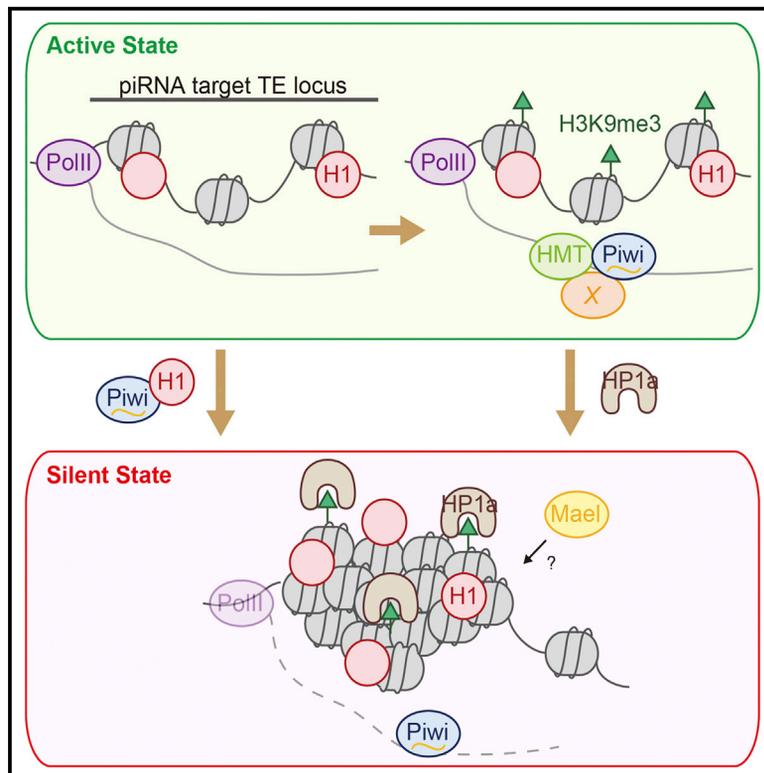


Piwi Modulates Chromatin Accessibility by Regulating Multiple Factors Including Histone H1 to Repress Transposons

Graphical Abstract



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In Brief

Piwi-piRNA complexes silence transposons in animal gonads. Iwasaki et al. identify linker histone H1 as a component of nuclear Piwi-piRNA complexes and show that Piwi-piRNA complexes require both H1 and HP1a to repress targets and that the transcriptional silencing is correlated with the chromatin state rather than H3K9me3 marks.

Highlights

- Piwi forms a complex with H1 to regulate H1 association to target transposon loci
- H1 regulates Piwi target transposons independently of H3K9me3 modification
- Piwi transcriptionally silences its targets by modulating chromatin accessibility
- H1 and HP1a interdependently regulate chromatin accessibility at Piwi target loci

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Piwi Modulates Chromatin Accessibility by Regulating Multiple Factors Including Histone H1 to Repress Transposons

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SUMMARY

PIWI-interacting RNAs (piRNAs) mediate transcriptional and post-transcriptional silencing of transposable element (TE) in animal gonads. In *Drosophila* ovaries, Piwi-piRNA complexes (Piwi-piRISCs) repress TE transcription by modifying the chromatin state, such as by H3K9 trimethylation. Here, we demonstrate that Piwi physically interacts with linker histone H1. Depletion of Piwi decreases H1 density at a subset of TEs, leading to their derepression. Silencing at these loci separately requires H1 and H3K9me3 and heterochromatin protein 1a (HP1a). Loss of H1 increases target loci chromatin accessibility without affecting H3K9me3 density at these loci, while loss of HP1a does not impact H1 density. Thus, Piwi-piRISCs require both H1 and HP1a to repress TEs, and the silencing is correlated with the chromatin state rather than H3K9me3 marks. These findings suggest that Piwi-piRISCs regulate the interaction of chromatin components with target loci to maintain silencing of TEs through the modulation of chromatin accessibility.

INTRODUCTION

Movement of mobile DNA elements such as transposable elements (TEs) can result in deleterious mutations on the host genome (Goodier and Kazazian, 2008). piRNAs are a class of small non-coding RNAs that associate with PIWI proteins and guide piRNA-induced silencing complexes (piRISCs) to target and repress TEs (Ge and Zamore, 2013; Iwasaki et al., 2015). PIWI proteins and piRNAs are expressed almost exclusively in the animal germline, and defects of Piwi-piRNA silencing result in activation of TEs and a sterile phenotype, underlining the importance of TE regulation and maintenance of genome integrity.

Among three PIWI proteins expressed in the *Drosophila* ovary, Aubergine (Aub) and AGO3 cleave complementary TE transcripts by small RNA-guided endoribonuclease (Slicer) activity

in the cytoplasm of germline cells (Brennecke et al., 2007; Guna-wardane et al., 2007). In contrast, Piwi is located in the nucleus of both germline cells and surrounding somatic cells (Cox et al., 2000). TE silencing by Piwi operates at the transcriptional level in a slicer-independent manner (Klenov et al., 2011; Le Thomas et al., 2013; Rozhkov et al., 2013; Saito et al., 2010; Sienski et al., 2012; Wang and Elgin, 2011). piRNAs form a complex with Piwi at the cytoplasmic Yb-body, and only Piwi proteins loaded with a piRNA are imported into the nucleus to silence their targets. Within the nucleus, Piwi-piRISCs repress TEs transcriptionally, and H3K9 trimethylation (H3K9me3) coincides with silenced TEs. Piwi is expressed in the cell line Ovarian Somatic Cell (OSC) (Saito et al., 2009). Earlier studies showed that OSC reproduces the Piwi-piRNA pathway observed in somatic cells of *Drosophila* ovaries (Dönertas et al., 2013; Ishizu et al., 2015; Muerdter et al., 2013; Ohtani et al., 2013; Post et al., 2014; Saito et al., 2009, 2010; Sienski et al., 2012), and therefore the cell line represents a very powerful system to elucidate how Piwi-piRISCs repress TEs.

Heterochromatin is characterized by H3K9me3 repressive epigenetic marks (Nakayama et al., 2001; Rea et al., 2000; Schotta et al., 2002), associated with Heterochromatin protein 1 (HP1a in *Drosophila*) (Bannister et al., 2001; Eissenberg et al., 1990; Lachner et al., 2001). Spread of H3K9me3 marks leads to heterochromatin formation on target TE loci (Le Thomas et al., 2013; Rozhkov et al., 2013; Sienski et al., 2012; Wang and Elgin, 2011). Maelstrom (Mael), DmGTSF1/Asterix (Arx), and HP1a are putative Piwi cofactors (Brower-Toland et al., 2007; Czech et al., 2013; Dönertas et al., 2013; Le Thomas et al., 2013; Muerdter et al., 2013; Ohtani et al., 2013; Sienski et al., 2012; Wang and Elgin, 2011), and recent genetic screening studies suggested several other factors are involved in Piwi-mediated transcriptional silencing (Czech et al., 2013; Handler et al., 2013; Muerdter et al., 2013). Mael and DmGTSF1/Arx regulate RNA polymerase II recruitment and RNA levels of Piwi target TEs (Dönertas et al., 2013; Muerdter et al., 2013; Ohtani et al., 2013; Sienski et al., 2012). HP1a regulates many TEs including those targeted by Piwi, and loss of Piwi reduces HP1a association to Piwi target TE loci (Brower-Toland et al., 2007; Le Thomas et al., 2013; Wang and Elgin, 2011). However, depletion of piRNA pathway genes leads to different outcomes at the level of H3K9me3 (Dönertas et al., 2013; Klenov et al., 2014; Muerdter et al., 2013; Ohtani et al., 2013; Sienski et al., 2012). Piwi

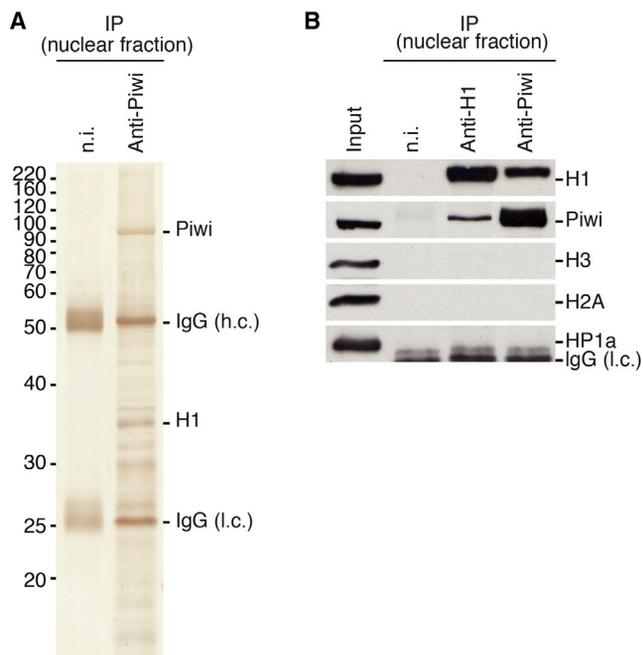


Figure 1. H1 Physically Associates with Nuclear Piwi

(A) Silver staining of nuclear protein components in the anti-Piwi immunoprecipitated complex. Immunoprecipitation was performed using OSC nuclear extracts under stringent conditions. h.c., heavy chain; l.c., light chain of the antibody.

