Ph.D Thesis

Studies on the *P*-element piRNAs associated with hybrid dysgenesis in *Drosophila melanogaster*

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Contents

General introduction

Ⅰ. Transposon

Transposable elements (TEs) are mobile DNA sequences. They occupy a substantial fraction of eukaryotic genomes; approximately 45% of the human genome (Lander *et al*. 2001), 37.5% of the mouse genome (Waterston *et al.* 2002), and 12% in *Drosophila* (Kidwell and Lish. 1997). TEs are classified two types. TEs in class 1 move via an RNA intermediate with reverse transcriptase. TEs of class 2, which are called DNA transposons, transpose via a DNA intermediate through cut-and-paste mechanism (Craig *et al.* 2002). Many effects of TE insertions have been shown, as regulation of gene expression, increased recombination rate, and unequal crossover. Although their mobilization provides genetic variations and drives genome evolution (Bennetzen *et al.* 2000; Britten *et al.* 2010), TEs exert deleterious effects on the host. For example, mobility of a TE in *Drosophila melanogaster* (*D. melanogaster*) causes germline abnormalities known as hybrid dysgenesis. The host counteracts this deleterious effect through various pathways, including Piwi-interacting small RNAs (piRNAs) (Saito *et al.* 2006; Brennecke *et al.* 2007).

Ⅱ. piRNA

piRNAs are generated from particular genomic loci called piRNA clusters, which consist of many TEs (Fig. 1). Two types of piRNA clusters are identified in *D. melanogaster*—dual-strand piRNA clusters and unistrand piRNA clusters (Brennecke *et al*. 2007; Yamanaka *et al.* 2014). In germ line cells, transcription occurs in both directions in the dual-strand piRNA cluster to produce long

precursor single-stranded RNAs, which are subsequently chopped into 24- to 35-nt RNAs (or 23- to 35-nt RNAs) called primary piRNAs. These are loaded onto Piwi-family proteins to direct the cleavage of complementary RNAs, including TE mRNAs. The product RNAs are then loaded onto a Piwi-family protein to aid in the cleavage of complementary-based RNA, a reaction known as the “ping-pong cycle” (Brennecke *et al*. 2007; Gunawardane *et al*. 2007; Klattenhoff and Theurkauf 2008). These piRNAs exhibit a ping-pong signature that is partially characterized by complementary sequences that overlap the 10th nucleotide A of the sense strand with the 5′ U of the antisense strand and that can be amplified through the cycles. In somatic cells, long precursor RNAs are transcribed in a single direction from the unistrand piRNA cluster. Because TEs are inserted into the unistrand piRNA clusters oriented predominantly from antisense to precursor transcription, they can serve as a source of TE-derived antisense piRNAs, which are used by the PIWI protein to induce repressive chromatin modification (Malone *et al.* 2009; Saito *et al*. 2010; Olivieri *et al*. 2010; Dennis *et al.* 2013; Ross *et al.* 2014; Iwasaki *et al.* 2016). Owing to these biogenesis pathways, piRNAs are generated and retained in the cytosol, although a fraction of them are transported into the nucleus.

Ⅲ. *P*-elements and P-M hybrid dysgenesis

The *P* element is a DNA transposon, and their copies in the *Drosophila melanogaster* genome include structurally complete and incomplete variants. The autonomous complete elements, which are 2,907 base pairs in length, encode an 87 kDa transposase that is expressed in the germline cells (O’Hare

and Rubin 1983; Rio *et al.* 1986; Engels *et al.* 1987). 66 kDa-repressor (type I repressor), which was soma-specific splicing variant retaining the IVS3 sequence, also act as repressor of *P*-element transposition in germline (Simmons *et al.* 2002). The *KP* elements, non-autonomous incomplete variants with a deletion of nucleotides 808–2060, are present ubiquitously in natural populations (Brack *et al.* 1987; Rasmusson *et al.* 1993; Itoh *et al.* 2007) and supply the most common type II repressor protein that inhibits *P*-element transposition (Rio, 1990; Rasmusson *et al.* 1993; Lemaitre *et al.* 1993; Andrews and Gloor 1995; Simmons *et al.* 1996 Lee *et al.* 1996; Simmons *et al.* 2002; Sameny and Locke 2011).

P elements are responsible for a phenomenon called “P-M hybrid dysgenesis.” Progeny of a cross between an M-strain female with no *P* element and a P-strain male carrying complete *P* elements demonstrate increased frequencies of *P*-element transposition, which results in germline cell abnormalities, such as gonadal dysgenesis (GD) with sterility, chromosomal breaks, mutations, and male recombination (Kidwell *et al.* 1977; Engels and Preston 1997; Rubin *et al.* 1982; Preston and Engels 1996). Therefore, P-strain males have a high ability to mobilize *P* elements in their progeny (high *P* inducibility).

When P-strain males are mated with P-strain females, *P*-element mobilization in the germline cells of their progeny is prevented by maternally deposited repressors (Engels and Preston, 1979; Simmons *et al.* 2016). The defect of suppressing *P*-element transposition when used as a mother is called *P* susceptibility, which is low for P strains and high for M strains (Kidwell 1981; Bingham *et al.* 1982; Anxolabéhère *et al.* 1984; Kidwell 1985; O’Hare *et al.* 1992;

Itoh *et al.* 1999). It has been proposed that the GD phenotype of daughters (i.e., *P* susceptibility) is largely determined by cytosolic factor(s) in the oocyte of their mothers rather than by the genotype of either the daughters or mothers. Thus, the oocytes are distinguished as "cytotypes." M-strains females produce oocytes of "M cytotype," which produce dysgenic daughters when crossed with a P strain, whereas females of P strains produce oocytes of "P cytotype," which produce normal daughters. The major molecular entity that determines the P-M cytotype in oocytes has been proposed to be cytosolic *P*-element piRNA, which is inherited by the daughters to suppress *P* transposition (Brennecke *et al.* 2008; Khurana *et al.* 2011). It has also been reported that *P* mobilization in the progeny is controlled by other factors, such as proteins produced from full-length (type I, 66-kDa repressors) and internally deleted elements (type II, *KP* repressors) (Black *et al.* 1987; Rasmusson *et al.* 1993; Lemaitre *et al.* 1993; Andrews and Gloor 1995; Simmons *et al.* 1996; Simmons *et al.* 2002; Simmons *et al.* 2015; Simmons *et al.* 2016).

Ⅳ. Hybrid dysgenesis of M' and Q strains

M′ and Q strains, which show different P–M phenotypes from P strains, are currently the most common in the natural populations in Eurasia, Africa, Australia, and the Far East (Bonnivard and Higuet 1999; Itoh *et al.* 2004; Ignatenko *et al.* 2015). Progeny of M'- or Q-strain females, which are crossed with P-strain males, referred to as "M′ or Q progeny" here. Progeny of M'- or Q-strain males, which are crossed with P-strain females, referred to as "M′ or Q

hybrid" here. Hybrid dysgenesis for both "M' or Q progeny" and "M' or Q hybrid" was explained below.

Ⅳ-1. Dysgenesis of M' and Q progeny

Although M′ progeny allows transposition of *P* elements in the germline cells (high *P* susceptibility and M cytotype), the M′ strains possess many copies of *P* elements in the genome (Anxolabéhère *et al.* 1984; Kidwell 1985; Itoh *et al.* 1999). The Q strain carries *P* elements and have an ability to repress *P* mobilization in their progenies (low *P* susceptibility and P cytotype) (Kidwell 1981; Bingham *et al.* 1982; O'Hare *et al.* 1992). In wild-type strains, previous studies show that *KP* elements are associated with repression (Black *et al.* 1987; Jackson *et al.* 1988). It has been proven that *KP* polypeptides repress *P* transposition in M′ strains (Rasmusson *et al.* 1993; Lemaitre *et al.* 1993; Andrews and Gloor 1995; Simmons *et al.* 1996). By contrast, in both M′ and Q strains, only a weak correlation was observed between the types of genomic *P* elements and the phenotypes of the P–M system (Itoh and Boussy 2002; Itoh *et al.* 2007; Onder and Bozcuk 2012; Onder and Kasap 2014). *KP*-mediated repression and piRNA-mediated repression are also confounded (Kelleher 2016). It has been proved that weak piRNA-mediated repression enhances *KP*-mediated repression (Simmons *et al.* 2015; Simmons *et al.* 2016). Therefore, a major factor affecting the different *P* susceptibilities in the M′ and Q progenies remain unrevealed.

Ⅳ-2. Dysgenesis of Q and M' hybrid

When M-strain females are crossed with Q- or M′-strain males, *P* transposition is prevented, although the mechanisms are not fully elucidated. Thus, the Q and M′ strains have low *P* inducibility in spite of the presence of *P* elements in their genomes. It has been reported that *KP* and *SR* polypeptides produced from non-autonomous incomplete *KP* and *SR* elements, respectively, in the paternally inherited chromosomes play a role in regulating *P* transposition (Lee *et al.* 1998; Castro and Carareto 2004). The positional effects are also involved in regulating *P* inducibility (Ronsseray *et al.* 1998; Fukui *et al.* 2008); however, it is unknown whether *P*-element piRNAs produced from the paternally inherited chromosomes (zygotic piRNAs) play a role in the regulation of *P* transposition in the progeny. In particular, it is largely unclear how zygotic piRNAs are produced in Q and M′ hybrids and whether they influence the P-M phenotypes.

Ⅴ. Outline of this thesis

In chapter 1, It was proved that the level of *P*-element piRNAs was a determinant for dividing strain types between M′ and Q, using M' and Q progeny. It was also shown that the levels of *P*-element piRNA were varied between natural populations. In detail, to elucidate the molecular basis of the suppression of *P* elements in M' and Q progeny, I analyzed the mRNA and piRNA levels of *P* elements in the F1 progeny between males of a P strain and nine-line females of M′ or Q strains. The levels of *P*-element mRNA in both the ovaries and F1 embryos were higher in M′ progenies than in Q progenies, indicating that the M′ progenies have a weaker ability to suppress *P*-element

expression. The level of *P*-element mRNA was inversely correlated to the level of piRNAs in F1 embryos. Importantly, the M′ progenies were characterized by a lower abundance of *P*-element piRNAs in both young ovaries and F1 embryos. The Q progenies showed various levels of piRNAs in both young ovaries and F1 embrys despite all of the Q progenies suppressing *P*-element transposition in their gonad.

In chapter 2, I proved that the zygotic piRNAs derived from paternal *P* elements were associated with the GD of Q and M' hybrids, and that the level of production of piRNAs influenced by the genomic constitution of the paternal *P* elements were associated with the GD score of F2 progeny. In detail, to elucidate the molecular basis of *P*-element suppression from zygotic factors, I investigated the genomic constitution and *P*-element piRNA production derived from fathers. As a result, I characterized males of naturally derived Q, M′, and P strains. The amounts of piRNAs produced in the father's testes, F1-hybrid embryos, and F1-hybrid ovaries varied among strains and were influenced by the characters of piRNA clusters that harbored the *P* elements. Importantly, the Q strains produced higher levels of piRNAs in the ovaries of young F1-hybrids, which is consistent with restricted *P*-element mobilization in these ovaries. The highest expression of *KP elements* inserted into a transcriptionally active region were shown in the M′-strain. Interestingly, the zygotic *P*-element piRNAs, but not *KP* element mRNA contributed to the variation in immunity among the granddaughters against *P* transposition.

Figure 1

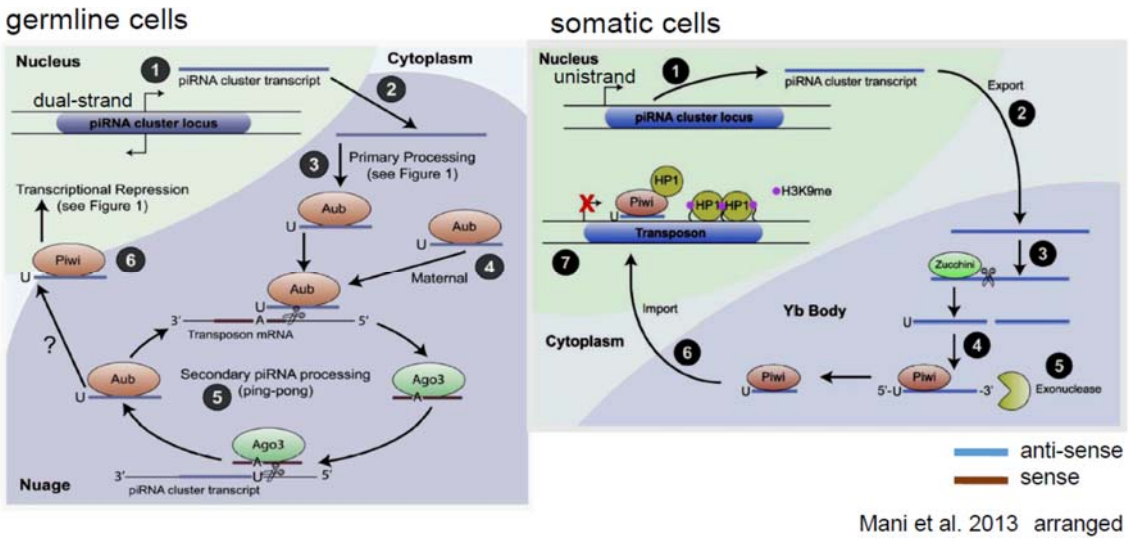

Figure legend

Figure 1. piRNA biogenesis in both germ line cells and somatic cells

Blue lines show anti-sense piRNAs and brown lines show sense piRNAs. Aub, Piwi and Ago3 are PIWI-family proteins.

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Chapter 1

Diversity of *P*-element piRNA production among M' and Q strains and its association with P-M hybrid dysgenesis in *Drosophila melanogaster*

1-1 Introduction

Transposable elements (TEs) occupy a substantial fraction of eukaryotic genomes, and their mobilization causes insertional mutations. Therefore, although such mobilization could provide genetic variations and drive genome evolution (Bennetzen *et al.* 2000; Britten *et al.* 2010), TEs could also inflict deleterious effects on the host. Piwi-interacting small RNAs (piRNAs), which are generally 23–35 nucleotides (nt) in length, suppress the expression of TEs (Brennecke *et al*. 2007). The piRNAs can be generated via primary pathways and ping-pong biogenesis (Siomi *et al.* 2011). In the primary pathway, long precursor RNAs are produced from genomic loci, chopped into 23- to 35-nt RNAs (called primary piRNAs), and loaded onto the Piwi-family of protein(s). In the ping-pong biogenesis, which is known as the ping-pong amplification cycle, the piRNA-bound Piwi-family of proteins cleaves an RNA that is complementary to the bound piRNA. The cleavage occurs at the site 10-nt away from the 5′ end of the guide piRNA, and the 3′ end of the cleaved RNA is trimmed to give a 23- to 35-nt RNA (ping-pong piRNA), which are loaded onto a Piwi-family protein to guide the next round of this complementarity-based RNA cleavage. Therefore, the two RNA species (ping-pong pairs) show a characteristic 10-nt complementarity in the respective 5′ regions, referred to as a “ping-pong signature.” If a primary piRNA has a sequence antisense to a TE, it can guide the cleavage of the mRNA of the TE. Moreover, both primary and ping-pong piRNAs can guide the introduction of repressive chromatin modifications at genomic sites complementary to them. Both the primary and ping-pong biogenesis are active in germline cells in *Drosophila* (Brennecke *et al*. 2007;

Gunawardane *et al*. 2007; Klattenhoff and Theurkauf 2008) and in other organisms (Kawaoka *et al*. 2011; Siomi *et al.* 2011). However, in the *Drosophila* soma, only the primary pathway is utilized to generate piRNAs (Malone *et al*. 2009; Saito *et al*. 2010; Olivieri *et al*. 2010; Dennis *et al*. 2013; Ross RJ *et al*. 2014; Iwasaki *et al*. 2016).

The *P* element is a DNA transposon, and their copies in the *Drosophila melanogaster* genome include structurally complete and incomplete variants. The autonomous complete elements, which are 2,907 base pairs in length, encode an 87 kDa transposase that is expressed in the germline cells (O'Hare and Rubin 1983; Rio *et al.* 1986; Engels *et al.* 1987). In *D. melanogaster*, crossing between females lacking *P* elements (M strain) and males carrying them (P strain) leads to the transposition of *P* elements in the F1 progeny (referred to as M progeny here), which causes abnormalities in the germline cells, such as gonadal dysgenesis (GD) with sterility, mutations, chromosomal breaks, and male recombination (Kidwell *et al.* 1977; Engels and Preston 1980; Rubin *et al.* 1982; Preston and Engels 1996). This phenomenon is known as P–M hybrid dysgenesis. In contrast, when P-strain females are mated with P-strain males, *P*-element mobilization is prevented by maternally deposited piRNAs in the germline cells and early embryos, which are laid by P-strain mothers but not P-progeny mothers (referred to as F1 embryos of P progenies) (Brennecke *et al*. 2008). A female's capacity to allow *P*-element transposition is defined as *P* susceptibility, which is low in the P strain but high in the M strain.

