Current Biology 21, 1373–1379, August 23, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.06.057

Report

piRNA Production Requires Heterochromatin Formation in *Drosophila*

Prashanth Rangan,[1] Colin D. Malone,[1,2] Caryn Navarro,[1,3] Sam P. Newbold,[1] Patrick S. Hayes,[1] Ravi Sachidanandam,[4] Gregory J. Hannon,[2] and Ruth Lehmann[1,*]

[1]Department of Cell Biology, Howard Hughes Medical Institute and Kimmel Center for Biology and Medicine, Skirball Institute, New York University School of Medicine, New York, NY 10016, USA

[2]Watson School of Biological Sciences and Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

[3]Department of Medicine (Genetics Program), Boston University School of Medicine, 72 East Concord Street, Boston, MA 02118, USA

[4]Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029, USA

Summary

Protecting the genome from transposable element (TE) mobilization is critical for germline development. In *Drosophila*, Piwi proteins and their bound small RNAs (piRNAs) provide a potent defense against TE activity. TE targeting piRNAs are processed from TE-dense heterochromatic loci termed piRNA clusters. Although piRNA biogenesis from cluster precursors is beginning to be understood, little is known about piRNA cluster transcriptional regulation. Here, we show that deposition of histone 3 lysine 9 by the methyltransferase *dSETDB1* (*egg*) is required for piRNA cluster transcription. In the absence of *dSETDB1*, cluster precursor transcription collapses in germline and somatic gonadal cells and TEs are activated, resulting in germline loss and a block in germline stem cell differentiation. We propose that heterochromatin protects the germline by activating the piRNA pathway.

Results and Discussion

Germline stem cells (GSCs) are unique in that they self-renew but also generate progeny that differentiate into gametes, which give rise to the next generation. During GSC differentiation, the genome is acutely vulnerable to chromosomal breaks due to either meiotic recombination or transposable element (TE) mobilization [1, 2]. To maintain germline genome integrity, organisms have evolved two distinct pathways: checkpoint monitoring of double-stranded DNA breaks, and transposon suppression via a specialized RNA interference (RNAi) system involving Piwi proteins and their bound small RNAs [3, 4]. There are two RNAi-based systems that utilize small RNAs to target TEs for degradation: the Piwi-interacting small RNA (piRNA) pathway, and the endogenous small interfering RNA (endo-siRNA) pathway. piRNAs act as the primary defense in the germline [5], whereas siRNAs act throughout the organism [6, 7]. In germline cells of the *Drosophila* gonad, piRNAs are bound by three Piwi-clade Argonaute proteins: Piwi, Aubergine (Aub), and Argonaute 3 (AGO3). Piwi and Aub bind piRNAs complementary to active TEs and target them for degradation. This cleavage leads to the production of new piRNAs, which are loaded into AGO3 and target cleavage of antisense transposon transcripts, which produces more small RNAs for Aub and Piwi to bind. These antisense transcripts are derived from piRNA clusters, which typically reside in subtelomeric and pericentric heterochromatin regions and contain a high density of fragmented, ancient transposon copies [8, 9]. TEs are also silenced by piRNAs in somatic tissues of the gonad where only Piwi is expressed [10, 11]. Although piRNA clusters predominantly produce piRNAs, many also produce significant levels of endo-siRNAs [12, 13], which are processed via a genetically independent machinery. How these transcripts are generated from piRNA clusters is unclear.

To determine whether heterochromatin formation and integrity may play a role in TE regulation during oogenesis, we examined the distribution of trimethylated histone 3 lysine 9 (H3K9me3). This prototypic repressive mark initiates heterochromatin formation by recruiting heterochromatin protein 1 (HP1) (Figure 1A) [14]. We focused on the GSCs and their niche located at the anterior tip of the ovary in a region called the germarium (Figure 1B) [15, 16]. During GSC division, one daughter cell maintains contact with the niche and continues to divide as a stem cell while the other differentiates. As the differentiating daughter cell (precystoblast) moves away from the niche, it initiates expression of the differentiation factor Bag-of-marbles (Bam) (Figure 1B) [17, 18]. Subsequently, this daughter, now called the cystoblast, undergoes four mitotic divisions to form an interconnected 16-cell germline cyst, producing one oocyte and 15 supporting nurse cells (Figure 1B) [15, 16]. We observed that although H3K9me3 signal was low in GSCs, as soon as the differentiating daughter left the niche, we could detect prominent, discrete nuclear H3K9me3-positive foci (Figure 1C). Once established, these H3K9me3 foci, which typically associated with DAPI-dense regions, persisted throughout oogenesis (see Figures S1A–S1B2 available online). We termed these foci "*r*epressive *c*hromatin *c*enters" (RCCs). RCCs possess heterochromatic character in that they contain not only H3K9me3 but also other heterochromatin markers such as HP1 and trimethylated histone 4 lysine 20 (H4K20me3) (Figure 1D) [14, 19, 20].

To correlate H3K9me3 with heterochromatic regions in vivo, we asked whether RCCs are located in proximity to centromeric and telomeric regions. Centromeres can be visualized by immunohistology of CENP-A H3-like proteins (CID) [21], and the telomeric caps by HP1 origin recognition complex subunit 2 (HOAP) [22]. When we stained for RCCs, by H3K9me3 or H4K20me3, we found a partial overlap with HOAP and a close association with CID in germline cells throughout oogenesis (Figures 1E and 1F). Repressive foci similar to RCCs were also present in the somatic cells that surround and associate with germline cells throughout oogenesis (Figures S1B–S1B2). This suggests that telomeres, centromeres, and their adjacent regions are organized into large heterochromatic structures in the gonads (Figure 1G).