(B) Western blot showing H1, Piwi, H3, H2A, and HP1a protein levels in the nuclear complex immunoprecipitated from OSCs using indicated antibodies. Mouse immunoglobulin G (IgG) (n.i.) was used for control immunoprecipitations.

See also [Figures S1](#) and [S2](#).

knockdown (KD) or DmGTSF1/Arx-KD decreases H3K9me3 levels at Piwi target TE loci, whereas Mael-KD does not, suggesting that H3K9me3 per se is not the final silencing mark for transcriptional gene silencing mediated by Piwi-piRNA complexes. Thus, it is unclear how H3K9me3 mark contributes to Piwi-piRISC-mediated TE silencing, and the final effector of this transcriptional silencing pathway is unknown.

Chromatin compaction by heterochromatin results in repression of genes encoded at corresponding regions ([Li and Reinberg, 2011](#)). Linker histone H1 confers higher-order organization to chromatin by direct binding with nucleosomes, making genes inaccessible to specific transcription factors to prevent their expression ([Buttinelli et al., 1999](#); [Laybourn and Kadonaga, 1991](#); [Pennings et al., 1994](#)). However, interaction of H1 with nucleosomes is only transient; its nucleosome residing time is modulated by posttranslational modifications in H1 or by nuclear proteins that interact with H1 or compete with H1 for nucleosome binding sites ([Bustin et al., 2005](#); [Misteli et al., 2000](#)). Recently, H1 was reported to be required for TE repression in *Drosophila* ([Lu et al., 2013](#); [Vujatovic et al., 2012](#)). H1 may recruit Su(var)3-9 methyltransferase to TE loci, resulting in the addition of H3K9me3 marks ([Lu et al., 2013](#)). However, a systematic understanding of how H1 is recruited to specific TE loci and how H3K9me3 modification induces TE silencing is unclear.

In this study, we identified H1 as a component of a nuclear Piwi complex. Using OSCs, we found that loss of Piwi results in a specific decrease of H1 density at Piwi target TE loci and their surrounding genomic regions, suggesting that Piwi enforces association of H1 to its target TE loci. Depletion of H1 derepresses a variety of TEs and their surrounding genes, including those normally silenced by Piwi-piRISCs, without affecting the density of H3K9me3 mark and HP1a at target TE loci. We demonstrated that chromatin accessibility at Piwi target TE loci is modulated by H1. Notably, depletion of HP1a also resulted in increased chromatin accessibility at Piwi target TE loci. These findings suggest that Piwi-piRISCs adopt interdependent actions of H1 and HP1a to maintain silencing of the TE state by modulating the chromatin state.

RESULTS

Piwi Forms a Complex with Linker Histone H1

To gain insights into the molecular pathway leading to Piwi-piRISC-mediated TE silencing, we immunopurified Piwi-associated complexes from OSC nuclear lysates. These complexes were subjected to SDS-PAGE gel electrophoresis followed by silver staining. Several specific protein bands were observed, and mass spectrometric analyses identified a 34-kDa band as linker histone H1, suggesting that H1 associates with Piwi in the nucleus ([Figures 1A](#) and [S1A](#)). To confirm the H1-Piwi association, we produced a specific monoclonal antibody against H1. Western blotting showed a discrete band at 34 kDa in OSC and ovary lysates but not in OSCs where H1 was depleted by RNAi, indicating that the antibody specifically recognizes H1 ([Figure S1B](#)). Using the antibody, we immunopurified H1-associated complexes from OSC nuclear lysates or *Drosophila* ovary lysates. Western blots of immunoprecipitates confirmed that Piwi, but not the core nucleosome proteins, histone H3 and H2A or HP1a, was present in the H1-associated complex ([Figures 1B](#), [S1C](#), and [S1D](#)). Thus, although H1 is a highly charged abundant nuclear protein, it specifically interacts with Piwi ([Figure S1E](#)).

We also performed an in vitro pull-down assay using recombinant H1 fused to glutathione S-transferase (GST-H1) and crude OSC lysates ([Figure S1F](#)). Piwi was detected in GST-H1 pull-down products, confirming the association in vitro. Because it is unlikely that GST-H1 is modified at specific H1 amino acid residues, which may occur in vivo ([Bonet-Costa et al., 2012](#)), specific protein modifications do not appear to require this interaction. Moreover, the addition of DNase or RNase did not affect interactions between H1 and Piwi ([Figure S1G](#)).

H1 Is Required for TE Silencing

To explore whether H1 is involved in Piwi-piRISC-mediated transcriptional silencing of TEs in OSCs, we depleted H1 by RNAi ([Figure S2A](#)). We isolated total RNA from 4-day KD samples, performed RNA-sequencing (RNA-seq) analysis, and compared RNA expression profiles with control EGFP RNAi. Overall, 82 (63.6%) TE transcripts of 129 annotated TEs were increased >2-fold, whereas 999 (7.1%) protein-coding genes were upregulated >2-fold in H1-depleted cells, revealing a major repressive effect of H1 on OSC TE expression ([Figure 2A](#)). Furthermore, qRT-PCR of *mdg1*, a TE regulated by the somatic Piwi-piRNA

