The Epigenetic Trans-Silencing Effect in Drosophila Involves Maternally-Transmitted Small RNAs Whose Production Depends on the piRNA Pathway and HP1

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Abstract

Background: The study of P transposable element repression in Drosophila melanogaster led to the discovery of the Trans-Silencing Effect (TSE), a homology-dependent repression mechanism by which a P-transgene inserted in subtelomeric heterochromatin (Telomeric Associated Sequences, “TAS”) has the capacity to repress in trans, in the female germline, a homologous P-lacZ transgene located in euchromatin. Phenotypic and genetic analysis have shown that TSE exhibits variation in ovaries, displays a maternal effect as well as epigenetic transmission through meiosis and involves heterochromatin (including HP1) and RNA silencing.

Principal Findings: Here, we show that mutations in squash and zucchini, which are involved in the piwi-interacting RNA (piRNA) silencing pathway, strongly affect TSE. In addition, we carried out a molecular analysis of TSE and show that silencing is correlated to the accumulation of lacZ small RNAs in ovaries. Finally, we show that the production of these small RNAs is sensitive to mutations affecting squash and zucchini, as well as to the dose of HP1.

Conclusions and Significance: Thus, our results indicate that the TSE represents a bona fide piRNA-based repression. In addition, the sensitivity of TSE to HP1 dose suggests that in Drosophila, as previously shown in Schizosaccharomyces pombe, a RNA silencing pathway can depend on heterochromatin components.

Introduction

Mobilization of transposable elements (TEs) is regulated by complex mechanisms involving proteins encoded by the TEs themselves, as well as heterochromatin formation and small RNA silencing mechanisms [1–11]. Genomic sites containing full-length or defective copies of TEs have been identified which are sufficient to establish complete repression of the other copies of the same family scattered throughout the genome. For example in Drosophila, the flamy/COM locus, located in pericentromeric heterochromatin, represents various families of “Type I” TEs (retrotransposons which transpose via an RNA intermediate) [12–15] and the TAS (Telomeric Associated Sequence) region of sub-telomeric heterochromatin houses strong regulatory P elements (“Type II” TEs whose transposition occurs via a DNA intermediate) [2,16–18]. The flamy/COM locus represses expression of gypsy, zeno, and Idefix in somatic follicle cells, thereby preventing transfer of these retrotransposons to the oocyte [19,20]. By contrast, P element repression by telomeric P copies takes place in the germline of both sexes [17,18,21,22] and it is in this tissue that all P element transposition steps take place [3,23,24]. It has been shown recently that the RNA silencing pathways implicated in both the germline and somatic follicle cells of the ovary rely on the piwi-interacting RNA (piRNAs) silencing pathway [8], although the mechanisms at work in these two tissues differ since some actors of the piRNA machinery are present only in the germline [25–28].

The study of the mechanism of P element repression in the germline, elicited by telomeric P copies, has been facilitated by the use of P-transgenes instead of natural P transposons. The P-lacZ transgene carries an in-frame fusion of the N-terminal region of the transposase with the E. coli lacZ gene and can be used as an enhancer-trap [29]. It has been shown that the presence of one or two copies of P-lacZ in TAS, can repress another P-lacZ copy in trans, irrespective of the genomic location of the latter copy [30–32]. This repression occurs in the female germline (nurse cells and oocytes), but not in the somatic follicle cells [32]. This phenomenon, termed “Trans-Silencing Effect” (TSE) [30], thus allows the precise study of the genetic and phenotypic properties of piRNA-based repression in the context of the germline. It has been
shown that TSE displays a maternal effect, epigenetic transmission through meiosis and variegation between egg chambers when repression is incomplete [31,33]. TSE was also shown to be affected by mutations in genes involved in heterochromatin formation (including HP1) and the piRNA silencing pathway [33]. In particular, TSE was shown to be completely abolished by mutations affecting aubergine, armitage, homeless (spindle-E) and a partial dose effect of piwi was also found [33]. All these genes have been shown to be necessary for the production of piRNAs in the germine [25].

