Piwi maintains germline stem cells and oogenesis in "Drosophila" through negative regulation of Polycomb group proteins

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The "Drosophila melanogaster" Piwi protein regulates both niche and intrinsic mechanisms to maintain germline stem cells, but its underlying mechanism remains unclear. Here we report that Piwi interacts with Polycomb group complex PRC1 and PRC2 in niche and germline cells to regulate ovarian germline stem cells and oogenesis. Piwi physically interacts with the PRC2 subunits Su(z)12 and Esc in the ovary and in vitro. Chromatin coimmunoprecipitation of Piwi, the PRC2 enzymatic subunit E(z), histone H3 trimethylated at lysine 27 (H3K27me3) and RNA polymerase II in wild-type and piwi mutant ovaries demonstrates that Piwi binds a conserved DNA motif at ~72 genomic sites and inhibits PRC2 binding to many non-Piwi-binding genomic targets and H3K27 trimethylation. Moreover, Piwi influences RNA polymerase II activities in "Drosophila" ovaries, likely via inhibiting PRC2.

We hypothesize that Piwi negatively regulates PRC2 binding by sequestering PRC2 in the nucleoplasm, thus reducing PRC2 activity12,13. E(z), the enzymatic subunit of PRC2, methylates H3K27 (refs. 14–17). PRC2 is known to function in the establishment and maintenance of the germline18,19 and in Caenorhabditis elegans20,21. PRC2 is recruited by Jarid2 to its targets22,23 and is antagonized by (es)BAF24, demonstrating both positive and negative inputs to PRC2 function in the maintenance and differentiation of stem cells. Here we report a new epigenetic mechanism mediated by Piwi and PRC2 in regulating germline stem cells.

**RESULTS**

**piwi, corto and Polycomb group genes genetically interact**

We previously showed that reducing corto activity partially rescues germline stem cell maintenance in piwi mutant ovaries5. This finding, together with the known interactions between Corto and PcG proteins6–8, led us to investigate whether corto mutations achieve this partial rescue by affecting PcG activity. We first analyzed H3K27me3 levels in wild-type and corto mutant ovaries. Immunofluorescence and immunoblotting showed that H3K27me3 levels were drastically reduced in corto mutant ovaries (Fig. 1b,c). Recombinant Corto protein did not affect the histone methyltransferase activity of PRC2 (Supplementary Fig. 1a). These results suggest that Corto is required for trimethylation of H3K27 but does not directly influence PRC2 methyltransferase activity in the ovary. We then analyzed whether reducing the activity of Pc (encoding a subunit of the PRC1 complex) would rescue the piwi mutant defects. This rescue was previously not observed5, presumably because the piwi2 chromosome used in that study contained the Irregular facets (If) mutation5, which was used as a chromosome marker but somehow blocked suppression. To avoid the potential effect of the If mutation and/or background mutation in the homozygous mutant, we used the piwi1/piwi2 transallelic combination without the If mutation to repeat our previous experiments on genetic suppression of piwi by corto, kni, mod(mdg4) or Mi-2 mutations5. We observed partial but significant rescue of germline stem cells in the...
piwi1/piwi2 mutant by two mutant alleles of Pc and a mutant allele of E(z) (encoding a PRC2 subunit; Fig. 1d,e). Transgenic short hairpin RNAs (shRNAs) reducing Pc, E(z) or Esc protein levels in adult flies (Supplementary Fig. 1b,d) also partially rescued oogenesis in ovaries in which Piwi levels were reduced by an shRNA targeting piwi mRNA for degradation (Supplementary Fig. 1c,e,g). These data indicate that PRC2 and PRC1 negatively interact with Piwi to regulate oogenesis.

To further characterize the effects of PcG genes on ovarian germline stem cell and oogenesis, we analyzed germline stem cell by immunofluorescence labeling the Hubi-Tai (Hts) protein to visualize the spectrosome (a germline stem cell) and cystoblast-specific organellae, Vasa to mark germ cells and Traffic Jam (Tj) to mark somatic cells. Reducing PcG gene activity by introducing one allele with mutations for corto, E(z) or Pc partially but significantly rescued germline stem cells (Fig. 1f,g), germlarial organization (Supplementary Fig. 1h) and egg chamber development (Supplementary Fig. 1i) of the piwi1/piwi2 mutants (homozygous PcG mutations were lethal). This rescue indicates genetic interactions between Piwi and PcG proteins.

Even more specifically, Pc mutation only suppressed gtwm and ZAM in group III. To exclude the possibility that the elevated expression of transposons in the mutants is due to increased soma to germline ratios in mutant ovaries, we quantified Vasa (germ cell) and Tj (somatic cell) expression by RT-qPCR and immunoblotting, normalized by Gapdh expression. The relative abundance of germ cells and somatic cells was approximately the same in all of the mutant ovaries (Supplementary Fig. 2b,c). Therefore, PcG proteins influence Piwi-mediated transposon silencing to various extents, underscoring the negative genetic interactions between Piwi and PcG proteins.

