

The JIL-1 histone H3S10 kinase regulates dimethyl H3K9 modifications and heterochromatic spreading in *Drosophila*

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In this study, we show that a reduction in the levels of the JIL-1 histone H3S10 kinase results in the spreading of the major heterochromatin markers dimethyl H3K9 and HP1 to ectopic locations on the chromosome arms, with the most pronounced increase on the X chromosomes. Genetic interaction assays demonstrated that *JIL-1* functions in vivo in a pathway that includes *Su(var)3-9*, which is a major catalyst for dimethylation of the histone H3K9 residue, HP1 recruitment, and the formation of silenced heterochromatin. We further provide evidence that JIL-1 activity and localization are not affected by the absence of *Su(var)3-9* activity, suggesting that *JIL-1* is upstream of *Su(var)3-9* in the pathway. Based on these findings, we propose a model where JIL-1 kinase activity functions to maintain euchromatic regions by antagonizing *Su(var)3-9*-mediated heterochromatinization.

KEY WORDS: JIL-1 kinase, Chromatin, Histone modifications, HP1, *Drosophila*

INTRODUCTION

Higher order chromatin structure is important for epigenetic regulation and control of gene activation and silencing. In eukaryotes, a considerable proportion of the genome is packaged into constitutive heterochromatin or facultative heterochromatin that represents transiently condensed and silenced euchromatin (Schotta et al., 2003). Formation of heterochromatin and repression of transcription involves covalent modifications of histone tails and/or the exchange of histone variants (Swaminathan et al., 2005). Current evidence suggests that a major pathway in the establishment of heterochromatin is initiated by the RNAi machinery that marks prospective heterochromatic regions (Volpe et al., 2002; Pal-Bhadra et al., 2004; Verdel et al., 2004). This leads to the deacetylation of histone H3K9, followed by the dimethylation of this residue and the recruitment of HP1 (Lachner et al., 2001; Nakayama et al., 2001; Ebert et al., 2004). Thus, dimethylation of histone H3K9 and the presence of HP1 serve as major chromatin modification markers for the presence of transcriptionally silenced chromatin (Fischle et al., 2003; Swaminathan et al., 2005). However, it should be noted that HP1 recently has been demonstrated to also play a role in euchromatic gene regulation that is not linked to histone H3K9 dimethylation (Piacentini et al., 2003; Cryderman et al., 2005).

Ebert et al. (Ebert et al., 2004) recently identified the *Su(var)3-1* mutations as alleles of the *JIL-1* locus that antagonize the expansion of heterochromatin formation in *Drosophila*. JIL-1 is a tandem kinase that localizes specifically to euchromatic interband regions of polytene chromosomes (Jin et al., 1999). Analysis of *JIL-1* null and hypomorphic alleles showed that *JIL-1* is essential for viability, and that reduced levels of JIL-1 protein lead to a global disruption of chromosome structure (Jin et al., 2000; Wang et al., 2001; Zhang et al., 2003; Deng et al., 2005). These defects are correlated with severely decreased levels of histone H3S10 phosphorylation (pH3S10), providing evidence that JIL-1 is the predominant kinase regulating the phosphorylation state of this residue at interphase (Wang et al., 2001).

However, as the *Su(var)3-1* alleles generate proteins with COOH-terminal deletions that are dominant gain-of-function mutations, the experiments of Ebert et al. (Ebert et al., 2004) did not directly address the normal function of JIL-1. In this study, we show that the reduction in JIL-1 protein levels and histone H3S10 phosphorylation caused by hypomorphic or null loss-of-function alleles of the *JIL-1* locus results in the spreading of the major heterochromatin markers dimethyl H3K9 (dmH3K9) and HP1 to ectopic locations on the chromosome arms, with the most pronounced increase on the X chromosomes. Furthermore, genetic interaction assays demonstrated that JIL-1 functions antagonistically to *Su(var)3-9*, which is the major catalyst for dimethylation of the histone H3K9 residue (Schotta et al., 2002). These findings suggest a model where JIL-1 kinase activity functions to mark euchromatic domains, and counteract heterochromatinization and gene silencing at ectopic locations by *Su(var)3-9*-mediated histone H3K9 dimethylation and HP1 recruitment.

MATERIALS AND METHODS

Drosophila stocks

Fly stocks were maintained according to standard protocols (Roberts, 1998). Canton-S was used for wild-type preparations. The *JIL-1²²*, *JIL-1²⁶⁰* and *JIL-1⁶⁹* alleles have been described by Wang et al. (Wang et al., 2001) and by Zhang et al. (Zhang et al., 2003). The GFP-lacI transgenic stock 128.1, and the *lac* operator repeats transgenic stock 4D5 were the generous gifts of Dr L. Wallrath (University of Iowa, Iowa City). The application of the GFP-lacI and *lac* operator repeat stocks for X chromosome identification in *JIL-1* mutant backgrounds is described by Deng et al. (Deng et al., 2005). *Su(var)3-1³/TM3 Sb Ser* stocks were obtained from the Bloomington Stock Center, whereas the *Su(var)3-9¹* and *Su(var)3-9²* stocks were from the Umeå Stock Center. The molecular lesion of *Su(var)3-1³* was determined by PCR mapping and sequencing, as described by Zhang et al. (Zhang et al., 2003). Recombinant *JIL-1²⁶⁰ Su(var)3-9¹* chromosomes were identified by generating recombinants as described by Ji et al. (Ji et al., 2005), except that the dominant *Su(var)3-9¹* phenotype was selected for in a *w^{msd}* background, and the presence of *JIL-1²⁶⁰* was confirmed by PCR as described by Zhang et al. (Zhang et al., 2003). Balancer chromosomes and markers were as described by Lindsley and Zimm (Lindsley and Zimm, 1992).