In the present study, we explore further the genetic and molecular properties of TSE with regard to the piRNA-based mechanism of repression. We first tested the effect on TSE of mutations in squash (squ) and zucchini (zuc), encoding two putative nucleases which have been shown recently to be involved in the piRNA pathway [25,34]. SQUASH and ZUCCHINI both interact with AUBERGINE and mutants exhibit dorso-ventral patterning defects similar to those associated with aub mutations. Mutations in squ and zuc induce the transcription upregulation of Het-A and TART telomeric retrotransposons and result in the loss of piRNAs in the germine [25,34]. We first show that the loss of function of squ and zuc has a very strong negative effect on TSE. Second, we provide the first molecular support of the mechanism of TSE showing that trans-silencing is correlated to the presence of lac_\alpha_ small RNAs in ovaries, the levels of these small RNAs being strongly affected by mutations in squ and zuc. Third, we show that accumulation of these small RNAs in ovaries is also sensitive to a mutation affecting HP1 levels. These results open the possibility of a functional reciprocal dependence between heterochromatin formation and RNA silencing in Drosophila. Thus TSE in the fly could parallel the “self-reinforcing loop” of RNA silencing and heterochromatin previously shown to occur in Schizosaccharomyces pombe [35–37].

**Materials and Methods**

**Experimental conditions**

All crosses were performed at 25°C and involved 3–5 couples in most of the cases. All ovary lac_\alpha_ expression assays were carried out using X-gal overnight staining as described in Lemaitre et al. 1993 [21], except that ovaries were fixed for 6 min [33].

**Transgenes and strains**

P-lac_\alpha_ fusion enhancer trap transgenes (P-1152, BQ16) contain an in-frame translational fusion of the E. coli lac_\alpha_ gene to the second exon of the P transposable gene and contain rosy [41] as a transformation marker [38]. The P-1152 insertion (FBti0005700) comes from stock #11152 in the Bloomington Stock Center and was mapped at the telomere of the X chromosome (site 1A); this stock was previously described to carry a single P-lac_\alpha_ insertion in TAS [30]. However, in our #11152 stock, we have mapped two P-lac_\alpha_ insertions in the same TAS unit and in the same orientation which might have resulted from an unequal recombination event duplicating the P-lac_\alpha_ transgene [33]. P-1152 is homozygous viable and fertile. BQ16 is located at 64C of euchromatin of the third chromosome [32] and is homozygous viable and fertile. P-1152 shows no lac_\alpha_ expression in the ovary, whereas BQ16 is strongly expressed in the nurse cells and in the oocyte.

Lines carrying transgenes have M genetic backgrounds (devoid of P transposable elements), as do the multi-marked balancer stocks used in genetic experiments and the strains carrying mutations used for the candidate gene analysis. The Canton' line was used as a control line, completely devoid of any P element or transgene (true “M” line).

**Mutations used for the candidate gene analysis**

Su(pan)205, squash (squ) and zucchini (zuc) are located on chromosome 2. Loss of function is lethal in the case of Su(pan)205, female sterile in the case of squash and zucchini.

Su(pan)2-505 (or Su(pan)2-505) was X-ray induced and corresponds to a null allele of Su(pan)205 since it only encodes the first ten amino acids of the HP1 protein [39], zuc and squ alleles were isolated from an EMS screen [40].

**Statistical analysis**

The levels of TSE produced in flies of different genotypes were compared using the non-parametric Mann-Whitney test, conducted on TSE percentages per ovary.

**RNase protection assays (RPA)**

Small RNAs from adult flies were extracted using the Ambion mirVanaTM miRNA isolation kit. Per each condition, 400 ovaries were used for RNA extraction. Aliquots of 4 μg of small RNAs were used in RPA experiments. The radiolabelled RNA probe homologous to the 5’ region of P-lac_\alpha_ was 150 nt long (position 600 to 750 of the P1ArB transgene [Ftbp0000160]). After purification, probes with a specific activity of 5×10^6 cpm were used. We used the Ambion mirVanaTM miRNA detection kit for RPA experiments. Hybridization was performed overnight at 42°C and digestion of single-stranded RNA was carried out for 45 minutes at 37°C with RNase A/RNase T1. After RNase inactivation, protected fragments were precipitated and separated on a 15% acrylamide/polyacrylamide (19:1) gel running in 0.5×TBE. Protected fragments were detected by autoradiography after 4 weeks of exposure.