M′ and Q strains, which show different P–M phenotypes from P strains, are currently the most common in the natural populations in Eurasia, Africa,

Australia, and the Far East (Bonnivard and Higuet 1999; Itoh *et al*. 2004; Ignatenko *et al*. 2015). Although M′ progeny allows transposition of *P* elements in the germline cells (high *P* susceptibility), the M′ strains possess many copies of *P* elements in the genome (Anxolabéhère *et al.* 1984; Kidwell 1985; Itoh *et al.* 1999). The Q strain carries *P* elements and have an ability to repress *P* mobilization in their progenies (low *P* susceptibility) (Kidwell 1981; Bingham *et al.* 1982; O'Hare *et al*. 1992). In contrast to the P strain, males of the M′ and Q strains have no ability to induce transposition of *P* elements in their progeny (low *P* inducibility). In wild-type strains, previous studies show that *KP* elements, which are nonautonomous incomplete elements, are associated with repression (Black *et al.* 1987; Jackson *et al.* 1988). It has been proven that *KP* polypeptides repress *P* transposition in M′ strains (Rasmusson *et al*. 1993; Lemaitre *et al.* 1993; Andrews and Gloor 1995; Simmons *et al.* 1996). By contrast, in both M′ and Q strains, only a weak correlation was observed between the types of genomic *P* elements and the phenotypes of the P–M system (Itoh and Boussy 2002; Itoh et al 2007; Onder and Bozcuk 2012; Onder and Kasap 2014). In our previous study, I proved that one line of M′ strain, named OM5 (see methods), have many KP elements in transcriptionally active sites and only a few autonomous P elements in inactive sites of their genomes (Fukui *et al.* 2008). *KP*-mediated repression and piRNA-mediated repression are also confounded (Kelleher 2016). Previously, it has been proved that weak piRNA-mediated repression enhances *KP*-mediated repression (Simmons *et al.* 2015; Simmons *et al.* 2016). Therefore, a major factor affecting the different *P* susceptibilities in the M′ and Q progenies remain unrevealed. It is possible

that there are two hypotheses in the P–M system of M′ and Q strains as described below: (1) While neither strain contains active *P* elements to induce hybrid dysgenesis, the Q strains produce a greater number of piRNAs that enact maternal repression. (2) While M′ strains do not contain active *P* elements to induce hybrid dysgenesis, the Q strains repress dysgenesis both maternally and paternally through *KP*-mediated repression.

To study whether the production of piRNAs is involved in the difference in *P* susceptibility between M′ and Q progenies, I examined the expression levels of *P*-element piRNAs in the ovaries and whole F1 embryos. This was done by generating progenies from crossing males of a P strain and females of nine wild-type strains of the M′ or Q phenotype. I tested 2- to 3-day-old ovaries of the hybrids. These are considered to be affected by piRNAs derived from the maternally inherited *P* elements because Khurana *et al.* showed that ovaries of 2- to 4-day-old hybrids generated by a cross between M-strain females and Har males produce no piRNAs. Moreover, the 2- to 3-day-old ovaries of hybrids were suitable for the evaluation of repression of P activity since they possess zygotic *P* elements from Har in their genome. Whole F1 embryos of hybrids were used for the same reasons as ovaries. The results revealed diversity in the expression levels of *P*-element piRNAs, which were correlated with mRNA expression. Importantly, I found that the production of *P*-element piRNAs was a factor dividing *P* susceptibility between the M′ and Q strains and that these piRNA production show different characters between natural strains.

1-2 Materials and Methods

Fly stocks

Nine isofemale *Drosophila melanogaster* lines were used: OM5, FIZ12 (FIZ-12-11), KY25 (KY-13-25), KY98 (KY-13-98), KY3 (KY-02-003), KY101 (KY-02-101), HKH (Hikone-H 1957), MSO12 (MSO-12-41), and KY74 (KY-02-074). Flies were maintained on a standard cornmeal medium at 25°C in the laboratory throughout this investigation. The exception was for the GD test, where Harwich (Har) males and Canton S (CS) females were used as standard P and M strains, respectively. I used Har females as a control. These females had the capacity to repress paternal *P*-element transposition by maternally deposited *P*-element piRNAs (Brennecke *et al.* 2008).

Gonadal dysgenesis (GD) test

GD tests were used to determine the strain types in the P–M system (Engels and Preston 1979; Kidwell and Novy 1979). Two kinds of crosses, A* (tested females × Har males) and A (CS females × tested males), were performed at 28°C. By analyzing more than 50 F1 females for each line, the GD score was calculated as the percentage of females having dysgenic ovaries. The P–M strain type was determined based on GD scores in the cross A* (indicating susceptibility of *P* transposition) and those in the cross A (indicating *P* inducibility). The criteria for M′ strains were <10% GD in cross A and >10% GD in cross A*. The criteria for Q strains were <10% GD in both crosses (Kidwell 1983) (see Table 1). KY25, KY98, MSO12, and FIZ12 were tested first. I retested KY3, HKH, KY101, KY74, and OM5, because these lines had undergone many generations since the previous GD tests (Itoh *et al.* 2001).

RNA preparation

To accurately analyze the correlation bet en the number of *P*-element piRNAs and the expression level of *P*-element mRNA, both small RNAs and total RNAs were prepared from same sample, as described below. Total RNA was extracted from 2- to 3-day-old ovaries or 0- to 24-h F1 embryos with the miRNeasy kit (Qiagen). Small RNAs were separated using the RNeasy MinElute Cleanup Kit (Qiagen). 0- to 24-h embryos were generated by 30–40 couples of cross A* kept in bottles on dishes. Eight ovaries of 2- to 3-day-old F1 females were dissected. These ovaries were generated by approximately 20 couples kept in bottles for 4–7 days at the GD-inducing temperature of 28°C (Engels and Preston 1979; Kidwell and Novy 1979). In OM5 × Har, I used equal numbers of complete and dysgenic ovaries.

Small RNA sequencing

The small RNA libraries were produced using 1 μg of small RNAs with the Truseq small RNA sample preparation kit (Illumina). After PCR amplification, products of approximately 150 bp were collected from a 6% polyacrylamide gel. Single-end 50-bp sequencing of these libraries was carried out on MiSeq (Illumina).

Analysis of the obtained piRNA sequence was performed as previously described (Brennecke *et al.* 2008; Malon *et al.* 2009; Khurana *et al.* 2011) using the CLC Genomics Workbench (detailed protocol is described in https://www.qiagenbioinformatics.com/support/manuals/). After trimming of the

adaptor sequence by Transcriptomics Analysis in g_x, were moved the reads corresponding to 2SrRNA, which were included in a considerable ratios (average of 92% of total reads). To see how much of the sequencing libraries corresponded to 2SrRNAs, I examined the number of total reads, 23- to 30-nt piRNAs and 186 TE-derived 23- to 30-nt piRNAs (Table S1). Reads that were mapped to rRNAs, tRNAs, and snoRNAs were removed. The remaining reads were mapped to the *D. melanogaster* genome (Release R22) using Download Genome in g_x. RNA reads of 23–35 nts that did not match miRNA sequences in miRBase (Kozomara and Griffiths-Jones 2014) were defined as piRNAs. These sequences were then mapped to *P*-element sequences (O'Hare and Rubin 1983) and 186 transposons (total TEs) (Repbase) by Map Reads to Reference in g_x. For normalization across the samples, the read numbers of piRNAs mapped to *P* elements were divided by the total number of miRNA reads and multiplied by one million. This gave the reads per million (RPM miRNA reads). Ping-pong signatures were analyzed by per scripts (Brennecke *et al.* 2007; Ichiyanagi *et al.* 2011, Ichiyanagi *et al.* 2014).

RT-PCR and quantitative RT-PCR

cDNA was synthesized by superscript III reverse transcriptase (Invitrogen) using total RNA and oligo-dT primer. Quantitative amplification of cDNA was performed in duplicate using SYBR Green quantitation (Toyobo) on a 7000 HT Fast Real-Time PCR System (Applied Biosystems; forward and reverse primers: 5′-GTGGGAGTACACAAACAGAGTCCTG-3′ and 5′-CGTATCTGCGTGTCCGTGA AGA-3′). The level of *P*-element mRNA was normalized to that of RP49 mRNA (forward and reverse primers: 5′

-CGGATCGATATGCTAAGCTGT and 5′-GCGCTTGTTCGATCCGTA) (Dourlen *et al.* 2012).

Statistical analysis

The Pearson product-moment correlation test and hierarchical cluster analysis were performed using R. For the hierarchical cluster analyses in Figs. 1E and 3B, I used the hclust function in R (ver. 3.0.2) with the furthest neighbor method.

1-3 Results

GD test revealed two lines of M′ and seven lines of Q strains

To test their capacity to regulate the paternally inherited *P* elements in F1 ovaries, females of nine natural strains were crossed to Har males (P strain) having high *P* inducibility (cross A*). The GD scores (fraction of their daughters showing dysgenic ovaries, see Methods) in cross A* indicate the *P* susceptibility of the test strain (Table 1). F1 progeny of KY25, KY98, KY3, KY101, HKH, MSO12, and KY74 displayed GD scores of 0 to 10%, indicating that *P*-element transposition was highly repressed in their ovaries (Table 2). In contrast, OM5 and FIZ12 showed GD scores of more than 10%, indicating *P*-element transposition activity in their ovaries. I also analyzed ovaries of F1 progeny from cross A, where males of each strain were crossed to CS females (M stain) with *P* susceptibility. In all tests, F1 progeny displayed GD scores less than 1% (Table 2), indicating that *P* inducibility is very limited in the nine strains.

I classified these nine lines into two types according to the GD scores. Seven strains (KY25, KY98, KY3, KY101, HKH, MSO12, and KY74) showed low *P*

susceptibility and low *P* inducibility, and thus they were Q strains. The other two strains (OM5 and FIZ12) were classified as M′ strains due to their high *P* susceptibility and low *P* inducibility.

Various levels of ping-pong-paired piRNAs derived from *P* elements in ovaries of young dysgenic progenies

The GD test above showed that progenies from the M′ strains (M′ progenies) displayed higher *P* susceptibilities than those from the Q strain (Q progenies) and the P strain (P progenies). To examine the possibility that this variation is due to the difference in the expression level of *P*-element piRNAs in germline cells of the F1 progenies, I performed deep sequencing of small RNAs present in the ovaries of 2- to 3-day-old progenies of crosses between Har males and M′ or Q females. After removal of miRNAs and fragments of functional RNAs, small RNAs of 23- to 35 nt in length were mapped to the sequences of *P* elements to identify *P*-element piRNAs (Fig. 1A).

In all cases, I detected *P*-element-derived piRNAs in both sense and antisense directions. These piRNAs were mapped mainly to exons 0 and 1, showing that there is some sequence similarity between lines. The M′ progenies (OM5 and FIZ15) produced the lowest numbers of piRNAs compared with the Q and P progenies, except for HKH. Such a low abundance was specific to the *P* element because the total TE-derived piRNAs in the M′ progenies were comparable with those in others (Table 2). To study whether the detected piRNAs are generated via ping-pong biogenesis in germline cells, I analyzed the overlap between sense and antisense piRNAs (Fig. 1B). Indeed, a peak at 10 bp

was evident in all cases, which suggested that a substantial fraction of the piRNAs were produced via ping-pong biogenesis. Interestingly, abundance of ping-pong-paired piRNAs were less in the M′ progenies compared with the Q and P progenies, suggesting that the ability of M′ progenies to amplify and maintain piRNAs in the germline cells is weaker than that of Q and P progenies (Fig. 1C). The Q progenies expressed various amounts of ping-pong piRNAs. These amounts were comparable with those in the P progenies and highlight that the higher ability to repress the *P* element is associated with a higher expression of ping-pong-paired piRNAs in the ovaries. In particular, KY101 progenies showed quite high amounts of ping-pong-paired piRNAs produced from *P* elements.

I next determined the levels of *P*-element mRNA in these ovaries by reverse transcription followed by quantitative PCR (qRT-PCR). The average expression levels of ovarian *P*-element mRNA was 0.1-fold lower than in embryonic *P*-element mRNA in 10 progenies. The mRNA levels varied between the progenies, with a tendency for the M′ progenies to show higher expression than the Q progenies (Fig. 1D). Furthermore, I repeated the qRT-PCR three to five times in four lines of M′ and Q strains and ensured that there was significantly higher expression of *P*-element mRNA in M′ (OM5) progenies compared with that in Q progenies (KY3, KY101 and KY74; p = 0.03, 0.003 and 0.05, respectively; Fig. 4). However, ovaries of KY3 (Q) progenies showed a high score of standard deviation (SD = 0.3). This suggests that individuals of KY3 progenies differ in their expression level of *P* elements. Importantly, the two M′ progenies were clustered in hierarchical clustering of *P*-element mRNA and

P-element ping-pong piRNA expression levels (Fig. 1E). These results favor an idea that the level of ping-pong-paired piRNAs is one determining factor for the expression level of *P* elements in natural populations.

M′ progenies were characterized by a low ability to produce ping-pong-paired piRNAs and high levels of *P*-element expression in the ovaries. While Q progenies were distinguished from M′ progenies by the amount of ping-pong-paired piRNAs and the levels of *P*-element expression, they showed variable levels of expression of piRNAs and mRNA.

Various levels of ping-pong-paired piRNAs derived from *P* elements in F1 embryos of progenies

To study the possible involvement of piRNAs in the regulation of the paternally inherited *P* elements during embryogenesis of the F1 progeny, I next analyzed *P*-element piRNAs and mRNA in whole F1 embryos (<24 h after hatching) of progenies of cross A*. It has been proven that *P*-element piRNAs produced in F1 embryos of hybrids between M-strain females and Har are very limited (Khurana *et al.* 2011). In contrast, I detected *P*-element piRNAs in whole F1 embryos of M′, Q, and P progenies (Table 2). There was a considerable variation in the abundance. The M′ progenies again showed the lowest abundance of *P*-element piRNAs although they produced total TE-derived piRNAs at levels similar to those in the Q and P progenies (Table 2). Analysis of sense and antisense piRNAs revealed that ping-pong-paired piRNAs are generally lower in whole F1 embryonic bodies than in ovaries. In particular, the two M′ progenies, in addition to KY98, KY3, and HKH progenies, produced a fewer number of

ping-pong-paired piRNAs (Fig. 2B and C). It is possible that some of the strange discrepancies with ovarian piRNAs from the same lines are caused by the limited power to accurately estimate the ping-pong fraction. This could be due to the production level of total-TE-derived piRNAs in F1 embryonic bodies being less than those in the ovaries (Table 2). Therefore, the level of total *P*-element piRNAs was evaluated to compare differences between lines, as below.

I investigated whether the expression of *P*-element mRNA was associated with the production of piRNAs derived from *P* elements in whole F1 embryos of the natural strains. I quantified *P*-element mRNA in the F1 embryonic bodies. This revealed that *P*-element expression is somewhat higher (not significantly) in M′ progenies compared with Q progenies (Fig. 2D). I repeated qRT-PCR three times in five lines of M′ , Q, and P strains and ensured that there was a significantly higher expression of *P*-element mRNA in M′ (OM5) progenies compared with those in the Q progenies (KY3, KY101, and KY74; $p < 0.05$) (Fig. 5A and B). Furthermore, 10 lines were classified into P, M′ , and Q strains, and it was determined that the mRNA expression level was negatively correlated to the expression level of total *P*-element piRNAs ($R = -0.88$, $p < 0.01$; Fig. 2D). I made sure that this negative correlation between the total *P*-element piRNAs and the mRNA level was analyzed by three biological replicates for five progenies ($R = -0.9$, $p < 0.05$; Fig. 5). These results suggest that cells in the F1 embryonic bodies produce piRNAs mainly via the primary pathway and that these primary piRNAs play a role in *P*-element regulation during embryogenesis.

M′ strains were characterized by the lowest production of

ping-pong-paired piRNAs in both young adult ovary and F1 embryonic bodies

The above results showed a tendency that ping-pong-paired *P*-element piRNAs in the ovary and the total *P*-element piRNAs in F1 embryos are less in the M′ progenies than in the Q and P progenies. To reveal whether there were clear differences in the amount of piRNAs derived from *P* elements between the M′ progenies and others, I did clustering analysis *P*-element ping-pong piRNAs production in the ovaries and total *P*-element piRNAs in F1 embryos of progenies. Actually, M′ progenies were characterized by the lowest production of *P*-element piRNAs in both the young adult ovary and in F1 embryonic bodies. For the Q and P progenies, KY101, Har, KY25, KY98, and KY3 showed higher production of *P*-element *P*-element piRNAs in young adult ovaries, while HKH, MSO12, and KY74 produced higher levels of *P*-element piRNAs in the F1 embryos (Fig. 3).

1-4 Discussion

Although the natural population of D. melanogaster generally carries P elements in their genome, the progeny displays a different resistance capacity against P elements as introduced upon hybridization with typical P strains. Here, I showed that the M′ strains distinguished from the Q strains by low levels of *P*-element piRNA production in both the ovaries and the F1 embryos of dysgenic progenies, and that this is associated with a low ability to suppress *P*-element transcription. This character of M′ strains is likely related to their high level of GD, which is linked to *P*-element transposition. In contrast, it was shown that the

Q progenies produced various degrees of *P*-element piRNAs. This could confer the ability to resist *P*-element expression in embryonic bodies. However, such varied production of *P*-element piRNAs among Q progenies did not induce different levels of GD.

