Paramutation in *Drosophila* Requires Both Nuclear and Cytoplasmic Actors of the piRNA Pathway and Induces *Cis*-spreading of piRNA Production

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ABSTRACT Transposable element activity is repressed in the germline in animals by PIWI-interacting RNAs (piRNAs), a class of small RNAs produced by genomic loci mostly composed of TE sequences. The mechanism of induction of piRNA production by these loci is still enigmatic. We have shown that, in *Drosophila melanogaster*, a cluster of tandemly repeated *P-lacZ-white* transgenes can be activated for piRNA production by maternal inheritance of a cytoplasm containing homologous piRNAs. This activated state is stably transmitted over generations and allows *trans*-silencing of a homologous transgenic target in the female germline. Such an epigenetic conversion displays the functional characteristics of a paramutation, *i.e.*, a heritable epigenetic modification of one allele by the other. We report here that piRNA production and *trans*-silencing capacities of the paramutated cluster depend on the function of the *rhino*, *cutoff*, and *zucchini* genes involved in primary piRNA biogenesis in the germline, as well as on that of the *aubergine* gene implicated in the ping-pong piRNA amplification step. The 21-nt RNAs, which are produced by the paramutated cluster, in addition to 23- to 28-nt piRNAs are not necessary for paramutation to occur. Production of these 21-nt RNAs requires *Dicer-2* but also all the piRNA genes tested. Moreover, cytoplasmic transmission of piRNAs homologous to only a subregion of the transgenic locus can generate a strong paramutated locus that produces piRNAs along the whole length of the transgenes. Finally, we observed that maternally inherited transgenic small RNAs can also impact transgene expression in the soma. In conclusion, paramutation involves both nuclear (Rhino, Cutoff) and cytoplasmic (Aubergine, Zucchini) actors of the piRNA pathway. In addition, since it is observed between nonfully homologous loci located on different chromosomes, paramutation may play a crucial role in epigenome shaping in *Drosophila* natural populations.

KEYWORDS gene regulation; *trans*-generational epigenetics; noncoding small RNAs; mobile DNA; *Drosophila*

GENOMES must confront the presence of a large fraction of mobile DNA whose activity can result in severe deleterious effects on chromosome stability and gametogenesis. In the germline of animals, a system of genomic traps exists into which any transposable element (TE) can insert, thereby

generating loci that contain a catalog of potentially dangerous sequences that have to be repressed (Brennecke *et al.* 2007; Pane *et al.* 2011; Iwasaki *et al.* 2015). In the *Drosophila melanogaster* germline, most of these loci are transcribed in both directions (dual-strand clusters) and undergo noncanonical transcription and RNA processing (Mohn *et al.* 2014; Zhang *et al.* 2014). This results in production of noncoding small RNAs having the capacity to target the transcripts of the homologous, potentially active, TE copies scattered throughout the genome. These small RNAs are called PIWI-interacting RNAs (piRNAs) and repress TE activity at both the transcriptional and post-transcriptional levels (Sato and Siomi 2013; Weick and Miska 2014). piRNA biogenesis in the germline involves a nuclear and a cytoplasmic step. In the nucleus,

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piRNA-producing cluster transcription requires in most of the cases the presence of the HP1 paralog Rhino associated with Deadlock and Cutoff on the locus, forming the so-called RDC complex (Klattenhoff *et al.* 2009; Pane *et al.* 2011; Mohn *et al.* 2014; Zhang *et al.* 2014). In the cytoplasm, transcripts produced by the piRNA locus are sliced in an optically dense region surrounding the nucleus, called the nuage, and small RNAs (23–28 nt) loaded on Piwi or Aubergine proteins are produced (primary piRNAs). Further, piRNAs loaded on Piwi enter the nucleus and target euchromatic TE copies to induce their transcriptional repression via heterochromatin formation, which involves HP1 (Wang and Elgin 2011; Sienski *et al.* 2012; Le Thomas *et al.* 2013; Rozhkov *et al.* 2013). In contrast, piRNAs loaded on Aubergine remain in the nuage and target homologous transcripts being exported from the nucleus, which are produced by both homologous TE copies and the piRNA locus. This produces secondary piRNAs loaded on Aubergine or Ago3, other PIWI proteins (Weick and Miska 2014; Iwasaki *et al.* 2015) and results in a piRNA amplification process called ping-pong amplification (Brennecke *et al.* 2007; Gunawardane *et al.* 2007). Finally, piRNA loaded on Piwi can also be produced downstream of the ping-pong amplification step by a slicing process that can spread on targeted RNA, increasing both piRNA quantity and diversity (Han *et al.* 2015; Mohn *et al.* 2015; Siomi and Siomi 2015). Upstream of this complex machinery, the presence of Rhino on the piRNA-producing locus is particularly important since it appears sufficient to promote processing of transcripts by the piRNA machinery (Klattenhoff *et al.* 2009; Zhang *et al.* 2014). How Rhino is addressed to a piRNA-producing locus is still unclear.

In *Drosophila*, in contrast to *Caenorhabditis elegans* (Ruby *et al.* 2006; Batista *et al.* 2008), the production of piRNAs by a piRNA locus in the germline does not appear to be only genetically determined, *i.e.*, no specific sequence motif or structure has been identified that is sufficient to promote piRNA production by DNA adjacent to this sequence. Conversely, using piRNA-producing loci that repress *P*-transposable elements (Ronsseray *et al.* 1996), it was shown that maternal transmission of piRNAs together with the piRNA locus can stimulate production of piRNAs by paternally inherited *P*-elements scattered through the genome (Brennecke *et al.* 2008). In addition, analysis of ageing effects on *I*-transposable element repression capacities showed that the amount of *I*-homologous piRNAs in adult ovaries is correlated to the amount of homologous piRNAs deposited in embryos (Grentzinger *et al.* 2012). Furthermore, it was shown that *de novo*, long-term activation of a piRNA locus can be achieved in *Drosophila* by maternal transmission of homologous piRNAs, without transmission of the initial piRNA donor locus. Under these conditions, there is emergence of a new autonomous and stable piRNA locus (de Vanssay *et al.* 2012). Indeed, a transgenic cluster of *P*-element deriving transgenes (called *BX2*), inert for repression and piRNA production, can be activated by crossing with females bearing a transgenic cluster (called *T-1*) that produces abundant piRNAs in both orienta-

tions homologous to the *BX2* cluster (de Vanssay *et al.* 2012). Once activated, this new piRNA cluster, called *BX2**, is stable over generations ($n > 100$) in absence of the inducer *T-1* locus and is able to produce abundant transgenic sense and antisense piRNAs. Cytoplasm of oocytes produced by these females can again *de novo* activate piRNA production by a paternally inherited inactive *BX2* cluster (de Vanssay *et al.* 2012). This recurrent epigenetic conversion process presents all the features of a “paramutation” process, a phenomenon previously described in plants (Brink 1956; Coe 1959) and recently in worms (Shirayama *et al.* 2012; Sapetschnig *et al.* 2015), and described as “an epigenetic interaction between two alleles of a locus, through which one allele induces a heritable modification of the other allele without modifying the DNA sequence” (Brink 1956; Chandler 2007). It was shown further that a *BX2** locus has a modified chromatin structure, showing an increase in H3K9 methylation with regard to the nonparamutated *BX2* locus (Le Thomas *et al.* 2014). This example of paramutation thus results in communication within a genome between homologous clusters allowing one cluster to transfer piRNA production capacity to a previously inert cluster. Paramutation may play an important role in establishment of TE repression following a genetic invasion since master sites of repression could activate piRNA secondary sites thus reinforcing their repressive capacity. Investigating the mechanism of *de novo* activation of piRNA-producing loci is therefore important for understanding aspects of epigenome shaping in general.

We have therefore analyzed the functional requirements and properties of a *BX2** paramutated locus. This locus allows the investigation of the autonomous properties of a piRNA-producing locus, *i.e.*, independently of the influence of homologous sequences present in the genome that could interfere with the process. The *BX2** locus was previously shown to produce, not only abundant transgenic piRNAs, but also 21-nt RNAs inferred to be small-interfering RNAs (siRNAs), but the precise nature of these 21-nt RNAs and their functional role in silencing capacities were not elucidated. Here, we show first that *BX2** silencing and piRNA production capacities depend on the *rhino* and *cutoff* genes, involved in the noncanonical transcription of piRNA-producing loci, and on the *aubergine* and *zucchini* genes, involved in piRNA cytoplasmic processing. In addition, transgenic 21-nt RNA production depends on *Dcr-2*, which characterizes them as siRNAs but they are not necessary for paramutation to occur. Surprisingly, production of these siRNAs depends also on the piRNA pathway. Moreover, paramutation can occur between only partially homologous loci within a genome and is accompanied by a rapid spreading of the capacity to produce piRNAs to the nonhomologous sequences within the paramutated locus. Finally, an impact of maternally inherited small RNAs linked to paramutation is also observed in somatic cells. These findings allow us to characterize more precisely the molecular mechanism of paramutation in animals and to propose that it is a key factor in epigenome shaping in natural populations.

