Abstract

Highly repetitive and transposable element rich regions of the genome must be stabilized by the presence of heterochromatin. A direct role for RNA interference in the establishment of heterochromatin has been demonstrated in fission yeast. In metazoans, which possess multiple RNA–silencing pathways that are both functionally distinct and spatially restricted, whether RNA silencing contributes directly to heterochromatin formation is not clear. Previous studies in Drosophila melanogaster have suggested the involvement of both the AGO2-dependent endogenous small interfering RNA (endo-siRNA) as well as Piwi-interacting RNA (piRNA) silencing pathways. In order to determine if these Argonaute genes are required for heterochromatin formation, we utilized transcriptional reporters and chromatin immunoprecipitation of the critical factor Heterochromatin Protein 1 (HP1) to monitor the heterochromatic state of piRNA clusters, which generate both endo-siRNAs and the bulk of piRNAs. Surprisingly, we find that mutation of AGO2 or piwi increases silencing at piRNA clusters corresponding to an increase of HP1 association. Furthermore, loss of piRNA production from a single piRNA cluster results in genome-wide redistribution of HP1 and reduction of silencing at a distant heterochromatic site, suggesting indirect effects on HP1 recruitment. Taken together, these results indicate that heterochromatin forms independently of endo-siRNA and piRNA pathways.

Introduction

In D. melanogaster, an estimated one-third of the genome is composed of repetitive and noncoding sequences associated with a condensed form of chromatin known as heterochromatin. Heterochromatin is characterized by repeat-rich sequences, hypoacetylation of histone tails, and dimethylation of histone H3 on lysine 9 (H3K9me2) [1]. A conserved nonhistone Heterochromatin Protein 1 (HP1) is a critical component of heterochromatin, localizing predominantly at and near centromeres but also residing at telomeres, and the Y and fourth chromosomes. These regions tend to be rich in transposable elements (TEs), which must be suppressed in order to maintain genomic stability but can serve a cellular function, particularly in the case of Het-A and TART at the telomeres (reviewed in [2]).

The phenomenon of position-effect variegation (PEV) provided the first glimpse into the role of heterochromatin in gene silencing in Drosophila. When a normally euchromatic gene is relocated near heterochromatin, variegated expression results from variable levels of heterochromatin spreading over the gene in each cell. Screens for dominant mutations that either suppress {Suppressor of variegation [Su(var)]} or enhance {Enhancer of variegation [E(var)]} PEV were performed to identify key components of heterochromatin. For example, mutation of Su(var)9-3, which encodes an H3K9 methyltransferase, was identified in a large screen for modifiers of PEV [3]. Accordingly, loss of HP1, encoded by Su(var)2-5, causes increased expression of a gene subject to PEV while an extra copy has the reverse effect [4].

Pioneering genetic and biochemical studies in Schizosaccharomyces pombe have shed considerable light on mechanisms of heterochromatin assembly. The RNA interference (RNAi) machinery was found to play a key role in heterochromatin formation by detecting the transcription of specific DNA repeats located at the mating type locus and the centromere and subsequently nucleating heterochromatin. For example, double-stranded RNAs (dsRNA) produced by bidirectional transcription of pericentromeric repeats are processed by the RNAse III endonuclease Dicer1 into short interfering RNAs (siRNAs) [5]. The Argonaute1 PAZ and PIWI domain protein binds these siRNAs as part of the RNA-induced transcriptional silencing complex (RITS) [6]. Loading of RITS with siRNA and recruitment of the complex to the site of dsRNA transcription requires the Clr4 histone methyltransferase, which methylates H3K9 [7]. This methylation mark serves as a binding site for Swi6, a fission yeast homolog of HP1, leading to heterochromatin establishment and spreading. Importantly, heterochromatin can also be nucleated independently of RNAi by other mechanisms. For example, in the absence of RNAi the ATF/CREB stress-activated proteins promote heterochromatin formation at the mating type locus [8], and the Taz1 protein can establish HP1 recruitment to telomeres [9]. These studies exemplify the redundancy of RNAi and additional mechanisms with respect to the formation of heterochromatin.
Author Summary

One role for silent heterochromatin is to preserve the integrity of the genome by stabilizing regions rich in repetitive sequences and mobile elements. Compaction of repetitive sequences by heterochromatin is needed to prevent genome rearrangement and loss of genetic material. Furthermore, uncontrolled movement of mobile elements throughout the genome can result in deleterious mutations. In fission yeast, one important mechanism of heterochromatin establishment occurs through RNA interference, an RNA–dependent gene silencing process. However, it is unclear whether a direct role for RNA silencing in heterochromatin formation is conserved throughout evolution. In the fruit fly, Drosophila melanogaster, which harbors multiple RNA–silencing pathways that are both functionally distinct and spatially restricted, previous studies have suggested the involvement of the endogenous small interfering RNA (endo-siRNA) and Piwi-interacting RNA (piRNA) pathways in heterochromatin formation. These small RNA silencing pathways suppress the expression of mobile elements in the soma or in both somatic and germline tissues, respectively. Utilizing complementary genetic and biochemical approaches, we monitored the heterochromatin state at discrete genomic locations from which both types of these small RNAs originate in endo-siRNA or piRNA pathway mutants. Our results indicate that heterochromatin can form independently of these two small RNA silencing pathways.

All RNA silencing pathways are characterized by the activity of an Argonaute effector protein that binds directly to small RNA. The five Argonautes in Drosophila can be divided into two families based on homology. The AGO subfamily includes AGO1 and AGO2, and the Piwi subfamily consists of Piwi, Aubergine (Aub), and AGO3 (reviewed in [10]). AGO2 and AGO3 are expressed throughout the fly while piwi, aub, and AGO3 are expressed mainly, although not exclusively, in the gonad [11–13]. AGO1 is required for the microRNA pathway, which regulates mRNA expression and functions chiefly through translational repression. Protecting against exogenous double stranded RNA, AGO2 associates with 21–22 nt siRNA produced by Dicer-2 (Dcr-2), and this pathway is required for viral immunity and a robust RNAi response [14,15]. In addition, AGO2 also binds endogenous siRNAs (endo-siRNAs), the majority of which silence the expression of TEs outside of the gonad [16–19].

