Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery

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Supporting Online Material

Materials and Methods

Fly strains. The following fly strains carrying mutations in genes that participate in the RNAi pathway were used in this analysis: lines (1) w^1; $piwi^1$/*CyO*, (2) w^1; $piwi^2$/*CyO*, (3) aub^{QC42} *cn bw*/*CyO*, and (4) w^1; $aub^{\Delta P\text{-}3a}$/*CyO* were provided by H. Lin (Duke University, Durham, North Carolina), and lines (5) *mwh* ry^{506} $hls^{P[lacZ,ry+]3987}$ *e*/*TM3*, ry^{RK} *Sb e*, (6) *w*; $hls^{\Delta 125}$ *e*/*TM3, Sb Ser e*, (7) *ru st* hls^{E616} *e ca*/*TM3, Sb* e^s, (8) *cv-c* hls^{E1} *sbd*/*TM3*, ry^{RK} *Sb* e^s, and (9) *ry cv-c* hls^{DE8} *sbd*/*TM2, Ubx ry e* were provided by the C. Berg laboratory (University of Washington, Seattle, Washington). Further description of the mutations can be found in Flybase (http://flybase.bio.indiana.edu).

***mini-white* arrays**: To determine the effect of *homeless* mutations on *mini-white* arrays, each allele of *homeless* was crossed with a multiple balancer stock (*y* w^{67c23}; *SM6a*/*In (2LR) Gla; MKRS*/ *TM3, Ser*). Three different *mini-white* stocks (6-2 *mini-w*; *BX2* and *DX1*) were also combined with the same stock. The F1 males of the former cross were then mated with F1 females of the latter carrying opposite balancers for chromosomes 2 and 3. A mass culture was set up with F2 females (*y* w^{67c23}/*y* w^{67c23}; *BX2*/ *SM6a; hls*/*TM3 Ser*) and F1 males (*y* w^{67c23}; *BX2*/ *Gla;* +/ *MKRS*). The progeny with no

balancer second chromosome, which are homozygous for the *mini-white* array, were selected for pigment assay. The *DX1* and *6-2 mini-w* stocks were combined with *homeless* alleles in a similar manner. To test the effect of homozygous mutations on *mini-white* arrays, the F2 females of the above crosses were mated to their male siblings or F2 males from the other crosses carrying different alleles of the same gene.

Both the *mini-white* arrays and *piwi* are situated on chromosome 2. We initially recombined the *mini-white* arrays and the two *piwi* mutations. For pigment assays, the flies with a *mini-white* array and a *piwi* mutation linked on a second chromosome that were heterozygous for a second chromosome balancer were mated with males of a recombined stock carrying the same or a separate *piwi* allele. The progeny with no balancer chromosome carry the two copies of the *mini-white* array and two alleles of the *piwi* mutation. To test the effect of heterozygous *piwi* alleles, the recombinant chromosomes heterozygous with a second chromosome balancer were mated to the respective *mini-white* stock and the appropriate genotype selected.

To measure eye pigmentation, the heads of 40 flies of each genotype were manually dissected. The amount of red eye pigment was estimated as previously described (*S1*). The isolated heads were homogenized in one ml of methanol, acidified with 0.1% HCl and centrifuged. The absorbance of the supernatant was measured at 480 nm.

To identify larvae of various genotypes, the three different *mini-white* stocks (6-2 *mini-w*; *BX2* and *DX1*) were transferred to the translocation stock *y* w^{67c23}*; T(2;3) CyO TM6, Cy Tb*, which serves as a balancer for chromosomes 2 and 3. In parallel, in order to identify larvae carrying two copies of a *mini-white* array together with the hetero-allelic

combinations of *homeless*, *E1*/*E616* and *DE8*/*E1*, we transferred each array and the two *homeless* alleles, each in combination with the respective *mini-white* array, to the translocation stock *y* w^{67c23}*; T(2;3) CyO TM6, Cy Tb*. For example, in a *y* w^{67c23}; *BX2; hls*/*CyO TM6, Cy Tb* stock, the Tubby (*Tb*) larvae are heterozygous for the complex balancer and the normal larvae are homozygous for *BX2 hls*/ *BX2 hls*. Crossing together the two lines with different *homeless* alleles produces larvae homozygous for *BX2* and heteroallelic for *hls*, which can be identified as the Tb^+ larvae. The two *piwi* recombinants for each *mini-white* array were also transferred to this translocation stock and then crossed together to generate larvae homozygous at the *mini-white* location but heteroallelic for *piwi*.