*Correspondence: ruth.lehmann@med.nyu.edu

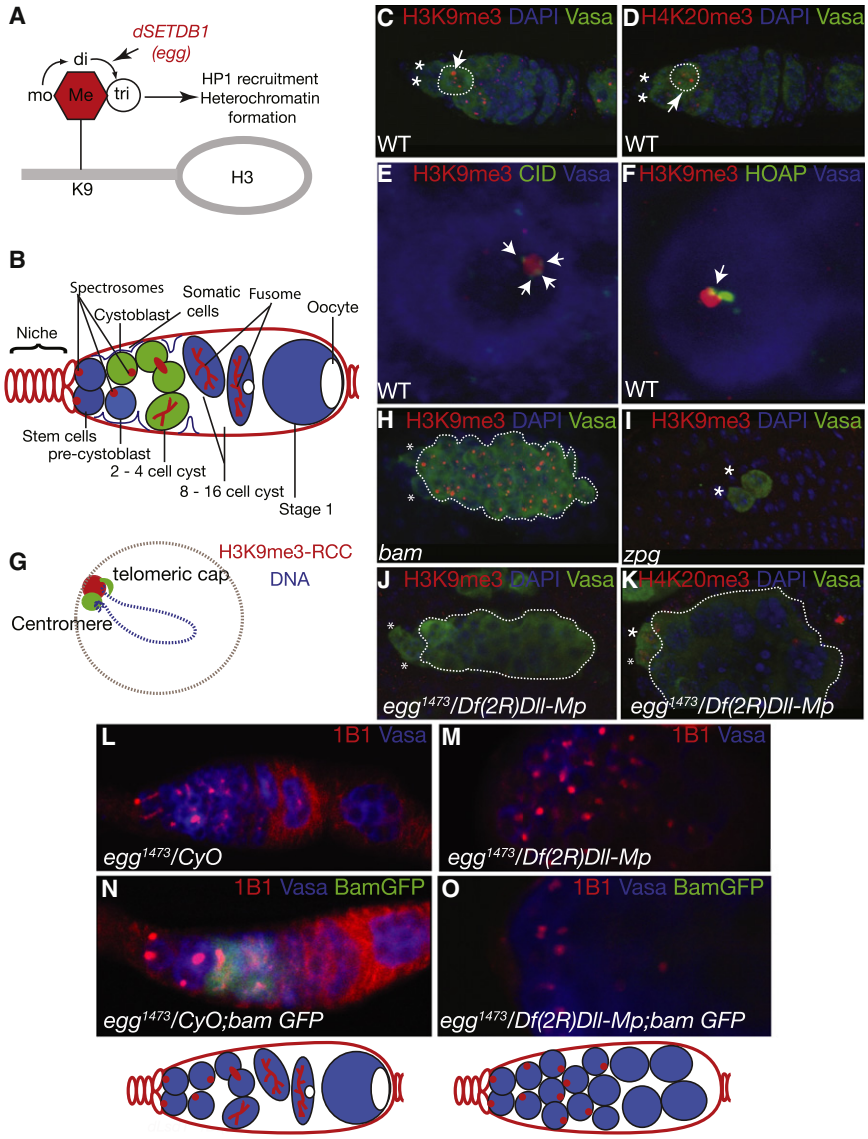

Figure 1. dSETDB1 Mediates Formation of Heterochromatic Repressive Chromatin Centers in the Precystoblast and Is Required for Germline Stem Cell Differentiation

(A) Schematic of *dSETDB1* function.

(B) Schematic of the germline stem cell (GSC) niche.

(C and D) Germaria of wild-type (WT) marked by Vasa (green), DAPI (blue), and anti-H3K9me3 (red) (C) anti-H4K20me3 (red) (D) showing discrete foci in the germline (white arrow). White asterisks mark GSCs; dotted white line marks the precystoblasts. Wild-type precystoblasts contain 3.6 ± 0.4 nuclear foci with one large focus (n = 15).

(E and F) Repressive chromatin centers (RCCs) associate with centromere marker CID (green) (E) (n = 20) and the telomeric cap marker HOAP (F) (n = 80), showing the proximity of these regions to RCCs.

(G) Schematic of a RCC (red), showing large heterochromatic domain associated with centromeric and telomeric regions (green).

(H and I) Germaria of *bam* (H) and *zpg* (I) mutants stained for anti-Vasa (green), anti-H3K9me3 (red), and DAPI (blue) show that GSCs lack prominent RCCs.

(J and K) egg^{1473}/*Df(2R)Dll-Mp* mutant germaria stained for anti-Vasa (green) and DAPI (blue) lack RCCs detected by anti-H3K9me3 (J) and anti-H4K20me3 (K).

(L and M) Germaria of egg^{1473}/CyO (L) and egg^{1473}/*Df(2R)Dll-Mp* (M) stained for anti-Vasa (blue) and anti-1B1 (red), which marks the spectrosomes, show that *dSETDB1* mutants accumulate undifferentiated cells (2.7 ± 0.8, n = 20 and 7.9 ± 3.5, n = 46, respectively).

(N and O) Germaria of egg^{1473}/CyO (N) and egg^{1473}/*Df(2R)Dll* (O) that also carry a Bam:GFP transgene stained for anti-1B1 (red), anti-Vasa (blue), and anti-GFP (green) show that *dSETDB1* mutant germaria do not express Bam GFP. Bottom schematics summarize confocal images above.