Suppression of TEs is especially imperative in the gonad in order to limit the propagation of unwanted mutations and is achieved principally by the activity of the Piwi subfamily proteins. Piwi, Aub, and AGO3 bind to 23–30 nt RNAs termed Piwi-interacting RNAs (piRNAs) that are predominantly derived from genomic locations termed piRNA clusters [20,21]. These piRNA producing loci are mainly pericentromeric and enriched in transposon sequences. From these and previous studies, it became clear that the piRNA pathway exists to eliminate TE transcripts in the gonad [22–24]. Based on comparative sequence analysis of piRNAs immunopurified from the ovary, the “ping-pong” or “amplification loop” model for germline piRNA biogenesis was proposed [20–23]. Precursor transcripts from piRNA clusters, derived from either one or both strands [25], give rise to piRNAs bound by Piwi, Aub, or AGO3. Those piRNAs antisense to a homologous TE transcript can result in its cleavage, and this event defines the 5’ end of a secondary piRNA that can then bind and cleave an antisense piRNA cluster transcript, and the cycle can continue. Piwi appears to play a minor role in ping-pong piRNA amplification [25,26], which is thought to occur primarily in the cytoplasmic nuage where Aub and AGO3 localize [20,23,27]. In contrast, Piwi resides in the nucleus [28]. Production of precursor transcripts at certain piRNA clusters that give rise to piRNAs from both sense and antisense strands (dual-strand clusters) is dependent on the germline specific HP1 homolog Rhino [29]. Rhino functions specifically in the ping-pong pathway, acting upstream of Aub and AGO3 but not Piwi.

Piwi independently serves an additional role in the silencing of certain TEs expressed in somatic follicle cells surrounding the ovary. This somatic piRNA pathway depends on Piwi alone and therefore does not undergo ping-pong amplification [25,26,30]. The flamenco (flam) piRNA cluster, which controls the gypsy, ZAM, and Idefix retrotransposons [31,32], is one of the major sites of primary piRNA production [25,26,30,33]. Piwi associates with piRNAs generated by flam and other piRNA clusters and has been proposed to cleave homologous TE transcripts using its Slicer activity [22].

Previous studies suggest that one or more RNA silencing pathways may participate in transcriptional TE silencing by inducing heterochromatin formation. First, mutation of AGO2 results in pleiotropic cellular defects in early embryos including mislocalization of HP1 and the histone H3 variant CTD, which binds specifically the centromere [34]. Later in development, AGO2 mutants display mislocalization of HP1 on polytene chromosomes of the larval salivary gland [35]. Additionally, silencing of a pericentromeric transcriptional reporter is relieved when the maternally derived pool of AGO2 is reduced. Despite these defects, AGO2 mutant flies develop normally and are fertile, suggesting that these defects are mild and can be compensated by other mechanisms.

Several pieces of evidence implicate piRNA pathways in establishment or maintenance of heterochromatin in the soma. First, mutation of piwi, aub, or spn-E, encoding an RNA helicase required for the germline piRNA pathway [24,26], results in defects in heterochromatine silencing and visible changes in heterochromatin localization. These mutants reduce silencing of pericentromeric transcriptional reporters and exhibit mislocalization of HP1 and H3K9me2 in salivary gland polytene chromosomes [36]. Moreover, a recent study identified HP1 as an interactor of Piwi in yeast two-hybrid screens [11]. The two proteins coimmunoprecipitate from embryonic nuclear lysate and display partially overlapping localization patterns in polytene chromosomes. Furthermore, both proteins associate specifically with the chromatin of transposable elements, 1360 and the F element. Based on their findings, the authors propose that Piwi could serve as a recruitment platform for HP1 binding. This model appears not to be applicable to the 3R-TAS subtelomeric region, a site of Piwi chromatin association and piRNA production [21]. Mutation of piwi results in an increase of HP1 association and an increase of transcriptional silencing at 3R-TAS. It remains an open question whether other sites in the genome could serve as Piwi-dependent HP1 recruitment sites.

In other metazoans, it is similarly unclear whether RNA silencing can establish heterochromatin directly. A recent study in chicken indicates that a 16 kb constitutive heterochromatin domain that separates the folate receptor gene and the β-globin locus is maintained by a Dicer and Argonaute 2 (cAg02) dependent mechanism [37]. Intriguingly, cAg02 was shown to associate with the heterochromatic domain by chromatin immunoprecipitation (ChIP) suggesting a direct effect. However, it is not known whether this represents a general mechanism to maintain heterochromatin.
In this study, we investigated whether HP1 association with heterochromatin in Drosophila is mediated by either the AGO2 dependent endo-siRNA pathway or by piwi dependent piRNA pathways. Using transcriptional reporters and ChIP, we show that piRNA clusters are subject to heterochromatin silencing and bound by HP1. Interestingly, mutation of AGO2, piwi or aub results in increased silencing at piRNA clusters and an increase in HP1 association with these loci. Furthermore, loss of piRNA production at a single piRNA locus results in global redistribution of HP1 and a reduction of silencing at a distant heterochromatic site. Therefore, our results indicate that HP1 can associate with chromatin independently of both endo-siRNA and piRNA pathways.