Piwi, Corto and PRC2 physically interact in the ovary
To determine whether the genetic interaction between piwi and the PcG genes reflects physical interaction of the encoded proteins, we used antibodies to Piwi and E(z) and a newly generated antibody to Corto to perform coimmunoprecipitation from ovarian extracts (Supplementary Fig. 3a,b). Corto, Piwi and E(z) coimmunoprecipitated with one another, yet the closest homolog of Piwi, Aubergine (Aub), did not coimmunoprecipitate with E(z) or Corto (Fig. 2a,b). Pc, a PRC1 subunit, also did not interact with Piwi or Corto (Fig. 2a,b). In addition, immunofluorescence microscopy of Piwi and E(z) demonstrated their highly overlapping pattern of colocalization within the nucleus, as shown by line-scan profiles and high Pearson correlations for the Piwi and E(z) signals (r = 0.95 and r = 0.92, respectively; Fig. 2c, magnified sections). Furthermore, in vitro–reconstituted
PRC2 complex (Supplementary Fig. 3c) coimmunoprecipitated with purified recombinant Piwi protein (Supplementary Fig. 3d), demonstrating direct PRC2–Piwi interaction (Fig. 3a) and supporting the hypothesis that Piwi and PRC2 interact in ovarian cells.

We next investigated which PRC2 subunits interact with Piwi and Corto in the Drosophila ovary and human HEK293 cells. In HEK293 cells, FLAG-Piwi separately coimmunoprecipitated with Myc-Esc and Myc-Su(z)12 but not with Myc-Corto, Myc-E(z) or endogenous human SUZ12—a negative control (Fig. 3b). Furthermore, Piwi coimmunoprecipitated with E(z) from ovarian extract (Fig. 2a,b), where all PRC2 subunits are present, but not from HEK293 extract (Fig. 3b), where endogenous Esc and Su(z)12 proteins are absent. These results indicate that Esc and Su(z)12 mediate interaction between Piwi and PRC2.

We then used HEK293 and Drosophila S2 cells to identify the domains of Piwi, Su(z)12 and Esc that mediate the interactions. Residues 160–257 of Myc-Piwi (fragment d in Fig. 3c and Supplementary Fig. 3d) interacted with residues 380–500 of FLAG-Su(z)12 (Fig. 3c) and 1–176 of Myc-Piwi (fragment c in Fig. 3d and Supplementary Fig. 3f, g). Furthermore, Piwi interacted with residues 1–150 of FLAG-Esc (fragment ii in Fig. 3d and Supplementary Fig. 3h). Therefore, the N-terminal domain of Piwi interacts with Esc and Su(z)12. Because this domain also binds to Tudor family members, it may act as a scaffold for interaction with Piwi partners.

The Piwi-interacting region of Su(z)12 falls inside the C2H2 zinc-finger domain that interacts with nucleic acids. The Piwi-interacting domain of Esc corresponds to the N-terminal region of the WD40 domain that binds to H3K27me3 and propagates H3K27me3 signal through mitotic cycles. To investigate whether Piwi regulates the H3K27 trimethylation activity of PRC2, we purified recombinant Piwi protein and recombinant PRC2 complex for an in vitro assay.

Figure 3e) interacted with residues 380–500 of FLAG-Su(z)12 (fragment VI in Fig. 3c and Supplementary Fig. 3f). Residues 1–176 of Myc-Piwi (fragment c in Fig. 3d and Supplementary Fig. 3f, g) interacted with residues 1–150 of FLAG-Esc (fragment ii in Fig. 3d and Supplementary Fig. 3h). Therefore, the N-terminal domain of Piwi interacts with Esc and Su(z)12. Because this domain also binds to Tudor family members, it may act as a scaffold for interaction with Piwi partners.

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methyltransferase assay. The presence or absence of Piwi did not affect the enzymatic activity of PRC2 (Fig. 3e).

