# *Drosophila* CAF-1 regulates HP1-mediated epigenetic silencing and pericentric heterochromatin stability

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## Summary

Chromatin assembly factor 1 (CAF-1) was initially characterized as a histone deliverer in the process of DNA-replication-coupled chromatin assembly in eukaryotic cells. Here, we report that CAF-1 p180, the largest subunit of *Drosophila* CAF-1, participates in the process of heterochromatin formation and functions to maintain pericentric heterochromatin stability. We provide evidence that *Drosophila* CAF-1 p180 plays a role in both classes of position effect variegation (PEV) and in the expression of heterochromatic genes. A decrease in the expression of *Drosophila CAF-1 p180* leads to a decrease in both H3K9 methylation at pericentric heterochromatin regions and the recruitment of heterochromatin protein 1 (HP1) to the chromocenter of the polytene chromosomes. The artificial targeting of HP1 to a euchromatin location leads to the enrichment of *Drosophila* CAF-1 p180 at this ectopic heterochromatin, suggesting the mutual recruitment of HP1 and CAF-1 p180. We also show that the spreading of heterochromatin is compromised in flies that have reduced *CAF-1 p180*. Furthermore, reduced *CAF-1 p180* causes a defect in the dynamics of heterochromatic markers in early *Drosophila* embryos. Together, these findings suggest that *Drosophila* CAF-1 p180 is an essential factor in the epigenetic control of heterochromatin formation and/or maintenance.

**Key words:** *Drosophila*, CAF-1 p180, Heterochromatin, Histone modification, Position effect variegation

## Introduction

In eukaryotic cells, DNA is packaged in repeated units of nucleosomes, which are further organized into chromatin, a template for gene expression and genetic inheritance. Whereas euchromatin is less condensed and gene rich, heterochromatin is considered highly condensed, gene poor and less accessible to proteins such as transcription factors and the DNA repair machinery. Heterochromatin is also associated with a number of repressive covalent modifications of histone tails (Grewal and Elgin, 2007). At least two mechanisms are responsible for the highly compact state of heterochromatin: pericentric heterochromatin is dependent on heterochromatin protein 1 (HP1) (Eissenberg and Elgin, 2000) and intercalary heterochromatin is dependent on the polycomb group (PcG) of proteins (Belyaeva et al., 2008; Zhimulev and Belyaeva, 2003). HP1 and Pc recognize their respective target sites with discriminating specificities. Whereas HP1 prefers platforms with histone H3 bearing trimethylated Lys9, Pc targets trimethyl-H3K27 loci (Fischle et al., 2003). Thus, H3K9 methylation is considered a typical hallmark of pericentric heterochromatin (Lachner et al., 2003).

The epigenetic information carried by the heterochromatin structures has to be transmitted to daughter cells after cell division in what is known as replication-coupled epigenetic memory (Wallace and Orr-Weaver, 2005). The faithful inheritance of hallmarks such as H3K9me3, H4K20me3 and HP1 on the daughter strands of DNA ensures the integrity and stability of heterochromatin. Therefore, some of the factors that function in the replication process are likely to participate in the re-establishment of heterochromatin. For instance, proliferating cell nuclear antigen (PCNA), origin recognition complex (ORC), anti-silencing factor 1 (ASF1) and chromatin assembly factor 1 (CAF-1) have been suggested to be good candidate regulators of heterochromatin formation after cell division (Bell et al., 1995; Dillin and Rine, 1997; Henderson et al., 1994; Moshkin et al., 2002; Murzina et al., 1999). But precisely how these factors participate in the processes of heterochromatin formation and/or maintenance at a molecular or cytological level remains poorly understood.

CAF-1, a highly conserved three-subunit complex, was originally identified as a histone chaperone that deposits histones at newly synthesized DNA (Smith and Stillman, 1989). However, recent studies suggested the epigenetic involvement of CAF-1 in the re-establishment of chromatin after DNA replication and/or repair. Furthermore, it has been proposed that CAF-1 might play a crucial role in organizing the actions of the heterochromatin components during heterochromatinization (for reviews, see Groth et al., 2007; Wallace and Orr-Weaver, 2005). This proposal was based on emerging in vivo evidence that CAF-1 dysfunction can cause heterochromatin abnormality in various organisms such as mouse (Houlard et al., 2006), *Xenopus* (Quivy et al., 2001), *Drosophila* (Song et al., 2007), *Saccharomyces cerevisiae* (Monson et al., 1997), *Schizosaccharomyces pombe* (Dohke et al., 2008) and *Arabidopsis* (Ono et al., 2006; Schonrock et al., 2006).

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In this report, we investigate the roles of CAF-1 in heterochromatin formation and/or maintenance in *Drosophila*. This is a system that offers a variety of powerful tools for studying epigenetic inheritance, including the well-documented position effect variegation (PEV) models for genetic interaction studies, the polytene chromosomes (which allow high-resolution cytological studies) and early embryos for studying the dynamics of heterochromatin establishment. Our recent genetic studies suggested that mutations in *CAF-1 p180*, the largest subunit of *Drosophila CAF-1,* can suppress PEV in *Heidi* flies (Song et al., 2007). Here, we extend this observation by demonstrating that a *CAF-1 p180* mutation can suppress several additional PEV models. Importantly, heterochromatic gene expression is also dependent on full function of *Drosophila* CAF-1 p180. Our results further show that a reduction in *CAF-1 p180* expression leads to a decrease in HP1 association, H3K9 methylation and other repressive epigenetic markers at the pericentric heterochromatin, particularly at the boundary of heterochromatin and euchromatin. The participation of CAF-1 p180 in heterochromatinization is also evident in an artificial system in which HP1 is targeted to an endogenous euchromatic location. Genetic interaction studies reveal an antagonistic relationship between *Drosophila CAF-1 p180* and *JIL-1*, which is important for maintaining euchromatic histone H3S10 phosphorylation. This suggests that *Drosophila* CAF-1 directly participates in the demarcation between heterochromatic and euchromatic domains. This suggestion is further supported by the direct demonstration of a role for CAF-1 p180 in the spreading of methylated H3K9. Together, our findings underscore the role of *Drosophila CAF-1 p180* in maintaining repressive epigenetic markers to control heterochromatin formation and stability.

