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RESEARCH ARTICLE

Histone H3S10 phosphorylation by the JIL-1 kinase in pericentric heterochromatin and on the fourth chromosome creates a composite H3S10phK9me2 epigenetic mark

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Abstract The JIL-1 kinase mainly localizes to euchromatic interband regions of polytene chromosomes and is the kinase responsible for histone H3S10 phosphorylation at interphase in *Drosophila*. However, recent findings raised the possibility that the binding of some H3S10ph antibodies may be occluded by the H3K9me2 mark obscuring some H3S10 phosphorylation sites. Therefore, we have characterized an antibody to the epigenetic H3S10phK9me2 double mark as well as three commercially available H3S10ph antibodies. The results showed that for some H3S10ph antibodies their labeling indeed can be occluded by the concomitant presence of the H3K9me2 mark. Furthermore, we demonstrate that the double H3S10phK9me2 mark is present in pericentric heterochromatin as well as on the fourth chromosome of wild-type polytene chromosomes but not in preparations from *JIL-1* or *Su(var)3-9* null larvae. Su(var)3-9 is a methyltransferase mediating H3K9 dimethylation. Furthermore, the H3S10phK9me2 labeling overlapped with that of the non-occluded H3S10ph antibodies as well as with H3K9me2 antibody labeling. Interestingly, when a Lac-I-Su(var)3-9 transgene is overexpressed, it upregulates H3K9me2 dimethylation on the chromosome arms creating extensive ectopic H3S10phK9me2 marks suggesting that the H3K9 dimethylation occurred at euchromatic H3S10ph sites. This is further supported by the finding that under these conditions euchromatic H3S10ph labeling by the occluded antibodies was abolished. Thus, our findings indicate a novel role for the JIL-1 kinase in epigenetic regulation of heterochromatin in the context of the chromocenter and fourth chromosome by creating a composite H3S10phK9me2 mark together with the Su(var)3-9 methyltransferase.

Keywords JIL-1 kinase · H3S10 phosphorylation · H3K9 dimethylation · Heterochromatin · Fourth chromosome · Occluded antibody · *Drosophila*

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Introduction

JIL-1 is the kinase responsible for histone H3S10 phosphorylation (Jin et al., 1999; Wang et al., 2001; Li et al., 2013) whereas Su(var)3-9 is a methyltransferase mediating H3K9 dimethylation (Schotta et al., 2002) at interphase in *Drosophila*. Mutational analyses have shown that *JIL-1* is essential for viability (Wang et al., 2001; Zhang et al., 2003) and that a reduction in JIL-1 kinase activity leads to a global disruption of polytene chromosome morphology (Wang et al., 2001; Deng et al., 2005). Furthermore, evidence has been presented suggesting that H3S10 phosphorylation functions to indirectly regulate transcription by counteracting H3K9 dimethylation and gene silencing (Zhang et al., 2006; Deng et al., 2010; Wang et al., 2011a; 2011b; 2012). Antibody labeling studies have indicated that H3S10 phosphorylation by the JIL-1 kinase mainly occurs at euchromatic interband regions of polytene chromosomes and is enriched about two fold on the male X-chromosome (Jin et al., 1999; 2000; Wang et al., 2001). However, a recent survey of commercially available H3S10ph antibodies suggested that some of these antibodies, in contrast to previously used antibodies, could recognize the H3S10ph mark in pericentric heterochromatin and on the fourth chromosome in addition to in the euchromatic

interbands (Cai et al., 2008). This raised the possibility that the binding of some H3S10ph antibodies may be occluded by the presence of the H3K9me2 mark. In this study, using an antibody to the double H3S10phK9me2 mark, we demonstrate that this mark indeed is present in pericentric heterochromatin as well as on the fourth chromosome of wild-type polytene chromosomes with little or no labeling detectable on the chromosome arms. Thus, taken together, our data imply the existence of a novel mechanism for regulating the interactions between kinase and methyltransferase activity in the context of pericentric heterochromatin and the fourth chromosome that promotes creation of the double H3S10phK9me2 mark in contrast to on the chromosome arms where the single marks are likely to reside on separate histone tails.

Materials and methods

Drosophila melanogaster stocks

Fly stocks were maintained at 25 °C according to standard protocols (Roberts 1998) and Canton S was used for wild-type preparations. The *JIL-1*^{z2} null allele is described in Wang et al. (2001) as well as in Zhang et al. (2003). The *Su*(*var*)*3-9*^{06} null allele is described in Schotta et al. (2002). The *LacI-JIL-1-ΔCTD* transgenic fly line is described in Li et al. (2013) and the *LacI-Su*(*var*)*3-9* line in Boeke et al. (2010) with expression driven using the *Sgs3-GAL4* driver (obtained from the Bloomington Stock Center) introduced by standard genetic crosses.