**Piwi binds DNA via an evolutionarily conserved sequence motif**

The interaction between Piwi and PRC2 prompted us to investigate genome-wide binding of Piwi in ovarian cells. The Piwi-piRNA complex was shown to bind to specific genomic sites, but these sites have not been definitively mapped across the genome. We generated a new antibody to Piwi capable of chromatin immunoprecipitation (Supplementary Fig. 4a-d) to map Piwi binding sites in the ovarian genome. We analyzed biological triplicates of Piwi chromatin immunoprecipitation and sequencing (ChIP-seq) in wild-type ovarian cells, with biological triplicates of Piwi ChIP-seq in piwi mutants as a negative control (Fig. 4a and Online Methods). We used the QuEST peak caller to identify 155, 72 and 76 Piwi enrichment peaks relative to input for the three replicates. Piwi peaks showed high overlap across replicates, with 42 shared peaks (binomial $P < 1 \times 10^{-16}$; Fig. 4b). These peaks were enriched at transcription start sites across the genome (Fig. 4a). Representative Piwi binding patterns at CG34256 and Fhos show enrichment of Piwi binding at the transcription start sites (Fig. 4c). De novo MEME motif analysis of the peaks identified an enriched motif, [A/G][A/A][T/A][CGC][4-nt spacer][A/G][A/A][T/A][CGC], consisting of two [A/G][A/A][T/A][CGC] direct-repeat cores separated by 4 nt (Fig. 4d), hereafter referred to as the Piwi binding motif (PBM). The PBM was enriched across all three Piwi ChIP-seq replicates and present in all 42

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**Figure 4** Genome-wide Piwi binding patterns and Piwi EMSA. (a) Distribution of Piwi-bound regions relative to the transcription start site (TSS). (b) Venn diagram showing the overlap of ChIP-seq peaks across triplicate experiments. (c) Representative Piwi binding patterns at the CG34256 and Fhos genes in wild-type and piwi mutant ovaries. (d) Sequence motif enriched within Piwi peaks identified using the MEME tool. Conservation using PhyloP scores is shown. (e) Pie charts show recovery of the PBM within Piwi ChIP-seq peaks: 100% (42/42) for three-way replicated overlapping sites, 41% (63/155) for replicate 1 peaks, 84% (64/76) for replicate 2 peaks and 80% (57/72) for replicate 3 peaks. (f) Gene Ontology terms of the genes that were up- or downregulated by the piwi mutations; triplicate RNA-seq samples were used. The bar graphs represent $-\log_{10}$ ($P$ value). WT, wild type. (g) EMSA analysis of Piwi binding to the PBM in 32P-labeled oligonucleotides. Red and green arrows indicate a shift and a supershift, respectively. Ab, antibody; Mut, mutant; ssDNA, single-stranded DNA.
Figure 5  Piwi inhibits PRC2 binding to chromatin and PRC2-mediated H3K27 trimethylation. (a) Circos plot showing H3K27me3 and E(z) binding patterns (enrichment scores calculated by ChiP-seq counts within non-overlapping 10-kb bins) in wild-type and piwi mutant ovarian cells. Selected genes and peaks are labeled. (b) Venn diagrams showing overlap of enriched regions between the indicated ChIP-seq data sets. (c) Comparison of replicate E(z) and H3K27me3 ChIP-seq data sets from wild-type and piwi mutant ovaries. (d) Confocal images of H3K27me3 and DAPI for wild-type and piwi mutant ovarioles. H3K27me3 signals (normalized to DAPI signals) in piwi mutant ovaries were ~2.05-fold those in wild-type ovaries. Scale bars, 10 µm. (e) Twofold serial dilutions of histone extract from wild-type and piwi mutant ovaries analyzed by immunoblotting to H3K27me3 and histone H3. H3K27me3 signals (normalized to histone H3 signals) in piwi mutant extracts were increased by ~2-fold as compared to those in wild-type extracts. (f) Gene Ontology analysis of gene targets with H3K27me3 signal. The bar graphs represent −log_{10} (P values). TF, transcription factor.
shared peaks (Fig. 4e). Increased phylogenetic conservation scores at the motif cores (Fig. 4d) support the functional importance of this motif.

To determine whether the PBM indeed possesses higher Piwi binding affinity, we performed in vitro EMSA analysis (Fig. 4g). Purified recombinant Piwi protein bound to PBM-containing sequences in the *Fhos* locus, the first intron of *srp* and the CG18135 promoter but not to their mutant forms (Supplementary Fig. 4e). Moreover, Piwi exhibited stronger binding to the single-stranded sequences (Supplementary Fig. 4f). This binding was not abolished by RNase A or RNase H treatment (Supplementary Fig. 4f), indicating that it is piRNA independent. In addition, only antibody to Piwi and not other

![Figure 6](https://example.com/figure6.png)
antibodies resulted in a supershift in our EMSA experiments (Fig. 4g, left). Using the Phos sequence as an example, binding was inhibited by unlabeled wild-type oligonucleotide but not by the mutant sequence, used as a competitor to the labeled sequence (Fig. 4g, middle). These results indicate the specific binding of Piwi to its genomic targets via the PBM.

Because the PAZ domain in Piwi binds to piRNA, we tested it for DNA binding by the above EMSA analysis. This domain indeed bound to DNA (Fig. 4g, right), which indicates that the Piwi PAZ domain likely binds to the PBM in vivo. However, we could not detect Piwi enrichment at piRNA target sites such as the gypsy locus (data not shown). This may indicate that PBM- and piRNA-mediated Piwi binding are mutually exclusive and that piRNA-mediated Piwi binding to the genome is too weak to be enriched under our ChIP-seq conditions.