Interestingly, M′ progenies of the two lines, which showed moderate scores of GD in cross A*(10%–30%) indicating partial repression of *P* transposition, produced *P*-element piRNAs in young adult ovaries at some degree. In I–R hybrid dysgenesis, the levels of *I*-element piRNAs inversely correlated with dysgenic scores (Ryazansky *et al.* 2017). While it has been reported that other repressive factors for *P*-element transposition, such as proteins produced from full-length (type I, 66-kDa repressors) and internally deleted elements (type II, *KP* repressors), play a role in germline cells to some degree, our results suggest that the level of *P*-element piRNAs in the M′ progenies is one major determinant of the *P* susceptibility, which is in addition to the P–M phenotype in M′ strains, as shown in the I–R system. Further studies are necessary to investigate M′ strains having various levels of *P* susceptibility. Why the M′ progenies are not able to produce abundant *P*-element piRNAs despite the presence of *P* elements in their maternal genomes? It is thought that piRNAs are inherited from the oocytes of the mothers and is imparted to the F1 progenies. These inherited piRNAs act to prime the ping-pong amplification cycle in the germline cells of the daughters. Thus, it is possible that the maternal lineage of the M′ strains does not produce abundant piRNAs. To produce both primary and ping-pong piRNAs, a genomic situation is required where *P* element(s) are located in the piRNA clusters (Brennecke *et al.* 2007). Therefore, the copy

number of *P* elements in the piRNA clusters is likely less in the genomes of the M′ strains, resulting in a reduced level of *P*-element piRNA production. Previously, it has been proven that autonomous complete P elements in M′ strains are transcriptionally inactive (Fukui *et al.* 2008). Therefore, the other possibility is that such P elements are repressed in M′ -strain parents and may not contribute to resistance against P elements introduced upon hybridization with typical P strains. Future studies, such as piRNA profiling of oocytes of mothers, will evaluate these possibilities.

For the Q strains, despite their resistance to paternal *P* elements, there was considerable variation in the mRNA and piRNA expression levels of *P* elements in both the ovary and the F1 embryonic bodies. Therefore, in Q strains, the molecular basis of production of *P*-element piRNAs affecting the P–M phenotype is likely different from that in I–R hybrid dysgenesis. In particular, progeny of KY101 showed higher production of *P*-element ping-pong-paired piRNAs in the ovaries, suggesting that piRNAs act as a main suppressor during oogenesis. F1 embryos of MSO12 and KY74 progenies produced abundant *P*-element piRNAs, including ping-pong-paired piRNAs, and lower levels of *P*-element mRNA. This suggested that piRNAs act as one of the main suppressors during embryogenesis. Other Q progenies were classified into two groups that were characterized by KY101 and KY74, as discussed above. They allowed the expression of the *P*-element mRNA at levels similar to those in the M′ progenies. This would imply that other factors, such as protein repressors, are involved in the repression of *P*-element transposition in the Q progenies (Castro and Carareto 2004). It is also possible that individuals could differ in their

sensitivity to germline P activity (higher for M′ progenies and lower for Q progenies), resulting in different severities of hybrid dysgenesis under equivalent levels of transpositional activity. Furthermore, whole F1 embryos are composed of germ line cells producing ping-pong piRNAs and somatic cells producing antisense piRNAs. Thus, further studies are required to address the varied expression of both *P*-element piRNAs and mRNA in Q progenies, including the effect from embryonic somatic cells and germ line cells. Interestingly, Har progeny was in the same group as KY101 progeny, which showed a higher production of P-element ping-pong-paired piRNAs in the ovaries. It is possible that those Q and P progenies have P elements inserted into germ-specific piRNA clusters, which produce ping-pong-paired piRNAs. Thus, in the ovaries of Q and P progenies, ping-pong-paired piRNAs likely act to suppress P elements introduced upon hybridization with typical P strains. On the other hand, males of the P strain have a high ability to mobilize P elements in their progeny when they are mated with M-strain females; this is in contrast to what is found in the Q strain. Therefore, the P strain may possess many P elements in active expression sites of the genome. Another possibility is that the P strain produces lower levels of zygotic piRNAs derived from paternal P elements. More investigation into the insertion site of P elements and P inducibility is required. Furthermore, since *P*-element-derived piRNAs exhibited similar sequences in all lines, piRNA biogenesis may not differ between lines.

In conclusion, our results suggest that piRNA abundance explains coarse phenotypic differences between M' and Q cytotypes with respect to P-repression, but not more modest differences between Q strains. Whether this piRNA

variation originates from genetic diversity, such as copy number and location of *P* elements, or from long-term inheritance of small RNAs may be an interesting question. Moreover, our results evoke an interesting possibility that the suppression mechanisms of TEs including piRNAs are varied in natural populations.

1-5 Figures

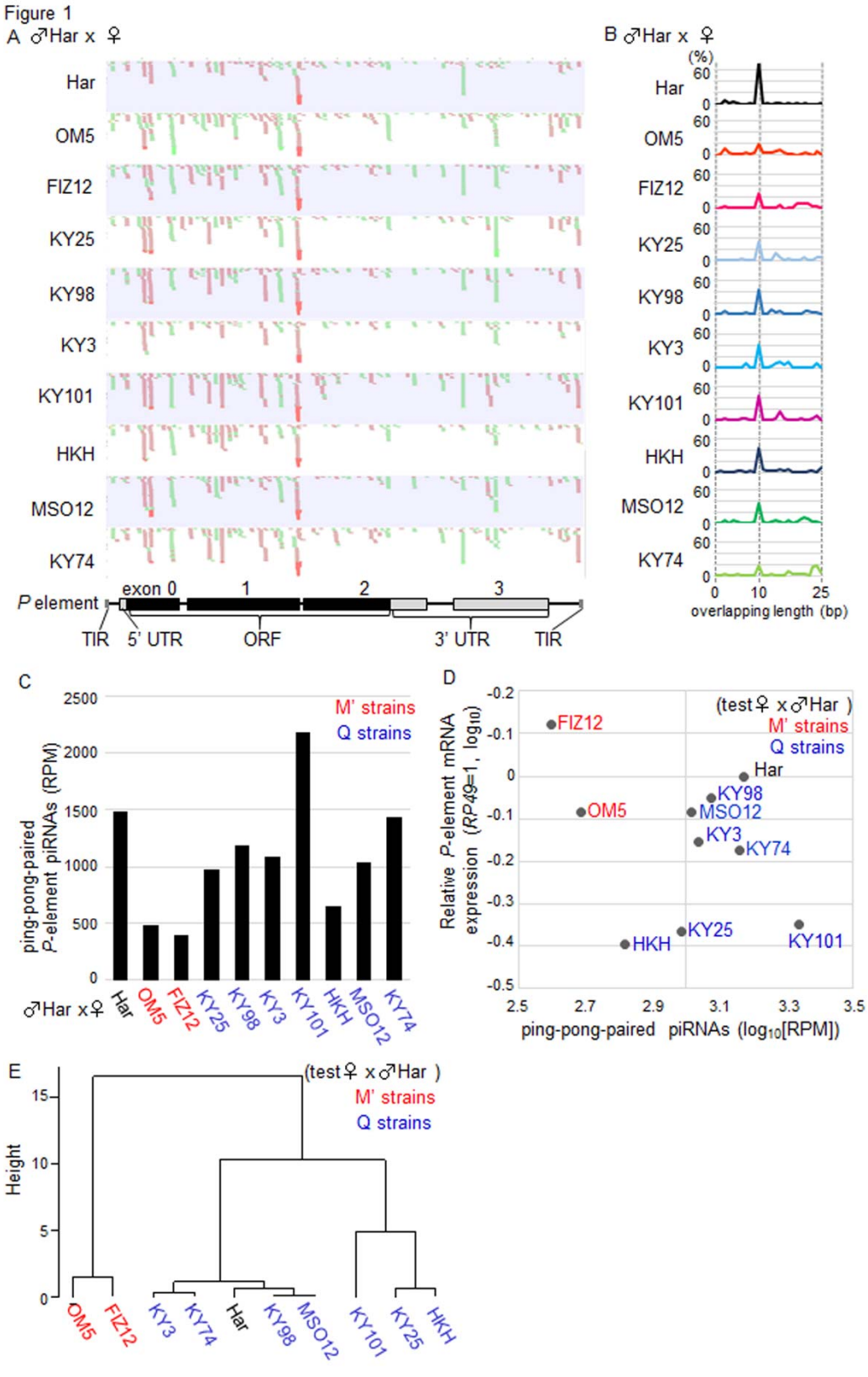


Figure 1
A ♂Har x ♀
Har
OM5
FIZ12
KY25
KY98
KY3
KY101
HKH
MSO12
KY74
P element
exon 0
1
2
3
TIR
5' UTR
ORF
3' UTR
TIR
B ♂Har x ♀
(%)
60
0
0
10
25
overlapping length (bp)
C
2500
2000
1500
1000
500
0
ping-pong-paired P-element piRNAs (RPM)
M' strains
Q strains
♂Har x♀
Har
OM5
FIZ12
KY25
KY98
KY3
KY101
HKH
MSO12
KY74
D
(test♀ x♂Har)
M' strains
Q strains
Relative P-element mRNA expression (RP49=1, log10)
-0.2
-0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
2.5
2.7
2.9
3.1
3.3
3.5
ping-pong-paired piRNAs (log10[RPM])
E
(test♀ x♂Har)
M' strains
Q strains
Height
15
10
5
0
OM5
FIZ12
KY3
KY74
Har
KY98
MSO12
KY101
KY25
HKH

Figure 2

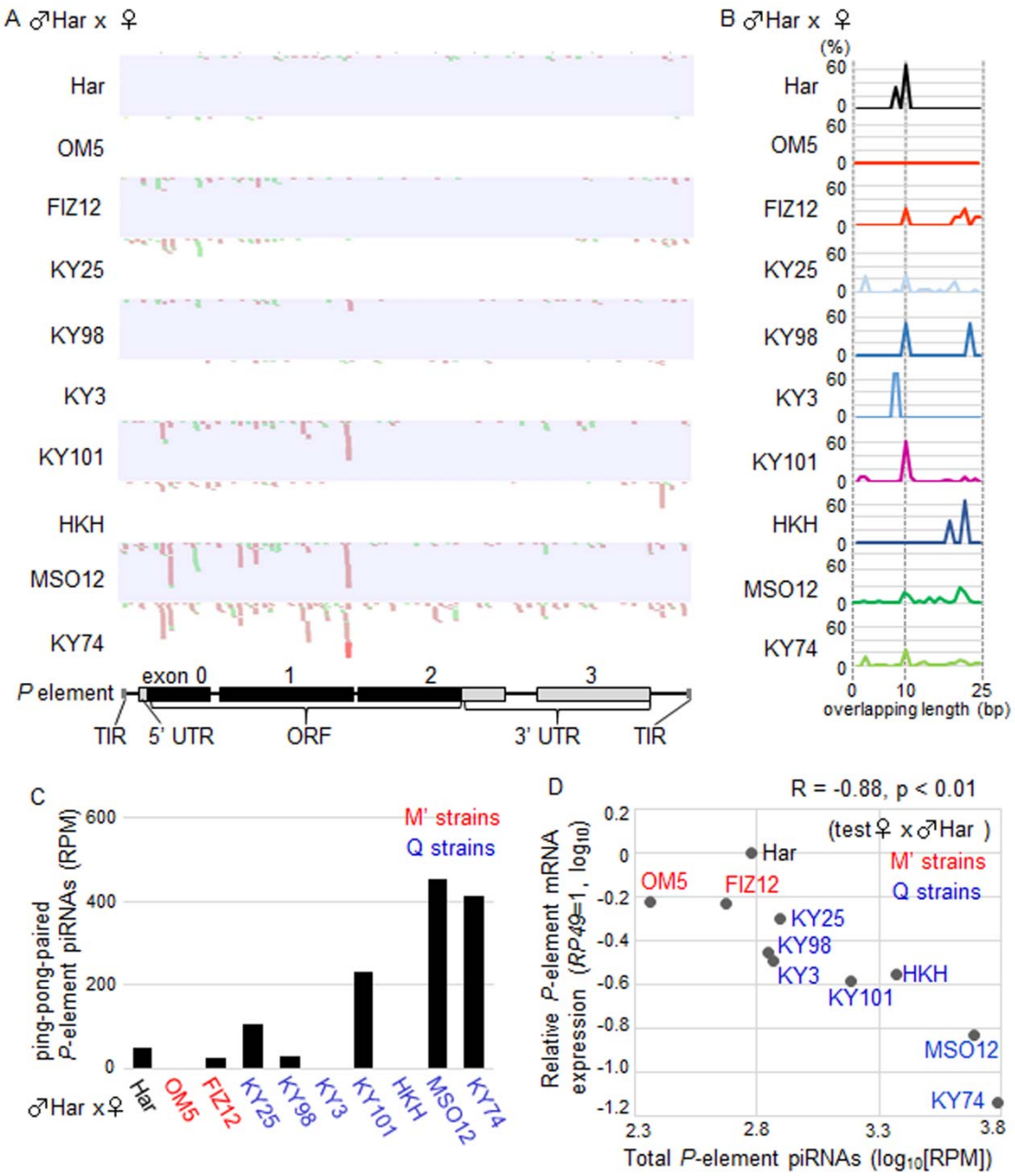

A ♂Har x ♀
Har
OM5
FIZ12
KY25
KY98
KY3
KY101
HKH
MSO12
KY74
P element
exon 0
1
2
3
TIR
5' UTR
ORF
3' UTR
TIR
B ♂Har x ♀
(%)
60
0
overlapping length (bp)
C
ping-pong-paired P-element piRNAs (RPM)
600
400
200
0
M' strains
Q strains
♂Har x♀
D
R = -0.88, p < 0.01
(test♀ x♂Har)
Relative P-element mRNA expression (RP49=1, log10)
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
2.3
2.8
3.3
3.8
Total P-element piRNAs (log10[RPM])

Figure 3

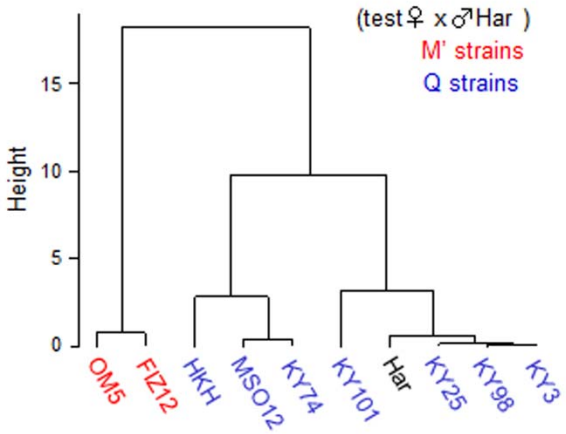


Figure 4

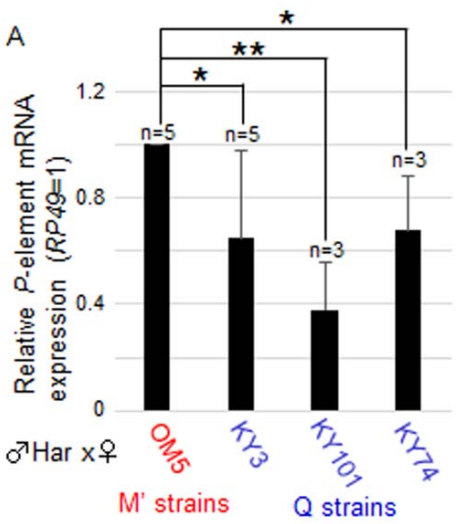
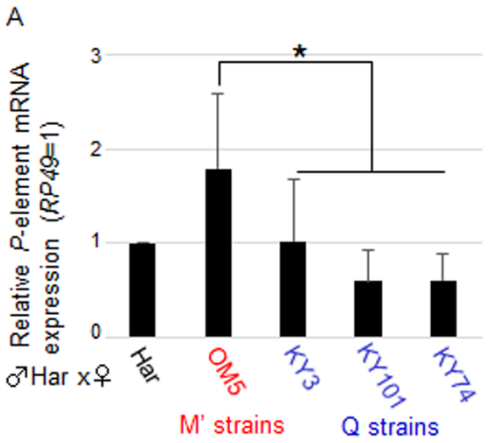


Figure 5



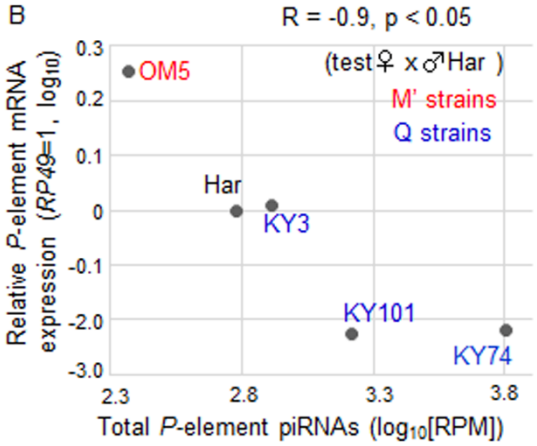


Table 1

Strain types in the P-M **system**

P susceptibility	*P* inducibility	
high: >10%GD	low: <10%GD	strain type
low: <10%GD	high: >10%GD	
high	low	M’
low	low	Q
low	high	P
high	low	M (*P*-elements (-))

Drosophila melanogaster is divided into the four strain types by GD ratios. *P* susceptibility shows the regulatory capacity against the *P*-elements and *P* inducibility exhibits the ability to transpose *P*-elements in progeny.

Table 2

GD ratios and total *P*-element piRNAs production in the progeny

Test strain	GD[a] (%) cross A* (♀test x ♂Har)	GD[a] (%) cross A (♀CS x ♂test)	Deduced strain type	*P*-element piRNAs (RPM)[bb] cross A* (♀test x ♂Har)		total-TE piRNAs (RPM)[bb] cross A* (♀test x ♂Har)	
				Ovaries	F1 embryos	Ovaries	F1 embryos
OM5	28.3	0	M'	5,137	233	1,105,901	139,280
FIZ12	13.3	1	M'	6,108	522	1,158,677	138,991
KY25	0	0	Q	7,333	740	1,428,653	130,670
KY98	0	1	Q	9,356	830	1,502,704	143,827
KY3	2.5	0	Q	8,049	818	1,060,199	144,297
KY101	0	0	Q	18,009	1,662	1,958,902	109,059
HKH	0	0	Q	4,989	2,421	1,048,057	417,233
MSO12	0	0	Q	8,941	5,200	1,351,448	521,266
KY74	0.8	0	Q	9,077	6,336	1,343,427	425,912
Har	0	100	P	7,780	604	1,090,850	99,772

[a] Percentage of gysgenic ovaries from cross A* (test female x Har male) and cross A (CS female x test male). [b] piRNA reads were divided by miRNA reads, expressed as reads per million miRNA reads (RPM) in the progeny from cross A*.