**Results**

**Functional assay for the Trans-Silencing Effect in zuc and squ mutants**

Given the role of squash and zucchini in the piRNA pathway [25,34], the effect of mutant alleles of these genes on TSE was tested (Figure 1). For a given assay, a P-1152 telomeric silencer was combined with a P-lac_\alpha_ target expressed in the female germline, in the absence (TSE positive control), or presence of mutant alleles of the candidate gene. The P-1152 silencer was inherited, in each case.
from a homozygous P-1152 female. The first gene tested was zucchini (Fig. 1C). The TSE positive control produced a strong repression (Fig. 1B, TSE = 86.6%, n = 1650), whereas females having a heteroallelic zw3^{SG63}/zw1^{HE17} genotype showed a complete loss of repression (Fig. 1C, TSE = 0.0%, n = 2650). The same result was found for females having the reciprocally inherited heteroallelic 

\[ zw3^{HE17}/zw1^{SG63} \] 

combination (i.e. the mutant alleles were inherited by the reciprocal parent: TSE = 0.0%, n = 1600, data not shown).

The same analysis was performed for squash and the heteroallelic

\[ sq^{HE47}/sq^{PP32} \] 

genotype showed reduced TSE (Fig. 1D, TSE = 56.2%, n = 1000). The reciprocally-inherited heteroallelic genotype, 

\[ sq^{PP32}/sq^{HE47} \] 

showed a very similar result (TSE = 56.6%, n = 2200, data not shown). The percentage of TSE observed for each of the two kinds of heteroallelic squash mutant females (\[ sq^{HE47}/sq^{PP32} \] and \[ sq^{PP32}/sq^{HE47} \]) was compared to that observed for the TSE positive control (Fig. 1B), using the non-parametric Mann-Whitney test. In both cases the difference is highly significant (\( P<0.001 \)). By contrast, for both zw3 and sq4, no significant effect on TSE was detected for the heterozygous mutants (\[ zw3^{HE17}/zw1^{HE17} \], \[ zw3^{SG63}/zw1^{SG63} \]) and otherwise wild-type, heterozygous or homozygous females (Fig. 1A, lanes 1 and 3, respectively). This analysis shows that telomeric squash silencer transgenes produce la^{-}

\[ zucchini \] 

small RNAs in the ovary and that loss of function of \( sq4 \) and \( zw3 \) has a strong negative effect on the accumulation of these \( la^{-}zucchini \) small RNAs. In addition, as for the TSE assay, no dose effects for \( sq4 \) or \( zw3 \) were observed on \( la^{-}zucchini \) small RNAs accumulation.

Accumulation of \( la^{-}zucchini \) small RNAs in ovaries is correlated with the maternal effect of TSE