Results
Heterochromatin-dependent transcriptional silencing at piRNA clusters
We sought to determine if HP1 is recruited to heterochromatin by AGO2 or Piwi. The majority of genomic regions that produce the bulk of piRNA, termed piRNA clusters, are pericentromeric and rich in transposable elements [20,21]. These regions also produce endo-siRNA [16–19], and due to their proximity to the centromere, may be heterochromatic and serve as platforms for Argonaute mediated HP1 recruitment. In order to test genetically whether pericentromeric piRNA clusters are heterochromatic, we examined a collection of fly lines bearing P element transgene insertions in or close proximity to four piRNA producing loci, *flam*, 80EF, 42AB, and 3BC. The P elements contain a *mini-white* transcriptional reporter that was assayed for expression in the adult eye. Genomic locations of these transgene insertions are indicated in relation to previously identified small RNAs immunoprecipitated with Piwi, Aub/AGO3, and AGO2 respectively from various cell types (Figure 1, Figure S1) [16,17,20,21]. Lines harboring P elements inside or in the vicinity of a piRNA cluster exhibit variegating coloration of distinct eye facets similar to PEV, suggesting the presence of variably spreading heterochromatin at their sites of insertion (Figure 2, Table 1). Interestingly, insertions within a piRNA cluster that display high *mini-white* expression without variegation harbor *SUPor-P* constructs, which contain Suppressor of Hairy wing (Su(Hw)) insulator sequences that flank and likely protect the Suppressor elements that flank and protect the piRNA cluster. We further tested whether the transcriptional reporters at piRNA clusters in somatic tissues of RNA silencing mutants
In order to further examine the heterochromatic nature of piRNA clusters at higher resolution, ChIP assays were performed in adult heads to assess HP1 association with two piRNA clusters, *flam* and 80EF, in the soma. Genomic locations of primer sets that uniquely amplify regions spanning these piRNA clusters are indicated in Figure 1A and 1B. As positive controls, primers for two transposable elements known to recruit HP1, TART, a telomere-specific non-LTR retrotransposon, and 1360, a DNA transposon were also tested [39–40]. Euchromatic genes *hsp26* and *yellow* were also included in the analysis as negative controls for HP1 association. In wild type fly heads, HP1 is observed at or near locations that give rise to piRNAs and endo-siRNAs at both *flam* and 80EF loci. ChIP was performed using α-HP1 antibodies in chromatin prepared from wild type heads, and the amount of DNA associated was determined by quantitative PCR using specific primer sets. As expected, low levels of *hsp26* and *yellow* were immunoprecipitated with HP1, while TART and 1360 levels are enriched above the euchromatic genes by over six-fold (Figure 3). At *flam*, HP1 associates with the majority of regions that produce high levels of piRNAs or endo-siRNAs approximately two to three-fold over the euchromatic sites (Figure 3A, primer sets 1–15). Similarly, at 80EF, HP1 immunoprecipitates piRNA and endo-siRNA producing regions two to three-fold higher than the negative controls indicating the presence of heterochromatic marks at these loci (Figure 3B, primer sets G-M). Regions flanking these areas display approximately one to two-fold enrichment over euchromatic sites, which may be due to tapering of HP1 spreading (Figure 3B, primer sets A-F and N-P). ChIP using antibodies directed against the chromatin insulator protein Su(Hw) verified its presence at known insulator sequences *gypsy* and 1A-2 [41] but only background levels at TART, 1360, and piRNA clusters, indicating the specificity of HP1 association at these sites (Figure S3). Rabbit IgG negative control immunoprecipitations yielded negligible amounts of DNA for all sites tested (<0.3% input).
Figure 1. Schematic representation of three top piRNA clusters. Genomic locations of small RNAs, primer sets used for ChIP, and P element insertions at (A) *flam* piRNA cluster on chromosome X, (B) *80EF* piRNA cluster on chromosome 3L, and (C) *42AB* piRNA cluster on chromosome 2R. Sequence datasets derived from previous studies were mapped to the genome using Bowtie software allowing two or zero mismatches [57]. Piwi-
Consistent with the transcriptional reporter assay, RNA silencing mutants display elevated levels of HP1 at piRNA clusters. ChIP of HP1 was performed in piwi1/piwi2 mutant heads, and similar levels at positive and negative controls were obtained compared to wild type (Figure 3). In contrast, at the flam locus, a two to five-fold increase in HP1 levels is observed at the centromere proximal side of the locus compared to wild type (Figure 3A, primer sets 6–15). Little change in HP1 recruitment is observed at the centromere distal end of flam in piwi1/piwi2 mutants (Figure 3A, primer sets 1–5). At 80EF, HP1 levels increase two to three-fold in piwi1/piwi2 mutants compared to wild type across all primer sets examined (Figure 3B, primer sets A–P).

In order to address differences in strain background and potential accumulation of TEs in piwi mutant strains, we performed ChIP assays comparing piwi1/piwi2 mutants to a piwi1/+ heterozygous strain and obtained similar results (Figure S4).

ChIP experiments performed in AGO251B mutant heads show a similar overall increase of HP1 at piRNA clusters compared to piwi1/piwi2 mutants. Levels of HP1 at hsp26, yellow, TART, and 1360 are similar in AGO251B mutants and wild type while differences are apparent at piRNA clusters (Figure 3). At flam, AGO211B mutants display a two to seven-fold increase of HP1 association with the centromere proximal side compared to wild type (Figure 3A, primer sets 6–15). At the centromere distal end, no significant changes in HP1 levels are detected (Figure 3A, primer sets 1–5). For 80EF, AGO211B mutants show similar levels of HP1 to wild type at the centromere distal end (Figure 3B, primer sets A-D) while an approximately two to five-fold increase of HP1 is detected in the remainder of the regions tested (Figure 3B, primer sets E–P). Moreover, ChIP assays in AGO211B homozygous mutants compared to an AGO211B/+ heterozygous strain produced similar results (Figure S5). Similar to AGO211B mutants, Der-2115A mutants show an increase of HP1 at regions that produce small RNAs compared to wild type (Figure S2B and S2C). HP1 protein levels in wild type, piwi1/piwi2, and AGO211B fly heads are similar indicating that the increased chromatin association observed is not due to an increased amount of HP1 (Figure S6). The increased HP1 chromatin association with piRNA clusters in RNA silencing mutants compared to wild type is consistent with increased silencing of P element insertions, and these results suggest that at least some of the observed effects on reporter gene expression in RNA silencing mutants are due to chromatin related events. Taken together, these data suggest an
antagonistic effect of Piwi, Aub, andAGO2 on HP1 recruitment to chromatin in somatic tissue.

**HP1 also associates with piRNA clusters in ovaries**

Given the evidence that transposable elements are mainly silenced in the gonad via piRNA pathways and in the soma via the endo-siRNA pathway, we wanted to determine whether HP1 also associates with piRNA clusters in gonadal tissues. Therefore, we investigated HP1 recruitment to piRNA clusters in wild type ovaries by ChIP. As in heads, low levels of hp26 and yellow are immunoprecipitated with HP1, whereas TART and 1360 loci are enriched above the euchromatic genes by over ten-fold (Figure 4). At the flam locus, a four to fifteen-fold increase over the euchromatic sites in HP1 levels is observed at most sites at the centromere proximal side of the locus (Figure 4A, primer sets 4-15). Similarly, at 80EF, HP1 immunoprecipitates small RNA producing regions two to twenty-fold higher than euchromatic sites indicating the presence of heterochromatic marks at these loci (Figure 4B, primer sets A-P). Rabbit IgG negative control immunoprecipitations yielded negligible amounts of DNA for all sites tested. We were unable to immunoprecipitate DNA at levels above background from either heads or whole ovaries using multiple antibodies to Piwi, Aub, AGO3, and AGO2 that have been used in previous studies for immunoprecipitation or immunofluorescence (data not shown) [11,22,23,42].