Figure 1 Effect of mutations affecting the piRNA or siRNA pathways on *trans*-silencing and small RNA production capacities of a *BX2** cluster. Upper box: Mating scheme used to analyze the effect of mutations (*mut*) affecting *aubergine*, *Dicer-2*, *rhino*, *cutoff*, and *zucchini* on *BX2** ovarian small RNA production and *trans*-silencing capacities. As the *BX2* cluster, all genes are located on chromosome 2. (Left) Heterozygous *BX2** *aub*⁻, *BX2** *Dcr-2*⁻, *BX2** *rhi*⁻, or *BX2** *cuff*⁻, or *BX2** *zuc*⁻ females were crossed with heterozygous males carrying a mutant allele of the same gene to generate either heterozygous control or loss-of-function females for deep sequencing of ovarian small RNAs. (Right) The same heterozygous females were crossed with heterozygous males carrying a mutant allele of the same gene plus a *P-lacZ* target transgene (*BQ16*) to measure the effect of the mutation tested on *BX2** *trans*-silencing capacities (TSE %). TSE was quantified by determining the percentage of egg chambers with no *lacZ* expression in the germline. In the genotypes presented, maternally inherited chromosomes (chromosomes 2 and 3) are indicated above the bar. Cy is a balancer chromosome carrying a dominant phenotypic marker. (A–L) In each case, the genotype tested is indicated, and the percentage of TSE is given below the genotype with the total number of egg chambers assayed in parentheses. Nonparamutated *BX2* ("naive") and *BX2** at generation 83 were analyzed as controls. Histograms show the length distributions of ovarian small RNAs matching the *P-lacW* locus. Positive and negative values correspond to sense and antisense reads, respectively. Plots show the abundance of 19- to 29-nt small RNAs matching the *P-lacW* locus. Analysis of the effect of *aub*, *rhi*, *cuff*, and *zuc* mutations shows that *BX2** silencing capacities, ovarian *P-lacW* 23- to 28-nt piRNA, but also 21-nt RNA production depend on the piRNA pathway. *Dcr-2* loss of function strongly affects production of *P-lacW* homologous 21-nt RNAs without affecting that of 23- to 28-nt RNAs or *trans*-silencing capacities. *P-lacW* 21 nt are not necessary for the maintenance of the paramutated state and their biogenesis depends on both *Dcr-2* and the piRNA pathway. TSE results for *Dcr-2* mutants (E and F) are reprinted from de Vanssay *et al.* (2012).

Materials and Methods

Experimental conditions

All crosses were performed at 25° and involved three to five couples in most cases. *lacZ* expression assays were carried out using X-gal overnight staining as described in Lemaitre *et al.* (1993), except that ovaries were fixed for 8 min. *Trans*-silencing was quantified as previously described by determining the percentage of egg chambers with no *lacZ* expression in the germline (Josse *et al.* 2007). It is referred to as the *trans*-silencing effect percentage (TSE %).

Dissection and RNA extraction

Total RNAs were extracted using TRIzol (Life Technologies) as described in the reagent manual (http://tools.lifetechnologies.com/content/sfs/manuals/trizol_reagent.pdf). For the RNA precipitation step, 100% ethanol was used instead of isopropanol. For ovarian small RNAs, 25 pairs of ovaries were manually dissected for each sample in PBS 1×.

Pigment dosage

Head pigments were quantified as described in Weiler and Wakimoto (2002). For each genotype, three replicates were

measured. For each replicate, five heads of 4- to 6-day-old females were manually dissected for the dosage. Absorbance was measured at 480 nm.

Transgenes and strains

P-lacZ fusion enhancer-trap transgenes: *P-1152* and *BQ16*, contain an in-frame translational fusion of the *Escherichia coli lacZ* gene to the second exon of the *P*-transposase gene and a *rosy* transformation marker (O'Kane and Gehring 1987). The *P-1152* insertion was mapped to the telomere of the X chromosome (cytological site 1A) and consists of two *P-lacZ* insertions in the same telomeric-associated sequence unit (TAS) (Karpen and Spradling 1992) and in the same orientation (Josse *et al.* 2007). *P-1152* is homozygous viable and fertile. *BQ16* is located at 64C in euchromatin of the third chromosome and is homozygous viable and fertile. *P-1152* shows no *lacZ* expression in the ovary; *BQ16* is strongly expressed in the nurse cells and in the oocyte (Josse *et al.* 2007). *RS3* is a *P-FRT-white* transgene (FBtp0001534). It is inserted in the TAS of the 3R chromosomal arm (cytological site 100E3). It is homozygous viable and fertile (DGRC, Kyoto no. 123282). *P-1152* and *RS3* induce a strong TSE and produce abundant ovarian transgenic piRNAs

Figure 2 Paramutation by a cytoplasm devoid of siRNAs. (A) $BX2^* Dcr-2^{-/-}$ females were crossed to $BX2 Dcr-2^{+/+}$ (naive) males to generate G_1 females having paternally inherited a $BX2$ locus and maternally inherited piRNAs but no 21-nt siRNAs homologous to the $P[lacW]$ cluster. In addition, these G_1 females paternally inherited a $Dcr-2^+$ allele. These G_1 females were crossed with $Dcr-2^+$ males to establish a $BX2^* Dcr-2^{+/+}/Dcr-2^+$ line in which no maternal $P[lacW]$ siRNAs have been initially introduced in G_0 . Cy is a balancer chromosome carrying a dominant phenotypic marker. Maternal chromosomal complement is written above the bar. (B) At generations 0, 1, and 2, deep sequencing of ovarian small RNAs was performed and silencing capacity was controlled in parallel by crossing females with males carrying the $BQ16$ target transgene and scoring TSE in female progeny. The genotype tested is indicated and TSE percentages and ovarian 19- to 29-nt RNAs are presented as in Figure 1. As expected, G_1 and G_2 females show complete silencing capacities. The same was true for successive G_n generations (G_6 , TSE = 100%, $n = 450$; G_{10} , TSE = 100%, $n = 1100$). Despite the lack of maternal $P[lacW]$ siRNA maternal transmission in G_0 , such ovarian small RNAs appear already in G_1 females and increase in G_2 in $BX2^* Dcr-2^{+/+}$ females (dashed arrow). Therefore, maternal inheritance of $P[lacW]$ homologous piRNAs, but no siRNAs, results in a strong and stable paramutated $BX2^*$ locus and in immediate production of both $P[lacW]$ piRNAs and siRNAs in females carrying a $Dcr-2^+$ allele.

(Roche and Rio 1998; Josse *et al.* 2007; de Vanssay *et al.* 2012; Dufourt *et al.* 2014) and this study (see Figure 3A). The *Lk-P(1A)* line carries two full-length *P*-elements inserted in TAS at the *X*-chromosome telomere (Ronsseray *et al.* 1991).

These telomeric *P*-insertions derive from a chromosome present in a natural population (Biemont *et al.* 1990) and induce very strong repression of *P*-element transposition and *P*-induced hybrid dysgenesis (Ronsseray *et al.* 1991, 1996).

Figure 3 Paramutation of a *BX2* locus by partially homologous piRNAs loci. *BX2* males carrying a *P{lacW}* naive cluster were crossed to females carrying partially homologous *P*-transgenes inserted in telomeric piRNA-producing loci at the heterozygous state. Female progeny were recovered that carry the *BX2* locus but did not carry the telomeric transgenes. These *G*₁ females inherited a cytoplasm carrying piRNA homologous to the telomeric transgenes, which therefore cover only partially the *BX2* *P{lacW}* transgene sequence. Lines were established and the *trans*-silencing capacities of these putative *BX2** lines were tested in subsequent generations, as in Figure 1 and Figure 2. In addition, deep sequencing of ovarian small RNAs was performed in *G*₃, *G*₅, and *G*₁₀. (A, left and middle) Structure of the *P-1152* and *RS3* telomeric *P*-transgenes used and of the *BX2* *P{lacW}* transgenes. Colored thick lines below the transgenes indicate the sequences shared by the *P{lacW}* and telomeric transgenes. The *P-1152* line carries two *P-lacZ-rosy* (*P{larB}*) transgenes inserted in the telomeric associated sequences (TAS) of the X chromosome. *RS3* carries a *P-FRT-white* transgene in the TAS of the 3R chromosomal arm. These two lines induce a strong TSE. (A, right) Ovarian piRNA production in females that carry a maternally inherited *P-1152* or *RS3* locus at the heterozygous state (*G*₁ females from crosses between *P-1152* and *RS3* females with males devoid of transgene) is mapped on the *P{lacW}* transgene. (B) Three types of replicate *BX2** sublines were generated: Sublines that inherited in *G*₁ (1) a cytoplasm from females heterozygous for the *P-1152* locus (*P*⁻¹¹⁵²*BX2* sublines, in orange); (2) a cytoplasm from females heterozygous for the *RS3* locus (*RS3**BX2* sublines, in green); and (3) a cytoplasm from females heterozygous for both the *P-1152* and *RS3* loci (*P*⁻¹¹⁵²+*RS3**BX2* sublines, in purple). *Trans*-silencing capacities of various *BX2** lines are shown with regard to generations (TSE %, *n* > 500 in all assays). Names of replicate sublines are given below the graphs. (C) Deep sequencing of ovarian small

RNAs in various *BX2** sublines at generations 3, 5, and 10. Plots show the abundance of 19- to 29-nt small RNAs matching *P{lacW}*. TSE assays show that silencing capacities vary among replicate *BX2** lines but strong and stable paramutated lines can be recovered with the three types of *G*₀ cytoplasmic inheritance (B). Ovarian small RNAs were analyzed for a subset of the *BX2** lines showing strong silencing capacities. The name of the line is indicated on the left of the graph. Deep sequencing analysis shows that piRNAs corresponding to the whole length of *P{lacW}* can be produced as early as generation 3 (C), showing that piRNA production by a paramutated locus can rapidly extend to the part of the locus that did not originally receive maternally inherited piRNAs.

Lk-P(1A) females produce abundant *P*-homologous ovarian piRNAs (Brennecke *et al.* 2008). The repressive capacity of the *Lk-P(1A)* line was verified immediately prior to the experiments reported in Supporting Information, Figure S6 and a very strong repression capacity was found (data not shown).

***P{lacW}* clusters:** The *BX2* line carries seven *P-lacZ-white* transgenes, including at least one defective copy, inserted in tandem and in the same orientation at cytological site 50C on the second chromosome (Dorer and Henikoff 1994). The transgene insertion site is located near the

mRpL53 gene in an *Ago1* intron (de Vanssay *et al.* 2012). This site is not a piRNA-producing locus, as observed for instance in the deep-sequencing dataset from *P-1152* or *w¹¹¹⁸* ovaries (data not shown). The *P-lacZ-white* construct contains the *P-lacZ* translational fusion and is marked by the mini-*white* gene (*P{lacW}*, FBtp0000204, 10,691 bp). The *T-1* line derives from X-ray treatment of *BX2* (Dorer and Henikoff 1997). *T-1* has chromosomal rearrangements including translocations between the second and third chromosomes. *DX1* was generated during the same *P-lacZ-white* transposase-mediated remobilization at 50C than *BX2* and carries six *P-lacZ-white* transgenes, one of which is in opposite orientation to the others, at the same genomic site as *BX2* (Dorer and Henikoff 1994, 1997). After overnight staining, weak *lacZ* expression is detected in the follicle cells of *BX2*, *T-1*, and *DX1* female ovaries, presumably because of a position effect at 50C, but no staining is observed in the germline (data not shown). The three *P{lacW}* clusters show variegated repression of *white* in the eyes due to repeat induced gene silencing (RIGS) (Dorer and Henikoff 1994, 1997). The extent of variegated repression of *white* is different between the clusters (*T-1* >> *DX1* > *BX2*). The *BX2* cluster was not used to assay the somatic phenotypic impact of piRNA maternal inheritance since RIGS-induced repression is too low in this case.