Immunohistochemistry

Standard polytene chromosome squash preparations were performed as in Cai et al. (2010) using 1- or 5-min fixation protocols, and acid-free squash preparations were done following the procedure of DiMario et al. (2006). Antibody labeling of these preparations was performed as described in Johansen and Johansen (2003) and in Johansen et al. (2009). Primary antibodies used in this study include rabbit anti-H3S10ph (Epitomics, Active Motif, and Cell Signaling), mouse anti-H3S10phK9me2 (Millipore), rabbit anti-H3K9me2 (Millipore), mouse anti-H3K9me2 (Abcam), rabbit anti-histone H3 (Cell Signaling), rabbit anti-JIL-1 (Jin et al., 1999), and chicken anti-JIL-1 (Jin et al., 2000). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype-specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. The final preparations were mounted in 90 % glycerol containing 0.5 % *n*-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a high resolution Spot CCD camera. Confocal microscopy was performed with a Leica confocal TCS SP5 microscope system using a PL APO 63X/1.40 oil objective. Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images, nonlinear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes.

Immunoblot analysis

Protein extracts were prepared from dissected third instar larval salivary glands homogenized in a buffer containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2 % Triton X-100, 0.2 % NP-40, 2 mM Na_3VO_4, 1 mM PMSF, and 1.5 μg/ml aprotinin. Proteins were separated by SDS-PAGE and immunoblotted according to standard procedures (Sambrook and Russell (2001)). For these experiments we used the Bio-Rad Mini PROTEAN III system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse, anti-chicken or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3,000) for visualization of primary antibody. Antibody labeling was visualized and digitized using a ChemiDoc-It®TS2 Imager (UVP,LCC).

Results and discussion

The aim of this study was to re-examine H3S10 phosphorylation in interphase polytene chromosome preparations in the context of determining the distribution of the H3S10phK9me2 double epigenetic mark. Towards this end, we double labeled polytene squash preparations with a mAb to H3S10phK9me2 as well as with antibodies to H3S10ph and H3K9me2. The antibodies used in this study are listed in Table 1. As illustrated in Fig. 1, the H3S10phK9me2 mAb strongly labeled the chromocenter and the fourth chromosome with little or no labeling visible on the chromosome arms. In order to verify that the antibody indeed recognized the H3S10phK9me2 double mark, we labeled *JIL-1* and *Su*(*var*)*3-9* null mutant chromosome preparations (Wang et al., 2001; Zhang et al., 2006) that eliminated H3S10 phosphorylation and most H3K9me2 dimethylation (Schotta et al., 2002; Deng et al., 2007), respectively. As shown in Fig. 1, in neither case was there any detectable antibody labeling, thus validating the specificity of the antibody. It is well established that H3K9me2 is present at the chromocenter and the fourth chromosome (Schotta et al., 2002); however, whether H3S10 phosphorylation also occurs at these sites has been previously unresolved because some antibodies showed labeling whereas others did not (Cai et al., 2008). To resolve this issue, we double labeled chromosome squash preparations with H3S10phK9me2 antibody and with three different commercially available H3S10ph

Table 1 Antibodies

Antibody	Manufacturer	Catalog #	Lot #	Note
Anti-H3S10ph				
Rabbit pAb	Active Motif	39253	8308001	non-occluded
Rabbit mAb	Epitomics	1173-1	C-02-25-10	non-occluded
Rabbit mAb	Cell Signaling	3377S	3	occluded
Anti-H3K9me2				
Mouse mAb	Abcam	1220	765084	
Rabbit pAb	Millipore	07-441	608038250	
Anti-H3S10phK9me2				
Mouse mAb	Millipore	05-1354	1798298	

antibodies from Active Motif (rabbit pAb), Cell Signaling (rabbit mAb), and Epitomics (rabbit mAb). The results showed that two of these antibodies (from Active Motif and Epitomics) were non-occluded and robustly labeled the chromocenter and the fourth chromosome in a pattern overlapping that of the H3S10phK9me2 mAb. This is illustrated in Fig. 2a for the Epitomics antibody. In contrast, while the Cell Signaling antibody labeled H3S10ph in the interbands of the chromosome arms there was little or no labeling of pericentric chromatin or of the fourth chromosome (Fig. 2b), strongly suggesting that labeling of this antibody was occluded by the concomitant presence of the H3K9me2 mark.

Since mutant analysis of *JIL-1* null preparations suggests that JIL-1 is the interphase H3S10ph kinase (Wang et al., 2001) this raises an issue that has not been previously addressed directly, namely whether JIL-1 localizes to the chromocenter and the fourth chromosome. We therefore reanalyzed chromosome squash preparations labeled with JIL-1 antibody. As illustrated in Fig. 3 JIL-1 is clearly present in a banded pattern on the fourth chromosome similar to that of the chromosome arms, whereas JIL-1 antibody labeling of the chromocenter is intermediate between that of the interband regions where JIL-1 levels are high and that of banded regions where JIL-1 levels are low or absent. Taken together with the absence of JIL-1 antibody labeling of these sites in *JIL-1* null preparations (Wang et al., 2001), these findings strongly suggest that JIL-1 is localized to pericentric chromatin and the fourth chromosome and participates with Su(var)3-9 in creating a composite H3S10phK9me2 mark.