Immunohistochemistry

Third instar salivary gland 'smush' preparations of polytene nuclei were prepared essentially as described by Wang et al. (Wang et al., 2001). Polytene chromosome squash preparations were performed as described

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by Kelley et al. (Kelley et al., 1999), using the 5-minute fixation and antibody-labeling protocol described by Jin et al. (Jin et al., 1999). Primary antibodies used include: affinity-purified Hope rabbit antiserum raised against JIL-1 residues 886-1013 (Jin et al., 1999); anti-tubulin mAb (Sigma); histone H3 goat antiserum (Santa Cruz); phospho-histone H3S10 rabbit antiserum (Upstate Biotechnology); dmH3K9 rabbit antiserum (Upstate Biotechnology); anti-HP1 mAb C1A9 (Developmental Studies Hybridoma Bank, University of Iowa); anti-MSL-1 rabbit antiserum [generous gift of Drs M. Kuroda (Harvard Medical School, Boston, MA) and R. Kelley (Baylor College of Medicine, Houston)]; and anti-GFP chicken IgY (Aves Laboratory). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype-specific Texas Red-, TRITC- and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. The final preparations were mounted in 90% glycerol containing 0.5% *n*-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a high resolution Spot CCD camera. Confocal microscopy was performed with a Leica confocal TCS NT microscope system equipped with separate Argon-UV, Argon and Krypton lasers, and the appropriate filter sets for Hoechst, FITC, Texas Red and TRITC imaging. A separate series of confocal images for each fluorophore of double-labeled preparations were obtained simultaneously with *z*-intervals of, typically, 0.5 μ m, using a PL APO 100 \times /1.40-0.70 oil objective. A maximum projection image for each of the image stacks was obtained using the ImageJ software (<http://rsb.info.nih.gov/ij/>). In some cases, individual slices or projection images from only two to three slices were obtained. Images were imported into Adobe PhotoShop, where they were pseudocoloured, image processed and merged. In some images, non-linear adjustments were made for optimal visualization of the Hoechst labeling of chromosomes.

Immunoblot analysis

Immunoblot analysis was performed as described by Wang et al. (Wang et al., 2001) and Zhang et al. (Zhang et al., 2003), using extracts from third-instar larvae of the specified genotype. For the quantification of immunolabeling, digital images of exposures of immunoblots on Biomax ML film (Kodak) were analyzed using the ImageJ software, as previously described (Wang et al., 2001). In these images, the grayscale was adjusted such that only a few pixels in the wild-type lanes were saturated. The area

of each band was traced using the outline tool and the average pixel value determined. Levels in mutant larvae were determined as a percentage relative to the level determined for wild-type control larvae, using tubulin or histone H3 levels as a loading control.

RESULTS

Decreased JIL-1 activity results in ectopic spreading of histone H3K9 dimethylation and HP1

To investigate the distribution of heterochromatin in *JIL-1* mutant backgrounds, we labeled *Drosophila* polytene squash preparations with an antibody specific to the histone H3K9 dimethylation (dmH3K9) heterochromatin marker (Jacobs et al., 2001; Delattre et al., 2004; Ebert et al., 2004) (Fig. 1A-D). In wild-type preparations, histone H3K9 dimethylation is found at the chromocenter, in a banded pattern on the fourth chromosome, and in a few locations on the chromosome arms (Delattre et al., 2004; Ebert et al., 2004) (Fig. 1A). However, in the *JIL-1²/JIL-1^{h9}* mutant background there was a striking spreading of histone H3K9 dimethylation to the chromosome arms that was most pronounced on the X chromosome (Fig. 1B). The same distribution was also observed in squash preparations from both male and female *JIL-1²* homozygous null larvae (Fig. 1C,D). In addition to the changes in the distribution of the histone H3K9 dimethylation marker, *JIL-1* mutant polytene chromosomes exhibit a range of morphological defects, as previously described by Wang et al. (Wang et al., 2001) and Deng et al. (Deng et al., 2005). There is a shortening and folding of the chromosomes, with a non-orderly intermixing of euchromatin and the compacted chromatin characteristic of banded regions (Fig. 1B-D). The extreme of this phenotype is exhibited by the male X polytene chromosome, where no remnants of coherent banded regions can be observed (Deng et al., 2005) (Fig. 1B,C).