To identify larvae homozygous for the various mutations depicted in Figure 4 that do not carry the *mini-white* transgenes, the collection of alleles was introduced into stocks with the same second-third translocation balancer, which allows phenotypic recognition of homozygous or heteroallelic mutant individuals.

Fourth chromosome *white* insertions: The effect of these mutations on position effect variegation was studied using two lines carrying variegating *P* element inserts, marked by the *white* gene, on the fourth chromosome (*S2*). In line 118E-10, the *P* element is inserted in the centromeric region, and in line 39C-12 the insert is located in the banded region, just proximal to *Hcf*. The *P* element lines were made using the recipient stock *y* w^{67c23}. To minimize possible background effects, standard genetic crosses were performed to replace all of the chromosomes, except for the chromosome with a given mutation and the corresponding balancer chromosome, with the chromosomes from the *y* w^{67c23} line. Heterozygous flies were obtained by crossing

virgin females carrying the variegating inserts to males with RNAi pathway mutations. Where homozygous or heteroallelic combinations were assessed, crosses were carried out in both directions, so that each allele came either from the mother or the father. As no parental effects were observed, these results were combined to generate the data shown in Figure 2.

To measure eye pigmentation, 4 samples of 10 males (3-4 days post eclosion) were homogenized in 0.5 ml of 0.01M HCl in ethanol; the homogenate was placed at 4°C overnight, warmed at 50°C for 5 min, clarified by centrifugation, and the OD at 480 nm of the supernatant was recorded (*S3*). Mean values (bar) are reported in comparison with the value for the +/+ control stock, with the standard error indicated (thin line).

Immunostaining of the polytene chromosomes: Salivary glands were dissected from third instar larvae and treated as described (*S4*). The chromosomes were fixed in a solution containing 3.7% formaldehyde and 1% Triton X-100 in phosphate buffered saline (PBS) (pH 7.2) for approximately two minutes and transferred to 45% acetic acid containing 3.7% formaldehyde for 2-5 min. The chromosomes were squashed and the slide was placed on dry ice with subsequent removal of the coverslip. The chromosomes were washed twice for 10 min with PBS and finally with PBS, 1% bovine serum albumin, 0.2% Triton X-100, 0.2% azide (PBT) for 30 min. The chromosomes were incubated overnight at 4˚C with primary antibodies in PBT buffer. For HP1 and HP2 proteins, chromosomes were labeled with rabbit anti-HP1 antibody (1:100) or rabbit anti-HP2 (*S5*) antibodies (1:100) as well as mouse anti-Sex Lethal M-18 (University of Iowa Hybridoma Bank) antibodies (1:50). Rabbit anti-H3-mK9 from Upstate Biotechnology was used (1:100) to detect H3 methylated at K9. For double labeling of HP1 and H3-

