

# A chromatin insulator driving three-dimensional Polycomb response element (PRE) contacts and Polycomb association with the chromatin fiber

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**Regulation of gene expression involves long-distance communication between regulatory elements and target promoters, but how this is achieved remains unknown. Insulator elements have been proposed to modulate the communication between regulatory elements and promoters due to their ability to insulate genes from regulatory elements or to take part in long-distance interactions. Using a high-resolution chromatin conformation capture (H3C) method, we show that the *Drosophila gypsy* insulator behaves as a conformational chromatin border that is able to prohibit contacts between a Polycomb response element (PRE) and a distal promoter. On the other hand, two spaced *gypsy* elements form a chromatin loop that is able to bring an upstream PRE in contact with a downstream gene to mediate its repression. Chromatin immunoprecipitation (ChIP) profiles of the Polycomb protein and its associated H3K27me3 histone mark reflect this insulator-dependent chromatin conformation, suggesting that Polycomb action at a distance can be organized by local chromatin topology.**

Coordinated spatial and temporal control of eukaryotic gene expression involves numerous regulatory elements required to enhance, silence, or insulate gene transcription. Many of these elements are involved in long-range intra- or interchromosomal interactions with their target gene promoter, suggesting that 3D chromosome organization is a key component of gene expression regulation (1–3). Therefore, the exploration of the interplay between chromatin conformation and the spatial relation of different regulatory elements is essential to understand the mechanisms underlying gene regulation.

Polycomb group (PcG) proteins are pleiotropic gene silencing factors that play important roles in development and disease (4–6). In *Drosophila*, PcG proteins are recruited to chromatin through Polycomb response element (PRE) sequences that can be located several tens of kilobases away from their target genes. PREs recruit the PRC2 complex, which trimethylates lysine 27 of histone H3 (H3K27me3), a mark that is recognized by PcG proteins of the PRC1 complex to bring about gene silencing (7). The function of PcG factors has also been suggested to involve higher-order chromatin organization of PREs (8, 9), but the mechanisms linking 3D chromatin folding to Polycomb distribution on chromatin are unknown.

Insulators have a unique place in gene expression regulation because they are able to organize the relationship between other regulatory elements and promoters (10–12). They are classified in two groups depending on their properties. Boundaries act as “roadblocks” that prevent spreading of epigenetic factors or histone marks, exemplified by their ability to block heterochromatin propagation. Enhancer blockers disrupt the communication between an enhancer and a promoter when they are located between these two elements. In addition, some of these insulators can be “bypassed,” restoring enhancer–promoter communication when a second copy of the insulator is interposed (13–15). Some insulators are involved in chromatin looping, suggesting that chromatin conformation might be a key feature of their functions (3, 16–19). However, the role of insulators in regulating chromatin topology and function is still not understood.

Using a transgenic system, we have investigated the impact of one or two copies of the *gypsy* insulator on chromatin fiber topology and analyzed the ability of this insulator to restrict or target PcG-mediated silencing to promoters. With the development of a high-resolution chromosome conformation capture (H3C) method, we show that one *gypsy* insulator directionally constrains chromatin topology. This constraint enables it to restrict PRE contacts within the chromatin region located on the same side with respect to the insulator. On the other hand, two insulators form a spatially isolated loop inducing chromatin contacts between an upstream PRE and a downstream reporter gene. These data suggest that insulator function relies on its ability to compartmentalize chromatin fibers. Finally, chromatin immunoprecipitation (ChIP) profiles of the Polycomb protein and its associated H3K27me3 histone mark reflect the chromatin conformation restrictions imposed on the PRE by the insulator, suggesting that PcG-mediated silencing is driven by the chromatin fiber topology.

## Results

**Two *gypsy* Insulators Form a Chromatin Loop.** Using a transgenic system carrying a PRE and two insulators from the *gypsy* retrovirus (20) that could be excised using FLP and CRE recombinases (Fig. 1A), we previously showed by ChIP that one *gypsy* insulator is able to protect the expression of downstream reporter genes from PcG proteins acting at a distance from an upstream PRE (Fig. 1A, *Top* and *Middle*) (14). In contrast, two *gypsy* insulators allow PRE-bound PcG proteins to silence the downstream reporter gene (Fig. 1A, *Bottom*), suggesting the possibility that the bypass of the two insulators may depend on the 3D arrangement of the two insulators and may involve chromatin contacts. The use of conventional 3C methods was, however, precluded by the small size of the region of interest (15 kb, with relevant DNA elements separated by 1 or a few kilobases), as well as the need to quantify precisely the variations in chromatin interaction frequency over these short distances. We thus developed an improved method, termed H3C, optimizing several steps in the standard 3C procedure (21–24) (Fig. S1). Specifically, chromatin was digested with the 4-base cutter DpnII, allowing an approximate eightfold increase in spatial resolution (Fig. S2). Furthermore, we established a qPCR method for rigorous, absolute quantification of ligation products, allowing their abundance to be expressed as a percentage of input (Figs. S3 and S4), and found that extensive cross-linking conditions (30 min with 3% formaldehyde) maxi-