DNA motif binding by Piwi does not regulate gene expression

To investigate the role of Piwi in regulating gene expression in ovarian cells, we performed RNA sequencing (RNA-seq) in piwi1/piwi2 and wild-type fly ovaries in triplicate. RPKM (reads per kilobase per million) expression values were highly consistent across the triplicates ($r^2 = 0.99$; Supplementary Fig. 5a,b), indicating the high reproducibility of the gene expression data. We identified 899 genes statistically upregulated and 1,036 genes statistically downregulated in the piwi1 mutant using $t$-tests and a $P$-value cutoff of 0.001 (Supplementary Fig. 5c). PANTHER Gene Ontology analysis showed that the upregulated in piwi mutant cells were modestly enriched for cell adhesion and developmental processes (Fig. 4f) and that the genes downregulated in piwi mutant cells were highly enriched for primary metabolism, translation and the cell cycle (Fig. 4f). Notably, the expression of genes bound by Piwi was not strongly affected in the mutant, suggesting that genomic Piwi binding may not have a strong influence on gene expression.

Although Piwi binding was observed in promoter regions, it was not strongly associated with nucleosome-bound DNA (Supplementary Fig. 4d) or promoters marked by histone H3 trimethylated at lysine 4 (H3K4me3) or H3K27me3. Most nuclear Piwi was not bound to DNA or chromatin. Thus, Piwi may regulate genes indirectly via its association with PRC2 complexes that are not bound to chromatin.

Piwi negatively regulates PRC2 binding to chromatin

Given the interactions between Piwi and Pi proteins and the participation of Piwi in Pcg-mediated transgene silencing, we investigated whether Piwi regulates PRC2 binding to chromatin and H3K27me3 marks by analyzing total E(z) levels, E(z) binding to the genome, and the genomic H3K27me3 profile in wild-type and piwi mutant ovarian cells. Immunoblotting analysis indicated that piwi mutation does not affect total E(z) protein levels in ovarian cells (Supplementary Fig. 6a). Next, we conducted ChIP-seq analysis for E(z) and H3K27me3 in wild-type and piwi mutant ovarian cells (Fig. 5a). A new antibody to E(z) was generated and tested for this purpose (Supplementary Fig. 6b). E(z) and H3K27me3 signals showed genome-wide colocalization in both wild-type and mutant ovaries (Fig. 5a). The shared regions had higher enrichment scores than regions unique to each data set (Fig. 5b and Supplementary Fig. 7), indicating threshold effects rather than true differences in Piwi binding. Of note, H3K27me3-associated regions were highly enriched for genes encoding transcription factors or involved in developmental processes (Fig. 5f).

We then examined the effects of Piwi on E(z) and H3K27me3 enrichment on chromatin. E(z) and H3K27me3 localization across the genome was not affected in piwi mutant ovaries (Fig. 5a,b); however, E(z) levels were uniformly 25–33% higher in piwi mutants in three of the four replicates (Fig. 5c). E(z) and H3K27me3 levels at individual PRC2 genomic targets were also significantly higher in the piwi mutant as measured by ChIP analysis and quantitative PCR (qPCR) ($P < 0.001$; Supplementary Fig. 6d). To confirm this analysis, we compared the levels of E(z) and H3K27me3 in wild-type and piwi mutant ovarian cells using immunofluorescence microscopy and immunoblotting. The H3K27me3 immunofluorescence and immunoblotting signals were twofold higher in the piwi mutant than in wild-type cells (Fig. 5d,e), whereas H3K4me3 signals were unaffected by piwi mutation (Supplementary Fig. 6e). These results confirm our ChIP-seq findings, which indicated that Piwi negatively regulates both PRC2 binding to chromatin and H3K27me3 levels. Together with our biochemical results, these findings indicate that the association between Piwi and PRC2 likely occurs away from the chromatin and in the nucleoplasm. We therefore propose that Piwi binds to PRC2 in the nucleoplasm to compete against PRC2 binding to chromatin.