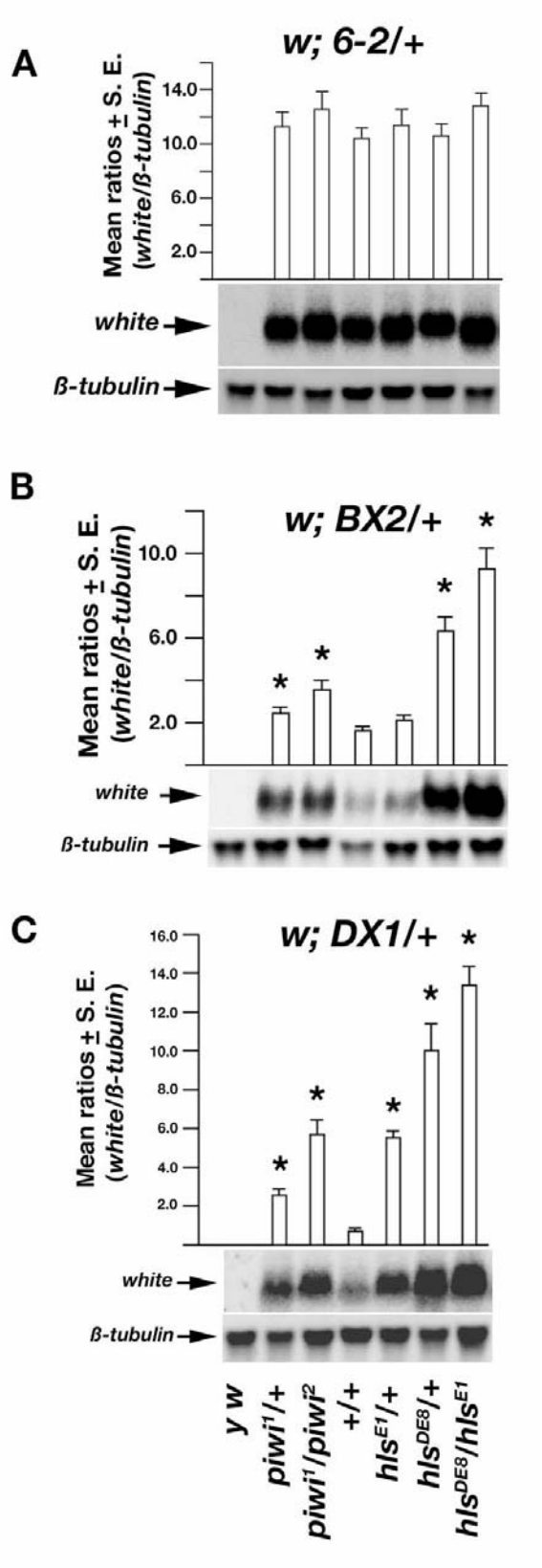
mK9, mouse monoclonal C1A9 anti-HP1 antibodies (*S6*) were used (1:100). To determine the specificity of methylated H3-K9, primary antibodies against the di and tri methylated forms (from T. Jenuwein) were applied to chromosome mixtures, using rabbit anti-H3-tmK9 antibodies (1:50) or rabbit anti H3-dmK9 antibodies (1:50) (*S7*) and mouse anti-SXL antibodies (1: 50). For detection of H3-mK27 residues, the fixed chromosomes were probed with primary rabbit H3-tmK27 (1:50) or H3-dmK27 (1:50) (*S7*) and mouse anti-SXL antibodies (1:50). After two 10 min washes in PBS and a 30 min incubation in PBT and 2% goat serum, the chromosomes were incubated with fluorochrome conjugated secondary antibodies for 3 hrs at room temperature in complete darkness. The slides were then incubated with RNase (50 ng/ml) for 30 min at 37°. Finally, the preparations were washed twice in PBS for 10 min each and air-dried in the dark. Secondary antibody for SXL was FITC-conjugated goat anti-mouse; for HP1, Cy5-conjugated goat anti-mouse or anti-rabbit; and for HP2, Cy5 conjugated goat anti-rabbit (Jackson ImmunoResearch). Preparations were finally mounted with a mixture of Vectashield mounting media with propidium iodide (PI) (1.5μg/ml). The slides were examined with a Bio-Rad 600 confocal microscope using a 100X oil lens.

Northern blot: Northern analysis was performed as previously described (*S8*). The northerns were probed for *white* mRNA and subsequently for *tubulin,* as a loading control, as described (*S6*).