In order to determine the degree of change in the levels of chromatin modification markers in *JIL-1* mutant backgrounds, we analyzed immunoblots of protein lysates from wild-type, *JIL-1²/JIL-1^{h9}* and *JIL-1²/JIL-1^{z2}* third instar larvae. The immunoblots were probed with anti-phH3S10, anti-dmH3K9, anti-histone H3 and anti-tubulin antibodies. As illustrated in Fig. 1E, both *JIL-1²/*

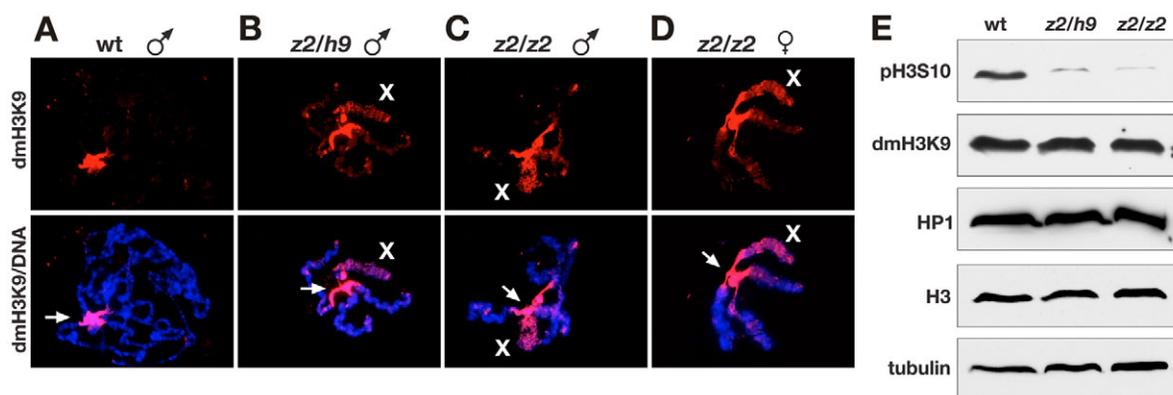


Fig. 1. Histone H3K9 dimethylation spreads to ectopic locations in *JIL-1* null and hypomorphic mutant backgrounds. (A-D) Polytene squash preparations labeled with anti-dmH3K9 antibody (red) and with Hoechst (DNA, blue). The upper panels show the anti-dmH3K9 labeling alone, whereas the lower panels show the composite images with the Hoechst labeling. The X chromosomes are indicated by X; the chromocenter with an arrow. In wild-type (wt) preparations, dmH3K9 labeling is mainly localized to the chromocenter (A). In *JIL-1²/JIL-1^{h9}* (*z2/h9*, B) and in *JIL-1²* homozygous (*z2/z2*) male (C) and female (D) mutants, the dmH3K9 labeling is clearly upregulated on the X chromosomes and to a lesser extent on the autosomes. In the *JIL-1* mutants the morphology of the polytene chromosomes is perturbed with coiled and compacted chromosome arms. (E) The level of histone H3S10 phosphorylation is greatly decreased in *JIL-1* null and hypomorphic alleles, whereas the levels of dmH3K9, HP1, histone H3 and tubulin are comparable to wild type. Immunoblots were performed on extracts from wild-type (wt), *JIL-1²/JIL-1^{h9}* (*z2/h9*) and *JIL-1²* homozygous (*z2/z2*) larvae. The immunoblots were labeled with anti-phospho-histone H3S10 (pH3S10), anti-dmH3K9, anti-HP1, anti-histone H3 (H3) and anti-tubulin antibodies.

JIL-1^{h9} (15.2±4.0%, n=4) and *JIL-1^{z2}/JIL-1^{z2}* (9.4%±5.0%, n=6) mutants showed greatly reduced levels of phosphorylated histone H3S10, when compared with that in wild-type larvae, indicating that *JIL-1^{z2}/JIL-1^{h9}* has the properties of a strong hypomorph. By contrast, the levels of both dmH3K9 and HP1 were at or near wild-type levels (Fig. 1E). In the *JIL-1^{z2}/JIL-1^{z2}* null background, the level of dmH3K9 was at 109.0±31.0% (n=9) of the wild-type level, and HP1 was at 99.3±17.8% (n=9). Thus, in *JIL-1* mutant backgrounds the level of phosphorylated histone H3S10 was dramatically reduced, whereas the levels of the heterochromatin markers dmH3K9 and HP1 were unaffected.