TSE was shown to exhibit a maternal effect: crossing females carrying a telomeric transgene with males carrying a target transgene produces \( G_1 \) females which show strong TSE, whereas the reciprocal cross produces \( G_1 \) females showing only weak TSE [31–33]. TSE also shows maternal inheritance since this maternal effect presents a remanence which can extend through six generations following the reciprocal \( G_2 \) crosses [33]. TSE is therefore, at least in part, epigenetically transmitted through meiosis. TSE maternal inheritance can also be observed in the ovaries of females which carry the P-1152 telomeric silencer locus and, if so, whether the production of these small RNAs requires the \( sq4 \) and \( zw3 \) functions. We used an RNAse protection assay to detect \( la^{-}zucchini \) small RNAs in ovaries from females carrying two copies of P-1152 and otherwise wild-type, heterozygous or homozygous mutants for \( sq4 \) and \( zw3 \). Ovaries from the M line Canton \( ^{\text{x}} \) were also analyzed as a negative control. RNAse protection analysis allowed detection of two abundant small RNAs in ovaries from homozygous P-1152 females (Fig. 2A, lane 5 and Fig. 2B, lane 1 – arrows to the right of the autoradiography), which were not detected in M females (Fig. 2A, lane 6 and Fig. 2B, lane 2). Females heterozygous for \( sq4 \) or \( zw3 \) mutant alleles, also exhibited abundant accumulation of the \( la^{-}zucchini \) small RNAs of the same size in ovaries (Fig. 2A, lanes 2 and 4, respectively). By contrast, these RNAs were almost undetectable for females heteroallelic for mutant alleles of \( sq4 \) or \( zw3 \) carrying two copies of \( P-1152 \) (Fig. 2A, lane 1 and 3, respectively). This analysis shows that telomeric \( P-la^{-}zucchini \) silencer transgenes produce \( la^{-}zucchini \) small RNAs in the ovary and that loss of function of \( sq4 \) and \( zw3 \) has a strong negative effect on the accumulation of these \( la^{-}zucchini \) small RNAs. In as much as for the TSE assay, no dose effects for \( sq4 \) or \( zw3 \) were observed on these small RNAs accumulation.

Silencing is correlated to the accumulation of \( la^{-}zucchini \) small RNAs in ovaries whose production is sensitive to squash and zucchini mutations

Since TSE is highly sensitive to mutations in genes involved in the piRNA silencing pathway, we tested whether \( la^{-}zucchini \) small RNAs were present in ovaries of females which carry the P-1152 telomeric silencer locus and, if so, whether the production of these small RNAs requires the \( sq4 \) and \( zw3 \) functions. We used an RNAse protection assay to detect \( la^{-}zucchini \) small RNAs in ovaries from females carrying two copies of P-1152 and otherwise wild-type, heterozygous or homozygous mutants for \( sq4 \) and \( zw3 \). Ovaries from the M line Canton \( ^{\text{x}} \) were also analyzed as a negative control. RNAse protection analysis allowed detection of two abundant small RNAs in ovaries from homozygous P-1152 females (Fig. 2A, lane 5 and Fig. 2B, lane 1 – arrows to the right of the autoradiography), which were not detected in M females (Fig. 2A, lane 6 and Fig. 2B, lane 2). Females heterozygous for \( sq4 \) or \( zw3 \) mutant alleles, also exhibited abundant accumulation of the \( la^{-}zucchini \) small RNAs of the same size in ovaries (Fig. 2A, lanes 2 and 4, respectively). By contrast, these RNAs were almost undetectable for females heteroallelic for mutant alleles of \( sq4 \) or \( zw3 \) carrying two copies of \( P-1152 \) (Fig. 2A, lane 1 and 3, respectively). This analysis shows that telomeric \( P-la^{-}zucchini \) silencer transgenes produce \( la^{-}zucchini \) small RNAs in the ovary and that loss of function of \( sq4 \) and \( zw3 \) has a strong negative effect on the accumulation of these \( la^{-}zucchini \) small RNAs. In addition, as for the TSE assay, no dose effects for \( sq4 \) or \( zw3 \) were observed on these small RNAs accumulation.

Accumulation of \( la^{-}zucchini \) small RNAs in ovaries is correlated with the maternal effect of TSE

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presence of a telomeric silencer alone, i.e., in the absence of the target transgene [32]. We thus tested if lacZ small RNA detection in ovaries parallels the maternal effect of TSE. RNAse protection analysis allowed detection of the two abundant small RNAs in G_1 females produced by the two (P-1152 x M) reciprocal crosses, but the intensity of the signal obtained with the progeny of the female P-1152 x male M cross (cross TSE-) was higher than that of the progeny of the reciprocal cross which induces only a weak level of TSE (cross TSE+) (Fig. 2B, lanes 3 and 4). This difference becomes particularly clear, when a comparison is made between the signal intensities of the aspecific bands shown below the lacZ small RNAs. However, the signal from TSE- females is not null, a result which is consistent with the weak but non null level of TSE (around 10%) which can be induced in this cross [32,33]. In conclusion, the presence of lacZ small RNAs in ovaries is detected in a manner which is correlated to the maternal effect of TSE.