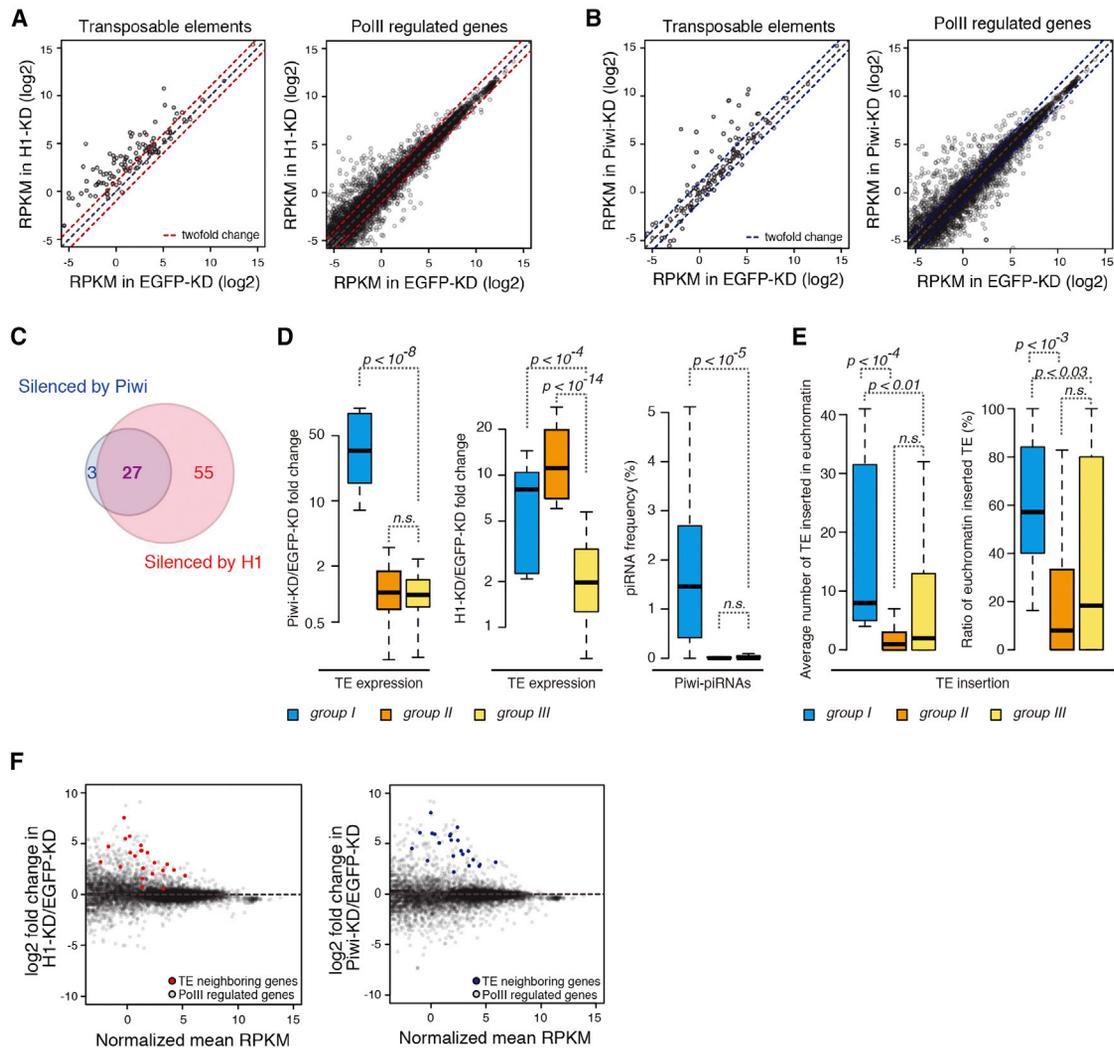


Figure 2. H1 Is Essential for Silencing of TEs Targeted by the Piwi-piRNA Pathway

(A) Scatterplot of RPKM values (log₂) for 129 TEs (left) or 14,145 Pol II-regulated genes (right) in EGFP-KD (control, x axis) or H1-KD (y axis) samples examined by RNA-seq. Red diagonal lines indicate 2-fold change.

(B) Scatterplot as in (A) for EGFP-KD (x axis) or Piwi-KD (y axis) samples. Blue diagonal lines indicate 2-fold change.

(C) Venn diagram displaying the number of >2-fold upregulated TEs upon depletion of Piwi (blue) or H1 (red).

(D) Boxplots showing fold changes in the expression of group I, II, and III TEs based on RNA-seq upon Piwi (left)- or H1 (center)-KD. Boxplot on the right shows frequency of piRNAs targeting group I, II, and III TEs. Boxplot whiskers show maxima and minima. p values were calculated by Wilcoxon rank-sum test, and y axis is log₂ scale. n.s.; not significant ($p > 0.05$).

(E) Boxplots, as in (D), show average number of TEs inserted in euchromatin (left) or ratio of euchromatin-inserted TEs (right) in group I, II, and III TEs.

(F) MA plot showing changes in RNA abundance for set of Pol II-regulated genes upon H1 (left)- or Piwi (right)-KD based on RNA-seq. Twenty-two genes with TE insertions either in the gene body or close proximity (<5 kb away) and upregulated <4-fold in Piwi-KD cells are plotted in red (left) or blue (right), while other genes are plotted in gray.

See also Figures S2 and S3.

pathway (Sienski et al., 2012), confirmed that its expression was activated as much as 80-fold in H1-KD cells (Figure S2B). Under conditions where endogenous H1 was depleted, expression of *myc*-H1r, which was designed to be RNAi insensitive, rescued *mdg1* repression (Figure S2C). Thus, activation of TE expression results from H1 protein decrease in OSCs. H1 loss did not affect the nuclear localization of Piwi (Figure S2D). Moreover, expression levels of piRNAs, which were severely decreased by the loss of Piwi were unaffected by H1 loss (Figure S2E).

We confirmed this by deep sequencing of Piwi-associated piRNAs upon control (EGFP) and H1-KD (Figures S2F and S2G). Levels of piRNAs mapped to the somatic piRNA cluster *flamenco* and the genic piRNA locus *traffic jam*, and those mapped to TEs remained unchanged upon H1-KD (Figures S2H–S2J), indicating that general piRNA biogenesis does not require H1. An exception was an increase of piRNAs mapped to *roo*, which was consistent with a previous study (Lu et al., 2013). Expression of *roo* TE, however, was unchanged by either H1- or Piwi-KD (Figure S3). These

results suggest that H1 is involved in the silencing step, rather than piRNA biogenesis, of the Piwi-piRNA pathway.

We delineated the region of H1 responsible for TE silencing. H1 is a tripartite protein consisting of a central globular DNA-binding domain flanked by lysine-rich regions at both N- and C-terminal domains (Figure S2K). Absence of C-terminal or N-terminal regions had no effect on nuclear localization (Figure S2L), and deletion of the C-terminal but not N-terminal region failed to rescue *mdg1* repression, indicating the requirement of the C-terminal region for TE silencing (Figure S2M). This C-terminal region of the H1 protein is sufficient for Piwi binding (Figure S2N). However, the same region of H1 is also known to be important for condensing chromatin in general (Zhou et al., 2013, 2015). We also found that the N-terminal region (amino acids 1–130) of the Piwi protein is essential for H1 interaction (Figure S2O). The N-terminal region (amino acids 1–72) of Piwi is known to be responsible for its nuclear localization (Saito et al., 2009), and, as we expected, Piwi lacking the N-terminal region (amino acids 1–130) was unable to localize to the nucleus (data not shown). These results show that the C-terminal region of H1 and the N-terminal region of Piwi interact with each other. However, it is difficult to definitively rule out the possibility that the regions responsible for this interaction are also essential for other functions.

H1 Regulates TEs, Including Those Targeted by the Piwi-piRNA Pathway

Over 60% of TE transcripts were increased >2-fold in H1-depleted cells (Figure 2A). To compare transcript levels in H1- or Piwi-depleted OSCs, we analyzed our RNA-seq data. Consistent with previous studies (Ohtani et al., 2013; Sienski et al., 2012), only a limited number of TE families (30/129, 23.3%) were increased >2-fold upon Piwi-KD (Figure 2B), almost all of which (27/30, 90%) were increased in H1-KD cells (Figures 2A–2C). Based on RNA-seq data, we classified TEs into three groups. TEs without mapped reads were excluded, because they are likely to be “dead” TEs. Thirteen TEs in group I exhibited >6-fold increase of mRNA expression in Piwi-KD cells. The 6-fold increase was used as a threshold based on the average fraction of piRNA frequency per TEs (Figure S3A, top). Of the remaining TEs, 29 upregulated expression >6-fold in H1-KD and were classified as group II. Other TEs ($n = 76$) were categorized as group III and underwent moderate changes of expression upon Piwi- or H1-KD (Figure S3B). Importantly, the Piwi-KD fold-change threshold was compatible with the fraction of TEs regulated by H1 (Figure S3A, bottom), supporting that H1 correlates with Piwi-regulated TE silencing and has a role in the piRNA pathway. Group II TEs were significantly repressed by H1 but not Piwi, implying that H1 may discriminate TEs in a piRNA-dependent (group I) or -independent manner (group II), while some TEs were independent of regulation by Piwi or H1 (group III) (Figures 2D and S3C). Selected examples of each group were analyzed by qRT-PCR to confirm the RNA-seq data (Figure S3D).

Both group I and group II TEs were regulated by H1, but only group I TEs were targeted by piRNAs (Figure 2D). Thus, we investigated features to separate these two groups. Most TEs in group I were long terminal repeat (LTR) elements (92.3%), except for the DNA element *transib2* (Figure S3C). In contrast,

only 58.6% of group II TEs were LTR elements. Genomic insertion sites and numbers of each TE indicated that group I TEs had significantly larger numbers of insertions in euchromatic regions compared to group II TEs ($p < 10^{-4}$) (Figure 2E, left). Additionally, the ratio of euchromatic insertions against the total number of the genomic insertions was higher for group I TEs ($p < 10^{-3}$) (Figure 2E, right). Thus, group I TEs are mostly LTR elements, which accumulate in euchromatic regions, whereas group II TEs tend to consist of various elements inserted in both euchromatic and heterochromatic regions.