It has been demonstrated that dimethylation of histone H3K9 leads to the recruitment of HP1, and that these two markers are colocalized at heterochromatic sites (Lachner et al., 2001; Nakayama et al., 2001; Schotta et al., 2002). We therefore performed double labeling with antibodies to these markers to determine whether HP1 also spreads to the same ectopic locations as does dmH3K9 in *JIL-1* mutant backgrounds. For these experiments, we used the polytene salivary gland ‘smush’ preparation that employs the modified whole-mount staining technique first described by Wang et al. (Wang et al., 2001). This preparation does not afford the resolution of regular squash preparations; however, it has an advantage in that it avoids the acetic acid treatment, which, in our experience, can adversely affect the quality and reproducibility of antibody labeling. Fig. 2A,B show polytene nuclei from male and female wild-type and *JIL-1^{z2}/JIL-1^{z2}* third instar larvae labeled with anti-HP1 and anti-dmH3K9 antibodies, and with Hoechst to visualize the DNA. In wild-type nuclei, the dmH3K9 marker and HP1 were colocalized, with the labeling largely confined to the chromocenter, whereas in *JIL-1^{z2}/JIL-1^{z2}* mutants both markers had spread to ectopic locations in an overlapping pattern, with the highest levels being found on the X chromosomes. To verify that it indeed was the X chromosome that showed the most pronounced increase, we performed double labeling with anti-HP1 and anti-MSL-1 antibodies. MSL-1 is a member of the MSL (male specific lethal) dosage compensation complex and is found only on the male X chromosome (Kuroda et al., 1991; Kelly and Kuroda, 1995). Fig. 2C shows that, apart from the chromocenter, there is no significant level of HP1 on the wild-type male X chromosome, whereas it is clearly distributed on the male X chromosome in *JIL-1^{z2}/JIL-1^{z2}* mutant nuclei. In order to verify the distinct increase on the female X chromosome in *JIL-1^{z2}/JIL-1^{z2}* mutants, we labeled polytene squash preparations from the transgenic line 4D5 (Danzon and Wallrath, 2004), which contains *lac* operator repeats integrated onto the X chromosome that can be detected by GFP-lacI binding (Belmont, 2001; Deng et al., 2005), with anti-dmH3K9 and anti-GFP antibodies. The labeling shown in Fig. 2D demonstrates that the ectopic spreading of dmH3K9 in females is preferentially located on the X chromosome.

JIL-1^{Su(var)3-1} alleles act differently from *JIL-1* loss-of-function alleles

Ebert et al. (Ebert et al., 2004) recently demonstrated that *JIL-1^{Su(var)3-1}* alleles are dominant gain-of-function mutations that are likely to antagonize the expansion of heterochromatin. However, in contrast to *JIL-1* loss-of-function alleles, *JIL-1^{Su(var)3-1}* homozygous mutants exhibit no change in the distribution of the heterochromatin marker dmH3K9 or in histone H3S10 phosphorylation (Ebert et al., 2004). We confirmed these results in heteroallelic mutants with only one copy of *JIL-1^{Su(var)3-1[3]}* together with the null *JIL-1^{z2}* allele (Fig. 3). The *JIL-1^{Su(var)3-1[3]}*

