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piRNA-mediated transgenerational inheritance of an acquired trait

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The maintenance of genome integrity is an essential trait to the successful transmission of genetic information. In animal germ cells, piRNAs guide PIWI proteins to silence transposable elements (TEs) in order to maintain genome integrity. In insects, most TE silencing in the germline is achieved by secondary piRNAs that are produced by a feed-forward loop (the ping-pong cycle), which requires the piRNA-directed cleavage of two types of RNAs: mRNAs of functional euchromatic TEs and heterochromatic transcripts that contain defective TE sequences. The first cleavage that initiates such an amplification loop remains poorly understood. Taking advantage of the existence of strains that are devoid of functional copies of the LINE-like I-element, we report here that in such Drosophila ovaries, the initiation of a ping-pong cycle is exclusively achieved by secondary I-element piRNAs that are produced in the ovary and deposited in the embryonic germline. This unusual secondary piRNA biogenesis, detected in the absence of functional I-element copies, results from the processing of sense and antisense transcripts of several different defective I-element. Once acquired, for instance after ancestor aging, this capacity to produce heterochromatic-only secondary piRNAs is partially transmitted through generations via maternal piRNAs. Furthermore, such piRNAs acting as ping-pong initiators in a chromatin-independent manner confer to the progeny a high capacity to repress the I-element mobility. Our study explains, at the molecular level, the basis for epigenetic memory of maternal immunity that protects females from hybrid dysgenesis caused by transposition of paternally inherited functional I-element.

[Supplemental material is available for this article.]
Sterile Female) female progeny of I mothers and R fathers can produce enough piRNAs to achieve I-element repression; conversely, the reciprocal cross generates dysgenic females (called SF, for sterile females) that do not produce detectable amounts of I-element piRNAs and therefore accumulate high amounts of I-element messenger RNAs in the vicinity of the nucleus where retrotransposition can occur (Chambeyron et al. 2008). The maternal contribution was assumed to correspond to the deposition of I-element piRNAs in the embryonic germline of RSF females (Brennecke et al. 2008).

Previous genetic studies reported that the fertility of SF females can be improved by various aging and thermic treatments applied not only on the SF females themselves but also on their R mothers (Bucheton 1978). The effect of such treatments is maternally inherited but is progressively lost over generations (Bucheton 1979; Bucheton et al. 2002; Chambeyron and Bucheton 2005). Environmental factors can therefore modify the epigenetic characteristics of R females, as an acquired trait that can be partially transmitted to the progeny. We thus hypothesized that piRNA populations could be major players in this transgenerational epigenetic inheritance.

We report here the detection of secondary I-element piRNAs in the ovaries of R and SF females derived from aged ancestors. We also show that the production of R-specific secondary piRNAs, in the absence of any functional I-element, results from ping-pong amplification loops between many defective heterochromatic I-elements. We present evidence that the ability to produce secondary piRNAs in the ovaries of R females is an epigenetic trait that, once acquired upon aging treatment, can be transmitted through several maternal generations. Indeed, our data demonstrate that the maternal deposit of piRNAs in the embryonic germ cells directly primes the amplification loop of homologous secondary piRNAs in the ovaries of the progeny, without affecting the primary biogenesis.

Results

An inheritable age-induced defense system against functional I-element expression

To study the molecular basis of the partial rescue of SF sterility after ancestor aging of R strains, we split the Cha R strain into two fly stocks. The “short-generation stock” was bred with flies that were always obtained from young parents (3-d-old females); the “long-generation stock,” with flies continuously obtained from aged parents (25-30-d-old females for at least five generations) (Fig. 1A; Supplemental Methods).

We then crossed 3-d-old R females from both stocks with inducer males of the isogenic Cha-I strain (see Methods; Pelisson and Bregliano 1987) and assessed the fertility of the progeny (Fig. 1A,B). SF females with aged R maternal ancestors (for at least five generations) were significantly more fertile than SF females with young R maternal ancestors (P = 10^{-12} (Fig. 1B). This fertility increase correlated with I-element repression in the SF germline. Indeed, in situ hybridization experiments on whole-mount ovaries showed that l-element transcripts were accumulated at the anterior pole of oocytes in sterile SF females, whereas they were not detected in fertile SF flies, the ancestors of which were aged (Fig. 1C). Therefore, we designated the short-generation stock as “L” and the long-generation one as “H” (for respectively Low and High capacity to repress the l-element).