Figure legends

Figure 1. Expression of piRNA and mRNA of *P* elements in adult ovaries of F1 progenies in cross A*. (A) Small RNA reads (23–35 nt in length) mapped to the sense (green) and antisense (red) strands of the *P* element are shown on the *P*-element structure (bottom). Har (top) was a P strain and used as a control. (B) Frequencies of length (0–25 bp, *x*-axis) of overlapping regions between sense and antisense small RNAs (23–35 nt) identified in ovaries of F1 progenies. An overlap of 10 bp is a signature of piRNA pairs produced via the ping-pong cycle. (C) The expression levels of ping-pong-paired piRNAs in F1 ovaries normalized by miRNA (reads per million [RPM] miRNAmiRNA reads). The strain names of mothers are shown in black (P), red (M′), and blue (Q). (D) The relationship between the log expression levels of mRNAs (*y*-axis) and ping-pong-paired piRNAs (*x*-axis) of *P* elements in F1 ovaries. The strain names of mothers are shown in black (P), red (M′), and blue (Q). The Pearson's correlation efficient is shown on the top. (E) A tree of hierarchical clustering of the nine natural strains and the Har strain based on the data shown in panel C. The strain names of mothers are shown in black (P), red (M′), and blue (Q). The M′ strains are clustered together.

Figure 2. Expression of piRNA and mRNA of *P* elements in F1 embryonic bodies of F1 progenies in cross A*. (A) Small RNA reads (23–35 nt) mapped to the sense (green) and antisense (red) strands of the *P* element are shown on the *P*-element structure (bottom). Har (top) was a P strain as a control. (B) Frequencies of length (0–25 bp, *x*-axis) of overlapping regions between sense and antisense small RNAs (23–35 nt) identified in F1 embryos. An overlap of 10

bp is a signature of piRNA pairs produced via the ping-pong cycle. (C) The expression levels of ping-pong-paired piRNAs in F1 ovaries (reads per million [RPM] miRNa reads). The strain names of mothers are shown in black (P), red (M′), and blue (Q). (D) The relationship between the log expression levels of mRNAs (*y*-axis) and piRNAs (*x*-axis) of *P* elements in F1 ovaries. The strain names of mothers are shown in black (P), red (M′), and blue (Q). The Pearson's correlation efficient is shown on the top.

Figure 3. Characterization of the natural strains based on piRNA levels in F1 progenies. Relationship between the expression levels (RPM) of *P*-element ping-pong-paired piRNAs in F1 ovaries and total *P*-element piRNAs in embryos. Hierarchical clustering of the nine strains and the Har.

Figure 4. Expression of mRNA of *P* elements in F1 ovaries of progenies of file lines

The expression levels of *P*-element mRNA in F1 ovaries (*RP49*=1). The strain names of mothers are shown in black (P), red (M') and blue (Q) (* $p<0.05$, **$p<0.01$).

Figure 5. Expression of piRNA and mRNA of *P* elements in F1 embryonic bodies of progenies of file lines

(A) The expression levels of *P*-element mRNA in F1 embryos (*RP49*=1). The strain names of mothers are shown in black (P), red (M') and blue (Q) (* $p<0.05$). (B) The relationship between the log expression levels of mRNAs (y-axis) and piRNAs (x-axis) of *P* elements in F1 embryos. The strain names of mothers are shown in black (P), red (M') and blue (Q). The Pearson's correlation efficient is shown on the top. (qRT-PCR was repeated three times in five lines (n =3).

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Chapter 2

Association of zygotic piRNAs derived from paternal *P* elements with hybrid dysgenesis in *Drosophila melanogaster*

2-1 Introduction

Transposable elements (TEs) are major structural constituents **of** eukaryotic genomes. Although their mobilization provides genetic variation and drives genome evolution (Bennetzen *et al.* 2000; Britten *et al.* 2010), TEs exert deleterious effects on the host. For example, TE mobility in *Drosophila melanogaster* causes germline abnormalities known as hybrid dysgenesis (see below for details). The host counteracts this deleterious effect through various pathways, including Piwi-interacting small RNAs (piRNAs).

piRNAs are small non-coding RNAs that are generally 24–35 nucleotides (nt) long and act to suppress TE expression (Brennecke *et al.* 2007). piRNAs are generated from particular genomic loci, called piRNA clusters that consist of many TEs. Two types of piRNA clusters have been identified in *D. melanogaster*; dual-strand and unistrand clusters are dominant in germline cells and somatic cells, respectively. In the dual-strand piRNA cluster, transcription occurs in both directions to produce long precursor single-stranded RNAs that are subsequently chopped into 24- to 35-nt RNAs. These are loaded onto Piwi-family proteins to direct the cleavage of complementary RNAs, including TE mRNAs. The cleaved RNAs are then loaded onto a Piwi family protein to aid in the cleavage of complementary based RNA, a reaction known as the “ping-pong cycle.” In the unistrand piRNA cluster, long precursor RNAs are transcribed in a single direction. Because TEs are inserted predominantly into the unistrand piRNA clusters in the reverse orientation to the precursor transcription, they can serve as a source of TE-derived antisense piRNAs that are used by the PIWI protein to induce repressive chromatin modification

(Malone *et al.* 2009; Saito *et al*. 2010; Olivieri *et al*. 2010; Dennis *et al.* 2013; Ross *et al.* 2014; Iwasaki *et al.* 2016). Owing to these biogenesis pathways, piRNAs are generated and retained primarily in the cytosol, although a fraction of them are transported into the nucleus. One hundred forty-two piRNA clusters have been identified in the *D. melanogaster* genome, but piRNA production levels supplied by these clusters are highly variable (Brennecke *et al.* 2007). Clusters with high piRNA production are called active piRNA clusters, while others are referred to here as low activity piRNA clusters.

P elements are DNA transposons that propagate in the *D. melanogaster* genome and include both structurally complete and incomplete variants. Autonomous 2,907-bp complete elements encode an 87-kDa transposase, for which expression can be detected in germline cells (O'Hare and Rubin 1983; Rio *et al.* 1986; Engels *et al.* 1987). *P* elements are responsible for a phenomenon called "P-M hybrid dysgenesis." Progeny of a cross between an M-strain female with no *P*-element and a P-strain male carrying complete *P* elements demonstrate increased frequencies of *P*-element transposition resulting in germline cell abnormalities. These abnormalities can include gonadal dysgenesis (GD) with sterility, chromosomal breaks, mutations, and male recombination (Kidwell *et al.* 1977; Engels and Preston 1980; Rubin *et al.* 1982; Preston and Engels 1996). Therefore, although recent reports argue against the involvement of the *P* transposition in GD (Dorogova *et al.* 2017 and Ignatenko *et al.* 2015), previous reports indicate that P-strain males have a high ability to mobilize *P* elements in their progeny (high *P* inducibility), and M-strain females are not able to repress *P* transposition (high *P* susceptibility) (Kidwell 1981;

Bingham *et al.* 1982; Anxolabéhère *et al.* 1984; Kidwell 1985; O'Hare *et al.* 1992; Itoh *et al.* 1999).

When P-strain males are mated with P-strain females, *P*-element mobilization in the germline cells of their progeny is prevented by maternally deposited repressors (Engels, 1979; Simmons *et al.* 2016); therefore, P-strain females have low *P* susceptibility. It has been proposed that the GD phenotype in female progeny (i.e., *P* susceptibility) is determined largely by cytoplasmic factor(s) in the maternal oocytes, rather than by the genotype of either the daughters or the mothers. Thus, the oocytes are distinguished as "cytotypes." M-strains females produce oocytes of "M cytotype," which produce dysgenic daughters when crossed with a P-strain male. P strain females produce oocytes of "P cytotype," which produce normal daughters. The major molecular entity that determines the P-M cytotype in oocytes has been proposed as a cytosolic *P*-element piRNA that is inherited by the daughters to suppress *P* transposition (Brennecke *et al.* 2008; Khurana *et al.* 2011). It also has been reported that *P* mobilization in progeny is controlled by other factors, such as proteins produced from full-length (type I, 66-kDa repressors) and internally deleted elements (type II, *KP* repressors) (Black *et al.* 1987; Rasmusson *et al.* 1993; Lemaitre *et al.* 1993; Andrews and Gloor 1995; Simmons *et al.* 1996; Simmons *et al.* 2002; Simmons *et al.* 2015; Simmons *et al.* 2016). The *KP* elements, non-autonomous incomplete variants with a nucleotide deletions at 808–2060, are present ubiquitously in natural populations (Brack *et al.* 1987; Rasmusson *et al.* 1993; Itoh *et al.* 2007) and supply the most common type II repressor protein that inhibits *P*-element transposition (Rio, 1990; Rasmusson *et al.* 1993; Lemaitre *et al.* 1993; Andrews

and Gloor 1995; Simmons *et al.* 1996 Lee *et al.* 1996; Simmons *et al.* 2002; Samany and Locke. 2011).

The Q and M′ strains have distinct characteristics from P and M strains and are of great interest. The M′ strains carry *P*-element copies or *P*-element-like copies in their genomes, but they behave as M strains. Thus, when M′-strain females are crossed with P-strain males, *P*-elements are transposed. The Q strains also carry P elements that are not mobilized, even upon paternal transmission. The difference between M′ and Q is the P susceptibility; when Q-strain females are crossed with P-strain males, *P* transposition is prevented. In a previous study, I proved that the M′-strain progeny produced lower levels of maternal piRNAs than the Q-strain progeny (Wakisaka *et al.* 2017). On the other hand, when M-strain females are crossed with Q- or M′-strain males (Q or M′ hybrids), *P* transposition is prevented, although the mechanisms are not fully elucidated. Thus, the Q and M′ strains have low *P* inducibility, despite the presence of *P* elements in their genomes. The Q and M′ strains are most common in the natural populations in Eurasia, Africa, Australia, and the Far East (Bonnivard and Higuet 1999; Itoh *et al.* 2004; Ignatenko *et al.* 2015). It has been reported that *KP* and *SR* polypeptides, produced from non-autonomous incomplete *KP* and *SR* elements, respectively, and found on the paternally inherited chromosomes, play an important role in regulating *P* transposition (Lee *et al.* 1998; Castro and Carareto 2004). The positional effects also are involved in regulating *P* inducibility (Ronsseray *et al.* 1998; Fukui *et al.* 2008); however, it is unknown whether *P*-element piRNAs produced from the paternally inherited chromosomes (zygotic piRNAs) play a role in the regulation of *P* transposition in

the progeny. In particular, it is largely unclear how zygotic piRNAs are produced in Q and M′ hybrids, and whether they influence the P-M phenotypes. Furthermore, although the abilities of the F2 hybrids to suppress *P* transposition are considerably varied (Kidwell 1983, Kidwell *et al.* 1988), it is unknown how a male genome contributes to the immunity of the produced granddaughters.

In the present study, I used four fly lines from wild-sampled Q, M′, and P strains as paternal lines, and then analyzed the following points to elucidate the paternal effects on the P-M phenotype: (1) the effects of the paternally inherited genome on the cytotype of F1 oocytes, (2) the fraction of each *P*-element type (e.g., *FP*, *KP*, and non-*KP*) present in the respective genomes, (3) the expression levels of *P* and *KP* elements, (4) the genomic positions of their insertions and the transcriptional activity of these insertion sites, (5) the number of *P* elements embedded in each piRNA cluster, and (6) the amount of piRNA production in whole embryos and ovaries of the F1 progeny, obtained by crossing with an M-strain female. As a result, I revealed that the paternally inherited Q and M′ genomes can serve as sources of zygotic piRNAs in the progeny, even at young ages; the amounts vary depending on the *P* elements embedded in the piRNA clusters. These zygotic piRNAs acted to reduce the amount of *P*-element mRNA. Furthermore, these piRNAs affected the P-M phenotypes of the F2 progenies. Thus, upon paternal inheritance, the Q and M′ genomes can co-transmit these *P*-element piRNA–generating immunity loci with complete *P* elements. In addition, high ratios of *KP* element transcription in the Q and M′ genomes likely are associated with the repressive transcriptional states of genomic regions surrounding *P* elements and appear to play a regulatory role. Moreover, the

Q-strain males conferred immunity against *P*-element transposition to their granddaughters, which not only underscores the important role of piRNA cluster-inserted *P* elements in the regulation of *P*-element transposition, but also offers a genetic basis for the prevalence of Q-type flies in natural populations.

2-2 Materials and Methods

Fly stocks

The following nine isofemale *D. melanogaster* lines were used: M′-OM5 (Fukui *et al.* 2008) as the M′ strain; Q-KY74 (KY-02-074) and Q-KY101 (KY-02-101) as the Q strains, and Q-HKH (Hikone-H 1957) (Gamo *et al.* 1990) as the Q strains in part. Harwich (P-Har) males and Canton S (M-CS) females were used as standard P and M strains, respectively.

Gonadal dysgenesis test

GD tests were used to determine *P* inducibility and *P* susceptibility in the P-M system (Engels and Preston 1979; Kidwell and Novy 1979) Two types of crosses were performed as follows: cross A (M-CS females x tested males) and cross A* (tested females x P-Har males). One- to four-day-old hybrid females of each line were dissected at same time. By analyzing approximately 50 F1 or 100 F2 hybrid females from each line, the GD score was calculated as the percentage of females having dysgenic ovaries. For the analysis of F1 hybrid GD scores, test males were crossed with M-CS and maintained at 28°C where GD becomes obvious. For the analysis of F2 hybrid GD scores, F1 hybrid were maintained at 25°C because they are fertile at this temperature, then the F1 hybrid females

were crossed with P-Har and the F2 progeny was incubated at 28°C.*P* inducibility was determined by GD scores in cross A. The criteria for low *P* inducibility was GD < 10.0%. *P* susceptibility was determined in cross A*. The criteria for low *P* susceptibility was GD < 10.0% in cross A* (Kidwell 1981).

PCR and quantitative PCR

Genomic DNA was extracted from whole bodies of 20–40 flies from each line with standard methods (Sambrook *et al.* 1989). These DNAs were used for polymerase chain reaction (PCR) as a template with two sets of primers: one to amplify total *P* elements and the other to amplify non-*KP* elements. The PCR products then were sequenced. Quantitative amplification of DNA was performed, using primer pairs specific to the *KP* element and total *P* element, respectively. The single-copy *RP49* gene was used for normalization (Dourlen *et al.* 2012). Details are shown in the supplementary material.

Deep sequencing of the *P*-element insertion site

The genomic insertion sites for *P* elements were amplified according to the protocol of Tsukiyama *et al.* (2013) with minor modifications. The genomic DNA extracted from 40 adult flies was digested with *Hha*I or *Taq*I (TaKaRa, Japan) and ligated to overhanging adapters. Using these ligation products as a template, PCR was performed with primers specific to the adaptor and to *P* elements respectively. Nested PCR was performed to specifically amplify P-element-containing PCR fragments. About 300- to 600-bp-long *Hha*I and *Taq*I products were purified from an agarose gel, and used for preparation of deep

sequencing libraries with the TruSeq DNA PCR-Free LT Library Prep Kit (Illumina, California, USA). Pair-end 250-bp sequencing was performed on the MiSeq system (Illumina). Details are shown in the supplementary file.

Analysis of insertion site

The obtained deep-sequencing data was analyzed, as previously described (Khurana *et al.* 2011), using the CLC Genomics Workbench (QIAGEN Bioinformatics, Denmark; detailed protocol: https://www.qiagenbioinformatics.com/support/manuals/) with minor modifications. Reads with no *P*-element sequence were discarded. Adaptor sequences were removed by the "transcriptome analysis" function in $g_x$and then the sequences were mapped to the *D. melanogaster* genome (Release 5) using the "download genome" function in g_x to identify insertion sites for *P* elements. To normalize the occupancyof each insertion site in the population, the number of reads supporting respective insertion sites was divided by the total reads. The transcriptional states of the identified genomic sites were analyzed with *D. melanogaster* Genome Browser in ModENCODE (Generic Genome Browser, v. 2.52; GMOD), and the read numbers of *P* elements inserted into piRNA clusters were analyzed according to Brennecke *et al.* (2007).

Analysis of piRNA clusters

The piRNA clusters were divided into two groups (unistrand and dual-strand) according to percentages of piRNA strand distribution, as reported by Brennecke *et al.* (2007). The 142 genomic locations are shown as sites of abundant piRNA

generation in *Drosophila* ovaries. If both sense and antisense strands in the piRNA cluster are >20.0%, I considered the piRNA cluster to be “dual-stranded,” with the ability to produce both strands of piRNA. Others were considered unistrand. The active piRNA clusters are the top 15 clusters ranked by the number of cluster-unique piRNAs (Brennecke *et al.* 2007).

RNA preparation

Total RNA was extracted from 0-h to 24-h embryos from forty cross A couples with the miRNeasy Kit (QIAGEN), and small RNAs were separated using the RNeasy MinElute Cleanup Kit (QIAGEN). F1 females from 20 couples were grown at the GD-inducing temperature of 28°C for 4–7 days (Engels and Preston 1979; Kidwell and Novy 1979), and then total RNA was extracted from the approximately 8 normal ovaries of those female progeny at 2- to 3-days old. The dysgenic ovaries of P-Har hybrids were dissected from approximately 100 females. The testes were dissected from 60 males of each line, and total RNA was extracted from the pooled testes.