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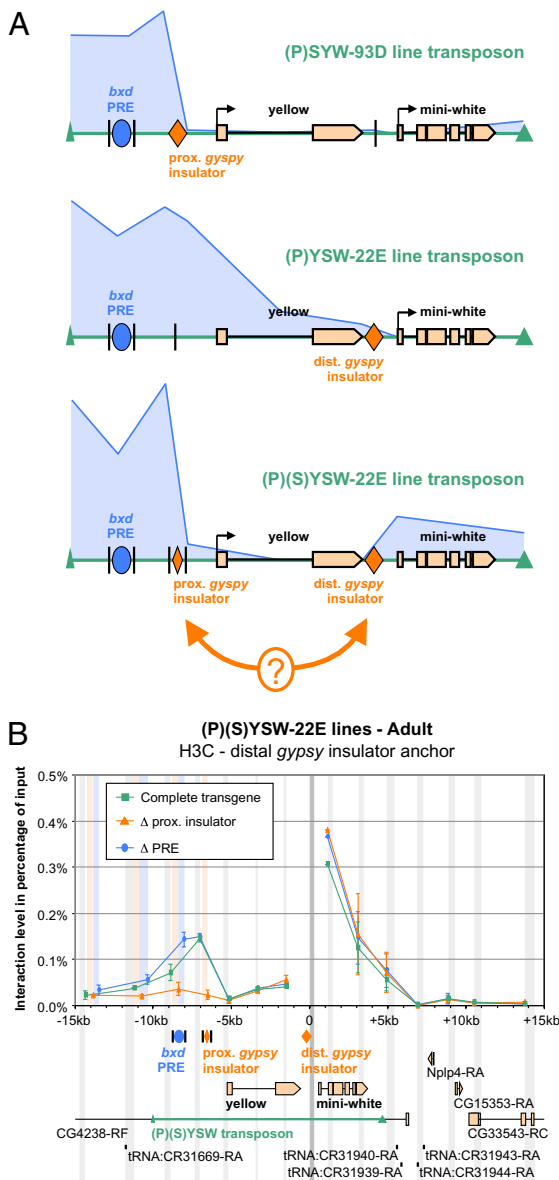
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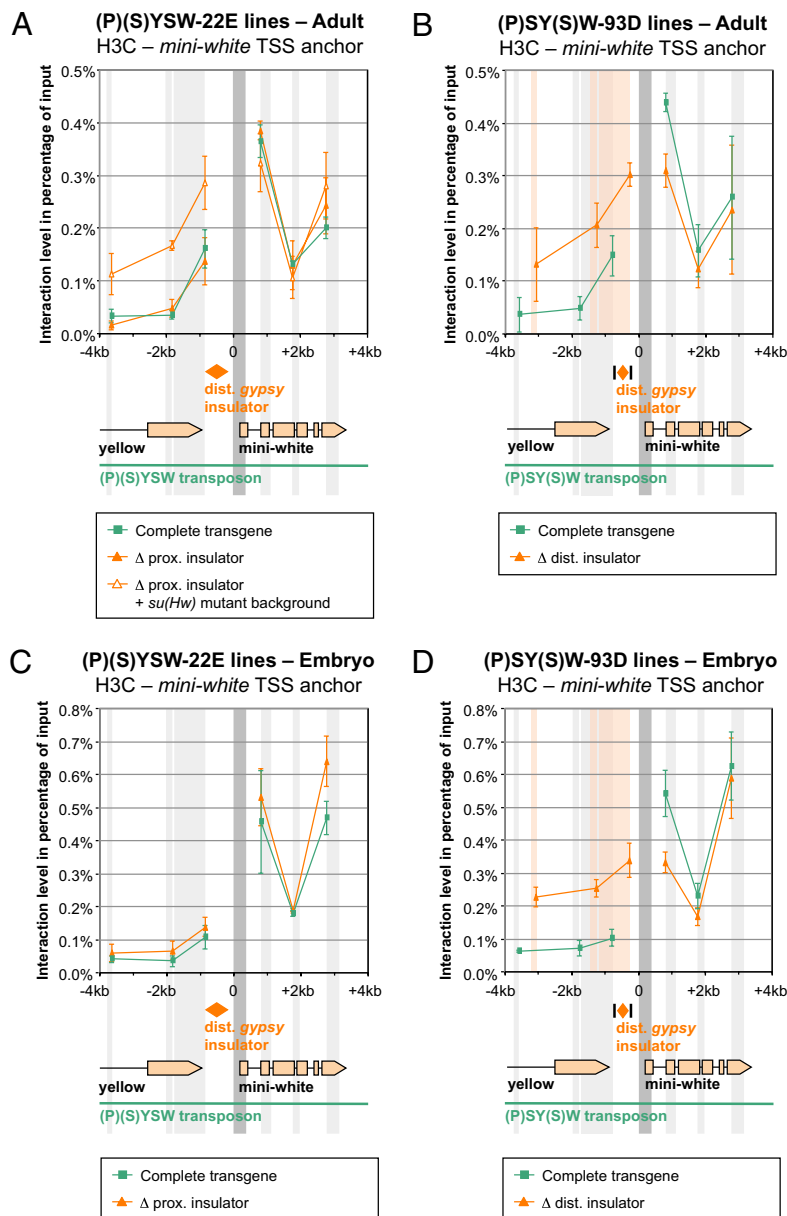
**Fig. 1.** Two *gypsy* insulators trigger the formation of a chromatin loop. (A) Illustration of the transgenic system used in this study and of the distribution of PcG proteins along the transgene in different conditions (14). Transgene DNA is shown by green lines, and the edges of the transgenes are indicated by green triangles. Corresponding *Drosophila* line names are indicated above each transposon. Reporter genes, *gypsy* insulators, and PREs are represented, respectively, by salmon-pink boxes, orange diamonds, and blue ovals. Lox and FRT sites allowing, respectively, the excision of insulators and PREs are indicated by vertical black lines. Active promoters are shown by arrows and the Polycomb protein (PC) binding profiles are indicated by blue curves. The question mark in the bottom indicates the putative interaction between the two insulators. (B) H3C profile of the (P)(S)YSW-22E line and its derivative without the proximal insulator or the PRE, analyzed at adult stage on a 30-kb region centered on an anchor fragment located just downstream of the distal insulator. Transgenes and surrounding genomic regions are drawn to scale. DpnII fragments selected for this analysis are indicated by light gray bands. The anchor fragment is highlighted in dark gray. Fragments whose distances to the anchor changed due to excision of the proximal insulator and the PRE are indicated, respectively, in light orange and in light blue. Error bars represent the SD of the means of two independent experiments.