These results indicate that in these two isogenic lineages, the capacity to repress I-element expression is tightly linked to the ancestor aging treatment.

Establishment of an efficient piRNA-mediated silencing of the l-element in the dysgenic female progeny of aged ancestors

We next compared the ovarian piRNA populations of 3-d-old isogenic SF (SF_{3d}) females (with young R ancestors) and SFH (SF_{H3d}) females (with aged R ancestors). In order to enhance the quality of
our libraries, we used an anion exchange chromatography strategy to purify protein-associated small RNAs as previously described (see Supplemental Methods; Lau et al. 2009). This approach depletes RNA degradation products and decreases the amount of 28S rRNA, the main Drosophila contaminant, in sequenced libraries (Supplemental Fig. S1). To account for differences in sequencing quality and depth, we normalized the small RNA populations to 1 million piRNAs as previously published (Handler et al. 2011; see Methods; Supplemental Methods). This normalization strategy is optimal for minimizing the differences between libraries (Supplemental Fig. S2).

The levels of ovarian piRNAs from each of the 85 major TEs were highly similar in both isogenic libraries (Fig. 2A). As expected, the 1-element showed the largest difference, with a 2.9-fold increase in 1-element piRNA levels in SFH3d ovaries compared with SF3d ovaries (Fig. 2B, Supplemental Table S1). This increase concerned both sense and antisense 1-element piRNA populations along the whole 1-element sequence (Supplemental Fig. S3A). Both perfectly matching 1-element piRNAs (mainly resulting from post-transcriptional processing of functional 1-element transcripts) and the mismatching 1-element piRNAs (exclusively coming from defective 1-element transcripts) were increased (Supplemental Fig. S3B). Moreover, 1-element piRNAs exhibited a classic ping-pong signature in SFH3d ovaries but not in SF3d ovaries, and this feature was correlated with 1-element repression (Fig. 2C). This finding is consistent with previous data showing a link among 1-element piRNA accumulation, ping-pong signature, and 1-element repression (Brennecke et al. 2008; Chambeyron et al. 2008). The ping-pong signatures for other TEs with dominant germline expression in ovaries from SFH3d and SF3d were not increased (Supplemental Fig. S7A).

Figure 2. Aging treatment boosts the production of 1-element piRNAs in the next generations. (A,D) Pairwise comparison of the TE piRNA levels in ovaries from 3-d-old isogenic flies (SF3d, SFH3d, and RL3d, RH3d) that have young or aged ancestors, respectively. Scatter plots display correlation between normalized piRNA abundances for each of the 85 most highly targeted Drosophila TEs (up to four mismatches allowed between reads and RepBase sequence). (B,E) Normalized counts of ovarian 1-element piRNAs shows that they are increased in the offspring of females with aged ancestors. (C,F) A ping-pong signature for the 1-element could only be detected in the progeny of aged ancestors.
Finally, bioinformatic analysis of previously published small RNA deep sequencing data (Brennecke et al. 2008) showed that, based on the amount of I-element piRNAs and the ping-pong signature, RF13d females were similar to fertile RSF females, obtained by crossing R males with 1 females (Fig. 2B,C; Supplemental Fig. S3C). Conversely, SF13d females (characterized by low I-element piRNA amount and absence of ping-pong signature) (Fig. 2B,C) were comparable to previously described sterile SF females (Supplemental Fig. S3C).

In summary, these observations demonstrate that the aging of R ancestors over several successive generations results in a piRNA-mediated repression of I-element expression in a SF cross that is almost as efficient as the repression previously described in a RSF cross.