Lines carrying transgenes have M genetic backgrounds (devoid of *P*-transposable elements), as do the multimarked balancer stocks used in genetic experiments. The *w¹¹¹⁸* and Canton^y lines were used as controls completely devoid of any *P*-element or transgene. Crosses performed with *BX2*, *T-1*, or *DX1* were performed with females carrying the cluster at the heterozygous state because of the sterility (*BX2*, *DX1*) and lethality (*T-1*) induced by transgene clusters.

Two strong hypomorphic mutant alleles of *aubergine* (*aub*) induced by EMS were used. Both of them are homozygous female sterile and TSE was previously shown to be abolished by a heteroallelic combination of these alleles (Josse *et al.* 2007). *aub^{QC42}* comes from the Bloomington Stock Center (stock no. 4968) and has not been characterized at the molecular level (Schupbach and Wieschaus 1991). *aub^{N11}* has a 154-bp deletion, resulting in a frameshift that is predicted to add 16 novel amino acids after residue 740. *Dicer-2^{L811fsX}* (*Dcr-2^{L811fsX}*) is a loss-of-function allele induced by EMS, which has a sequence variant at residue 811 resulting in a stop codon (Lee *et al.* 2004). It is homozygous viable and fertile. *rhino⁰²⁰⁸⁶* (*rhi²*) results from a *P{PZ}* insertion (*P-lacZ-rosy*) at nucleotide 267. The *P{PZ}* transgene is oriented in the opposite direction to that of *rhi* transcription (Volpe *et al.* 2001) (Bloomington no. 12226). *rhi^{KG00910}* (*rhi^{KG}*) is due to the insertion of a *P{SUPor-P}* transgene, which carries the *yellow* and *white* transformation markers (Gene Disruption Project 2001, Bloomington no. 13161). *cutoff^{WM25}* (*cuff^{WM25}*) and *cuff^{QQ37}* alleles were isolated from an EMS screen (Schupbach and Wieschaus 1991). *cuff^{WM25}* corresponds to the replacement of the first methionine by a lysine and *cuff^{QQ37}* has a T to A substitution at position 786 that could result in a splicing alteration (data not shown). *zucchini*

(*zuc*) alleles were isolated from an EMS screen (Schupbach and Wieschaus 1991). *zuc^{HM27}* contains a stop codon at residue 5 and *zuc^{SG63}* a substitution of histidine 169 with a tyrosine in the conserved HKD domain presumably involved in nuclease activity. TSE was previously shown to be abolished by a heteroallelic combination of these alleles (Todeschini *et al.* 2010). The crosses performed to generate heteroallelic mutant *BX2** females are indicated in Table S1. When information was available, the most severe allele was introduced maternally. Lines carrying mutations of *zuc* and *cuff* were kindly provided by Attilio Pane and Trudi Schupbach and *aub^{N11}* was kindly provided by Paul Macdonald.

All the alleles described above are located on the second chromosome and are maintained over a Cy balancer chromosome. Additional information about mutants and stocks are available at FlyBase: <http://flybase.bio.indiana.edu/>.

Deep sequencing analyses

A small RNA fraction, from 18 nt to 30 nt in length, was obtained by separating it on a denaturing polyacrylamide gel from total RNA extracted from dissected ovaries. This fraction was used to generate multiplexed libraries with Illumina TruSeq Small RNA Library preparation kits (RS-200-0012, RS200-0024, RS-200-036, or RS-200-048) at Fasteris (<http://www.fasteris.com>). A house protocol based on TruSeq, which reduces 2S RNA (30 nt) contamination in the final library, was performed (except for libraries whose GRH number is below no. 40). Libraries were sequenced using Illumina HiSeq 2000 and 2500. Sequence reads in fastq format were trimmed from the adapter sequence 5'-CTGTAGGCAC CATCAATCGTA-3' (GRH12, GRH13, GRH14, GRH17) or 5'-TGGAAATCTCGGGTGCCAAG-3' (other samples) and matched to the *D. melanogaster* genome release 5.49 using Bowtie (Langmead *et al.* 2009) and to the sequences of the *P*-element constructs *P{lacW}* (FlyBase ID FBtp0000204) as indexed reference. Only 19- to 29-nt reads matching the reference sequences with 0 or 1 mismatch were retained for subsequent analysis. For global annotation of the libraries (Table S1), we used the release 5.49 of fasta reference files available in FlyBase, including transposon sequences (dmel-all-transposon_r5.49.fasta) and the release 20 of miRNA sequences from miRBase (www.mirbase.org).

Sequence length distributions, small RNA mapping, and small RNA overlap signatures were generated from bowtie alignments using Python and R (www.r-project.org/) scripts, which were wrapped and run in a Galaxy instance publicly available at <http://mississippi.fr>. Tools and workflows used in this study may be downloaded from this Galaxy instance. For library comparisons, read counts were normalized (Table S1) to the total number of small RNAs that matched the *D. melanogaster* genome (release 5.49) and did not correspond to abundant cellular RNAs (tRNAs and miscRNAs). For small RNA mapping (Figure 1, Figure 2, and Figure 3 and Figure S1, Figure S3, and Figure S6), we took into account only RNA reads that uniquely aligned to *P{lacW}* or the 42AB locus.

Distributions of piRNA overlaps (ping-pong signatures, Figure S2 and Figure S5) were computed as first described in Klattenhoff *et al.* (2009) and detailed in Antoniewski (2014). Thus, for each sequencing dataset, we collected all the 23- to 28-nt RNA reads matching *P{lacW}* or the 42AB locus whose 5' ends overlapped with another 23- to 28-nt RNA read on the opposite strand. Then, for each possible overlap of 1–28 nt, the number of read pairs was counted. Distributions of siRNA overlaps (Figure S3) were computed using a similar procedure, except that 20- to 21-nt RNA reads were collected instead of the 23- to 28-nt RNA reads. The distributions of piRNA/siRNAs overlaps (Figure S3) were computed by collecting separately the 20- to 21-nt and 23- to 28-nt RNA reads matching *P{lacW}* or the 42AB locus and counting for each possible overlap of 1–21 nt the number of read pairs across these two distinct read datasets. To plot the overlap signatures, a z-score was calculated by computing, for each overlap of 1 to *i* nucleotides, the number *O(i)* of read pairs and converting it, using the formula $z(i) = (O(i) - \text{mean}(O)) / \text{standard deviation}(O)$.

Data availability

Small RNA sequences have been deposited at the European Nucleotide Archive (ENA) (<http://www.ebi.ac.uk/ena>) under accession number PRJEB11491.

Results

Effect of mutation affecting the piRNA or siRNA pathway on BX2* small RNA production and silencing capacities

Paramutated *BX2** females were previously shown to produce 23- to 28-nt and 21-nt ovarian sense and antisense small RNAs homologous to the cluster of *P-lacZ-white* transgenes (called *P{lacW}*) and to induce complete silencing of homologous transgene expression in the female germline (de Vanssay *et al.* 2012). This homology-dependent silencing, which reflects piRNA functionality, is quantified using the *trans*-silencing effect (TSE) assay (Josse *et al.* 2007) in which the percentage of repressed egg chambers is measured. The 23- to 28-nt, but not 21-nt, RNAs presented the molecular signature of the ping-pong amplification step of the piRNA pathway. This strongly suggests that production of the 23- to 28-nt RNAs is dependent on the piRNA pathway, whereas that of the 21-nt RNAs would depend on the siRNA pathway. Moreover, the TSE capacities of the *BX2** paramutated locus were shown to be completely impaired by loss of function of *aubergine* (*aub*) involved in the piRNA ping-pong step, whereas they were insensitive to loss of function of *Dicer-2* (*Dcr-2*) involved in the siRNA pathway (de Vanssay *et al.* 2012). This suggested that the 21 nt would not be necessary for *BX2** silencing capacities.

We thus performed deep sequencing of ovarian small RNAs of mutant females for *aub* and *Dcr-2* and extended the analysis to *rhino* (*rhi*) and *cutoff* (*cuff*), which are nuclear actors

involved in piRNA loci noncanonical transcription (Mohn *et al.* 2014; Zhang *et al.* 2014), and to *zucchini* (*zuc*), which is involved in the cleavage of piRNA precursors and secondary piRNAs in the cytoplasm (Ipsaro *et al.* 2012; Nishimasu *et al.* 2012; Voigt *et al.* 2012; Han *et al.* 2015; Mohn *et al.* 2015). All these genes are located, as *BX2* is, on chromosome 2. For each gene tested, a recombinant chromosome carrying a *BX2** locus and a mutated allele was generated. Females heterozygous for this recombinant chromosome were crossed with heterozygous males, carrying one mutant allele of the corresponding tested gene, to generate control heterozygote, loss-of-function heteroallelic (*aub*, *rhi*, *cuff*, *zuc*) and homozygote (*Dcr-2*) females for deep sequencing of ovarian small RNAs (Figure 1, upper box, and Table S1). In addition, females heterozygous for recombinant chromosomes were crossed with heterozygous males carrying a corresponding mutated allele and a target *P-lacZ* transgene expressed in the female germline to investigate the effect of the mutation tested on the *trans*-silencing capacities of *BX2** (Figure 1, upper box). As controls, we analyzed small RNAs from ovaries of *BX2** females at generation 83 (*G*₈₃) after the paramutation process and of *BX2* nonparamutated females (called “*BX2* naive”). *BX2** lines maintained at 25° show a very strong stability through generations, as tested every five generations, showing a complete repression capacities (for example *G*₈₃: TSE = 100%, *n* = 1500; *G*₁₁₅: TSE = 100%, *n* = 1150). Figure 1 shows control females together with heterozygous and homozygous females for loss-of-function mutations for each gene tested, in terms of their capacity for *trans*-silencing (TSE %) and for production of ovarian small RNAs homologous to the *P{lacW}* transgene. As expected, the *BX2* naive cluster showed no silencing capacities (TSE = 0%, *n* = 2000) and did not produce significant amounts of *P{lacW}* homologous 23- to 28-nt or 21-nt RNAs (Figure 1A) whereas the *BX2** paramutated cluster continued to induce complete *trans*-silencing and to produce high levels of *P{lacW}* homologous 23- to 28-nt and 21-nt RNAs 83 generations after the paramutation process (Figure 1B). *aub* loss of function resulted in the complete loss of 23- to 28-nt RNAs homologous to the *P{lacW}* transgene (Figure 1D), when compared to heterozygous *aub* females (Figure 1C). Surprisingly, *aub* loss of function also affected production of the 21-nt RNAs by the *P{lacW}* cluster (Figure 1D). This did not result from a genomic background effect on the siRNA pathway in *aub* mutant females since the production of endogenous siRNAs at the *esi-1* locus was not affected (Figure S4, D). *Dcr-2* loss of function resulted in a strong decrease of 21-nt RNAs homologous to the *P{lacW}* transgene but the production of corresponding 23- to 28-nt RNAs was not affected, nor their ping-pong signature (Figure 1F and Figure S2J). Analysis of mutations affecting *rhi*, *cuff* or *zuc* produced similar results to that of *aub* mutations. TSE percentages showed that *rhi*, *cuff*, or *zuc* loss of function resulted in loss of *BX2** silencing capacities (Figure 1, H, J, and L), whereas no effect was found with *rhi*, *cuff*, and *zuc* heterozygotes (Figure 1, G, I, and K). *rhi*, *cuff*, and *zuc* loss of function also