Although Piwi binding may not have a strong influence on gene expression, Piwi-mediated inhibition of PRC2 likely affects genes that are important for germline stem cells and oogenesis. RNA-seq analysis of PRC2-bound genes in wild-type and piwi mutant ovarian cells demonstrated that 27 of the 202 downregulated genes affect germline formation, whereas only 12 of the 354 upregulated genes have germline function ($P = 0.01$; Supplementary Fig. 8). Further, the 12 upregulated genes affected the germ line only when they were downregulated but not when they were upregulated (Supplementary Fig. 8). Therefore, wild-type Piwi likely inhibits PRC2 activities to promote the expression of 27 PRC2-bound genes. A notable PRC2-bound gene downregulated by piwi mutation is pum, which is required for germline stem cell division.1,3

Piwi-PcG interaction influences RNA Pol II activities

To investigate whether reduced PRC2 binding to chromatin and reduced H3K27me3 levels promote RNA Pol II transcription, we profiled genomic RNA Pol II localization by ChIP-seq in ovarian cells from wild type (four replicates) and piwi (three replicates), piwi; E(z)/+ (four replicates), piwi; Pci/+ (four replicates) and piwi; corto/+ (four replicates) mutants. Representative Pol II tracks at the cibr locus showed a typical Pol II localization pattern (Fig. 6a). Pol II ChIP-seq replicates for the same genotype were normalized and compared to each other, showing high correlation coefficients ($r^2 = 0.8$) and supporting good reproducibility of the RNA Pol II binding signals (Supplementary Fig. 9).

### Table 1 RNA Pol II gene ontology analysis

| Pol II increased in piwi mutant versus wild type | Observed | Expected | $P$ value |
|-----------------------------------------------|----------|----------|-----------|
| Protein folding                               | 12       | 2        | $1 \times 10^{-5}$ |
| Response to stress                            | 12       | 2        | $1 \times 10^{-4}$ |
| Cytoskeleton                                  | 14       | 4        | $1 \times 10^{-5}$ |

| Pol II increased in piwi; corto/+ mutant versus wild type | Observed | Expected | $P$ value |
|----------------------------------------------------------|----------|----------|-----------|
| Response to stress                                       | 12       | 2        | $1 \times 10^{-5}$ |
| Response to stimulus                                     | 17       | 4        | $1 \times 10^{-4}$ |
| Protein folding                                          | 9        | 1        | $1 \times 10^{-5}$ |
| Apoptosis signaling                                      | 5        | 1        | $1 \times 10^{-5}$ |

| Pol II increased in piwi; E(z)/+ mutant versus wild type | Observed | Expected | $P$ value |
|----------------------------------------------------------|----------|----------|-----------|
| Response to stress                                       | 6        | 1        | $1 \times 10^{-5}$ |

| Pol II increased in piwi; Pci/+ mutant versus wild type | Observed | Expected | $P$ value |
|---------------------------------------------------------|----------|----------|-----------|
| Response to stress                                       | 9        | 1        | $1 \times 10^{-5}$ |
Comparison of wild-type and mutant replicates identified 195 genes with a statistically significant increase in RNA Pol II levels and 214 genes with a statistically significant decrease in RNA Pol II levels in the piwi mutant (P < 0.001, t test; Fig. 6b). However, repeat sequences did not show significant changes in the mutant. Overall, on the basis of correlation values relative to data in wild-type ovaries, we conclude that mRNA Pol II levels in the piwi mutant differ most from those in wild type, followed by the piwi; corto/+; piwi; E(z)/+ and piwi; Pcl/+ mutants (Fig. 6b). This pattern supports the idea that Piwi-PRC2 interaction influences RNA Pol II activity and that genetic loss of the E(z) and Pc proteins partially rescues the effects of piwi mutation on RNA Pol II activity.

We next determined which genes are influenced by Piwi-PcG protein interaction. Although no particular type of genes showed preferentially decreased RNA Pol II binding in the mutants, ‘protein folding’ and ‘response to stress’ genes (Table 1) showed preferentially increased Pol II binding in the mutants. Our analysis demonstrated that piwi mutation likely affects genes related to cellular stress response and translation. Mutations in corto, E(z) and Pc can partially attenuate Pol II binding changes in the piwi mutant, consistent with the involvement of PRC1 and PRC2 in the regulation of RNA Pol II binding. Collectively, our results indicate that Piwi indirectly influences RNA Pol II binding at hundreds of genes in Drosophila ovarian cells, partly by negatively interacting with the PcG mechanism.