Ping-pong amplification can occur between defective I-elements in R strains

The production of secondary I-element piRNAs in RSF females was assumed to depend on the piRNAs inherited from their 1 mothers (Brennecke et al. 2008). We therefore investigated whether I-element piRNAs accumulated in the R mothers of SF13d females by comparing the ovarian piRNA populations of R13d (short -generation stock) and R13d (long-generation stock) 3-d-old flies. In contrast to the relatively small differences in piRNA accumulation observed for the 85 studied TEs (Fig. 2D, Supplemental Table S1), I-element piRNA levels were 3.2-fold higher in R13d than in R13d ovaries (Fig. 2E). This increase concerned both sense and antisense I-element piRNA populations along the I-element sequence (Supplemental Fig. SSA). The increase of I-element piRNAs by maternal aging was not associated with changes in PIWI protein levels (Supplemental Fig. S4). Moreover, for the first time, we detected a ping-pong signature for the I-element in R ovaries (R13d in Fig. 2F), indicating that aging over several generations results in active I-element secondary biogenesis in the progeny.

To further characterize this phenomenon, we split the I-element piRNA populations into piRNAs that had at least one sequenced ping-pong partner (i.e., another piRNA containing a 10-mer; see Methods) and all the others (Fig. 3A). The first class is likely to correspond to secondary piRNAs, while the piRNA class without a sequenced ping-pong partner consists of both primary piRNAs and secondary piRNAs, the ping-pong partners of which were not sequenced due to the limited sequencing depth. As shown in Figure 3A and Supplemental Figure SSB, the increase of I-element piRNA level in R13d ovaries was likely due to ping-pong amplification.

The production of I-element piRNAs with ping-pong partners exhibited a strong nucleotide-strand bias (80% of antisense piRNAs had a uridine [U] at position 1 and 80% of sense piRNAs had an adenosine [A] at position 10) (Supplemental Fig. SSC). So, in R strains that are devoid of functional I-element, sense secondary piRNAs mainly had an adenosine at position 10; a feature previously observed for I-element piRNAs produced in 1 strains by processing of functional I-element transcripts (Brennecke et al. 2008). Hence, the ping-pong amplification in R strains could be achieved by heterochromatic-defective I-element transcripts behaving just like the functional transcripts of euchromatic I-elements in 1 strains, by producing sense I-element piRNAs with an adenosine at position 10.

To determine whether a specific defective I-element fragment played the role of the “functional-like” I-element in R13d ovaries and could thus potentially trigger an efficient secondary biogenesis through the production of 1A sense piRNAs, we computed the amount of secondary piRNAs originating from each of the I-element defective fragments that have been annotated in the Drosophila genome (Fig. 3B). Here we identified that, in R13d ovaries, many I-element defective fragments produce secondary piRNAs in sense and antisense orientation according to the functional I-element sequence (Fig. 3B; Supplemental Fig. S6). Interestingly, we also determined that the ping-pong loops between heterochromatic-defective I-element transcripts mainly occur between transcripts that do not originate from the same heterochromatic-defective fragment (Supplemental Fig. S5D). Indeed, 90% of the secondary piRNAs uniquely mapping to the genome have ping-pong partner(s) that belong to a defective I-element transcript coming from other genomic loci.

The ping-pong loop increase between heterochromatic-defective fragments is specific to I-element as secondary piRNAs coming from other germinal TEs do not seem to be affected (Supplemental Fig. S7B).

The 42AB cluster is considered to be the major producer of genome-unique I-element piRNAs and a major regulatory locus for the I-element (Brennecke et al. 2008). We thus asked whether 42AB-derived I-element piRNAs were also highly sensitive to ancestor aging. Ancestor aging only induced a 2.2-fold increase of 42AB-derived I-element piRNAs in R13d compared with R13d whereas the other genome-unique I-element piRNAs showed a 3.7-fold increase (Supplemental Fig. S5E). These results indicate that (1) despite their strong piRNA production, defective I-elements from the 42AB cluster were not more sensitive to ancestor aging than defective I-elements belonging to the other producer loci such as those located in 2RHet and U chromosome arms; and (2) the increase of the secondary piRNA population in the R13d library originated from most of, if not all, the defective I-element fragments (Fig. 3B).

Our observations provide evidence for a cryptic secondary piRNA biogenesis pathway that is boosted in the R female progeny of aged ancestors and involves many defective I-element fragments.