## Results

### *Drosophila CAF-1 p180* suppresses PEVs that affect euchromatic genes and enhances PEVs that affect heterochromatic genes

Euchromatic gene expression is silenced when the gene is translocated to the heterochromatin block or nearby region (Grewal and Elgin, 2002; Spofford, 1967; Wustmann et al., 1989); this is the basis of PEV for euchromatic genes. We have surveyed a variety of PEV models and examined their response to *Drosophila CAF-1 p180* mutation. These models include both class I PEV rearrangements, which cause aberrant expression of endogenous genes such as *Stubble* and *brown* (Fig. 1A, columns 2 and 3), and class II PEV rearrangements, which are generated by transgene insertions such as *mini-white* and *lacZ* (Fig. 1A, columns 1 and 4). Our results show that all these PEVs are suppressed by *CAF-1 p180* mutation, indicating a general suppressor function for *CAF-1 p180* on PEVs. The endogeneous heterochromatic gene expression relies on factors that maintain the heterochromatic environment, disruption of which leads to the silencing of these genes. This phenomenon is known as PEV affecting heterochromatic genes (Eberl et al., 1993; Howe et al., 1995; Schulze et al., 2005). To determine whether *Drosophila CAF-1 p180* is important not only for PEVs that affect euchromatic genes, but also for PEV models that affect heterochromatic genes, two mutations, *72* and *1-166-37*, which belong to the *lethal 2* group of heterochromatic genes (Schulze et al., 2005), were examined for their genetic interactions with *CAF-1 p180*. One copy of the *lethal 2* mutation, either *72* or *1-166-37*, combined with the inversion *In(3L)C90*, a defined PEV model that relocates the heterochromatic gene *lethal 2* near a euchromatin block, results in the posterior wing-margin phenotype (Fig. 1B) (Schulze et al., 2005). Our results show that both the penetrance and expressivity of this phenotype were enhanced by reduced *CAF-1 p180* gene expression (Fig. 1B) (supplementary material Table S1). In another experiment, *lethal 2* mutants display wing-margin defects in the weak *Notch* allele $N^{55e11}$ background (Schulze et al., 2005), which can also be further enhanced in the presence of one copy of a *CAF-1 p180* mutation (supplementary material Fig. S1).

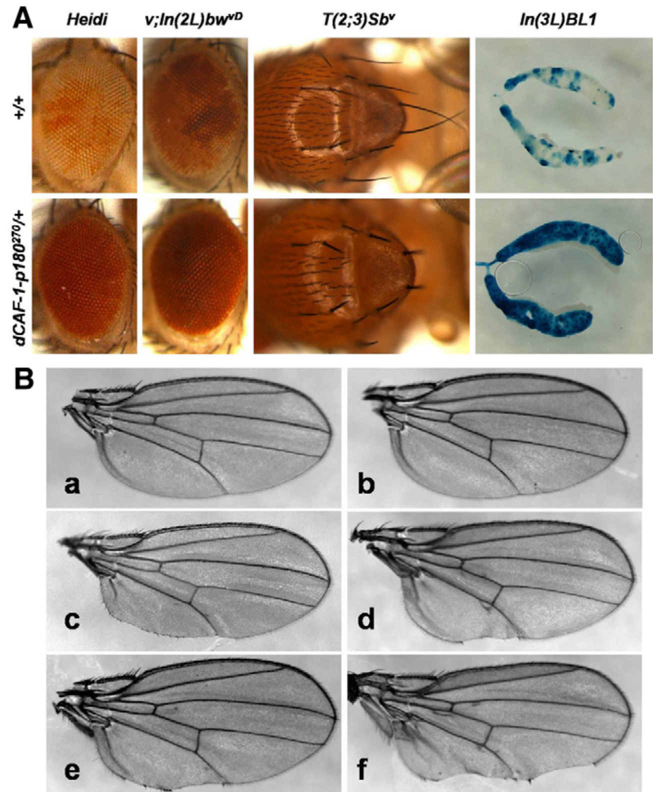


**Fig. 1. Involvement of *Drosophila CAF-1 p180* in heterochromatin-mediated gene silencing and activation of heterochromatic gene expression.** (**A**) *CAF-1 p180* mutation suppresses a variety of PEV rearrangements [*mini-white*, *brown*, *Stubble* (statistical results are shown in supplementary material Table S3), *lacZ*]. (**B**) Wing-margin phenotype enhancement showing the genetic interactions between the *lethal 2* group of heterochromatic genes and *CAF-1 p180*. The heterochromatic PEV is enhanced by *CAF-1 p180* mutation for both expressivity (here) and penetrance (supplementary material Table S1). (a) *In(3L)C90/+*; (b) *dCAF-1-p180*$^{270}$*/+; In(3L)C90/+*; (c) *1-166-37/In(3L)C90*; (d) *72/In(3L)C90*; (e) *dCAF-1-p180*$^{270}$*/+; 1-166-37/In(3L)C90*; (f) *dCAF-1-p180*$^{270}$*/+; 72/In(3L)C90.*

To evaluate the molecular effects of *Drosophila* CAF-1 p180 on heterochromatic gene expression, we quantified the expression levels of a series of heterochromatic genes using quantitative real-time reverse transcription (real-time qRT-PCR). We tested genes on both the second chromosome (*light* and *concertina*) and the third chromosome (*Dbp80*). Our results show that reduced *CAF-1 p180* decreased the expression of these tested heterochromatic genes (supplementary material Fig. S2) without affecting the expression of euchromatin-resident genes *actin*, *rp49*, *Su(var)2-5* and *Su(var)3-9* (supplementary material Figs S2 and S3). Taken together, these results demonstrate that *CAF-1 p180* gene expression is important for different PEVs that affect either euchromatic or heterochromatic genes. This suggests that this factor has an integral

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role in maintaining proper heterochromatin functions in gene expression.

### *Drosophila CAF-1 p180* is an epigenetic PEV regulator that genetically interacts with HP1 and has an antagonistic relationship with *JIL-1*

PEV is regulated by a number of heterochromatic and euchromatic factors, such as HP1, SU(VAR)3-9, JIL-1 and Rpd3 (Bao et al., 2007; Czermin et al., 2001; Ebert et al., 2004; Krauss and Reuter, 2000; Lerach et al., 2006). HP1, a constitutive heterochromatin component, has been well documented for its function in heterochromatin formation (Clark and Elgin, 1992; Kourmouli et al., 2004; Schotta et al., 2004). Increased HP1 levels through the *Hsp70–HP1* transgene led to increased variegations (Fig. 2A,B). The *CAF-1-p180*$^{270}$/+*;;Hsp70-HP1*/+ double heterozygous flies exhibited less *mini-white* expression compared with *dCAF-1-p180*$^{270}$/+ flies in the *Heidi* background (Fig. 2A,B), suggesting that increasing HP1 counteracts the effects caused by CAF-1 p180 reduction in heterochromatin-induced gene silencing.