DISCUSSION

There are two major epigenetic repression mechanisms that involve histone modification, mediated by HP1 and PcG complexes, respectively. We showed previously that Piwi is involved in the HP1-mediated mechanism in which the Piwi-piRNA complex recruits HP1 and histone H3 lysine 9 (H3K9) methyltransferase to genomic sites for epigenetic regulation. Here we further demonstrate that Piwi negatively regulates PcG proteins and H3K27 trimethylation. The Piwi-PRC2 interaction appears to occur mostly, if not exclusively, in the nucleoplasm, sequestering PRC2 away from its chromatin targets. This leads to a genome-wide reduction in H3K27me3 levels that influences transcription by RNA Pol II. This negative regulation represents a new mechanism of epigenetic programming required for germline stem cell self-renewal, oogenesis and transposon suppression. This regulation may be germ cell specific, as overexpression of Piwi in all somatic cells did not result in observable defects and therefore possibly does not affect PRC2 function. Our study, in addition to reporting an antagonistic interaction between (es)BAF and PRC2 (ref. 24), demonstrates the importance of inhibiting PRC2 binding to the genome for the fine-tuning of chromatin levels of H3K27me3 in ovarian cells. Although our study highlights the cooperation of PRC1 and PRC2 in their interaction with Piwi in regulating germline stem cell maintenance (Fig. 1e–g), PRC1 and PRC2 can exhibit different effects on germ cell development. For example, reduction in PRC1 activities by Pc knockdown does not markedly affect germ cells, yet reduction of PRC2 activities by E(z) knockdown drastically affects germline stem cell differentiation or oocyte to nurse cell specification. These differences indicate that the distinct protein compositions of the PRC1 and PRC2 complexes render them overlapping but not identical functions.

Our in vitro and in vivo analyses also indicate that Piwi can bind to DNA independently of piRNAs and that this function appears to be separate from the PRC2 inhibition activity of Piwi. Whether Piwi binds to specific genomic sites has been a contentious issue. Our analysis indicates that Piwi protein has ~72–155 binding peaks in ovarian cells (Fig. 4a,b). This binding activity is apparently independent of piRNA and prefers single-stranded DNA as the substrate (Supplementary Fig. 4f). Although this mode of binding does not appear to be involved in PcG protein regulation and its role remains unknown, the similarity of the PBM to that in C. elegans indicates that this binding activity might be conserved across species.

We hypothesize that Piwi inhibits PRC2 binding to its genomic targets by sequestering PRC2 in the nucleoplasm. This is accomplished by Piwi associating with the Su(z)12 and Esc PRC2 subunits (Figs. 2 and 3, and Supplementary Fig. 3) independently of piRNA. In addition, this sequesteration might lead to inhibition of H3K27 trimethylation, dissociation of PRC2 complexes or piRNA-mediated degradation of long noncoding RNAs (lncRNAs) associated with PRC2 (refs. 44,45) (Fig. 6c). Extensive biochemical, genetic and genomic characterizations would be necessary to distinguish these possibilities.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequencing data were deposited in the Sequence Read Archive (SRA) under accession PRJNA289709.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.C.P. and H.L. designed the project, analyzed the data and wrote the manuscript. J.C.P. conducted all of the experiments except for those listed in the Acknowledgments. A.V. produced all bioinformatics results in the paper and participated in manuscript writing. N. performed the initial bioinformatics analysis that helped guide the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Buffers. Buffer A contains 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1× protease inhibitors (Roche) and 1 mM DTT. Buffer B contains 3 mM EDTA, 0.2 mM EGTA, 1× protease inhibitors (Roche) and 1 mM DTT. Buffer D contains 20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 1× protease inhibitors (Roche), 1 mM DTT and 300 mM KCl for protein extraction. PBS contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.4 mM KH₂PO₄, pH 7.4. Triton X-100 is abbreviated “T.” EMD buffer contains 25 mM Tris, pH 8, 80 mM NaCl, 35 mM KCl and 0.5 mM DTT.

Fly stocks. All fly stocks were raised at 20°C, except the RNA interference (RNAi) flies, which were raised at 25°C. The piwi mutant flies are transheterozygous piwi¹/piwi²; both are loss-of-function alleles, and 10–20% of piwi¹/piwi² flies have ovarioles. The wild-type strain is w¹¹¹⁸. The corto mutations are transheterozygous for alleles 420 (from F. Peronnet) and L1. The E(z) mutation is allele 63 (from R. Jones). The Pc mutations are alleles 1 and 3. Other fly stocks, listed in Supplementary Table 1, were obtained from the Bloomington Drosophila Stock Center or the Vienna Drosophila RNAi Center. Fly crosses were performed as described in Fly stocks. No randomization or blinding was used to determine which flies were analyzed or processed.

Immunofluorescence. Dissected ovaries were fixed with 3% paraformaldehyde in PBS, washed and permeabilized in 0.5% T/PBS for four nights. The ovaries were then blocked with 2% BSA in PBS, incubated in primary and secondary antibody solutions overnight, and washed with 0.2% T/PBS for >2 h. The ovaries were then stained with DAPI, washed, mounted on slides and imaged with the Leica TCS SP5 Spectral Confocal Microscope.

Antibodies. The names, sources and dilution conditions for all antibodies used are listed in Supplementary Table 2.

Histone extraction. Dissected ovaries (>50 pairs) were homogenized in 1.5-ml tubes, and proteins were extracted for 10 min using 400 mM KCl buffer D. Resultant pellets were extracted with 0.2 M HCl overnight at 4°C. Extract was neutralized by the addition of 1.5 M Tris-HCl, pH 8.8, and then analyzed by immunoblotting.