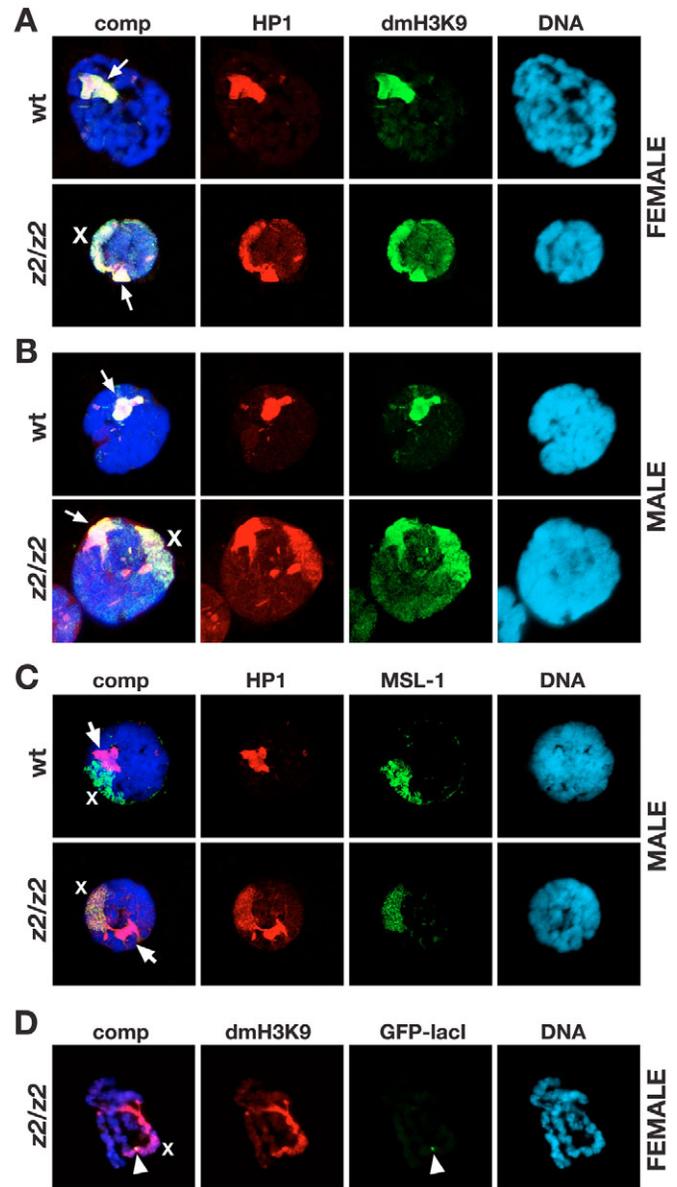


Fig. 2. HP1 and dmH3K9 are upregulated on both male and female X chromosomes in *JIL-1* null mutants. (A–C) Confocal images of whole-mount polytene nuclei from male and female third instar larvae. The X chromosome is indicated by X; the chromocenter by an arrow. (A,B) Triple labeling with anti-HP1 (red) and anti-dmH3K9 (green) antibodies, and with Hoechst (DNA, blue), of female (A) and male (B) wild-type (wt) and *JIL-1^{z2}* homozygous (*z2/z2*) mutant polytene nuclei. (A) In the female, both HP1 and dmH3K9 are upregulated on the X chromosome, and to a lesser degree on the autosomes in *JIL-1^{z2}* homozygous nuclei. (B) In the male, both HP1 and dmH3K9 are upregulated on the X chromosome, and to a lesser degree on the autosomes in *JIL-1^{z2}* homozygous nuclei. (C) Triple labeling with anti-HP1 (red) and anti-MSL1 (green) antibodies, and with Hoechst (DNA, blue), of male wild-type (wt) and *JIL-1^{z2}* homozygous (*z2/z2*) mutant polytene nuclei. MSL-1 labeling identifies the male X chromosome. (D) Identification of the female X chromosome by a GFP-lacI fusion protein targeted to 256 *lacO* repeats inserted into the 4D5 interband region of polytene X chromosomes in a *JIL-1^{z2}* homozygous (*z2/z2*) mutant. The preparation was triple labeled with anti-dmH3K9 (red) and anti-GFP (green) antibodies, and with Hoechst (DNA, blue). The position of the *lacO* repeats identifying the X chromosome is indicated (arrowhead). Composite images (comp) are shown to the left.

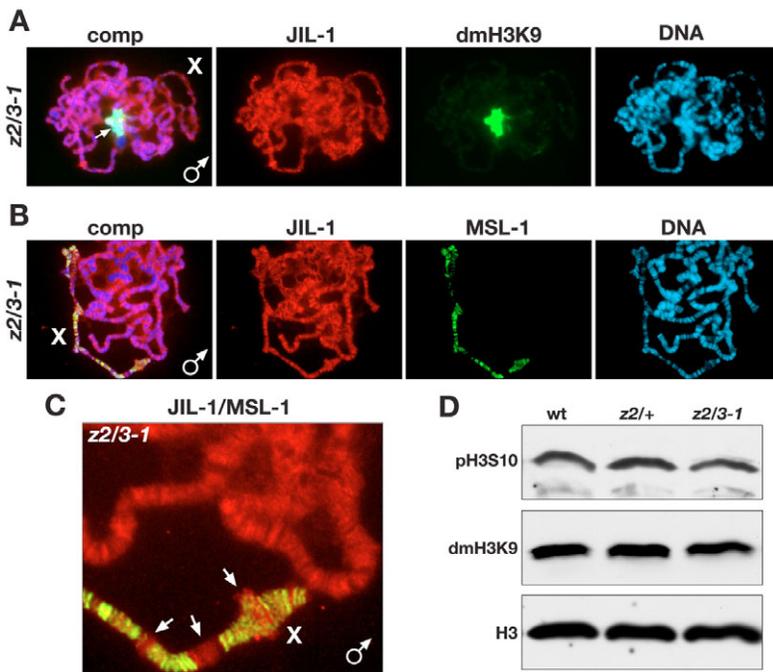


Fig. 3. The $JIL-1^{Su(var)3-1[3]}$ protein is mislocalized but does not affect dmH3K9 distribution or levels of histone H3S10 phosphorylation. (A, B) Triple labeling with anti-JIL-1 (red) and anti-dmH3K9 (A) or anti-MSL-1 (B) (green) antibodies, and with Hoechst (DNA, blue), of a male $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ polytene squash preparation. The X chromosome is indicated by X; the chromocenter by an arrow. (A) The dmH3K9 antibody labeling is mainly localized to the chromocenter. (B) The $JIL-1^{Su(var)3-1[3]}$ protein is not upregulated on the X chromosome, which is identified by MSL-1 antibody labeling. (C) Higher magnification image of the preparation in B, showing the relative distribution of JIL-1 (red) and MSL-1 (green) antibody labeling. The $JIL-1^{Su(var)3-1[3]}$ protein is localized to the chromosomes, but the antibody labeling is diffuse and associates with both interband and banded regions. The arrows indicate regions on the X chromosome where the $JIL-1^{Su(var)3-1[3]}$ protein is localized to ectopic locations not overlapping with MSL-1 labeling. (D) The level of histone H3S10 phosphorylation in $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ heteroallelic mutants is comparable to that in $JIL-1^{z2}/+$ heteroalleles. Immunoblots were performed on extracts from wild-type (wt), $JIL-1^{z2}/+$ ($z2/+$) and $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ ($z2/3-1$) third instar larvae. The immunoblots were labeled with anti-phospho-histone H3S10 (pH3S10), anti-dmH3K9 and anti-histone H3 (H3) antibodies.