Depletion of piRISC components influences the expression of a subset of protein-coding genes near TE insertions (Ohtani et al., 2013; Sienski et al., 2012). To assess the role of H1, we analyzed RNA-seq data of 22 genes, for which TE insertions were either in the gene body or in close proximity (<5 kb away) and which were upregulated in Piwi-KD OSCs (Sienski et al., 2012). As expected, >90% (20 of 22) of genes with a 4-fold change upon Piwi-KD were also highly upregulated upon H1-KD (Figure 2F). Moreover, variation in expression of TE neighboring genes was highly comparable between H1- and Piwi-KD cells ($r = 0.95$; Figure S3E). Collectively, these data strongly support the notion that H1 is a component of the Piwi-piRNA-mediated silencing pathway.

Piwi Regulates Association of H1 to Piwi-piRNA Target TE Loci

To elucidate how H1 functions in Piwi-mediated TE silencing, we verified H1 binding to chromatin in OSCs. Because H1 association with DNA inhibits nucleosome mobility and transcription in vitro (Laybourn and Kadonaga, 1991; Pennings et al., 1994), and that H1 is required for Piwi-dependent transcriptional silencing (Figure 2), we hypothesized that Piwi may act upstream of H1 by regulating its association with the target TE locus. To test this, we studied the association of H1 with TE loci using chromatin immunoprecipitation sequencing (ChIP-seq) analysis under Piwi-KD. Piwi-KD reduced H1 density throughout the entire TE consensus sequences on group I but not group II or III TEs (Figure 3A). We further confirmed the group I TE (*mdg1*)-specific decrease in the H1 ChIP signal upon Piwi-KD by ChIP-qPCR analyses (Figure S4A). These data indicated that Piwi is specifically required for H1 to associate with group I TEs.

Previously, Piwi-KD was shown to increase the occupancy of Pol II within an ~15-kb region flanking euchromatic TE insertions (Sienski et al., 2012). We analyzed the H1 density in regions flanking euchromatic TE insertions and found that the ~15-kb extension feature was also observed in H1 density (Figure 3B) on group I, but not group II or group III TE insertions, indicating that the H1 association with group I TEs is controlled by the Piwi-piRNA pathway. Because we focused on several representative TEs in each group (Figures 3A and 3B), we analyzed ChIP-seq data of the complete set of group I, II, and III TEs to determine whether Piwi-regulated H1 association with TE loci was reflected within the entire set of group I TEs. Comparison of ChIP-seq signal fold changes demonstrated a significant decrease in H1 occupancy upon Piwi loss for group I TEs compared with group III TEs ($p < 0.02$) (Figure 3C). Moreover, H1 occupancy was further decreased ($p < 10^{-3}$) when we collected TEs derepressed >20-fold upon Piwi loss (group I-20; Figure S3C). By contrast, no

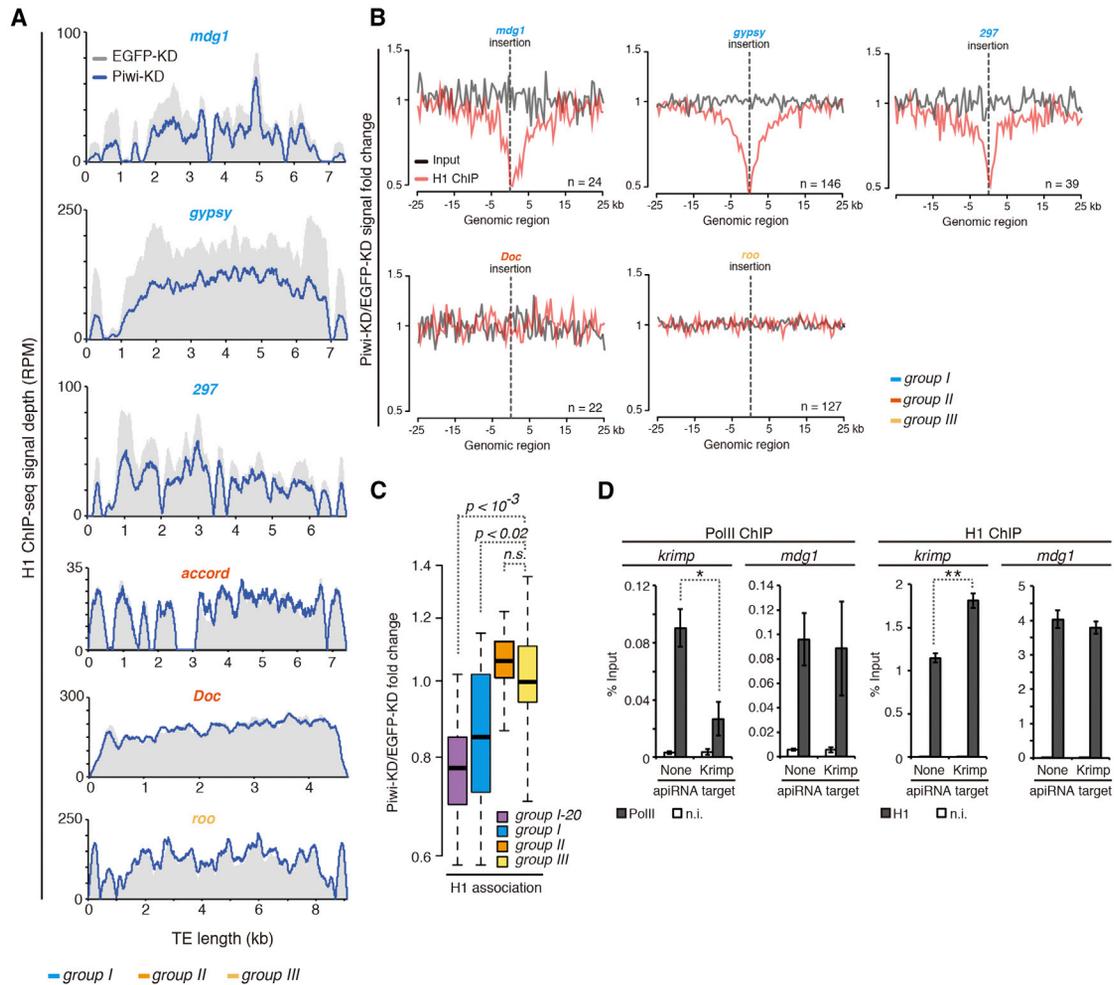


Figure 3. Association of H1 with piRNA Target TE Loci Is Regulated by Piwi

(A) Density plots for normalized H1 ChIP-seq signals over consensus sequences from group I, II, and III TEs in EGFP (control)- or Piwi-KD cells. ChIP signal depths on EGFP (gray)- and Piwi (blue)-KD samples are shown. Note that *accord* had only one euchromatic insertion, and therefore metaplot could not be calculated.

(B) Metaplots showing signal of H1 ChIP-seq for genomic regions around euchromatin insertions of group I, II, and III TEs. Fold-change in Piwi-KD compared with EGFP-KD is shown for H1 ChIP (red) and Input (gray) signals. y axis is \log_2 scale.

(C) Boxplot, as in Figure 2, showing fold changes in the association of H1 with group I, II, and III TEs upon depletion of Piwi, based on ChIP-seq. Group I-20 TEs are a population of group I TEs whose expression fold change was >20 in Piwi-KD samples compared with EGFP-KD samples.

(D) ChIP-qPCR analysis of RNA PolIII (left) and H1 (right) occupancy on the *krimp* and *mdg1* promoter region, following expression of *krimp* targeting artificial piRNA (apiRNA). apiRNAs expressed without target genes (None) were used as a negative control. Error bars represent means \pm SEM values of three independent experiments. * $p < 0.05$; ** $p < 0.005$.

See also Figure S4.

significant difference was observed between group II and III TEs (Figure 3C), whose expression levels were unaffected by Piwi-KD (Figure 2D). Thus, the Piwi-regulated H1 occupancy on TE loci correlated with the degree of TE expression regulated by Piwi and supported our notion that H1 density on group I TEs is regulated by the Piwi-piRNA pathway.

To corroborate our data, we applied an artificial piRNA (apiRNA) production system that targets and transcriptionally silences a gene of interest in OSCs (Figure S4B, left) (Ishizu et al., 2015). We confirmed that the expression of apiRNAs against *krimper* (*krimp*), a protein coding gene expressed in naive OSCs, decreased Krimper protein levels and occupancy of Pol

II (Figures 3D, left, and S4B, right). Analysis of the level of H1 association with the *krimp* promoter region indicated that H1 density increased 1.6-fold by expression of apiRNA (Figure 3D, right) without affecting levels of *mdg1* TE. These results showed that apiRNAs induce the association of H1 with a target gene in a sequence-specific manner.

