. These studies support a model whereby histone deacetylase works together with the Rb protein to control cell cycle progression.

Other studies have disclosed how chromatin modifying complexes, such as the ATP-dependent chromatin remodeling complexes and the histone acetyltransferase complexes, are orchestrated to work together with other general transcription machinery during gene expression. The induction of IFN-β gene expression by viral infection is a good example. The IFN-β enhanceosome, containing multiple transcription factors, is first recruited to the promoter to the IFN-β promoter after viral infection. Histone acetylase GCN5 complex is then recruited to acetylate histone proteins. After histone acetylation, the CBP-PolII holoenzyme complex is bound, followed by the binding of the SWI/SNF chromatin remodeling complex. Finally, the joining of TFIID to the promoter leads to transcription (Agalioti et al., 2000).

Another good example of coordinated action of chromatin modifying activities and general transcription machinery is the developmental and cell cycle specific control of HO endonuclease gene expression in yeast (Cosma et al., 1999). The HO endonuclease mediates the α to a (or a to α) mating type switch. The expression of HO is restricted to the “mother cell” at the late G1 phase of the cell cycle. Therefore, the mating type can only be switched in the mother cell. The recruitment of transcription factors, SWI/SNF chromatin remodeling complex, and SAGA histone acetyltransferase complex, takes place in an orderly way. The
transcription factor SWI5p is bound to the promoter first, followed by the SWI/SNF chromatin remodeling complex. Later, the SAGA acetylation complex is recruited to the promoter. At last, these factors are joined by the transcription factor SBF and PolII to activate transcription of the HO gene.

**Dosage compensation in *Drosophila melanogaster***

In many species, females have two X chromosomes and males have only one X. The difference in X chromosome number is sometimes important for sex determination during early development. However, many of the X-chromosome encoded proteins are equally required in both sexes, therefore, an equity of X chromosome gene expression must be achieved (Kelley and Kuroda, 1995; Kelley and Kuroda, 2000; Lucchesi, 1996). To reach this equity, distinct mechanisms are used in different species (Fig 5A). In mammals, one of the two X chromosomes in females is painted by the *Xist* RNA, highly condensed to form heterochromatin-like architecture in interphase, and transcriptionally inactive (Kelley and Kuroda, 1995). The random selection of either the maternal or the paternal X chromosome for silencing during early development is controlled by the subtle balance of *Xist* and *Tsix* expression from opposite directions at the same locus (Lee et al., 1997).

In *C. elegans*, the two X chromosomes in the cells of hermaphrodites are specifically bound by the *dpy* (or *dumpy*) gene products and the expression of each of the two X chromosomes is reduced to half of that of the male X chromosome. One of these DPY proteins, DPY-27, shares high homology with the SMC (structural maintenance of chromosomes) family of proteins that are components of the condensation complex in *Xenopus*, suggesting that higher order chromatin structure might be modified to allow transcription to occur at a reduced level (Chuang et al., 1994; Hirano, 1995; Hirano et al., 1995).
Fig 5. Mechanisms of dosage compensation. (A) Different mechanisms have evolved to reach dosage compensation. In mammals, transcription from one of the female X chromosome is mostly shut down by condensing the chromosome to form a heterochromatin-like structure during interphase. In C. elegans, the expression of both hermaphrodite X chromosomes is reduced to roughly half of that of the male single X chromosome to achieve an equality. In D. melanogaster, equity is reached by doubling the gene expression from the single male X chromosome. (B) The formation and assembly of the MSL complex is restricted to the male cells. In female cells, the SXL protein blocks the translation of msl-2 mRNA, therefore, a stable MSL complex is not formed. In males, there is no translation inhibitor, SXL, thus the MSL2 protein is produced. Our current model is that the MSL-2 protein is assembled with MSL-1, -3, MOF, MLE, JIL-1 and two non-encoding RNAs, roxl and rox2, to form a 2MD complex that is specifically associated with the male X chromosome to regulate histone modifications, such as H4 lys16 acetylation, H3 Ser10 phosphorylation and lys14 acetylation, and chromosome structure in order to reach dosage compensation.
Mechanism of Dosage compensation

### Mammals
- $X_X = 1$

### C. elegans
- $XX = \frac{1}{2} + \frac{1}{2} = 1$

### Drosophila
- $XX = 1 + 1 = 2$

#### Hypotranscription
- Female

- msl-2 (mRNA)
- Sxl

#### Hypertranscription
- Male

- Compensation complex
- rox1
- MLE
- MSL2
- MSL3
- JIL1
- MSL1
- MOF

- H4K16
- H3S10
- H3S10-PK14

- Dosage compensation
In *D. melanogaster*, the male X chromosome is decondensed due to the function of a group of genes, collectively called the msl (male specific lethal) genes. Expression of genes on the male X chromosome is doubled to reach an equivalent level with that of the two female X chromosomes. Genetic studies have identified five genes that are required for the viability of males. The proteins encoded by these genes, Male specific lethal-1, -2, -3, Maleless, and Male-absent on the first (MOF), form a 2MD protein complex, termed the MSL dosage compensation complex, which binds to hundred of bands on the male polytene X chromosomes in salivary gland nuclei. There are an extensive number of publications concerning the molecular characterization and functional studies of each of these proteins (Maleless or MLE (Gorman et al., 1993; Kuroda et al., 1991; Lee et al., 1997); MSL-1 (Palmer et al., 1993; Scott et al., 2000); MSL-2 (Copps et al., 1998; Kelley et al., 1995); MSL-3 (Gorman et al., 1995); MOF (Akhtar and Becker, 2000; Hilfiker et al., 1997; Smith et al., 2000)). By studying genes highly expressed in the nervous system, two non-encoding RNA genes, *roxl* and *rox2*, were identified whose RNAs are specifically associated with the male X chromosome and are components of the dosage compensation complex (Franke and Baker, 1999; Meller et al., 1997; Amerin and Axel, 1997). The *roxl* and *rox2* genes themselves are located on the X chromosome and are two of the approximate 35 entry sites for the binding of the MSL complex. Interestingly, the *roxl* gene inserted to autosomal sites can still nucleate and induce extensive spreading of the MSL complex on the autosomes in a certain range from the insertion site. This raised a plausible model to explain how the MSL complex binds to the male X chromosome. After transcription, the *rox* RNAs are only able to diffuse locally and form some nucleation sites for the MSL dosage compensation complex on the male X. Therefore, the MSL complex is spread a certain distance from the *roxl* and *rox2* transcription sites (Kelley et al., 1999). Are there more *rox*-like RNAs? Only time will tell.
There is quite a collection of functional protein domains represented in the MSL complex. The MLE protein has high similarity with RNA helicase and possesses RNA binding motifs. MSL1 has an acidic region at its N-terminus, which is a characteristic of transcription activation domains. The MSL3 protein has two chromodomains that are often found in protein factors responsible for transcriptional repression. MSL2 has a zinc-binding ring finger domain that is also found in proteins that maintain repressive chromatin structure. MOF encodes a chromodomain containing histone acetyltransferase of the MYST family, which is likely to be the acetylase responsible for the acetylation of H4 at the Lys16 site (Bone et al., 1994; Smith et al., 2000). The finding that the DNA binding domain of Gal4 fused with MOF could activate the expression of a reporter gene with GAL4 binding sites in its promoter in yeast directly placed MOF in a pathway that leads to transcription (Akhtar and Becker, 2000). The ability of MOF to activate transcription in this test is dependent on its active HAT domain, suggesting H4 Lys16 acetylation is a signature for gene expression and is probably a positive code for dosage compensation as well. However, the two-fold increase of gene expression on the male X must be regulated precisely. Therefore, the finding of both repression and activation domains in the dosage compensation complex should not be a surprise (Lucchesi, 1996).

How can MSL complex function be restricted to the males? The translational regulation of MSL2 by Sex lethal (SXL) might be the key (Kelley et al., 1997). SXL is a female specific RNA binding protein that regulates alternative RNA splicing in the sex determination cascade. In the case of MSL-2, however, the binding of SXL to the msl-2 RNA inhibits translation instead of causing alternative splicing. Because of the presence of SXL in females, the MSL2 protein is not made and the dosage compensation complex fails to form and to bind to the X chromosome. Conversely, because there is no SXL in males, the MSL2 protein is produced and the MSL complex is formed on the male X chromosome (Fig 5B).
The study of JIL-1 in our lab suggests that JIL-1 is involved in the dosage compensation process. The JIL-1 protein level is elevated on the male X chromosome, while it remains evenly distributed on the X chromosome and autosomes in females (Jin et al., 1999). That the distribution of JIL-1 on the male X overlaps with that of the MSL proteins and JIL-1 could be coimmunoprecipitated with MSL proteins suggest JIL-1 can associate with the MSL complex (Jin et al., 2000; Kelley and Kuroda, 2000). That the JIL-1 immunocomplex can phosphorylate the Ser10 site in a synthetic H3 N-terminal peptide in vitro (unpublished data from Y. Wang) and the level of Ser10 phosphorylation is decreased in JIL-1 mutants suggest that JIL-1 is involved in the H3 Ser10 phosphorylation pathway. Furthermore, the levels of both H3 Ser10 phosphorylation and H3 double modification (Ser10 phosphorylation and Lys14 acetylation) are elevated on the male X chromosome, which was detected by antibody staining. In addition, the pattern of modified H3 isoform staining colocalizes with that of the JIL-1 staining. Our data suggest Ser10 phosphorylation and Lys14 acetylation on H3 could coexist with the H4 Lys16 acetylation as the histone code for dosage compensation (Wang et al., 2001).
CHAPTER 3. MATERIALS AND METHODS

Fly stocks

All of the fly stocks were maintained according to standard methods in Roberts, 1986. The mutant phenotypes not discussed here can be found in Lindsley and Zimm, 1992 and on fly base (http://www.flybase.org). Oregon R flies were used as wild type in the studies described in this dissertation. The EP(3)3657/TM6B Tb stock was provided by Dr. Todd Laverty at the Berkeley Drosophila Genome Project. The w; Δ2-3 Sb/TM2 Ubx stock, used as a transposase source for imprecise P-element excision, was a generous gift of Dr. Linda Ambrosio. The GFP-JIL-1 transgenic lines were previously described in Jin et al., 1999. Flies were maintained at room temperature, however, to improve polytene chromosome squashes, larvae were grown at 18°C.

Antibodies

The antibodies used include:

JIL-1 antibodies:

ODIN (a rabbit polyclonal antibody made by our laboratory)
Hope (an affinity purified rabbit polyclonal antibody made by our laboratory)
1008/1007 (purified chicken polyclonal IgY antibody)
V5 mAb (a mouse monoclonal antibody against a V5 epitope from Invitrogen Inc.)

MSL antibodies: (generous gifts from Dr. Kuroda and Dr. Kelley)

MSL1 antibody (affinity purified rabbit polyclonal antibody against MSL1)
MSL2 antibody (affinity purified rabbit polyclonal antibody against MSL2)
MSL3 antibody (affinity purified goat polyclonal antibody against MSL3)
Modified histone antibodies:

H4Ac16 antibody (affinity purified rabbit polyclonal antibody against the H4 isoform acetylated at K16 available from Serotec Inc.)

pH3S10 antibody (affinity purified rabbit polyclonal antibody against the H3 isoform phosphorylated at S10 sites available from Upstate Biotechnology Inc. or Cell Signaling Technology Inc.)

pH3S10Ac14 antibody (affinity purified rabbit polyclonal antibody against the double modified H3 by phosphorylation at S10 and acetylation at K14 available from Upstate Biotechnology Inc.)

Other antibodies:

Lamin Dm0 antibody R836 (rabbit polyclonal antibody against Dm0, a generous gift of Dr. Fisher)

Lamin Dm0 mAb T40 (a mAb against Dm0, a generous gift of Dr. Paddy and Dr. Saumweber)

Tubulin mAb (a mAb against α-tubulin available from Sigma)

7F1 mAb (a mAb made in our laboratory using GST-J1 (the C-terminal region of SW59) as antigen)

G39 mAb (a mAb against leech Gliarin made by our laboratory (Xu et al., 1999))

Histone H3 antibody (a goat polyclonal antibody against the C-terminal sequence of histone H3 available from Santa Cruz Biotech.)

**PCR and primers**

Primers were designed using the Oligo 5 program and synthesized by the DNA Sequencing and Synthesis Facility at Iowa State University. Primers used were listed in Appendix A. The PCR reaction was done following the instruction in Sambrook and Russel,
A typical 50ul PCR reaction included 5ul of 10xPCR buffer, 8ul of 1.25mM dNTPs, 3ul of each primer (5pmol/ul), 2.5 units of Taq DNA polymerase, appropriate amount of DNA template, and H$_2$O. In order to sequence the products, PCR amplified fragments were sometimes cloned into the pGem-T vector (Promega Inc.).