See also Figure S1

To more precisely determine the developmental sequence of RCC formation, we analyzed two genetic mutants: a GSC differentiation-defective mutant, *bam*, and a mutant that lacks differentiated progeny, *zero population growth* (*zpg*) [17, 18, 23]. In the wild-type, GSCs are maintained by Decapentaplegic (DPP), a *Drosophila* BMP2/4 ortholog secreted from the niche [24]. Activation of the Dpp receptor Thickveins (Tkv) directly represses transcription of *bam* [25]. In *bam* mutants, single undifferentiated germ cells accumulate into a tumorous mass. Based on the expression of Dpp reporters, only those germ cells closest to the niche, the GSCs, respond to the Dpp signal, whereas the precystoblasts do not [26, 27]. Consistent with our observations in the wild-type ovary, in *bam* mutant ovaries we detected low H3K9me3 signal in a few cells that corresponded to GSCs as judged by their proximity to the niche, whereas the tumor composed of precystoblasts displayed prominent RCCs (Figure 1H). In *zpg* mutant germaria, precystoblasts die upon completing cell division, leaving only GSCs [17, 23]. Again, we observed that GSCs in *zpg* mutant germ cells showed little H3K9me3 staining (Figure 1I). We conclude that prominent heterochromatin marks are established in the germline as GSCs differentiate.

We next asked whether mutants in *Drosophila* H3K9 methyltransferase affect RCC formation and GSC differentiation. We chose to study *dSETDB1* (*egg*), one of two *Drosophila* H3K9 methyltransferases that had previously been shown to be required for deposition of H3K9me3 in the germarium and for female fertility [14, 28]. We found that H3K9me3 and H4K20me3 were absent in *dSETDB1* mutants (Figures 1J and 1K) [14]. To determine whether *dSETDB1* is required for the GSC-to-cystoblast transition, we stained wild-type and *dSETDB1* mutant ovaries with antibodies against the germ cell marker Vasa and the spectrosome marker 1B1. Spectrosomes are present in GSCs, the intermediate precystoblast, and the differentiating cystoblast (Figure 1B) [29]. 1B1 also stains the fusome, a branched organelle found in differentiating multicellular cysts (Figure 1B) [29]. *dSETDB1* mutant ovaries contained an increased number of spectrosome-positive cells compared to wild-type (Figures 1L and 1M) and failed to develop fusome-containing cysts (Figure 1M). The undifferentiated cells in *dSETDB1* mutants did not express the

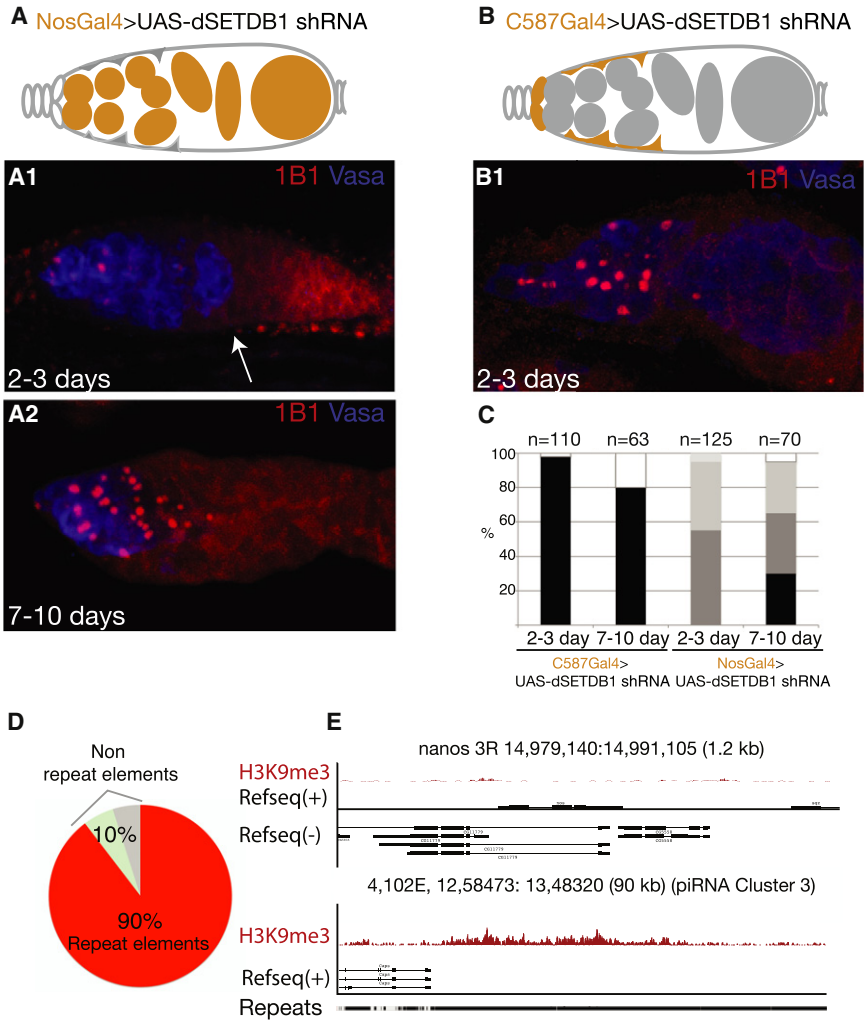

Figure 2. dSETDB1 Is Required in Both Soma and Germline, and piRNA Clusters Carry H3K9me3 Marks

(A) Schematic of expression pattern of *nos-Gal4* in the germarium.

(A1) Germaria after germline-specific *dSETDB1* knockdown show a block in differentiation at the cyst stage after 2–3 days (white arrow).

(A2) After 7–10 days, germaria show an accumulation of undifferentiated cells monitored by spectrosomes (red).

(B) Schematic of expression pattern of *c587-Gal4* in the germarium.

(B1) Germaria after soma-specific *dSETDB1* knockdown show an accumulation of undifferentiated cells as soon as 2–3 days after eclosion.