Piwi and H1 Regulate Targets by Modulating Chromatin Accessibility

Stable association of H1 functions in the compaction of chromatin into higher order structures, resulting in regulation of the encoded genes (Buttinelli et al., 1999; Laybourn and Kadonaga,

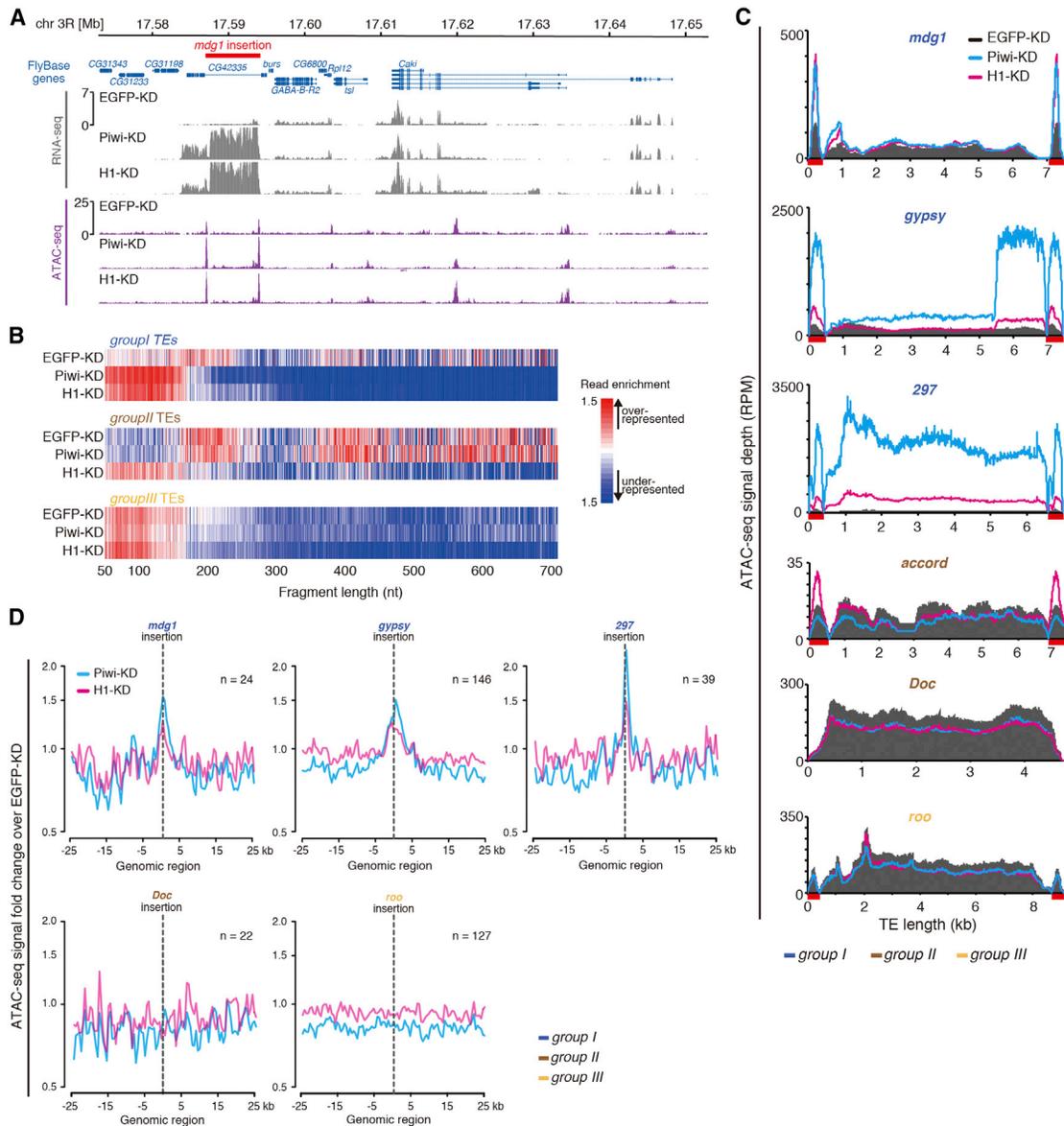


Figure 4. Piwi and H1 Modify Chromatin Accessibility to Silence Their Target TEs

(A) RNA-seq and ATAC-seq signal densities at genomic regions neighboring *mdg1* insertion are shown for OSCs with EGFP (control)- Piwi- or H1-KD. FlyBase protein-coding genes are indicated at the top, together with chromosome position.

(B) Read enrichments for three groups of TEs upon Piwi or H1-KD. Distributions of paired-end sequencing fragment sizes were calculated, and reads were normalized by percent maximal within each group of TEs. Enrichment was calculated relative to the genome-wide set of fragment sizes.

(C) Density plots for normalized ATAC-seq signals over consensus sequences from group I, II, and III TEs in EGFP-, Piwi- or H1-KD cells. ATAC-seq signal depths on EGFP (black)-, Piwi (blue)-, and H1 (pink)-KD samples are shown. LTR regions are shown by red line under x axis.

(D) Metaplots showing signal of ATAC-seq signal for genomic regions around euchromatin insertions of group I, II, and III TEs. Fold change in Piwi (blue)- or H1 (pink)-KD compared to EGFP-KD is shown. y axis is log₂ scale.

See also Figure S4.

1991; Pennings et al., 1994). We hypothesized that Piwi-mediated recruitment of H1 resulted in chromatin compaction, leading to downregulation of Piwi-piRNA target TE expression. We performed transposase-accessible chromatin (ATAC)-seq analysis (Buenrostro et al., 2013) to profile open chromatin structures. ATAC-seq probe chromatin accessibility with Tn5 transposase, which integrates into accessible chromatin re-

gions. Therefore, genomic regions with open chromatin regions, such as transcription start sites (TSSs), result in accumulated ATAC-seq reads, whereas chromatin-dense regions result in decreased ATAC-seq signals (Figure 4A). Notably, only TSSs and not the gene body of transcribed genes had increased ATAC-seq signals, suggesting that ATAC-seq may be used to detect TSSs of coding genes. Indeed, we determined

ATAC-seq peaks from EGFP-, Piwi-, and H1-KD samples, and peaks commonly detected in EGFP- and Piwi/H1-KD samples were enriched in genomic regions neighboring TSSs (Figure S4C). In contrast, most peaks detected specifically in Piwi/H1-KD samples corresponded to intron or intergenic regions where TEs are enriched, rather than TSSs neighboring regions of genes (Figures S4C and S4D). Therefore, we further analyzed ATAC-seq signals at TE regions. First, we checked ATAC-seq fragment size, because accessible chromatin regions are enriched for short fragments, whereas regions with low chromatin accessibility are depleted of short fragments and enriched for phased multinucleosomal inserts (Buenrostro et al., 2013). The increase of short fragments for group I TEs after depletion of Piwi or H1 was detected, suggesting that Piwi and H1 are responsible for the regulation of chromatin accessibility at group I TEs. In contrast, increased shorter fragments were observed only in H1-KD samples for group II TEs, and Piwi or H1-KD did not affect the fraction size for group III TEs (Figure 4B). At steady state (EGFP-KD sample), shorter fragments were relatively under-represented in group II TEs compared to group I TEs. In agreement with the result showing that group I consists of larger fractions of TEs with euchromatic insertions (Figure 2E), these data suggest that group I TEs are located at regions with higher chromatin accessibility compared to group II TEs. Group III TEs tend to consist of TEs with shorter ATAC-seq fragment sizes, suggesting that these TEs are inserted in open chromatin regions. Group III TEs may be more ancient, degenerated TEs with lower transcriptional activity caused by mutations in their promoter region, or regulation by other mechanisms, and no longer pose a threat to *Drosophila*.