Small RNA sequencing

Small RNA libraries were prepared using 1 μg of small RNAs with the TruSeq Small RNA Sample Preparation Kit (Illumina). After PCR amplification, products of approximately 150 bp were extracted from a 6% polyacrylamide gel. Single-end 50-bp sequencing of these libraries was performed using the MiSeq system (Illumina). The obtained small RNA reads were analyzed and annotated as described previously (Wakisaka *et al.* 2017).

RT-PCR and quantitative RT-PCR

cDNAs were synthesized by superscript III reverse transcriptase (Invitrogen) using total RNA and an oligo-dT primer. Quantification of cDNAs was performed by real-time PCR using primer pairs specific to the *KP* element and total *P* element, respectively. Details are shown in supplementary materials.

Statistical analyses

Pearson product–moment correlation tests were conducted using R (ver. 3.0.2).ierarchical cluster analyses were conducted using R and Excel with the hclust function (the furthest-neighbor method). Student *t* tests were conducted using Excel.

Data availability

Sequence data are available at DDBJ under the accession number, PRJDB5877.

2-3 Results

The effects of the paternal genome on the P-M system

To study the effects of the paternally inherited genome on the mobility of *P* elements in progeny, fly lines of Q (Q-KY74 and Q-KY101), M′ (M′ -OM5), and P strains (P-Har) were analyzed. When males from these lines (i.e., fathers; test strain) were crossed with females from the *P*-element-susceptible M-CS strain (i.e., mothers), the F1 progeny showed 100% GD for P-Har males, and 0% GD

for the others (Fig. 1A, B), as previously reported (Fukui *et al.* 2008; Wakisaka *et al.* 2017), confirming that P-Har males have high *P* inducibility; Q-KY74, Q-KY101, and M′ -OM5 males had no *P* inducibility, despite carrying *P* elements. These results suggest that some repressive factors are co-inherited with *P* elements from M′ and Q fathers, and then expressed in F1 ovaries.

It is possible that these repressive factors also affect the cytotype of oocytes of F1 hybrids. To examine this, I performed GD tests for F2 hybrids from a cross between F1 hybrid females and P-Har males. If the paternally inherited genome served as a source of cytoplasmic repressive factors in the F1 oocytes, the F2 hybrids should have shown resistance against *P* elements (i.e., low GD score). Interestingly, F2 hybrids [(M-CS x test males) females x P-Har males] showed considerable variability in GD scores (Fig. 1B). When M′ -OM5 was used as a test strain, F2 hybrid offspring showed a GD score of 100%. Thus, although the M′ -OM5 genome inhibited repressive factors of *P* transposition in F1 ovaries (see above), it did not alter the cytotype of F1 oocytes. In contrast, when Q-KY101 was used as a test strain, F2 female offspring showed a very low GD score (2%), suggesting that the Q-KY101 genome conferred the *P*-resistant cytotype to the F1 oocytes. The Q-KY74 F2 hybrid offspring also demonstrated a low GD score (38%), although not as low as Q-KY101 F2 hybrids. When P-Har males were used as test males, the F2 progeny showed a high GD score, as seen previously (Kidwell *et al.* 1988). It should be noted that the F1 hybrids resulting from crosses between M-CS females and P-Har males were fertile when grown at 25°C, and that GD tests were conducted at 28°C to enhance dysgenic effects.

To determine the paternally inherited factors that contributed to the suppression of *P* transposition in F1 and F2 hybrid offspring, I examined the expression levels of *P*-element piRNAs and those of *KP*-element mRNAs, both of which are known to be main repressors in germ line cells

The Q and M′ strains possessed high ratios of *KP* elements and low fractions of *FP* elements

First, I characterized the *P*-element copies in the fly lines Q-KY74, Q-KY101, M′-OM5, and P-Har by PCR using two sets of primers designed for total *P* and non-*KP* elements, respectively (see Fig. 2A for primer design). The "total *P*" primers allowed for amplification of both *FP* elements and incomplete (internally deleted) *P* elements, but if incomplete elements were predominant in the genome, the *FP* element amplicon (2,526 bp) would not be produced efficiently. The "non-*KP* primers" allowed for amplification of *FP* elements, even in the presence of a large number of *KP* elements (e.g., Q-KY74, Q-KY101, and M′-OM5). Using the total-*P* primers, I detected an amplicon (2,526 bp, sequence confirmed) of *FP* elements from P-Har genomic DNA (Fig. 2A). DNA from strains Q-KY74, Q-KY101, and M′-OM5 revealed a faint *FP*-element band and a thick *KP*-element band (789 bp). The presence of *FP* elements in the Q-KY74, Q-KY101, and M′-OM5 genomes were confirmed by PCR using the non-*KP* primers (Fig. 2A); these genomic DNAs revealed an *FP* amplicon (2,206 bp), along with amplicons from incomplete variants. These results indicate that Q-KY74, Q-KY101, and M′-OM5 indeed carry *FP* elements, although the vast majority of their genomic *P* elements are *KP* elements.

To determine relative copy numbers of *P* elements in the four genomes, I performed real-time PCR with primers that amplified *P* copies (Fig. 2B and Fig. 8; three biological replicates for each line). Results revealed that these strains contained similar numbers of *P*-element copies, although the copy number in the Q-KY74 genome was somewhat (0.7-fold) lower than those in the other genomes. It should be noted, however, that standard deviations were large, indicating that *P*-element copy numbers varied significantly among individual flies of the same line. To determine relative copy numbers of *KP* elements, I performed real-time PCR with *KP*-specific primers (Fig. 2C; three biological replicates for each line). The Q-KY74, Q-KY101, and M′-OM5 genomic DNAs amplified *KP* elements, while the P-Har genomic DNA did not. Although copy numbers varied between individuals of the same lines, as was the case with *P* elements, the M′-OM5 genome showed a higher ratio of *KP* copy numbers (73% of total *P* elements) than the two Q strains (~50% of total *P* elements), which is consistent with our previous results from M′-OM5 using Southern blotting (Fukui *et al.* 2008).

These results suggest that the number of *P* elements in the genome is not attributable to the differences in GD scores from F2 hybrids among the P, M′ and Q strains. However, regarding F1 hybrids, the strains that showed low GD scores (M′ and Q) possessed high ratios of *KP* elements in their genomes. This suggests the contribution of paternally inherited *KP* elements in the suppression of the *P*-element expression and/or transposition in F1 hybrids. As a result, I chose to investigate the expression of *P* elements, including *KP* elements, as shown below.

The Q strains possessed high percentages of *P* elements inserted into repressive regions

To investigate whether chromosomal environments of *P*-element insertion sites in the paternal genome influence the level of *P*-element expression in F1 hybrid ovaries, I first determined the *P*-element insertion sites in the respective genomes. I then inferred the transcriptional states of the surrounding regions in ovaries of a *D. melanogaster* reference line. To identify the insertion sites, I digested genomic DNA (pooled for 40 adults) with restriction enzymes, and ligated an adaptor DNA to the ends. Junction regions between *P* elements and their flanking sequences were amplified using a *P*-specific primer, an adaptor-specific primer, and the ligated DNA. The PCR products were then subjected to paired-end deep sequencing and mapped onto the reference *D. melanogaster* genome (Release 5). This identified a number of insertion sites in the respective genomes (Fig. 3A). I noticed that the number of sequencing reads significantly differed among loci. This variability most likely stemmed from differences in occupancies of the respective sites. For insertion sites with low coverage, it is conceivable that only a fraction of the individuals carried the insertions (i.e., insertional polymorphism among individuals of the same line).

Next, the transcriptional states of the flanking regions were categorized into active-expression regions (score >0) and silent-expression regions (score =0) (Fig. 3A), according to the RNA-seq data from 4-day-old ovaries in *D. melanogaster* Genome Browser (ModENCODE) (Langmead 2010; Graveley *et al.* 2011). Using these data, the numbers of *P*-element copies inserted in the

respective transcriptional states were calculated. I determined the fraction of read numbers (rather than number of insertion sites) mapped in the two regions so that the occupancy of respective insertion sites was taken into account (Fig. 3A). In the P-Har genome, about half of the *P* elements were inserted into active-expression regions. Because nearly all *P* elements are *FP* elements in P-Har (Fig. 1A), it is likely that the *FP* copies inserted in the active-expression regions were expressed in F1 overies, resulting in high *P* inducibility. On the other hand, in the Q-KY74 and Q-KY101 genomes, more than three-quarters of the *P* elements (including *FP* and *KP*) resided in silent-expression regions, consistent with showing no *P* inducibility (Fig. 1B, F1 GD scores); however, this could not completely explain the variable GD scores for both F1 and F2 hybrids. In M′-OM5, about half of the *P* elements resided in active-expression regions, although they were not dysgenic.

The M′ strain expressed higher levels of *KP* elements, while the Q strain transcribed lower levels of *P* elements

Next, I quantified *P*-element mRNA in 2- to 3-day-old ovaries of F1 hybrids, using real-time PCR. As expected from the features of *P* insertion sites, P-Har hybrids showed a high expression of the *P* element (Fig. 3B). *P*-element expression in M'-OM5 hybrids was also high, and notably, it was 5-fold and 10-fold higher than expression in the Q-KY74 and Q-KY101 hybrids, respectively ($p < 0.01$; Fig. 3B). Given that the number of *P*-element copies inserted in transcriptionally active regions differed only by 2.5-fold between the M′ and Q strains, some factors other than genomic loci were likely responsible for the very

low expression levels of *P* elements in the hybrid offspring from Q-KY74 and Q-KY101 strains.

I determined the levels of *KP*-element mRNA in the same samples (2- to 3-day-old ovaries of F1 hybrids) using *KP*-specific primers (Fig. 3C). While P-Har hybrids did not express detectable *KP* mRNA, M′-OM5, Q-KY74, and Q-KY101 hybrids did show *KP* expression; M′-OM5 hybrids expressed higher levels of *KP*-element mRNA than both Q-KY74 and Q-KY101 ($p<0.01$; Fig. 3C). Although the abovementioned genomic site identification (shown in Fig. 3A) did not discriminate between *FP* and *KP*, a substantial number of *KP* elements should have been in active-expression regions in the M′-OM5 genome; therefore, higher levels of *KP* expression in M′-OM5 are consistent with the abundant insertions in active-expression regions in this strain of flies.

As mentioned above, I noticed that the M′-OM5 hybrids expressed a substantial amount of *P*-element mRNA, although they were not dysgenic. The non-dysgenic phenotype could be due to the concomitant expression of the *KP* repressor mRNA in the ovaries. Using the data from the four strains and an additional Q strain, Q-HKH, a simple comparison of *KP*-element mRNA expression and GD scores from the F1 hybrids for the respective strains did not indicate a strong correlation (Fig. 3D) like that seen in F2 hybrids (Fig. 3E). However, I noted that F1 hybrids of the Q strains expressed *P*-element piRNAs, another repressor molecule (see below); therefore, it remains possible that the *KP* mRNA plays an important role in the prevention of GD in M′ F1 hybrids. I will discuss this possibility later.

Thus, the relative abundance of *P*-element insertions in active-expression

regions is roughly correlated to the level of *P*-element mRNA expression, but it did not fully account for the difference in expressions between the M′ and Q strains. Thus, other factor(s) also should be involved in the control of the *P*-element mRNA levels and the P-M phenotype. Such factors may involve piRNAs.

The Q strains had more copies of *P*-element in piRNA clusters

piRNAs are produced from the transcripts of piRNA clusters (Brennecke *et al.* 2007). To reveal whether the level of *P*-element piRNAs in F1 hybrid ovaries was affected by the number of *P*-element copies inserted into the clusters in paternal genomes, I first re-analyzed the insertion site data of the four lines in view of the number of *P*-element reads identified in the piRNA clusters. The characteristics of piRNA clusters (genomic locations, piRNA-transcriptional directions, and piRNA-producing activities) were compared to the data presented in Brennecke *et al.* (2007; see “Methods”). Here, active piRNA clusters have been defined as the top 15 clusters, ranked by the number of unique piRNAs they provide, whereas the remaining clusters have been defined as low activity piRNA clusters.

In all lines analyzed, some *P*-element copies were located in piRNA clusters (Fig. 4A), but not all lines harbored *P* elements in the same clusters; only Q-KY74 and P-Har had *P*-element insertions in the same100F piRNA cluster. The fraction of *P*-element reads in clusters to the total *P*-element reads, as well as real read numbers (Fig. 9), was higher in Q-KY74 and Q-KY101 than in M′-OM5 and P-Har (Fig. 4B). To compare the characteristics of *P*-harboring

piRNA clusters across the lines, I analyzed the number of *P*-element insertions in each piRNA cluster (Fig. 4C). The Q-KY74 genome harbored many *P* elements in unistrand piRNA clusters, but none in dual-strand piRNA clusters. In particular, I found six copies of antisense-oriented *P* elements inserted into the active 100F unistrand cluster (rank 11) (Fig 10). Q-KY101 carried two copies of antisense-oriented *P* elements in an active dual-strand piRNA cluster, 38C (rank 5), as well as a copy of sense-oriented *P* element in a low activity unistrand piRNA cluster. M′-OM5 carried several copies of both sense- and antisense-oriented *P* elements in active and low activity dual-strand piRNA clusters, but the low number of reads mapped to these suggests that the insertions are polymorphic within the strain (Figs. 4C, S2). P-Har had a single copy of sense-oriented *P* element in an active dual-strand piRNA cluster and three copies of both sense- and antisense-oriented *P* elements in active and low activity unistrand piRNA clusters, all with low read numbers (Figs. 4C, S3).

The number of *P*-element copies in piRNA clusters was comparable among the P, M′ and Q strains; however, when read numbers were compared, the fraction of *P*-element reads in clusters to total *P*-element reads was 4- to 5-fold higher in the Q strains than in P and M′ strains (Fig. 4B). These results suggest that the Q strains carry higher occupancies of piRNA-cluster-embedded *P*-element copies within their populations.

The Q hybrids produced higher levels of *P*-element piRNAs derived from paternal *P* elements

To characterize piRNAs produced in F1 hybrids, I deeply sequenced small

RNAs in 2- to 3-day-old ovaries from Q, M′ , and P F1 hybrids. After removing the miRNAs and fragments of functional RNAs, small RNAs of 24–to 35-nt long were mapped onto the *P* element sequence to identify the *P*-element piRNAs. Because the mother (the M-CS strain) in this cross had no *P*-element in her genome, all *P*-element piRNAs detected in F1 hybrids should have derived from the paternally inherited genome. The analysis revealed the presence of zygotic piRNAs in F1 ovaries of all fly lines (Fig. 5A). The abundances of *P*-element piRNAs differed between the lines, and had a significant positive correlation to the occupancy-adjusted *P* element copy numbers within the piRNA clusters ($R = 0.95$, $p<0.05$; Pearson's product–moment correlation test; Fig. 5B). Thus, the two Q hybrids produced >3-fold more abundant *P*-element piRNAs than the M′ and P hybrids (Fig. 5C). In these Q hybrids, the amounts of sense and antisense piRNAs were similar. For the Q-KY101 hybrids, the very active dual-strand cluster, 38C, likely served as a source of sense and antisense piRNAs. Although the Q-KY74 hybrids harbored nine copies of *P* elements in a unistrand cluster, with eight having an antisense orientation to the cluster transcription, they also produced both sense and antisense piRNAs. It is possible that the *P*-element mRNA was cleaved to serve as sense piRNAs.

Theoretically, it is possible that the detected piRNAs in F1 ovaries were inherited directly from the father's sperm. To examine this, the small RNAs in testes were also deeply sequenced for the four fly lines. In all lines, testes produced 5- to 10-fold more abundant *P*-element piRNAs than F1 ovaries (Fig. 11); but, in any case, most of the sequences did not closely identify with those in the ovaries of female progeny (Fig. 5A). Therefore, I believe the piRNAs

detected in the F1 ovaries were produced mostly *de novo*, rather than inherited from parental sperm. Consistently observed in Q hybrids, the levels of *P*-element piRNAs were extremely low (<10 RPM) immediately after fertilization (in 0–24 h whole embryos, Fig. 5D), and then increased to >1000 RPM （in 2- to 3-day old ovaries, Fig. 5C）, most likely by *de novo* production. Although the degree of increase was less, P and M′ hybrids showed similar trends along the same time line.

In summarizing the results of piRNA analysis, I emphasize that the Q-KY74 and Q-KY101 male parents conferred the ability to produce abundant *P*-element piRNAs in ovaries to their progeny, which well reflects the low GD scores.

GD scores from both F1 and F2 hybrid progeny were associated with piRNA production in young F1 ovaries

To elucidate the relatioship between piRNA production capabilities and GD scores, I first analyzed the relationship between *P*-element mRNA expression and *P*-element piRNA production in 2- to 3-day-old ovaries from F1 hybrids. As shown in Fig. 6A, mRNA expression levels had a significant negative correlation with piRNA expression levels ($R = -0.97$, $p = 0.004$; **Pearson's** product–moment correlation test), suggesting that these piRNAs negatively regulate the levels of *P*-element mRNAs in young hybrid ovaries. Importantly, all three Q hybrids were distinguished from P and M′ strains because they expressed high amounts of piRNAs and very low amounts of mRNAs (Fig. 6B).