The stimulation of secondary piRNA biosynthesis by maternal aging does not result from increased levels of I-element piRNA precursors

Several chromatin-associated proteins were reported to modulate the efficiency of piRNA biogenesis in the Drosophila germline at the level of transcription and/or processing of the heterochromatic piRNA precursors (Klattenhoff et al. 2009; Rangan et al. 2011; Zamparini et al. 2011). We therefore asked whether the increased production of secondary piRNAs and its maternal inheritance could be due to modulation of transcription or chromatin modifications of defective I-elements in R13d ovaries compared with R13d ovaries.

We first compared the steady-state RNA levels of specific regions that contain defective I-elements and are affected by ancestor aging (I1667-1, I1667-2, U, and 2RHet), of other regions of the 42AB cluster (CL1A, CL1B), and of the control (vasa). By use of quantitative real-time PCR amplification after random reverse transcription of total RNA from R13d and R13d ovaries, we were unable to detect any significant differences in the steady-state RNA levels after ancestor aging (P > 0.05) (Fig. 4A). We then compared the chromatin state of these regions in R13d and R13d ovaries by performing chromatin immunoprecipitation experiments with antibodies against chromatin marks that are assumed to reflect the transcriptional activity of these genomic loci (Fig. 4B). Again, we did not detect any significant differences in the enrichment of
Figure 3. Evidence for ping-pong amplification between defective \textit{l-element} fragments. (A) Density profile of piRNAs matching the \textit{l-element} (up to four mismatches) when they are split into piRNAs with ping-pong partners (green) and other piRNAs (blue). (B) Most of the \textit{l-element} defective fragments contributed to the ping-pong–mediated amplification of \textit{l-element} piRNAs in the offspring of aged ancestors. Each horizontal bar indicates one of the \textit{l-element}–defective fragments annotated in the Release 5 genome sequence that were aligned to the functional \textit{l-element} sequence and sorted by chromosomal positions (the \textit{l-element} fragments located in the 42AB cluster [orange]). Green bar diagrams indicate the abundance of their corresponding piRNAs with ping-pong partners in the two libraries (RH$_{3d}$ and RL$_{3d}$ ovaries). (Left) Total piRNA levels, including read counts of piRNAs mapping to several defective \textit{l-elements} weighted by their mapping number; (right) levels of piRNAs that map uniquely to each defective \textit{l-element}. 

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trimethylated histone 3 lysine 9 (H3K9me3) \( (P > 0.05) \), which has been reported to be associated with piRNA cluster transcription (Rangan et al. 2011); in the enrichment of dimethylated histone 3 lysine 4 (H3K4me2; \( P > 0.05 \)), which has been described as a mark of transcriptionally active chromatin (Klenov et al. 2011); or in the enrichment of RNA polymerase II (Pol II) (\( P > 0.05 \)), which might reflect the transcription level of a genomic region.

If ancestor aging results in an increase of the level of the piRNA precursors containing \( I \)-element fragments, then an increase of the level of piRNAs from genomic loci flanking defective \( I \)-element fragments in the same precursor should also be observed. We thus determined the amount of genome-unique piRNAs mapping to \( 1 \)-kb bins flanking the \( 5' \) or \( 3' \) end of annotated \( I \)-element–defective fragments in both \( R^{I}_{3d} \) and \( R^{H}_{3d} \) ovary libraries (see Methods) (Fig. 5; Supplemental Table S2; Supplemental Figs. S8, S9). First, the change of piRNA level in these bins was on average 1.1-fold, a significantly lower variation than the average of 3.3-fold increase observed for the associated defective \( I \)-elements (\( P = 10^{-8} \)) (Fig. 5A). Second, we did not detect any significant correlation between the changes in the piRNA levels mapping to individual \( I \)-element fragments and those of their flanking sequences (Spearman’s correlation coefficient, 0.43; \( P = 10^{-8} \)) (Fig. 5B).

Thus, we conclude that the increase of piRNA level in \( R^{H}_{3d} \) did not spread from the genomic loci that contain defective \( I \)-elements toward the flanking regions (that contain defective copies of many other TEs), in agreement with the observation of absence of chromatin changes after ancestor aging (Fig. 4).

Our data therefore suggest that the effect of ancestor aging on secondary piRNA production is not mediated by chromatin changes of the \( I \)-element piRNA producers.