Distributions of ATAC-seq fragments within TE consensus sequences were analyzed upon Piwi and H1-KD. Depletion of Piwi and H1 increased ATAC-seq signal density for group I TEs ($p < 10^{-5}$, $p < 10^{-3}$) (Figures 4C and S4E). Increased ATAC-seq signals in Piwi- and H1-KD suggest that both modify chromatin accessibility at group I TE loci. Although the distribution of ATAC-seq fragments on each TE consensus sequence suggests a greater effect of Piwi than H1 on chromatin accessibility (Figure 4C), this may be explained by differences in efficiency of RNAi-KD (Figure S2A) or the other factor acting in parallel with H1 for modulation of chromatin accessibility. The distributions of ATAC-seq reads upon Piwi/H1-KD were commonly increased at LTR regions for each member of group I TEs. However, the distribution on the internal portion differed among TEs. We also profiled chromatin accessibility in regions flanking euchromatic TE insertions and found that the ~5-kb extension feature was observed in ATAC-seq tag density (Figure 4D) on group I, but not group II or group III TE insertions, consistent with the result of H1-ChIP-seq analysis. This indicated that the effect on the state of chromatin also spreads to the flanking region of group I, but not group II or group III TE insertions. Together, these results show that the Piwi-piRNA pathway regulates its targets by recruiting H1 to modify chromatin accessibility.

H1 Is Required for TE Silencing but Not H3K9me3 Modification

To address how H1 affects chromatin states, we analyzed H3K9me3 signals by ChIP-seq. Consistent with previous studies

(Ohtani et al., 2013; Sienski et al., 2012), Piwi-KD significantly decreased H3K9me3 signals on group I TE loci (Figure 5A). By contrast, we observed only a slight difference in H3K9me3 density between H1-KD and EGFP-KD on group I TEs ($p < 0.04$) (Figures 5A and S5A, right). Moreover, no significant differences were observed for group I-20 TEs compared with group III TEs (Figure S5A, right), showing that, unlike Piwi-KD (Figure S5A, left), H1-KD does not significantly affect H3K9me3 levels. Meta-analysis of TE insertion sites and closer inspection of loci that harbor TE insertions showed that H1-KD does not affect H3K9me3 signals (Figures 5B and 5C), in contrast with earlier models suggesting that H1 recruits H3K9 methyltransferase Su(var)3-9 to chromatin (Lu et al., 2013). Increased Pol II occupancy on group I TEs in H1-KD cells (Figure S5B), and upregulation of TEs and their neighboring genes coupled with open chromatin structure and unaffected H3K9me3 marks upon depletion of H1, suggests that modulation of chromatin accessibility by H1 is responsible for transcriptional silencing, and that H3K9me3 itself is not the final silencing mark.

Several factors have been identified in the Piwi-piRNA pathway, including Mael and HP1a, which regulate expression of Piwi-piRNA target TEs but not H3K9me3 marks (Le Thomas et al., 2013; Sienski et al., 2012; Wang and Elgin, 2011). Because H1 acts downstream of or in parallel to H3K9me3, we analyzed the relationship between H1 and these factors within the Piwi-piRNA pathway. We performed H1 ChIP-seq analysis upon depletion of Mael or HP1a. Mael-KD did not result in a significant decrease of H1 ChIP-seq signals distributed at group I, II, and III TEs, and effects on neighboring regions of group I TE insertion sites were limited (Figures S5C–S5F). HP1a-KD resulted in a relatively mild decrease of H1 distribution at group I TEs and their neighboring regions (Figures S5G–S5J). For example, reads mapped to *mdg1*, *gypsy*, or 297 consensus TE decreased to 83.0%, 78.3%, or 76.0% upon HP1a-KD, where it decreased to 64.3%, 58.4%, or 69.7% upon Piwi-KD (Figures 3A and S5G). Additionally, Mael and HP1a were not detected in the complex containing Piwi and H1 (Figures 1B and S1E), suggesting that recruitment of H1 to group I TE loci is independent of Mael or HP1a.

H1 and HP1a Interdependently Modulate Chromatin Accessibility in the Piwi-piRNA Pathway

Previously, it was shown that H1 interacts with HP1a in mammals and *Drosophila* (Hale et al., 2006; Lu et al., 2013; Nielsen et al., 2001) and that H3K9me stabilizes the binding of HP1a to chromatin using mammals and yeast models (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001; Nakayama et al., 2001). To analyze whether HP1a in the Piwi-piRNA pathway is regulated by H1 or H3K9me3 marks, we performed HP1a ChIP-seq analysis upon Piwi- or H1-KD. Consistent with previous studies showing that association of HP1a on several Piwi target TEs are regulated by Piwi (Wang and Elgin, 2011), and recruitment of HP1a to artificial piRNA target loci (Le Thomas et al., 2013), we observed the downregulation of HP1a signals upon Piwi-KD specifically at group I TEs ($p < 10^{-3}$) (Figures 6A and S6A), suggesting that HP1a also acts downstream of Piwi. However, H1-KD did not affect HP1a signal levels at the same loci (Figures 6A and S6A), and the effect of

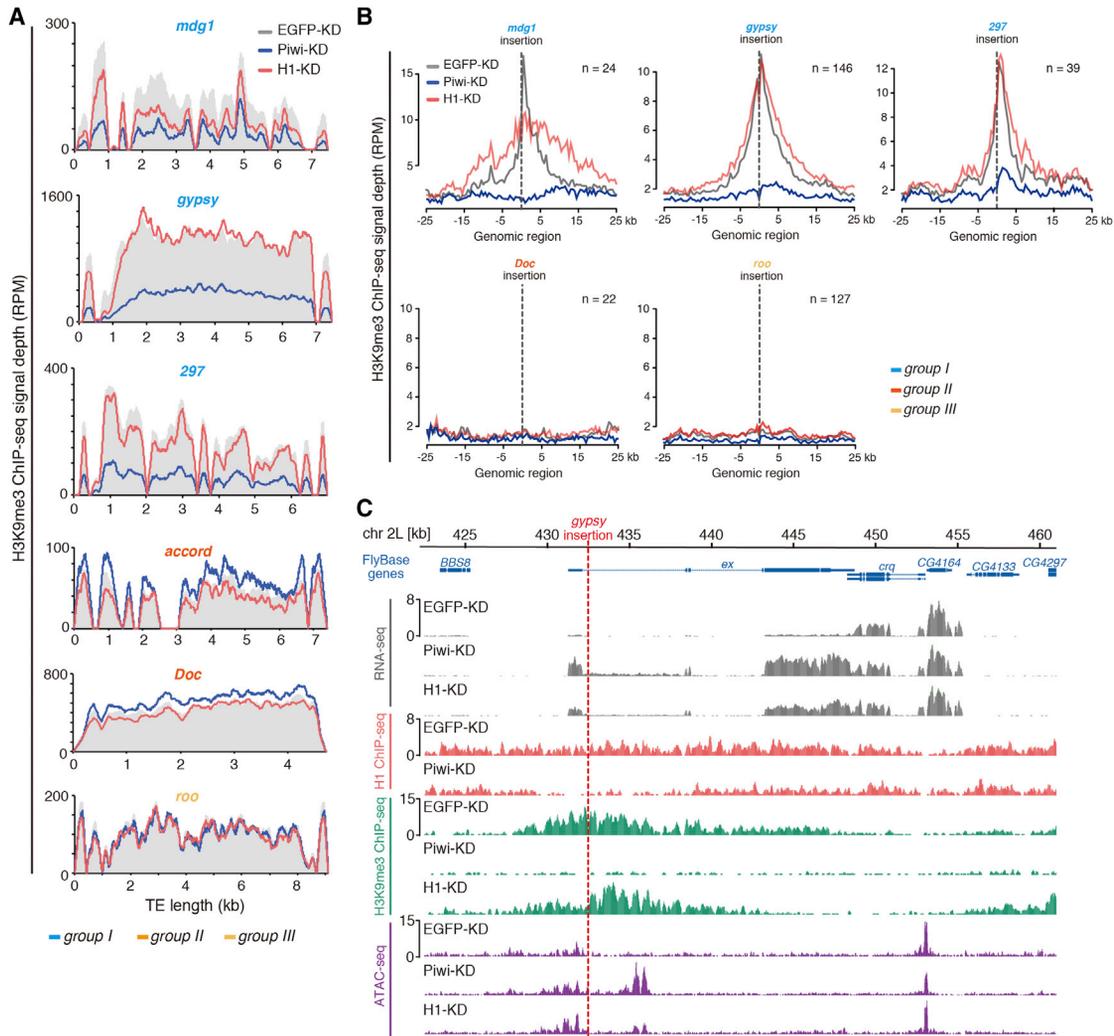


Figure 5. H1 Is Responsible for Transcriptional Silencing and Chromatin Accessibility but Not for H3K9me3 Marks

(A) Density plots for normalized H3K9me3 ChIP-seq signals over consensus sequences from group I, II, and III TEs in EGFP (control)-, Piwi-, or H1-KD cells. ChIP signal depths on EGFP (gray)-, Piwi (blue)-, and H1 (red)-KD samples are shown. x axis of LTR regions are highlighted in red.