These results, together with those from *KP*-element expression analysis (Fig. 3C), suggest that *P*-element piRNAs and *KP* elements are involved in the P-M

phenotype. To test this, I performed a multiple regression analysis for GD scores (the objective variable) with the amounts of *P*-element piRNAs and *KP*-element mRNAs from 2- to 3-day-old F1 ovaries (explanatory variables). This revealed that the amounts of both *P*-element piRNAs and *KP*-element mRNAs were effective explanatory variables (for *P*-element piRNAs, partial regression R = −0.99, t = 21.7, p = 0.02; for *KP*-element mRNA, partial regression R = −0.84, t = 18.5, p = 0.03; Fig. 6C). Negative R coefficients indicated that the amounts of both *P*-element piRNAs and *KP*-element mRNAs were suppressors of the dysgenesis, and hence repressors of the *P* transposition. Similar t-values suggest that the effectiveness of the *P*-element piRNAs and *KP*-element mRNAs was similar to each other, while the partial R coefficients, at nearly −1, suggest that high expression of only one of these is sufficient to suppress the dysgenesis. Indeed, for hybrids of low GD scores (M′-OM5, Q-KY101, Q-KY74, and Q-HKH), M′ hybrids showed high *KP* expression in ovaries with low piRNA expression, while Q hybrids showed high piRNA expression with low *KP* expression.

As previously stated (shown in Fig. 1B), GD scores of F2 hybrids also varied between fly strains. Here Q strains showed lower GD scores (2−38%), whereas the P and M′ strains showed 83−100% GD. Thus, using multiple regression I analyzed whether the amounts of the *P*-element piRNAs and *KP*-element mRNAs in F1 ovaries affected the GD phenotype of F2 females (Fig. 6F). Again, the amount of *P*-element piRNA in F1 ovaries was an effective explanatory variable (partial regression R = −0.85, p = 0.02) for the F2 phenotype. The amount of *KP* mRNA was also an explanatory variable (p = 0.04); however, its effectiveness on the GD phenotype was much weaker (partial regression R =

0.24). The t-value for the *KP* mRNA amounts was indeed >3-fold less than that for piRNA amounts (1.4 vs. 5.0); therefore, the *KP* mRNA in the mothers' ovaries also affected the GD phenotype of F2 offspring, but the effectiveness was not as pronounced as that observed for the F1 offspring. As a result, I have concluded that *P*-element piRNAs in F1 mother's ovaries have a large, and possibly major, impact on the GD phenotype of her daughters. Indeed, in a single regression analysis, the piRNA amounts in the F1 mother's ovaries alone explained well the GD scores of her F2 hybrid offspring (Fig. 6D). I have noted in Figs. 6D and 6E that the Q strains clustered together.

2-4 Discussion

Although naturally living flies generally carry *P* elements in their genomes, males of the Q and M′ strains in this study demonstrated a higher capacity to suppress the mobilization of introduced *P* elements than the P strain when hybridization occurred with M-strain females. Here, I show that such low *P* inducibility in the three lines of the Q (two lines) and M′ strains was associated with repressive factors derived from the paternal genomes. In particular, I found that the low *P* inducibility in two lines of the Q-strain was strongly associated with a higher level of zygotic piRNAs in young F1 ovaries that contributed to the regulation of *P*-element expression. Interestingly, I found that the level of zygotic piRNAs depended on the *P*-element insertion sites in paternal genomes, and that these piRNAs conferred immunity against *P* transposition in the next generation (F2 hybrids). On the other hand, the low *P* inducibility in one M′-strain line was associated with a higher expression of *KP* elements in F1

ovaries due to a higher copy number of *KP* elements in the paternally inherited genome, where some of *KP* elements are likely inserted into the actively transcribed regions, in addition to the silencing of *FP* elements by harboring them in the transcriptionally inert sites, as previously shown (Fukui *et al.* 2008). However, *KP* mRNA levels in the mother's ovaries did not efficiently protect the daughter's ovaries from *P*-induced dysgenesis. Srivastav and Kelleher (2017) showed that *P* inducibility weakly correlated with the number of *P*-element copies in the genome although the relationship between *P* inducibility and *P*-element insertion sites remains to be explored. In this study, I revealed that, in addition to thesimple copy number, transcriptional activity, and piRNA production ability in the regions surrounding *P*-element copies are important factors.

Q-KY101 was characterized by low *P*-element expression, and the highest expression of zygotic *P*-element piRNAs in young F1 ovaries; these qualities are associated with high numbers of *P*-element copies harbored in piRNA clusters. In particular, the two *P*-element copies harbored in the 38C piRNA cluster highly active in the germline cells likely account for the highest levels of sense and antisense piRNAs. The offspring of this strain had a very low GD score in F2 ovaries. Assuming that these piRNA-producing *P*-element copies segregate randomly, the F2 phenotype could not be explained solely by the genotype. This suggests that the piRNAs produced in F1 oocytes were deposited to repress the *P*-element expression in F2 progeny (Fig. 7). These results argue that GD of F2 progeny was suppressed by the genomes of their grandfathers carrying *P* elements in their dual-strand (germ-specific) piRNA clusters.

Q-KY74 also is characterized by low *P*-element expression, and high expression of zygotic *P*-element piRNAs in young F1 ovaries, which reflects high *P* element copy numbers harbored in piRNA clusters. While this strain also had a higher number of antisense-oriented *P*-element copies inserted into unistrand piRNA clusters, which are dominant in somatic cells, the Q-KY74 hybrids expressed both-strand zygotic piRNAs in young ovaries. It is possible that, in the young F1 ovaries of Q-KY74 hybrids, many zygotic antisense piRNAs produced from unistrand (soma-specific) clusters induce the production of sense piRNAs by cleaving *P*-element mRNAs. Interestingly, the Q-KY74 strain showed a relatively low (38%) GD score in F2 hybrids. Like Q-KY101, this may be due to piRNA deposition from F1 oocytes (Fig. 7); however, it has been shown that unistrand piRNA clusters are not active in germ line cells (Yamanaka *et al.* 2012). Previous reports have shown that the piRNAs produced in germline and somatic cells affect each other (Akkouche *et al.* 2013; Gonzalez J *et al.* 2015). Moreover, Malone *et al.* (2009) demonstrated that the low production of antisense piRNAs correlated with the weak deposition of maternal suppressors in F1 progenies, while high both-strand piRNA production correlated with strong deposition of maternal suppressors in F1 progenies. Therefore, there is a possibility that piRNA production in F1 oocytes can be reinforced partially by piRNAs from the unistrand clusters in ovarian somatic cells, and these oocyte piRNAs are deposited, to some degree, into F2 hybrids (Fig. 7). This means that the difference in the GD scores of F2 hybrids from the Q-KY74 strain and the Q-KY101 strain may stem from a difference in the abundance of the piRNA load in F1 oocytes. Although this presents the possibility of a non-Mendelian

inheritance, the moderate GD score of the Q-KY74 strain is explained potentially by Mendelian inheritance as well. Further investigation on the correlation between dysgenic phenotype, piRNA levels, and *P*-element loci of individual F2 hybrids will address this issue.

M′-OM5 hybrids are characterized by low levels of zygotic piRNAs and the active transcription of paternally inherited *P* elements and *KP* elements. Thus, the low levels of piRNAs are not sufficient to decrease the levels of *P*-element mRNA. This is consistent with our previous study on maternal strain effects, where M′-strain females allowed only low levels of piRNAs in F1 hybrids, resulting in *P* susceptibility (Wakisaka *et al.* 2017). However, even with paternally inherited *P* elements considered, the F1 hybrids still presented a low GD score. The genome carried many *KP* elements, some of which resided in transcriptionally active regions, allowing higher *KP*-element expression; therefore, the low *P* inducibility is most likely ascribed to the co-inherited *KP* elements (Fig. 7). However, there is a caveat. In a previous study, all *FP* elements were likely imbedded in transcriptionally silent genomic regions in the M′-OM5 strain, showing low *P* inducibility (Fukui *et al.* 2008). Therefore, I propose that, if an active *P*-element is present in the paternally inherited genome, an active *KP* element(s) is required to be co-inherited to suppress the *P*-element activity. Even in such a case, the *KP* elements in the F1 genome would be diluted in F2 hybrids; sufficient amounts of *KP* mRNA are not produced in F2, resulting in GD.

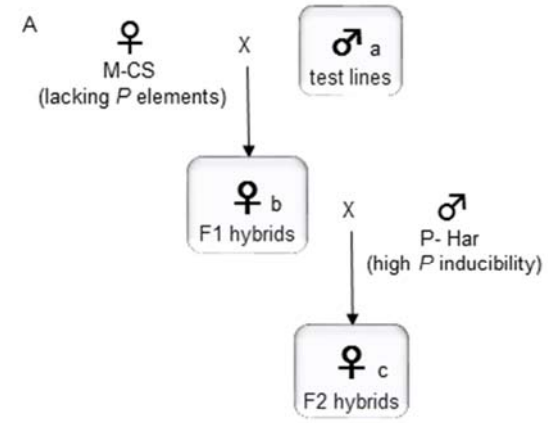
I demonstrated that, in 2- to 3-day-old hybrid ovaries of P-Har, high *P* inducibility was associated with low levels of zygotic *P*-element piRNAs, which is

consistent with a previous report by Khurana *et al.* (2011). Their low piRNA expression is likely because the P-Har genome carries low copy numbers of *P*-elements in piRNA clusters. High percentages of GD in F2 hybrids were affected by this low production of *P*-element piRNAs in F1. It should be noted that the GD score of the P-Har F2 hybrids was 83%, and not 100 %, meaning that some progeny had the ability to counteract the *P* transposition. This suggests an involvement of suppressors other than piRNAs, which should be elucidated by further studies.

I concluded below. Using the *P*-element as a model, our results revealed the importance of zygotically produced piRNAs from the paternal genome to suppress TE activity in *D. melanogaster* progeny. In addition to the well-characterized effects of maternally deposited piRNAs, our results also evoke an interesting possibility that individual TE locations and their insertional polymorphism in natural populations direct the various expressions of piRNAs, leading to variability in the immunizing capacity of their granddaughters against TEs. In nematodes, studies have shown that piRNAs are inherited over many generations (Ashe *et al.* 2012). To explore the host-TE battle in natural populations, interesting questions to be addressed include: (1) whether and to what extent the piRNA-producing ability is inherited across generations, (2) whether the transcriptional states of individual TEs are affected by other copies, and if so, (3) whether the altered transcriptional state is inherited, like paramutation (de Vanssay *et al.* 2012), as paramutation often involves a class of small RNAs.

2-5 Figures

Figure 1



B

line name of a	P-Har	M'-OM5	Q-KY74	Q-KY101
GD score of F1 hybrids b (%)	100 M-CS x P-Har	0 M-CS x M'-OM5	0 M-CS x Q-KY74	0 M-CS x Q-KY101
GD score of F2 hybrids c (%)	83 (M-CS x P-Har) xP- Har	100 (M-CS x M'-OM5) x P-Har	38 (M-CS x Q-KY74) x P-Har	2 (M-CS x Q-KY101) x P-Har

F1 hybrids: n = 50
F2 hybrids: n = 100-116

Figure 2

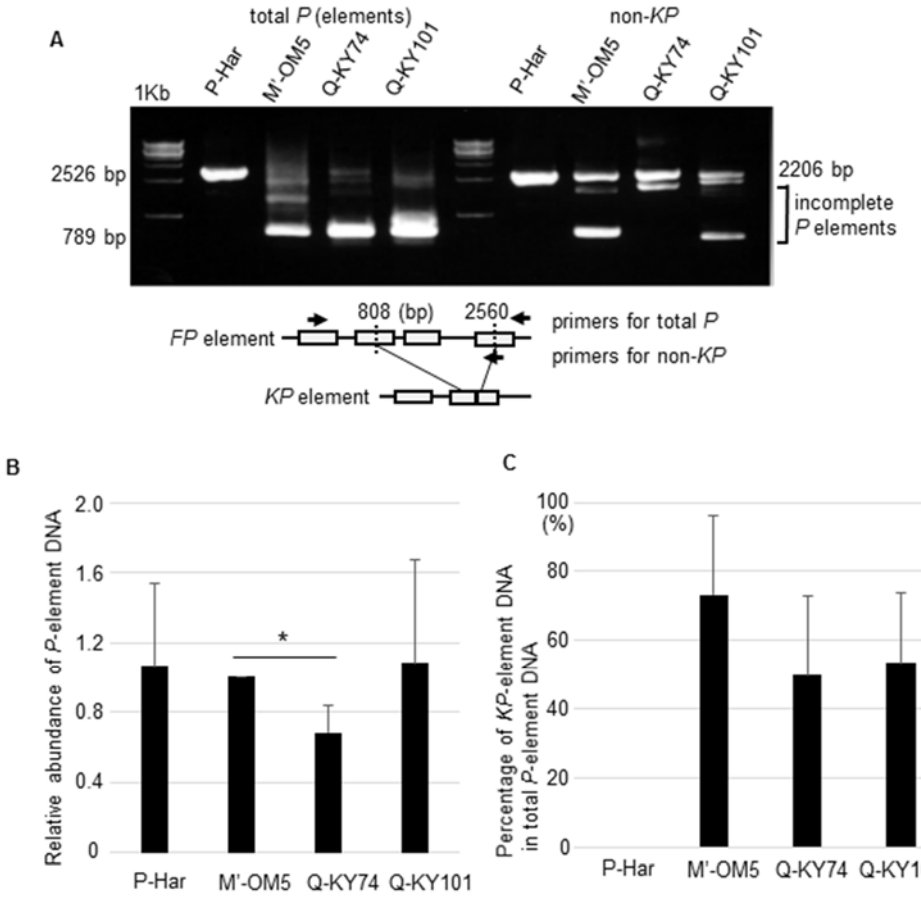

A
total P (elements)
non-KP
1Kb
P-Har
M'-OM5
Q-KY74
Q-KY101
P-Har
M'-OM5
Q-KY74
Q-KY101
2526 bp
789 bp
2206 bp
incomplete
P elements
808 (bp)
2560
FP element
KP element
primers for total P
primers for non-KP
B
Relative abundance of P-element DNA
2.0
1.6
1.2
0.8
0.4
0
*
P-Har
M'-OM5
Q-KY74
Q-KY101
C
Percentage of KP-element DNA in total P-element DNA
100 (%)
80
60
40
20
0
P-Har
M'-OM5
Q-KY74
Q-KY101

Figure 3

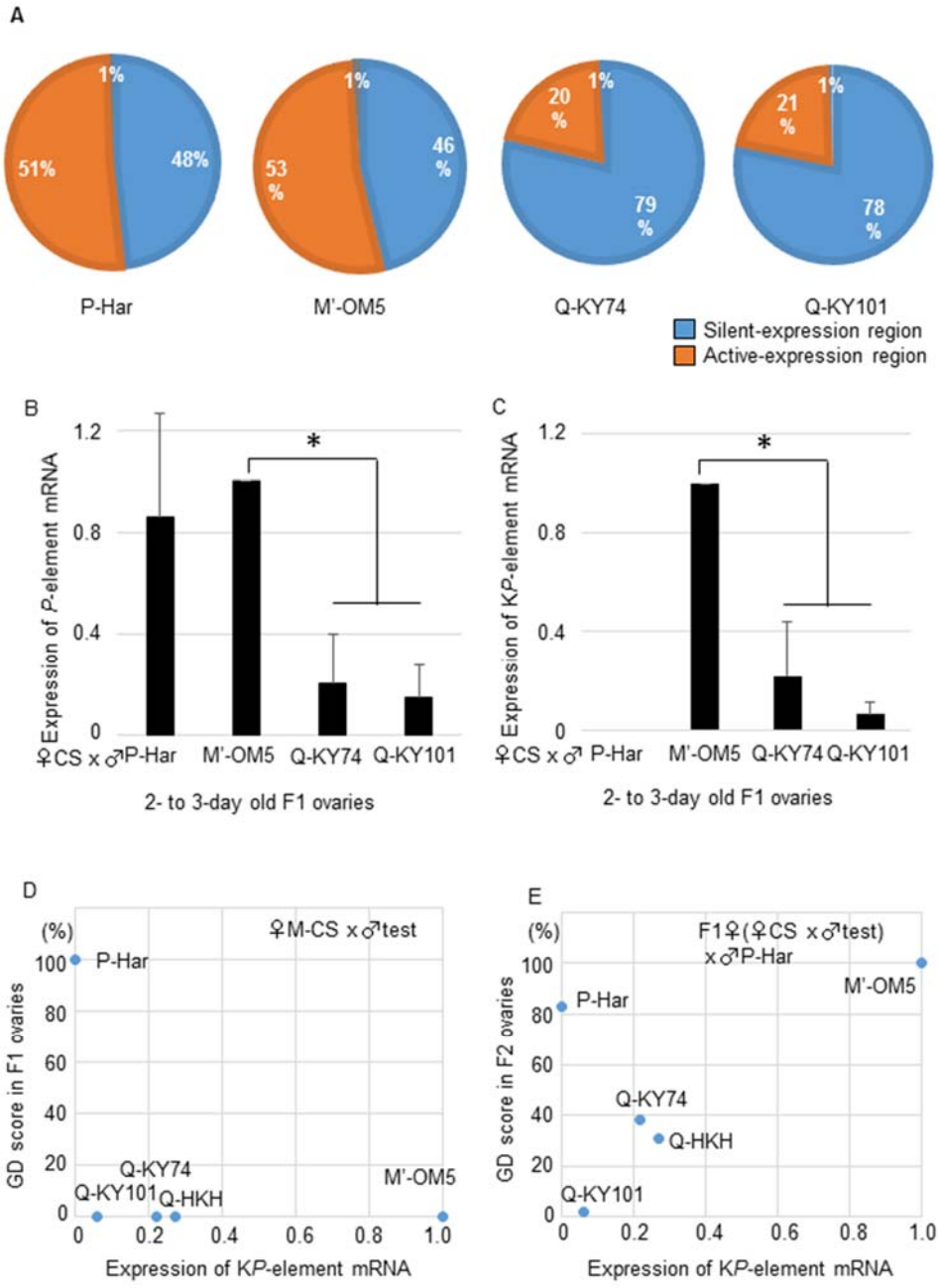

A
1%
48%
51%
1%
46
%
53
%
1%
20
%
79
%
1%
21
%
78
%
P-Har
M'-OM5
Q-KY74
Q-KY101
Silent-expression region
Active-expression region
B
Expression of P-element mRNA
1.2
0.8
0.4
0
*
♀CS x ♂P-Har
M'-OM5
Q-KY74
Q-KY101
2- to 3-day old F1 ovaries
C
Expression of KP-element mRNA
1.2
0.8
0.4
0
*
♀CS x ♂ P-Har
M'-OM5
Q-KY74
Q-KY101
2- to 3-day old F1 ovaries
D
(%)
♀M-CS x ♂test
P-Har
GD score in F1 ovaries
100
80
60
40
20
0
Q-KY74
Q-KY101
Q-HKH
M'-OM5
0
0.2
0.4
0.6
0.8
1.0
Expression of KP-element mRNA
E
(%)
F1♀(♀CS x ♂test)
x ♂P-Har
M'-OM5
P-Har
GD score in F2 ovaries
100
80
60
40
20
0
Q-KY74
Q-HKH
Q-KY101
0
0.2
0.4
0.6
0.8
1.0
Expression of KP-element mRNA