The effect of ancestor aging is transmitted to the next generations through maternally deposited embryonic piRNAs

We then hypothesized that maternally deposited embryonic piRNAs could be responsible for the de novo ovarian amplification of secondary piRNAs, thus explaining the non-Mendelian inheritance of the age-induced defense system. In order to determine whether...
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Figure 5. Ancestor aging does not affect piRNA production of genomic loci flanking defective I-element fragments. (A) Boxplots display the fold change in the level of genome-unique piRNAs mapping to defective I-element fragments (right) and to associated flanking 1-kb bins (left). Only genomic loci producing at least one piRNA per million total sequenced piRNAs in both R\textsuperscript{3d} and R\textsuperscript{3d} ovary libraries were considered. (Dashed line) The one-fold change value. Wilcoxon signed-rank test \( P = 10^{-5}, n = 26 \). (B) Correlation between the fold change in the level of genome-unique piRNAs mapping to each defective I-element fragment and the fold change in the level of genome-unique piRNAs mapping to their associated flanking 1-kb bins (Spearman’s rank correlation test: \( \rho = -0.15; P = 0.43, n = 26 \)).

Overall, our data show that the memory of increased accumulation of secondary I-element piRNAs in aged ovaries is likely to be transmitted to the next generations through the increased deposition of these piRNAs in the embryonic germline at each generation.

Discussion

Aging triggers the production of heterochromatic-only I-element secondary piRNAs in the next generations

piRNAs contribute to the maintenance of genome integrity in the Drosophila female germline by repressing complementary TEs and thereby preventing dysgenisis. According to the ping-pong model, these antisense piRNAs result from the slicing of transcripts from the heterochromatic-defective TEs by ARGONAUTE3 (AGO3). AGO3 is guided by sense piRNAs that are themselves produced by the reciprocal piRNA-dependent cleavage of functional euchromatic TE RNAs (Brennecke et al. 2007; Gunawardane et al. 2007). Here we show that a ping-pong amplification can occur in the total absence of euchromatic I-elements (Fig. 2F) involving many defective I-elements (Fig. 3B, Supplemental Fig. S6).

One of the interesting questions raised by our observations is whether or not this previously unknown heterochromatic-only secondary piRNA production is specific to the I-element. We speculate that such a secondary biogenesis might also exist for the other TEs but produced only very few secondary piRNAs compared to the ping-pong loop involving heterochromatic TE precursors and TE mRNAs. This might explain the weak influence of ancestor aging on the secondary piRNA populations of all TEs, except the I-element, which is not present as a functional element in the Cha-R genome.

Once acquired upon aging treatment, this ability to produce heterochromatic-only I-element secondary piRNAs can be transmitted to the next generation of young females (Fig. 6) in a chromatin-independent manner (Fig. 4, Figure 5). The memory of ancestor aging is strong enough to promote a significant reduction of dysgenisis in the granddaughters of aged females (Fig. 1).

Khurana et al. (2011) have recently described another kind of ancestor aging memory in the P-M hybrid dysgenesis system, which is based on the mobilization of resident functional elements like the hsc-element into piRNA clusters in aged dysgenic female ovaries. However, the P-M dysgenic aging effect is not reversible and leads to de novo piRNA production in the progeny of aged females in contrast to the piRNA-dependent aging memory here described.

Maternally deposited piRNAs as vectors of short-term maternal inheritance (Fig. 7)

In the ping-pong model as described for some TEs like R\textit{1b}, the primary piRNAs produced by heterochromatic RNA precursors initiate the secondary piRNA biogenesis (Brennecke et al. 2007; Handler et al. 2011). However, for other TEs, like the F and Doc elements, the production of secondary piRNAs is much less affected by mutations of the primary piRNA pathway than in the case of R\textit{1b}, suggesting that they can rely on maternal piRNAs as ping-pong loop initiators (Malone et al. 2009; Handler et al. 2011). Whether these maternal F and Doc piRNAs are essential or have only a redundant role with primary piRNAs in initiating the ping-pong cycle is unknown.

The observation that, in the R\textsuperscript{3d} strain, the I-element secondary biogenesis pathway is very inefficient (Fig. 2F) suggests that I-element primary piRNAs are not sufficient to frequently initiate
the ping-pong loop. This conclusion is also supported by the finding (Fig. 3A; Supplemental Fig. SSB) that the important increase of secondary piRNAs in the RH strain did not seem to result from an increase in the putative primary piRNAs (those without sequenced ping-pong partner).