**HP1 chromatin association is not affected greatly by depletion of Piwi in somatic ovarian follicle cells**

We wished to address whether HP1 association with piRNA clusters is dependent on Piwi in the gonad, which express high levels of both proteins. Due to a complete loss of germ cells and the severe underdevelopment of ovarian tissue in piwi mutants, it was not possible to obtain enough mutant material to perform ChIP. Therefore, we examined the recruitment of HP1 to chromatin in an ovarian somatic follicle cell line (OSC) that expresses Piwi but not Aub or AGO3 and produces only primary piRNAs, a large proportion of which derive from the flam locus [30]. The majority of Piwi was depleted from OSC cells by siRNA-mediated knockdown, and depletion of Piwi does not affect HP1 or Lamin protein levels compared to mock transfected cells (Figure 5A).

Subsequently, we investigated HP1 recruitment to piRNA clusters by ChIP in OSC cells. In mock treated cells, low levels of hp26 and yellow are immunoprecipitated with HP1, while TART and 1360 loci are enriched above the euchromatic genes by 1.5- to over two-fold (Figure 5B and 5C). Two additional TEs tested, gypsy and mdg1, are immunoprecipitated at similar levels to TART with HP1 (Figure 5B-5E). At flam, HP1 associates with the piRNA cluster similar to TE levels (Figure 5B and 5C). Despite much lower piRNA production from the 80EF cluster in OSC compared to flam [30], HP1 associates with piRNA producing regions of 80EF at similar levels to flam and TEs (Figure 5C, primer sets A-P). Overall, the HP1 recruitment profile in OSC is similar to that of heads and whole ovaries albeit at lower relative levels. In Piwi knockdown cells, no significant differences are seen for HP1 recruitment to all sites compared to mock treated cells except a two-fold decrease at the 1360 element. Rabbit IgG negative control immunoprecipitations yielded low amounts of DNA for all sites tested (<0.06% and <0.07% input for mock and Piwi knockdown cells, respectively).

Importantly, Piwi association with chromatin is detectable in OSC cells, but its profile differs from that of HP1. In mock treated cells, antibodies directed against Piwi [22] immunoprecipitate euchromatic sites at levels similar to that of TEs (Figure 5D and 5E). Furthermore, the majority of regions producing piRNA at flam is also immunoprecipitated at comparable levels to both

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**Table 1. Expression of mini-white in fly lines harboring P element insertions in four top piRNA clusters.**

| Insertion       | piRNA cluster | Genomic coordinates of insertion | Variegation | Inside piRNA cluster |
|-----------------|---------------|----------------------------------|-------------|----------------------|
| P{EPgy}2DIPy1360 | flam          | X:21,501,171 [-]                | Yes         | No                   |
| P{SUPor-P}flam  | flam          | X:21,505,285 [-]                | No          | No                   |
| P{GT}4flam      | flam          | X:21,502,538 [-]                | Yes         | No                   |
| PBac{WH}CGC2230 | 80EF          | 3L:23,237,018 [+                | Weak        | No                   |
| PBac{PB}c06482  | 80EF          | 3L:23,286,922 [-]               | Yes         | Yes                  |
| PBac{PB}CG0474  | 80EF          | 3L:23,849,420 [+                | No          | No                   |
| P{GT}1BG01672  | 42AB          | 2R:2,370,529 [-]                | No          | Yes                  |
| P{EPgy}2EY08366 | 42AB          | 2R:2,129,510 [+                | Yes         | No                   |
| P{X}{d}02126    | 42AB          | 2R:2,129,452 [-]                | Weak        | No                   |
| P{SUPor-P}PB{G}22714 | 42AB   | 2R:2,133,438 [-]                | No          | No                   |
| P{SUPor-P}KG09351 | 42AB     | 2R:2,160,357 [-]                | No          | Yes                  |
| PBac{WH}I04291  | 42AB          | 2R:2,228,280 [-]                | Yes         | Yes                  |
| P{EPgy}2EY1034  | 38C           | 2L:20,205,306                   | Yes         | Yes                  |
| P{X}{d}02757    | 38C           | 2L:20,174,968 [+                | Yes         | Yes                  |
| PBac{WH}I03548  | 38C           | 2L:20,165,746                   | Yes         | Yes                  |
| P{SUPor-P}KG05288 | 38C     | 2L:20,166,034 [+]               | No          | Yes                  |
| PBac{PB}e03575  | 38C           | 2L:20,121,359 [-]               | Weak        | No                   |
| P{SUPor-P}CG02342 | 38C     | 2L:20,120,504 [-]               | No          | No                   |

The genomic coordinates for four top piRNA clusters were defined as previously determined by Brennecke et al., 2007 [20]. The genomic coordinates of the P-element insertions were confirmed by PCR with primers specific to the P-elements and flanking genomic sequences.

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**Figure 4.** Chromatin association with piRNA clusters in gonadal tissues.

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**Figure 5.** Chromatin association with piRNA clusters in OSC cells.

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**Figure 5A.** Chromatin association with piRNA clusters in OSC cells.

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**Figure 5B.** Chromatin association with piRNA clusters in OSC cells.

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**Figure 5C.** Chromatin association with piRNA clusters in OSC cells.
euchromatic sites and TEs (Figure 5D). Moreover, levels of Piwi association with 80EF is akin to that of flam, while several sites in both flam and 80EF clusters show particular enrichment of Piwi up to three-fold compared to the average association with other sites tested (Figure 5D and 5E). In Piwi knockdown cells, Piwi chromatin association drops two to five-fold, down to background levels at all sites except for some residual association with two sites in or near the flam locus. Mouse IgG negative control immunoprecipitations yielded low amounts of DNA in comparison to α-Piwi immunoprecipitations in mock treated cells for all sites tested (<0.04% and <0.02% input for mock and Piwi knockdown cells, respectively). We conclude that in ovarian somatic follicle cells, reduction of the total pool of Piwi as well as the chromatin bound fraction does not affect HP1 association with piRNA

Figure 3. HP1 associates with chromatin at piRNA clusters, and its levels increase in RNA-silencing mutants. ChIP at (A) flam and (B) 80EF piRNA clusters in wild type (blue), piwi1/piwi2 (yellow), and AGO251B (red) mutants from adult heads with antibodies specific to HP1. Percent input immunoprecipitated is shown for each primer set, and error bars indicate standard deviation of quadruplicate PCR measurements. 