Western blot: Fifteen adult flies in each genotype were homogenized in 100μl Laemmli loading buffer containing protease inhibitors as described (*S8*). The crude extracts were boiled for 5 min and centrifuged to remove debris. 200 μg of protein were loaded per well of a 6% stacking and 8% running PAGE-SDS gel. Proteins were transferred to a

nylon membrane using an electro-blot apparatus at 120 mA for 1.5 to 2 hrs. The membrane was blocked overnight in 1X Tris buffered saline (TBS) (pH 8.0) plus 5% nonfat milk. The diluted mouse monoclonal HP1 antibodies (C1A9) (1: 3000) or rabbit anti-H3-mK9 antibodies (1:1000) (Upstate Biotechnology) were added to the incubation solution (1X TBS plus 2.5% nonfat milk) for 60-90 min. Subsequently, blots were incubated with secondary antibodies in the same solution for one hour. The amount of HP1 or H3-mK9 in each genotype was detected using horseradish peroxidase-conjugated detection ECL Kits (Amersham Pharmacia). The blots were reprobed with mouse anti-tubulin antibodies (E7, University of Iowa Hybridoma Bank) (1:500); tubulin was detected using the ECL detection method. The images were converted to Pict files to estimate the band intensities.

Fig. S1.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w; 6-2/+
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10.0
6.0
2.0
white
ß-tubulin
B
w; BX2/+
Mean ratios ± S. E. (white/ß-tubulin)
10.0
6.0
2.0
*
white
ß-tubulin
C
w; DX1/+
Mean ratios ± S. E. (white/ß-tubulin)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
*
white
ß-tubulin
y w
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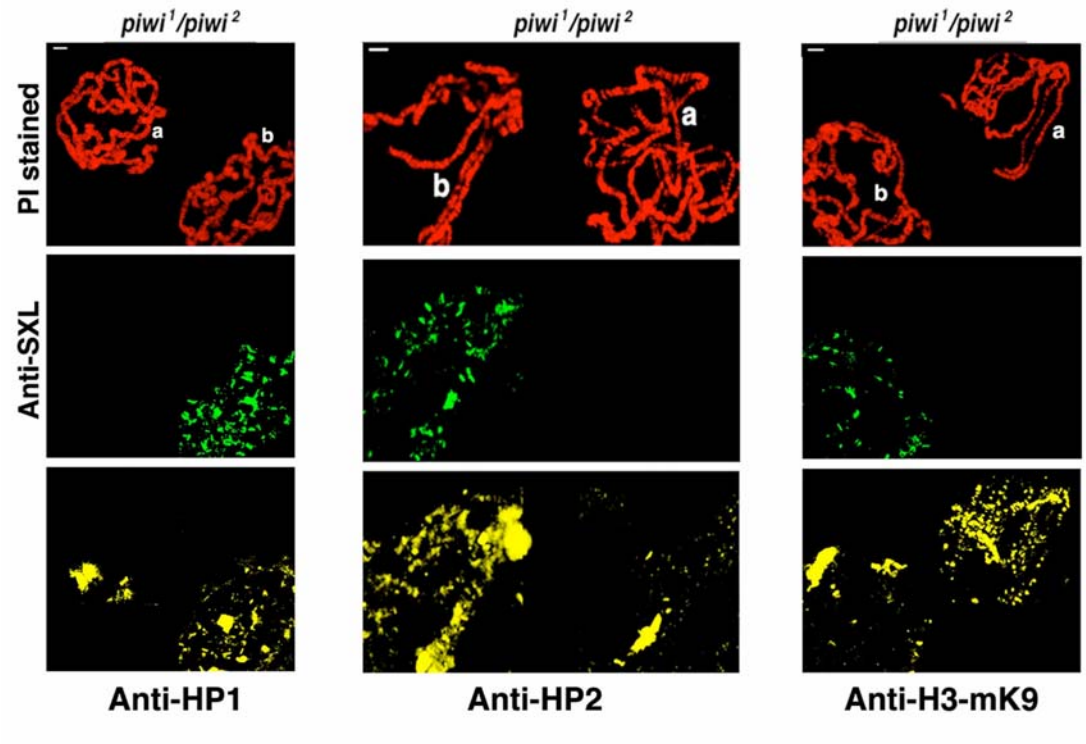
hlsE1/+
hlsDE8/+
hlsDE8/hlsE1