Accumulation of lacZ small RNAs is sensitive to HP1 dose

TSE was shown previously to be sensitive not only to mutations in genes involved in the piRNA pathway, but also to mutations in genes involved in heterochromatin formation, such as Su(var)205 which encodes HP1 [33]. For Su(var)205, a particularly clear dose effect on TSE was observed. We thus tested if the presence of lacZ small RNAs in ovaries is affected in P-1152 females having only one dose of the Su(var)205 gene compared to wild-type. RNAse protection was performed as previously on females carrying two copies of P-1152 and heterozygous for Su(var)205, an amorphic allele of Su(var)205. Figure 2B (lane 5) shows that the level of small RNAs detected for females having two copies of P-1152 and only one dose of Su(var)205 is strongly reduced when compared to P-1152 wild-type females (Fig. 2B, lane 1). Indeed, with one dose of HP1, the level of these small RNAs is comparable to that of females carrying a single paternally-inherited P-1152 copy (Fig. 2B, lane 4). Under these two latter conditions, comparable low levels of TSE were also found [33]. Therefore, the effect of mutations affecting HP1 on TSE [33], as for sqz and zuc mutations, can be correlated to a significant reduction in the accumulation of small RNAs in ovaries produced by the telomeric P-1152 silencer locus.

Figure 2. TSE is correlated with the presence of small RNAs whose production depends on the piRNA pathway and HP1. (A–B) RNAse protection was carried out using a lacZ sense riboprobe (150 nt) hybridized to RNAs extracted from ovaries from 3–6 day-old females. Data concerning the 20–30 nt region are shown together with aspecific bands used as a loading control (shown below). Canton^{1} was used as an M strain, (devoid of any P element or P transgene). (A) Small RNA detection and effect of mutations in squash and zucchini. WT corresponds to P-1152 females which are wild-type for both squ and zuc. Two small RNAs (arrows) are highly abundant in females carrying the P-1152 telomeric TSE silencer at the homozygous state (WT), but are not detected in ovaries of females devoid of the TSE- squash or zucchini functions. Thus, accumulation of lacZ small RNAs occurring in P-1152 ovaries requires sqz and zuc functions. (B) TSE maternal effect and effect of mutations affecting HP1. TSE- indicates that this cross allows a strong TSE in G_{1} females due to the maternal transmission of the telomeric P-1152 silencer, whereas TSE+ means that only a weak TSE is recovered from this cross in which P-1152 is inherited paternally. P-1152 homozygous females and M females were analyzed as positive and negative controls, respectively. The two most abundant small RNAs are indicated by arrows. A strong signal for these small RNAs is obtained for P-1152 WT females having inherited a P-1152 telomeric TSE silencer maternally (TSE-), as well as for P-1152 homzygous females carrying one null allele of Su(var)205 which encodes HP1. Therefore, accumulation of lacZ small RNAs is correlated to the maternal effect of TSE and depends on HP1 dose.

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Discussion

Trans-Silencing Effect, a typical piRNA germline repression mechanism

Trans-silencing was previously shown to be strongly impaired by mutations affecting several components of the piRNA silencing pathway (AUBERGINE, ARMITAGE, HOMELESS, PIWI) [8,33]. By contrast, TSE was not impaired by mutations affecting R2D2, a component of the siRNA pathway [33,41], or LOQUACIOUS, a component of both the miRNA and endo-siRNA pathways [33,42–44]. This indicates that TSE likely involves the piRNA silencing pathway, a hypothesis which is consistent with the fact that TSE is restricted to the germline [32], the tissue in which the “canoncal” piRNA pathway functions [25,26]. Furthermore, SQUASH and ZUCCHINI were found to interact with AUBERGINE and to localize to the nurse cell nuclei, which also contains AUBERGINE and ARMITAGE and appears to be involved in RNA silencing [34]. sqh and zuc mutations were also shown to affect piRNA production in ovaries at the cytological 42AB repetitive sequence cluster, a typical piRNA-producing genomic region [25]. Regarding TE repression in the germline, sqh and zuc mutants were found to derepress transcription of the telomeric retrotransposons Het-A and TART [34] and of the I factor, a retrotransposon involved in a Drosophila system of hybrid dysgenesis [45,46]. It is noteworthy that the I factor and the Het-A retrotransposons have also been found to be sensitive to abu, armi and het (spn-E) [5,45,47]. The genetic analysis reported here shows that TSE is also highly sensitive to zuc and sqh mutations (Figure 1). TSE is therefore sensitive to mutations affecting all the genes of the germline piRNA pathway tested and thus appears to represents a bona fide piRNA-based repression.