Image acquisition and quantification. Confocal images were acquired with a Leica TCS SP5 Spectral Confocal Microscope, with equal intensity and exposure across samples when imaging the same protein of interest. Immunoblotting images were obtained with the Gel Logic 2200 Imaging System. Image quantification was accomplished using the measure function of ImageJ software to obtain total signals for the region or band of interest and then standardizing the background-subtracted signal to either Coomassie band signal for immunoblotting or DAPI signal for immunofluorescence.

RNA extraction and RT-qPCR. Total RNA was extracted with TRIzol, 2 µg of RNA was reverse transcribed (by high-capacity cDNA reverse transcription kit, Applied Biosystems) and qPCR analyses were performed with iQ SYBR Green Supermix in the CFX96 system (Bio-Rad). Primers are listed in Supplementary Table 3.

Protein extraction and immunoprecipitation. Dissected ovaries (>50 pairs) were homogenized in 1.5-ml tubes, and proteins were extracted using 300 mM KCl buffer D. Equal amounts of ovarian extract were incubated with antibodies overnight, followed by incubation with Protein A–conjugated Dynabeads (Invitrogen) for 3 h. Beads were washed with 0.05% T/PBS, and immunoprecipitates were eluted with 2× loading buffer.

Protein pulldown using HEK293 or S2 cell extract. HEK293 cells (American Type Culture Collection, CRL-1573) were transfected with DNA constructs (in pcDNA3.1(+); primers listed in Supplementary Table 4) using Lipofectamine 2000 (Life Technologies). Drosophila S2 cells (Drosophila Genome Resource Center, stock 6) were transfected with DNA constructs (in pAMW or pAFW from the Drosophila Genome Resources Center; primers listed in Supplementary Table 4) using Cellex® II (Life Technologies). Both cell lines were mycoplasma free, as tested by MycoGuard Mycoplasma PCR Detection kit (GeneCopeia, MD-P-T-050). Twenty-four to forty-eight hours after transient transfection, the cells were washed and lysed with 300 mM KCl buffer D. Extracts (diluted to 150 mM KCl) were incubated with beads linked to antibody for FLAG or Myc (Sigma-Aldrich) for 2 h, the beads were washed with 0.05% T/PBS and eluates were collected for analysis.

Recombinant protein preparation, pulldown and in vitro histone methylation assays. The ORFs for E(z), Su(z)12 and Esc were cloned into pFastBac using the Bac-to-Bac N-His TOPO cloning kit (Life Technologies) for baculovirus generation via Bac-to-Bac baculovirus expression systems (Life Technologies). N-terminally His-tagged proteins were isolated by HisPur Ni-NTA resin (Thermo Scientific, PI-88221) from S9 extracts for cells coinfected with baculoviruses encoding E(z), Su(z)12 and Esc. Protein complexes were reconstituted from the eluate and analyzed by Coomassie staining. HEK293 cells were transfected with FLAG-Piwi-pcDNA3.1(+). FLAG-Piwi recombinant proteins were isolated from HEK293 cell extract using beads linked to antibody for FLAG (Sigma-Aldrich), beads were washed, and complexes were eluted with 0.2 mg/ml 3×FLAG peptide (Sigma-Aldrich) at 28°C and analyzed by Coomassie staining. For pulldown, 1 µg of FLAG-Piwi protein and 10 µg of PRC2 complex were incubated in 150 µl of 0.1% T/PBS for 20 min at room temperature, and 2 µl of guinea pig antibody to Piwi was used. In vitro histone methylation assays were performed according to Peng et al. 22.

Chromatin immunoprecipitation. Dissected ovaries were fixed in 1% paraformaldehyde in PBS, homogenized and sonicated in a Bioruptor (Diagenode). The ChIP procedure was performed according to Boyer et al. 46, using 100–300 µg of chromatin for each immunoprecipitation. ChIP-qPCR signals were calculated as the percentage of input. Primer sequences are included in Supplementary Table 5.

EMSA analysis. Full-length FLAG-Piwi protein was isolated from HEK293 cell extract, and the PAZ domain was isolated from BL21 bacterial extract. Recombinant proteins (10–40 ng) were incubated with 10 fmol of 32P-labeled (by T4 PNK, NEB) oligonucleotides in 10 µl of EMSA buffer (10 mM HEPES, pH 7.9, 80 mM NaCl, 35 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1× protease inhibitors (Roche) and 1 mM DTT) for 20 min at room temperature, and 2 µl of gel shift reaction was analyzed. Gels were run for 50 min at 120 V and underwent autoradiography. Oligonucleotide sequences are listed in Supplementary Table 6.