(C) Quantification of the phenotypes at 2–3 days and 7–10 days in soma- and germline-specific knockdown of *dSETDB1*: germaria containing tumors (black), germaria degenerating after 16-cell cyst stage (dark gray), germaria degenerating before stage 6 (light gray), and ovarioles that had lost stem cells (white).

(D) Distribution of repressive marks on various repeat elements (red) expressed as a percentage of total H3K9me3 marks (all mappers).

(E) Representative examples of the distribution of repressive marks showing enrichment of repressive marks on the fourth chromosome telomeric piRNA cluster (bottom panel) compared to *nanos* (top panel). Repeat elements as annotated by repeat master are shown as black lines.

See also Figure S2.

differentiation marker Bam but did express phosphorylated Mad (pMad), the mediator of the Dpp signal and a marker of GSC in the wild-type (Figures 1N and 1O; Figures S1C–S1F), suggesting an accumulation of "GSC-like" cells [26, 27]. To determine whether these GSC-like cells were capable of differentiating, we forced expression of Bam under the control of a heat-shock promoter, and we observed the development of multicellular cysts as revealed by the formation of fusomes (Figures S1G and S1H). The accumulation of GSC-like cells was not due to change in niche size or increased GSC division (Figures S1I and S1J) [28]. We conclude that *dSETDB1* is required for Bam-dependent differentiation of GSCs.

Because we observed repressive marks in both the soma and the germline, we wanted to determine where *dSETDB1* is required for GSC differentiation. We performed tissue-specific knockdown of *dSETDB1* using an inducible short hairpin RNA (shRNA) [30]. We crossed flies carrying shRNA-dSETDB1 under the control of the GAL4-responsive UAS promoter with flies carrying the germline-specific *nos-Gal4::VP16* (Figure 2A) [31] or *C587-Gal4*, which drives GAL4 in the somatic inner sheath cells that intermingle with germ cells (Figure 2B) [32]. We found that reducing dSETDB1 levels in either tissue resulted in females that laid few eggs (Figure S2A; Table S1). To determine the cause of this ovarian defect, we stained for Vasa and 1B1. For the germline knockdown, we observed an age-dependent increase in the phenotype, as has been observed with other shRNAs using germline-specific drivers [30]. In 2- to 3-day-old flies, GSCs were present and we observed a few differentiating cysts, most of which degenerated, leaving only a few mature egg chambers (Figures 2A1–2C), which frequently developed into eggs with dorsoventral patterning defects (spindle phenotype) (Table S1). In 7- to 10-day-old flies, undifferentiated germ cells accumulated as seen in the genetic mutant (Figures 2A2–2C). For the somatic knockdown, we observed an age-independent accumulation of undifferentiated cells resembling the *dSETDB1* mutant phenotype (Figures 2B1 and 2C). Based on these results, we conclude that *dSETDB1* expression in both germline and germarial somatic cells is required for oogenesis and differentiation.

To gain more insight into the germline and somatic function of dSETDB1, we followed RCC formation using the heterochromatin marker H4K20me3 [19, 33]. In the few late-stage egg chambers that formed in 2- to 3-day-old flies from the germline *dSETDB1* knockdown, we observed a loss of H4K20me3 staining specifically in the germline but no change in the soma, indicating that loss of germline *dSETDB1* does not affect H4K20me3 foci in somatic cells (Figures S2B–S2D2). However, in the somatic knockdown (Figures S2E–S2E2), we observed a loss of repressive marks in both the germline and soma, indicating that the function of dSETDB1 in the soma affects germline RCC formation, possibly by blocking GSC differentiation. Thus, *dSETDB1* acts to regulate heterochromatin formation in the germline directly and indirectly through its somatic function.

To identify genomic targets of *dSETDB1* in oogenesis, we profiled genome-wide H3K9me3 occupancy by chromatin immunoprecipitation combined with massively parallel DNA sequencing (ChIP-seq) in wild-type ovaries. We used the entire ovary because RCCs, once formed, are stably maintained throughout oogenesis. After chromatin immunoprecipitation with H3K9me3 antibody, the recovered DNA was cloned, sequenced, and mapped to the *Drosophila* genome. H3K9me3 was highly enriched at transposons and other repetitive loci (~90%) (Figure 2D). Because of the repetitive nature of transposable elements, we analyzed uniquely mapping sequence reads to the genome and compared these to the actively transcribed germline gene *nanos* as a negative control. We found that piRNA clusters, which are mostly present in the pericentric or subtelomeric heterochromatin, but not actively transcribed genes such as *nanos*, were marked by H3K9me3 (Figure 2E; Figures S3A–S3C).

To determine how H3K9 methylation affects piRNA production, we cloned and sequenced small RNAs from *dSETDB1* mutant ovaries, which lack RCCs, and compared them to *bam* mutants, which display a morphologically similar phenotype but form prominent RCCs, as a control (Figures 1H–1K). To account for differences in sequencing quality and depth, we normalized small RNAs to gene-derived, antisense-mapping endo-siRNAs, similar to previously published analyses [10]. piRNA (23–29 nt) levels were significantly reduced in *dSETDB1* mutant ovaries (Figures 3A–3C), along with endo-siRNAs derived from major germline and somatic piRNA clusters (Figure 3D). This loss of endo-siRNAs is specific to piRNA clusters, because production from a euchromatic endo-siRNA-producing locus, *esi-2* [12, 13, 34], which is also marked by H3K9me3, increased in *dSETDB1* mutants (Figures S3D and S3E). This increase in *esi-2* endo-siRNAs is likely due to the loss of silencing chromatin marks at this locus, allowing for increased transcription and, likewise, increased siRNA production. This is in contrast to the heterochromatic piRNA clusters, which show a dramatic loss of both siRNAs and piRNAs from all clusters examined, suggesting a direct defect in cluster transcription rather than an effect on downstream transcript processing by either the siRNA or piRNA machinery. Therefore, we sought to directly test the effect of *dSETDB1* loss on piRNA cluster transcription.