**Peptides**

Two peptides, H3N25 (ARTKQTARKSTGGKAPRKQLATKAA) and H3N25S10A (ARTKQTARKATGGKAPRKQLATKAA) were synthesized by the Protein Facility at Iowa State University. H3N25 is the H3 N-terminal 25 residues, while H3N25S10A has an S10 to A10 substitution. These two peptides were used in *in vitro* immunocomplex kinase assays.

**Molecular cloning**

Many molecular methods not described in this chapter can be found in Sambrook and Russel (2000) or in a Protocols and Applications Guide from Promega Inc. Plasmid preparation was done using the Wizard minipreps kit (Promega). DNA fragment gel recovery was done following the GeneClean kit protocol (Midwest Scientific Inc).

**GST fusion protein expression in E. coli and purification**

cDNA fragments or PCR products of interest were cloned in frame into the pGEX4T vectors (Amersham Pharmacia Biotech). The cloned fragment was sequenced to confirm the correct reading frame and sequence. To express GST fusion proteins, *E. coli* strain BL21 was often used to produce more full length fusion proteins. Cells with the cloned insert were grown to OD$_{600}$ of about 1.0, then the inducer IPTG was added to a concentration of 0.1mM. After induction of 4-6 hours, cells were harvested and sonicated in PBS supplemented with protease inhibitors (aprotinin, 1.5ug/ml, PMSF, 1mM). Cell lysate was clarified by
centrifugation at 13,000 rpm for 12 min at 4°C. Total protein solution was incubated with glutathione agarose beads for 4-6 hours with continuous mixing at 4°C. Bound protein was washed with 20 times bead volume of ice cold PBS supplemented with protease inhibitors. Protein retained on the beads can be used as affinity matrix for pull down experiment or can be eluted with glutathione solution to purify fusion protein. Many GST fusion protein constructs made were described in the results and discussion (Chapter 4). A list of constructs can be found in Appendix B.

**Antibody generation**

The purified GST fusion protein, GST-J1, was injected into BALB/c mice at 21 day intervals to generate mAbs. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and monospecific hybridoma lines were established and used to produce antibody using standard procedures (Harlow and Lane, 1988). This work was carried out by the Hybridoma Facility at Iowa State University.

**S2 cell culture and gene expression in S2 cells**

The *Drosophila* Schneider 2 (S2) cell line was grown in the Shields and Sang M3 insect medium (Sigma) supplemented with L-glutamine and antibiotics. S2 cell transfection was carried out using a calcium and phosphate method described in the instruction manual (*Drosophila* Expression System, Invitrogen). The establishment of stably transfected cell lines and protein expression were done following the instruction manual. Total S2 cell lysate was prepared in IP buffer (20mM Tris.HCl, 10mM EDTA, 1.0mM EGTA, 150mM NaCl, 0.2% Triton X-100, 0.2% NP-40, 2mM Na3VO4, 1mM PMSF, and 1.5ug/ml aprotinin, pH 8.0). Cell lysate was prepared from healthy and rapidly growing S2 cells. S2 cells were collected by spinning for 3-5min at 3000rpm, resuspended in IP buffer to a final concentration of 1-
2x10^7 cells/ml. Cells were broken to dissolve proteins by sonication 2-3 times, 30 seconds each, with 1 min interval. The insoluble cell debris was removed by spinning for 15min at 12,000rpm at 4°C. Cell lysate could be stored at -80°C for months.

**In vitro immunocomplex kinase assays**

Buffers used for in vitro immunocomplex kinase assays include IP buffer (20mM Tris.HCl, 10mM EDTA, 1.0mM EGTA, 150mM NaCl, 0.2% Triton X-100, 0.2% NP-40, 2mM Na_3VO_4, 1mM PMSF, and 1.5ug/ml aprotinin, pH 8.0) and kinase assay buffer (20mM Hepes, pH7.4, 10mM MgCl_2, 2mM Na_3VO_4, 1mM DTT). S2 cell lysate was prepared as described above. Total histone proteins or purified histone H3 was purchased from Boehringer-Mannheim. The H3N25 and H3N25S10A peptides were described above. If the cell lysate has been stored at -80°C for some time, some proteins may become insoluble. Lyaste was centrifuged to remove any insoluble materials before using. Three tubes with 1ml IP buffer and 5ul of 50% protein-G beads slurry into each were prepared. The protein-G beads were quickly washed three times with 1ml IP buffer to remove ethanol in which the beads were preserved. 300ul IP buffer was added to each tube and 10ul ODIN anti-serum or 10ul preimmune rabbit serum were added to two tubes separately to pre-bind the antibody to the beads. The third tube was used as a control for mock IP. In order to pre-clear the S2 cell lysate, 100ul of 50% protein-G beads slurry was washed three times with IP buffer and combined with 600ul of S2 lysate, which should contain about 6x10^6 cells. Pre-clearing and pre-binding were continued for about 4 hours at 4°C with continuous mixing. The pre-bound antibodies on protein-G beads were washed three times quickly with 1ml of IP buffer to remove unbound antibody. The pre-cleared lysate was transferred to a new tube after spinning and brought up to a total volume of 900ul with IP buffer. 300ul of the precleared S2 lysate, which had about 2x10^6 cells, was combined with ODIN protein-G beads, preimmune
protein-G beads, or protein-G beads only. The immunoprecipitation was continued for another 4 hours with continuous mixing. After IP, the immunocomplex was washed 5 times with 1ml IP buffer for 10min each, and three times with 1ml kinase assay buffer for 10 min each. After extensive washing, the immunocomplex was divided into two tubes. In some experiments, one aliquot was used for western blots and the other was used for kinase reactions. In the peptide kinase assays, one aliquot was used for H3N25 peptide and the other was used for H3N25S10A peptide. Kinase assays were performed as follows. The kinase assay buffer was removed after washing. 24ul of kinase buffer was added to each tube, together with a substrate, 3ul (12ug) of H3N25 peptide, 3ul H3N25S10A (12ug) peptide, or 5ul (25ug) of histone H3, and 3ul γ-32P-ATP (10uCi/ul). The reactions were carried out for 30min with mixing at room temperature. After the reactions were finished, the reaction mixture was applied to P81 paper, dried briefly, and then washed three times with a large amount of 0.6% phosphoric acid for 5 min each and 1 time with acetone for 3 min. Paper squares were put into 5ml of scintillation buffer and the amount of incorporated 32P was determined by scintillation counting. If histone H3 was used as a substrate, proteins in the reaction were separated on SDS-PAGE. The gel was stained, dried, and exposed to X-ray film to detect phosphorylation signal.

Co-immunoprecipitation (co-IP)

For co-IP, an appropriate amount of antibody of interest was coupled to 5ul of protein G sepharose beads (Sigma, capacity, 1.5ug IgG/ul of beads) for 4 hours at 4°C on a rotating wheel in 250ul of IP buffer (20mM Tris.HCl, 10mM EDTA, 1.0mM EGTA, 150mM NaCl, 0.2% Triton X-100, 0.2% NP-40, 2mM Na3VO4, 1mM PMSF, and 1.5ug/ml aprotinin, pH 8.0). If the amount of antibody used was over the binding capacity of the protein G beads, excess antibody was washed away with IP buffer. S2 cell lysate (about 2-3x10^6 cells in 200ul
IP buffer for each IP) prepared in IP buffer was pre-cleared by incubating with 5μl normal sera of appropriate species and 20μl protein G beads for 2 hours at 4°C. Precleared cell lysate and protein-G beads pre-loaded with antibodies were combined and incubated overnight at 4°C with continuous mixing. After immunoprecipitation, the protein G beads were washed 3 times 10 min with 1ml of IP buffer. The immunocomplex was analyzed by SDS-PAGE and western blot. Signals on western blots were detected with ECL reagents (Amersham-Pharmacia).

**In-vitro GST pull down assays**

For *in-vitro* protein-protein interaction assays, about 0.5-1μg of GST or a GST-fusion protein of interest expressed in *E. coli* were coupled with glutathione agarose beads. The GST protein was often used as a negative control. S2 cell lysate was pre-cleared by incubating with glutathione agarose beads for 4 hours at 4°C with continuous mixing. The coupled beads were incubated with 300μl of pre-cleared S2 cell lysate (containing about 1x10⁶ cells) at 4°C for 6 hours on a rotating wheel. Then the beads were washed 4 times of 10 min with 1ml of IP buffer and proteins retained on the beads were analyzed with SDS-PAGE and western blots. Signals on western blots were detected with ECL reagents (Amersham-Pharmacia).

**Immunostaining**

Polytene chromosome staining was performed following the protocol from Dr. Kuroda with slight modifications as follows. The most photogenic polytene chromosomes were produced with larvae grown in uncrowded vials at 18°C, which were periodically given modest supplements of yeast and water. The best chromosomes were obtained from the first wave of larvae to climb up the side of the vial. The salivary glands were dissected under a
dissecting microscope in PBS. The dissected salivary glands were fixed in formaldehyde and 50% acid as follows. First, 4 ml of 5x formaldehyde (0.74g paraformaldehyde in 4 ml of H₂O with 28ul of 1N KOH) was made by warming the solution at 65°C to dissolve paraformaldehyde. Dissected salivary glands were first fixed in Fix 1 solution (1xPBS, 1% Triton X-100, 1xformaldehyde in H₂O) for about 45 seconds, then fixed in Fix 2 solution (50% glacial acetic acid, 1xformaldehyde in H₂O) for about 2 min. After the above fixation, salivary glands were transferred to lactoacetic solution (16% lactic acid, 50% acetic acid in H₂O). About 15ul of lactoacetic solution was transferred together with the fixed salivary gland to a siliconized cover slip. The cover slip with the salivary gland was picked up using a clean (or a polylysine treated) slide. The polytene chromosomes were gently spread by gently tapping with the eraser end of a pencil and carefully squashed between several layers of lens paper. The slides were frozen in liquid nitrogen, allowed to warm up for 3-5 seconds, and the coverslip was removed with a clean razor blade, and immediately placed into ethanol to dehydrate for 1 hour at room temperature. The slides were rehydrated in PBT (PBS with 0.2% Triton X-100), 2 times 30 min. After this, the slides were blocked in blocking solution (usually 5% NGS (normal goat serum) in PBT) for 2-3 hours at 4°C. Primary antibodies were appropriately diluted according to their titer in blocking solution and incubated with the slides overnight at 4°C. After washing for 3 times 10 min, the slides were stained with appropriate secondary fluorescence coupled antibodies diluted in the blocking solution for 2 hours at room temperature in dark. The slides were washed, stained with Hoechst (0.2ug/ml in PBS) for several minutes and washed in PBS for several minutes, then mounted and observed under a fluorescent microscope. Fluorescence signal was collected using the Scion image program and imported to the Adobe Photoshop program in which the image was processed and pseudocolored.
Embryo staining was performed essentially as previously described (Walker et al., 2000). Embryos (0-5 hour) were dechorionated in a 50% Chlorox solution for about 2 min, washed with 0.7M NaCl/0.2% Triton X-100, and fixed in a 1:1 heptane/fixative mixture for 20 min with vigorous shaking at room temperature. The fixative was either 4% paraformaldehyde in PBS or Bouin’s fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid). Vitelline membranes were removed by shaking embryos in heptane/methanol at room temperature for 30 seconds. Rehydration and antibody staining was done as described above for polytene chromosome staining.

Whole mount salivary gland nuclei staining was performed by fixing the nuclei with paraformaldehyde in PBS. Briefly, salivary glands were dissected in PBS. About 5 pairs of salivary glands were placed in the center of a slide along with 50ul of PBS. The salivary glands were gently compressed under a cover slip by sliding the cover slip back and forth several times. The cover slip was then removed and 50ul of the Fix 1 solution (1x PBS, 1% Triton X-100, 1x formaldehyde in H2O) was added and covered by a piece of parafilm. The tissue was fixed at 4°C for 20 min and brought to room temperature for 5 min, then washed in PBT (PBS with 0.2% of Triton X-100) 3 times, 10 min each. The slides were blocked, stained, and observed as described above for polytene chromosomes.

**Western blot analysis**

Western blots were carried out essentially as described in Harlow and Lane, 1988. Briefly, protein samples of S2 cell lysate or total larvae extract were prepared in IP buffer. Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, incubated with a specific antibody using appropriate dilution overnight at 4°C. The secondary antibody was either an HRP-labeled anti-rabbit IgG or an HRP-labeled anti-mouse IgG (Bio-Rad). The signals were detected with the ECL kit (Amersham Pharmacia).
Fly viability assays and rescue experiments

To assay the embryo hatching rate, embryos produced by wild type (Oregon R) or homozygous EP(3)3657 parents were collected on apple juice agar plates. The hatching rate was scored and recorded 24 and 48 hours after egg collection under a dissection microscope. The hatching rate was calculated as the percentage of the embryos hatched out to become larvae. The pupae or larvae of homozygous mutants can be distinguished from their heterozygous siblings by not carrying the tubby marker. The larvae or pupae carrying a tubby marker are short and fat, while those without the tubby marker are slim and long. Thus, the non-tubby pupae, which are the JIL-1 homozygous mutants, were collected into separate vials to observe eclosion rate. The number of flies hatched from pupae was recorded every day until no more flies emerged. The eclosion rate was calculated as the percentage of number of hatched flies out of the number of total pupae collected. To perform rescue experiments, a GFP-JIL-1 (GF29.1, carrying a P[hs83-GFP-JIL-1, w+] transgene on the second chromosome was used to cross with JIL-1 mutants (Jin et al., 1999). The hatch rate of embryos and eclosion rate of pupae of JIL-1 mutants carrying this GFP-JIL-1 transgene were observed. To compare the hatch rates and eclosion rates of the wild type, mutants, and rescued mutants statistically, a $\chi^2$ test was used.