The presence of lacz small RNAs in ovaries of females carrying a TSE silencer was therefore investigated using RNase protection analysis. In addition, maternal or paternal transmission of the telomeric silencer was compared. Indeed, TSE was previously shown to have a maternal effect, i.e., strong repression occurs only when the telomeric silencer is maternally inherited, whereas a paternally-inherited telomeric silencer has weak or null repression capacities [32,33,48]. More precisely, it was shown genetically that TSE requires inheritance of two components, a cytoplasmic component plus a chromosomal copy of the transgene, but these two components can be transmitted separately [33]. Indeed, a paternally-inherited telomeric transgene can be “potentiated” by a maternally-inherited cytoplasm from a female bearing a silencer. This interaction also functions between telomeric silencers located on different chromosomal arms [32].

The RNase protection analysis reported here shows that: 1- P-1152, a telomeric P-lacz silencer produces small lacZ RNAs in ovaries (Figure 2A-B); 2- P-1152 lacZ small RNA accumulation is negatively affected in sqh and zuc mutants (Figure 2A); 3- maternal transmission of P-1152 leads to accumulation of higher levels of these small RNAs than that observed upon paternal P-1152 transmission (Figure 2B). We have reproduced these results with independent RNase protection assays (two experiments for the effect of each mutant and three experiments for the maternal effect). The size of the small RNAs detected here appears smaller (around 22–23 nt) than that corresponding to piRNAs as characterized by deep sequencing (23–28 nt, [8]), but they are consistent with piRNAs as detected by RNase protection assays in other studies [49]; this can result from the RNase protection protocol which tends to reduce the size of the RNAs detected. In conclusion, our results strongly suggest that the lacz small RNAs in P-1152 oocytes may correspond to cytoplasmically-transmitted piRNAs mediating the maternal effect of TSE, as well potentiating a paternally-inherited telomeric silencer [33].

Towards a mutual dependence between RNA silencing and heterochromatin formation

TSE was previously shown to be sensitive to mutations affecting HP1 since a negative, dose-dependent, effect on TSE was found with two loss of function alleles of Su(var)205 (including Su(var)2-505) [33]. RNase protection analysis shows here that lacz small RNA accumulation is also negatively affected by the dose of HP1 (Figure 2B). Although we cannot exclude that this effect may be indirect, this opens the possibility that some piRNA-producing loci depend on the presence of HP1 itself at the locus to produce piRNAs. A similar model was recently proposed for rhino, a HP1 homolog, mutations of which strongly reduce the production of piRNAs by dual strand piRNA-producing loci [28]. The authors propose that rhino is required for the production of the long precursor RNAs which are further processed to produce primary piRNAs. Note that in their study, rhino mutants were shown to have a drastic effect on the production of piRNAs by the X-chromosome TAS locus [28]. A similar situation may therefore exist for HP1 at this locus and, if so, it would be interesting to characterize more precisely the function of HP1 in the production of piRNAs at the TAS locus.