Figure 4 Paramutagenicity of a partially homologous paramutation. $BX2^*$ lines having, at G₀, maternally inherited piRNA homologous to a part of the $BX2$ $P\{lacW\}$ transgene sequence, and showing strong silencing capacities at the 20th generation (Figure 3), were tested for their ability to *de novo* paramutate a naive $BX2$ locus. (A) The name of the line is indicated beside the G₀ female genotype. Maternal chromosomal complement is written above the bar. Cy and CyRo1 are balancer chromosomes devoid of transgenes and carrying different dominant markers. These $P-1152 BX2^*$, $RS3 BX2^*$, and $P-1152+RS3 BX2^*$ females were crossed with males carrying a $BX2$ naive cluster and G₁ progeny having paternally, but not maternally, inherited a $BX2$ cluster were crossed to establish lines. These lines carry a second order paramutated locus. (B) These lines were subsequently tested for *trans*-silencing capacities at generations 1, 4, and 36. The TSE percentage is indicated with the number of egg chambers counted in parentheses. Strong silencing capacities are observed over generations showing that $P-1152 BX2^*$, $RS3 BX2^*$, and $P-1152+RS3 BX2^*$ are strongly paramutagenic.

resulted in complete loss of ovarian $P\{lacW\}$ piRNA production together with an almost complete loss of 21-nt RNAs homologous to the transgene (Figure 1, H, J, and L). As expected, these mutants exhibited no effect on the produc-

tion of endogenous siRNAs at the *esi-1* locus (Figure S4) but production of piRNAs by the 42AB typical dual-strand piRNA locus was affected (Figure S1). In conclusion, $BX2^*$ silencing capacities and $P\{lacW\}$ homologous piRNA production depend on all the piRNA pathway genes tested, including nuclear actors, and do not depend on *Dcr-2*. Interestingly, *Dcr-2*-dependent 21-nt RNAs' production by the cluster also depends on all the piRNA pathway genes tested. $BX2^*$ is thus a typical dual-strand piRNA cluster, requiring the primary and secondary piRNA pathways, which allows the study of the properties of a piRNA cluster, independently from any influence of endogenous homologous sequences in *trans*.

Production of ovarian transgenic siRNAs does not require siRNA maternal inheritance

Our previous work (de Vanssay *et al.* 2012), combined with the results in Figure 1F, shows that $BX2^*$ silencing properties and production of piRNAs homologous to the $P\{lacW\}$ transgene do not require maternal inheritance of siRNAs. Results in Figure 1 show that presence of ovarian $P\{lacW\}$ 21-nt siRNAs depends, in addition to *Dcr-2*, on the function of all piRNA pathway genes tested. We asked next if production of $P\{lacW\}$ ovarian siRNAs requires maternal inheritance of such siRNAs. We thus generated $BX2^*$ females carrying a paternally inherited *Dicer-2* wild-type allele and maternally inherited $P\{lacW\}$ homologous piRNAs but no siRNAs, because their G₀ mothers were homozygous for *Dcr-2* mutations (Figure 2). As expected (de Vanssay *et al.* 2012), these G₁ *Dcr-2* heterozygous females showed complete silencing capacities. Deep sequencing shows that, in addition to ovarian $P\{lacW\}$ piRNAs, they produce $P\{lacW\}$ siRNAs. Further, these G₁ females were crossed with *Dcr-2*^{+/+} males and increased ovarian $P\{lacW\}$ siRNA levels were found in $BX2^*$ G₂ females having recovered a *Dcr-2*^{+/+} genotype. Therefore, production of ovarian $P\{lacW\}$ siRNAs by females having a functional siRNA pathway does not require homologous siRNA inheritance and can be induced by inheritance of solely homologous piRNAs.

Paramutation of a locus only partially covered by maternally inherited piRNAs can be stable and is accompanied by cis-spreading of piRNA production capacity

Targeting of transgenes or natural transposable elements by piRNAs can lead to spreading of piRNA production to sequences adjacent to the targeted elements (Muerdter *et al.* 2011; Olovnikov *et al.* 2013; Shpiz *et al.* 2014). We tested if spreading could be observed for a silencer locus induced by paramutation. Telomeric transgenes inserted in subtelomeric heterochromatin can show strong *trans*-silencing capacities (Roche and Rio 1998; Ronsseray *et al.* 2003; Josse *et al.* 2007; Josse *et al.* 2008; Dufourt *et al.* 2014) and produce abundant piRNAs in the germline (Muerdter *et al.* 2011; de Vanssay *et al.* 2012). These transgenes have a different structure than the $P\{lacW\}$ transgene of $BX2$ but share some common sequences (de Vanssay *et al.* 2012). It was therefore possible to

produce females, having paternally inherited a *BX2* naive locus and maternally inherited cytoplasm containing piRNAs homologous to only a subset of the *P{lacW}* transgene structure. Two telomeric transgenic loci were used (Figure 3). The *P-1152* line carries two copies of the *P-lacZ-rosy* transgene (*P{larB}*) in the telomeric associated sequences (TAS) of the *X* chromosome (Josse *et al.* 2007). The *P-1152* telomeric locus was the canonical silencer locus used in previous *trans*-silencing effect studies (Roche and Rio 1998; Josse *et al.* 2007, 2008; Todeschini *et al.* 2010; Pöyhönen *et al.* 2012). *RS3* carries a *P-FRT-white* transgene in the TAS of the third chromosome (3R arm). It also induces a strong TSE (Dufourt *et al.* 2014). Thus, *BX2 P{lacW}* transgenes shares *P*-element and *lacZ* sequences with *P-1152* and shares *P*-element and *white* sequences with *RS3*. In both cases, a part of the *P{lacW}* sequence is missing in the telomeric transgenic construct (see Figure 3A). In order to transmit the cytoplasm, but not the telomeric transgenes, females heterozygous for these telomeric transgenes were generated. We further crossed *BX2* males, from a nonparamutated line, with females heterozygous for each or both telomeric transgenes. *G*₁ females having inherited the *BX2* locus, but not the telomeric transgenes, were recovered and potentially paramutated *BX2* lines were established and analyzed for *trans*-silencing and ovarian piRNA production capacities over generations. Figure 3B shows that *G*₁ females that carried a paternally inherited *BX2* cluster and recovered cytoplasm containing piRNAs produced by partially homologous transgenes, can show strong or complete silencing capacities. However, the efficiency was not as strong as that of paramutation experiments in which the *P{lacW}* transgenes of the *BX2* paramutated locus inherit piRNAs homologous to the entire transgene length, which always result in a complete and stable paramutation (de Vanssay *et al.* 2012 and data not shown). For the *BX2** lines activated by piRNAs homologous to the *P-1152* transgenes (called *P-1152BX2**), two lines (QA5 and QA6) showed very strong silencing capacities after >30–50 generations (QA5 is at 100% at each generation tested), which appeared even more stable than those reported in de Vanssay *et al.* (2012). One line (BB5) exhibited a period of strong silencing capacity followed by a reduction in silencing and one line showed partial silencing capacities (BB6). For the *BX2** lines activated by piRNAs homologous to the *P-white* transgene (called *RS3BX2**), one line (QA8) established a long-term strong level of repression, while the other line (BB7-4) showed diminishing repression over time. For the *BX2** lines activated by piRNAs homologous to both *P-1152* and *RS3* transgenes (called *P-1152 + RS3BX2**), the situation resembled that of lines activated by *P-1152* cytoplasm, with one line showing complete repression capacity (QA1) and other lines showing intermediate repression levels. An additive effect was not observed since these *P-1152 + RS3BX2** lines did not appear stronger than the *P-1152BX2** lines despite the fact that small RNAs covering most of the sequence were maternally transmitted in *G*₀. We conclude that paramutation by partially homologous transgenes is globally less efficient than para-

mutation by fully homologous transgenes. However, piRNAs produced by partially homologous transgenes can induce stable and strong paramutation we call “partially homologous paramutations.”

For each type of *G*₀ maternal inheritance, lines showing strong silencing capacities were chosen and ovarian small RNA production was analyzed in *G*₃, *G*₅ and *G*₁₀ by deep sequencing (Figure 3C and Figure S5). Figure 3C shows that, as early as generation 3, the three types of *G*₀ maternal inheritance resulted in strong production of small RNAs covering the entire *BX2 P{lacW}* transgene length. Thus, partially homologous paramutations are associated with rapid and stable spreading of piRNA production by nonhomologous sequences of the targets, similarly to what was found for target transgenes (Muerdter *et al.* 2011; Olovnikov *et al.* 2013; Han *et al.* 2015; Mohn *et al.* 2015) or transposable elements (Shpiz *et al.* 2014).