*JIL-1* encodes the H3S10 phosphokinase that phosphorylates H3S10 to prevent heterochromatin expansion and maintain the euchromatin state (Ebert et al., 2004; Wang et al., 2001; Zhang et al., 2006). The enhancement effect of *JIL-1* on the PEV in *Heidi* flies is genetically antagonized by *CAF-1 p180* (Fig. 2A,B). The antagonistic effect between *CAF-1 p180* and *JIL-1* is also revealed by the following lines of evidence. First, *JIL-1* mutation enhances the small-eye phenotype induced by *ey-Gal4*-driven overexpression of *UAS-CAF-1-p180* (supplementary material Fig. S4). Second, the lethality of homozygous *JIL-1* mutants can be partially rescued by reducing *CAF-1 p180*, with an efficiency of 17.6%. Third, at the cytological level, ectopic spreading of HP1 and dimethylated H3K9 at the chromocenter of the polytene chromosomes in *JIL-1* mutants was suppressed by removal of one copy of *CAF-1 p180* (Fig. 2C).

### Depletion of *Drosophila CAF-1 p180* results in reduction of HP1 recruitment and of heterochromatic methylated H3K9

Our experiments described thus far suggest that CAF-1 p180 plays a crucial role in maintaining proper gene expression patterns that are dependent on an intact heterochromatin structure. HP1 and H3K9 methylation are two major markers of pericentric heterochromatin (Fanti and Pimpinelli, 2008; Hiragami and Festenstein, 2005; Schotta et al., 2002). To directly investigate whether the levels of these markers are altered in *CAF-1 p180* mutants, we focused on the heterochromatic chromocenter of the polytene chromosomes and evaluated the effects of CAF-1 p180 on the local densities of these heterochromatin markers. For these experiments, we altered CAF-1 p180 levels through two distinct means. Reduced *CAF-1 p180* expression was achieved using a UAS-RNAi transgene under the control of either *act-Gal4* for global knock down or a salivary-gland-specific *Gal4* driver, *SGS-Gal4*, for tissue-specific knock down. The efficiency of the RNAi transgenic lines was verified by RT-PCR (supplementary material Fig. S2, right column). In addition, overexpression of *CAF-1 p180* was achieved through the use of the *UAS-CAF-1-p180* transgene under the same Gal4 control used in the knockdown experiments. Fig. 3 shows that, in *CAF-1 p180* knockdown larvae (*CAF-1-p180-KD*), the heterochromatic chromocenter region has lower levels of HP1, and di- and tri-methylated H3K9 compared with the wild-type larvae. Western blot analyses of the salivary gland extracts

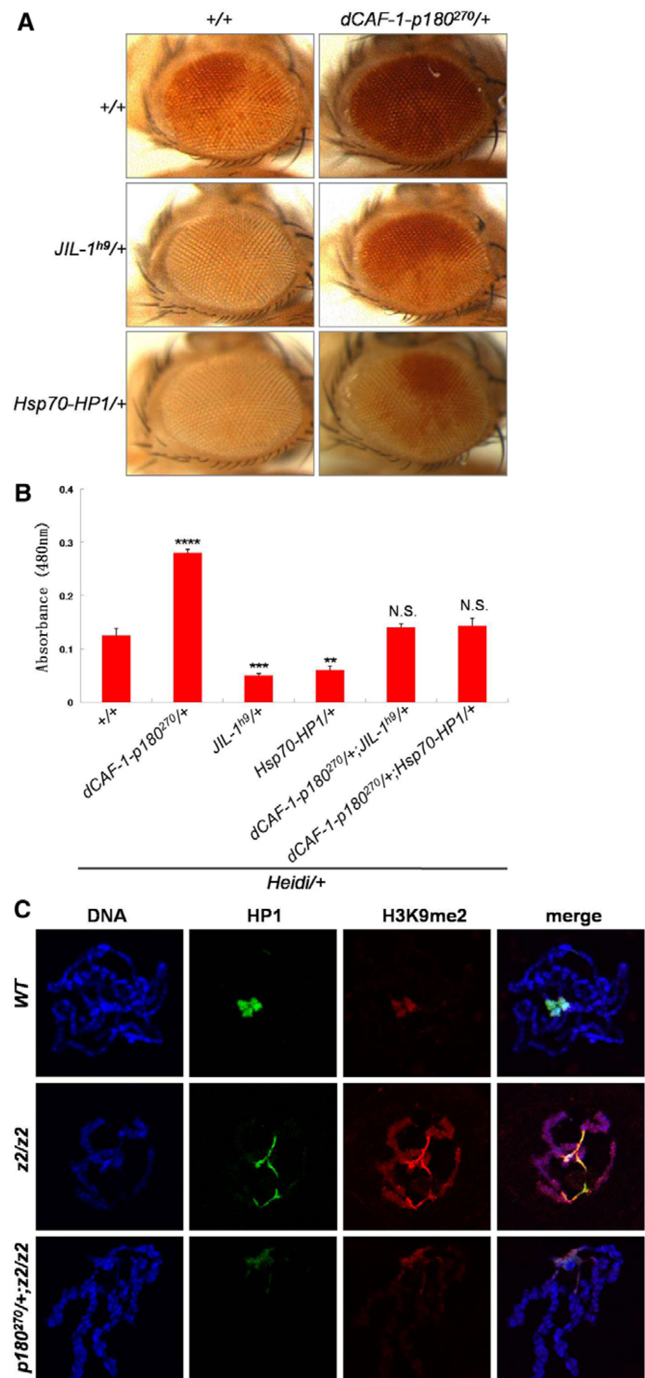


**Fig. 2. *Drosophila CAF-1 p180* genetically interacts with *HP1* and *JIL-1*.** (**A**) *Drosophila CAF-1 p180* counteracts the *Heidi* PEV enhancement induced by *JIL-1* mutation (middle row) or overexpression of *HP1* (bottom row). Eyes of the *Heidi* flies in wild-type, heterozygous *JIL-1*$^{h9}$ and heterozygous *Hsp70-HP1* genetic backgrounds, as indicated on the left. Eyes of such flies with an additional copy of the *CAF-1 p180*$^{270}$ mutation are shown on the right. Flies bearing the *Hsp70–HP1* transgene were reared at 29°C. All the genotypes are in the $w^{-/-}$ and *Heidi* background, in which the *mini-white* reporter is juxtaposed next to pericentric heterochromatin, resulting in random expression inactivation. (**B**) Eye pigment quantification of corresponding genotypes. Error bars represent s.d. $**P<0.01$. N.S. not significant. (**C**) HP1 and H3K9me2 localization in *JIL-1*$^{z2}$ and *CAF-1 p180*$^{270}$ mutant larvae. The polytene chromosome squashes were immunostained with anti-HP1 (green), anti-H3K9me2 (red) and DAPI (blue).