allele is a point mutation that introduces a premature in-frame stop codon, leading to a JIL-1 protein with a 166-amino acid truncation of the COOH-terminal domain. In order to determine the distribution of the truncated $JIL-1^{Su(var)3-1[3]}$ protein in relation to other chromatin constituents, we double-labeled polytene squashes from $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ larvae with either anti-dmH3K9 or anti-MSL-1 antibody, and with anti-JIL-1 antibody (Fig. 3A-C). Fig. 3A shows that one copy of the $JIL-1^{Su(var)3-1[3]}$ allele is sufficient to prevent ectopic spreading of the dmH3K9 marker, which is localized at the chromocenter as in wild-type preparations. Interestingly, one copy of the $JIL-1^{Su(var)3-1[3]}$ allele is also sufficient to largely rescue the abnormal chromosome morphology of $JIL-1$ null mutants, including the male X chromosome. This is despite the finding that the $JIL-1^{Su(var)3-1[3]}$ protein is mislocalized on the chromosomes when compared with wild-type JIL-1. Fig. 3A,B shows that although the $JIL-1^{Su(var)3-1[3]}$ protein is associated with the chromosomes, the antibody labeling is diffuse, does not define clear banded regions, and overlaps with Hoechst-labeled banded regions, a distribution that was never observed for the wild-type JIL-1 protein (Jin et al., 1999; Wang et al., 2001). Furthermore, the $JIL-1^{Su(var)3-1[3]}$ protein is not upregulated on the male X chromosome (Fig. 3B) and is present along the chromosome arms at ectopic locations. This is shown at higher magnification in Fig. 3C, in a double labeling with JIL-1 and MSL-1 antibodies. In wild-type preparations, MSL-1 and JIL-1 are colocalized on the male X chromosome (except for the telomere) (Jin et al., 2000); however, as indicated by the arrows in Fig. 3C, the $JIL-1^{Su(var)3-1[3]}$ protein is associated with many regions of the X chromosome not labeled by MSL-1 antibody. Immunoblot analysis of protein lysate from wild-type, $JIL-1^{z2}/+$ and $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ larvae show that the level of histone H3S10 phosphorylation in $JIL-1^{z2}/JIL-1^{Su(var)3-1[3]}$ mutants is comparable to that of $JIL-1^{z2}/+$ larvae, suggesting that $JIL-1^{Su(var)3-1[3]}$ mutants have normal JIL-1 kinase activity (see also Ebert et al., 2004). Taken together, these data suggest that the COOH-terminal domain of JIL-1 is required for proper

chromosome localization, and indicate that the dominant gain-of-function effect of the $JIL-1^{Su(var)3-1[3]}$ allele (Ebert et al., 2004) may be attributable to JIL-1 kinase activity at ectopic locations.

JIL-1 functions in the same pathway as *Su(var)3-9*, which encodes the major histone methyltransferase for histone H3K9 dimethylation in *Drosophila*

Su(var)3-9, a histone methyltransferase (Rea et al., 2000; Shotta et al., 2002), *Su(var)2-5*, HP1 (Eissenberg and Elgin, 2000), and *Su(var)3-7* (Delattre et al., 2004), a 1169 amino acid zinc-finger protein, are all inherent components of heterochromatin in *Drosophila* (Ebert et al., 2004). These proteins physically interact and in a genetic hierarchy *Su(var)3-9* is epistatic to the other two genes (Schotta et al., 2002; Ebert et al., 2004). Furthermore, *Su(var)3-9* has been shown to catalyze most of the dimethylation of the histone H3K9 residue (Schotta et al., 2002). We therefore performed immunolocalization and genetic interaction studies with the *Su(var)3-9¹* and *Su(var)3-9²* loss-of-function alleles (Reuter et al., 1986; Tschiersch et al., 1994) in order to determine whether *Su(var)3-9* also controls *JIL-1* activity. However, in polytene squashes from *Su(var)3-9¹/Su(var)3-9²* heteroallelic larvae, JIL-1 localization in both male and female chromosomes was indistinguishable from that observed in wild-type chromosomes (Fig. 4A-D). By immunoblot analysis of protein extracts from *Su(var)3-9¹/Su(var)3-9²* heteroallelic larvae, we verified that the dmH3K9 level was dramatically reduced (Fig. 4E). The data further indicate that histone H3S10 phosphorylation is at wild-type levels in this mutant background (Fig. 4E). These results suggest that JIL-1 activity and localization are not affected by the absence of *Su(var)3-9* activity, which is in contrast to the finding that the distribution of *Su(var)3-9* methyltransferase activity is altered in *JIL-1* null or hypomorphic mutants.

To determine whether *Su(var)3-9* and JIL-1 genetically interact in the same pathway in vivo, we explored interactions between mutant alleles of *Su(var)3-9* and *JIL-1* by generating double-mutant individuals. Because *Su(var)3-9* and *JIL-1* are both located on the