Further, the paramutagenicity of *P-1152BX2**, *RS3BX2**, and *P-1152+RS3BX2** females was tested. Figure 4 shows that maternal transmission of cytoplasm produced by these females, combined to paternal transmission of a *BX2* naive locus, resulted in female progeny showing strong *trans*-silencing properties. These capacities are transmitted over generations, as tested until *G*₃₆. Thus, paramutation mediated via maternal inheritance of piRNAs homologous to a subregion of the targeted locus can be strong and stable and can generate strongly paramutagenic loci.

Maternally transmitted small RNAs can affect an adult somatic phenotype

piRNAs are maternally transmitted not only to the posterior pole of *Drosophila* embryos but also to more anterior regions where they can be implicated in gene regulation. Indeed, piRNAs homologous to transposable elements (*roo*, 412) have been implicated in *nanos* mRNA decay at the anterior part of the embryo by a mechanism involving mRNA deadenylation (Rouget *et al.* 2010). Furthermore, a high number of maternal RNAs were shown to undergo piRNA-dependent destabilization in the soma during maternal-to-zygotic transition in embryos (Barckmann *et al.* 2015). In addition, piRNAs can have an effect in somatic cells of the gonads. Indeed, in *D. simulans*, expression of the *tirant* transposable element in ovarian follicle cells was shown to be affected by *tirant* homologous piRNA maternal transmission (Akkouche *et al.* 2013). Finally, maternal depletion of Piwi in *D. melanogaster* impacted *white* variegation in adult eyes (Gu and Elgin 2013). Therefore, piRNA maternal deposition can impact not only the germline of progeny but also somatic tissues. During the course of experiments performed to study paramutation in the germline using the *T-1* line as paramutagenic line (de Vanssay *et al.* 2012), we detected impact of *P{lacW}* homologous small RNA maternal inheritance on somatic tissues, which we further investigated.

The *T-1* paramutagenic line, which carries seven *P{lacW}* copies inserted in tandem (Dorer and Henikoff 1994, 1997), was shown to produce abundant ovarian piRNAs including

piRNAs homologous to the *white* transformation marker (de Vanssay *et al.* 2012). The *white* gene is involved in eye pigment transport and its loss of function results in white eyes instead of red ones (Figure 5B). The *T-1* cluster shows strong variegation for the *white* marker, i.e., stochastic ON-OFF *white* repression from cell to cell (RIGS) (Dorer and Henikoff 1994, 1997). RIGS is associated with heterochromatin formation on tandem repeats, which includes *de novo* binding of HP1 at the locus (Fanti *et al.* 2003). In the *T-1* line, RIGS-induced variegation was enhanced by X-ray-induced chromosomal rearrangements. We first investigated the potential somatic effects of *P{lacW}* piRNA maternal inheritance by performing reciprocal crosses between *T-1/Cy* and *white* mutant individuals from different genetic backgrounds and comparing female progeny eyes from these crosses (eye images and pigment amounts). Figure 5A shows the very clear difference observed for all the three backgrounds tested (*w*¹¹¹⁸, *w*¹¹¹⁸ *Cy/Xa*, and *yw*^c). Females having maternally inherited the *T-1* cluster presented stronger *white* repression than females having paternally inherited the cluster. Note that in each case the two types of females have strictly the same genotype. The differences were confirmed by analysis of pigment levels (Figure 5A).

We further tested the effect of *P{lacW}* homologous piRNA maternal inheritance on the eye phenotype in flies carrying another *P{lacW}* cluster called *DX1*. This cluster has six tandem *P{lacW}* copies, one of which is in opposite orientation to the others (Dorer and Henikoff 1994, 1997). This cluster shows a low level of variegation at 25° but variegation is stronger at higher temperatures presenting almost a fully white phenotype at 29° (data not shown). *DX1* has no *trans*-silencing capacities (Ronsseray *et al.* 2001; de Vanssay *et al.* 2012). The two reciprocal crosses were first performed between *DX1* and *w*¹¹¹⁸; *Cy/Xa* individuals to test for the existence of a maternal effect of *DX1* inheritance on its eye phenotype and no significant difference was observed between the two types of progeny for *white* expression (Figure 5B). Further *DX1* individuals were crossed with *T-1* individuals and female progeny having inherited *DX1*, but not *T-1*, were analyzed. Figure 5B shows that *DX1* females, having maternally inherited *T-1* cytoplasm, i.e., containing transgenic small RNAs, presented eyes with a stronger level of *white* repression than *DX1* females having inherited *DX1* cytoplasm. Thus, *T-1* maternal cytoplasm induced *DX1* repression in adult heads. Moreover, females produced by crossing *DX1* females and *T-1* males showed significantly stronger *white* repression than those produced by crossing *DX1* females and *w*¹¹¹⁸; *Cy/Xa* males. This likely resulted from genetic background effects since no paternal inheritance from *T-1* *P{lacW}* piRNAs was previously detected (de Vanssay *et al.* 2012). In contrast, the difference observed between the progeny of reciprocal crosses of *DX1* and *T-1* individuals can be attributed to piRNA maternal transmission since these two types of female progeny have the same genotype. Consequently, a repressive effect of a *T-1* cytoplasm, which carries a high level of *P{lacW}* piRNAs, can be observed in

progeny adult eyes with both the *T-1* and *DX1* *P{lacW}* clusters as a read-out. To understand this small RNA somatic impact in progeny, it will be necessary to test it in zygotes, which are mutants for small RNA silencing pathways or chromatin-based repression pathways.

Discussion

The present results establish the paramutated *BX2** locus as a typical piRNA dual-strand cluster whose *de novo* small RNA production involves nuclear and cytoplasmic actors of the piRNA pathway. Among the small RNAs produced by the *BX2** locus, the 23- to 28-nt piRNAs, but not the 21-nt RNAs, are required for paramutation. Finally, a stable paramutation can be generated between partially homologous loci that are not allelic.

Implication of the primary piRNA pathway in *BX2** capacities

Mutant analysis shows thus that *aubergine*, *rhino*, *cutoff*, and *zucchini* are required for a paramutated locus to exhibit piRNA production capacities and *trans*-silencing. Two main molecular models, one nuclear, one cytoplasmic, have been proposed for the paramutation process (de Vanssay *et al.* 2013; Le Thomas *et al.* 2014). Considering a cytoplasmic paramutation model, it can be proposed that a *trans*-generational nuage-to-nuage transfer of information can be the only factor that explains the acquisition of silencing capacities by the *BX2** paramutated locus. It was previously shown by qRT-PCR that the *BX2* naive (nonparamutated) locus is transcribed in both orientations (de Vanssay *et al.* 2012). Thus, during paramutation, piRNAs loaded on Aub or Ago3 in the nuage of *T-1* or *BX2** female nurse cells could be transferred to the nuage of zygotic primordial germ cells (PGCs) having paternally inherited a *BX2* naive locus, resulting in *de novo* targeting of *BX2* *P{lacW}* transcripts by the ping-pong machinery and accounting by itself for the acquisition of silencing capacities by the *BX2** paramutated locus. In a nuclear paramutation model, maternally transmitted piRNAs, loaded on Piwi, could enter into PGC nuclei and activate *de novo* targeting of *BX2* naive transcript by the primary piRNA pathway via promoting the binding of the Rhino-Deadlock-Cutoff complex on the *P{lacW}* cluster. This model is suggested by the fact that deposition of Rhino at several piRNA producing loci depends on Piwi (Mohn *et al.* 2014). It is also strongly supported by ChIP analysis, which showed that a *BX2** cluster, as tested in G₁ ovaries of the paramutation experiment, exhibited an increase in H3K9 methylation levels when compared to a naive *BX2* locus (Le Thomas *et al.* 2014). H3K9me3 residues can indeed be targeted by the chromodomain of Rhino, resulting in RDC complex stabilization on the cluster. The mutant analysis reported here provides the functional assay that shows that *rhino*, *cutoff*, and *zucchini* are necessary for *BX2** *trans*-silencing and piRNA production capacities (Figure 1). Therefore, paramutation-linked repressive properties do not solely depend on cytoplasmic ping-pong

Figure 5 Somatic impact of maternally transmitted small RNAs. The effect of *P{lacW}* small RNA maternal inheritance on *P{lacW}* cluster expression in adult eyes was tested using two different transgene clusters, *T-1* (A) and *DX1* (B), as a read-out. *T-1*, but not *DX1*, has *trans*-silencing capacities, correlated with the presence of abundant ovarian piRNAs. The G_0 cross is indicated above the picture and was performed at 25°. Green female symbol indicates cytoplasmic inheritance containing *P{lacW}* piRNAs. The genotype (chromosome 2) of the G_1 females analyzed is given below the picture. *Cy* and *Xa* are chromosomes devoid of transgenes and carrying dominant markers. The maternally inherited chromosome is written above the bar. (A and B) Pigment levels (absorbance at 480 nm) are shown beside the pictures. Numbers in abscissa indicate the genotype analyzed (reported directly on the head picture). For pigment level dosages, Canton^v and *w¹¹¹⁸* female heads served as WT and *w¹¹¹⁸* null mutant controls, respectively. Control pictures are boxed in B. Bars indicate the standard error. Comparisons were performed using the Student's *t*-test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Maternal inheritance of *P{lacW}* small RNAs produced by a *T-1* female results in increased silencing of both *T-1* and *DX1* expression in the eye.

activation resulting from nuage-to-nuage *trans*-generational transfer of information. These properties depend also on the primary piRNA pathway, which is linked to the binding of the RDC complex on the locus (Mohn *et al.* 2014; Zhang *et al.* 2014). Analysis focused on the *T-1* paramutagenic locus, which produces a high level of ovarian *P{lacW}* piRNAs, strongly reinforces this model (Le Thomas *et al.* 2014). Indeed, ChIP analysis performed on ovaries of G_1 females produced by the two reciprocal crosses between *T-1* and wild-type (devoid of transgene) individuals showed that maternal inheritance of the *T-1* cytoplasm is associated with an increase of Rhino and Cutoff binding on the *T-1 P{lacW}* cluster in progeny ovaries (Le Thomas *et al.* 2014). Paramutation associated repressive properties also depend on the Zuc and on Aub activities (Figure 1), which take place in the nuage. In conclusion, chromatin analyses (Le Thomas *et al.* 2014) and functional analyses performed with mutants (Figure 1) complement each other to show that *trans*-generational induction of piRNA-mediated silencing by maternally inherited piRNAs involves nuclear and cytoplasmic steps of the piRNA pathway, including those involved in the atypic transcription of the piRNA loci. Thus, during paramutation, the paramutated locus is modified and remains so over generations.