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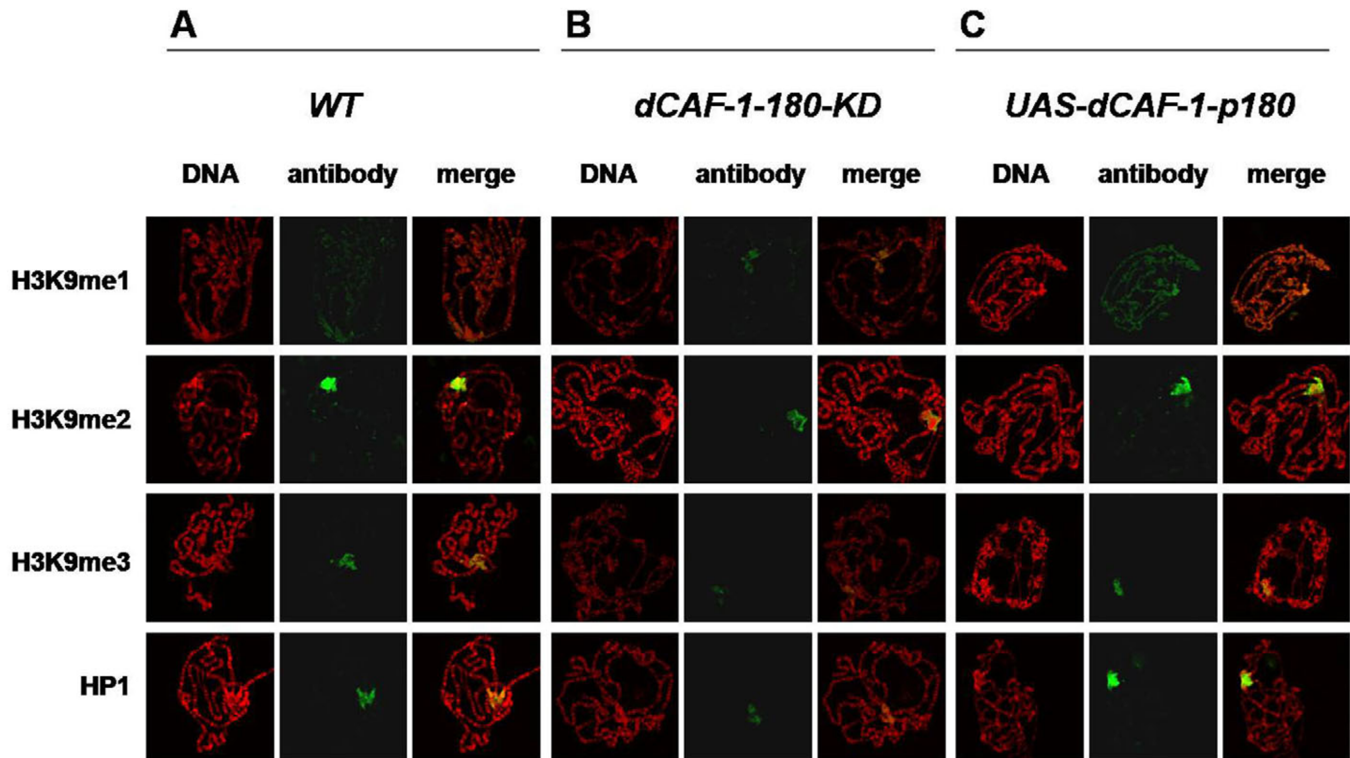


**Fig. 3. Reduced expression of *Drosophila CAF-1 p180* affects the status of pericentric H3K9 methylation and HP1 association with *Drosophila* polytene chromosomes.** (**A**) Wild-type Canton-S salivary gland polytene chromosomes probed with anti-mono-, anti-di-, anti-tri-methylated H3K9 and anti-HP1 antibodies. (**B**) Reduced *CAF-1 p180* results in less H3K9 di- and tri-methylation in the chromocenter heterochromatin region, as well as less HP1 recruitment. The epigenetic hallmarks are visualized with corresponding antibodies (green). DNA was stained with DAPI (red). (**C**) Ectopic expression of *CAF-1 p180* driven by either *SGS-Gal4* or *actin-Gal4*. Polytene chromosomes were labeled with anti-mono-, anti-di-, anti-tri-methylated H3K9 and anti-HP1 antibodies.

obtained from wild-type and *CAF-1-p180-KD* larvae confirmed the reduction in di- and tri-methylation of H3K9 upon *CAF-1 p180* depletion (supplementary material Fig. S5). The local decrease in HP1 and di- and tri-methylated H3K9 was not due to alterations in *Su(var)3-9* or *Su(var)2-5* expression in the presence of *CAF-1 p180* mutation (supplementary material Figs S3 and S2). Furthermore, wild-type and mutant *CAF-1 p180* have comparable bulk levels of mono-, di- and tri-methylated H3K9, as detected by western blots (data not shown), indicating that CAF-1 p180 mutation does not affect the total cellular methylation level of H3K9. In *CAF-1 p180* overexpression animals, although the signals of di- and tri-methylated H3K9 did not change significantly (Fig. 3), we did detect intensified staining for mono-methyl H3K9 along the entire chromosome and an increased HP1 density at the chromocenter. Together, these results suggest that *CAF-1 p180* plays a pivotal role in the maintenance of pericentric heterochromatin markers.

### *Drosophila CAF-1 p180* regulates the stability of repressive epigenetic modifications

Heterochromatin formation and spreading have been shown to be a stepwise process. Our results show that H2Av replacement, which is thought to be an early-stage event of the pericentric heterochromatin formation hierarchy, was unaffected by *CAF-1 p180* abrogation (see below). However, as shown by the results described in the previous section, downstream events such as HP1 association and H3K9 methylation are affected by CAF-1 p180. To investigate how *CAF-1 p180* might affect H3K9 methylation at a molecular level during heterochromatin spreading, we took advantage of the genetically well documented *white* rearrangement, $w^{m4}$, in which *white* and its neighboring genes are variably silenced by heterochromatinization. Chromatin immunoprecipitation was employed to examine the level of H3K9 dimethylation along the rearranged *white* region. As shown in Fig. 4, H3K9 methylation (H3K9me2) at the heterochromatin–euchromatin boundary loci of A, B, D, E and *white* is reduced in $w^{m4}$ flies that carry a *CAF-1 p180* mutation compared with wild-type counterparts. In similar tests, *CAF-1 p180* mutation had no effect on methylation levels either in the deep heterochromatic ribosomal DNA (rDNA) region (high level) or in the euchromatic *actin* region (low level). Because $w^{m4}$ flies had comparable histone H3 abundance in both the wild type and *CAF-1 p180* heterozygous background, global nucleosomal occupancy does not appear to be affected by *CAF-1 p180* mutation (supplementary material Fig. S6). Collectively, these results show that the H3K9 methylation status at distinct locations within the $w^{m4}$ region is sensitive to *CAF-1 p180*, directly reflecting position-dependent molecular changes within a heterochromatin–euchromatin boundary region. In addition to methylated H3K9, H4K20 tri-methylation, a constitutive heterochromatin hallmark that is established by SUV4-20 (Schotta et al., 2004), was also similarly affected by *CAF-1 p180* knock down (Fig. 5, lower panels). Collectively, these results suggest that *CAF-1 p180* is necessary for heterochromatin spreading and for stabilizing multiple downstream repressive epigenetic modifications.