Piwi ChiP-seq analysis. Sequencing reads were mapped against the reference dm3 assembly (RS assembly, April 2006) using the Novoalign read mapper (flags: -o SAM -o Sync -S 4000 -s 10 -p 7, 10 0.4, 2 -t 120 --k). We retained only uniquely mapping reads for further analyses.

Piwi peaks were identified by the QuEST peak caller 36 using the following settings: (i) transcription factor ChIP-seq, (ii) ChIP enrichment: 3, ChIP-to-background enrichment: 2, ChIP extension enrichment: 1. The de novo Piwi motif was identified using the MEME 47 and FIMO 48 tools. Three-way Piwi overlapping sites were calculated using a 200-bp distance cutoff.

DNAseq analysis. DNAseq reads from three wild-type replicates and three piwi mutant replicates were processed using the DNA Nexus cloud platform, including mapping reads to RefSeq transcript annotations and calculation of RPKM values. We calculated the Student’s t-test P value for each gene using the triplicate measurements for piwi mutant and wild-type samples and report up- or downregulated genes using a P-value cutoff of 0.001. Differentially expressed genes were further analyzed using the Gene Ontology system in PANTHER 37.

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H3K27me3 and E(z) ChIP-seq analysis. Sequencing reads from H3K27me3 ChIP-seq, E(z) ChIP-seq or input data sets were mapped against the reference dm3 assembly (R5 assembly, April 2006) using the Novoalign read mapper (flags: -o SAM -o Sync -S 4000 -s 7, 10 0.4, 2 -t 120 --k). We retained only uniquely mapping reads for further analyses.

We used the QuEST peak caller to identify genomic regions where H3K27me3 or E(z) signals were enriched. When running QuEST, we used the following sets of parameters: (i) histone ChIP-seq, (ii) ChIP enrichment: 3, ChIP-to-background enrichment: 1.5, ChIP extension enrichment: 1.

H3K27me3 and E(z) scatterplots were generated by calculating the number of reads falling into H3K27me3 or E(z) regions and normalizing to the expected counts before plotting. Linear fit was performed in R.

Circos plots. Circos plots were generated using the Circos tool by (i) calculating ChIP-seq reads in 10,000-bp bins, (ii) converting counts to an enrichment score by normalizing counts to the expected read counts within the 10,000-bp bins and (iii) applying the Circos tool to the resulting enrichment scores.

RNA Pol II analysis. RNA Pol II ChIP-seq data sets were generated for the following fly strains: wild type (four replicates), piwi mutant (four replicates), with one replicate removed owing to its very low correlation with other piwi replicates, piwi; Corto/+ mutant (four replicates), piwi; E(z)+/+ mutant (four replicates), and piwi; Pcl/+ mutant (four replicates), with one replicate not considered owing to its very low correlation (<0.6) with the other three piwi; Pcl/+ replicates.

First, 1,551 RNA Pol II binding peaks were identified using the strongest Pol II ChIP-seq data set (piwi; Corto+) with the QuEST tool. Then, for each ChIP-seq data set, we counted the number of RNA Pol II ChIP-seq reads within ±300 bp of each Pol II peak. We plotted replicate data sets after log transformation of tag counts and calculated correlations between replicates, which confirmed good experimental replication. For each mutant, we designated one RNA Pol II data set to calculate the scaling factor, necessary to average across the replicates scaling factor = median(Pol II tags in replicate 2/Pol II tags in replicate 1)

Next, we adjusted the Pol II tag counts in that replicate according to the scaling factor or used the original signals for the reference replicate. For each RNA Pol II binding region for each mutant, we calculated the mean binding signal (by averaging scaled signals) and sample variance (using scaled signals). We then calculated the correlations of RNA Pol II binding signals between mutants (Supplementary Fig. 5a) as well as between the mutant and wild-type strains (Supplementary Fig. 5b).

To identify regions with evidence of differential RNA Pol II binding between a given mutant strain and the wild-type strain (Fig. 6b), we first scaled RNA Pol II signals between that mutant (for example, piwi) and the wild-type strain according to the formula

piwi scaling factor = median(Pol II signal from piwi / Pol II signal from wild type)

The scaling factor was used to adjust the mean and variance of the Pol II signal for that mutant. We then identified Pol II peaks that increased in strength in the mutant in comparison to the wild-type strain (Pol II increased) and determined the statistical significance of these changes by calculating z scores and using an appropriate cutoff (z ≥ 1.5) to select regions showing increased Pol II binding in that mutant. Similarly, we identified Pol II peaks that decreased in strength in the mutant versus the wild-type strain (z ≤ −1.5). Each Pol II binding region was assigned to a nearby gene promoter, and sets of sites showing statistically significant increased (or decreased) RNA Pol II binding signals were further analyzed using the PANTHER Gene Ontology classification system to determine enriched gene sets in that mutant as compared to the wild-type strain.

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