To determine whether the loss of piRNAs in *dSETDB1* mutants is due to a loss of precursor transcription, we carried out strand-specific qPCR to measure transcript levels from the germline-specific 42AB cluster, the germline- and soma-expressed cluster 2, and the soma-specific *flamenco* cluster [35]. The 42AB cluster contains a mixture of sense- and antisense-oriented transposon fragments that requires bidirectional transcription to ensure antisense transposon transcript production for the piRNA pathway [8]. We found that production of at least one strand of the 42AB cluster was affected in *dSETDB1* mutants compared to *bam* mutants. In cluster 2 and *flamenco*, which contain transposon fragments oriented in the same direction, unidirectional transcription is sufficient to generate antisense transposon precursors [8]. We also observed diminished transcription in *dSETDB1* mutants compared to *bam* mutants (Figure 3E). qPCR measuring native transcript levels in *bam* mutants probably underrepresents wild-type levels of cluster transcripts because they are processed by the active piRNA and siRNA pathways. Thus, *dSETDB1* is required for transcription of both bi- and unidirectionally transcribed clusters, in both germline and somatic tissues of the gonad. This is in stark contrast to what is seen for the HP1 homolog, *Rhino*, which is selectively required for the transcription of only bidirectional clusters, and specifically so in germline cells [35]. Therefore, we have identified a unifying mark dictating piRNA cluster transcription prior to piRNA production in germline and somatic cells. Our results are consistent with Rhino recognizing H3K9me3 at bidirectional germline clusters, but there must then be an alternative Rhino-independent mechanism directing somatic and unidirectional germline cluster transcription. These results strongly suggest that repressive marks deposited by dSETDB1 are required for transcription from all major piRNA clusters, although how these marks are targeted to piRNA clusters remains unknown.

Finally, we wanted to assess whether loss of piRNA function in *dSETDB1* mutants also results in increased TE levels, as has been shown for piRNA pathway mutants involved in posttranscriptional piRNA processing. We assessed TE levels in *dSETDB1* mutant ovaries, again using *bam* mutants as a control. In *dSETDB1* mutants, we found higher levels of both germline (HeT-A and TART) and somatic (gypsy and ZAM)-expressed transposons by qPCR of steady-state transcript levels (Figure 3F). To determine whether dSETDB1-dependent H3K9me3 protects germline and somatic cells separately, we knocked down *dSETDB1* in either germline or somatic cells of the ovary as before. As expected, germline knockdown led to the preferential derepression of germline transposons, whereas somatic knockdown resulted in the selective derepression of somatic transposons (Figure 3G). Therefore, *dSETDB1* plays a critical role in silencing transposons in both the germline and somatic cells of the ovary.

Our results indicate that *dSETDB1* is required for transposon control in the germline and ovarian soma by positively regulating piRNA cluster transcription through the deposition of H3K9me3. To test directly whether transposon derepression in either the germline or soma is sufficient to cause a block in GSC differentiation, we utilized two models of hybrid dysgenesis. First, we used the germline-specific *P* element model of hybrid dysgenesis, which results in derepression of *P* element DNA transposons and progeny sterility when a male carrying a copy of an active *P* element transposon (*Harwich*) is crossed to a female devoid of *P* elements (*w1118*) [36, 37]. This is attributed to the absence of maternally supplied piRNAs capable of silencing this transposon [38]. Second, to test for somatic derepression, we used *flamenco* mutant lines, which ablate production from the primary somatic piRNA cluster, *flamenco*, resulting in derepression of gypsy-family transposable elements [39, 40]. We found that in both cases, germaria accumulated undifferentiated cells similar to those observed in *dSETDB1* mutants (Figures 4A, 4B, and 4D). As with *dSETDB1* mutants, these undifferentiated cells did not express Bam but did stain positive for pMad (Figures 4A and 4D–4D2). These results indicate that transposon mobilization alone in either germline or soma is sufficient to cause a block in GSC differentiation. Additionally, we observed that the loss of GSC differentiation during *P* element dysgenesis could be relieved by removing the *Chk-2* kinase, suggesting that transposon derepression activates a double-stranded DNA break checkpoint (Figures 4B and 4C) [1]. Interestingly, it is known that through viral packaging, some gypsy-family elements maintain the ability to infect germline cells from the surrounding soma [39, 41], leaving open the possibility that the GSC differentiation block in *flamenco* mutants is due to gypsy invasion and mobilization within differentiating germline cells. Alternatively, loss