In double-labeled preparations with antibody to the double H3S10phK9me2 mark (in green) and with antibodies to either the single H3S10ph mark or the single H3K9me2 mark (in red) (Fig. 4), the labeling of the double mark would be expected to coincide with that of the single marks as indicated by a yellow color. However, in such preparations as illustrated in Fig. 4, there is very little yellow color that normally would indicate co-localization. Rather, the labeling, while congruent, occurred in separate interspersed patches of adjacent labeling as shown in Fig. 4a for a confocal section from a double labeling with H3S10phK9me2 and H3K9me2 antibody and in Fig. 4b for a double labeling with H3S10phK9me2 and H3S10ph non-occluded antibody of an acid-free fixed preparation. We speculate that this is the result of a second form of occlusion where only one of the antibodies to either the double mark or the single mark can bind to their respective epitopes at a time because of the close proximity of the epitopes.

It has been proposed that the epigenetic H3S10ph mark functions to counteract heterochromatization by participating

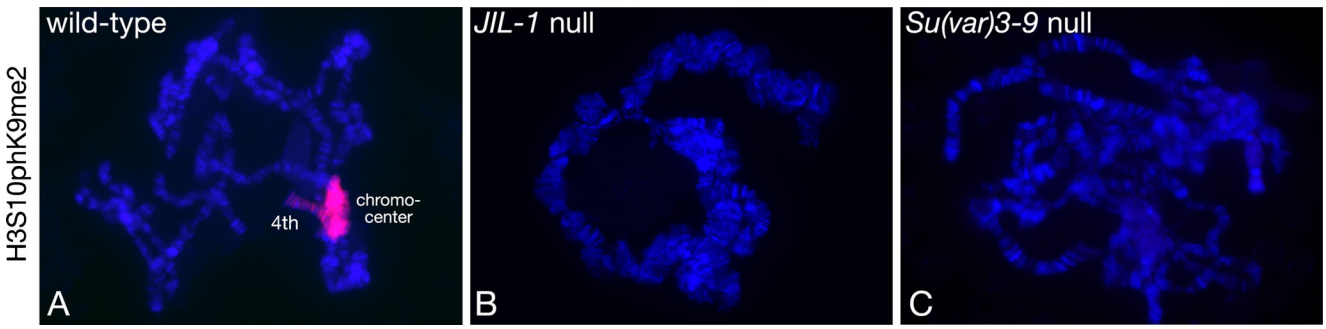

Fig. 1 The H3S10phK9me2 double epigenetic mark is present on the chromocenter and the fourth chromosome. **a**–**c** Polytene chromosome squash preparations labeled with anti-H3S10phK9me2 antibody (*red*) and with Hoechst (DNA in *blue*) from wild-type (**a**), *JIL-1* null ($JIL\text{-}1^{z2}/JIL\text{-}1^{z2}$) (**b**), and *Su(var)-3-9* null ($Su(var)3\text{-}9^{06}/Su(var)3\text{-}9^{06}$) (**c**) third instar larvae

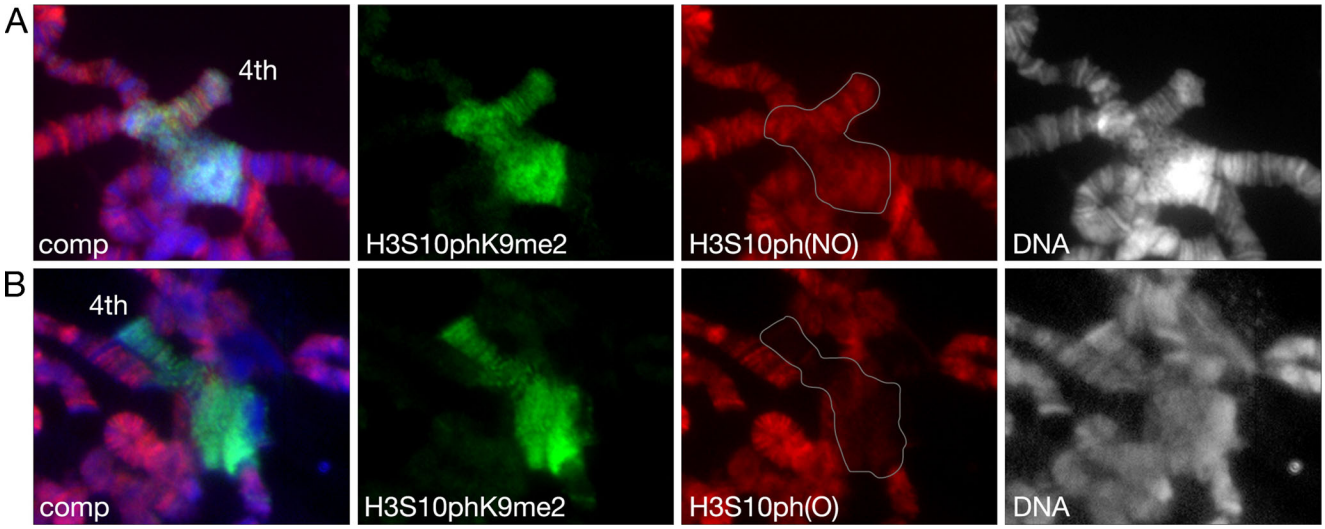

Fig. 2 Labeling of the chromocenter and fourth chromosome by occluded and non-occluded H3S10ph antibodies. **a** Triple labeling with non-occluded (NO, Epitomics) H3S10ph antibody (*red*), with H3S10phK9me2 antibody (*green*), and with Hoechst (DNA in *blue*/*gray*) of a wild-type polytene chromosome squash preparation. **b** Triple labeling with occluded (O, Cell Signaling) H3S10ph antibody (*red*), with H3S10phK9me2 antibody (*green*), and with Hoechst (DNA in *blue*/*gray*) of a wild-type polytene chromosome squash preparation. The location of the chromocenter and the fourth chromosome is outlined in *gray*