Fig. S2.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piwi1/piwi2
piwi1/piwi2
PI stained
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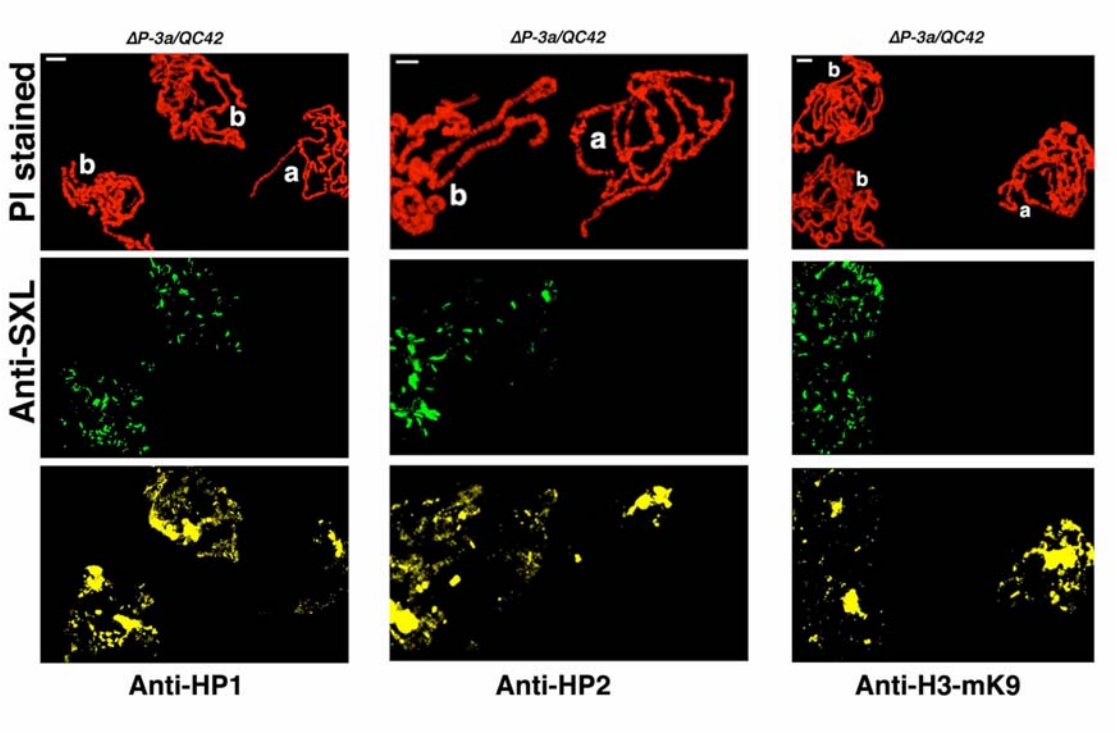
a
b
a
b
a
b
Anti-HP1
Anti-HP2
Anti-H3-mK9

Fig. S3.