Yeast two hybrid

Yeast two-hybrid analysis is a powerful tool to study protein-protein interaction. In yeast two-hybrid screening, a protein (or a part of a protein) of interest was fused with the Gal4-BD (DNA binding domain) and transformed into yeast strains with inducible reporter genes containing the Gal4 binding sites. If another protein fused with the Gal4-AD (activation domain) can interact with the protein of interest and was co-transformed into the
yeast cells, the Gal4-AD was brought to the promoter of reporter genes via protein-protein interaction. This would lead to the expression of reporter genes.

In order to search for proteins that can interact with JIL-1, four JIL-1 two hybrid constructs were made, including BD-NTD (N-terminal domain), BD-KDI (kinase domain I), BD-KDII (kinase domain II), and BD-CTD (C-terminal domain). The BD-CTD was used to screen a Drosophila 0-2 hour early embryonic yeast two-hybrid library (a generous gift from Dr. Ambrosio) following the protocol from Stratagene. The BD-KDI was used to screen a Drosophila 0-18 hour yeast two-hybrid library following the protocol from Clontech. The candidate interacting proteins identified from two-hybrid screens were further tested using other protein-protein interaction methods.

Bioinformatics

Homolog sequence searching was performed using the BLAST program (Altschul et al., 1990) at NCBI (National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov). The GeneSeqer program (Usuka et al., 2000) was used to search for EST sequences from a certain genomic region (http://deepc2.zool.iastate.edu/cgi-bin/gs.cgi). The homologous sequence alignments using the Clustalw program (Thompson et al., 1994) were performed at http://www2.ebi.ac.uk/clustalw. The analysis of the genomic sequence of lola for zinc finger containing regions was performed in the Microsoft word'98 program using the “find” function to search consensus sequences, CXXC, HXXXXC, and HXXXXH ('X' can be any amino acid). Fly genome information can be accessed from the flybase website (http://www.flybase.org).
CHAPTER 4. RESULTS AND DISCUSSION

The JIL-1 immunocomplex can phosphorylate histone H3 \textit{in vitro}

The JIL-1 kinase belongs to the MSK subfamily of AGC kinase family which includes the cyclic nucleotide-dependent kinases (PKA and PKG), and diacylglycerol dependent protein kinase C (PKC). This family of kinase preferentially phosphorylates Ser/Thr sites surrounded by basic residues (Morrison, et al., 2000). Because JIL-1 is associated with chromosomes (Fig 2 and 3 in Chapter 1, and Jin et al., 1999), it likely has access to histones whose tails have many Ser/Thr surrounded by basic residues. Therefore, histone proteins were tested as possible substrates for the JIL-1 immunocomplex immunoprecipitated from Schneider 2 cell lysate. In \textit{in vitro} kinase assays, histone H3 was phosphorylated by the JIL-1 immunocomplex but not by the immunocomplex of PIRS (pre-immune rabbit serum), suggesting that histone H3 was a putative substrate of the JIL-1 pathway (Fig 6B). In addition, a protein with a molecular weight close to the size of JIL-1 in the JIL-1 immunocomplex was phosphorylated in \textit{in vitro} kinase assays, suggesting JIL-1 may possess autophosphorylation activity (Fig 6A). These data led to a proposal of histone H3 as a substrate candidate for the JIL-1 immunocomplex and possibly of the JIL-1 kinase itself (Jin et al., 1999).

Studies of MSK1 and Rsk-2, two close homologs of JIL-1, showed that these two kinases could phosphorylate histone H3 (Thomson et al., 1999; Sassone-Corsi et al., 1999). Moreover, MSK1 can phosphorylate the Ser10 site of H3 in \textit{in vitro} immunocomplex kinase assays (Thomson et al., 1999). Recent evidence indicated that Rsk-2 was required for EGF stimulated H3 Ser10 phosphorylation \textit{in vivo} (Trivier et al., 1996; Sassone-Corsi et al., 1999). In fibroblast cells from Rsk-2 mutant mice, H3 Ser10 phosphorylation was not induced after EGF stimulation, but was restored by the introduction of a Rsk-2 transgene. These data
Fig 6. Kinase assays with JIL-1 immunocomplex. (A) JIL-1 putatively has autophosphorylation activity. The JIL-1 complex was immunoprecipitated by a JIL-1 polyclonal antibody, ODIN. After kinase reactions, part of the product was applied for a western blot to detect the JIL-1 protein; part of the product was separated by SDS-PAGE, stained with Coomassie Blue, dried and exposed to X-ray film to detect the phosphorylation signal. There was a phosphorylated band on the X-ray film of the size corresponding to that of JIL-1 detected on western blot. The arrow indicates the heavy chain of rabbit IgG that was detected on western blot. (B) The JIL-1 immunocomplex phosphorylated H3 strongly (right lane) but the immunocomplex of PIRS did not (left lane), which was shown in the upper panel where the phosphorylated substrates were separated by SDS-PAGE and detected with an X-ray film. The lower panel indicated that similar amount of H3 protein was used in both reactions as shown by Coomassie Blue staining of the same SDS-PAGE gel exposed the X-ray film. (C) The JIL-1 immunocomplex phosphorylated the Ser10 site of a synthetic peptide, H3N25. The peptide sequences of H3N25 and H3N25S10A were shown with an arrow indicating the S10A substitution. The H3N25 peptide was efficiently phosphorylated by the JIL-1 immunocomplex, showing a signal of incorporated $^{32}$P of about 40000±11100 (lane 1), while the signal of incorporated $^{32}$P on H3N25S10A peptide was decreased more than six fold to only 6300±800 (lane 3). In the negative control group, where the immunocomplex of the PRIS was tested with the H3N25 peptide as substrate, the amount of $^{32}$P signal detected was 4400±2100 (lane 2) (modified from Jin et al., 1999).
Odin kinase assay western

Histone H3

A

B

C

H3N25: ARTKQTARKSTGGKAPRKQLATKAA
H3N25S10A: ARTKQTARKATGGKAPRKQLATKAA

H3N25 JIL-1 ip
H3N25 preimmune ip
H3N25S10A JIL-1 ip
placed Rsk-2 downstream in the EGF-MAP kinase pathway that regulates histone H3 phosphorylation in response to extracellular mitogen. Since JIL-1 has high homology to MSK1 and Rsk-2, it was of interest to study whether JIL-1 might also be involved in the H3 Ser10 phosphorylation pathway. To address this question, we designed two peptides: H3N25 contains the N-terminal 25 amino acids of H3 sequence, while H3N25S10A has the same sequence as H3N25 except for the Ser10 residue was replaced by an alanine (Fig 6C). In *in vitro* immunocomplex peptide kinase assays, the JIL-1 immunocomplex demonstrated strong kinase activity toward the wild type peptide, H3N25, (Fig 6C, Lane 1) but very low kinase activity toward the mutant peptide, H3N25S10A (Fig 6C, Lane 3), showing about 6 times decrease. As a negative control, the immunocomplex of PIRS (pre-immune rabbit serum) was tested with wild type peptide as substrate (Fig 6C, Lane 2). The phosphorylation signals in lanes 2 and 3 are roughly comparable. These data suggest that the JIL-1 immunocomplex can preferentially phosphorylate the Ser10 site in a synthetic peptide.

One limitation of immunocomplex kinase assays is the possibility that other kinases may be co-immunoprecipitated with the kinase of interest, making it hard to ascribe the phosphorylation activity detected to the kinase immunoprecipitated by the antibody. An alternative approach that could avoid this problem is to express the kinase to high level in an expression system and purify the kinase by biochemical methods. Future efforts to express and purify an active JIL-1 kinase would have many benefits for the JIL-1 study.

**JIL-1 interacts with the MSL dosage compensation complex**

The higher level of JIL-1 protein on the male X chromosome (Fig 3, D and E) and the colocalization of JIL-1 and the MSL proteins (Fig 4) suggest JIL-1 could play a role in dosage compensation. Is there any direct protein-protein interaction between JIL-1 and proteins in the MSL dosage compensation complex in addition to their colocalization? To
address this question, two approaches, co-immunoprecipitation (co-IP) and the GST-fusion protein pull down (PD) assays, were used to examine possible interactions between JIL-1 and the MSL proteins.

The MSL proteins can form a stable 2MD protein complex as shown by gel-filtration chromatography and co-IP studies (Copps et al., 1998). Therefore, if JIL-1 interacts with any of the MSL proteins, it is very likely other proteins in the MSL complex could also be recovered in in-vitro protein interaction studies, such as co-IP and GST-PD assays. Using the V5 antibody against a JIL-1-V5 fusion protein expressed in the S2 (Schneider 2) cells to immunoprecipitate JIL-1, both MSL-1 and MSL-2 were coimmunoprecipitated with JIL-1 (Fig 7B). Conversely, anti-MSL-1, -2, and -3 antibodies were able to co-IP JIL-1-V5 fusion protein as well (Fig 7A). In control experiments, the nonspecific antibody could not co-IP the JIL-1-V5 fusion protein, suggesting the co-IP results are specific to the antibodies used. To carry out in vitro GST-PD protein-protein interaction assays, five separate GST fusion proteins were prepared, GST-JIL-1, GST-NTD (N-terminal domain), GST-KDI (kinase domain I), GST-KDII (kinase domain II), and GST-CTD (C-terminal domain) (Fig 7C). The GST-JIL-1, GST-KDI, and GST-KDII fusion proteins bound to glutathione agarose beads could pull down MSL proteins, but not the GST, GST-CTD, or GST-NTD (Fig 7D). These data suggested that there is physical interaction between JIL-1 and the MSL complex. Furthermore, the fact that two domains of the JIL-1 protein can interact with the MSL complex suggests JIL-1 may interact with more than one protein of the MSL complex or more than one domain of the same protein. The involvement of multiple protein interaction sites in the same protein was found as a common theme for protein interaction in huge protein complexes. For example, different parts of the MSL1 protein interact with different proteins of the MSL complex (Scott et al., 2000). Currently, we do not know which protein(s) of the MSL complex directly interacts with JIL-1. Further study of JIL-1 and MSL
Fig 7. Interaction of JIL-1 and the MSL protein(s) of the dosage compensation complex. (A) The antibodies, anti-MSL-1, -2 and -3, can co-IP JIL-1-V5 fusion protein expressed in S2 cells (lane 3). For the control in lane 2, either normal rabbit serum was used for anti-MSL-1, -2 antibodies or normal goat serum was used for anti-MSL3 to match with the species where the specific antibodies were produced. The controls showed that JIL-1-V5 was specifically coimmunoprecipitated by anti-MSL antibodies but not the negative control serum. S2 cell lysate loaded in lane 1 was used to indicate the size of the JIL-1-V5 protein. (B) The V5 mAb that immunoprecipitates the JIL-1-V5 fusion protein can also co-IP MSL proteins, as detected by the anti-MSL-1, and -2 antibodies on western blots. The MSL proteins were not detected in the negative control lane 2, where the mAb V5 was used to immunoprecipitate from S2 cells that were not transfected with the JIL-1-V5 expression vector. S2 cell lysate was loaded in lane 1 to indicate the size of the proteins. (C) A schematic drawing of the GST fusion proteins made in E.coli for the GST-pull down study. The amino acid residues included in the fusion proteins are numbered at the end of each construct. (D) GST-JIL-1 (lane 3), GST-KDI (lane 5), GST-KDII (lane 6) can pull down MSL proteins, but not the GST alone (lane 2), GST-NTD (lane 4), or GST-CTD (lane 7). Lane 1 is the S2 cell lysate to indicate the size of the MSL1 or MSL3 protein (modified from Jin et al., 2000).
A

S2 lysate  control  MSL1 ip

MSL2 ip

MSL3 ip

JIL-1 immunoblots

B

S2 lysate  control  JIL-1 ip

MSL1 immunoblot

MSL2 immunoblot

C

JIL-1

GST-JIL-1

GST-NTD

GST-KDI

GST-KDII

GST-CTD

D

S2 lysate  GST  GST-JIL-1  GST-NTD  GST-KDI  GST-KDII  GST-CTD

MSL1

MSL3
interaction with purified proteins or using the two-hybrid system in yeast may yield a better understanding of how JIL-1 interacts with the MSL complex.

