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Insect small non-coding RNA involved in epigenetic regulations

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Small regulatory RNAs can not only guide post-transcriptional repression of target genes, but some of them can also direct heterochromatin formation of specific genomic loci. Here we review the published literature on small RNA-guided epigenetic regulation in insects. The recent development of novel analytical technologies (deep sequencing and RNAi screens) has led to the identification of some of the factors involved in these processes, as well as their molecular mechanism and subcellular localization. Other findings uncovered an additional mode of epigenetic control, where maternally inherited small RNAs can affect phenotypes in a stable, transgenerational manner. The evolutive history of small RNA effector proteins in insects suggests that these two modes of regulation are variably conserved among species.

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Introduction

Small regulatory RNAs in eukaryotes typically direct post-transcriptional repression of specific genes. However, some small RNAs are involved in transcriptional regulation, directing chromatin modifications that epigenetically control gene expression. These phenomena have been thoroughly explored in insects. This review will summarize current knowledge on the mode of action of insect small RNAs in epigenetic regulation.

Characterization of small RNA-mediated phenomena in animals revealed the co-existence of three major pathways (see [1] for a review). Each class of small RNAs is loaded onto a protein of the 'Argonaute' family, which contains the 'Ago' and 'Piwi' protein subfamilies [2]. In one pathway, named 'RNA interference' ('RNAi'), long double-stranded RNAs are cleaved by an enzyme named Dicer, generating diverse populations of small interfering RNAs ('siRNAs') that tend to cover the double-stranded trigger sequence in a head-to-tail fashion. In an alternate pathway, microRNAs ('miRNAs') are generated from discrete genomic loci, whose transcripts fold into short ($\approx$ 70 nt long) hairpins. These hairpin-folded precursors are cleaved by the RNases Drosha and Dicer, liberating homogeneous RNA species, with most small RNA products being derived from a given locus having the exact same sequence. Both siRNAs and miRNAs are loaded onto effector proteins belonging to the 'Ago' subfamily. Finally, a third pathway generates small RNAs in a Dicer-independent manner, which are then loaded onto proteins of the 'Piwi' subfamily. These Piwi-interacting RNAs ('piRNAs') are mostly expressed in the gonad. Of note, piRNAs were initially called 'rasiRNAs' (repeat-associated siRNAs) [3].

Since the discovery of small RNA-mediated gene regulation, the implication of small RNAs in guiding chromatin changes has been demonstrated in several model organisms such as plants [4], fungi [5,6] and ciliates [7]. These early discoveries in such diverse eukaryotic lineages suggested that small RNA-mediated heterochromatinization could be a conserved feature among eukaryotes, particularly in insects. Indeed, several studies soon reported chromatin defects in *Drosophila* when the biogenesis or action of small RNAs is perturbed (see [8–12] for the key initial reports).

It is now clear that piRNAs are the primary small RNAs mediating chromatin modifications. The implication of Dicer (which does not generate piRNAs) and Piwi proteins (which are only loaded with piRNAs) in a common phenotype became hard to rationalize, and it is likely that some of the reported observations are not due to a direct effect of small RNAs on homologous chromatin sequences. For example, heterochromatic marks increase, rather than decrease, on the tested loci in adult fly heads when RNAi or the piRNA pathway is perturbed, suggesting that the effect of small RNAs in that organ is indirect [13]. The RNAi machinery is also dispensable for endogenous Polycomb-mediated heterochromatinization [14].

Whereas a direct role of the RNAi pathway in guiding chromatin modification is now questioned, the involvement of piRNAs is supported by many lines of evidence. In addition to chromatin modifications, piRNAs in insects have also been shown to promote extrachromosomal forms of epigenetic inheritance. This review will focus on the biogenesis and mode of action of piRNAs in these various forms of epigenetic regulation. Most available data were obtained in *Drosophila melanogaster*, but comparative

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genomics and pioneer biochemical work in additional insect systems help draw a general picture of small RNA-guided epigenetic modifications in insects.

Genomics of piRNAs in insects

piRNAs are extremely diverse in sequence, and individual piRNA sequences tend to be of very low abundance. Consequently, the total piRNA repertoire, even in well-studied species like *D. melanogaster*, is still far from being completely documented, despite years of high-throughput sequencing of ovarian small RNAs, and millions of published fly piRNA sequences [15]. piRNAs tend to originate from specialized genomic loci, the so-called 'piRNA clusters', which are usually located in heterochromatic regions, and whose size typically ranges from a few kilobases (kb) to more than 200 kb [16,17].