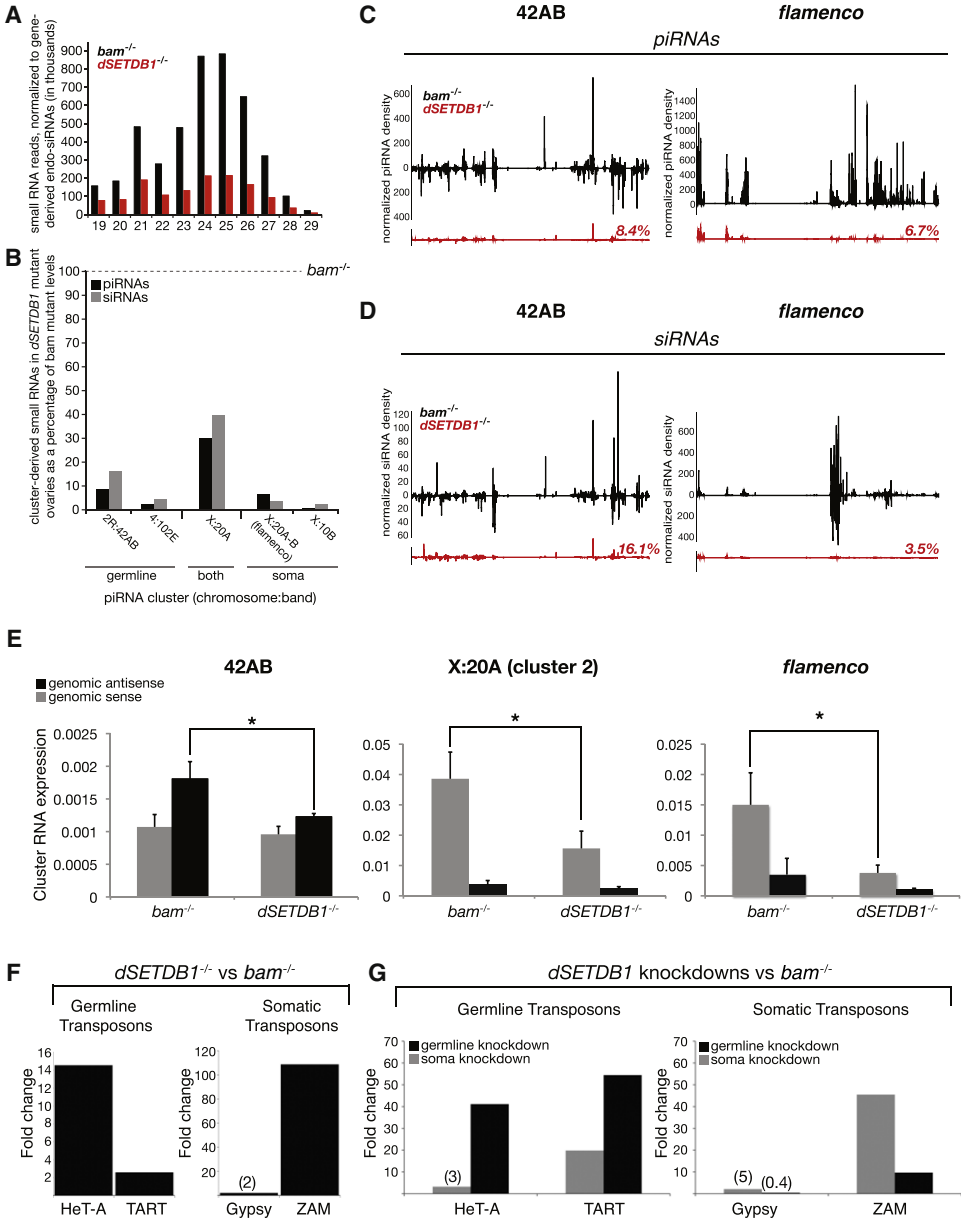

Figure 3. dSETDB1 Is Required for piRNA Production and Transposon Control

(A) Size profiles of small RNA populations in *bam* (black) and *dSETDB1* (red) mutant ovaries. Sequence reads were normalized to number of gene-derived, antisense-mapping endo-siRNAs.

(B) Quantification, via bar graph, showing the log2-fold changes in total normalized, uniquely mapping cluster-derived small RNA (piRNA + siRNA) levels, comparing *dSETDB1* to *bam* mutant levels.

(C) piRNAs (23–29 nt) that can be assigned to a unique genomic location, normalized to antisense, genic endo-siRNAs, from *bam* (black) and *dSETDB1* (red) mutant ovaries, plotted across 42AB and *flamenco* piRNA clusters. Reads above the x axis indicate mapping sense to the genomic sequence; reads below indicate antisense mapping.

(D) Endo-siRNAs that can be assigned to a unique genomic location, normalized to antisense, genic endo-siRNAs, from *bam* (black) and *dSETDB1* (red) mutant ovaries, plotted across 42AB and *flamenco* piRNA clusters.

(E) qPCR measuring transcripts from genomic sense (gray) and genomic antisense (black) in *dSETDB1* mutants and *bam* mutants, showing reduction of antisense transcripts from the 42AB cluster and sense transcripts from cluster 2 and *flamenco* in *dSETDB1* mutants.

(F) Germline transposons (*HeT-A*, *TART*) and somatic transposons (*gypsy*, *Zam*) measured by qPCR are specifically upregulated in *dSETDB1* mutants relative to *bam* mutant.

(G) Germline transposons (*HeT-A*, *TART*) and somatic transposons (*gypsy*, *Zam*) measured by qPCR are preferentially upregulated in the germline and somatic knockdowns, respectively.

All qPCR experiments were carried out in triplicate; error bars represent standard error. See also Figure S3.

of piRNA production from the *flamenco* locus, as in *piwi* mutants, could result in the loss of somatic cells that surround germ cells and provide cues for differentiation. Thus, transposon upregulation in the germline and soma in *dSETDB1* mutants is sufficient to cause a loss of differentiation phenotype.