**Identification and generation of a series of JIL-1 mutant alleles**

As the genome sequence of the JIL-1 locus was available from the NCBI (National Center for Biotechnology Information) DNA database, the cDNA sequence of JIL-1 was compared with the genomic sequence using the BLAST program (Adams et al., 2000). The JIL-1 gene contains eight exons separated by seven introns, which are scattered in about 15kb region of the genome (Fig 8A). The regions encoding the protein, including the regions that encode KDI (kinase domain I) and KDII (kinase domain II), are indicated below whereby the starting methionine is located in the second exon. By searching the DNA database at NCBI using the cDNA sequence of JIL-1, a flanking sequence of an EP element insertion site was found, which indicates an EP element was inserted at the end of exon I about 700bp upstream of the starting methionine (Fig 8B). An EP element carrying a Gal4 regulated promoter was used to produce fly lines that drive protein overexpression in a controllable and tissue specific manner to look for phenotypes caused by protein overexpression (Rørth et al., 1998). In this JIL-1 EP element insertion line (referred to as EP3 or EP(3)3657), although the EP element blocked the normal transcription of JIL-1 mRNA, the JIL-1 protein was expressed at about 11.4±1.6% (n=4) of that of the wild type (Fig 8C). To generate additional mutant alleles of JIL-1, especially a JIL-1 null, Dr. Kristen M. Johansen and Weiguo Zhang carried out imprecise P-element excision experiment to screen for P-element excision events that took away a part of the JIL-1 genomic DNA. Weiguo mapped the part of genomic DNA removed by the imprecise P-element excision events using PCR (Fig 8B). Two JIL-1 alleles identified in this screen, Z60 and Z2, which have only about 3.0±0.8% (n=4) and 0% of JIL-1 protein expression, respectively, were selected for furthe
Fig 8. Identification and generation a series of JIL-1 mutant alleles. (A) A diagram of the JIL-1 genomic map. JIL-1 has eight exons separated by seven introns that are located in a 15kb region. The position of exons is indicated by black boxes and the cutting sites of several major enzymes are indicated at the top. The regions of the gene that encode the JIL-1 protein, including KDI and KDII, are indicated at the bottom. (B) The original EP element represented by a triangle in the EP3 allele was inserted about 700bp upstream of the starting methionine in exon II. Two more JIL-1 mutant alleles, Z60 and Z2, were generated from the original EP3 by imprecise P-element excision. The regions carried away by the EP element are indicated by dashed lines. (C) The JIL-1 protein expression level is reduced to about 10% in EP3, 3% in Z60, and 0% in Z2 in this western blot detected with affinity purified anti-JIL-1 Hope antibody. The amount of tubulin loaded in each lane is roughly equal as detected with anti-tubulin mAb. (D) JIL-1 protein expression is reduced to an undetectable level in Z2 (lane 2). The amount of JIL-1 in Z2 was restored by a GFP-JIL-1 transgene on the second chromosome (lane 3) to a comparable level with the wild type JIL-1 as detected on western blots with the Hope antibody (modified from Wang et al., 2001).
analysis (Fig 8C). In the Z60 allele, the 5' end of the EP element, the upstream regulatory region, and most of the exon I of JIL-1 were removed as depicted by dashed line in fig 8B. In the Z2 allele, the 3' end of the EP element, intron I, exon II, and most of intron II, including the starting methionine, were removed (Fig 8B). The next methionine in the JIL-1 cDNA sequence is in the middle of KDI, therefore, the Z2 allele should be a JIL-1 null, which was confirmed by western blots analysis (Fig 8C). To carry out rescue experiments in the JIL-1 mutants to confirm the mutant phenotypes were caused by JIL-1, we used a GFP-JIL-1 transgene on the second chromosome, which is a slightly slower moving band on western blots than that of JIL-1 due to the added GFP moiety (Fig 8D). The GFP-JIL-1 was expressed at a comparable level to that of endogenous JIL-1, making it suitable for the rescue experiments. These three alleles, EP3, Z60, and Z2, comprise a series of JIL-1 mutants from hypomorph, strong hypomorph, to null and are very useful to study the JIL-1 function in vivo.

**JIL-1 is essential for the viability of both males and females; the male viability is more sensitive to the level of JIL-1 protein**

By studying the JIL-1 mutant phenotypes, we found the viability of both males and females is greatly reduced as the level of JIL-1 protein is decreased (Table 1). The eclosion rate of homozygous EP(3) pupae produced by heterozygous parents was 81%, which was dropped to 69% for homozygous EP(3) pupae produced by homozygous parents. This reduction of eclosion rate in EP(3) pupae produced by homozygous parents may be caused by a lower level of maternal JIL-1 product, as the heterozygous parents have a normal copy of JIL-1 to encode maternal product, while homozygous parents do not. In the first generation of homozygous Z60, in which the JIL-1 protein level was reduced to 3%, the eclosion rate was only 0.5%. The eclosion rate further dropped to 0% in homozygous Z2
Table 1. The viability and male/female ratio are affected in JIL-1 mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Eclosion rate (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of observed M/F&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>EP(3)/TM6 Tb</td>
<td>ND</td>
<td>107 (331/308)</td>
</tr>
<tr>
<td>EP(3)/EP(3) &lt;sup&gt;c&lt;/sup&gt;</td>
<td>81</td>
<td>73 (206/354)</td>
</tr>
<tr>
<td>EP(3)/EP(3) &lt;sup&gt;d&lt;/sup&gt;</td>
<td>69</td>
<td>48 (467/968)</td>
</tr>
<tr>
<td>Z60/Z60</td>
<td>0.5</td>
<td>32 (22/59)</td>
</tr>
<tr>
<td>Z2/Z2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>GFP-JIL-1;EP(3)</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
<td>97 (484/500)</td>
</tr>
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</table>

<sup>a</sup> Eclosion rate (%) = number of flies eclosed/number of pupae collected x 100
<sup>b</sup> % of observed M/F = number of males observed/number of females observed x 100
<sup>c</sup> EP3 flies were produced by heterozygous parents
<sup>d</sup> EP3 flies were produced by homozygous parents
<sup>e</sup> ND = not determined
pupae when there was no JIL-1 protein detectable. These data demonstrated that JIL-1 is important for the viability of both males and females. This is also consistent with the global distribution of JIL-1 protein on the X chromosome and autosomes in both sexes, suggesting JIL-1 plays a general function in addition to its putative function in dosage compensation.

Since JIL-1 protein is elevated on the male X chromosome and JIL-1 can interact with the MSL complex, we intentionally counted and compared the number of surviving males and females of the homozygous JIL-1 mutants to see whether the reduced levels of JIL-1 can affect the viability of males and females differently. Interestingly, the number of males was less than that of females, suggesting males are more sensitive to the reduction of JIL-1 protein (Table 1). For example, the number of males in the first generation of homozygous EP3 was 73% of that of females and was dropped to 48% in the second generation of homozygous EP3. We reasoned that the decrease of male to female ratio could be caused by the lower level of JIL-1 maternal product in the second generation of EP(3) as well. In the small number of surviving homozygous Z60, in which the JIL-1 protein level is about 3% of wild type, males were only 32% percent of females. In addition, the male to female ratio was recovered to 97%, close to that of the wild type, when the GFP-JIL-1 was introduced into the EP(3) mutant. These data support that JIL-1 plays a role in dosage compensation, which is in agreement with its elevated level on the male X chromosome and its interaction with the MSL proteins. The importance of JIL-1 for the viability of both males and females also explains why JIL-1 was not identified in the genetic screens looking for a male specific lethal phenotype.

**JIL-1 is required for early embryonic development**

Although the eclosion rate of homozygous EP3 pupae produced by homozygous parents is 69%, the hatching rate of embryos of homozygous parents is only 4% (16/400).
showing a dramatic decrease from the observed 83% (333/402) hatching rate of wild type, Oregon R, flies with statistical difference (P<0.001). To investigate the cause of this reduced hatching rate, the embryos produced by homozygous EP3 parents were stained with Hoechst to label DNA in chromosomes and with tubulin antibody to visualize tubulin in mitotic apparatus. We found a series of mutant embryonic phenotypes from abnormal to roughly as normal as that of the wild type embryos, which reflects the 4% hatching rate of these embryos, indicating the variability of embryonic phenotypes could be due to the roughly 10% of JIL-1 protein expression left in these animals. Chromosomes in the abnormal mutant embryos were disintegrated (Fig 9B), fragmented (9C), and sometimes in big clumps (9E). In contrast, chromosomes in wild type embryos at this stage should be nicely arranged and evenly distributed (Fig 9A). By double labeling of embryos with Hoechst and tubulin, we found, in some embryos, there were many centrosomes separated from the big clumps of chromosome and dispersed in the embryos (fig 9D, E, and F). In some other embryos, the microtubule spindles with aberrant morphology were formed and associated by the disintegrated and fragmented chromosomes (fig 9G, H, and I). These observations suggest JIL-1 may play a role in early embryonic development and is involved in the maintaining of the integrity of chromosomes in the early embryos. However, how the low level of JIL-1 could induce these mitotic defects is still a question for future study. One possible explanation is the mitotic defects are caused by the disorganization of the higher order chromatin structure during interphase (see next section), which activated certain cell cycle checkpoint(s) to block the mitotic progress and eventually led to the disintegrating of chromosomes in mutant embryos. Nevertheless, other possibilities, such as JIL-1 plays a direct role in mitosis, may exist as well.

Interestingly, some forms of abnormal chromosome morphology observed in EP3 mutant embryos are very similar to that found in the mutant of pitkin, a dominant gain-of-
Fig 9. JIL-1 is required for early embryonic development. (A) A wild type embryo at the early developmental stage was stained with Hoechst to show the chromosome morphology and evenly distributed nuclei that have migrated to the surface of the embryo. (B and C) Mutant EP3 embryos were stained with Hoechst. Chromosomes in these embryos were either disintegrating (B) and/or severely fragmented (C). (D, E, and F) Double labeling of embryo with Hoechst and anti-tubulin antibody showed that DNA was in some big clumps and small fragments (E) and centrosomes stained by tubulin antibody were uncoupled from DNA and dispersed in the embryos. (G, H, and I) Double labeling of embryo with tubulin antibody and Hoechst, abnormal microtubule spindles pointed out by arrows (G) were formed on the aberrant chromosomes (modified from Wang et al., 2001).
function mutation that enhances position-effect variegation and regulates chromatin function (Kuhfittig et al., 2001). This suggests multiple protein factors may be involved in maintaining chromatin structure in early embryos. Moreover, a recent study showed that JIL-1 is haploid insufficient to maintain the normal transmission of a *Drosophila* minichromosome, suggesting JIL-1 regulates the faithful segregation of the chromosomes during cell division (Dobie et al., 2001).

**JIL-1 is required for maintaining higher order polytene chromosome structure**

The distribution of JIL-1 protein on polytene chromosomes encouraged us to examine the polytene chromosomes in the JIL-1 mutants. Using Hoechst to label DNA and MSL2 antibody to stain MSL2 on the male X chromosome, we found that the polytene chromosome structure in JIL-1 mutants was severely distorted, suggesting JIL-1 is required for the maintenance of higher order chromatin structure in salivary gland nuclei (Fig 10). The wild type polytene chromosomes have long and extended arms with defined bands stained by Hoechst and MSL-2 antibody (Fig 10 A, B, and C). However, the length of the polytene chromosome was decreased, and the banding pattern of Hoechst and MSL2 disappeared to degrees correlating with the reduced levels of JIL-1 protein in the mutants. The architecture of polytene chromosomes from homozygous EP3 was slightly changed (Fig 10E) and more severely altered in the Z60 and Z2 mutants (Fig 10H, K and N). In homozygous EP3 males, both the Hoechst pattern and MSL2 staining pattern were subtly changed with a lower number of discernible bands on the slightly shortened X chromosome (Fig 10 D, E, and F). In homozygous Z60 and Z2 males, the X chromosome morphology was strongly perturbed with total disappearance of banding pattern of Hoechst and MSL2 (Fig 10 G, H, I, J, K, and L). In homozygous Z60 mutant, the chromosome structures varied from strongly abnormal to
Fig 10. JIL-1 is required for maintaining higher order polytene chromosome structure. (B, E, H, K, and N) Hoechst staining of polytene chromosomes from wild type, EP3, Z60, Z2 males, and a Z2 female, respectively. (C, F, I, L, and O) MSL2 antibody staining of polytene chromosomes with their genotype labeled. (A, D, G, J and H) The composite figures of Hoechst and MSL2 antibody staining with the genotype labeled (modified from Wang et al., 2001).
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<th></th>
<th>composite</th>
<th>Hoechst</th>
<th>MSL2 antibody</th>
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<tbody>
<tr>
<td>male</td>
<td>X wt</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>X z60</td>
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less abnormal, which is probably due to the residual amount (about 3%) of JIL-1 left. However, in the Z2 null mutant, the higher order structure of polytene chromosomes was affected to roughly the same degree as the example shown in fig 10K. Noteworthy is that the male X chromosome structure was especially affected in the JIL-1 mutants but the female X chromosome is affected to the same degree as autosomes (compare 10K and 10N). The male X in homozygous Z2 totally lost the bands stained by Hoechst and MSL2, suggesting the unique male X chromosome is more sensitive to the reduced level of JIL-1. Whether this is the reason that male viability is more sensitive to the JIL-1 function, however, is still unknown. Furthermore, that the association of MSL2 with the male X did not disappear in the JIL-1 null Z2 suggests JIL-1 is not required for the binding of MSL-2 to the male X.