mize the signal-to-background ratio of chromatin interactions in the distance range of few kilobases (Fig. S5). Finally, we tested the requirement for chromatin dilution before ligation and found

that, surprisingly, this step is not essential (Figs. S6 and S7 and SI Materials and Methods).

We used H3C to map the chromatin conformation along the transgene and surrounding region, using an anchor fragment located just downstream of the distal insulator (Fig. 1B; the proximal insulator is defined as the one closest to the PRE and the distal insulator as the one farthest from the PRE), close to the *mini-white* reporter gene promoter. As expected, on the right side of the insulated domain, interactions were strong with neighboring chromatin fragments and decreased gradually in the *mini-white* gene and the flanking genomic region. Strikingly, the level of interactions was dramatically reduced on the other side of the distal insulator (left of the anchor in Fig. 1B). However, high-frequency interactions were restored just upstream of the proximal insulator, including the PRE. These data demonstrate that the PRE is in physical contact with the *mini-white* gene, in perfect agreement with the strong *mini-white* silencing and PcG binding profile observed in this transgenic line (Fig. S8 and ref. 14). Contacts with the region between the two insulators are absent, suggesting that the locus adopts a looped conformation. Homologous chromosomes extensively pair in *Drosophila* throughout development. In principle, this pairing could favor interactions of a PRE with distal promoters *in trans*, but we discount interchromosomal contacts as being a major factor in reporter gene regulation, as the insulators' influence on the phenotypes is virtually identical when the adults are homozygous or heterozygous for the transgenes in these and other previously studied lines (15) (Fig. S8). Another possible effect due to pairing might be that a heterozygous transgene could loop out to allow the rest of the chromosome to pair with its homolog. In such a looped-out structure some of the elements may get in close proximity, mimicking pairing of two insulators. However, the fact that two insulators are bypassed while one insulator blocks the PRE in a heterozygous state (Fig. S8) indicates that insulator-mediated looping is the dominant determinant of the observed phenotypes and chromatin conformations. Finally, the excision of the PRE did not affect the interaction profile, suggesting that the contacts of the PRE with the anchor fragment are mainly driven by the insulators. Indeed, the excision of the proximal insulator abolished this interaction, in agreement with loss of *mini-white* silencing (14) (Fig. S8). Thus, insulator bypass and preferential chromatin loop structure formation depend on the pairing of the two insulators (Fig. 1B).