in a dynamic balance between factors promoting repression and activation of gene expression (Ebert et al., 2004; Zhang et al., 2006; Deng et al., 2007; Wang et al., 2011b; Girton et al., 2013). In this model JIL-1 kinase activity antagonizes Su(var)3-9 activity, keeping H3K9 dimethylation levels low relative to H3S10 phosphorylation levels at actively transcribed interband regions. However, it is not known whether JIL-1 and Su(var)3-9 actively compete at the same nucleosomal histone tails. To explore this question, we overexpressed a LacI-Su(var)3-9 construct in third instar salivary glands and double labeled polytene chromosome squash preparations with H3S10phK9me2 and H3K9me2 or

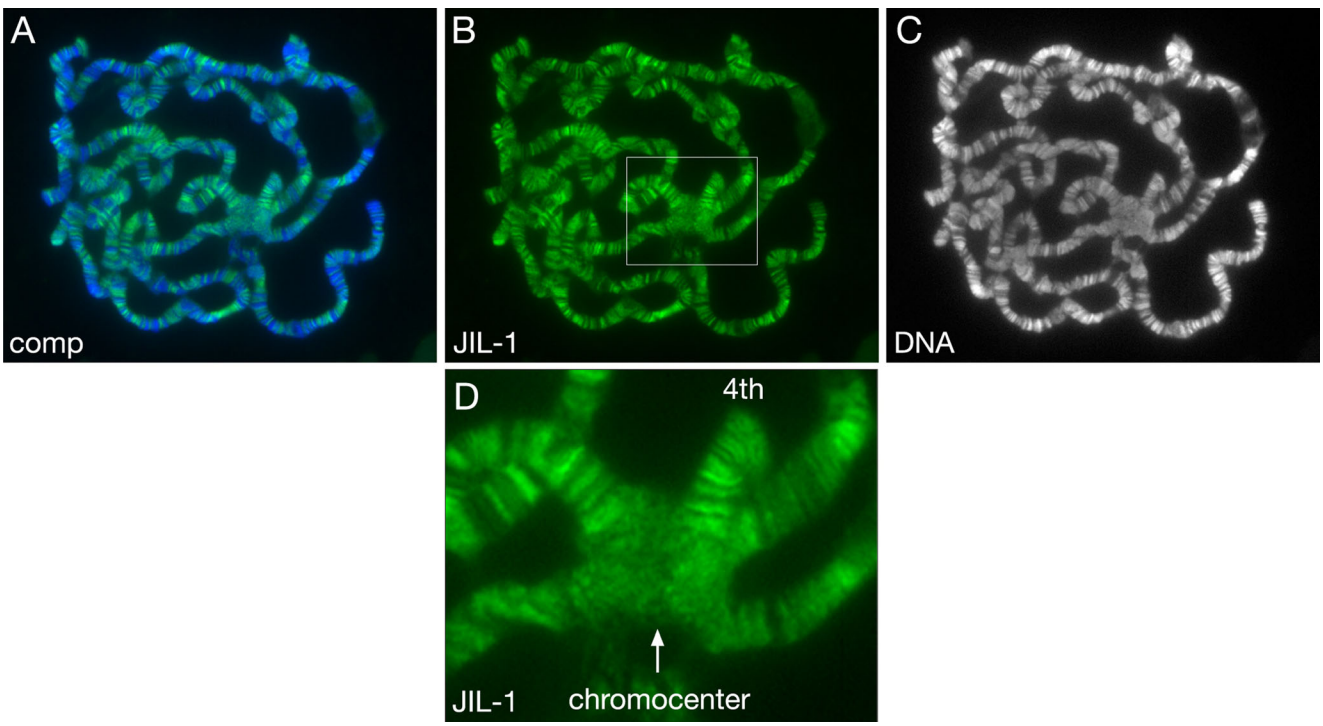

Fig. 3 The JIL-1 kinase is present on the chromocenter and the fourth chromosome. **a**–**c** Polytene chromosome squash preparation labeled with JIL-1 antibody (*green*) and with Hoechst (DNA in *blue*/*gray*). **d** Higher magnification image of the area indicated by the *white box* in (**b**)