The above results support that JIL-1 function is required for maintaining normal higher order polytene chromosome structure. This notion is further confirmed by the rescue experiments with a GFP-JIL-1 transgene on the second chromosome. The GFP-JIL-1 introduced to the Z2 mutant was localized to polytene chromosomes with a pattern similar to that of JIL-1, with an elevated level on male X chromosomes and a lower level on autosomes in males, which was detected by anti-JIL-1 antibody staining (Fig 11D). Chromosome structure was restored to the level of wild type with long and extended arms, and defined Hoechst and MSL2 staining patterns (Fig 11C and D). The structural difference between the rescued chromosome and the mutant chromosome could be easily perceived (Fig 11A compared with Fig 11C).

As discussed in the literature review, the regulation of chromatin structure could have a profound effect on the function of chromatin, such as gene expression. Therefore, the perturbed chromosome structure described above may have a profound effect on the chromatin function. Based on the chromosomal territory theory, the surface area of chromatin
Fig 11. The perturbed polytene chromatin structure was rescued by a GFP-JIL-1 transgene. (A) The polytene chromosome structure in the Z2 mutant was strongly perturbed. The X-chromosome was labeled by MSL2 antibody. (B) There is no JIL-1 antibody staining on polytene chromosomes in the Z2 mutant. (C) The chromosome structure was restored by the GFP-JIL-1 transgene in the Z2 mutant, so was the MSL2 staining pattern. (D) GFP-JIL-1 was localized similarly as native JIL-1 on polytene chromosomes, which was upregulated on the male X and distributed with lower level on autosomes. (JIL-1, white; MSL2, red; Hoechst, green) (modified from Wang et al., 2001).
is important for efficient transcription. Therefore, the shortened polytene chromosomes with reduced surface areas would be unfavorable for transcription if the above theory were correct. Furthermore, the modification of histones has been shown to regulate chromatin structure and functions in various ways (Cheung et al., 2000). Could the phenotype observed in JIL-1 be correlated with histone modification given the fact that the JIL-1 immunocomplex can phosphorylate histone H3 in vitro (see next two sections)?

The H3 Ser10 phosphorylation and H3 Ser10 phosphorylation-Lys14 acetylation levels are increased on the male X chromosome

That the JIL-1 immunocomplex could phosphorylate the H3 Ser10 residue in vitro and the reports that RSK-2 and MSK1, two of the JIL-1 homologs in higher eukaryotes, could phosphorylate the Ser10 site of H3 in vivo encouraged us to study whether JIL-1 is involved in the Ser10 phosphorylation in vivo as well (Sassone-Corsi et al., 1999; Thomson et al., 1999; Cheung et al., 2000; Clayton et al., 2000). To answer this question, we applied a commercially available antibody that recognizes the H3 isoform phosphorylated at the Ser10 residue. The staining of polytene chromosomes with this antibody by following a traditional acid squash protocol yielded inconsistent results that varied from different lots of antibody. We reasoned that the acid treatment might have destroyed the epitopes or interfered with antibody performance. Therefore, we developed an alternative method to stain the polytene nuclei by compressing the salivary gland gently under a cover slip and fixing with paraformaldehyde/PBS. The staining signal of pH3S10 (phospho-Ser10 of H3) with this method is consistent and repeatable with antibodies from two independent sources, making it suitable for our study.

Double labeling of salivary gland polytene nuclei with both JIL-1 antibody and the pH3Ser10 antibody showed that the pH3Ser10 signal was enriched on the male X
chromosome stained by the JIL-1 antibody (Fig 12C; Jin et al., 1999). In addition, the signal of pH3Ser10 staining overlaps with that of JIL-1 staining as shown by the predominant yellow signals in the composite fig 12A. It has long been known that H4Ac16 colocalizes with the MOF protein on the male X chromosome. Did nature do the parallel trick to have the pH3S10 colocalize with a kinase, JIL-1? Nonetheless, the colocalization data further linked JIL-1 function to the H3 Ser10 phosphorylation pathway.

Recent studies have demonstrated that Ser10 phosphorylation and Lys14 acetylation are synergistically regulated processes (Clayton et al., 2000; Cheung et al., 2000b; Rea et al., 2000). The phosphorylated H3 N-terminus can be more efficiently acetylated by the GCN5 family of histone acetyltransferases (HATs), and vice versa. To analyze whether these two kinds of H3 modifications were synergistically regulated on the male X as well, we applied a pH3S10Ac14 antibody that recognizes the double modified H3 with phosphorylated Ser10 and acetylated Lys14 (pH3S10Ac14). It was found that the double modified H3 isoform was also enriched on the male X chromosomes stained by JIL-1 antibody (Fig 12E, F) and was colocalized with the JIL-1 staining as indicated by the overwhelming yellow signal in the composite fig 12D. This finding leads to a plausible model that phosphoacetyl-H3 might configure a chromatin structure together with acetylated H4 at Lys16 to regulate dosage compensation (Fig 5). However, this model is simplified to fit with most of the reported data so far and needs to be further tested and modified in the future. Nevertheless, the combining of multiple histone modifications on the male X again emphasized the importance of covalent histone modifications in the various processes templated by DNA/chromatin.

The phosphorylation of H3 Ser10 is involved in two separate pathways. The interphase phosphorylation is correlated with gene expression (Sassone-Corsi et al., 1999; Cheung et al., 2000) and the mitotic phosphorylation is correlated with chromosome condensation and segregation (Wei et al., 1998). Correspondingly, there are two enzymatic systems that
Fig 12. The H3 Ser10 phosphorylation (pH3S10) and H3 Ser10 phosphorylation-Lys14 acetylation (pH3S10Ac14) levels are increased on the male X chromosome. (A, B, and C) A salivary gland polytene nucleus was stained with both JIL-1 antibody and anti-pH3S10 antibody. The pH3S10 signal was elevated on a chromosome (C) that was also labeled by the JIL-1 antibody (B). The labeling of JIL-1 antibody and that of the pH3S10 antibody largely overlap with each other as shown by predominant yellow color in the composite figure (A). (D, E, and F) A polytene nucleus was double labeled with both JIL-1 antibody and pH3S10Ac14 antibody, which recognizes the H3 isoform phosphorylated at Ser10 and acetylated at K14. The pH3S10Ac14 labeling (F) was elevated on a chromosome stained by JIL-1 antibody (E) and was predominantly colocalized with the staining of JIL-1 as shown by the predominant yellow color in the composite figure (D). (G, H, and I) Neuroblast squash of the Z2 mutant was stained with pH3S10 antibody and Hoechst. The pS10 signal was present on the condensed mitotic chromosomes but not in surrounding interphase nuclei (I) (modified from Wang et al., 2001).
control H3 Ser10 phosphorylation during interphase and mitosis. The MSK1 and RSK-2 kinases were found as the interphase Ser10 kinases (Sassone-Corsi et al., 1999; Thomson et al., 1999), while the Ipl1/aurora kinase was found as the mitotic Ser10 kinase (Hsu et al., 2000). Therefore, it is likely there are two enzymatic systems existing in D. melanogaster to control Ser10 phosphorylation at different stages of the cell cycle. To investigate whether mitotic Ser10 phosphorylation was affected in the JIL-1 mutant Z2, neuroblast squashes were stained with pH3S10 antibody. The phospho-Ser10 signal (Fig 12I) was present on the condensed mitotic chromosomes but not on the surrounding interphase nuclei (Fig 12H, data from W. Zhang). This result together with a recent report of the fly Aurora B kinase as the mitotic Ser10 kinase strongly argues that JIL-1 is not involved in the H3 Ser10 phosphorylation at mitosis but could be involved in the interphase H3 Ser10 phosphorylation signaling pathway.

The H3 Ser10 phosphorylation level in JIL-1 mutant larvae is greatly reduced

The converging data of in vitro kinase assays and in vivo colocalization studies of pH3S10 and JIL-1 support that JIL-1 might be involved in the Ser10 phosphorylation signal transduction pathway. This notion was further supported by western blot analysis using the pH3S10 antibody. To evaluate whether or to what extent, the Ser10 phosphorylation signal was reduced when the JIL-1 protein level was reduced or absent, western blots were carried out with total third instar larva extract of wild type, EP3, Z60, and Z2 animals. Samples were separated by SDS-PAGE and detected with pH3S10 antibody. Comparing with that of wild type, the phosphorylation level of H3 Ser10 was reduced to 40.0±17.4% (N=4), 19.4±14.4% (N=4), and 4.6±1.9% (N=4) in EP3, Z60, and Z2 respectively, albeit the amount of H3 protein in each extract was roughly equal. One example of such a western blot was shown in fig 13A. The degrees of reduction of pH3S10 were correlated with the decreased levels of
Fig 13. Histone H3 Ser10 phosphorylation level in JIL-1 mutant larvae was greatly reduced. (A) Total larvae extract from WT (wild type), EP3, Z60 and Z2 animals were separated by SDS-PAGE for western blots and probed with pH3S10 antibody. The pH3S10 signal was reduced to roughly 40%, 20%, and 5% in EP3, Z60 and Z2 respectively. Tubulin, lamin Dm0, and H3 antibodies were used to demonstrate that roughly equal amount of protein sample was loaded in each lane. (B) The reduced level of pH3S10 in homozygous Z2 (the middle lane) could be restored by a GFP-JIL-1 transgene in the Z2 mutant (the last lane).
JIL-1 protein in these animals. These data suggest the normal level of H3 S10 phosphorylation in third instar larvae is dependent on the JIL-1 signal transduction pathway.

To further confirm this idea, rescue experiments were employed with a GFP-JIL-1 transgene on the second chromosome. We found the reduced level of H3 Ser10 phosphorylation can be restored by the GFP-JIL-1 transgene, which was shown on western blots detected with the pH3S10 antibody (Fig 13B, data from W. Zhang).

Taken together, these data support a hypothesis that JIL-1 is involved in a signal transduction pathway regulating Ser10 phosphorylation in vivo. Because the H3 Ser10 phosphorylation level in Z2 was reduced to some 5% of that of wild type in the third instar larvae, and most of the cells at this developmental stage are in interphase, we propose that the JIL-1 pathway is the predominant signal transduction pathway regulating H3 Ser10 phosphorylation in interphase.

Applying the anti-pH3S10Ac14 antibody to study the level of double modified H3 in JIL-1 mutants, we found the amount of double modified H3 was also reduced in the JIL-1 mutant Z2 and could be restored by a GFP-JIL-1 transgene on the second chromosome (data not shown).

**Lamin Dm0, a candidate interacting with the C-terminal domain of JIL-1**

Nuclear lamin forms a fibrous network underneath the surface of the nuclear membrane, called nuclear lamina, which has long been thought as a structural component to support and maintain the shape of the nuclear envelope. However, more recent studies suggested that lamin and lamin associated proteins play a broad range of functions in the nuclei, such as participating in DNA replication and transcriptional regulation(Moir et al., 2000).
Nuclear lamin belongs to the family of intermediate filament proteins with an α-helical rod domain that can be divided into four segments, coil 1a, coil 1b, coil 2a and coil 2b (Johansen and Johansen, 1995). Lamin proteins can polymerize to form intermediate filaments by head-to-tail binding with each other (Hutchison et al., 2001; Stuurman et al., 1996). The nuclear lamina structure and nuclear envelope is subject to rapid disassembly at the beginning of mitosis and reassembled at the end of mitosis (Newport and Spann, 1987). The assembly/disassembly of lamina is at least partially controlled by the dephosphorylation/phosphorylation of nuclear lamin. Phosphorylated lamin protein demonstrates different solubility and polymerization behavior from unphosphorylated lamin protein (Lin and Fisher, 1990). The phosphorylation of lamin is governed by kinases, such as protein kinase A, C, and p34\(^{cdel}\), which lead to disassembly of the lamin filament. Conversely, the dephosphorylation of lamin is controlled by phosphatase PP1 (protein phosphatase 1), which leads to the reformation of the intermediate filament (Stuurman, 1997; Thompson et al., 1997).