**One *gypsy* Insulator Is Able to Locally Constrain Chromatin Fiber Topology.** In the absence of the proximal insulator, anchor contacts seemed to be precluded beyond the distal insulator, suggesting that one insulator is able to constrain chromatin fiber topology (Fig. 1B). To test the effect of the distal insulator on chromatin conformation, we analyzed in more detail a 7-kb region centered on an anchor fragment located at the *mini-white* transcription start site (TSS), both in adult flies (Fig. 2A and B) and embryos (Fig. 2C and D). Downstream of the insulator, the H3C profile of the *mini-white* gene is compatible with a loop between the TSS and the gene 3' end, as previously observed in yeast genes (25). As expected, this structure is formed independently of insulator function, as it was detected after excision of either the proximal or the distal insulator or by mutation of the *su(Hw)* gene that is required for *gypsy* insulator function (15, 26, 27). In contrast, upstream of the insulator the H3C signal is low and decreases gradually (Fig. 2A and B). The low signals observed in this direction suggest that the insulator is able to locally constrain chromatin topology. We then performed H3C after excision of the distal insulator or in a *su(Hw)* mutant background (Fig. 2A and B). Strikingly, both conditions induce an increase of 3C interactions as little as 850 bp upstream of the insulator, whereas no downstream changes are observed. Similar results were obtained when the analysis was repeated in embryos (Fig. 2C and D). These data show that one *gypsy* insulator is able to prevent

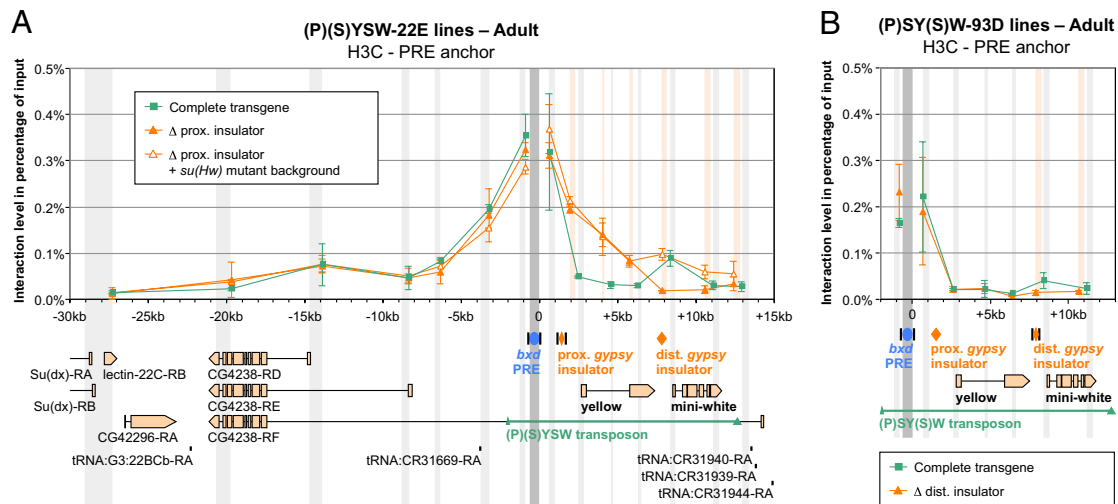


**Fig. 2.** One *gypsy* insulator is able to constrain chromatin fiber conformation. (A) H3C profile in the adult (P)(S)YSW-22E line and its derivative without the proximal insulator, wild type or mutant for *su(Hw)*, analyzed on a 7-kb region centered on an anchor fragment located in the *mini-white* TSS. (B) H3C profile as in A, but in the adult (P)SY(S)W-93D line and its derivative without the distal insulator. (C and D) H3C profiles as in A and B, but at the embryonic stage and only in a wild-type genetic background. All data are presented as described in Fig. 1B. Error bars represent the SD of the means of two independent experiments.

contacts between chromatin fragments when it is located between them, thus behaving as a topological chromatin border.