BX2* transgenic 21-nt RNAs are actual siRNAs whose production also depends on the piRNA machinery

Deep sequencing of ovarian small RNAs associated with both telomeric *P*-transgene insertions and to the *T-1* paramutagenic or *BX2** paramutated clusters showed abundant 21-nt transgene homologous RNAs, in addition to 23- to 28-nt piRNAs (de Vanssay *et al.* 2012). Both sense and antisense 21-nt RNAs were found that contained no ping-pong signatures. These 21-nt RNAs were proposed to be *Dicer-2* dependent. Here, ovarian small RNAs of *BX2**, *Dcr-2*^{-/-} females have been analyzed by deep sequencing. As expected, *BX2** 21-nt RNAs were almost absent in these *Dcr-2* mutant females showing that they are genuine siRNAs (Figure 1). Strikingly, all the piRNA pathway mutations tested also resulted in the loss of the transgene-derived 21-nt RNAs (Figure 1). This suggests that *P{lacW}* siRNAs could be generated downstream of the piRNA pathway. It was shown further that production of these transgene-derived siRNAs by a female requires piRNA, but not siRNA, maternal inheritance, confirming that their appearance is downstream of piRNAs (Figure 2). The 21-nt small RNA production appears to be a common feature for a number of piRNA-producing loci (Czech *et al.* 2008; Malone *et al.* 2009; Pane *et al.* 2011). *cuff* mutant analysis was shown to strongly affect 21-nt RNA production by a number of double-strand piRNA-producing loci, including the 42AB locus (Pane *et al.* 2011; Le Thomas *et al.* 2014). What mechanism could explain the 21-nt RNA dependence on the piRNA pathway? *P{lacW}* homologous siRNA biogenesis depends on all the piRNA actors tested, including for example Rhino, which is necessary for production of piRNA precursors; Zucchini, which is responsible for slicing

steps in the cytoplasm; and Aubergine, which is an actor of the ping-pong amplification of the pool of piRNA. One possibility is that *P{lacW}* siRNA production results from the fact that both sense and antisense RNAs, produced by this dual-strand cluster, are concentrated locally due to the piRNA machinery, for example in the nuage, leading to occurrence of double-strand RNAs, which can further be processed by *Dcr-2*. The piRNA mutants tested could alter the production of the piRNAs associated with Piwi, which are produced downstream of the ping-pong amplification step (Han *et al.* 2015; Mohn *et al.* 2015), and consequently reduce a positive feedback in the nucleus of these piRNAs (Mohn *et al.* 2014). This would reduce deposition of Rhino at several piRNA, producing loci (Mohn *et al.* 2014) and thus affect dual-strand transcription of the *BX2* locus, abolish the sense and antisense RNA concentration step, and siRNA production as well. However, a low level of *P{lacW}* 21-nt RNA is still detectable in *Dcr-2* homozygous mutant females (Figure 1F), which present a ping-pong signature (Figure S3J). If we analyze small RNAs produced by the 42AB locus, which is a typical dual-strand cluster piRNA locus, piRNAs are reduced or disappear in all piRNA pathway mutant backgrounds tested (*aub*^{-/-}, *rhi*^{-/-}, *cuff*^{-/-}, *zuc*^{-/-}, Figure S1, D, H, J, and L). The 21-nt RNAs with a ping-pong signature also disappear in these mutant backgrounds for the 42AB locus (Figure S3, H, P, T, and X). An intriguing variation is observed in the *zuc*^{-/-} background (Figure S1L), which showed reduction in piRNA levels and a dramatic increase in 21-nt RNA levels for the 42AB locus. This suggests that in the absence of Zuc, precursor transcripts coming from double-stranded piRNA clusters can be processed into 21-nt RNA instead of piRNAs. This could be achieved by *Dcr-2*, since this enzyme is responsible for the presence of the majority of the 21-nt RNAs coming from piRNA clusters (see above) and these 21-nt RNAs show no ping-pong signature (Figure S3X). Thus, for a given locus, two 21-nt RNA populations coexist in the *Drosophila* germline: a high level of *Dcr-2*-dependent siRNAs showing no ping-pong signature whose production could be in competition with that of piRNAs and a low level of *Dcr-2* independent 21-nt piRNAs showing a ping-pong signature.

Spreading of the piRNA production capacity within a piRNA-producing cluster can be completed within three generations

piRNA clusters behave as genomic traps in which new insertions of TEs lead to *de novo* production of piRNA homologous to the new insertions. The kinetics of appearance of this new piRNA production is not clear, but some functional evidence has been provided from previous studies concerning the repression capacity inheritance of the *I*-factor and of the *P*-element (Kidwell *et al.* 1977; Bucheton 1978; Engels 1979). In both systems, it appears that establishment of repressive capacities, following paternal inheritance of *I*- or *P*-elements carrying genomes in a cytoplasm devoid of *I*- or *P*-homologous piRNAs, is progressive and require several generations to be completed (Engels 1979, 1989; Bregliano *et al.* 1980;

Finnegan 1989; Rio 2002). For example, experiments were performed with a line [*Lk-P(1A)*] carrying only two regulatory *P*-elements inserted in a telomeric strong piRNA-producing locus (TAS) (Coen *et al.* 1994; Ronsseray *et al.* 1996). It was shown that paternal transmission of the telomeric *P*-element locus in a cytoplasm devoid of *P*-homologous piRNAs, followed by maternal inheritance of the telomeric *P*-elements in successive generations, requires four generations to progressively establish complete *P*-repression (Coen *et al.* 1994). Thus, during these generations, a progressive spreading must occur within the TAS locus for piRNA production capacity, which finally results in strong production of piRNAs homologous, not only to the TAS, but also to the *P*-element. A spreading process for piRNA production was also observed for the *flamenco* piRNA-producing locus, which functions in the somatic follicle cells that surround germline cysts (Muerdter *et al.* 2011). Further, analysis of previously established transgenic lines that carry an *I*-fragment within a *P*-transgene have shown that piRNA production capacity can spread within transgenes (Olovnikov *et al.* 2013). The capacity to produce piRNAs invaded the whole *I*-derived transgene and reached in some cases flanking sequences. A similar spreading process into flanking sequences was also found for natural transposon insertions (Shpiz *et al.* 2014). It is possible, however, that spreading processes inside or outside a transgene or a TE involve different mechanisms. However, in these last experiments, the capacity of targeted sequences to maintain piRNA production on their own, in absence of the endogenous homologous piRNA locus that stimulated them, was not tested. In this article, we show that *cis*-spreading of piRNA production on *BX2** is maintained over generations in absence of the original input, the telomeric transgenic locus. In addition, this spreading is completed within a few generations.

What mechanism could be responsible for the spreading process? Very recent studies have shown that piRNAs associated with Piwi can be produced downstream of piRNA generation by ping-pong amplification, which can increase piRNA sequence diversity (Han *et al.* 2015; Mohn *et al.* 2015). Indeed, piRNAs associated with Ago3 can target complementary RNA and induce production of phased piRNAs (27 nt) corresponding to the slicing of the 3' tail of the cleaved target RNAs. These phased "trailer" piRNA, once loaded on Piwi, can potentially return to the nucleus and increase both transcriptional repression of homologous targeted TEs or stimulate binding of Rhino on the piRNA-producing locus. One consequence of such an additional mechanism would be the adjustment of the force of repression to the level of transcription of active copies. Another would be the formation of novel piRNAs, which are *cis* to the sequence targeted initially by piRNAs. This phenomenon would explain why a *P-lacZ-white* transcript targeted by *P-lacZ* homologous piRNAs is able to produce piRNAs homologous to *white*. However, some observations remain to be explained. First, according to this model, when telomeric *P*-insertions are paternally introduced in a lineage and thereupon are maternally transmitted, establishment

of repression would be expected to require only one generation to be completed but in fact it was shown to be progressive over generations (Coen *et al.* 1994). It is possible that nuclear reinforcement mediated by trailer piRNAs loaded on Piwi on the piRNA-producing locus is necessary, and this reinforcement involves maternal transmission to be efficient and thus requires more than one generation to be completed. Second, we performed an additional paramutation experiment in which a paternally inherited *BX2* locus was introduced in a maternal cytoplasm produced by *Lk-P(1A)* females. In that experiment, no paramutation of the paternally transmitted *BX2* cluster was observed (Figure S6) despite maternal inheritance of *P*-element homologous piRNAs corresponding to 820 bp of homology of the 10,691 bp of the *P{lacW}* transgene. It remains possible, however, that production of such trailer piRNAs is limited by the propagation mechanism itself, making it possible to fill a gap of 4 kb (Figure 3) but not a gap of almost 10 kb (Figure S6).

Role of paramutation in TE repression establishment and stabilization in natural populations

The *Drosophila* genome contains >140 piRNA-producing loci, most of them composed of truncated versions of TEs (Brennecke *et al.* 2007). How have these loci been activated in natural populations to produce piRNAs? Paramutation may have played a key role in such a process. Once activated in a single fly by an unknown factor, which could be environmental (Schott *et al.* 2014), a first piRNA-producing locus may have progressively replaced the inactive allele within the population by a paramutation process similar to paramutation of a *BX2* naive locus by a *BX2** locus (de Vanssay *et al.* 2012). Further, the first locus activated may have activated other TE-accumulating loci, despite showing only partial homology and being located on a different chromosome, by a paramutation process similar to that of *BX2* by *P-1152* or *RS3* (Figure 3). These secondarily activated sites could maintain TE repression capacities in individuals from the population that did not inherit the first active piRNA locus if this first locus is not fixed in the population. With time, epigenetic communication between TE-accumulating loci may activate all "genetically" competent loci (sufficient number of tandem repeats, bidirectional transcription, etc.). Moreover, these partially homologous paramutations could also have played a role during migration processes between natural populations. Indeed, a fly bearing active loci arriving from one population into another one could activate loci present on the chromosomes of the recipient population, despite the existence of potential partial divergences between TE-accumulating loci in these different populations. Therefore, it is possible to propose that paramutation may play an important role in epigenome shaping of the *Drosophila* germline, especially because it can occur even when interacting loci partially diverge in their sequence.