At the beginning of M phase, the nuclear envelope is broken down to small nuclear envelope vesicles, which are reassembled to form the nuclear envelope at the end of mitosis. The assembly of the nuclear envelope is mediated by the binding of nuclear membrane vesicles to chromosomes. The fusion of these vesicles on the surface of chromosomes leads to the formation of the nuclear envelope, which encompasses all of the chromosomes (Pfaller et al., 1991; Ulitzur et al., 1997). Evidence showed that the attachment of nuclear membrane vesicles to chromosomes was mediated by the interaction between lamin and the N-terminal tails of histone H2A and H2B (Goldberg et al., 1999).

In mammals, there are three types of lamins, designated as lamin A, B, and C. The A and C type lamins are produced from the same gene by alternative splicing, share considerable homology with each other, and have relatively neutral isoelectric points
B type lamin, however, is quite acidic and was found to be associated with the DNA replication sites (Moir et al., 1994). In *D. melanogaster*, there is one major type of lamin, lamin Dm0, which is expressed widely and closely related to the B-type lamin in mammals. Flies also have an A (or C)-type lamin whose expression is limited to some cells at certain developmental stages (Riemer et al., 1995; Cohen et al., 2001). There are two RNA isoforms of lamin Dm0 produced by alternative splicing, which encode the same protein. The newly synthesized Dm0 protein is rapidly processed by post-translational modification to generate two proteins with molecular weight of 74KD and 76KD, respectively (Gruenbaum et al., 1988).

Recent studies of nuclear lamin associated proteins, such as lamin B receptor (LBR), lamin associated proteins (LAP1 and LAP2) and emerin, revealed that these proteins can interact with lamins and in many cases attach chromatin as well. Thus, these proteins offer a connection between the nuclear envelope and chromatin (Wilson et al., 2001). The interaction between LBR and HP1 (heterochromatin protein 1), a protein repressive for transcription, suggests nuclear lamin and/or its associated proteins might be involved in transcriptional regulation as well (Ye et al., 1997). However, the picture of how nuclear lamina and associated proteins regulate the nuclear function, such as transcription, DNA replication, and how lamins are involved in the internal nuclear structure organization is far from clear. The study for more lamin interaction proteins will provide more insight into the nuclear organization and function.

To better understand the function of JIL-1, a chromosomes/chromatin associated Ser/Thr kinase, we carried out yeast two-hybrid screens to search for proteins that can interact with JIL-1. By screening a 0-2 hour *Drosophila* early embryo yeast two-hybrid library (a generous gift from Dr. Linda Ambrosio), the CTD (C-terminal domain) of JIL-1 was found to interact with the fly lamin Dm0 protein. Of the seven positive clones identified
and sequenced from this yeast two-hybrid screen, two of them, C13g and C8d, were found to be two independent clones containing lamin Dm0 fragments of different length (Fig 14A). The C8d clone contains the coding sequence for residues 219-622 of Dm0, including the coil2a and coil2b regions and the C-terminal domain of Dm0, while the C13g clone contains the cDNA sequence encoding residues 270-622 of Dm0.

When the Gal4-BD-CTD (Gal4 DNA binding domain fused with the CTD) of JIL-1 and the AD-C8d (AD, Gal4 transcription activation domain) or the AD-C13g constructs were co-transformed into yeast, the lacZ reporter gene expression was turned on. This was shown in paper-lift color assays to measure the activity of LacZ (Fig 14B). As control, when the Gal4-BD-NTD (N-terminal domain) of JIL-1 construct was co-transformed with either of these two AD domain containing lamin Dm0 clones into yeast, the LacZ reporter gene was not expressed. These data suggest that there is specific interaction between the lamin Dm0 and the CTD of JIL-1 in yeast cells. To further investigate the interaction between JIL-1-CTD and lamin Dm0, we carried out GST-PD (pull down) assays with GST-CTD fusion protein expressed in E. coli. The GST-CTD fusion protein coupled on glutathione agarose could pull down lamin Dm0, which was detected on western blots with lamin Dm0 antibody (Fig 14C). In the control experiments, GST protein alone was unable to pull down Dm0. The interaction of JIL-1 and Dm0 was further studied by co-IP (immunoprecipitation) experiments. The lamin polyclonal antibody R836 (a generous gift from Dr. Paul Fisher) was able to co-IP JIL-1-V5 fusion protein expressed in the fly S2 cells, which was detected by the V5 mAb on western blot (Fig 14D). In control, normal rabbit serum could not co-IP JIL-1-V5 (Fig 14D).

The yeast two-hybrid and in vitro protein interaction assay data support the JIL-1 CTD may interact with lamin Dm0. However, it is not known whether this protein-protein interaction observed in the in vitro systems or in yeast cells is physiologically relevant. It has
Fig 14. Protein-protein interaction studies of JIL-1 and lamin Dm0. (A) A schematic drawing of the lamin Dm0 protein structure. The protein has 622 amino acids and contains a rod domain with four coil regions, coil 1a, 1b, 2a, and 2b. The corresponding lamin Dm0 fragments included in clone C8d and C13g were depicted below the diagram of the Dm0 protein. (B) The expression of LacZ reporter gene in yeast cells was induced by the interaction between the JIL-1 CTD (C-terminal domain) and lamin Dm0. From left to right, the positive control showed interaction between TRAF2 (TNFR associated factor 2) and CD40 (a kind of tumor necrosis factor receptor, Ni et al., 2000) shown by blue color produced by lacZ activity in the yeast cells in the paper lift assay. The interaction between C8d and CTD of JIL-1 turned on the LacZ expression (blue color). There was no LacZ expression in the yeast with C8d and NTD of JIL-1 (red color). There was interaction between the C13g and CTD of JIL-1 (blue). No interaction was found between NTD of JIL-1 and C13g (red). (C) The GST-CTD fusion protein coupled with glutathione agarose beads pulled down lamin Dm0, but the GST alone did not. S2 lysate was loaded on western blots to indicate the size of lamin proteins. (D) The anti-lamin R836 serum can co-IP JIL-1-V5 expressed in the S2 cells (right lane) but not the control normal rabbit serum (middle lane). S2 lysate was loaded in the left lane to indicate the size of JIL-1-V5.
long been thought that lamin protein is only distributed underneath the nuclear envelope but not in the nuclear interior. One reason that the interior lamin Dm0 was not observed for such a long time is the limitation of conventional fluorescent microscopy, which cannot distinguish the on focus signal and out-of-focus signal well. Therefore, the low level of internal signal inside the nucleus was considered as out-of-focus signal coming from the surrounding lamina staining. With the help of confocal microscope, the interior lamin Dm0 distribution in the salivary gland nuclei was reported. Moreover, the interaction of Dm0 and topoisomerase II was shown by co-IP assays (Fisher et al, 2001).

Nonetheless, the putative interaction between lamin Dm0 and JIL-1 is quite intriguing. Does this interaction exist in cells? Since JIL-1 is associated with chromatin, is this putative interaction involved in the regulation of chromatin structure? These questions might be very interesting to be explored in the future study of JIL-1. Considering the complexity of the nuclear structure, the interaction between nuclear proteins must be important to coordinate many nuclear events. The nucleus is organized into functional domains for transcription, RNA processing and DNA replication. The individual chromosomes occupy a certain territory that is separated by inter-chromosomal channels (Lamond and Earnshaw, 1998). Heterochromatin is often found around the nuclear envelope. Noteworthy is that the telomere regions of polytene chromosomes are associated with the nuclear envelope (Dr. Gary Karpen, personal communication), which are regions that the JIL-1 staining was also found (Fig 4). These observations suggest the internal nuclear structure and chromatin are deliberately organized and regulated. The finding of the interior localization of lamins and the discovery of the interaction between LBR (lamin B receptor) and chromatin associated HP1 suggest there is a cross-talk between the nuclear envelope and chromatin (Ye et al., 1997). The view of the nuclear envelope has been changed from a static structure to a more dynamic and functionally important network inside the nucleus.
Therefore, studies of lamin interacting proteins will help us to better understand the nuclear structure and function.

**SW59, one of the alternative splicing isoforms of the *lola* locus, might interact with the kinase domain I of JIL-1**

Like any development process, the development of the central nervous system is regulated by genes expressed at the right time and place. Interfering with gene expression has profound effects on normal development. Mutations in the *Drosophila* *lola* (*longitudinal lacking*) gene, which encodes putative transcription factors, cause severe defects in the development of the axon tracts of the central nervous system. In the embryos of a *lola* mutant, *lola*\(^{502}\), in which a P-element was inserted at the 5' end of the gene, the longitudinal tracts are broken at junctions of two adjacent segments and the two commissure tracts (cross tracts) of the same segment are fused with each other (Giniger et al., 1994). Two alternatively spliced RNA isoforms, *lola* long and *lola* short, were initially reported (Giniger et al., 1994). The two Lola proteins derived from these two RNA isoforms share a common region of 454 AAs (amino acids) including a conserved BTB domain (see below) and a NLS (nuclear localization sequence) (Fig 15A). Comparing the cDNA sequence with the genomic sequence of *lola*, we found that the two alternatively spliced isoforms share four exons, exon I, II, III, and IV, which encode the common part of the protein (Fig 15B). The starting methionine of the protein is contained in exon I. The most 5' exons of *lola* long and *lola* short are different: it is exon-5'altl in *lola* long and it is exon-5'altc in *lola* short (Fig 15B). Exon IV in *lola* long is 78bp and linked to a 45kb downstream exon, which encodes two zinc finger motifs. However, exon IV of *lola* short extends from the 78bp exon into adjacent downstream sequence including an early stop codon, therefore, the Lola short protein has only 467 AAs.
Fig 15. Lola protein isoforms and the genomic map of lola. (A) Alignment of protein sequences of Lola isoforms, including the published Lola short, Lola long, SW59, and the predicted Alt1, 2, 3, 4, and 5. The length of each protein is labeled at the end. The shared region by all isoforms is indicated by a black box. The position of the two zinc finger motifs in Alt2-5, SW59, and Lola long is labeled. The part of the SW59 sequence contained in KDI-J1 clone is underlined below SW59. All of alternatively spliced isoforms have a common N-terminus including a BTB domain and a NLS (nuclear localization signal). Lola short and Alt1 contain the shared sequence (454 residues) and 13 additional amino acids but do not have a zinc finger motif. The exon IV of lola short and Alt1 is extended from exon78 (a 78bp exon shared by all known isoforms and linked to unique zinc finger containing exons) into downstream sequence including an early stop codon. The sequence of the zinc finger motifs in lola long, SW59, and Alts are aligned at the bottom with the conserved residues labeled including the zinc binding residues. (B) A genomic map of lola with the known and predicted splicing isoforms. All splicing isoforms include exon I, II, III, and IV in their cDNAs, which encode the part of protein commonly shared. Alt2, 3, 4 and 5 were predicted to have different 5' exons, 5'alta, 5'altb, 5'altc, and 5'altd, respectively, which use the same zinc finger encoding exon that is different from that in SW59 or Lola long.
74

A

NLS

BTB

Lola short, Alt1 (467aa)

Alt2, 3, 4, and 5 (757aa)

SW59 (748aa)

Lola long (894aa)

GHPBPVSGRVYKLBsSLRNffiQKV

^G-KEgQFQBPEfflVYRAKQMHIGBMERAMKEKF

Alt2,3,4 and 5

RFHSyx.V™NKSYLRBRHLQRSMREHlGIEBRFK8EE»SSRFRRKYHMVRHLSKEGIPP

SW59

VYEgRHGKKYRFEASTLRFQNVGGKEWGHQBPYYPYKSQRGNLGIVVRKH3TDLP

Lola long

B

SW59

Alt2,3,4,5

Lola long

Lola short, Alt1

SW59

Alt2

Alt3

Alt4

Alt5

Lola long
and no zinc finger motifs. The phenotypes of lola mutants suggest it may regulate nervous 

system development. In agreement with this prediction, staining with a rabbit polyclonal 

antibody made against the shared region of Lola demonstrated that the protein was enriched 

in the nuclei of nervous system in later embryonic stages (Giniger et al., 1994).

The Lola long protein has two zinc finger motifs of a C2HC type and a C2H2 type. 

The C2H2 type zinc finger was often found, while the C2HC type zinc finger is quite 

uncommon and has only been found in a small number of proteins, including the Drosophila 

Su(Hw) (suppressor of hairy wing protein, Parkhurst et al., 1988) and snail (Boulay et al., 

1987). Each zinc finger motif has a conserved ββα structure with the conserved C and H 

residues coordinately binding a zinc ion (Wolfe et al., 2000). The zinc finger usually 

functions as a motif that determines the DNA binding specificity of a protein. However, in 

some proteins, the C2H2 zinc finger can mediate RNA-protein, or protein-protein interaction, 

suggesting that the zinc finger motif may have diverse functions (Wolfe et al., 2000).

The BTB domain shared by both the Lola long protein and the Lola short protein is a 

conserved domain with about 120 amino acids. It was so designated because this domain was 

originally found in Briac-à-brac, Tramtrack, and Broad-Complex. The crystal structure of the 

BTB domain from human PLZF (promyelocytic leukemia zinc finger) protein indicated that 

this domain consists mainly of β-sheets and α-helices and can form stable dimers by 

hydrophobic interactions (Ahmad et al., 1998). The BTB domain can mediate the formation 

of both homodimers and heterodimers and was estimated to present in 5-10% of human zinc 

finger containing proteins (Ahmad et al., 1998).