**PRE Contacts Can Be Organized by the *Su(Hw)* Protein Binding to the *gypsy* Insulator.** We next used H3C to assess the chromatin interaction landscape from an anchor fragment located within the PRE (Fig. 3). Upstream of the PRE, the 3C signal decreased gradually with distance (Fig. 3A), reaching background level at a distance of 20 kb, in agreement with previous observations for chromatin regions devoid of specific 3D interactions (28). As expected, this curve was not affected by either excision of the proximal insulator or mutation of *su(Hw)*. In contrast, the downstream interaction curve showed a sharp decrease after the proximal insulator that was not observed upstream of the PRE at the same distance. A second interaction peak was detected just after the distal insulator, demonstrating that the two insulators

interact by looping out the intervening domain, allowing the PRE to come into close proximity to the *mini-white* promoter. Excision of the proximal insulator (Fig. 3A) restored the symmetry between the downstream and the upstream interaction curves as far as the distal insulator. At that point, the interaction level dropped sharply to background level. The additional mutation of the *su(Hw)* gene induced a higher 3C signal in the *mini-white* gene close to the level observed with the presence of the two functional insulators. This result means that, even at a distance of 8 kb from the PRE, the distal insulator was still imposing a chromatin constraint that was removed upon mutation of the *su(Hw)* gene. Moreover, this result suggests that the PRE may contact the promoter of the repressed gene without the influence of an intervening insulator element, in agreement with previous observations (8, 9). These results are qualitatively reproduced in a second transgenic line, where the PRE–promoter interaction is



**Fig. 3.** The PRE interaction profile is modulated by *gypsy* insulators. (A) H3C profile in the adult (P)(S)YSW-22E line and its derivative without the proximal insulator, wild type or mutant for *su(Hw)*, analyzed on a 45-kb region with an anchor fragment located in the PRE. (B) H3C profile as in A, but in the adult (P)SY(S)W-93D line and its derivative without the distal insulator and restricted to the transgene. All data are presented as described in Fig. 1B. Error bars represent the SD of the means of two independent experiments.

present with two intervening *gypsy* elements, but abolished on removal of one of the insulators (Fig. 3B). The weaker interaction in this line may be accounted for by the use of identical primers despite a reversed orientation of the PRE in the second transgenic line. These data thus show that the topological constraints imposed on chromatin conformation by regulatory elements like insulators can dictate local chromatin architecture and composition by restricting access to distal chromatin or by modulating looping interactions. Moreover, they show that the chromatin constraint depends on Su(Hw) binding to the insulator rather than on the *gypsy* DNA sequence.

**PC and H3K27me3 ChIP Profiles Reflect 3D PRE Interactions.** Finally, we compared H3C profiles from the PRE anchor fragment to quantitative ChIP analysis for Polycomb (PC) and its associated histone mark, H3K27me3 (Fig. 4). H3K27me3 is deposited by the PRC2 complex (6, 29, 30) and the PRC1 component PC binds specifically to H3K27me3 via its chromodomain (31, 32). We found PC binding peaks at the PRE whereas H3K27me3 is locally reduced (Fig. 4C and D), in agreement with previous data suggesting that the core region of PREs is nucleosome depleted (33). Upstream of the PRE, in the (P)(S)YSW-22E lines, both PC and H3K27me3 levels decrease gradually in a way that is reminiscent of the PRE interaction profile in this region (Fig. 4A and C). The only difference between the two is that the PC profile is almost identical to the H3C profile, whereas H3K27me3 levels decrease more slowly. Downstream of the PRE, in the presence of two insulators, PC and H3K27me3 are found upstream of the proximal insulator and downstream of the distal insulator but not in the intervening region, in agreement with the chromatin contact data. Following the excision of the proximal insulator, PC and H3K27me3 profiles are remodeled in perfect agreement with the PRE contact redistribution. Indeed, symmetry between upstream and downstream profiles is restored until the remaining insulator that keeps the *mini-white* gene protected from both PRE contacts and PC or H3K27me3 association. Similar results were obtained when the analysis was repeated in the (P)SY(S)W-93D lines (Fig. 4B and D) and at the adult stage (Fig. S9). These data suggest that the PC and the H3K27me3 ChIP profiles reflect PRE contacts with its surrounding chromatin, arguing for the hypothesis that PRE action generally involves chromatin looping rather than PcG protein spreading.