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Supporting Information

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Paramutation in *Drosophila* Requires Both Nuclear and Cytoplasmic Actors of the piRNA Pathway and Induces *Cis*-spreading of piRNA Production

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Paramutation in *Drosophila* requires both nuclear and cytoplasmic actors of the piRNA pathway and induces *cis*-spreading of piRNA production.

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SUPPORTING INFORMATION

(Table S1, Figures S1-S6)

Library ID	1		2		3		4		5		6		7	
Library	GRH12		GRH49		GRH50		GRH20		GRH19		GRH13		GRH14	
Parental origin	<i>BX2</i> / <i>Cy</i> x <i>Canton</i> ⁺		<i>BX2</i> */ <i>CyRoi</i> x <i>w</i> ¹¹¹⁸		<i>BX2</i> */ <i>CyRoi</i> x <i>w</i> ¹¹¹⁸		<i>BX2</i> *, <i>aub</i> ^{0C42} / <i>Cy</i> x <i>aub</i> ^{N11} / <i>Xa</i>		<i>BX2</i> *, <i>aub</i> ^{0C42} / <i>Cy</i> x <i>aub</i> ^{N11} / <i>Xa</i>		<i>BX2</i> *, <i>Dcr-2</i> ^{1811fsX} / <i>Cy</i> x <i>Dcr-2</i> ^{1811fsX} / <i>Cy</i>		<i>BX2</i> *, <i>Dcr-2</i> ^{1811fsX} / <i>Cy</i> x <i>Dcr-2</i> ^{1811fsX} / <i>Cy</i>	
Female genotype	<i>BX2</i> / +		<i>BX2</i> * _{G83} / +		<i>CyRoi</i> / +		<i>BX2</i> *, <i>aub</i> ^{0C42} / <i>Xa</i>		<i>BX2</i> *, <i>aub</i> ^{0C42} / <i>aub</i> ^{N11}		<i>BX2</i> *, <i>Dcr-2</i> ^{1811fsX} / <i>Cy</i>		<i>BX2</i> *, <i>Dcr-2</i> ^{1811fsX} / <i>Dcr-2</i> ^{1811fsX}	
Dmel_matched	20 140 166	100.00%	15 675 824	100.00%	14 222 359	100.00%	12 774 247	100.00%	11 504 770	100.00%	25 425 639	100.00%	17 615 555	100.00%
miRNAs	6 848 463	34.00%	3 867 491	24.67%	3 207 689	22.55%	1 536 928	12.03%	2 097 790	18.23%	4 570 877	17.98%	3 659 767	20.78%
tRNAs	1 101 672	5.47%	365 007	2.33%	208 814	1.47%	129 576	1.01%	205 847	1.79%	1 044 735	4.11%	960 156	5.45%
ncRNAs	158 102	0.79%	97 362	0.62%	94 152	0.66%	103 080	0.81%	95 869	0.83%	130 168	0.51%	120 312	0.68%
miscRNAs	1 393 137	6.92%	763 262	4.87%	924 378	6.50%	7 952 990	62.26%	6 939 045	60.31%	6 909 892	27.18%	3 156 238	17.92%
Transposons	7 687 715	38.17%	8 021 967	51.17%	7 315 480	51.44%	2 184 449	17.10%	1 426 029	12.40%	9 460 422	37.21%	7 189 726	40.81%
Introns	935 677	4.65%	784 756	5.01%	754 263	5.30%	240 681	1.88%	190 169	1.65%	1 105 726	4.35%	827 244	4.70%
Transcripts	527 498	2.62%	351 601	2.24%	365 592	2.57%	200 782	1.57%	201 707	1.75%	540 473	2.13%	457 607	2.60%
Intergenic	1 307 958	6.49%	1 294 915	8.26%	1 217 106	8.56%	379 149	2.97%	2 88 815	2.51%	1 494 552	5.88%	1 117 847	6.35%
Remaining	179 944	0.89%	129 463	0.83%	134 885	0.95%	46 612	0.36%	59 499	0.52%	168 794	0.66%	126 658	0.72%
Effective depth	1 7645 357		14 547 555		13 089 167		4 691 681		4 359 878		17 471 012		13 499 161	
Normalisation factor	0.2470835		0.2996983		0.3330905		0.9292784		1		0.2495492		0.322974	

Library ID	8		9		10		11		12		13	
Library	GRH60		GRH61		GRH44		GRH43		GRH34		GRH33	
Parental origin	<i>BX2</i> *, <i>rhi</i> ² / <i>Cy</i> x <i>rhi</i> ^{KG} / <i>Cy</i>		<i>BX2</i> *, <i>rhi</i> ² / <i>Cy</i> x <i>rhi</i> ^{KG} / <i>Cy</i>		<i>BX2</i> *, <i>cuff</i> ^{0Q37} / <i>Cy</i> x <i>cuff</i> ^{N1WM25} / <i>Cy</i>		<i>BX2</i> *, <i>cuff</i> ^{0Q37} / <i>Cy</i> x <i>cuff</i> ^{N1WM25} / <i>Cy</i>		<i>BX2</i> *, <i>zuc</i> ^{HM27} / <i>Cy</i> x <i>zuc</i> ^{SG63} / <i>Cy</i>		<i>BX2</i> *, <i>zuc</i> ^{HM27} / <i>Cy</i> x <i>zuc</i> ^{SG63} / <i>Cy</i>	
Female genotype	<i>BX2</i> *, <i>rhi</i> ² / <i>Cy</i>		<i>BX2</i> *, <i>rhi</i> ² / <i>rhi</i> ^{KG00910}		<i>BX2</i> *, <i>cuff</i> ^{0Q37} / <i>Cy</i>		<i>BX2</i> *, <i>cuff</i> ^{0Q37} / <i>cuff</i> ^{WM25}		<i>BX2</i> *, <i>zuc</i> ^{HM27} / <i>Cy</i>		<i>BX2</i> *, <i>zuc</i> ^{HM27} / <i>zuc</i> ^{SG63}	
Dmel_matched	16 245 668	100.00%	11 825 256	100.00%	13 389 291	100.00%	13 685 445	100.00%	20 218 897	100.00%	15 281 858	100.00%
miRNAs	3 391 308	20.88%	2 750 213	23.26%	3 478 815	25.98%	4 264 109	31.16%	3 535 506	17.49%	3 707 069	24.26%
tRNAs	563 440	3.47%	381 717	3.23%	249 630	1.86%	1 047 588	7.65%	970 824	4.80%	973 116	6.37%
ncRNAs	274 114	1.69%	250 716	2.12%	84 967	0.63%	197 184	1.44%	145 664	0.72%	319 660	2.09%
miscRNAs	1 940 438	11.94%	4 441 776	37.56%	794 777	5.94%	3 122 174	22.81%	3 326 698	16.45%	6 507 497	42.58%
Transposons	7 247 574	44.61%	2 346 980	19.85%	6 625 525	49.48%	3 077 762	22.49%	9 174 921	45.38%	2 454 899	16.06%
Introns	813 565	5.01%	377 459	3.19%	558 367	4.17%	336 092	2.46%	968 061	4.79%	287 434	1.88%
Transcripts	542 936	3.34%	875 963	7.41%	569 531	4.25%	1 145 556	8.37%	440 817	2.18%	425 596	2.78%
Intergenic	1 321 210	8.13%	320 193	2.71%	881 608	6.58%	367 902	2.69%	1 475 029	7.30%	435 582	2.85%
Remaining	151 083	0.93%	80 239	0.68%	146 071	1.09%	127 078	0.93%	181 377	0.90%	171 005	1.12%
Effective depth	13 741 790		7 001 763		12 344 884		9 515 683		15 921 375		7 801 245	
Normalisation factor	0.3172714		0.6226828		0.3531728		0.4581781		0.2738380		0.5588695	

Library ID	14		15		16		17		18		19		20		21	
Library	GRH75		GRH76		GRH27		GRH30		GRH21		GRH58		GRH88		GRH22	
Parental origin	<i>BX2</i> *, <i>Dcr-2</i> ¹⁸¹¹ / <i>Dcr-2</i> ¹⁸¹¹ x <i>BX2</i> / <i>Cy</i>		<i>BX2</i> */ <i>Dcr-2</i> ¹⁸¹¹ x <i>Cy</i> / <i>Xa</i>		P1152 <i>BX2</i> */ <i>Cy</i>		P1152 <i>BX2</i> */ <i>Cy</i>		P1152 <i>BX2</i> */ <i>Cy</i>		RS3 <i>BX2</i> */ <i>Cy</i>		RS3 <i>BX2</i> */ <i>Cy</i>		RS3 <i>BX2</i> */ <i>Cy</i>	
Female genotype	<i>BX2</i> */ <i>Dcr2</i> ^{1811fsX} _{G1}		<i>BX2</i> */ <i>Cy</i> _{G2}		P1152 <i>BX2</i> */ <i>Cy</i> _{G3}		P1152 <i>BX2</i> */ <i>Cy</i> _{G5}		P1152 <i>BX2</i> */ <i>Cy</i> _{G10}		RS3 <i>BX2</i> */ <i>Cy</i> _{G3}		RS3 <i>BX2</i> */ <i>Cy</i> _{G5}		RS3 <i>BX2</i> */ <i>Cy</i> _{G10}	
Dmel_matched	16 947 093	100.00%	15 269 297	100.00%	17 430 597	100.00%	18 065 034	100.00%	15 429 273	100.00%	16 279 059	100.00%	16 920 341	100.00%	13 871 755	100.00%
miRNAs	3 997 672	23.59%	3 473 591	22.75%	2 828 264	16.23%	2 456 132	13.60%	4 060 488	26.32%	3 916 155	24.06%	4 046 793	23.92%	2 720 348	19.61%
tRNAs	620 994	3.66%	293 115	1.92%	111 433	0.64%	178 245	0.99%	191 691	1.24%	302 025	1.86%	505 384	2.99%	147 918	1.07%
ncRNAs	124 284	0.73%	91 470	0.60%	99 510	0.57%	126 380	0.70%	134 384	0.87%	156 772	0.96%	218 565	1.29%	102 794	0.74%
miscRNAs	1 588 023	9.37%	1 013 759	6.64%	8 991 044	51.58%	9 726 925	53.84%	4 286 124	27.78%	1 480 806	9.10%	1 984 125	11.73%	6 807 534	49.07%
Transposons	8 073 990	47.64%	7 698 603	50.42%	3 947 448	22.65%	4 110 704	22.76%	5 048 740	32.72%	7 717 454	47.41%	7 325 666	43.30%	3 049 544	21.98%
Introns	755 550	4.46%	867 737	5.68%	440 087	2.52%	437 666	2.42%	513 910	3.33%	842 832	5.18%	916 638	5.42%	312 114	2.25%
Transcripts	435 636	2.57%	350 353	2.29%	231 976	1.33%	262 210	1.45%	289 041	1.87%	384 269	2.36%	400 014	2.36%	196 380	1.42%
Intergenic	1 225 750	7.23%	1 324 711	8.68%	678 534	3.89%	675 281	3.74%	797 943	5.17%	1 314 860	8.08%	1 357 357	8.02%	464 318	3.35%
Remaining	125 194	0.74%	155 958	1.02%	102 301	0.59%	91 491	0.51%	106 952	0.69%	163 886	1.01%	165 799	0.98%	70 805	0.51%
Effective depth	14 738 076		13 962 423		8 328 120		8 159 864		10 951 458		14 496 228		14 430 832		6 916 303	
Normalisation factor	0.2958241		0.3122579		0.5235128		0.5343076		0.3981093		0.3007594		0.3021224		0.6303769	