Another lola isoform, SW59, was cloned in an enhancer trap screen looking for genes 

primarily expressed in the nervous system (Zhong, 1997; gene bank DNA sequence 

submission). The 5’ end exon of SW59 is exon-5’altc, an alternative exon encoding a 5’UTR 

region and predicted by the Drosophila genome project (see below), and the 3’ end exon of
SW59 is another zinc finger containing exon (Fig 15B). SW59 also contains the four-shared exons of *lola* long and *lola* short. The SW59 protein derived from the cDNA has 748 AAs with a region of 454 AAs shared with other Lola isoforms and a region of 294 AAs in its unique C-terminus (Fig 15A). Interestingly, the SW59 C-terminus also contains two zinc finger motifs of the C2HC type and the C2H2 type (Fig 15A).

Additionally, another zinc finger containing exon was predicted by the fly genome project. In the genome annotation, five alternative splicing isoforms of *lola*, Alt1, 2, 3, 4, and 5, were predicted (Fig 15B). The Alt1 isoform is the same as *lola* short. Alt2, 3, 4, and 5 have different 5' exons, exon-5'alta, -5'altb, -5'altc, and -5'altd, respectively, but have the same coding sequences. Surprisingly, the predicted zinc finger containing exon by the genome project also has the C2HC type and the C2H2 type zinc finger motifs. The zinc finger motif sequences of *Lola* long, SW59, and Alt2-5 are aligned to show the conserved residues including the residues that contribute to zinc binding (Fig 15A). It is quite intriguing to us that neither the SW59 isoform nor the *lola* long isoform was predicted by the fly genome project, suggesting that the splicing pattern of *lola* is very complicated.

Alternative splicing is a common phenomenon in higher eukaryotes and is considered as an important mechanism to greatly increase the number of proteins produced by a genome (Claverie, 2001; Graveley, 2001). It was surprising to many people that only about 30,000 genes were found in the first version of human genome, which is much lower than the previously estimated 80,000 genes. Compared with the some 20,000 genes in *C. elegans* and some 13,000 genes in *Drosophila*, one might think the number of genes in human should be higher considering the complexity of human body and mental behaviors. However, the number of proteins produced by a genome is not solely determined by the number of genes. For example, the *Drosophila* DsCAM (*Down syndrome cell adhesion molecule*) gene could possibly produce as many as 38,000 different Ig domain containing cell adhesion proteins...
(Schmucker et al., 2000). Hence, the protein diversity can be greatly increased by alternative splicing. However, this is also a great challenge for finding out all of the protein products of a certain organism in the post-genome era (Black, 2000).

To search for JIL-1 interacting proteins, the KDI (kinase domain I) of JIL-1 was used as a bait to screen a 0-18 hour *Drosophila* embryonic library. In this screen, we identified a clone, KDI-J1, which contains the C-terminal portion of the SW59, an alternative splicing isoform of the *lola* gene. The part of SW59 included in the KDI-J1 clone is underlined in Fig 15A.

To further investigate the putative interaction between JIL-1-KDI and the C-terminal domain of SW59, we generated a monoclonal antibody, 7F1, by immunizing mice with the GST-J1 fusion protein, which contains the unique SW59 C-terminus (294 AA) and about 28 AA from the shared regions of all isoforms. On western blots, 7F1 recognized two proteins with molecular weights of about 110KD and 200KD, respectively. These two proteins are designated as 7F1-100 and 7F1-200 in the following discussions. The expression of 7F1-200 and 7F1-100 demonstrated a dynamic pattern during development (Fig 16). On a developmental western blot with samples collected every 5 hours after egg-laying, the 7F1-100 protein was found to be expressed at a high level in the early embryos and then gradually reduced. After 20 hours of development, a time before the first instar larvae hatched out, the expression of 7F1-100 was detectable by western blot. In contrast, the 7F1-200 was transiently expressed in the early embryos, reduced in the 5-10 hour embryos and then expressed again at gradually increasing levels in the later developmental stages. The predicted molecular weight of SW59 is 117KD, which is very close to the 110KD molecular weight of 7F1-100. Therefore, the 7F1-100 protein is likely to be the protein predicted from the SW59 cDNA sequence. Then, what is this 7F1-200 protein? Several possibilities came into consideration: first, it could be an alternatively spliced isoform of *lola* containing an
Fig 16. Developmental western blot of the two proteins recognized by 7F1 mAb. The 7F1 mAb can recognize two proteins: the one with a molecular weight about 200KD is designated 7F1-200; the one with a molecular weight about 110KD is designated 7F1-100. Samples were collected every 5 hours after egg-laying. Lane 1, 0-5 hour embryos; lane 2, 5-10 hour embryos; lane 3, 10-15 hour embryos; lane 4, 15-20 hour embryos; lane 5, 20-25 hour embryos and larvae; lane 6, first instar larvae; lane 7, third instar larvae. The 7F1-100 protein was expressed in the early developmental stages and disappeared in the 20-25 hour embryos. In contrast, the 7F1-200 protein had a transient expression in the early embryos, was nearly disappeared in the 5-10 hour embryos, then expressed and maintained at a high level in the later developmental stages.
epitope recognized by 7F1; second, it could be another unknown protein that was cross-recognized by the 7F1 mAb. Right now, there is no convincing evidence to support either of these hypotheses. Future efforts to understand the molecular characteristics of this 7F1-200 protein will be required.

The yeast two-hybrid experiment suggested that there is putative interaction between the C-terminal part of SW59 and the KDI of JIL-1. In order to analyze this interaction, we carried out *in vitro* GST-pull down (GST-pd) experiments. The GST-J1 fusion protein, which contains the part of the SW59 sequence included in KDI-J1, was bound to agarose beads to do pull down experiments using the S2 cell lysate. As shown in the upper panel of fig 17A, the GST-J1 fusion protein could pull down JIL-1 but the GST protein alone could not.

To answer whether 7F1-200 can interact with JIL-1, we have carried out *in-vitro* co-IP (immunoprecipitation) assays. Since the 7F1-200 protein was detected by the 7F1 mAb in S2 cell lysate (Fig 17C), we performed co-IP experiments using the S2 cell lysate. Using 7F1 for co-IP assays, JIL-1 was co-immunoprecipitated and detected on western blots by affinity purified JIL-1 Hope antibody. As a control, a monoclonal antibody, G39, recognizing the Gliarin protein in leech (Xu et al., 1999), was unable to co-IP JIL-1 (Fig 17A, middle panel). Conversely, the JIL-1 polyclonal antibody ODIN could co-IP 7F1-200 from S2 cell lysate but the PIRS (preimmune rabbit serum) could not (Fig 17A, bottom panel). These data supported that there is putative interaction between JIL-1 and 7F1-200. Since JIL-1 was found to be a part of the MSL (male specific lethal) dosage compensation complex (Jin et al., 2000; Fig 7), the immunocomplex of 7F1 mAb was also checked for the presence of MSL1 by western blot. Interestingly, MSL1 was also found to be in the immunocomplex of 7F1 mAb (Fig 17B, bottom panel). Conversely, when 7F1 was used on western blots to probe the immunocomplex of MSL1 antibody, the 7F1-200 protein was found to be coimmunoprecipitated by the MSL1 antibody (Fig 17B, upper panel). These data suggest that
Fig 17. *In vitro* protein interaction studies. (A) Upper channel, the GST-J1 fusion protein containing the C-terminus of SW59 sequence was expressed in *E. coli* and bound to glutathione agarose beads for pull down experiments. The GST-J1 (GST-J1-pd) could pull down JIL-1 as detected on western blots by an affinity purified JIL-1 antibody, Hope. In the control (GST-pd), the GST protein alone did not pull down JIL-1. In the middle panel, 7F1 mAb (7F1 ip) made against J1 sequence was able to co-IP JIL-1 from S2 cell lysate as detected with the JIL-1 Hope antibody on western blots. As control (control ip), G39 mAb recognizing a leech protein, Gliarin, was unable to co-IP JIL-1 from S2 cell lysate. In the bottom panel, the anti-JIL1 polyclonal rabbit serum ODIN was able to co-IP 7F1-200 shown as a doublet bands on this gel. As control (control ip), normal rabbit serum could not co-IP 7F1-200. (B) Upper panel, the MSL1 antibody (MSL1 ip) could co-IP the 7F1-200 protein as detected by 7F1 mAb on western blots. In the control (control ip), the normal rabbit serum could not co-IP 7F1-200. Bottom panel, conversely, 7F1 mAb (7F1 ip) was able to co-IP MSL1 as detected by MSL1 antibody on western blots. As control (control ip), G39 mAb was not able to co-IP MSL1. The lysate (S2 cell lysate) was used for western blots to indicate the size of each protein. (C) The 7F1-200 protein could be detected from both the S2 cell lysate (S2) and from the dissected salivary glands associated with fat bodies (SG and FB).
the 7F1-200 protein could possibly interact indirectly with components of the MSL complex by interacting with JIL-1 or even directly with other components of the MSL complex.

In early embryos, 7F1 mAb can stain the nuclei of early embryos (Fig 18A) similarly as the Lola polyclonal antibody made against the common region of Lola (Giniger et al., 1994). Since 7F1-200 was also expressed in salivary glands and/or fat bodies (Fig 17C). The 7F1 mAb and JIL-1 antibody were used for double labeling on polytene chromosome squashes. The titer of 7F1 mAb tissue culture medium was not very high and the 7F1 staining signal on polytene chromosomes was not very strong. However, there are some discernible bands on the male X stained by 7F1 (Fig 18B). In the composite image, there is predominantly yellow signal on the male X chromosome, suggesting that 7F1-200 and JIL-1 were colocalized on the male X (Fig 18D). The immunostaining data and the co-IP data further suggested that JIL-1 and the 7F1-200 protein may interact with each other.

As discussed above, the splicing pattern of the *lola* locus seems to be very complicated. I was intrigued by the possible presence of more zinc finger containing exon(s) in the *lola* genomic region and did some bioinformatic analysis of the *lola* genomic DNA. There are about 45kb between exon IV (78bp long, referred as exon78 hereafter) shared by the known *lola* isoforms and the unique zinc finger encoding exon of *lola* long. There is another gene transcribed from the opposite direction not far downstream from the *lola* long 3' exon, suggesting that the *lola* long 3' exon is probably the 3' boundary of *lola*. Therefore, attention was focused on the 45kb region between the exon78 and the *lola* long 3' exon to look for alternative splicing. We searched for more putative zinc finger containing exons in this region by searching for the conserved pattern of zinc finger motifs. To do this, the genomic sequence of *lola* was translated into protein sequence in three open reading frames in the GCG program. Three consensus sequences of zinc finger motifs, CXXC ('X' stands for any residues), HXXXXC, and HXXXXH, were used to search the predicted peptide sequence. If
Fig 18. Immunostaining with 7F1 mAb and JIL-1 antibody. (A) The 7F1 mAb stains the early embryo nuclei (white signal). (B, C, and D) Double labeling of male polytene chromosomes with 7F1 mAb and JIL-1 antibody. The 7F1 staining is not very strong but has some discernible bands on the male X chromosome (B) The JIL-1 protein is found to form many bands on the male X chromosome. (C) In the composite fig D, the predominant yellow signal on the male X chromosome suggesting the overlaps between 7F1 staining and JIL-1 staining.
a region with a long continuous coding sequence (without a stop codon) contains the CXXC, HXXXXX, and HXXXXXH sequences, it was considered as a putative zinc finger encoding exon. In addition, the distance between the second C and the first H in a zinc finger motif is usually 12 residues, which offers a standard to judge whether the adjacent CXXC sequence and the HXXXXXH sequence are included in the same zinc finger. This analysis was done in the Microsoft word’98 program using the "find" function. In the first round of searching, there were thirteen putative zinc finger-containing exons identified, which were numbered from 1 to 13, including lola long (13), SW59 (5), and the exon predicted by the genome project (9). Reading through the sequence line by line by eye, two more putative zinc finger containing exons were found and designated as 10.5 and 12.5 because they were between 10 and 11, 12 and 13, respectively (Fig 19). Fig 20 is a clustalw alignment of the zinc finger sequences, in which the conserved zinc binding residues are red-colored. The zinc finger regions of exon 8 and 13 have very high identity, suggesting that they could recognize very similar DNA binding sequence. More interestingly, thirteen of the fifteen predicted exons contain sequence encoding two zinc finger motifs of the C2HC type and the C2H2 (or C2HC, number 10) type (Fig 20). Note the C2HC type zinc finger was not often found, therefore, the finding of so many putative C2HC type zinc fingers in the lola genome is quite unusual. The other two, number 4 and 12.5, have sequence to encode only one C2H2 type zinc finger. Another fascinating question is whether these putative exons are used for alternative splicing, i.e., if they were exons, they should be included in mRNA sequence. To analyze this question, the 45kb genomic sequence of lola gene was first used to search for EST sequences using the GenSequer program developed by Dr. V. Brandel's lab. We found fifteen clusters of EST sequences in the 45kb genomic region, suggesting that the presence of multiple alternative splicing isoforms in this region is likely (Fig 19, top line). Since the zinc finger encoding exons of lola long, SW59, and alt2-5 are spliced to the 5' exon78, we
Fig 19. The genomic map of the lola locus. The top line is the clusters of EST (expression sequence tag) identified in the GenSequer program, which were labeled by black boxes. The predicted putative zinc finger containing exons were numbered and shown on the second line with black boxes. PCR and EST evidence supported ten out of the fifteen putative zinc finger encoding domains were used as exons and joined with the exon\textsuperscript{78}, which were shown in this figure by linking the putative exon with exon\textsuperscript{78}. PCR evidence exists for 1, 2, 6, 9, 11, and 12.5. EST evidence exists for 3 (LD03274), 5 (SW59, LD31218, LD33478), 8 (LD29668), and 12.5 (LD14150).
EST clusters