## Discussion

**Insulator Function and Chromatin Conformation.** Long-distance communication between *cis* regulatory elements and their promoters may involve either chromatin looping or the ability of factors recruited to the regulatory element to scan the surrounding chromatin by mechanisms of linear spreading or tracking along the fiber until they reach the target promoter. 3C technology can distinguish between these mechanisms by detailed analysis of the interaction profile of a chromatin fragment with the surrounding chromatin fiber (34). This interaction profile reflects contact probabilities, thus giving a perception of the conformational constraints imposed on the chromatin fiber in a given locus (35). Using this technology, preferential contacts between regulatory elements and their target promoters have been shown to correlate with their regulatory function, suggesting that chromatin looping can be involved in long-distance regulatory phenomena (3, 16, 19). Our data demonstrate that the *gypsy* insulator functions via chromatin looping. However, it should be noted that our results cannot distinguish between mechanisms whereby homotypic interactions between the *gypsy* elements directly establish chromatin loops or whether transient contacts between elements tracking along the chromatin fiber are stabilized by such interactions.

A second feature of this element is that one copy of the insulator is able to prohibit contacts between adjacent chromatin fragments. Due to the remarkable resolution of H3C, we could detect inhibition of contacts between fragments spaced by <1 kb (Fig. 2) or by greater distances (we detected an effect as far away as 8 kb; Figs. 1 and 3). This topological constraint delimits the range of action of a PRE on its surrounding regions independently of the relative distances between the insulator and the PRE. How does the insulator function to prohibit interactions? A model, called enhancer decoy, suggests that an insulator would mimic a promoter architecture without actually being able to drive transcription (36). This outcome would predict that a regulatory element such as an enhancer or a PRE may interact with it but this interaction would not be functional. Whereas we did see strong homotypic interactions between insulators, our data did not show high contact levels between the PRE and the insulator. Instead, contact levels reduced just adjacent to it and were low at the insulator and on a 5-kb region downstream of it. This result suggests that insulator complexes are repulsive with respect to the PRE. Because the configuration in which the PRE can bypass two



absence of a neighboring PRE (14). This result shows that the *mini-white* promoter itself is not able to recruit PC. Therefore, the ChIP signal observed at this promoter strictly reflects 3D chromatin fiber interactions rather than DNA sequence-autonomous protein binding to their target chromatin sites. Because Su(Hw), as well as many other insulator proteins, binds more than a thousand genomic sites (38, 46–49), this kind of 3D looped chromatin organization may represent an important component modulating PRE-mediated silencing of gene expression.

## Materials and Methods

**Fly Methods.** Transgene cloning, germ-line transformation, and recombination-mediated excision of PREs and insulators are described in ref. 14. All transgenic lines used in this study carry a single copy of their construct and are homozygous for their transgene excepted when indicated. Lines mutant for *su(Hw)* were crossed with the line  $y^1, w^1; su(Hw)^{YTM6}, su(Hw)^f$ . Resulting lines carrying both *su(Hw)* mutations are homozygous for the transgene. Details of the crosses are available upon request. Flies were grown at 25 °C on standard medium for amplification and egg laying was done on plates with standard vinegar medium. Embryos and pupae were collected, respectively, in 0.03% Triton X-100, 0.4% NaCl, 12 h after the beginning of egg laying and 1–2 d after pupa formation. Adult flies were collected 2–4 d after hatching.

**Standard 3C and H3C Methods.** Standard 3C and H3C DNA preparation and quantification as well as procedures to optimize 3C parameters for establishment of H3C are described in *SI Materials and Methods*.

**ChIP Method.** ChIP on *Drosophila* embryos or adult flies was essentially performed as described in ref. 50 with minor modifications, outlined in *SI Materials and Methods*.

**Antibodies.** Antibodies for ChIP experiments (PC and H3K27me3) were diluted at 1:100. PC antibody is described in ref. 50; H3K27me3 antibody is from Upstate Biotechnology (07-449).

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