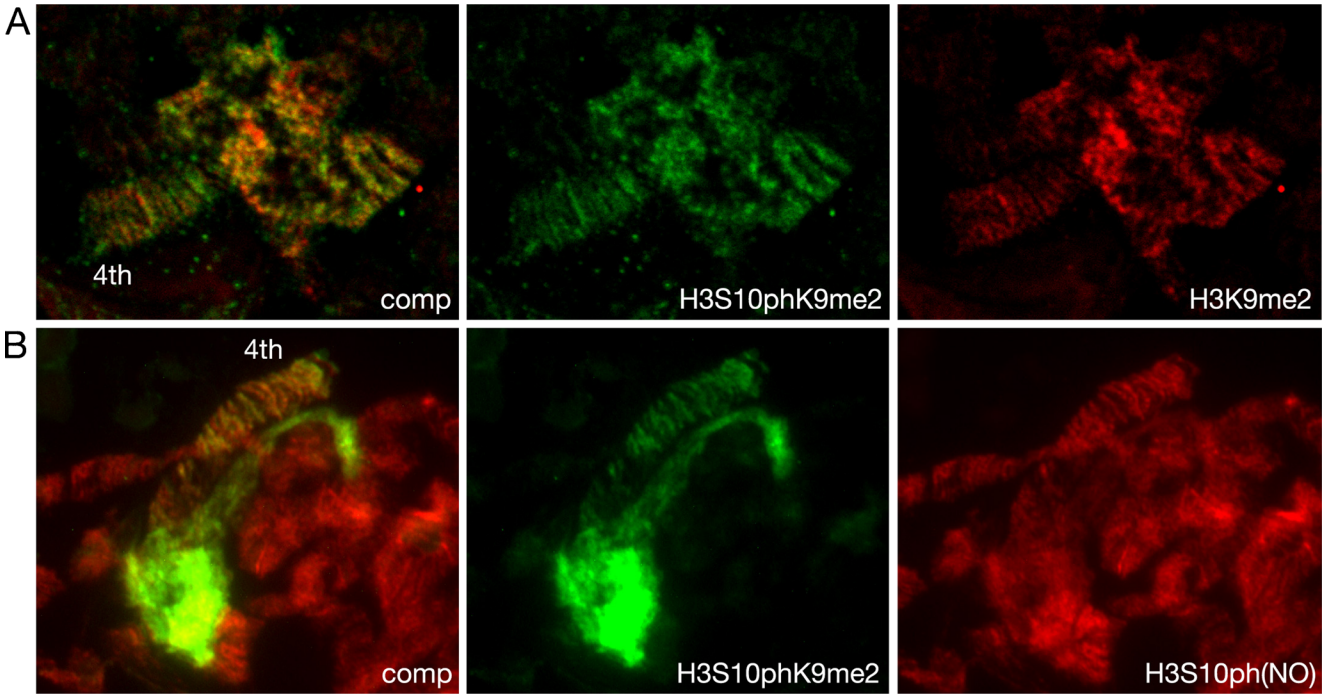

Fig. 4 Double labelings with H3S10phK9me2 antibody of the chromocenter and fourth chromosome together with H3K9me2 or H3S10ph antibody. **a** Confocal section of a polytene chromosome squash preparation double labeled with anti-H3S10phK9me2 antibody (*green*) and with H3K9me2 antibody (*red*). **b** Acid free fixed polytene chromosome squash preparation double labeled with anti-H3S10phK9me2 antibody (*green*) and with non-occluded (NO, Epitomics) H3S10ph antibody (*red*)