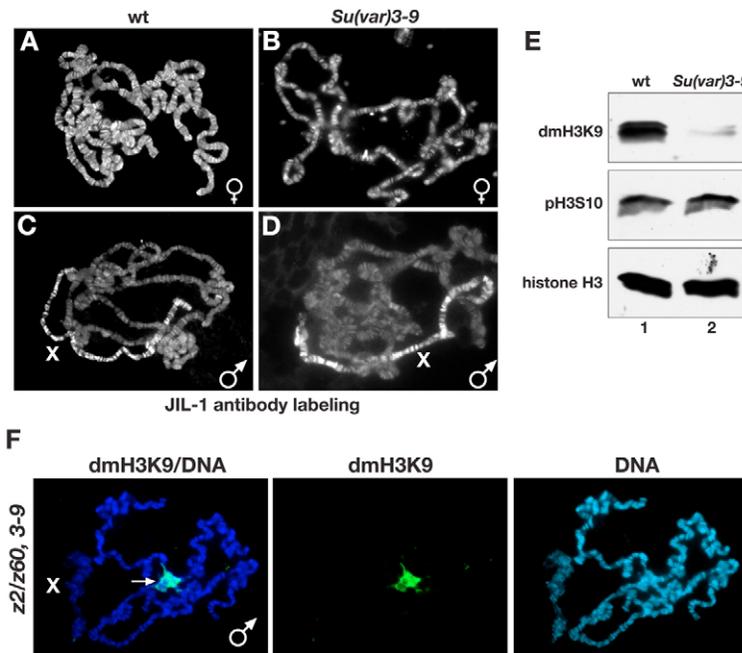


Fig. 4. JIL-1 localization and kinase activity is not affected in *Su(var)3-9* mutants.

(A–D) Polytene squash preparations from male and female wild-type (wt) and *Su(var)3-9¹/Su(var)3-9²* (*Su(var)3-9*) third instar larvae labeled with JIL-1 antibody. Chromosome morphology and JIL-1 localization in *Su(var)3-9¹/Su(var)3-9²* preparations were similar to in wild-type preparations. (E) dmH3K9 levels were greatly reduced in *Su(var)3-9¹/Su(var)3-9²* mutants, whereas histone H3S10 phosphorylation was at wild-type levels. Immunoblots were performed on extracts from wild-type (wt) and *Su(var)3-9¹/Su(var)3-9²* (*Su(var)3-9*) third instar larvae. The immunoblots were labeled with anti-dmH3K9, anti-phospho-histone H3S10 (pH3S10) and anti-histone H3 antibodies. (F) dmH3K9 did not spread from the chromocenter to ectopic locations in polytene squashes from *JIL-1^{z60}/JIL-1^{z60} Su(var)3-9¹* (*JIL-1^{z60}, 3-9*) larvae. The preparation was labeled with anti-dmH3K9 antibody (green) and Hoechst (DNA, blue). The X chromosome is indicated by X; the chromocenter by an arrow.

third chromosome, we first recombined the *Su(var)3-9¹* allele onto the *JIL-1^{z60}* chromosome. Subsequently, *JIL-1^{z60} Su(var)3-9¹/TM6 Sb Tb* males were crossed with *JIL-1^{z2}/TM6 Sb Tb* virgin females generating *JIL-1^{z2}/JIL-1^{z60} Su(var)3-9¹* progeny, identified as non-*Sb*. In control experiments in which *Su(var)3-9* activity was not altered, we crossed *JIL-1^{z60}/TM6 Sb Tb* males with *JIL-1^{z2}/TM6 Sb Tb* virgin females generating *JIL-1^{z2}/JIL-1^{z60}* progeny. *JIL-1^{z2}* is a null allele and *JIL-1^{z60}* is a strong hypomorph producing less than 0.3% of the levels of JIL-1 wild-type protein, resulting in semi-lethality of this heteroallelic combination (Wang et al., 2001; Zhang et al., 2003). Consequently, in the control crosses, we observed only one fly of the *JIL-1^{z2}/JIL-1^{z60}* genotype out of a total of 871 eclosed flies. However, in the double mutant combination (*JIL-1^{z2}/JIL-1^{z60} Su(var)3-9¹*) with one copy of the *Su(var)3-9¹* allele, the number of surviving flies with the *JIL-1^{z2}/JIL-1^{z60}* genotype increased dramatically to 243 out of a total of 789 eclosed flies. In this cross, one-third of the eclosed flies would be expected to be of the *JIL-1^{z2}/JIL-1^{z60} Su(var)3-9¹* genotype, assuming full rescue, indicating that the reduction of *Su(var)3-9* activity in these animals resulted in a 92% viability rate compared with a rate of 0.3% for *JIL-1^{z2}/JIL-1^{z60}* flies without the reduction in *Su(var)3-9* activity. Consistent with the observed rescue of viability, there was no detectable spreading of histone H3K9 dimethylation to ectopic locations in polytene squashes from *JIL-1^{z2}/JIL-1^{z60} Su(var)3-9¹* larvae, and the morphology of the polytene chromosomes was markedly improved (Fig. 4F).

Taken together, these results suggest that *JIL-1* is in the same pathway as *Su(var)3-9* in vivo, and that *JIL-1* functions to establish or maintain euchromatic chromosome regions by antagonizing heterochromatization mediated by *Su(var)3-9*-catalyzed dimethylation of histone H3K9. Furthermore, the data indicate that the lethality, as well as some of the chromosome morphology defects, observed in *JIL-1* null or hypomorphic mutant backgrounds may be the result of ectopic *Su(var)3-9* activity.