Lola short, Alt1

SW59 (5)

Alt2, 3, 4, and 5 (9)

Lola long (13)
Fig 20. Clustalw alignment of the 15 putative zinc finger (number 1-13, and number 10.5 and 12.5) encoding sequences in the lola locus. Thirteen of them contain two putative zinc finger motifs of a C2HC and a C2H2 (or C2HC in number 10) type. Two of them have only one putative zinc motif (number 4 and 12.5) of a C2H2 type. Notice that the sequence of number 8 and 13 are very similar with each other. The conserved C and H residues that bind a zinc ion were red-colored.
predicted that all the putative zinc finger encoding exons can be possibly spliced with the exon\textsuperscript{78}. Based on this prediction, we designed 13 pairs of primers for PCR to analyze whether these putative zinc finger containing exons are used for alternative splicing. The SW59 and \textit{lola} long were not included for designing primers because their cDNA sequences have been previously reported. The 5' primer was designed in the exon\textsuperscript{78}, which is the same for all of the primer pairs. The 3' primers were designed around the first stop codon of each of the putative zinc finger coding exons and are different from each other. PCR was done with plasmids isolated from a 0-18 hour \textit{Drosophila} yeast two-hybrid library, which contains the cDNA of 0-18 hour embryos. The PCR results showed that number 1, 2, 6, 9, 11, 12.5 exons were possibly spliced with the exon\textsuperscript{78}, which was confirmed by cloning and sequencing the PCR products. In addition to the PCR method, we have acquired EST (expression sequence tag) clones of the \textit{lola} locus from the fly EST stock center. Sequencing these EST clones with a primer in exon\textsuperscript{78}, number 3 and 8 exons were found to be alternatively spliced to encode mRNAs. Taken together, the presence of 10 different alternatively spliced zinc finger exons has been confirmed. 8 of them were found in this study (Fig 19). It is interesting to note that the zinc finger motif is important to determine the DNA binding specificity of these Lola isoforms. Therefore, different Lola isoform can have different DNA binding specificity and regulate different gene(s). However, whether the other five putative zinc finger containing exons, number 4, 7, 10, 11 and 12, are used for alternative splicing still requires further study.

In conclusion, the yeast two-hybrid study with the KDI of JIL-1 led our interest to the \textit{lola} gene. Intrigued by the complicated splicing pattern of \textit{lola}, we have examined the \textit{lola} locus for putative alternative splicing. The existence of at least 10 different zinc finger coding exons from this single gene again stressed that protein diversity can be greatly increased by the alternative splicing mechanism (Claverie, 2001; Graveley, 2001). The
enigmatic 7F1-200 protein can possibly interact with JIL-1 but its molecular nature is still unknown, which needs further study.
CHAPTER 5. CONCLUSIONS

JIL-1 mediates histone H3 Ser10 phosphorylation

The JIL-1 Ser/Thr kinase has high homology with members of the MSK and RSK subfamilies, which are found to be involved in the H3 Ser10 phosphorylation signal transduction pathway in mammalian systems (Sassone-Corsi et al., 1999; Thomson et al., 1999b). The phosphorylation of H3 Ser10 in combination with the acetylation at adjacent lysine residues likely defines the histone code deciphered by component(s) of the transcription complex for gene expression (Cheung et al., 2000b; Clayton et al., 2000). Furthermore, the H3 phosphorylation is also correlated with chromosome condensation and segregation. For example, when the Ser10 phosphorylation is prevented by a S10A substitution in *Tetrahymena*, normal mitotic chromosome segregation behavior is perturbed (Wei et al., 1999). Therefore, the effect of Ser10 phosphorylation is context dependent, i.e., is determined by other modifications of adjacent histone residues and/or by proteins that recognize the phosphorylated Ser10.

The *in-vitro* and *in-vivo* data strongly suggest that JIL-1 is involved in a signal transduction pathway leading to H3 Ser10 phosphorylation. In the *in-vitro* immunocomplex kinase assays, the JIL-1 immunocomplex was able to phosphorylate histone H3, showing preference to the Ser10 site in a synthetic peptide (Fig 6). *In vivo*, the Ser10 phosphorylated H3 isoform was elevated on the male X chromosome and colocalized with JIL-1 (Fig 12). In addition, the H3S10 phosphorylation level is greatly reduced in the third instar larvae of JIL-1 mutant animals, which is mostly comprised of interphase cells. The levels of reduction are correlated with the decreased levels of JIL-1 protein in the mutants. Moreover, the reduced pH3S10 level in the JIL-1 null Z2 can be restored by the introduction of a GFP-JIL-1 transgene.
In many species, there are two enzymatic systems controlling H3 Ser10 phosphorylation during interphase and during mitosis, respectively. We found that mitotic H3 Ser10 phosphorylation was present in the JIL-1 null mutant Z2, therefore, JIL-1 is likely to be mainly involved in the interphase H3S10 phosphorylation pathway. Furthermore, because H3S10 phosphorylation level is reduced to about 5% in the third instar larvae of JIL-1 null Z2, we reasoned that the JIL-1 pathway is the predominant interphase H3S10 phosphorylation pathway.

**JIL-1 is required for maintaining higher order chromatin structure**

In the JIL-1 strong hypomorph Z60 and null Z2, where the JIL-1 protein level is very low, or reduced to zero, the higher order polytene chromosome structure is severely perturbed (Fig 10). Generally, the chromosome arms in JIL-1 mutants are shorter and broader compared with those of wild type. The Hoechst and MSL2 antibody stained banding pattern has largely disappeared. The female X chromosome was affected to the same level as the autosomes. In contrast, the male X chromosome demonstrates higher sensitivity to the decreased level of JIL-1 protein, forming a structure that totally lost the Hoechst and MSL2 staining pattern, suggesting the open male X chromosome is more sensitive to the levels of JIL-1 protein reduction.

In the JIL-1 mutant embryos produced by the homozygous EP3 parents, the rapidly replicating and dividing chromosomes demonstrate various distorted morphologies. The chromosomes in these EP3 embryos were often disintegrated and fragmented, forming clumps. Abnormal microtubule spindles sometimes form on the chromosome remnants in the mutant embryos (Fig 9). Therefore, it seems likely that the loss of JIL-1 can lead to the disruption of both interphase chromatin structure and mitotic chromosome integrity. How could the partial loss of JIL-1 function lead to the formation of these abnormal
chromosome/chromatin structures and mitotic defects? One hypothesis is that the mitotic defects observed are a consequence of abnormal interphase chromosome structure. However, since JIL-1 is associated with the mitotic chromosomes during M phase, other mitotic function of JIL-1 might exist besides its interphase function.

The finding that the structure of all of the chromosomes, including that of male X chromosome and autosomes, is affected in the JIL-1 mutants, suggests that JIL-1 plays a more general role apart from its putative function on the male X chromosome for dosage compensation. This observation is in agreement with the global distribution of JIL-1, i.e., not only elevated on male X chromosome, but also present on the male autosomes and distributed evenly on all of the female chromosomes.

**JIL-1 plays a putative role in dosage compensation**

Previous studies have shown that the level of JIL-1 protein is increased on the male X chromosome and that JIL-1 is colocalized with MSL (male specific lethal) proteins on the male X chromosome (Fig 4; Jin et al., 1999). Furthermore, the *in vitro* protein-protein interaction studies demonstrated there was physical interaction between JIL-1 and components of the MSL dosage compensation complex (Fig 7; Jin et al., 2000), suggesting that JIL-1 may be involved in dosage compensation.

By analyzing and comparing the viability of male and female JIL-1 mutants, we found that the viability of both males and females was reduced in the mutant strains, suggesting JIL-1 has a general function that is required by both sexes (Table 1; Wang et al., 2001). We also found that, of the surviving homozygous JIL-1 progeny, females outnumber males, suggesting that JIL-1 may also play a role in the dosage compensation pathway. This male super sensitivity to JIL-1 function is consistent with the elevated JIL-1 levels on the male X chromosome and the concomitant intensified H3S10 phosphorylation levels on the
male X chromosome (Fig 12). The fact that JIL-1 is essential for the viability of both males and females also explains why JIL-1 was not identified in previous genetic screens looking for a male specific lethal phenotype.

Taking the above three conclusions together, we propose a model that the JIL-1 kinase is involved in a signal transduction pathway that regulates H3 S10 phosphorylation in interphase, which is required for maintaining higher order chromatin structure, and is involved in the dosage compensation pathway. However, the above model does not rule out that JIL-1 may be involved in other signal transduction pathways and can mediate phosphorylation of other substrates.

The *lola* gene locus has a complicated alternative splicing pattern

In the effort to find JIL-1 interacting proteins using the yeast two-hybrid method, an alternative splicing isoform of the *lola* gene, SW59, was identified. *Lola* gene encodes transcription factors with zinc finger motifs, which was reported to regulate nervous system development in early embryos (Giniger et al., 1994). In addition to studying the putative interaction between SW59 and JIL-1, we have found that this gene has a very complicated splicing pattern. Detailed analyses of the genomic sequence of this gene found there are at least fifteen putative zinc finger encoding exons. Thirteen out of the fifteen putative zinc finger exons can encode two zinc finger motifs of the C2HC type and the C2H2 type. The remaining two have conserved sequences for one C2H2 type zinc finger. Using PCR method and sequencing EST clones of the *lola* gene, we found ten out of these fifteen predicted zinc finger exons are actually used for alternative splicing. This suggests that at least ten different zinc finger proteins can be produced from the *lola* gene by alternative splicing. Since zinc fingers can determine the DNA binding specificity of the proteins, these proteins will each have different recognition sequences in terms of DNA binding. The implication of this
finding and other studies of alternative splicing (Schmucker et al., 2000) is that the number of proteins produced by the genome might be much higher than the number of genes. This has raised another important task for the post genomic era, which is to identify all the transcripts produced by a genome.
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## APPENDIX B. SELECTED CONSTRUCTS

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<td>GST-JIL-1</td>
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<td>GST-NTD</td>
<td>GST fusion protein of the N-terminal domain of JIL-1, including residues 1-182</td>
</tr>
<tr>
<td>GST-KDI</td>
<td>GST fusion protein of the kinase domain I of JIL-1, including residues 213-520</td>
</tr>
<tr>
<td>GST-KDII</td>
<td>GST fusion protein of the kinase domain II of JIL-1, including residues 580-880</td>
</tr>
<tr>
<td>GST-CTD</td>
<td>GST fusion protein of the C-terminal domain of JIL-1, including residues 893-1207</td>
</tr>
<tr>
<td>BD-NTD</td>
<td>Two hybrid construct of the N-terminal domain with Gal4-DNA binding domain, including residues 1-182 of JIL-1</td>
</tr>
<tr>
<td>BD-KDI</td>
<td>Two hybrid construct of the kinase domain I with Gal4-DNA binding domain, including residues 213-520 of JIL-1</td>
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<tr>
<td>GST-H1</td>
<td>GST fusion protein of Drosophila histone H1</td>
</tr>
<tr>
<td>GST-H2A</td>
<td>GST fusion protein of Drosophila histone H2A</td>
</tr>
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<td>GST-H2B</td>
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<td>GST-H3</td>
<td>GST fusion protein of Drosophila histone H3</td>
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<td>GST-H4</td>
<td>GST fusion protein of Drosophila histone H4</td>
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<tr>
<td>GST-J1</td>
<td>GST fusion protein of the C-terminal domain of SW59, including residues 426-748</td>
</tr>
<tr>
<td>JIL-1-V5</td>
<td>JIL-1-V5 fusion protein to express in S2 cells, including residues 34-1207 of JIL-1</td>
</tr>
<tr>
<td>BV-JIL-1</td>
<td>GST-JIL-1 fusion protein construct to express JIL-1 in baculovirus system, including residues 34-1207 of JIL-1</td>
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