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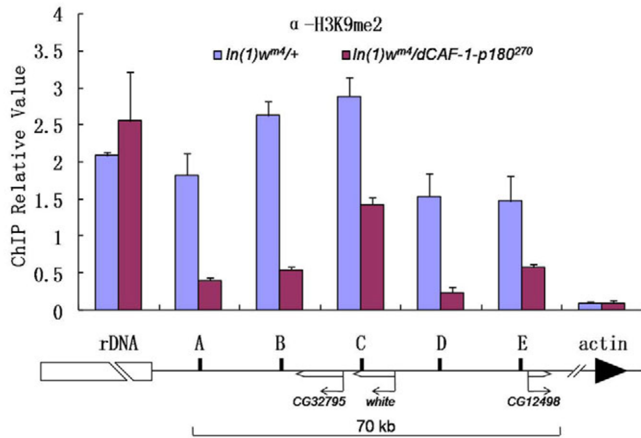


**Fig. 4. *Drosophila CAF-1 p180* mutation impairs heterochromatin spreading.** Chromatin immunoprecipitation (ChIP) analyses with anti-H3K9me2 from the euchromatic region and the *mini-white* reporter juxtaposed to the pericentric heterochromatin. In *CAF-1* $p180^{270}$ heterozygous flies, H3K9me2 di-methylation was reduced at the A, B, D, E and *white* loci. The ChIP relative value represents the amount of PCR product from the ChIPed DNA relative to that from the input sample. Error bars indicate s.d.

### *Drosophila CAF-1 p180* is involved in ectopic heterochromatin formation

It is known that heterochromatin formation is a complex process that might involve many different factors and steps. Li and colleagues reported that HP1 is sufficient to nucleate silent chromatin formation at some ectopic euchromatic sites (Li et al., 2003). This provides an important tool to allow us to determine whether CAF-1 p180 also participates in heterochromatin formation that is initiated by a simplified (albeit artificial) mechanism (Danzer and Wallrath, 2004; Li et al., 2003). In this system, expression of *lacI–HP1* induces silencing of *mini-white* reporters, such as lines *S9.2* and *157.1*, in which the reporters are inserted in the euchromatic regions (Fig. 6A) (Font-Burgada et al., 2008; Li et al., 2003). When reporter line *S9.2* was examined together with *lacI–HP1* in animals that have one copy of the *CAF-1 p180* mutation, the silencing of *mini-white* was suppressed, as judged by eye pigmentation (Fig. 6A). Fig. 6B shows that HP1 was ectopically associated with 1E4, 73A2-73A4 and 61F3 sites, and with the expression of *lacI–HP1* in lines *157.1*, *S9.2* and *157.4.112*, respectively. The ectopically expressed HP1 also led to additional CAF-1 p180 signals near the tethered site (Fig. 6B, arrowheads), suggesting local enrichment of CAF-1 p180 during HP1-induced ectopic heterochromatinization. These results, together with those shown in supplementary material Fig. S7, suggest the mutual recruitment of CAF-1 p180 and HP1 at heterochromatic sites.

### *CAF-1 p180* is required for early embryonic heterochromatin formation in *Drosophila*

To determine the dynamic effects of CAF-1 p180 on heterochromatin establishment during development, we analyzed the amount and patterns of HP1 and H3K9 methylation in early embryos. It has been reported that the differentiation of euchromatin and heterochromatin begins after several mitotic divisions and before zygotic gene expression (Vlassova et al., 1991). Zygotic *CAF-1 p180* is not expressed until late embryogenesis [(Klapholz et al., 2008) and our data, not shown], indicating that CAF-1 p180 during early embryonic development (e.g. cycle 14) is a maternal contribution (Song et al., 2007). Our results show that CAF-1 p180 has a uniform nuclear distribution at the multinucleate syncytium stages before mitotic cycle 10 (supplementary material Fig. S8A). Starting from the cellular blastoderm stage, CAF-1 p180 becomes enriched in the prospective heterochromatin region (arrows) and pole cells (arrowheads) (Fig. 7; supplementary material Fig. S8A). We therefore focused on the cellularization stage (cycle 14), when HP1 localization in the heterochromatin region is clearly visible, to examine HP1 and histone H3K9 methylation status. Notably, embryos from *CAF-1 p180* heterozygous females showed less intensive signals for CAF-1 p180 compared with the wild-type embryos (Fig. 7, arrows). In such embryos, the amount of both H3K9 trimethylation and HP1 in the apical heterochromatin regions appeared to be reduced upon the reduction of CAF-1 p180 protein (Fig. 7, arrows; supplementary material Fig. S8B). Meanwhile, the HP1 distribution pattern was also changed from punctate patterns in wild-type embryos to more diffuse patterns in the mutants (Fig. 7; supplementary material Fig. S8B). However, unlike HP1 and H3K9me3 signals, which are sensitive to maternal CAF-1 p180, the H3K9me2 foci did not exhibit any obvious differences in wild-type and *CAF-1 p180* mutant embryos (Fig. 7). These results

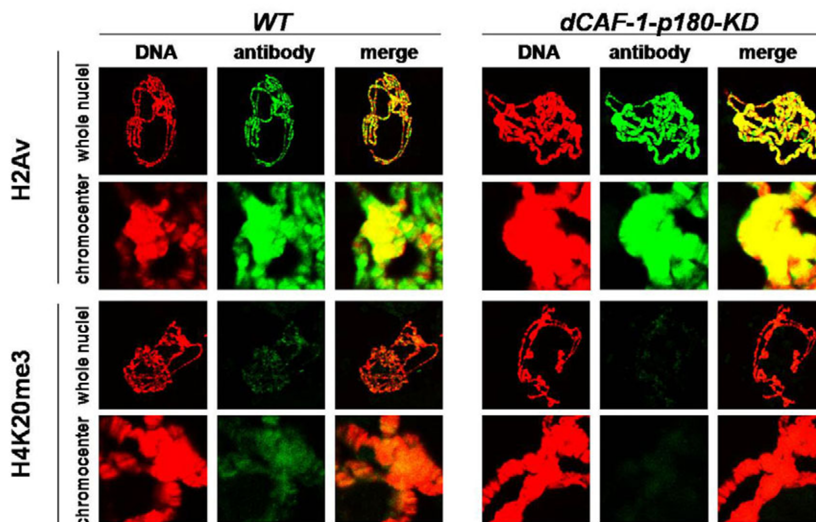


**Fig. 5. Reduced expression of *Drosophila CAF-1 p180* decreases the accumulation of trimethyl H4K20, but not H2Av, on the polytene chromosomes.** Epigenetic markers on polytene chromosomes of wild-type and *CAF-1 p180* knockdown larvae were recognized by anti-H2Av or anti-H4K20me3. The H2Av accumulation is not altered (upper two rows), whereas H4K20me3 is reduced in *CAF-1 p180* knockdown animals (the right lower panels).