Our data therefore suggest that the lack of maternally deposited I-element piRNAs is responsible for the absence of secondary piRNAs in the RH strain. Indeed, the ping-pong signature only becomes detectable in the ovaries of young daughters after the increase in the maternal secondary piRNA pool (i.e., in the eggs laid by the aged RH strain) (Fig. 2F). Moreover, in agreement with the requirement of maternal piRNAs for the initiation of the corresponding ping-pong loop, we observed a good correlation between the amounts of secondary, but not for putative primary piRNAs deposited in the embryo and those produced in the ovary (Fig. 6E). In contrast, the maternal deposition does not seem to influence the availability of I-element defective RNA precursors as indicated by the fact that (1) the steady-state RNA levels of I-element piRNA precursors remained unchanged (Fig. 4A); (2) we failed to detect any changes in chromatin that could be responsible for an increase in transcription and/or processing of the I-element piRNA producers (Fig. 4B); and (3) the increased ability of individual I-elements to produce piRNAs did not spread to the defective TE sequences present in their flanking sequences (Fig. 5).

Our model might also explain the establishment and maintenance of P-element repression which requires both chromosomal and extrachromosomal determinants (Ronsseray et al. 1993). When P-elements are paternally inherited in the absence of the maternal extrachromosomal determinants, it takes several generations to fully repress P-element in this naïve maternal lineage (Engels 1979). Since P-element piRNAs are maternally transmitted (Brennecke et al. 2008), we propose that, as for the I-element, a maternal piRNA pool of P-element secondary piRNAs is required to initiate an efficient ping-pong-dependent piRNA production. The progressive acquisition of the P-element repression could result from maternal deposition of steadily increasing levels of such ping-pong loop initiators at each generation.

We moreover speculate that, in the aged RH strain, the level of sense RNA precursors represents the limiting input of the secondary piRNA biogenesis, as the introduction of functional I-elements (mainly transcribed in the sense orientation) results in an additional production of I-element piRNAs in SFH females (Fig. 2B,E). This limitation, probably combined with partial maternal deposition and dilution of the maternal piRNA pool during oogenesis, could explain the reversibility of the acquired trait after several generations if the aging treatment is not maintained (Fig. 7).

Our model also explains why paternally inherited functional I-elements are not efficiently repressed in the ovaries of SF dysgenic females when the reactive mother does not provide enough initiator piRNAs. Nevertheless, the establishment of the I-element
repression is somewhat quicker than that of P-element (Engels 1979), since it generally takes only two maternal generations (Pelisson and Bregliano 1987). This might be due to the fact that, unlike M strains, R strains can produce low levels of maternally transmitted initiator heterochromatic piRNAs. When higher amounts of piRNAs are maternally deposited, as in the RH lineage described here, almost full repression can be reached in the first generation (SFH females). Note however that, as recently reported (Khurana et al. 2011), P-M dysgenic females, lacking any P-element piRNA, can, at the end of their life, produce enough piRNAs to completely repress their P-elements.

Long-term inheritance of the piRNA nucleotide-strand bias

Cloning and sequencing of piRNAs associated with PIWI proteins revealed protein-dependent nucleotide and strand bias. In the Drosophila ovaries, the PIWI and AUBERGINE (AUB) proteins preferentially load antisense 1U piRNAs coming from heterochromatic transcripts, and the AGO3 protein mainly loads sense 10A piRNAs derived from the cleavage by AUB of functional TE transcripts. The PIWI and AUB 1U bias is probably due to their 1U affinity, as shown for their SIWI silkworm homolog (Kawaoka et al. 2011b). The molecular basis for the PIWI and AUB antisense bias is not thoroughly understood.