Figure 4

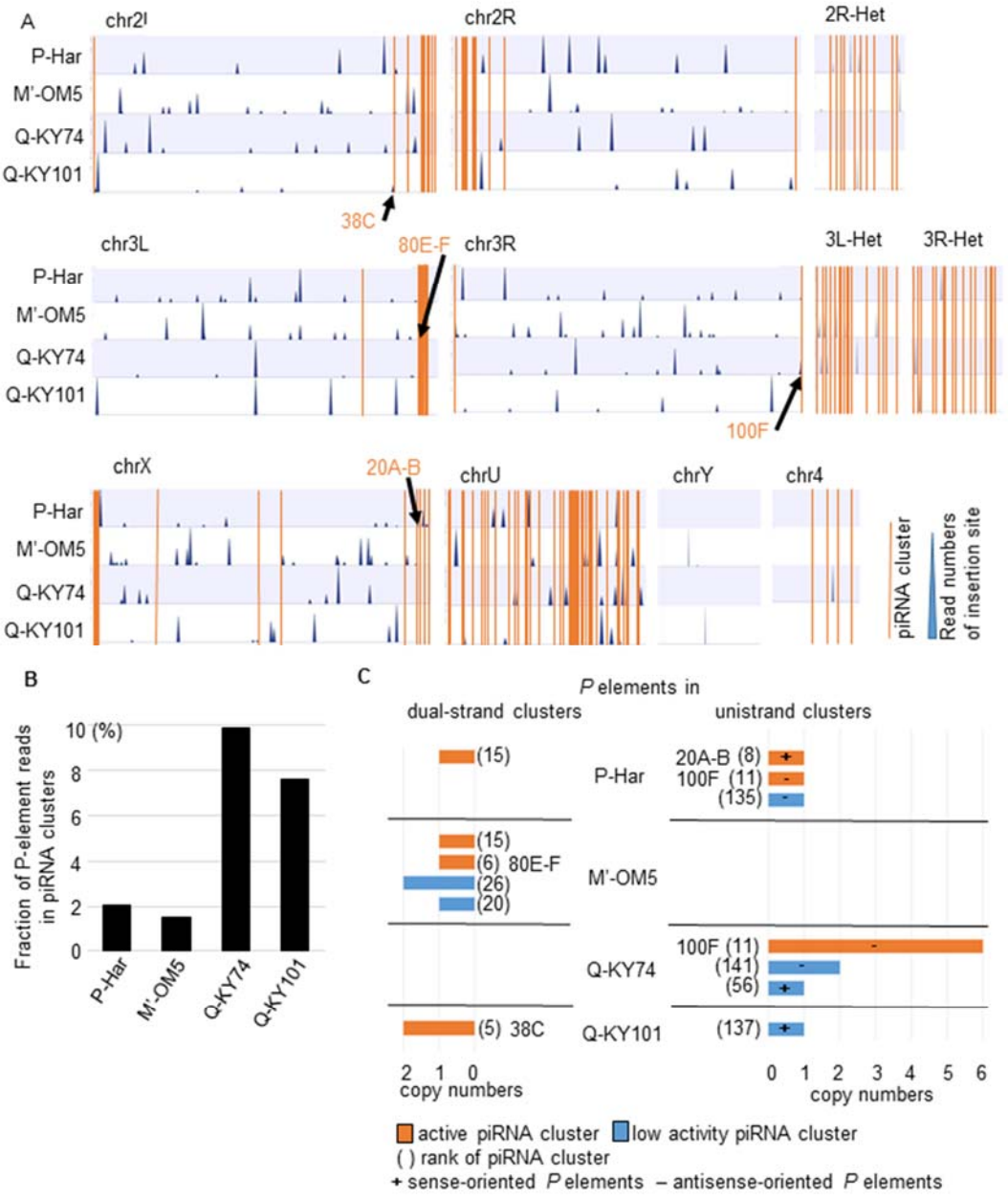

A
chr2l
chr2R
2R-Het
P-Har
M'-OM5
Q-KY74
Q-KY101
38C
chr3L
80E-F
chr3R
3L-Het
3R-Het
100F
chrX
20A-B
chrU
chrY
chr4
piRNA cluster
Read numbers of insertion site
B
Fraction of P-element reads in piRNA clusters
10 (%)
P-Har
M'-OM5
Q-KY74
Q-KY101
C
P elements in
dual-strand clusters
unistrand clusters
(15)
(15)
(6) 80E-F
(26)
(20)
(5) 38C
20A-B (8)
100F (11)
(135)
100F (11)
(141)
(56)
(137)
copy numbers
active piRNA cluster
low activity piRNA cluster
( ) rank of piRNA cluster
+ sense-oriented P elements – antisense-oriented P elements

Figure 5

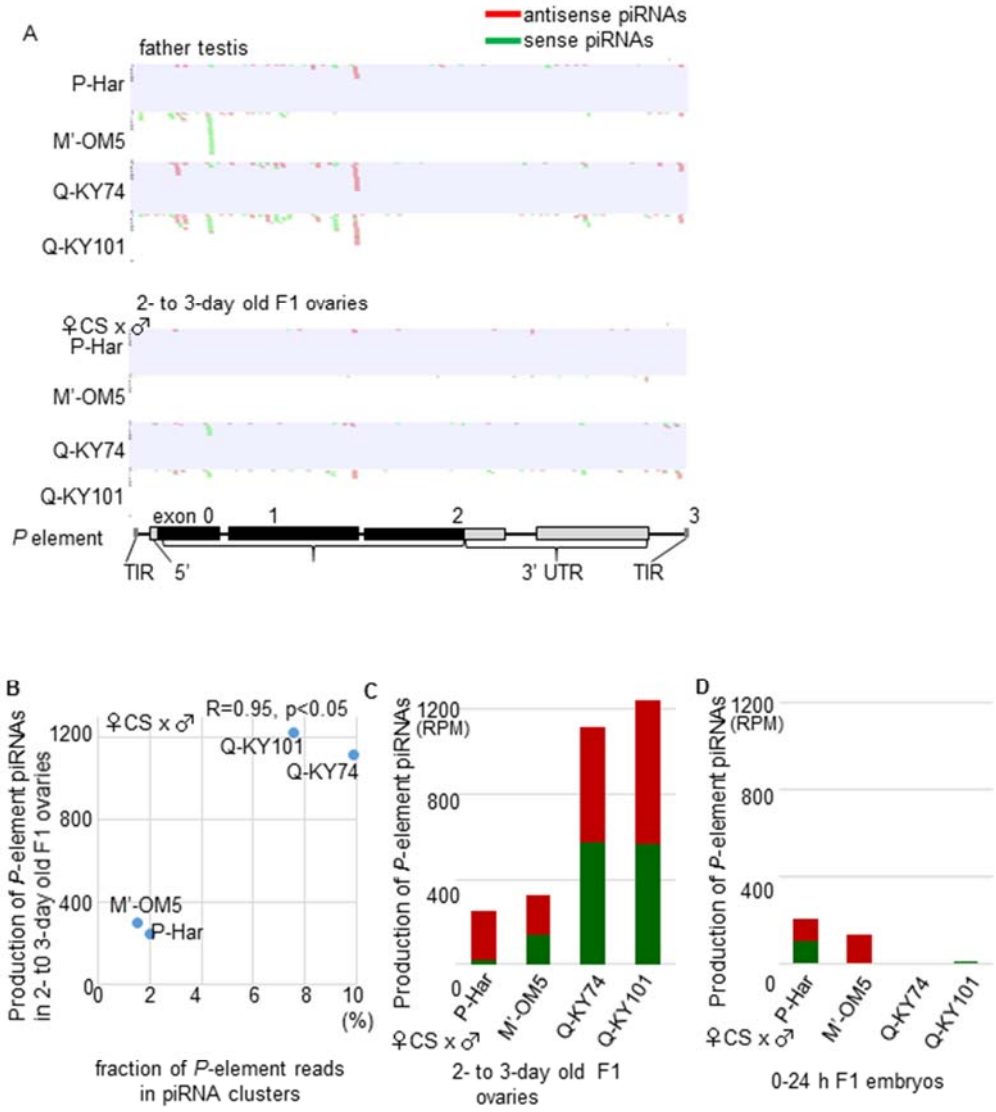
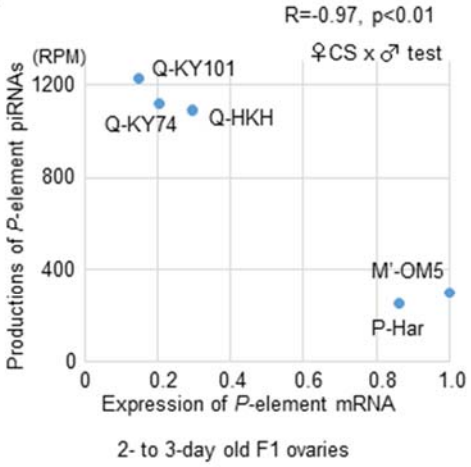
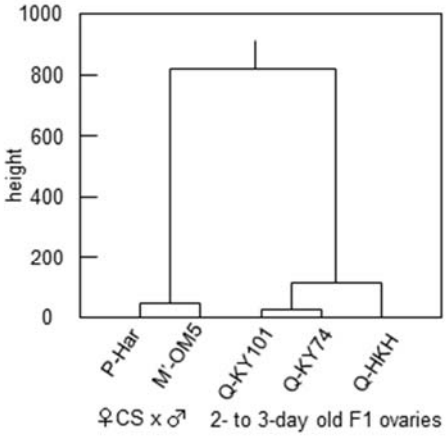

A
antisense piRNAs
sense piRNAs
father testis
P-Har
M'-OM5
Q-KY74
Q-KY101
2- to 3-day old F1 ovaries
♀CS x ♂
P-Har
M'-OM5
Q-KY74
Q-KY101
P element
exon 0
1
2
3
TIR
5'
3' UTR
TIR
B
♀CS x ♂
R=0.95, p<0.05
Q-KY101
Q-KY74
M'-OM5
P-Har
Production of P-element piRNAs in 2- t0 3-day old F1 ovaries
1200
800
400
0
0
2
4
6
8
10
(%)
fraction of P-element reads in piRNA clusters
C
Production of P-element piRNAs
1200
(RPM)
800
400
0
P-Har
M'-OM5
Q-KY74
Q-KY101
♀CS x ♂
2- to 3-day old F1 ovaries
D
Production of P-element piRNAs
1200
(RPM)
800
400
0
P-Har
M'-OM5
Q-KY74
Q-KY101
♀CS x ♂
0-24 h F1 embryos

Figure 6

A



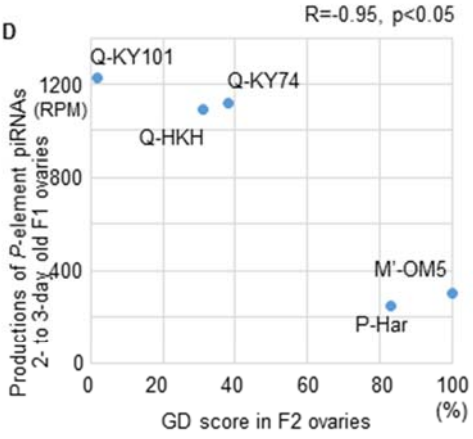
B



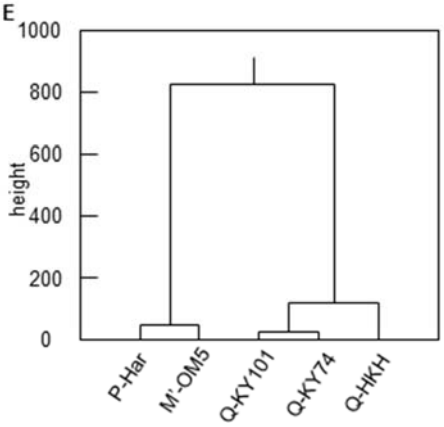
C

GD score of F1 as objective variable	Normalized partial regression coefficient	t value	p value	F value	Freedom-adjusted multiple correlation coefficient
KP-element mRNA expression in F1	-0.842	18.4644	0.029	289.381 (p = 0.034)	0.998278
P-element piRNA production in F1	-0.9892	21.691	0.021		

D



E



F

GD score of F2 as objective varible	Normalized partial regression coefficient	t value	p value	F value	Freedom-adjusted multiple correlation coefficient
KP-element mRNA expression in F1	0.2407	1.41418	0.037	19.8422 (p = 0.048)	0.97601
P-element piRNA production in F1	-0.851	4.99928	0.023		

Figure 7

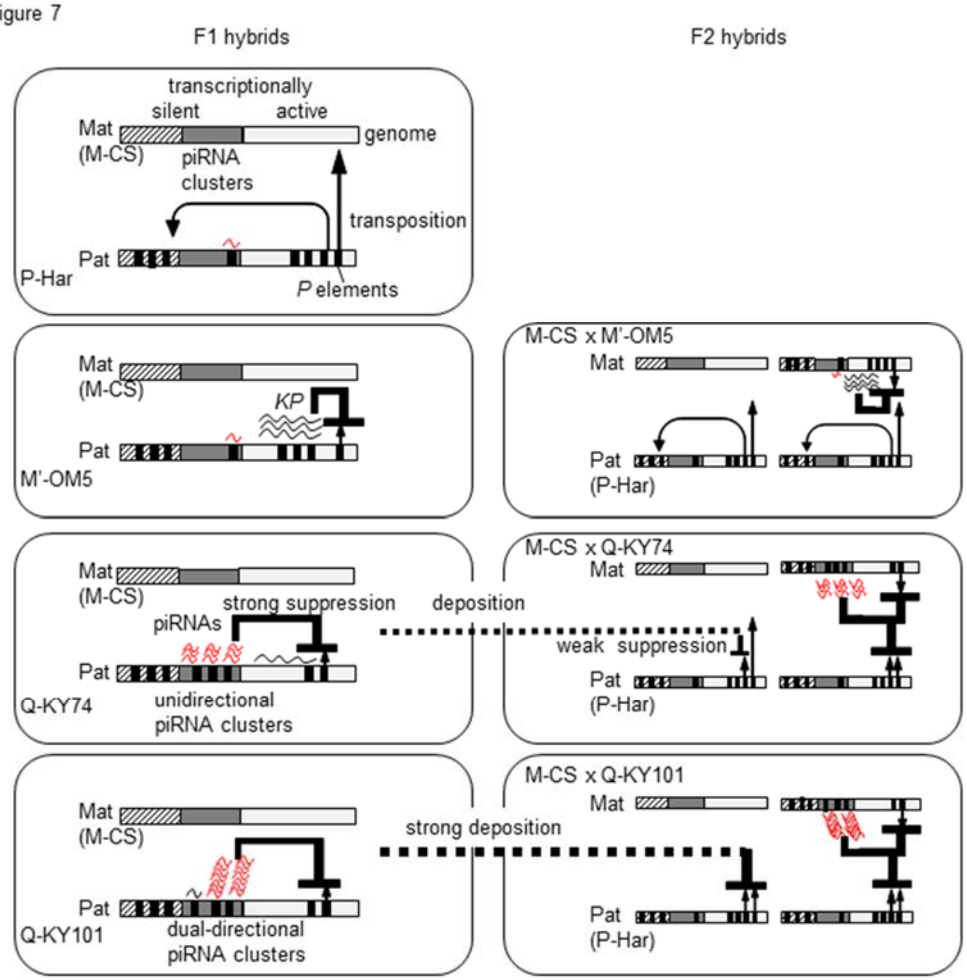

F1 hybrids
F2 hybrids
transcriptionally
silent
active
Mat
(M-CS)
genome
piRNA
clusters
transposition
Pat
P-Har
P elements
Mat
(M-CS)
KP
Pat
M'-OM5
M-CS x M'-OM5
Mat
Pat
(P-Har)
Mat
(M-CS)
strong suppression
deposition
piRNAs
Pat
Q-KY74
unidirectional
piRNA clusters
M-CS x Q-KY74
Mat
weak suppression
Pat
(P-Har)
Mat
(M-CS)
strong deposition
Pat
Q-KY101
dual-directional
piRNA clusters
M-CS x Q-KY101
Mat
Pat
(P-Har)