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Library ID	22		23		24		25		26		27		28	
Library	GRH84		GRH85		GRH23		GRH29		GRH31		GRH17		GRH97	
Parental origin	P1152+RS3 <i>BX2</i> */ <i>Cy</i>		P1152+RS3 <i>BX2</i> */ <i>Cy</i>		P1152+RS3 <i>BX2</i> */ <i>Cy</i>		LK-PIA <i>BX2</i> / <i>Cy</i>		LK-PIA <i>BX2</i> / <i>Cy</i>		<i>RS3</i> x <i>Canton</i> ^y		<i>P-1152</i> x <i>w</i> ¹¹¹⁸	
Female genotype	P1152+RS3 <i>BX2</i> */ <i>Cy</i> _{G3}		P1152+RS3 <i>BX2</i> */ <i>Cy</i> _{G5}		P1152+RS3 <i>BX2</i> */ <i>Cy</i> _{G10}		LK-PIA <i>BX2</i> / <i>Cy</i> _{G3}		LK-PIA <i>BX2</i> / <i>Cy</i> _{G5}		<i>RS3</i> / +		<i>P-1152</i> / +	
Dmel_matched	15 823 878	100.00%	50 523 384	100.00%	11 186 364	100.00%	21 593 154	100.00%	18 827 290	100.00%	20 591 093	100.00 %	9 737 043	100.00 %
miRNAs	2 503 425	15.82%	9 036 497	17.89%	1 603 513	14.33%	4 852 422	22.47%	3 165 469	16.81%	3 711 711	18.03 %	2 074 552	21.31 %
tRNAs	429 049	2.71%	1 515 203	3.00%	99 422	0.89%	128 814	0.60%	168 102	0.89%	863 587	4.19 %	48 616	0.50 %
ncRNAs	258 574	1.63%	757 628	1.50%	61 017	0.55%	108 460	0.50%	103 952	0.55%	122 807	0.60 %	35 478	0.36 %
miscRNAs	3 729 832	23.57%	9 508 301	18.82%	663 4861	59.31%	6 667 164	30.88%	9 859 094	52.37%	4 610 621	22.39 %	807 308	8.29 %
Transposons	6 282 282	39.70%	21 465 112	42.49%	2 072 874	18.53%	7 448 049	34.49%	4 096 768	21.76%	8 188 718	39.77 %	2 184 131	22.43 %
Introns	775 641	4.90%	2 496 136	4.94%	209 781	1.88%	709 770	3.29%	418 701	2.22%	1 003 326	4.87 %	21 5734	2.22 %
Transcripts	510 853	3.23%	1 423 633	2.82%	119 967	1.07%	336 981	1.56%	231 367	1.23%	484 108	2.35 %	160 894	1.65 %
Intergenes	1 201 771	7.59%	3 861 720	7.64%	338 746	3.03%	1 168 266	5.41%	676 687	3.59%	1 398 340	6.79 %	338 239	3.47 %
Remaining	132 451	0.84%	459 154	0.91%	46 183	0.41%	173 228	0.80%	107 150	0.57%	207 875	1.01 %	81 688	0.84 %
Effective depth	11 664 997		39 499 880		4 452 081		14 797 176		8 800 094		15 116 885		8 881 119	
Normalisation factor	0.3737573		0.1103769		0.9792899		0.2946425		0.4954353		0.2880898		0.4903685	

Table S1. Annotation of small RNA libraries

Small RNAs were prepared from ovaries of females of the indicated genotype (*Female genotype* row). The parental origin of these females is indicated in the *Parental origin* row (females x males). The Canton^y and the *w*¹¹¹⁸ strains are devoid of any transgene. Values for the different categories of sequences are expressed as their percentage of the total number of sequence reads that matched the *D. melanogaster* genome 5.49 in each library (Dm Matched row). For comparisons, libraries were normalized (normalization factor) relatively to the number of sequence reads aligning to the *D. melanogaster* genome but not to tRNAs or miscRNAs (effective depth). Library #5 has the lowest effective depth and was taken as reference to normalize the other libraries.

Figure S1. Effect of mutations affecting piRNA and siRNA pathways on small RNA production by the 42AB piRNA locus

(A-L) Ovarian small RNAs analysed in Figure 1 for *P{lacW}* homologous small RNAs were analysed for small RNAs homologous to the 42AB dual strand piRNA locus. Histograms show the length distributions of small RNAs matching the 42AB locus. Positive and negative values correspond to sense and antisense reads, respectively. Plots show the abundance of 19-29nt small RNAs. piRNA production by the 42AB locus is strongly affected by *rhi*, *cuff* and *zuc* mutations, partially affected by *aub* mutations and is not affected by *Dcr-2* mutations.

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Figure S2. Effect of mutations on 23-28nt RNA ping-pong signatures

A-AB. Panels show the relative frequency (z-score) of overlapping sense-antisense small RNA pairs in the subset of 23-28nt small RNAs matching *P{lacW}* or the 42AB locus as indicated. The genotype is indicated above the graph. Y-AB correspond to G₁ and G₂ females shown in Figure 2.

Fig. S3 - Hermant *et al.*

Figure S3. Effect of mutations on 20-21nt RNA abundance and ping-pong signatures

A-AB. Bar plots show the abundance of 20-21nt small RNAs matching *P{lacW}* or the 42AB locus in ovaries from females of the indicated genotype. Positive and negative values correspond to sense and antisense reads, respectively. Panels show the relative frequency (z-score) of overlapping sense-antisense small RNA pairs in the subsets of 20-21nt small RNAs (left) and of 20-21nt RNAs that pair with the indicated overlap with 23-28nt small RNAs (right), matching *P{lacW}* or the 42AB locus. Y-AB correspond to G₁ and G₂ females shown on Figure 2.

Figure S4. Effect of mutations on ovarian endo-siRNA production

A-N. Length distribution of 19-29nt small RNAs matching the *esi-1* endosiRNA-producing locus. Bar plots show the abundance of 20-21nt small RNAs on the *esi-1* locus. Positive and negative values correspond to sense and antisense reads, respectively. Production of ovarian *esi-1* 20-21nt RNAs is highly sensitive to mutants affecting *Dcr-2* but is not sensitive to mutants affecting the piRNA pathway.

Figure S5. piRNA distribution on partially homologous paramutations

The nature of the cytoplasm inherited in G_1 females is indicated at the top of the columns. The generation at which the test was conducted is indicated on the left of the rows. **(Graphs to the left)** Length distributions of small RNAs matching $P\{lacW\}$. Positive and negative values correspond to sense and antisense reads, respectively. **(Graphs to the right)** Relative frequency (z-score) of overlapping sense-antisense small RNA pairs in the subset of 23-28nt small RNAs matching $P\{lacW\}$.

A

		TSE percentage
<i>Lk-P(1A)</i>		100% (n=1400)
<i>BX2</i> naive		0% (n> 2000)
^{T-1} <i>BX2</i> *		100% (n> 2000)
<i>Lk-P(1A)</i> <i>BX2</i>	G₃	0% (n> 2000)
	G₅	0% (n> 2000)
	G₇	0% (n> 2000)
	G₁₁	0% (n> 2000)
	G₂₆	0% (n> 2000)

B

Figure S6. Effect of maternal transmission of piRNA, limited to the *P{lacW}* 820bp *P*-element sequence, on a *BX2* naive cluster

BX2 males carrying a *P{lacW}* naive cluster were crossed to females *Lk-P(1A)* which carry two complete *P*-elements inserted in a telomeric piRNA-producing locus at the heterozygous state. *Lk-P(1A)* females have a very strong capacity to repress *P*-element activity. Female progeny were recovered which carry the *BX2* locus but do not carry the telomeric *P*-copies. These *G₁* females inherited a cytoplasm carrying piRNA homologous to the complete *P*-element, which therefore covers only the first 587bp and the last 233bp of the *BX2* *P{lacW}* transgene sequence (10691bp). Lines were established and the silencing capacities of these putative ^{*Lk-P(1A)*}*BX2** lines were tested in subsequent generations using *BQ16*. (A). In addition, deep sequencing of ovarian small RNAs was performed in *G₃* and *G₅* (B). TSE assays show that the silencing capacities of ^{*Lk-P(1A)*}*BX2* lines are null at all generations tested. Accordingly, no *P{lacW}* homologous piRNAs were detected at generations 3 and 5. Inheritance of piRNAs homologous to 820bp out of the 10691bp of the *P{lacW}* transgene does not result in *BX2* paramutation.