In an attempt to explain these strand biases, it was proposed that functional euchromatic sense transcripts are physically isolated in specific subcellular domains where they would be selectively targeted by complementary AUB-associated piRNAs and presented to AGO3 for piRNA loading. Such domains might correspond, for instance, to the mouse pi-bodies, where TE transcript-derived piRNAs appear to be preferentially loaded by the PIWIL2 (also known as MILI) protein (Aravin et al. 2008).
Here, we show that a ping-pong signature could be readily detected in the absence of functional euchromatic I-elements (Fig. 2F). Surprisingly, in these R ovaries, only sense I-element ping-pong piRNAs exhibit a 10A bias (Supplemental Fig. 2C, SSC). As the secondary biogenesis in R strains occurs between many heterochromatin transcripts that contain various detectable I-element fragments in any orientation, how could AGO3 discriminate piRNAs, which are sense according to the I-element? An exciting hypothesis is that the bias originated a very long time ago during the first invasion of the Drosophila genome by functional I-elements. As PIWI and AUB localize to the posterior pole of the oocyte where the primordial germ cells of the embryo are formed (Harris and Macdonald 2001; Megosh et al. 2006), AUB-associated piRNAs were already maternally deposited as antisense 1U piRNAs, initiating the production of complementary sense 10A AGO3-associated piRNAs. Since then, the bias would have been faithfully transmitted in an egg-hen cycle.

Why do aged ovaries produce more I-element secondary piRNAs?

In this work, we took advantage of an increased level of I-element piRNAs in aged ovaries to study how this initial change is maternally inherited. However, we can only speculate about the mechanisms whereby aging itself can affect I-element piRNA biogenesis. In particular, we do not know whether a specific germ cell and/or oogenesis stage translates the effect of aging to I-element ovarian piRNAs. A priori, aging could affect the physiology of any ovarian tissue. Even germlinal stem cells (GSCs) were reported to age and die after a number of asymmetric divisions (Xie and Spradling 2000; Pan et al. 2007) generating one GSC and one cystoblast (i.e., the differentiated precursor of the future egg) (Spradling et al. 2001).

In our reactive strain-model, I-element piRNA biogenesis could be limited by the lack of both maternal (secondary) and zygotic (primary) initiator piRNAs. Aging is known to affect mitochondrial physiology (Schwarze et al. 1998; Das et al. 2001). Potential relationships have been recently described between mitochondria and primary piRNA biogenesis (Haase et al. 2010; Olivieri et al. 2010; Saito et al. 2010; Huang et al. 2011; Watanabe et al. 2011). Hence, a putatively improved primary piRNA production might explain why aged ovaries produce more secondary I-element piRNAs.

Alternatively, the increased piRNA production might not be directly due to cellular aging, but rather to the several cell divisions required to generate aged GSCs, like the several fly generations that are needed for the progressive establishment of the P-element repression. According to this hypothesis, the first ping-pong cycle would be initiated by maternally deposited piRNAs in the primordial germ cells, as described in the silkworm (Kawaoaka et al. 2011). Then, a round of secondary piRNA production, initiated by the piRNA pool inherited from the mother cell, would occur postmitotically in each GSC. A challenge for the future will be to test this cellular memory hypothesis.