Figure 8

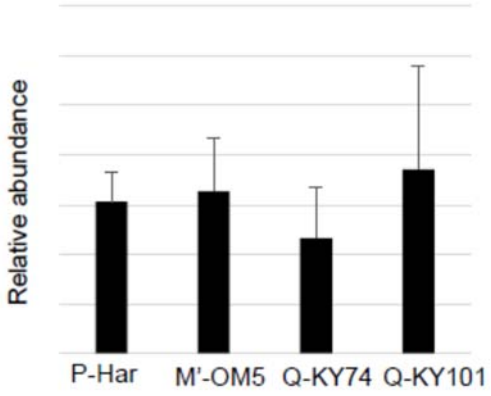


Figure 9

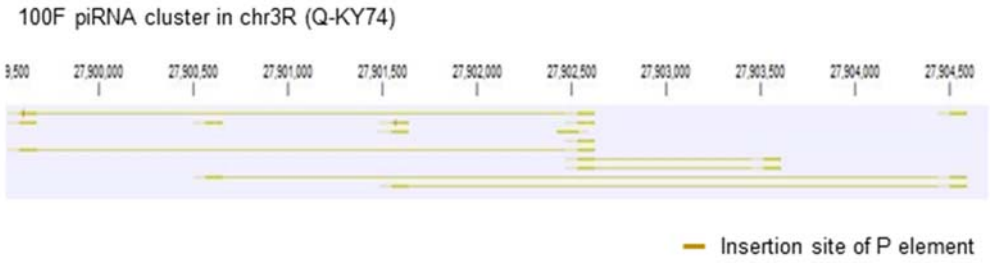


Figure 10

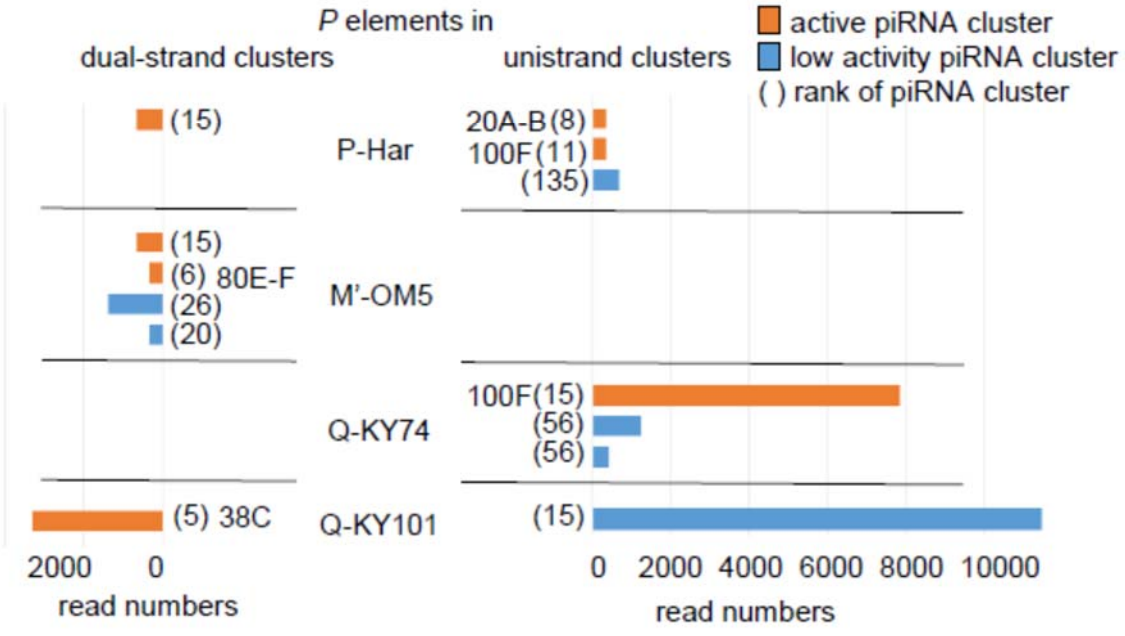


Figure 11

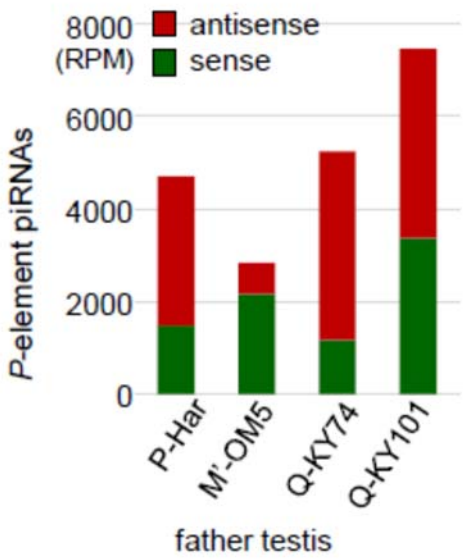

8000
(RPM)
6000
4000
2000
0
antisense
sense
P-element piRNAs
P-Har
M'-OM5
Q-KY74
Q-KY101
father testis

Figure 1. GD phenotypes of the F1 and F2 hybrids from the P-, M′ - and Q-strain males (A) Schematic representation of the experimental design for crosses. The test males (a) of the four line were crossed with the M-CS females to obtain F1 females (b), which were then crossed with P-Har males to obtain F2 females (c). (B) GD scores of F1 and F2 hybrids. Ovaries of approximately 50 (F1) and 100 (F2) flies were investigated to score the GD.

Figure 2. Genomic composition of *P* elements (A) Top: Results of PCR analysis amplifying total *P* elements (left, labeled with total *P* on the top) and non-*KP* elements (right, labeled with non-*KP*) using the respective genomic DNAs indicated on the top of each lane. The positions of DNA bands derived from *FP* (2526 bp) and *KP* (789 bp) elements produced in the "total *P*" PCR are indicated on the left. The positions of bands derived from the *FP* (2206 bp) and incomplete (internally deleted) elements produced in the "non-*KP*" PCR are indicated on the right. Bottom: Structure of the FP and KP elements and the primer design used for the PCR. The deletion junctions (at 808 and 2560 bp) of the KP element are indicated. (B) The copy numbers of *P* elements in the respective genomes relative to that in the M′ -OM5 genome. Star (*) indicates statistical significance ($p<0.05$). (C) The ratio of *KP* elements to the total *P*-element copies quantified by qPCR with KP- and total-P-specific primers.

Figure 3. Expression of *P*-element and *KP*-element mRNAs in ovaries and their relationships to GD scores (A) Fractions of silent- and active-expression regions that harbor *P* elements in the respective genomes. The fractions were calculated by dividing the number of sequencing reads that supported insertion

of the respective regions by the total sequencing reads to represent occupancy-adjusted copy numbers. 1% represents the fraction of unknown region that harbors *P* elements in the respective genomes. (B, C) The levels of *P*-element mRNA (B) and *KP*-element mRNA (C) in young F1 ovaries. The expressions were quantified by qRT-PCR and normalized by those of M′ -OM5 hybrids using qRT-PCR. Star (*) indicates statistical significance ($p<0.05$). (D, E) The relationship between the level of *KP*-element mRNAs in F1 ovaries (x-axis) and GD scores (y-axis) of F1 (D) and F2 (E) progenies.

Figure 4. Identification of *P*-element copies inserted into piRNA clusters (A) Genomic distributions of *P*-element insertion sites identified in the respective genomes (blue triangles). The height of blue triangles represents relative numbers of sequencing reads that supported insertion. Orange blocks show the piRNA (Brennecke *et al.,* 2007). Arrows indicate the active piRNA clusters harboring *P* elements. (B) The percentages of *P*-element reads identified in piRNA clusters in the total *P*-element reads. (C) Number of *P*-element copies in dual-strand (left) or unistrand (right) piRNA clusters. Active and low activity piRNA clusters are shown in orange and blue, respectively. Cluster names are shown with their rank of expression ability. The. + and - show the sense and antisense *P*-element insertion in relation to the transcription of the unistrand cluster.

Figure 5. Expression of *P*-element piRNAs derived from *P* elements in F1 hybrids. (A) Small RNA sequencing reads (24–35 nt long) in testes (upper) and F1 ovaries (lower) were mapped to the sense (green) and antisense (red) strands of the *P*-element. The *P*-element structure is schematically shown at the

bottom. (B) The positive relationship between occupancy-adjusted relative copy number of piRNA cluster-embedded *P* elements (the fraction of DNA reads supporting *P*-element insertion in clusters, x-axis) and the expression level of *P*-element piRNAs in F1 (CS female x test male) ovaries (y-axis). The Pearson's R and p values are indicated on the top. (C, D) The expression levels of *P*-element piRNAs in the ovaries of young (2- to 3 days old) F1 hybrids (C) and in 0-24 h whole embryos of F1 hybrid (D). *P*-element piRNA reads were normalized by miRNA reads (RPM, reads per million miRNA reads).

Figure 6. The *KP*-element mRNA and *P*-element piRNA in F1 ovaries are correlated to the GD scores in F1 and F2 hybrids (A) The relationship between the expression levels of piRNAs (y-axis) and mRNA (x-axis) of *P* elements in F1 ovaries. The Pearson's R and p values are shown on the top. (B) A dendrogram of the five natural strains constructed by hierarchical clustering based on the data shown in panel A. (C) The results of the multiple regression analysis for GD scores in F1 (as the objective variable) with the levels of *KP*-element mRNA and *P*-element piRNAs in F1 ovaries (as explanatory objectives). (D) The relationship between the expression levels of piRNAs in F1 hybrids (y-axis) and GD scores of F2 hybrids (x-axis). Pearson's R and p values are shown on the top. (E) A dendrogram constructed of the five natural strains by hierarchical clustering based on the data shown in panel D. (F) The results of the multiple regression analysis for GD scores in F2 (as the objective variable) with the levels of *KP*-element mRNA and *P*-element piRNAs in F1 ovaries (as explanatory objectives).

Figure 7. Proposed models for the mechanisms by which the respective

paternal genome protects daughters and granddaughters from *P*-element-induced gonadal dysgenesis

Schematic representation of transcriptionally active (light gray box) and silent (oblique-line box) regions, piRNA clusters (dark gray box), and *P* and *KP* elements (thick vertical lines) in the maternally (Mat) and paternally (Pat) inherited genomes, as well as their interactions in F1 and F2 hybrid ovaries. The name of the strain used as the male parent of the F1 progeny are indicated on the left. Short and long wavy lines represent *P*-element piRNAs and *P*-element mRNAs, respectively. Repressive effects are represented by a thick line with an inverted T (inverted T line), where thickness indicates the strength of suppression. Arrows show the transposition of *P* elements. Dotted lines from F1 to F2 hybrids show maternal deposition of piRNAs.

Figure 8: The relative abundance of *P* elements in genomes

P elements present in the respective genome was quantified by qPCR. Their abundance was normalized using the *RP49* gene.

Figure 9: *P* elements inserted into 100F piRNA cluster in KY74

Closed view the 100F piRNA cluster having 6 copies of *P* elements in Q-KY74. The nucleotide positions in chr3R are shown on the top.

Figure 10: *P*-element reads in piRNA clusters

The read numbers of deep sequencing data that suppot the *P*-element insertion in the respective piRNA clusters are shown for each fly genome. The clusters are categorized into dual-strand (left) and unistrand piRNA clusters (right). Active piRNA clusters are shown in orange, while low activity piRNA clusters are shown in light blue. The name of piRNA cluster is indicated if appreciable. The rank by

piRNA expression level is shown in parenthesis.

Figure 11: *P*-element piRNA abundance in testes

The *P*-element piRNA counts in testes of the respective strains are normalized by miRNA reads. RPM, million mapped miRNA reads. The abundance of sense (green) and antisense (red) piRNAs are colored.

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Supplement of methods

PCR and quantitative PCR

Genomic DNA was extracted from 20-40 whole flies from each line with standard methods as previously described (Sambrook *et al.* 1989). These DNAs were amplified using polymerase chain reaction (PCR) (Tks Gflex DNA polymerase; TaKaRa) using two sets of primers specializing in total-*P*-element (forward and reverse primers: 5′-TCAACGCAGATGCCGTACCT-3′ and 5′-CGTCGGCAAGAGACATCCACT-3′) and non-*KP* element (forward and reverse primers: 5′-TCAACGCAGATGCCGTACCT-3′ and 5′-CGACGTTTCGCGCTGCTAAT-3′), and then sequenced with Applied Biosystems big dye terminator v3.1 (Applied Biosystems) on Applied Biosystems 310 DNA sequencer (Applied Biosystems). Quantitative amplification of DNA was performed in duplicate using SYBR Green quantitation (TOYOBO and Applied Biosystems) on a 7000 HT Fast Real Time PCR System (Applied Biosystems) CFX96 Real Time System (BIO RAD), using primers specializing the *KP* element (forward and reverse primers: 5′-AACGTGACTGTGCGTTAGGT-3′ and 5′-TCAACATCGACGTTTCCACATC-3′) and non-*KP* element (forward and reverse primers: 5′-GGTGTCTCACGGCGGACTTA-3′ and 5′-CGACGTTTCGCGCTGCTAAT-3′). The level of *P*-element DNA was

normalized to that of RP49 DNA (forward and reverse primers: 5′-CGGATCGATATGCTAAGCTGT-3′, 5′-GCGCTTGTTCGATCCGTA-3′) (Dourlen *et al.* 2012).

Deep sequencing of the *P*-element insertion site

The genomic insertion site of *P* elements was amplified according to the protocol of Tsukiyama *et al.* (2013) with minor modifications. The genomic DNA (100 μg) extracted from 40 adult flies was digested with *Hha*I or *Taq*I (TaKaRa) and ligated to overhanging adapters (5′-CTCGTAGTCGGCACAGGATCACTCCGATACGC-3′, 5′-GTGCCGACTACGAGCG-3′ for *Hha*I and 5′-CGATCGTAGTCGGCACAGCATCACTCCGATACGCTAGCA-3′, 5′-ATGCTGTGCCGACTACGAT-3′ for *Taq*I; 0.25 pmol each) with 70U T4 ligase (TaKaRa). After purifying them with AMPure XP (Agencourt), primary PCR was performed with the standard protocol of 61.0°C for annealing, using 10 ng purified DNA, Tsk Gflex DNA polymerase (TaKaRa), and primers (5′-CACACTTCGGCACGTGAAT-3′, 5′-GCGTATCGGAGTGATCCT-3′ for *Hha*I products and 5′-GCTGTCTCACTCAGACTCAA-3′,

5′-TGCTAGCGTATCGGAGTGATG-3′ for *Taq*I products). Secondary PCR was performed at 62°C for annealing with 1/10 the volume of primary PCR products, KOD-Plus-Neo DNA polymerase (TOYOBO), and primers (5′-ACAAGCAAACGTGCACTGA-3′, 5′-GTGATCCTGTGCCGACTAC-3′ for *Hha*I products and 5′-CACTCGCACTTATTGCAAGCAT-3′, 5′-ATGCTGTGCCGACTACGAT-3′ for *Taq*I products).

For deep sequencing, the 300- to 600-bp-long *Hha*I and *Taq*I products were purified with the QIAquick Gel Extraction Kit (QIAGEN), followed by purification of the mixture of products with AMPure XP (Agencourt). The libraries were produced using 0.5–1.0 μg of them with TruSeq DNA PCR-Free LT Library Prep Kit (Illumina). Pair-end 500-bp sequencing of these libraries was performed on the MiSeq system (Illumina).

RT-PCR and quantitative RT-PCR

cDNAs were synthesized by superscript III reverse transcriptase (Invitrogen) using total RNA and an oligo-dT primer. Quantitative amplification of cDNA was performed in duplicate using SYBR Green quantitation (TOYOBO) on a 7000 HT Fast Real Time PCR System (Applied Biosystems) and CFX96 Real Tima System (BIO RAD). We used primers for *P* elements: forward

5′-GTGGGAGTACACAAACAGAGTCCTG-3′ and revers 5′-CGTATCTGCGTGTCCGTGAAGA-3′ and for *KP* elements: forward 5′-AACGTGACTGTGCGTTAGGT-3′ and revers 5′-TCAACATCGACGTTTCCACATC-3′. The level of *P*-element mRNA was normalized to that of RP49 mRNA (forward and reverse primers: 5′-CGGATCGATATGCTAAGCTGT-3′, 5′-GCGCTTGTTCGATCCGTA-3′, respectively) (Dourlen *et al.* 2012).

Achievements

Original reports

Keiko Wakisaka, Kenji Ichiyanagi, Seiko Ohno, Masanobu Itoh. 2017. Diversity of *P*-element piRNA production among M' and Q strains and its association with P-M hybrid dysgenesis in *Drosophila melanogaster*. Mob DNA. 8, 13

Keiko Wakisaka, Kenji Ichiyanagi, Seiko Ohno, Masanobu Itoh. 2018. Association of zygotic piRNAs derived from paternal P elements with hybrid dysgenesis in Drosophila melanogaster. Mob DNA. 6, 9:7.

Presentations in domestic meetings

Keiko Wakisaka, Masanobu Itoh. 2013 KP element piRNA repress the activity of P transposable element. Annual Meeting of the Genetics Society of Japan 2013. Yokohama Japan

Keiko Wakisaka, Masanobu Itoh. 2015 Diversity of P element piRNAs production

and mRNA expression in natural populations of *Drosophila melanogaster.* Annual Meeting of the Genetics Society of Japan 2015 Sendai Japan

Keiko Wakisaka, Kenji Ichiyanagi, Seiko Ohno, Masanobu Itoh. 2017 The association of zygotic piRNA derived from paternal *P* elements with hybrid dysgenesis in *Drosophila melanogaster*. Biochemistry and Molecular Biology Annual Meeting 2017. Kobe Japan

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June 2018

Keiko Wakisaka (Keiko Tsuji Wakisaka)