ΔP-3a/QC42
ΔP-3a/QC42
ΔP-3a/QC42
PI stained
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b
b
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b
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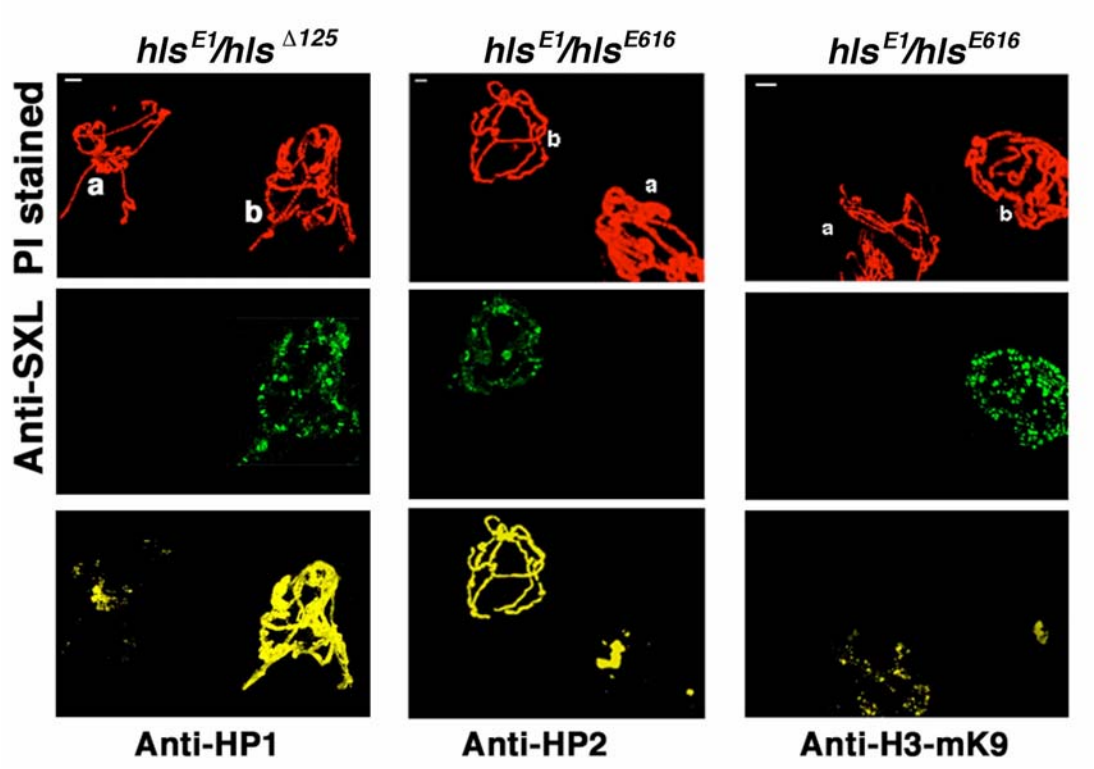
Anti-HP1
Anti-HP2
Anti-H3-mK9

Fig. S4.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hls^E1/hls^E616
hls^E1/hls^E616
PI stained
Anti-SXL
a
b
b
a
a
b
Anti-HP1
Anti-HP2
Anti-H3-mK9

Fig. S5.

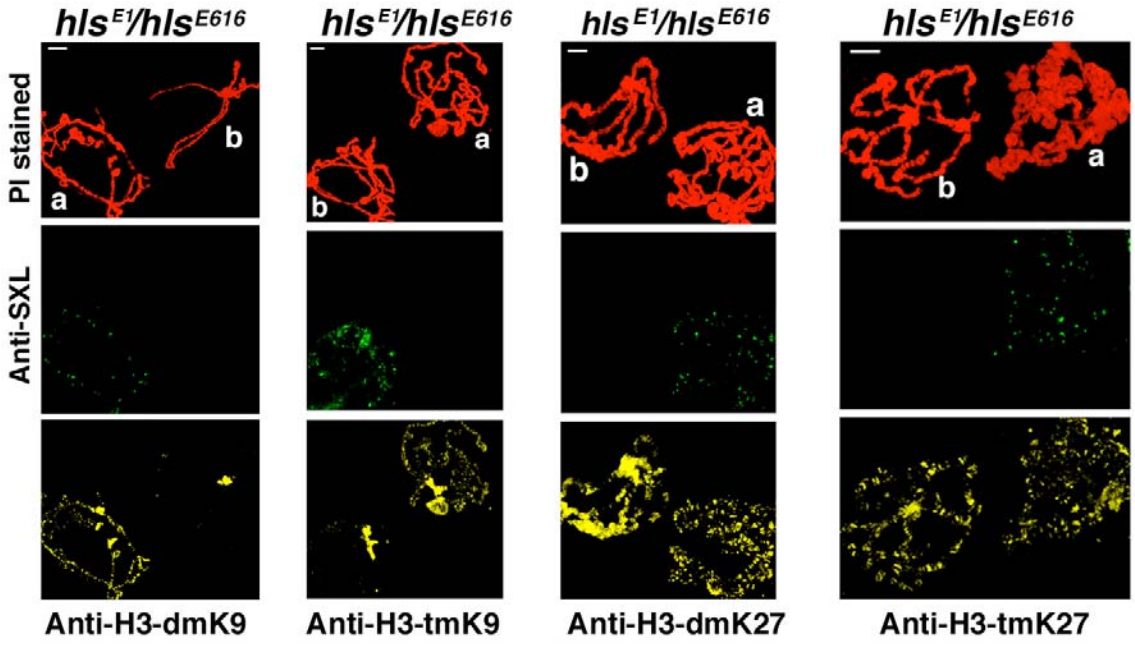
hls E1/hls E616
hls E1/hls E616
hls E1/hls E616
hls E1/hls E616
PI stained
Anti-SXL
a
b
a
b
a
b
a
b
Anti-H3-dmK9
Anti-H3-tmK9
Anti-H3-dmK27
Anti-H3-tmK27