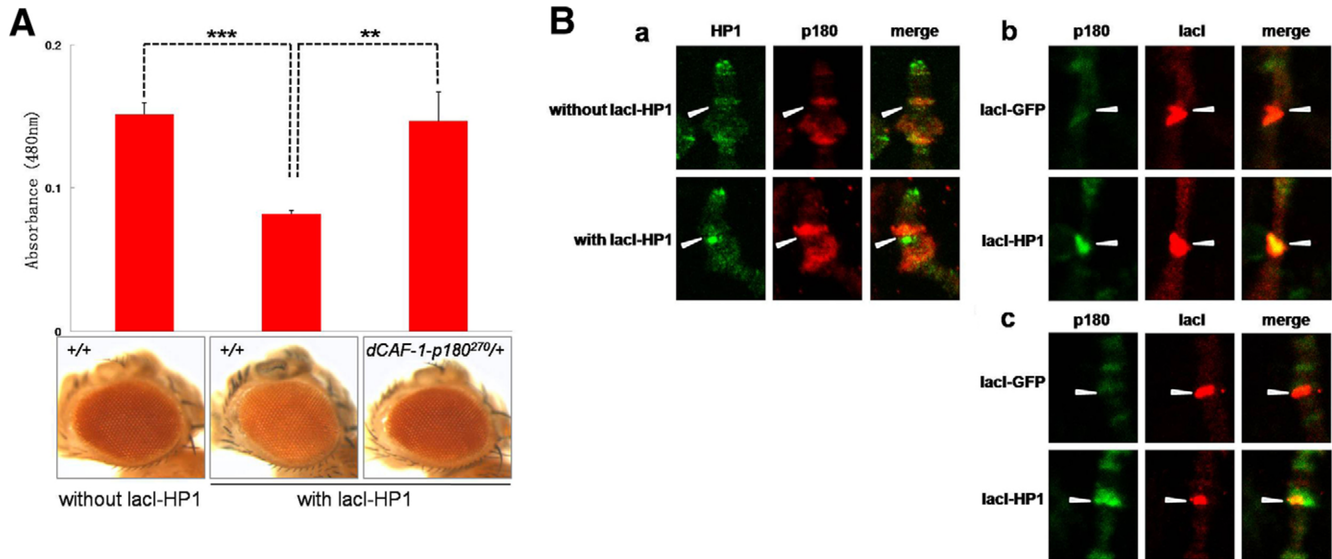


**Fig. 6. *Drosophila CAF-1 p180* mutation counteracts HP1-induced heterochromatinization and targeted HP1 induces CAF-1 p180 accumulation.** (**A**) The eye phenotype of flies that express LacI-tethered HP1 (see Li et al., 2003) in the background of one copy of the *CAF-1 p180* mutation (on the right) is compared with the tethered HP1 flies in a wild-type background (middle panel). Flies in all three panels carry the *S9.2* transgene, which contains *lac* repeats upstream of the *white* reporter gene. The eye pigmentation was quantified (see Materials and Methods section for details). Error bars represent s.d. from three independent measurements. Asterisks indicate statistical significance. (**B**) (a) Immunostaining of polytene chromosome showing that targeted HP1 (in green, arrowheads) (transgenic line *157.1*) induces ectopic accumulation around the targeting site of CAF-1 p180 that is fused with a DsRed tag (in red, arrowheads) [as described by Song et al. (Song et al., 2007)]. The *lac* repeats without LacI–HP1 (upper panels) show a low level of HP1 and CAF-1 p180, which might be due to the spontaneous localization of HP1 to repetitive sequences (Li et al., 2003). (b) Anti-lacI antibodies (in red, arrowheads) detect the tethered HP1 (lower panels) and GFP (control, upper panels) at 73A2-73A4 in the *S9.2* line. Extra CAF-1 p180 (in green, arrowheads) is induced and colocalizes with *lacI*-fused HP1 (yellow, arrowhead, lower right panel). Neither induction of CAF-1 p180 nor colocalization is observed when *lacI*–GFP is tethered at the same site. (c) Similar results to those presented in b are shown with *lacI* repeats at another site of 61F3. The reporter line is *157.4.112* (Li et al., 2003).

provide a first dynamic evaluation of the role of CAF-1 p180 with regards to heterochromatin markers during *Drosophila* development.

## Discussion

CAF-1 was originally isolated and characterized as a histone chaperone that brings histone H3–H4 heterodimers to newly synthesized DNA (Shibahara and Stillman, 1999; Smith and Stillman, 1989). On the basis of the findings described in this report, we propose that, in addition to being a histone deliverer, CAF-1 is also an integral factor in HP1-induced heterochromatin formation. Our results show that *CAF-1 p180* mutation affects all PEVs tested, as well as heterochromatic gene expression. CAF-1 p180 physically interacts with HP1 and knock down of its activity directly leads to alteration of the repressive heterochromatin markers (Fig. 3; supplementary material Fig. S7). Reduction of *CAF-1 p180* affects the spreading of H3K9 methylation. Furthermore, CAF-1 p180 antagonizes JIL-1 activity, which is known to maintain the euchromatic state of chromatin by preventing spreading. Importantly, a simplified (artificial) heterochromatin