HP1 was shown to be present at TAS [50,51]. A first possibility would be that HP1 stimulates transcription of the TAS locus as a classical transcription factor, independent of any heterochromatic role at this locus. Consistent with this, it was shown that PIWI, a partner of HP1 [52], promotes euchromatin histone modification and piRNA transcription at the third chromosome TAS [49]. The precise status of TAS, however, remains complex since some studies have shown that TAS exhibit some of the properties attributed to heterochromatin [53–55] and carry primarily heterochromatic histone tags [56]. Therefore, a second possibility would be that HP1 enhances the heterochromatic status of TAS in the germline, such that production of aberrant transcripts being processed into piRNAs is enhanced. This would result in a “heterochromatin-dependent RNA silencing pathway”. Examples of heterochromatin formation that depends on RNA silencing (“RNA-dependent heterochromatin formation”) have been described in numerous species including yeast [37], ciliates [57] and plants [58]. In Drosophila, this type of interaction has been described for variegation of pigment production in the eye linked to the insertion of the white gene in different types of heterochromatin structures [59,60], as well as for heterochromatin formation at telomeres in the germline [51]. Therefore, telomeric regions in fly may be submitted to both RNA-dependent heterochromatin formation [47,51] and heterochromatin-dependent RNA silencing. RNA silencing may favor heterochromatin formation that in turn potentiates RNA silencing, resulting in a functional positive loop between transcriptional gene silencing and post-transcriptional gene silencing. In such cases, RNA silencing and heterochromatin may not only reinforce each other but may also be functionally interdependent. Such bidirectional reinforcement between RNA silencing and heterochromatin formation was demonstrated in S. pombe since: 1- deletion of genes involved in RNA silencing were shown to derepress transcriptional silencing from centromeric heterochromatic repeats and was accompanied by loss of Histone 3 Lysine 9 methylation and Sex6 (a HP1 homolog) delocalization [57]; 2- Sex6 was found to be required for the propagation and the maintenance of the RNA Induced Transcriptional Silencing (RITS) complex at the mat locus, a complex involved in amplification of RNA silencing [35,61]. A positive loop between RNA silencing and heterochromatin
formation may therefore also be played in the *Drosophila* germline. According to this model, the epigenetic transmission of TSE through meiosis, i.e., six generations of maternal transmission of the silencer are required to elicit a strong TSE following maternal inheritance of a cytological devoi of lacZ piRNAs [33] would underlie progressive establishment of this loop. Note that RNAi-dependent DNA methylation in *Arabidopsis thaliana* was shown to occur progressively over several consecutive generations [62].

Since TSE can be considered as a sub-phenomenon within P regulation, it may underlie epigenetic transmission of the P element repression. P element mobilization is responsible for a syndrome of germline abnormalities, known as the “P-M” system of hybrid dysgenesis which includes a high mutation rate, chromosomal rearrangements, male recombination and an agametic temperature-sensitive sterility called GD sterility (Gonadal Dysgenesis) [63]. P-induced hybrid dysgenesis is repressed by a maternally inherited cellular state called the P cytotype [3,23,64,65]. The absence of P-repression is called M cytotype. G1 females produced from the cross (P cytotype females × M cytotype males) present a strong capacity for repression, whereas females produced from the reciprocal cross present a weak capacity for repression [64]. In the subsequent generations, cytotype is progressively determined by the chromosomal P elements but the influence of the initial maternal inheritance can be detected for up to five generations [64,66]. Therefore, P cytotype exhibits partial epigenetic transmission through meiosis. Furthermore, the identification and use of telomeric P elements as P cytotype determinants [2,16–18], has made it possible to show that P cytotype (like TSE) involves a strictly-maternally inherited component (called the pre-P cytotype) [67], is sensitive to mutations affecting HPI [2,68] and *aubergine* [1,69], and is correlated to maternal deposition of piRNAs [70]. Some of these properties are also found for the I factor which is responsible for the occurrence of another system of hybrid dysgenesis (“I-R” system) [45,46,71–73]. TSE therefore parallels germine regulation of TEcs (P, I, and does not resemble regulation of TEcs in the somatic follicle cells (*gypsy*, *ZAM*, *Idfis* [19,20]) for which no epigenetic transmission of repression capacities through meiosis has been described so far. It will be interesting to test if previously described cases of RNA-dependent heterochromatin formation show the reciprocal dependence, thus being able to form a positive loop.

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**Author Contributions**

Conceived and designed the experiments: ALT LT SR. Performed the experiments: ALT LT VS SR. Analyzed the data: ALT LT VS SR. Wrote the paper: SR.

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