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clusters and has a minimal effect on HP1 association with TE chromatin association.

Loss of piRNA production from a single cluster results in global HP1 mislocalization

We next sought to determine whether loss of piRNA production at a single piRNA cluster would affect HP1 recruitment to chromatin. Previous studies have shown that mutation of various RNA silencing components results in global mislocalization of HP1 on polytene chromosomes [35–36]. Mutation of flam has been previously shown to result in loss of piRNA production [20] and upregulation of the gypsy retroelement [32]. In order to obtain a genome-wide view of HP1 chromatin association in flam mutants, we examined the localization of HP1 to highly replicated salivary gland polytene chromosomes from either wild type or flam1 mutant third instar larvae by indirect immunofluorescence using α-HP1 antibodies. In wild type, HP1 localizes predominantly to a concentration of heterochromatin where the

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Figure 4. HP1 associates with chromatin at piRNA clusters in ovaries. ChIP at (A) flam and (B) 80EF piRNA clusters in wild type ovaries with antibodies specific to HP1 (blue) and normal rabbit IgG (red).

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centromeres of each chromosome coalesce, termed the chromocenter (Figure 6A, green). In contrast, flam\(^1\) mutants display expansion of HP1 at the chromocenter. Spreading of HP1 is apparent on the second and third chromosomes, but not on the X chromosome, where flam is located. As a reference, we also examined the localization of the chromatin insulator protein Mod(mdg4)\(^2.2\), which is unchanged in localization between wild type and flam\(^1\) (Figure 6A, red). The extent of HP1 chromocenter expansion is comparable to the level of HP1 expansion that we observe in spn-EhlsE1\(^{1/4}\) spn-EhlsE616 mutants (Figure S7). A lesser degree of HP1 expansion was also observed in flamBG02658/flamKG00476 mutants (data not shown). Finally, total HP1 levels are unchanged in flam\(^1\) whole flies compared to wild type (Figure S6).

These results indicate a global change in HP1 localization resulting from inactivation of a single piRNA cluster.

We reasoned that accumulation of HP1 at the chromocenter of flam\(^1\) mutants may result in an increase in silencing at pericentromeric sites. Therefore, the expression of transcriptional reporters at 42AB or 80EF piRNA clusters, which are located on different chromosomes from the flam locus, was examined in flam\(^1\) mutants. Compared to wild type, flam\(^1\) mutants harboring a P element insertion at either 42AB or 80EF piRNA clusters display mildly decreased pigmentation suggesting increased silencing at these distinct pericentromeric loci (Figure 6B).

**Mutation of the flam piRNA cluster suppresses heterochromatic silencing at a distant site**

Finally, to verify HP1 genome-wide redistribution in flam\(^1\) mutants, we examined the effect of flam\(^1\) on the silencing of a centromere distal heterochromatic site on a different chromosome. The DX1 transgene array consists of seven mini-white P elements with one inverted copy at a normally euchromatic site at 50C on chromosome 2R [43]. Due to this configuration, the array forms ectopic repeat induced heterochromatin and displays a variegated

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**Figure 5. Depletion of Piwi from ovarian somatic follicle cells does not affect HP1 recruitment to piRNA clusters.** (A) Western blotting of Piwi, HP1 and Lamin in OSC cells that were either mock treated (left lane) or treated with siRNA directed against piwi (right lane, Piwi KD). ChIP at flam (B,D) and 80EF (C,E) piRNA clusters in mock treated and Piwi KD OSC cells with antibodies specific to HP1 (B,C) or Piwi (D,E).

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phenotype similar to PEV that is dependent on HP1. Expression of the DX1 array was assessed based on variegation of eye pigmentation in wild type, heterozygous flam1/+ and homozygous or hemizygous flam1 mutants (Figure 6C). Due to a wide range of eye coloration, variegation was scored by categorization into five groups that ranged between Light (few pigmented facets) to Dark (almost all pigmented facets). For females, 3% of wild type was classified as Dark, while 29% of flam1/+ and 52% of flam1 mutants displayed the same high level of pigmentation. In males, 15% of wild type was scored as Medium-Dark or Dark while 40% of flam1

Figure 6. Mutation of the flam piRNA cluster results in global HP1 redistribution. (A) Wild type (left) and flam (right) polytene chromosomes stained with antibodies directed against HP1 (green) and a reference protein Mod(mdg4)2.2 (red). DNA is stained with DAPI (blue). Chromosome arms are labeled, and insets of the enlarged chromocenter are shown. (B) Adult eyes of wild type and flam1 mutants carrying a mini-white transgene inserted in 42AB (top row) and 80EF (bottom) piRNA clusters. (C) Degree of eye pigmentation due to expression of the DX1 transgene array at 50C on chromosome 2L, which undergoes repeat-induced heterochromatic silencing, in wild type, flam1/+, and flam1 female flies and wild type and flam1 male flies. Scoring of variegation in the eye is categorized into five groups that range between light (few pigmented facets) to dark (almost all pigmented facets). Percentage of flies falling into each category was graphed. Representative eyes are shown on right.

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males fell into these categories. These results indicate that mutation of *flam* can suppress heterochromatic silencing in *tomo*. Taken together with the HP1 centromeric expansion in polytene chromosomes and increased pericentromeric silencing in *flam* mutants, there appears to be a global redistribution of HP1 resulting from the loss of piRNA production from a single locus.