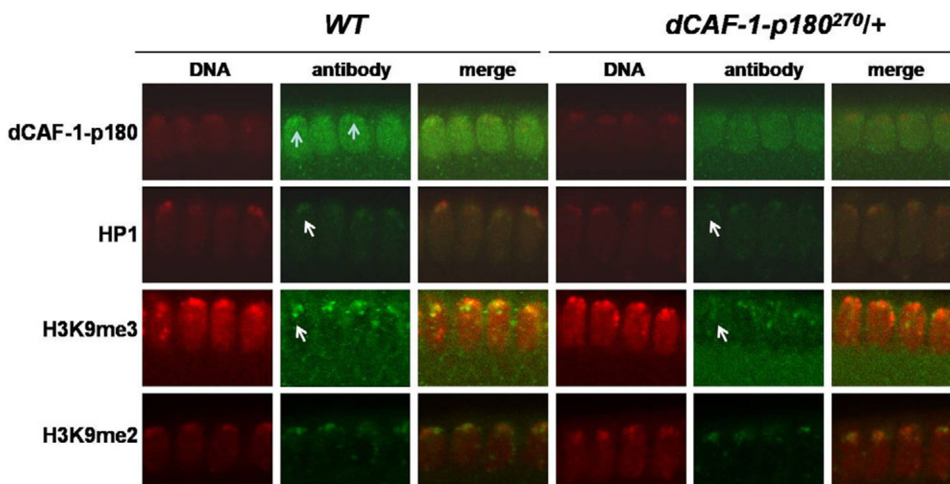


**Fig. 7. *CAF-1 p180* expression influences the abundance and cellular patterns of HP1 and methylated histone H3K9 in *Drosophila* early embryonic development.** Wild-type embryos and embryos produced by *dCAF-1-p180*/+ females were immunostained with anti-H3K9me2, anti-H3K9me3 and anti-HP1 antibodies. High-resolution images showing signals of CAF-1 p180, HP1 and H3K9 methylation in the blastoderm cells. White arrows indicate apical heterochromatin regions.

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formation process established solely by targeting HP1 to a euchromatic site is also dependent on CAF-1 p180. It remains to be determined whether the heterochromatin formation and histone chaperone activities of CAF-1 p180 reflect a common function or independent functions of the protein.

Heterochromatinization is a complex process that involves multiple regulatory factors. The first step of this process is thought to involve H2Av replacement with the assistance of remodeling and spacing factor (RSF) (Hanai et al., 2008; Swaminathan et al., 2005). Initiation can also be triggered by RNAi machinery through the recruitment of an RNA-induced transcriptional silencing (RITS) complex (Fagegaltier et al., 2009; Pal-Bhadra et al., 2004; Verdel et al., 2004) or by an artificially tethered HP1. Following deacetylation of H3K9 by Rpd3 (Czermin et al., 2001; Rudolph et al., 2007), SU(VAR)3-9 gains access to and methylates the H3K9 sites (Ebert et al., 2004), which represent a platform for HP1 binding (Lachner et al., 2001; Nakayama et al., 2001) and subsequent binding of SUV4-20, which trimethylates H4K20 (Kourmouli et al., 2004; Schotta et al., 2004). Crosstalk among histone modifications is required during heterochromatin formation and maintenance. For instance, H3S10 phosphorylation by JIL-1 prevents the spreading of H3K9 dimethylation, so that the chromatin acquires a euchromatic identity (Zhang et al., 2006). Loss of acetylation of H4K12 impairs H3S10 phosphorylation and enhances the extension of H3K9 dimethylation (Ciurciu et al., 2008). Our finding that *CAF-1 p180* genetically antagonizes *JIL-1* during heterochromatin spreading suggests that CAF-1 p180 might function in late steps of the sequential heterochromatinization process, affecting histone modifications such as H3K9 methylation and H4K20 methylation. This idea is consistent with a lack of detectable differences in H2Av incorporation at the chromocenter between wild-type and *CAF-1 p180* knockdown flies (Fig. 5, upper panels). Given the relationships between CAF-1 p180, JIL-1, H3K9 methylation and H4K12 acetylation in heterochromatin dynamics, we speculate that CAF-1 p180 is an important factor coordinating crosstalk involving methylation, phosphorylation and acetylation. To determine whether the reduction in H3K9 methylation upon decreasing *CAF-1 p180* is attributed to the impairment of SU(VAR)3-9, we examined the SU(VAR)3-9 level in *CAF-1 p180* knockdown animals and a reproducible, although slight, reduction of SU(VAR)3-9 was detected (supplementary material Fig. S9A). However, no physical interaction between CAF-1 p180 and SU(VAR)3-9 was detected in a co-immunoprecipitation experiment (supplementary material Fig. S9B). These results are consistent with the hypothesis that reduced HP1 levels caused by *CAF-1 p180* abrogation might result in the reduction of SU(VAR)3-9 levels.

It has been reported in several species that CAF-1 physically interacts with HP1 through its largest subunit (Dohke et al., 2008; Murzina et al., 1999; Quivy et al., 2004). This interaction was also detected in *Drosophila* in our experiments described here (supplementary material Fig. S7). HP1-binding partners are proposed to have the PXVXL motif (Thiru et al., 2004). Although this consensus motif is found in the CAF-1 p150 MOD1 interaction region (Murzina et al., 1999), CAF-1 p180 lacks such a motif, suggesting that the interaction between HP1 and CAF-1 p180 might be achieved through a degenerate PXVXL motif or other means (Eskeland et al., 2007). A physical interaction between CAF-1 p180 and HP1 can readily explain a variety of our findings, including the additional accumulation of HP1 caused by *CAF-1 p180* overexpression (Fig. 3) and the recruitment of CAF-1 p180 to an HP1-induced ectopic heterochromatin location (Fig. 6B). We suggest that the physical interaction represents a molecular basis for the observed mutual recruitment of CAF-1 p180 and HP1. In addition to HP1, the H3K9 monomethylation level of the polytene chromosomes is also affected by alterations of *CAF-1 p180* gene expression (Fig. 3), effects that are similar to those caused by *Drosophila Setdb1* gene expression alterations (Seum et al., 2007). Therefore, CAF-1 p180 probably interacts with not only HP1 but also other epigenetic factors, such as *Drosophila* SETDB1, to accomplish its functions in the process of heterochromatinization (Sarraf and Stancheva, 2004). Consistent with this notion, a protein complex that contains HP1, CAF-1, SETDB1 and methylated H3K9 has been reported (Loyola et al., 2009; Sarraf and Stancheva, 2004). It remains to be determined precisely how this and/or other complexes control both the inheritance and de novo formation of heterochromatin.