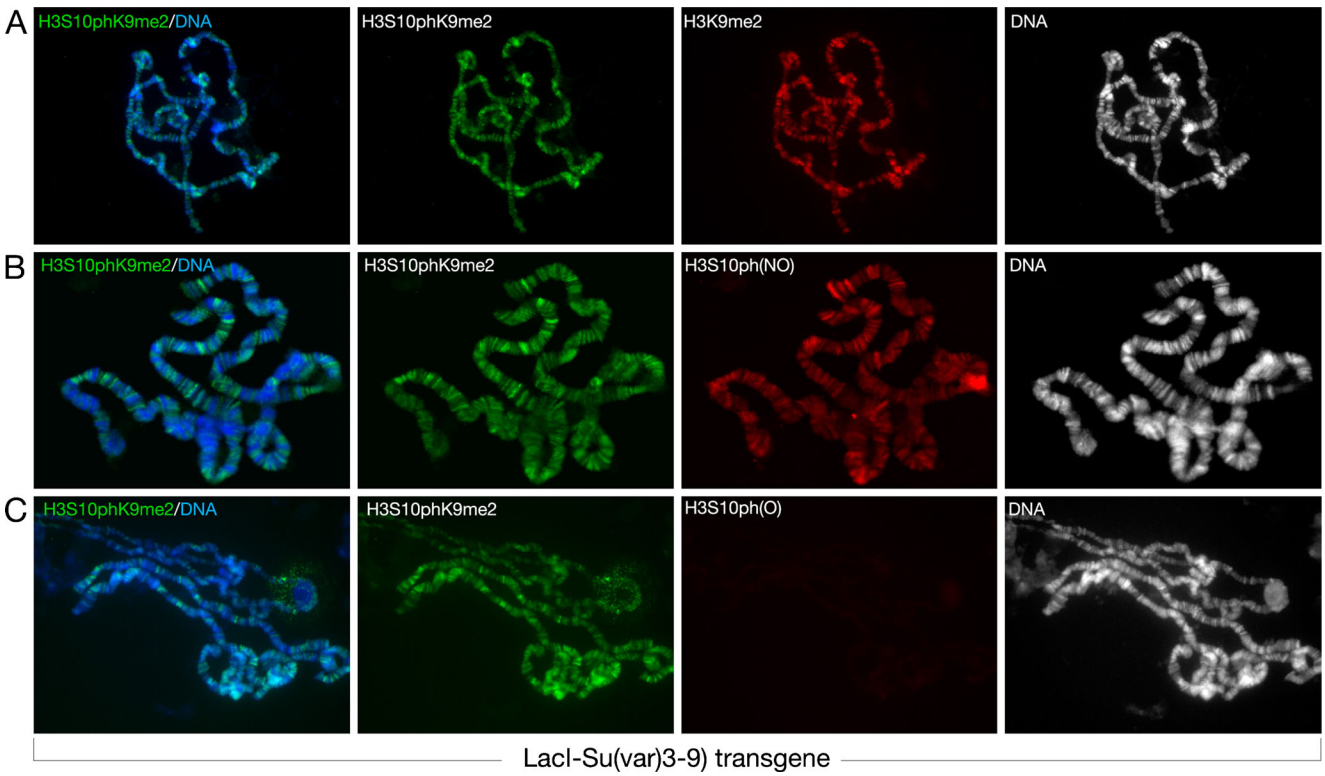

Fig. 5 Overexpression of the Su(var)3-9 methyltransferase leads to ectopic spreading of the H3S10phK9me2 mark to the chromosome arms. **a**–**c** Polytene chromosome squash preparations from third instar larvae expressing a LacI-Su(var)3-9 construct labeled with anti-H3S10phK9me2 antibody (*green*), with Hoechst (DNA in *blue*/*gray*), as well as with H3K9me2 antibody (*red*) in (**a**), with non-occluded (Active Motif) H3S10ph antibody (*red*) in (**b**), and with occluded (Cell Signaling) H3S10ph antibody (*red*) in (**c**)

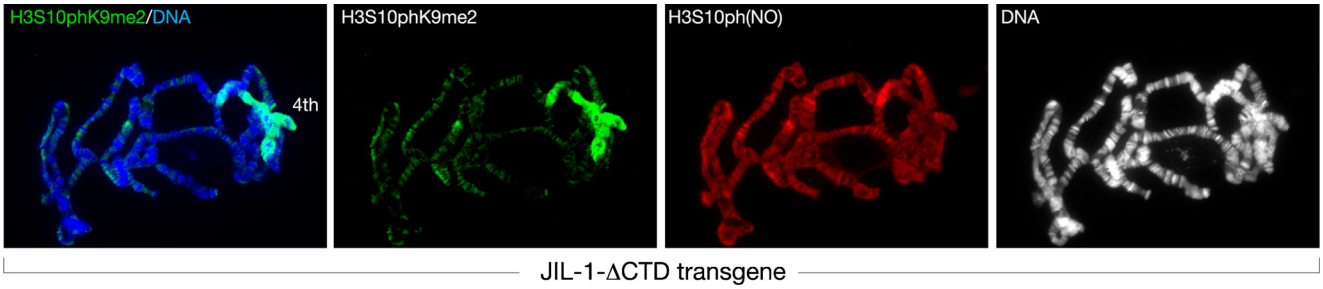

Fig. 6 Expression of a truncated version of JIL-1 that does not localize properly leads to ectopic spreading of the H3S10phK9me2 mark to the chromosome arms. Polytene chromosome squash preparation from a third instar larvae expressing a LacI-JIL-1-ΔCTD construct labeled with anti-H3S10phK9me2 antibody (*green*), with non-occluded (Active Motif) H3S10ph antibody (*red*), and with Hoechst (DNA in *blue*/*gray*)

H3S10ph antibody. Under these conditions H3K9 dimethylation was dramatically upregulated on the chromosome arms at interband regions as also indicated by robust antibody labeling for the H3S10phK9me2 double mark (Fig. 5a). That this H3K9me2 upregulation occurred at H3S10ph sites was further corroborated by the finding that labeling by occluded (Fig. 5c) but not non-occluded (Fig. 5b) H3S10ph antibody was abrogated. Conversely, we also expressed a truncated version of JIL-1 without the COOH-terminal domain (JIL-1-ΔCTD) that does not localize