(B) Metaplots showing H3K9me3 ChIP-seq signal for genomic regions around euchromatin insertions of group I, II, and III TEs. Normalized ChIP-seq reads in EGFP (gray)-, Piwi (blue)-, and H1 (red)-KD are shown.

(C) RNA levels (RNA-seq signal density), H1 association (H1 ChIP-seq signal density), H3K9me3 association (H3K9me3 ChIP-seq signal density), and chromatin structure (ATAC-seq signal density) at the genomic region flanking the *gypsy* insertion (red dashed line) are shown for OSCs with EGFP-, Piwi-, and H1-KD. FlyBase protein-coding genes are indicated at the top, together with chromosome position.

See also [Figure S5](#).

HP1a-KD on H1 distribution was limited compared to that of Piwi (Figures 3 and S5G–S5J). A similar effect was observed by meta-analysis of genomic regions surrounding euchromatic TE insertions (Figures 6B and S5H). Importantly, we observed a synergistic effect on the derepression of *mdg1* upon H1- and HP1a-KD (Figures 6C and S6B). These data suggest that H1 and HP1a play an interdependent role in the effector step of the Piwi-piRNA pathway.

Distribution of HP1a ChIP-seq signals upon Piwi- and H1-KD (Figures 6A and 6B) was similar to H3K9me3 ChIP-seq signals upon Piwi- or H1-KD (Figures 5A and 5B). Therefore, we studied the relationship between HP1a and H3K9me3 marks. We deter-

mined H3K9me3 peaks using H3K9me3 ChIP-seq data and analyzed the overlap of HP1a and H1 ChIP-seq signals, by displaying HP1a and H1 signals in a metaplot centered on H3K9me3 peak summit. Significant enrichment of HP1a signals was observed at H3K9me3 peaks, suggesting, as expected, that HP1a is probably recruited by H3K9me3 marks (Figure 6D). In contrast, H1 was not accumulated at H3K9me3-enriched sites (Figure 6D). Additionally, as previously reported (Sienski et al., 2012), obvious enrichment of H3K9me3 signals on TE loci and flanking regions of TE insertions was observed. HP1a signal was similarly enriched, whereas the enrichment was not detected for H1 signals (Figure S6C). Combined with data showing

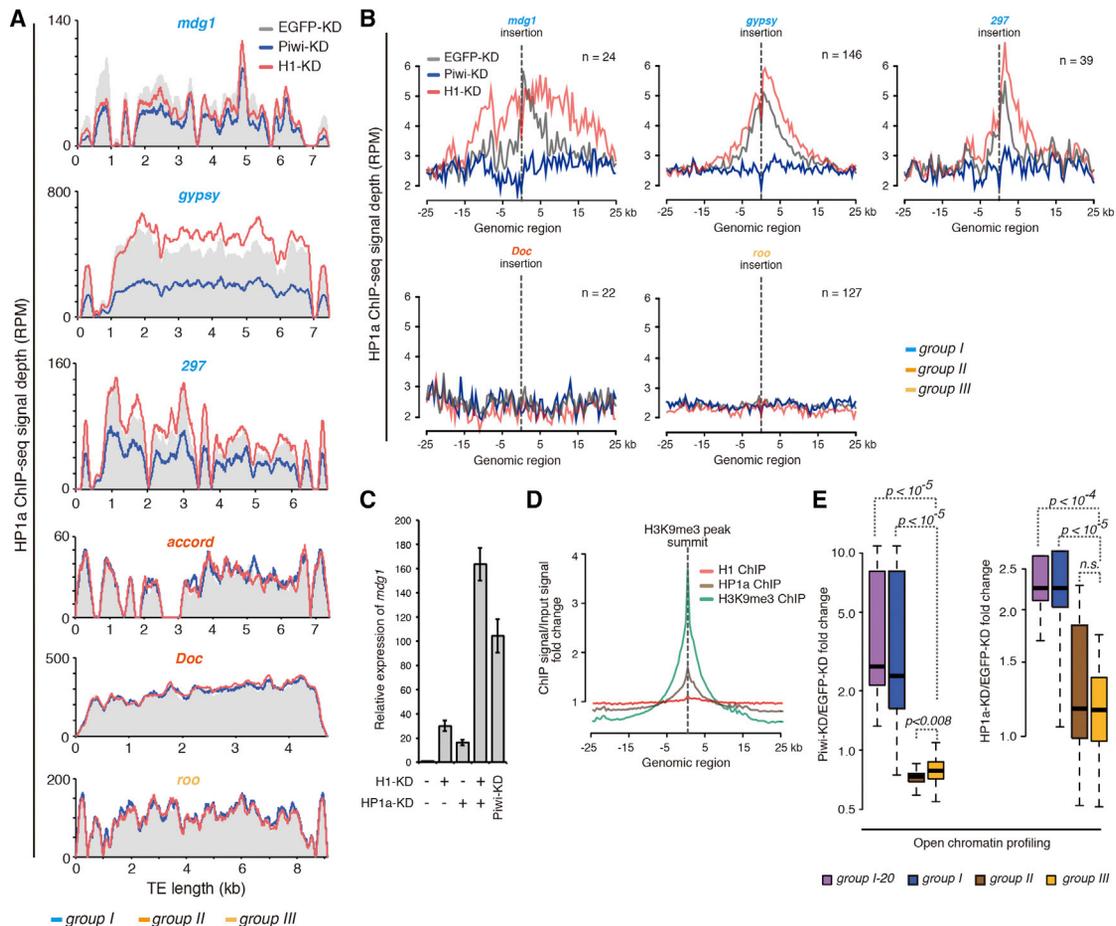


Figure 6. HP1a Functions in Parallel to H1 to Silence Piwi-piRNA Target TEs

(A) Density plots for normalized HP1a ChIP-seq signals over consensus sequences from group I, II, and III TEs in EGFP (control)-, Piwi-, or H1-KD cells. ChIP signal depths on EGFP (gray)-, Piwi (blue)-, and H1 (red)-KD samples are shown.

(B) Metaplots showing HP1a ChIP-seq signal for genomic regions around euchromatin insertions of group I, II, and III TEs. Normalized ChIP-seq reads in EGFP (gray)-, Piwi (blue)-, and H1 (red)-KD are shown.

(C) *mdg1* mRNA levels were quantified by qRT-PCR upon depletion of EGFP, H1, HP1a, and Piwi. Amount of siRNA efficient for limited KD conditions of H1 and HP1a were determined pre-experimentally. Error bars represent mean \pm SEM values of three independent experiments.

(D) Metaplots of H3K9me3, HP1a, and H1 signals normalized to the Input signal for the 50-kb window flanking the H3K9me3 peak summit.

(E) Boxplot, as in Figure 3, showing fold changes in normalized ATAC-seq reads for group I, II, and III TEs upon depletion of Piwi or HP1a. Number of reads shorter than 100 bp were counted.

See also Figures S5 and S6.

independency of HP1a and H1 distributions (Figures 6A–6C and S5G–S5J), our data indicate that the H3K9me3 mark itself may contribute to HP1a association, but not to H1 association with chromatin.

To check the dependency on chromatin accessibility for TE silencing by the Piwi-piRNA pathway, we performed ATAC-seq experiments upon HP1a- and Mael-KD. As for H1, loss of HP1a and Mael resulted in an increase of chromatin accessibility, supporting our hypothesis that compaction of chromatin structure is the final effector of Piwi-piRNA silencing (Figures 6E and S6D–S6J). Depletion of Mael did not alter H1 accumulation (Figures S5C–S5F) or H3K9me3 levels (Sienski et al., 2012), suggesting that Mael functions downstream or in parallel to H1 and H3K9me3 modifications to modify chromatin accessibility.