Methods

Drosophila stocks

Fly ovaries were dissected into ice Schneider's insect medium (Sigma) and homogenized in 500 μL buffer A (60 mM KCl, 15 mM NaCl, 15 mM Hepes, 0.5% Triton-X100, 10 mM sodium butyrate, complete “mini” EDTA-free protease inhibitor tablets; Roche) with 1.8% formaldehyde (Thermo Scientific) at room temperature (RT) using a Douncer with pestle “tight.” Total time for homogenization and cross-linking was exactly 10 min. Glycine was added to 125 mM final, and samples were incubated 3 min at RT and then chilled on ice. Samples were centrifuged (5 min, 4000 g at 4°C), and pellets were resuspended in buffer A with 125 mM glycine, incubated 5 min with agitation, and then centrifuged. The washing step was repeated two times with buffer A and another time with lysis buffer (140 mM NaCl, 15 mM Hepes, 1 mM EDTA, 0.5% sodium deoxycholate, 10 mM sodium butyrate, complete “mini” EDTA-free protease inhibitor tablets). Pellets were resuspended in 200 μL lysis buffer with 0.5% SDS, 0.5% N-lauroylsarcosine and incubated 3 h at 4°C on a rotating wheel. Samples were sonicated with a Bioruptor (Diagenode; high level, 30 sec on, 30 sec off, repeated 12 times with resuspension after six times) and centrifuged (10 min, 20,000 g at 4°C). Lysates were then 5× diluted to 0.1% SDS with lysis buffer. Protein G dynabeads (Invitrogen) were washed in PBS-0.1 mg/mL BSA three times, added to lysates and incubated 3 h at 4°C on a rotating wheel. Protein G dynabeads were then replaced by dynabeads coupled with antibody (anti-Pol II clone 4H8 [ab5408, Abcam], anti-H3K4me2 [no. 07-030, Millipore], and anti-H3K9me3 [ab8898, Abcam]) according to the manufacturer’s instructions and incubated overnight at 4°C on a rotating wheel. After incubation, beads were washed seven times for 10 min at 4°C; two times in RIPA buffer (150 mM NaCl, 50 mM Tris at pH 8.1, 0.1% SDS, 1% NP40), one time in high-salt buffer (500 mM NaCl, 50 mM Tris at pH 8.1, 0.1% SDS, 1% NP40), one time in LiCl buffer (250 mM LiCl, 50 mM Tris at pH 8.1, 0.5% sodium deoxycholate), and three times in Tris-EDTA (TE) (10 mM Tris at pH 8, 0.1 mM EDTA). Chromatin
was eluted by adding 100 μL of elution buffer 1 (10 mM EDTA, 1% SDS, 50 mM Tris-HCl at pH 8) and incubated 15 min at 65°C under vigorous shaking. Elution was repeated with 150 μL of elution buffer 2 (0.67% SDS in TE). The two supernatants were pooled and cross-linking of each immunoprecipitated complex was reversed by incubation of the eluate during 6 h at 65°C in a mix containing 1% SDS and 200 mM NaCl. After digestion with RNase A at 37°C for 1 h and proteinase K for 2 h at 50°C, DNA was purified by phenol-chloroform extraction (MP Biomedicals) and precipitated with ethanol. Biological triplicates were generated for each experiment and subjected to quantitative real-time PCR performed with ethanol. Biological triplicates were generated for each experiment with anti-Pol II and anti-H3K4me2 antibodies were normalized to rp49-positive control and to 1360-element for the anti-H3K9me3 antibody.

RNA extraction and qRT-PCR
Quantitative PCR was performed on LightCycler480 (Roche) using SYBR Green I Master (Roche). Total RNA was isolated from 50 ovary pairs by TRIzol extraction (Invitrogen). RNA was treated with Turbo DNase (Ambion) according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription of 2 μg total RNA using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers. Quantitative PCR was realized on LightCycler480 (Roche) using SYBR Green I Master (Roche). Each experiment was performed in biological triplicates with technical duplicates. RNA levels were normalized to the mRNA level of light.

Bioinformatic analysis of small RNA libraries
Our pipeline was based on the analytical strategy which has been previously described in Brennecke et al. (2008) and Handler et al. (2011; see Supplemental Methods). To quantify the non-I-element-derived piRNAs that flank defective I-elements, we first retrieved from the UCSC repeatmasker track genomic positions of all defective I-element fragments that were at least 1 kb apart. We then defined a 1-kb bin flanking each end of these defective I-elements and retrieved all piRNA sequences mapping uniquely to them. Annotation of defective TEs located in flanking bins was performed using the UCSC RepeatMasker track.

Statistical analyses
All statistical analyses were performed using R software (R Development Core Team 2010; http://www.R-project.org). For comparison of sample means (Figs. 1B, 4A, B, 5A; Supplemental Fig. S4), P-values were calculated using two-tailed Student t-test for samples displaying normal distribution (tested with Shapiro-Wilk test) and variance homogeneity (tested with Levene’s test) and using Wilcoxon signed-rank test for others. For correlation analyses (Figs. 5B, 6B–E), P-values were calculated using Spearman’s rank correlation test to avoid outlier effects.

Data access
Small RNA libraries generated in this study are available via the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE34506, data sets GSM850349 to GSM850354.

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Author contributions: T.G., C.B., and B.M. performed the experiments. C.A. performed all bioinformatic analysis, assisted by discussions with V.S. and A.P. A.F. and S.C. designed the research. C.A., A.P., T.G., and S.C. wrote the manuscript with the help of discussions with B.M., V.S., and C.B.

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