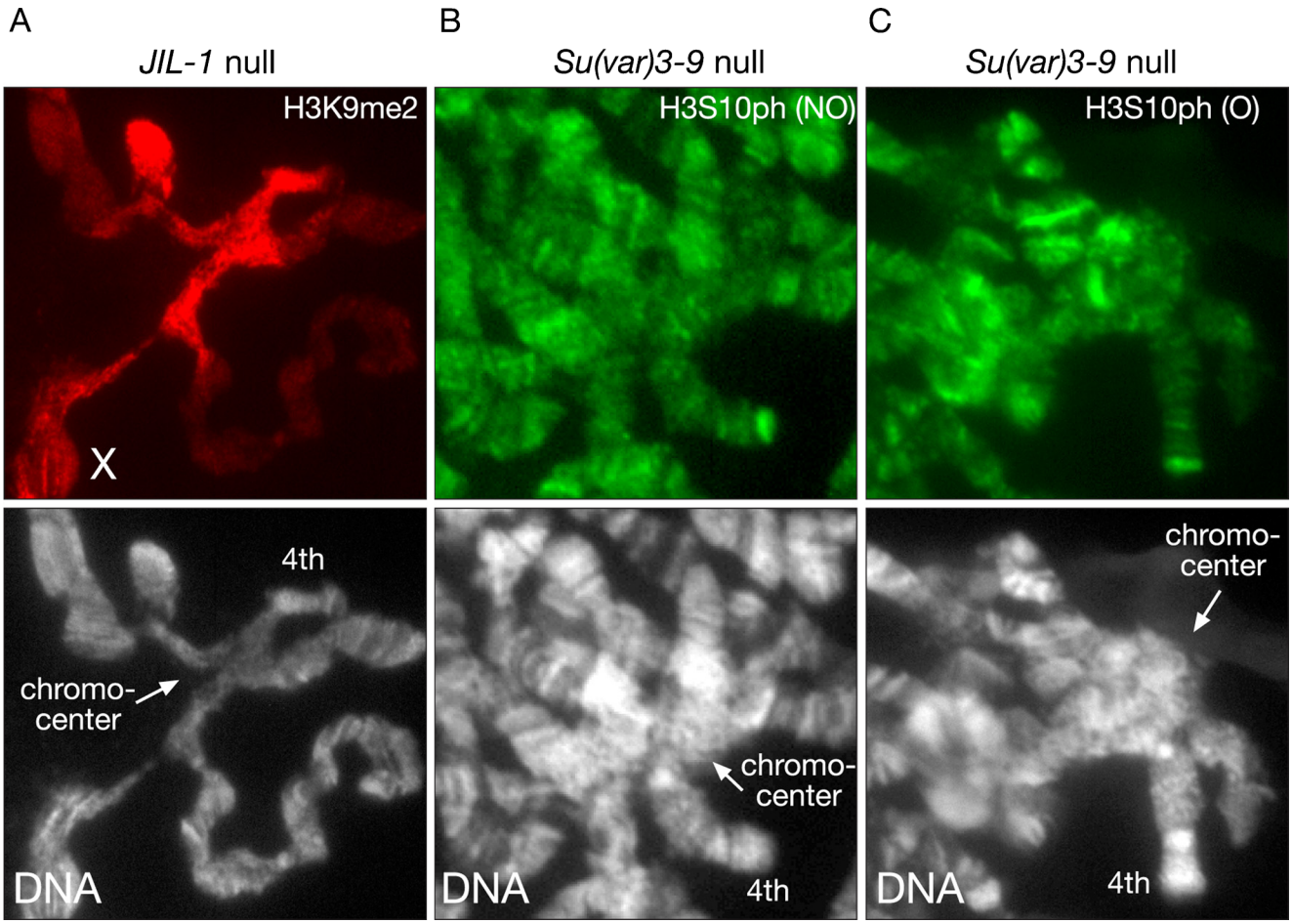

Fig. 7 Neither the H3K9me2 nor the H3S10ph mark depend on the other for deposition at pericentric chromatin. **a** Polytene chromosome squash preparations labeled with anti-H3K9me2 antibody (*red*) and with Hoechst (DNA in *gray*) from a *JIL-1* null ($JIL\text{-}1^{z2}/JIL\text{-}1^{z2}$) third instar larvae. The X chromosome is indicated by an X. **b** Polytene chromosome squash preparations labeled with anti-H3S10ph non-occluded (NO) antibody (*green*) and with Hoechst (DNA in *gray*) from a *Su*(*var*)*-3-9* null ($Su(var)3\text{-}9^{06}/Su(var)3\text{-}9^{06}$) third instar larvae. **c** Polytene chromosome squash preparations labeled with occluded anti-H3S10ph (O) antibody (*green*) and with Hoechst (DNA in *gray*) from a *Su*(*var*)*-3-9* null ($Su(var)3\text{-}9^{06}/Su(var)3\text{-}9^{06}$) third instar larvae

properly and phosphorylates H3S10 at ectopic sites (Bao et al., 2008; Li et al., 2013). As illustrated in Fig. 6, this also led to appearance of labeling for the H3S10phK9me2 double mark on the chromosome arms suggesting that some of the ectopic phosphorylation occurs at H3K9me2 sites.