DISCUSSION

In this study, we show that a reduction in the levels of the JIL-1 histone H3S10 kinase results in the spreading of the major heterochromatin markers dmH3K9 and HP1 to ectopic locations on

the chromosome arms, with the most pronounced increase being observed on the X chromosomes. However, overall levels of the dmH3K9 marker and HP1 were unchanged, suggesting that the spreading is accompanied by a reduction in the levels of pericentromeric heterochromatin in *JIL-1* hypomorphic mutant backgrounds. Genetic interaction assays demonstrated that *JIL-1* functions in vivo in a pathway with *Su(var)3-9*, which is a major catalyst for dimethylation of the histone H3K9 residue, HP1 recruitment, and formation of silenced heterochromatin (Schotta et al., 2002). We further provide evidence that *JIL-1* activity and localization are not affected by the absence of *Su(var)3-9* activity, suggesting that *JIL-1* is upstream of *Su(var)3-9* in this pathway. Based on these findings, we propose a model where *JIL-1* functions in a novel pathway to establish or maintain euchromatic regions by antagonizing *Su(var)3-9*-mediated heterochromatization.

According to the histone code hypothesis (Strahl and Allis, 2000) and the recently proposed binary switch model (Fischle et al., 2003), phosphorylation of a site adjacent to a methyl mark that engages an effector molecule may regulate its binding. JIL-1 phosphorylates the histone H3S10 residue in euchromatic regions of polytene chromosomes (Jin et al., 1999; Wang et al., 2001), raising the possibility that this phosphorylation at interphase prevents the recruitment of *Su(var)3-9* and/or the dimethylation of the neighboring K9 residue. This, in turn, would affect the binding of HP1, thus antagonizing the formation of silenced heterochromatin at interbands. That different regions of chromatin may have different combinations of posttranslational modifications controlling effector/histone interactions, as predicted by the histone code hypothesis (Strahl and Allis, 2000), is underscored by the finding that, in *JIL-1* null backgrounds, the level of the dmH3K9 marker and HP1 are preferentially increased on the male and female X chromosomes. It is well documented that the male X chromosome is unique because of the activity of the MSL dosage compensation complex and the MOF histone acetyltransferase, which leads to hyperacetylation of histone H4 (Bone et al., 1994; Hilfiker et al., 1997). However, comparable markers for the female X chromosome have yet to be discovered, and our results are the first indication that markers may exist that distinguish male and female X chromosomes

from autosomes, and that this difference may increase the affinity for Su(var)3-9. That the spreading of heterochromatic markers in the absence of JIL-1 occurs on both the male and female X chromosome further indicates that these changes are independent of dosage compensation processes.

Unfortunately, in this study, we could not directly address the possibility that the observed spreading of heterochromatin markers occurred preferentially to specific euchromatic sites. In *JIL-1* null and hypomorphic backgrounds, chromosome morphology is greatly perturbed, and there is an intermixing not only of euchromatin and the compacted chromatin characteristic of banded regions, but also of non-homologous chromatid regions, which become fused and confluent (Deng et al., 2005). Thus, we cannot rule out as an alternative hypothesis that JIL-1 activity may regulate boundary elements (West et al., 2002) that control the spreading of heterochromatic factors, or that the two mechanisms may act in concert. However, the spreading of the dmH3K9 marker and HP1 to ectopic locations on the chromosomes is likely to lead to heterochromatinization and repression of gene expression at these sites. Our results further suggest the possibility that the lethality of *JIL-1* null mutants may be due to the repression of essential genes at these ectopic sites as a consequence of the spreading of Su(var)3-9 activity. This hypothesis is supported by genetic interaction assays that demonstrated that the lethality of a severely hypomorphic *JIL-1* heteroallelic combination could be almost completely rescued by a reduction in *Su(var)3-9* dosage that prevented the ectopic dimethylation of histone H3K9.

It has recently been demonstrated that the *Su(var)3-1* alleles of *JIL-1* consist of dominant gain-of-function alleles that antagonize the expansion of heterochromatin formation (Ebert et al., 2004). However, we provide evidence that the underlying molecular mechanism of this antagonism is different from that occurring in the loss-of-function null and hypomorphic *JIL-1* alleles described in this study. *JIL-1^{Su(var)3-1}* alleles are characterized by deletions of the COOH-terminal domain that do not affect JIL-1 kinase activity or the spreading of heterochromatin markers (Ebert et al., 2004) (this study). Furthermore, our results indicate that the COOH-terminal domain of JIL-1 is required for proper chromosomal localization and that JIL-1^{Su(var)3-1} proteins are mislocalized to ectopic chromosome sites. Thus, we propose that the dominant gain-of-function effect of the *JIL-1^{Su(var)3-1}* alleles may be attributable to JIL-1 kinase activity at ectopic locations, possibly through the phosphorylation of novel target proteins (see also Ebert et al., 2004), or by mis-regulated localization of the phosphorylated histone H3S10 marker. Although the JIL-1^{Su(var)3-1} proteins are mislocalized, they still associate with chromosomes and phosphorylate the histone H3S10 residue, suggesting that other regions of the protein have a binding affinity for at least some of the substrates and interaction partners of JIL-1. This is supported by the finding that each of the two kinase domains of JIL-1 can interact with the MSL-complex in vitro (Jin et al., 2000).

In summary, we provide evidence that the JIL-1 kinase is a major regulator of histone modifications that affect gene activation, gene silencing and chromatin structure. Thus, it will be informative in future experiments to further explore the interaction of *JIL-1* with genes controlling heterochromatin formation, in order to gain a better understanding of the molecular mechanisms of epigenetic gene regulation.

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