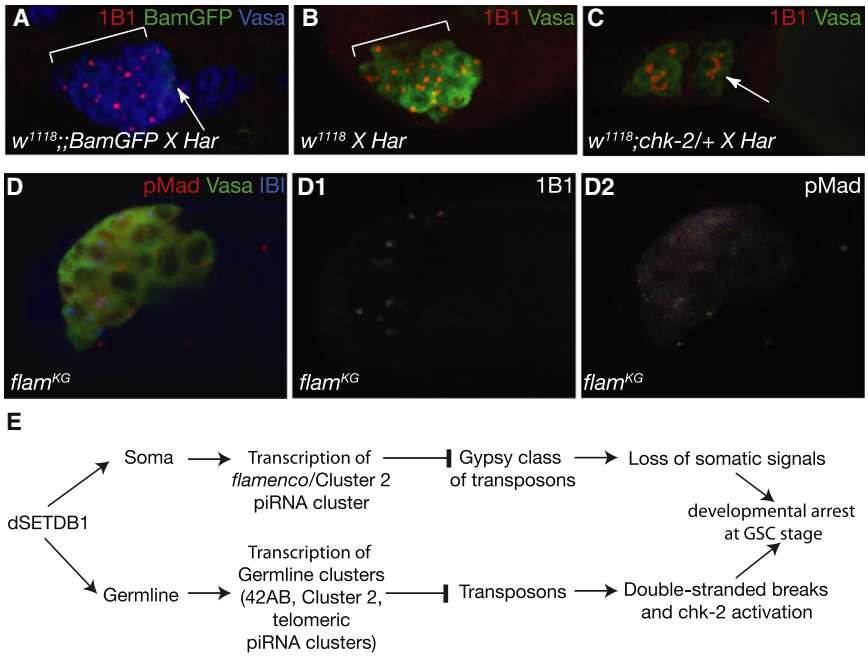

Figure 4. Upregulation of Transposons Is Sufficient to Cause a Block in Differentiation

(A) Germaria of progeny of *Harwich* males crossed to w^{1118} females carrying the *bam-GFP* transgene stained for anti-Vasa (blue), anti-1B1 (red), and anti-GFP (green) accumulate cells with spectrosomes (red) that do not express Bam (white bracket). Faint Bam-GFP expression is seen at the edge of these tumors (white arrow).

(B) Germaria of progeny of *Harwich* males crossed to w^{1118} females stained for anti-Vasa (green) and anti-1B1 (red) accumulate undifferentiated cells (white bracket).

(C) Removing a copy of *chk-2* from this cross partially rescues the block in differentiation, with fusomes (white arrow) connecting differentiating cyst cells.

(D–D2) Germaria of *flamenco* mutants stained for anti-1B1 (blue), anti-Vasa (green), and anti-pMad (red), showing an accumulation of "GSC-like" undifferentiated cells. (D1) and (D2) are 1B1 and pMad channels, respectively.

(E) Schematic of dSETDB1 regulating piRNA production from both somatic and germline clusters, which in turn suppress transposon expression. Transposon expression either activates a checkpoint in the germline or leads to loss of some somatic cells, leading to a block in GSC differentiation.

From *Drosophila* to humans, a large fraction of eukaryotic genomes contain transposable elements. Here, we find a novel role for *dSETDB1*-mediated heterochromatin formation in activating transcription of piRNA clusters and thus triggering piRNA-based control of transposon regulation (Figure 4E). Interestingly, this transcriptional upregulation of the germline piRNA pathway happens at the time when transcription is generally upregulated in the germline to permit differentiation [42], potentially also leading to increases in transposon transcription. Thus, production of piRNAs needed to keep transposon activity in check is timed to occur when they are likely most needed to protect the integrity of the genome of the next generation.

Accession Numbers

Small RNA libraries have been deposited at the NCBI Gene Expression Omnibus with the accession number GSE30086 (data sets GSM744619 and GSM744620). ChIP-Seq data have been deposited at the NCBI Gene Expression Omnibus with the accession number GSE30157 (data set GSM746836).

Supplemental Information

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.06.057.

Acknowledgments

We are particularly grateful to all members of the Lehmann laboratory for discussion and extensive comments on the manuscript, in particular Daria Siekhaus, Allison Blum, Saskia Houwing, and Thomas Hurd for comments and discussion. We also thank Alexander Stark for computational assistance. We thank the Transgenic RNAi Resource Project at Harvard Medical School (NIH/NIGMS grant R01-GM084947) for providing transgenic RNAi fly stocks used in this study. We thank Tulle Hazelrigg (*egg*) as well as the Bloomington Drosophila Stock Center and FlyBase for sharing fly stocks and antibodies. We thank Dan Vasiliauskas, Susan Morton, Tom Jessell, and Ed Laufer for the pMad antibody. P.R. is an Howard Hughes Medical Institute (HHMI) Research Associate. R.L. is an HHMI Investigator and a member of the Kimmel Center for Stem Cell Biology at NYU Langone Medical Center. G.J.H. is an HHMI Investigator and is funded in part by grants from the National Institutes of Health and a kind gift from Kathryn W. Davis.

Received: May 12, 2011
Revised: June 24, 2011
Accepted: June 28, 2011
Published online: August 4, 2011

References

1. Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., Cook, H.A., and Theurkauf, W.E. (2007). Drosophila rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev. Cell *12*, 45–55.
2. Theurkauf, W.E., Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., and Cook, H.A. (2006). rasiRNAs, DNA damage, and embryonic axis specification. Cold Spring Harb. Symp. Quant. Biol. *71*, 171–180.
3. Ghabrial, A., and Schüpbach, T. (1999). Activation of a meiotic checkpoint regulates translation of Gurken during Drosophila oogenesis. Nat. Cell Biol. *1*, 354–357.
4. Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. Cell *136*, 656–668.
5. Aravin, A.A., Hannon, G.J., and Brennecke, J. (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science *318*, 761–764.
6. Williams, R.W., and Rubin, G.M. (2002). ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. Proc. Natl. Acad. Sci. USA *99*, 6889–6894.
7. Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: An expanding universe. Nat. Rev. Genet. *10*, 94–108.
8. Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell *128*, 1089–1103.
9. Gunawardane, L.S., Saito, K., Nishida, K.M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., and Siomi, M.C. (2007). A Slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. Science *315*, 1587–1590.