An issue is whether H3K9 dimethylation is required for deposition of H3S10 phosphorylation at the chromocenter or vice versa. To answer this question we labeled *JIL-1* null chromosome squash preparations with H3K9me2 antibody and *Su*(*var*)*3-9* null preparations with H3S10ph antibody. As illustrated in Fig. 7, there was robust labeling by the respective antibodies in both scenarios. Interestingly, we found that in the *Su*(*var*)*3-9* null background the chromocenter and the fourth chromosome could be labeled with non-occluded (Fig. 7b) as well as with occluded (Fig. 7c) H3S10ph antibody. Thus, neither the H3K9me2 nor the H3S10ph mark was dependent on the other for deposition at pericentric chromatin. However, since it is known that ectopic spreading of histone modifications can occur in the mutant backgrounds (Zhang et al., 2006), it should be noted that these experiments do not resolve whether the observed H3K9 dimethylation or the H3S10 phosphorylation in the mutants took place at endogenous sites.

To verify the results obtained by immunocytochemistry and further validate the antibodies, we performed immunoblot analysis of protein extracts from salivary glands from the various experimental conditions (Fig. 8). The results showed (1) that there was no or little labeling by the H3K9me2 antibody used in the *Su*(*var*)*3-9* null background; (2) that both occluded and non-occluded H3S10ph antibody labeling was absent in the *JIL-1* null background; however, labeling by the occluded antibody, in contrast to the non-occluded, was greatly reduced in LacI-Su(var)3-9 expressing larvae; and (3) that labeling by the H3S10phK9me2 antibody was increased in LacI-Su(var)3-9 expressing salivary glands compared to wild-type, and that labeling was greatly reduced or absent in *Su*(*var*)*3-9* and *JIL-1* null salivary glands. These results strongly support the immunocytological findings.

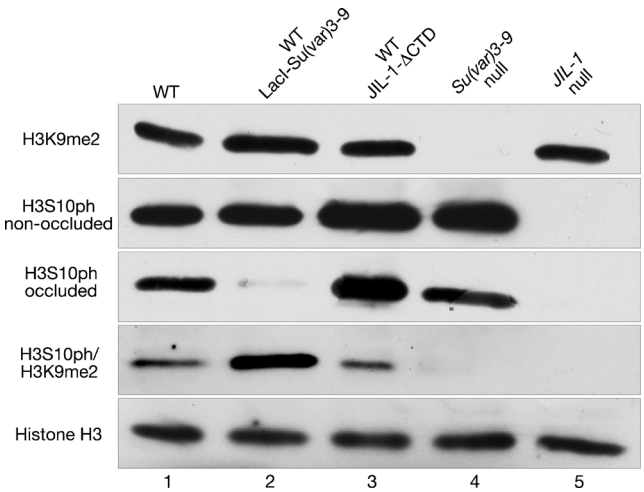

Fig. 8 Immunoblot analysis of histone modification antibodies. Immunoblots were performed on extracts from salivary glands of wild-type, LacI-Su(var)3-9 and LacI-JIL-1-ΔCTD expressing larvae as well as from *JIL-1* null ($JIL\text{-}1^{z2}/JIL\text{-}1^{z2}$) and *Su*(*var*)*3-9* null ($Su(var)3\text{-}9^{06}/Su(var)3\text{-}9^{06}$) larvae. The immunoblots were labeled with anti-H3K9me2 (Abcam), anti-H3S10ph non-occluded (Epitomics), anti-H3S10ph occluded (Cell Signaling), anti-H3S10phK9me2, and anti-histone H3 antibodies

In this study we have characterized an antibody to the epigenetic H3S10phK9me2 double mark as well as three commercially available H3S10ph antibodies. The results showed that for some H3S10ph antibodies their labeling can be occluded by the concomitant presence of the H3K9me2 mark. This underscores the need to verify the specificity and suitability of histone modification antibodies, as they are often poorly validated by the manufacturer (Cai et al., 2008; Wang et al., 2013). Furthermore, we demonstrate that the double H3S10phK9me2 mark is present in pericentric heterochromatin as well as on the fourth chromosome of wild-type polytene chromosomes but not in preparations from *JIL-1* or *Su*(*var*)*3-9* null larvae. The H3S10phK9me2 labeling overlapped with that of the non-occluded H3S10ph antibodies as well as with H3K9me2 antibody; however, conventional co-localization could not be demonstrated likely due to steric constraints on simultaneous binding of the respective antibodies to their closely apposed epitopes. Interestingly, when a LacI-Su(var)3-9 transgene was overexpressed it upregulated H3K9me2 dimethylation on the chromosome arms creating extensive ectopic H3S10phK9me2 marks, suggesting that the H3K9 dimethylation occurred at euchromatic H3S10ph sites. This was further supported by the finding that under these conditions euchromatic H3S10ph labeling by the occluded antibodies was abolished. These findings are consistent with the model that JIL-1 kinase activity under normal conditions antagonizes Su(var)3-9 activity by keeping H3K9 dimethylation levels low relative to H3S10 phosphorylation levels at actively transcribed interband regions on the chromosome arms (Ebert et al., 2004; Zhang et al., 2006; Deng et al., 2007; Wang et al., 2011b; Girton et al., 2013). However, it also implies the existence of a different mechanism for regulating the interactions between kinase and methyltransferase activity in the context of pericentric heterochromatin and the fourth chromosome that instead of competition promotes creation of the double mark. It will be of interest to determine the nature of this regulation and its functional importance in future studies.

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