Depletion of HP1a resulted in shorter ATAC-seq fragments that mapped to group I TEs (Figure S6D), and reads mapped to group I TEs significantly increased upon HP1a-KD ($p < 10^{-5}$) (Figures 6E and S6E). A similar effect was observed for flanking euchromatic regions of group I TE insertion sites (Figure S6F). Thus, HP1a is also indispensable for modification of chromatin accessibility, leading to silencing of TEs in the Piwi-piRNA pathway. As for H1, the impact of HP1a-KD on chromatin accessibility was not as potent as for Piwi-KD (Figures 6E and S6E). Because HP1a couples with H3K9me3 modification and functions in parallel to H1, we propose that Piwi regulates interdependent pathways, mediated by H1 and HP1a, to achieve highly dense chromatin compaction to maintain TE silencing (Figure S6K).

DISCUSSION

Modulation of Chromatin Accessibility by H1 and Piwi-piRNA Pathways

Based on our data, we propose a model where the association of Piwi-piRISCs with H1 enforces the sustained binding of H1 to target TE chromatin, which is probably mediated by the base-pairing of piRNAs with target nascent transcripts (Le Thomas et al., 2013; Post et al., 2014; Rozhkov et al., 2013; Sienski et al., 2012). This then exerts selective transcriptional silencing of TEs mediated by the regulation of chromatin accessibility (Figure S6K). H1 is distributed evenly throughout chromatin with a propensity for paucity at TSSs (Braunschweig et al., 2009), and the distribution of ATAC-seq reads shows clear enrichment at TSSs (Buenrostro et al., 2013). Consistently, our H1 ChIP-seq reads were depleted and ATAC-seq reads were accumulated at TSSs in OSCs (Figure S4C; data not shown). It is conceivable that H1 distributes evenly throughout chromatin and only genes depleted with H1 become transcriptionally active. We thus postulate that Piwi-target TEs may actively remove H1 from their chromatin loci by currently unknown mechanisms to activate their transcription. Indeed, H1 is depleted at target TE loci in Piwi-KD cells (Figure 3). Piwi-piRNA complexes actively recruit H1 to target TE chromatin loci and stabilize its association to modify the chromatin state at the loci to repress TEs.

Although H3K9me3 accumulation on target TE loci coincides with the continual activities of the Piwi-piRNA complexes, our results show that H1 is involved, in parallel with H3K9me3, in Piwi-piRISC-mediated TE silencing. The common feature of H1- and H3K9me3-mediated transcriptional silencing is the compaction of chromatin into higher-order structures. H3K9me3 modification is required for binding of HP1, which initiates heterochromatin formation (Grewal and Jia, 2007). Our data show that HP1a also regulates chromatin accessibility (Figures 6E and S6D–S6F). HP1a slightly affects H1 binding on TE loci (Figures S5G–S5J), suggesting that HP1a may stabilize chromatin formation, thereby maintaining H1 association with piRNA target loci. Furthermore, Mael is also responsible for modifying chromatin accessibility at Piwi target loci (Figures S6G–S6J). Thus, Piwi regulates mutually interdependent pathways that alter chromatin formation, suggesting that each pathway may induce repressive chromatin states to cooperatively reinforce TE silencing. This supports an obligatory functional relationship between H1, HP1a, and Mael as effectors of Piwi-mediated TE silencing.

Although most of the ATAC-seq peaks detected specifically in Piwi/H1-KD samples corresponded to TEs (Figures 4 and S4C), we also detected 84 genes with enriched ATAC-seq peaks in both Piwi- and H1-KD samples. Of these 84 genes, 18 were located near group I TEs. Gene Ontology analysis of the remaining genes (66 genes) suggested enrichment of terms such as “development of primary sexual characteristics” and “sex differentiation” (Figure S4D), which might be useful to study the role of Piwi in germline development.

Molecular Mechanism of TE Silencing in the Piwi-piRNA Pathway

Several important points remain to explain this pathway fully. Piwi was shown to regulate the association of HP1a and H3K9 methyl-

ation at piRNA target locus (Brower-Toland et al., 2007; Le Thomas et al., 2013; Wang and Elgin, 2011). Recently, Panoramix/Silencio was demonstrated to be responsible for the recruitment of H3K9me3 marks in this process (Yu et al., 2015; Sienski et al., 2015). Because our data suggest H1 and HP1a/H3K9me3 function interdependently in the Piwi-piRNA pathway (Figure 6), we speculate that Piwi regulates H1 in parallel to Panoramix/Silencio and H3K9me3, to modulate chromatin accessibility and induces silencing of their target TEs. Further analysis is necessary to reveal the precise relationship between H1 and Panoramix/Silencio.

The concept that H3K9me and HP1 serve as a recruiting platform for various effectors, including HDACs, which, in turn, mediate heterochromatic silencing, is well developed in the *S. pombe* system (Grewal and Jia, 2007). Moreover, loss of HDACs associated with the H3K9me-HP1 platform or defects in RNAi components involved in targeting H3K9me result in increased turnover of nucleosomes and loss of heterochromatic repression (Aygün et al., 2013). Piwi-bound H1 and effectors associated with H3K9me-HP1 might similarly prevent histone turnover to repress TEs. The changes detected in ATAC-seq may reflect changes in histone dynamics. Therefore, analysis of the effects of H1 and Piwi on histone turnover may provide a unifying theme underlying the assembly of repressive chromatin.

Piwi-piRNA complexes in OSCs repress only a subset of TEs (Figure 2A), consistent with previous studies (Le Thomas et al., 2013; Ohtani et al., 2013; Sienski et al., 2012). In line with a previous study (Lu et al., 2013), our data also suggest that H1 regulates TEs other than those targeted by the Piwi-piRNA pathway (Figure 2B), indicating that the overlap between TEs regulated by Piwi and H1 is rather limited. Previously, H1 was shown to regulate TEs ubiquitously, by recruiting H3K9 methyltransferase Su(var)3-9 (Lu et al., 2013). Our results indicated that loss of H1 did not affect H3K9me3 marks on Piwi-piRNA target TEs (Figure 5). Additionally, loss of Su(var)3-9 did not affect *mdg1* expression levels (data not shown), suggesting that piRNA-mediated TE regulation differs from ubiquitous TE regulation by H1, as far as Su(var)3-9 dependency is concerned. We therefore hypothesize that the Piwi-piRNA pathway utilizes the ability of H1 to silence TEs in order to silence their targets.

Overall, we proposed chromatin accessibility functions as the final effector of Piwi-piRNA-mediated TE silencing. Interdependent pathways are responsible for this: (1) recruitment of H1 by Piwi, and (2) H3K9me3-mediated recruitment of HP1a. These pathways coordinately regulate chromatin formation to regulate genome-wide piRNA target TEs.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

OSCs were cultured in Shields and Sang M3 Insect Medium (Sigma) supplemented with 10% fly extract, 10% fetal bovine serum, 0.6 mg/ml glutathione, and 10 mU/ml insulin as described (Niki et al., 2006; Saito et al., 2009). Transfection of siRNAs was performed using Cell Line 96-well Nucleofector Kit SF and 96-well Shuttle Device (Lonza). Details are given in the Supplemental Information.

Production of Anti-H1 Monoclonal Antibody

A 148-amino-acid C-terminal fragment of H1, fused with glutathione S-transferase (GST), was used as the antigen to immunize mice. Details are given in the Supplemental Information.

RNA-seq Analysis

Poly(A)⁺ RNA libraries were prepared as described (Ohtani et al., 2013) and analyzed by Illumina HiSeq (Hokkaido Systems Science). This yielded ~20–25 million genome-mapped reads in each sample transfected with siRNA. Computational analyses are described in Supplemental Information.

ChIP and ChIP-seq Analysis

ChIP was performed as described (Ohtani et al., 2013) with modifications. To prepare ChIP-seq libraries, DNA fragments from the ChIP experiment were sheared to ~200 bases using Covaris S220. These were used for library preparation with the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB) following the manufacturer's protocol. Details are given in the Supplemental Information.

ATAC Sequencing Analysis

ATAC sequencing (ATAC-seq) analysis was performed as described (Buenrostro et al., 2013, 2015) with some modifications. Details are given in the Supplemental Information.

ACCESSION NUMBERS

The accession number for the deep sequencing datasets reported in this paper is NCBI GEO: GSE81434.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2016.06.008>.

AUTHOR CONTRIBUTIONS

Y.W.I., M.C.S., H.S., and K.S. conceived of the project and designed the experiments. Y.W.I., K.M., H.I., and K.S. performed the experiments with the help of A.S. and Y.I. Y.W.I. performed the computational analyses. Y.W.I., M.C.S., H.S., and K.S. analyzed the data and wrote the paper.

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