Figure Legends for Supporting Online Material

Fig. S1. Northern analysis of repeat induced silencing. Total cellular RNA was extracted from adults of selected genotypes carrying various *mini-white* arrays with different combinations of *piwi* and *homeless* mutations. Northerns were performed in triplicate with measurement of *white* RNA relative to *B-tubulin*. Asterisks denote those means that are significantly different from the respective control (+/+, the *mini-white* array with normal alleles at *piwi* and *homeless*) at the 95% level of confidence. All genotypes are male and are noted in the figure.

Fig. S2. Comparative assessment of the impact of *piwi* mutations. Simultaneous squashes for homozygous *piwi* and control (Canton S) lines, probed using rabbit antibodies specific for HP1, HP2, and H3-mK9 (Upstate Biotechnology), counterstained with propidium iodide and viewed by confocal microscopy, are shown. The mixtures were made using females of one genotype and males of the other, as well as the reciprocal combination, so that staining with mouse antibodies against Sex-lethal (SXL), which is only present in females (*S9*), would allow one to assign unambiguously each nucleus to the proper genotype. The examples depicted were chosen because adjacent nuclei of different genotype were present in the same field of view; some, but not all, are represented in Figure 4. As illustrated in Figure 4, some decrease in histone H3 methylated at K9 is observed, and a small increase in the level of HP1 and HP2 associated with the euchromatic arms is noted. a = Canton S; b = mutant.

Fig. S3. Comparative assessment of the impact of *aubergine* mutations. Simultaneous squashes for homozygous *aubergine* and control (Canton S) lines, probed using antibodies specific for HP1, HP2, and H3-mK9 (Upstate Biotechnology), counterstained with propidium iodide and viewed by confocal microscopy are shown. The mixtures were prepared and probed with SXL antibody to identify the two genotypes as described in figure S2. Some decrease in histone H3 methylated at K9 is observed, and a small increase in the level of HP1 and HP2 associated with the euchromatic arms is noted. a = Canton S; b = mutant.

Fig. S4. Comparative assessment of the impact of *homeless* mutations. Simultaneous squashes for homozygous *homeless* and control (Canton S) lines, probed using antibodies specific for HP1, HP2, and H3-mK9 (Upstate Biotechnology), counterstained with propidium iodide and viewed by confocal microscopy are shown. The mixtures were prepared and stained with SXL antibody to identify the two genotypes as described in figure S2. As illustrated in Figure 4, a strong decrease in histone H3 methylated at K9 is observed, and HP1 and HP2 are redistributed along the euchromatic arms. a = Canton S; b = mutant.

Fig. S5. Specificity of H3 modification affected. Simultaneous squashes of homozygous *homeless* and control (Canton S) lines in the same field of view, stained using antibodies specific for different histone H3 modifications: dimethylation of K9 (H3-dmK9), trimethylation of K9 (H3-tmK9), dimethylation of K27 (H3-dmK27), and trimethylation of K27 (H3-tmK27) (*S7*). The mixtures were prepared and labeled with SXL antibody to identify the two genotypes as

described in figure S2. There is a significant loss of both H3 dimethylated at K9 and H3 trimethylated at K9, while there appears to be little change in the distribution of H3 modified by di- or trimethylation at K27. a = Canton S; b = mutant.

References and Notes:

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