**Discussion**

In this study, we tested directly whether the Argonautes AGO2 or Piwi recruit HP1 to chromatin. As candidate sites for Argonaute/HP1 interaction, we examined whether piRNA clusters may be heterochromatic using both genetic and molecular approaches. First, P elements inserted at or near pericentromeric piRNA clusters were assayed as transcriptional reporters, and these transgenes were found to display variegated expression that is increased in heterochromatin mutants. Next, ChIP with α-HP1 antibodies showed that HP1 associates with piRNA clusters at levels significantly above euchromatic sites. However, mutation of *piwi*, *aub*, or *AGO2* leads to a modest increase in silencing of transcriptional reporters as well as an increase of HP1 association at piRNA clusters in heads. In ovarian somatic follicle cells, in which both Piwi and HP1 are highly expressed, depletion of Piwi results in little or no change in HP1 recruitment to piRNA clusters and TE clusters. Furthermore, loss of piRNA production at a single locus results in expansion of HP1 at the centromere. In these *flam* mutants, silencing of a distant heterochromatic transgene array is reduced, further indicating a global redistribution of HP1 and suggesting indirect effects. Taken together, the results argue against direct recruitment of HP1 or maintenance of its association by AGO2 or Piwi in the soma.

**AGO2 and Piwi are not required for HP1 association at piRNA clusters**

Several reasons dictated the choice of piRNA clusters as the focus of our analyses. First, both endo-siRNAs and piRNAs are generated from these loci [16–21]. Next, we reasoned that at least some piRNA clusters are likely to be heterochromatic because of their strong bias toward TE-rich pericentromeric positions in the genome [20,21], in close proximity to the vast majority of HP1 localization. In fact, early cloning attempts determined that the *flam* locus is located in a repetitive, TE-rich heterochromatic region [44]. Furthermore, the pericentromeric position of these clusters likely coincides with the transition between euchromatin and heterochromatin, corresponding to the borders of HP1 spreading. This characteristic allows variegation assays, which monitor the variable spreading of HP1 and heterochromatin, to be extremely sensitive. ChIP assays at the borders of HP1 spreading would also likely be optimally sensitive to both local and overall changes in HP1 chromatin association. Finally, piRNA clusters contain enough unique sequence for specific primer design and monitoring by directed ChIP analysis.

Given that *AGO2* is the predominant Argonaute expressed outside the gonad that participates in the silencing of TEs in the soma, we tested whether AGO2 could recruit HP1 to chromatin in somatic tissue. Moreover, it has been shown that *AGO2* mutants exhibit mislocalization of HP1 [34,35]. However, our results show that mutation of *AGO2* results in a strong increase of silencing of transcriptional reporters at or near piRNA clusters and a mild increase of HP1 chromatin association in heads. Given the extent of increased silencing in the *AGO2* mutant compared to *piwi* or *aub* mutants, which accumulate HP1 on chromatin to a similar degree, a posttranscriptional step of silencing likely contributes to the negative effects observed on transcriptional reporters. *AGO2* mutants show a plethora of cellular defects during early nuclear divisions but develop normally and are fertile suggesting that effects on these various processes as well as HP1 localization are mild or otherwise compensated [34]. Therefore, *AGO2* is unlikely to be required for HP1 recruitment in this tissue.

Additionally, we find that HP1 association at piRNA clusters does not depend on the presence of Piwi. Our analysis of piRNA clusters included *flam*, a primary piRNA cluster, and *80EF*, a germline piRNA producing locus. We examined both *flam* and *80EF* clusters in somatic head tissue and ovaries, which are a mixed population of somatic follicle and germline derived cells. In heads, there is no apparent requirement for *piwi* with respect to HP1 recruitment to the piRNA clusters or to TEs that were examined.

In our study, Piwi chromatin association was detected only in OSC cells, and its presence is dispensable for HP1 chromatin association. The *flam* piRNA cluster produces high levels of primary piRNA in OSC while *80EF* is active for piRNA production in germ cells but not in OSC [25,26,30]. Nonetheless, Piwi associates with both the *flam* and *80EF* clusters at comparable levels, suggesting that the amount of piRNA production from a particular locus does not correlate with Piwi chromatin association. Furthermore, the pattern of Piwi chromatin association in OSC differs from that of HP1 in that there is no particular enrichment of Piwi at TEs above euchromatic sites and only a minor accumulation at a few sites in the *flam* and *80EF* piRNA clusters. When Piwi levels were reduced by siRNA knockdown, Piwi chromatin association was essentially abolished but HP1 recruitment was not affected except for a two-fold decrease over the 1360 element. Previous studies suggested that the 1360 element may be responsible for nucleating heterochromatin on the largely heterochromatic fourth chromosome and further showed that mutation of factors representing all RNA silencing pathways, *piwi*, *aub*, *80EF*, *Dcr-1*, and *Dcr-2*, affect 1360 dependent heterochromatic silencing [40,43]. Unlike the results in adult heads, no accumulation of HP1 over piRNA clusters was detected as a result of Piwi knockdown in OSC cells. This discrepancy may reflect differential effects in distinct cell types or the length of the Piwi knockdown in OSC cells, which was at least adequate to essentially eliminate Piwi chromatin association. In a related but independently derived ovarian somatic follicle cell line (OSS), Piwi and HP1 do not colocalize in the nucleus [33], and this finding supports the conclusion that Piwi does not direct HP1 recruitment in this cell type. Also consistent with our results, HP1 remains localized to the chromocenter in salivary gland polytene chromosomes in *piwi* null mutants [11,36]. We conclude that association of HP1 with chromatin can occur independently of *AGO2* and *piwi* in somatic tissue.

A previous study addressed the role of the germline piRNA pathway in HP1 association with transposable elements. The *spn-E* gene controls predominantly germline piRNA production but does not affect the somatic piRNA pathway [26]. ChIP was used to show that *spn-E* mutants display significantly decreased levels of H3K9me3 and HP1 at telomeric *Het-A* but similar to wild type HP1 levels at the *I-element and copia* TEs, which are distributed throughout the genome [46]. This modest reduction of HP1 at Het-A was apparent in ovaries but not in carcasses, which contain only somatic tissue. One caveat to this study is that ChIP was performed using primers that detect all TEs matching a particular sequence, thus measuring average HP1 and H3K9me levels on TEs across the genome. Nonetheless, this work suggests a limited role for the germline piRNA pathway in HP1 recruitment at the telomere.
Additional candidate platforms for Piwi-dependent HP1 recruitment

Several studies have shown that Piwi associates with at least some heterochromatic sites in the genome, but direct evidence that any of these sites serve as recruitment platforms for HP1 and subsequent spreading is lacking. The best characterized Piwi-associated site is the heterochromatic 3R-TAS subtelomeric region, which generates the abundant Piwi bound 20nt 3R-TAS piRNA. Surprisingly, the role of piwi at this location is transcriptional activation, as piwi mutants display increased transcriptional silencing of a nearby reporter transgene as well as an increase of HP1 association at 3R-TAS [21]. Likewise, we observe a mild corresponding increase in HP1 association and silencing at piRNA clusters in piwi mutants suggesting that piwi function could in fact oppose HP1 recruitment at multiple sites in the genome. Our results are consistent with the possibility that piRNA clusters act as boundaries to the spread of pericentromeric heterochromatin. The mechanism of Piwi dependent transcriptional activation has not been determined, but considering that Piwi interacts with the chromoshadow domain of HP1 [11], Piwi may compete for binding with other HP1 interactors such as Su(var)3-9 that promote heterochromatic silencing.