10. Malone, C.D., Brennecke, J., Dus, M., Stark, A., McCombie, W.R., Sachidanandam, R., and Hannon, G.J. (2009). Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary. Cell *137*, 522–535.
11. Li, C., Vagin, V.V., Lee, S., Xu, J., Ma, S., Xi, H., Seitz, H., Horwich, M.D., Syrzycka, M., Honda, B.M., et al. (2009). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell *137*, 509–521.
12. Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L., Zapp, M.L., Weng, Z., and Zamore, P.D. (2008). Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science *320*, 1077–1081.
13. Czech, B., Malone, C.D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., Perrimon, N., Kellis, M., Wohlschlegel, J.A., Sachidanandam, R., et al. (2008). An endogenous small interfering RNA pathway in Drosophila. Nature *453*, 798–802.
14. Yoon, J., Lee, K.S., Park, J.S., Yu, K., Paik, S.G., and Kang, Y.K. (2008). dSETDB1 and SU(VAR)3-9 sequentially function during germline-stem cell differentiation in Drosophila melanogaster. PLoS ONE *3*, e2234.
15. Spradling, A.C. (1993). Developmental Genetics of Oogenesis, *Volume 1* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
16. Gilboa, L., and Lehmann, R. (2004). How different is Venus from Mars? The genetics of germ-line stem cells in Drosophila females and males. Development *131*, 4895–4905.
17. Gilboa, L., Forbes, A., Tazuke, S.I., Fuller, M.T., and Lehmann, R. (2003). Germ line stem cell differentiation in Drosophila requires gap junctions and proceeds via an intermediate state. Development *130*, 6625–6634.
18. McKearin, D., and Ohlstein, B. (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. Development *121*, 2937–2947.
19. Ebert, A., Lein, S., Schotta, G., and Reuter, G. (2006). Histone modification and the control of heterochromatic gene silencing in Drosophila. Chromosome Res. *14*, 377–392.
20. Schotta, G., Ebert, A., and Reuter, G. (2003). SU(VAR)3-9 is a conserved key function in heterochromatic gene silencing. Genetica *117*, 149–158.
21. Blower, M.D., and Karpen, G.H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat. Cell Biol. *3*, 730–739.
22. Cenci, G., Siriaco, G., Raffa, G.D., Kellum, R., and Gatti, M. (2003). The Drosophila HOAP protein is required for telomere capping. Nat. Cell Biol. *5*, 82–84.
23. Tazuke, S.I., Schulz, C., Gilboa, L., Fogarty, M., Mahowald, A.P., Guichet, A., Ephrussi, A., Wood, C.G., Lehmann, R., and Fuller, M.T. (2002). A germline-specific gap junction protein required for survival of differentiating early germ cells. Development *129*, 2529–2539.
24. Xie, T., and Spradling, A.C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell *94*, 251–260.
25. Chen, D., and McKearin, D. (2003). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Curr. Biol. *13*, 1786–1791.
26. Kai, T., and Spradling, A. (2003). An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. Proc. Natl. Acad. Sci. USA *100*, 4633–4638.
27. Casanueva, M.O., and Ferguson, E.L. (2004). Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signaling. Development *131*, 1881–1890.
28. Clough, E., Moon, W., Wang, S., Smith, K., and Hazelrigg, T. (2007). Histone methylation is required for oogenesis in Drosophila. Development *134*, 157–165.
29. de Cuevas, M., and Spradling, A.C. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development *125*, 2781–2789.
30. Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., et al. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat. Methods *8*, 405–407.
31. Van Doren, M., Williamson, A.L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Curr. Biol. *8*, 243–246.
32. Zhu, C.H., and Xie, T. (2003). Clonal expansion of ovarian germline stem cells during niche formation in Drosophila. Development *130*, 2579–2588.
33. Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R., and Reuter, G. (2002). Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. EMBO J. *21*, 1121–1131.
34. Okamura, K., Chung, W.J., Ruby, J.G., Guo, H., Bartel, D.P., and Lai, E.C. (2008). The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs. Nature *453*, 803–806.
35. Klattenhoff, C., Xi, H., Li, C., Lee, S., Xu, J., Khurana, J.S., Zhang, F., Schultz, N., Koppetsch, B.S., Nowosielska, A., et al. (2009). The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell *138*, 1137–1149.
36. Kidwell, M.G., Kidwell, J.F., and Sved, J.A. (1977). Hybrid dysgenesis in Drosophila melanogaster: A syndrome of aberrant traits including mutation, sterility and male recombination. Genetics *86*, 813–833.
37. Castro, J.P., and Carareto, C.M. (2004). Drosophila melanogaster P transposable elements: mechanisms of transposition and regulation. Genetica *121*, 107–118.
38. Brennecke, J., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A., and Hannon, G.J. (2008). An epigenetic role for maternally inherited piRNAs in transposon silencing. Science *322*, 1387–1392.
39. Lécher, P., Bucheton, A., and Pélisson, A. (1997). Expression of the Drosophila retrovirus gypsy as ultrastructurally detectable particles in the ovaries of flies carrying a permissive flamenco allele. J. Gen. Virol. *78*, 2379–2388.
40. Mével-Ninio, M., Pelisson, A., Kinder, J., Campos, A.R., and Bucheton, A. (2007). The flamenco locus controls the gypsy and ZAM retroviruses and is required for Drosophila oogenesis. Genetics *175*, 1615–1624.
41. Pélisson, A., Song, S.U., Prud'homme, N., Smith, P.A., Bucheton, A., and Corces, V.G. (1994). Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the Drosophila flamenco gene. EMBO J. *13*, 4401–4411.
42. Buszczak, M., Paterno, S., and Spradling, A.C. (2009). Drosophila stem cells share a common requirement for the histone H2B ubiquitin protease scrawny. Science *323*, 248–251.