## Materials and Methods

### *Drosophila* stocks and genetics

Flies were reared on a cornmeal and agar medium at 25°C according to standard protocols. The *CAF-1-p180*$^{270}$ and *UAS-CAF-1-p180* flies have been described previously (Song et al., 2007). The deletion of the *CAF-1 p180* gene was generated by P-element imprecise excision (Song et al., 2007). The N-terminal portion (576 bp of DNA) of the region encoding CAF-1 p180 was deleted. The homozygous animals mostly die around 48 hours after hatching, with abnormally fragmented chromosomes and cumulative DNA damage (data not shown) (Klapholz et al., 2008). A GFP-marked balancer was used to distinguish heterozygous and wild-type animals from the hemizygotes at early stages. The *JIL-1*$^{h9}$ and *JIL-1*$^{z2}$ mutants were generous gifts from Kristen M. Johansen (Department of Biochemistry, Biophysics & Molecular Biology, Iowa State University, IA). *Heidi* flies were kindly provided by François Karch (Department of Zoology and Animal Biology, University of Geneva, Switzerland). *T(2;3)Sb*$^{v}$ was from Nicholas Dyson's laboratory (Department of Medicine, Massachusetts General Hospital Cancer Center, Boston, MA). The *In(3L)BLl* and *Hsp70–HP1* stocks were generously provided by Joel C. Eissenberg (Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, MO). The *lethal 2* mutants were kindly provided by Barry M. Honda (Department of Molecular Biology and Biochemistry, Simon Fraser University, Canada). *Rpd3-RNAi* stock was generously provided by Hao Li (Novartis Institutes for Biomedical Research, Cambridge, MA). The RNAi line of *CAF-1 p180* was obtained from the Vienna Drosophila RNAi Center (VDRC). The rest of the fly stocks that were used in this study were from the Bloomington *Drosophila* Stock Center.

For tethering experiments, the *lacI–HP1* transgenic flies described by Li and colleagues were crossed to *mini-white* reporter lines (kindly provided by Lori Wallrath, Department of Biochemistry, University of Iowa, IA). The crossed flies were heat shocked at 37°C daily (Li et al., 2003).

For genetic interaction between *CAF-1 p180* and *JIL-1*, *JIL-1*$^{z2}$*/TM6B* flies were mated to *CAF-1-p180*$^{270}$*/FM7C,Kr-GFP; JIL-1*$^{z2}$*/TM6B*. The *TM6* chromosome was marked by *Tubby*. Thus, the *JIL-1* homozygotes were identified as non-Tubby animals. The percentage of rescue efficiency was calculated as observed number of *JIL-1* homozygotes to the expected number, that is, observed number of non-Tubby animals×6/total number of the offspring. Rescued progeny were obtained from at least three independent crosses.

### Eye pigment measurement assay

To quantify eye pigmentation, 40 heads of female flies (2–3 days old) with proper genotypes were homogenized in a 1:1 mixture of chloroform and 0.1% ammonium hydroxide. The upper aqueous layer after centrifugation was applied to the absorbance measurement at 480 nm for each sample.

### Generation of the anti-CAF-1-p180 antibody

Polyclonal antibodies that recognize *Drosophila* CAF-1 p180 were raised against a purified, bacterially expressed fragment of amino residues 221–498 (Antiomics). The recombinant antigens were purified using Ni-NTA resins (Qiagen). Antibodies were affinity purified before using.

### Western blotting and immunoprecipitation

The embryos were dechorionated and larvae with correct genotypes were collected and frozen in liquid nitrogen or dissected before homogenization in buffer H (10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 0.25 M saccharose) and centrifugation. The nuclei were extracted for 30 minutes in nuclear extraction buffer (50 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF) in the presence of a protease inhibitor cocktail. The extracts were used either for immunoblot or for coimmunoprecipitation assays.

2×SDS buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 0.1% bromophenol blue, 20% 2-mercaptoethanol) was added to the extracts before the samples were boiled for 5 minutes and spun at maximum speed at room temperature for 5 minutes. The supernatants were applied to SDS-polyacrylamide gel and transferred to a PVDF membrane. For western blotting, the membranes were blocked for 1 hour at room temperature and incubated with anti-mono- (generously provided by Thomas Jenuwein, Max-Planck Institute of Immunobiology, Freiburg, Germany), anti-di- (generously provided by Thomas Jenuwein), anti-tri-methylated H3K9 antibodies, anti-HP1 (kindly provided by Lori Wallrath) and anti-histone H3 antibodies (Abcam) overnight at 4°C. Subsequently, after washing, the blots were processed with horseradish-peroxidase-linked anti-rabbit IgG and signals were detected using a SuperSignal West Pico Trial Kit (Thermo Scientific).

For coimmunoprecipitations, FLAG M2 antibodies were coupled to agarose beads (Sigma). The extracts were then incubated with the beads for 6 hours at 4°C. Subsequently, the beads were washed three times with IP buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 1% NP-40) and eluted with SDS-loading buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 0.05% bromophenol blue, 10% 2-mercaptoethanol). The anti-HP1 (1:1000) and anti-SU(VAR)3-9 (1:1000) antibodies were used for western blot. The detection was performed according to standard manufacturer's instructions (Thermo Scientific, ECL-Kit).

### Immunohistochemistry on polytene chromosomes and embryos

Wandering third instar larvae with correct genotypes were collected and dissected in cold Cohen buffer (10 mM $MgCl_2$, 25 mM $Na_2GlycerolPO_4$, 3 mM $CaCl_2$, 10 mM $KH_2PO_4$, 0.5% NP-40, 30 mM KCl, 160 mM sucrose). The salivary glands were removed and fixed in 2% formaldehyde for 3 minutes, and squashed in 45% acetic acid and 2% formaldehyde on poly-L-lysine-coated microscope slides. The slides were then frozen in liquid nitrogen. The chromosomes were labeled by anti-H4K20me3 (Upstate), anti-H2Av (generously provided by Robert Glaser, Wadsworth Center, State University of New York, Albany, NY), anti-lacI (Millipore) and other histone modification antibodies at 1:100 dilutions, followed by incubation with Cy3 or FITC-conjugated secondary antibodies. Images were taken using a confocal microscope (Leica SP5). The *Drosophila* embryos were fixed, permeabilized and probed with specific antibodies as previously described (He et al., 2008). Fluorescent secondary antibodies (1:200, Jackson ImmunoResearch) were used. The embryos were photographed with Leica confocal microscope SP5 as above.

### Chromatin immunoprecipitation

The fly heads with correct genotypes were fixed in 1.8% formaldehyde at 37°C for 15 minutes. Vortex was done briefly in between. The cross-linked chromatin was resuspended in RIPA buffer (140 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate). The extracts were sonicated to produce 500–1000 bp DNA fragments. 4 μg anti-dimethyl H3K9 antibodies was coupled to Dynabead protein A (Invitrogen). Then, sonicated lysates were added and rotated overnight at 4°C. Control IgG immunoprecipitations were performed in parallel. The chromatin samples were reverse cross-linked at 65°C for 5 hours. Genomic DNA was then extracted and used as templates for PCR with specific primers (supplementary material Table S2).

### RT-PCR

Total mRNA was isolated using the Trizol method and eluted with RNase-free water. The concentrations were determined using a biophotometer (Eppendorf). Reverse transcription was carried out using the First-Strand Synthesis System (Invitrogen) according to the standard manual. The in vitro reverse transcribed cDNAs were used as templates for further PCR reactions.

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