Functions for piwi outside of the gonad

The majority of Piwi protein is found in both somatic and germline tissues of the gonad, yet piwi clearly exerts an effect on non-gonadal somatic tissues as well. RT-PCR analysis shows that piwi transcript is readily detectable outside the gonad and in somatic cell lines [11,12], but Piwi protein is difficult to detect [11]. Nevertheless, mutation of piwi suggests important functions for this gene outside of the gonad. For example, piwi is essential for viability, and loss-of-function mutants display a variety of phenotypes manifest in various non-gonadal somatic tissues such as demonstrated in this study and others, which show a requirement for piwi in pairing-dependent silencing, nucleolar integrity, and chromatin insulator function [47–50]. For each of these chromatin related studies, it remains a possibility that even a small amount of maternally deposited Piwi could trigger early events in the oocyte or embryo that persist throughout development, manifesting phenotypes visible in adult somatic tissues.

HP1 redistribution in piRNA pathway mutants

Our results along with previous studies have demonstrated that HP1 mislocalizes from the chromocenter in a subset of piRNA pathway mutants. We found that polytene chromosomes of flam mutants exhibit expanded HP1 chromocenter distribution. This result is intriguing because the flam mutation affects a single piRNA cluster on the X chromosome but HP1 spreading to other chromosomes is apparent. A previous study detected spreading of HP1 to euchromatic arms especially in flam mutants [36], and we confirmed this result albeit to a lesser degree, with spreading being comparable to the extent seen in flam mutants. Perhaps the increase of TE expression in RNAi silencing mutants can stimulate HP1 recruitment and spreading from the centromere, which contains the highest concentration of TEs. In fact, transcription of pericentromeric repeats stimulates RNAi-dependent heterochromatin formation in fission yeast [51–53].

Redistribution of HP1 in RNAi silencing mutants may indirectly affect silencing at various heterochromatic locations in the genome. Seemingly inconsistent with HP1 spreading, syn-E, adh, and piwi mutants display decreased silencing of P element transgene arrays such as DX1 and single insertions at pericentromeric regions on chromosomes 2 and 4 [36]. In our study, we found that mutation of flam also results in loss of silencing at DX1, which is distant from the flam locus. This reduced silencing in trans could not be due to posttranscriptional events as there are no shared sequences between DX1 and the flam locus. Therefore, we consider the possibility that there exists a finite pool of HP1 that accumulates at the centromere in flam and other RNAi silencing mutants at the cost of reduced density and reduced silencing at other heterochromatic regions such as the transgene array, the fourth chromosome, and the telomere. The concept of a limited population of HP1 was suggested previously to explain the finding that the Y chromosome behaves as a suppressor of variegation by acting as a sink for HP1 [43].

Conclusions

Studies in multiple organisms have identified or suggested alternative mechanisms to RNAi silencing for the recruitment of HP1 to chromatin. In fission yeast, overlapping and redundant RNAi-dependent and independent mechanisms of heterochromatin formation have been elucidated. In mouse cells, HP1 localization to pericentromeric heterochromatin was found to be RNase A sensitive suggesting that an RNA moiety may be involved in HP1 recruitment [54]. Our data indicate that heterochromatin can form independently of RNA silencing in Drosophila. It will be interesting to determine if any of these alternative mechanisms of heterochromatin formation are conserved throughout evolution.

Materials and Methods

Drosophila stocks

Fly stocks were maintained at 25°C on standard cornmeal medium. Lines containing P[EP02]DPI1[3103623] and P[EPgy2]E108566 were obtained from the Bloomington Drosophila Stock Center, and a line harboring PBac(P)E06482 was obtained from the Exelixis Collection at Harvard Medical School. Genomic coordinates of these P-element insertions were confirmed by PCR with primers specific to the P-elements and flanking genomic backgrounds and scored against crosses to wi67c23 as a reference. For ChIP and immunofluorescence, Oregon-R was used as a wild type control. The y w f mal flam1/FM7c; CyO/Sp as a reference. For ChIP and immunofluorescence, Oregon-R was used as a wild type control. The y w f mal flam1/FM7c; CyO/Sp flies as a reference.

Transcriptional reporter and eye pigmentation assays

Eye pigmentation of 40 to 60 adult males six days of age was examined, and representative eye photos were taken. To quantify overall levels of eye pigmentation, the heads of 25 male flies of each genotype were dissected, and eye pigmentation was measured as previously described [36]. Briefly, heads were homogenized in 0.8 ml of methanol, acidified with 0.1% HCl and centrifuged. The absorbance of the supernatant was measured at 490 nm.

Chromatin immunoprecipitation

Adult fly heads or ovaries were dissected and crosslinked with 1.8% formaldehyde for 20 min at 23°C. Chromatin was fragmented to an average size of 300 bp by sonication and incubated with antibodies overnight at 4°C. Quantitative PCR was conducted on Applied Biosystems Real Time PCR system using SYBR Green incorporation (Affymetrix/USB). Amplicon
sizes ranged between 150 and 250 bp. Chromatin was immuno-
purified with the following antibodies: α-HP1 (Covance), α-
Su(Hw), α-Pwi (P3G11, a gift from M. Siomi), and normal rabbit
mouse IgG (Santa Cruz Biotechnology). A recombinant N-
terminal His-tagged fusion protein of the N-terminal of Su(Hw)
(amino acids 1–218, kind gift of M. Labrador) was purified from
E.coli on a nickel-agarose column and used to immunize guinea
pigs using standard procedures. Similar results were obtained
using the C1A9 α-HP1 antibody (Developmental Studies Hybrid-
om Bank), but lower quantities of RNA were obtained. Fifty to
one hundred fly heads and twenty five to fifty ovaries were used
per IP. The quantities of target genomic regions precipitated by
different antibodies were calculated as percent input based on
four-point standard curves constructed from input DNA for each
primer set. Standard deviation of each PCR performed in
quadruplicate was calculated to determine the error of measure-
ment. Two independent ChIP samples were analyzed, and similar
results were obtained. ChIP primers were designed to be unique,
detecting only sequences present in the

Results were obtained. ChIP primers were designed to be unique,
derived from in silico PCR. All primers (Table S1 and Table S2)
were checked for both specificity and efficiency by standard
agarose gel electrophoresis and real time PCR respectively.
Primers to piRNA clusters amplify significantly in the same DNA
dilution range as primers specific to hsp26 and yellow single copy genes
compared to high copy TE elements (Figure S8). Primers to the
flam locus were verified to amplify approximately two-fold more
DNA from female compared to male genomic DNA. A detailed
version of this protocol is available in Text S1.

Culture of OSC cell line and siRNA knockdowns

The OSC line was maintained and Pwi siRNA knockdown was performed as previously described [30]. Briefly, 3×10^6 Trypsinized

Cells were resuspended in 0.1 mL of Solution V of the Cell Line

Nucleofector Kit V (Amaxa Biosystems) and mixed with 200 pmol of siRNA duplex. Transfection was conducted according to the

manufacturer’s protocol using the nucleofector program T-029,
and the transfected cells were incubated at 25°C for 48 hrs. Protein knockdowns were verified by Western blotting, and ChIP
assays were performed on mock and pwi siRNA transfected cells
(5×10^6 cells per IP).

Immunostaining of polytene chromosomes

Preparation and immunostaining of salivary gland polytene

chromosomes was performed as described previously [55].
Primary antibodies directed against HP1 (Covance) and Mod/mdg4/2.2 (generated similarly as in [56]) and Alexa Fluor
488 labeled anti-guinea pig or Alexa Fluor 594 labeled anti-rabbit
secondary antibodies (Invitrogen-Molecular Probes) were used.
The chromosomes were viewed using a Leica epifluorescence
microscope and photographed using a Hamamatsu digital camera.

DX1 variegation assay

Eye pigmentation of 100 to 200 flies was scored. The scoring of
variegation was categorized into five groups: Light, Medium-
Light, Medium, Medium-Dark and Dark corresponding to the
percentage of pigmented facets. Percentage of flies falling into each
category was graphed. Representative eye photos were taken.

Supporting Information

Figure S1 Schematic representation of the 38C piRNA cluster
on chromosome 2L. Genomic locations of small RNAs and P
element insertions. Sequence datasets derived from previous
studies were mapped to the genome using Bowtie software
allowing two mismatches. Piwi-immunoprecipitated, Aub or
AGO3-immunoprecipitated and AGO2-immunoprecipitated
reads mapping to multiple locations in the genome are indicated in
red (with 2 mismatches allowed) and pink (with 0 mismatches
allowed) while uniquely mapping reads are in dark blue (with
2 mismatches allowed) and light blue (with 0 mismatches allowed).

Figure S2 Der-2 mutants display increased HP1 chromatin
association and increased silencing at piRNA clusters. ChIP at (A)
flam and (B) 80EF piRNA clusters in wild type (blue) and Der-
80EF/+ (orange) from adult heads with antibodies specific to
HP1. Values shown are percent input immunoprecipitated for
each primer set normalized to hsp26. Error bars indicate standard
deviation of quadruplicate PCR measurements. (C) Adult eyes of
wild type and Der-80EF/+ mutants carrying a mini-white transgene
inserted in close proximity to the flam piRNA cluster.

Figure S3 Su(Hw) does not associate with chromatin at piRNA
clusters in heads. ChIP at (A) flam and (B) 80EF piRNA clusters
in wild type with antibodies specific to Su(Hw) (blue) and rabbit
normal serum (yellow). Percent input immunoprecipitated is
shown. For each primer set, and error bars indicate standard
deviation of quadruplicate PCR measurements.

Figure S4 HP1 chromatin association levels are increased in
pwi mutants at piRNA clusters. ChIP at (A) flam and (B) 80EF piRNA
clusters in pwi1/+ (light grey) and pwi1/pwi2 (dark grey) from
adult heads with antibodies specific to HP1. Values shown are
percent input immunoprecipitated for each primer set normalized
to hsp26. Error bars indicate standard deviation of quadruplicate
PCR measurements.

Figure S5 HP1 chromatin association levels are increased in
AGO2 mutants at piRNA clusters. ChIP at (A) flam and (B) 80EF
piRNA clusters in AGO2+/- (light blue) and AGO2-/- (dark blue)
from adult heads with antibodies specific to HP1. Values shown are
percent input immunoprecipitated for each primer set normalized
to hsp26. Error bars indicate standard deviation of quadruplicate
PCR measurements.

Figure S6 HP1 protein levels in wild type, flam1, AGO2+/- and
pwi1/pwi2 fly heads. Total protein was extracted from twenty
adult heads by homogenization in RIPA buffer and separated by
SDS-PAGE. Immunoblotting of HP1 and Protein on Ecdysone
Puffs (Pep), a nuclear protein serving as a loading control, is
shown.

Figure S7 spn-E80EF/spn-E80EF mutants display accumulation of
HP1 at the chromocenter. Wild type (top) and spn-E80EF/spn-
E80EF (bottom) polytene chromosomes stained with antibodies
directed against HP1 (green) or a reference protein Mod/mdg4/2.2
(red). DNA is stained with DAPI (blue).

Figure S8 ChIP primer efficiency and specificity. PCR amplification
efficiency and specificity of ChIP primers at (A) flam and
(B) 80EF piRNA loci are graphed as a function of C\textsubscript{t} values over DNA concentration. Cycle threshold (C\textsubscript{t}) values of standard curves were found at: doi:10.1371/journal.pgen.1000880.s010 (0.05 MB PDF). Table S1: Primer set sequences used for ChIP at the "flam" piRNA cluster. Found at: doi:10.1371/journal.pgen.1000880.s008 (0.62 MB TIF). 

Table S2: Primer set sequences used for ChIP at the 80EF piRNA cluster. Found at: doi:10.1371/journal.pgen.1000880.s009 (0.05 MB DOC)

Text S1: Detailed ChIP protocol.

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