Drosophila piRNAs also frequently map on transposable elements (TE) or other repeated sequences [16,18]. Many TE sequences are actually embedded in piRNA clusters, although some piRNAs also originate from isolated, euchromatic copies of TEs. All in all, whether they are generated from piRNA clusters or from isolated TE copies, between one-third and two-thirds of *Drosophila* piRNAs map on TE sequences [15,16]. In a cell line derived from *Bombyx* ovary that expresses both Piwi proteins and piRNAs, 31% of the detected piRNAs map on annotated repeated genomic sequences [19]. It should be kept in mind that non-TE-matching RNAs in these experiments may be co-immunoprecipitated contaminants, or actual TE-matching piRNAs that map to non-anotated TE copies. Hence, these numbers may be an under-estimation of the real percentage of TE-matching piRNAs.

Several recent studies uncovered the surprising plasticity of piRNA clusters in evolution. Insertion of either a TE or a transgene containing fragments of a TE sequence can transform a genomic locus into a novel piRNA cluster [20,21]. As for pre-existing piRNA clusters, they can experience frequent rearrangements (insertions, deletions, recombinations) that alter the repertoire of generated piRNAs [22].

TE-matching piRNAs are involved in the repression of their cognate TEs. For example, mutants lacking either components of the piRNA pathway (see for example [18,23–28]) or functional piRNA clusters[1] [29,30] de-repress TEs in the germ line, which leads to massive transposon mobilization, double-strand DNA breaks [31] and, ultimately, sterility. Natural variability in piRNA cluster composition also permits the isolation of fly strains with diverse TE-repressing abilities [32,33].

As for piRNAs that do not arise from repeated genomic sequences, a fraction of them[2] map on the 3′ untranslated regions (UTRs) of annotated, non-transposable genes [34,35]. Since these piRNAs map on 3′ UTRs in the sense orientation, they are not complementary to the gene's mRNA and are therefore not expected to base-pair with these mRNAs. The function of such piRNAs, if any, remains elusive.

piRNA biogenesis

Unlike siRNAs and miRNAs, piRNAs are not generated by a Dicer enzyme [18,36]. Their biogenesis has been thoroughly studied in insects, with detailed genetic and biochemical studies in *D. melanogaster* and *Bombyx mori*.

The oocyte is transcriptionnally inactive, and does not express piRNAs. piRNAs are produced in additional germ line cells, called 'nurse cells' (see Figure 1), then deposited in the oocyte. Deep-sequencing analyses of *Drosophila* ovarian piRNAs revealed that piRNAs tend to be complementary to each other throughout their first 10 nucleotides. This sequence feature suggested an auto-ampliflying biogenesis pathway (named the 'ping-pong loop'), where a mature piRNA guides the cleavage of a complementary piRNA precursor, thus contributing to the maturation of another piRNA ([17,37]; see Figure 1). More precisely, piRNAs loaded on a Piwi protein named 'Aubergine' (Aub) tend to be complementary to piRNAs loaded on another Piwi protein, named 'Ago3'. As Aub-loaded piRNAs tend to have an uridine at their 5′-most position, Ago3-loaded piRNAs frequently have an adenosine at position 10. For those piRNAs that match transposon sequences, Aub-loaded piRNAs are usually antisense to the transposon sequence, while Ago3-loaded piRNAs are usually in the sense orientation.

Not only does the ping-pong loop produce mature piRNAs in an auto-amplifying fashion, but it also degrades TE mRNAs: RNAs cleaved by Aub (these RNAs are the precursors for Ago3-loaded piRNAs) are usually TE mRNAs. For a few exceptional TEs, Aub-loaded piRNAs are in the sense orientation and Ago3-loaded piRNAs are in the antisense orientation. But in both cases, TE mRNAs are chopped by either protein, hence degraded, resulting in a post-transcriptional repression of TEs. At the subcellular level, RNA cleavage by the ping-pong loop occurs in an electron-dense structure named 'nuage', that surrounds the nucleus of nurse cells (Figure 1).

This biogenesis mechanism, as well as these sequence biases, is extremely conserved. For example, piRNAs with a bias for uridine at position 1 and piRNAs with a bias for adenosine at position 10 are found in the

[1] *N.B.*: The *flamenco* piRNA cluster is also known as *COM*.

[2] That fraction can be evaluated in a *Drosophila* ovary somatic cell line, named 'OSS': 3′ UTR-matching piRNAs constitute ≈ 16% of the non-TE-matching piRNAs [35,38].

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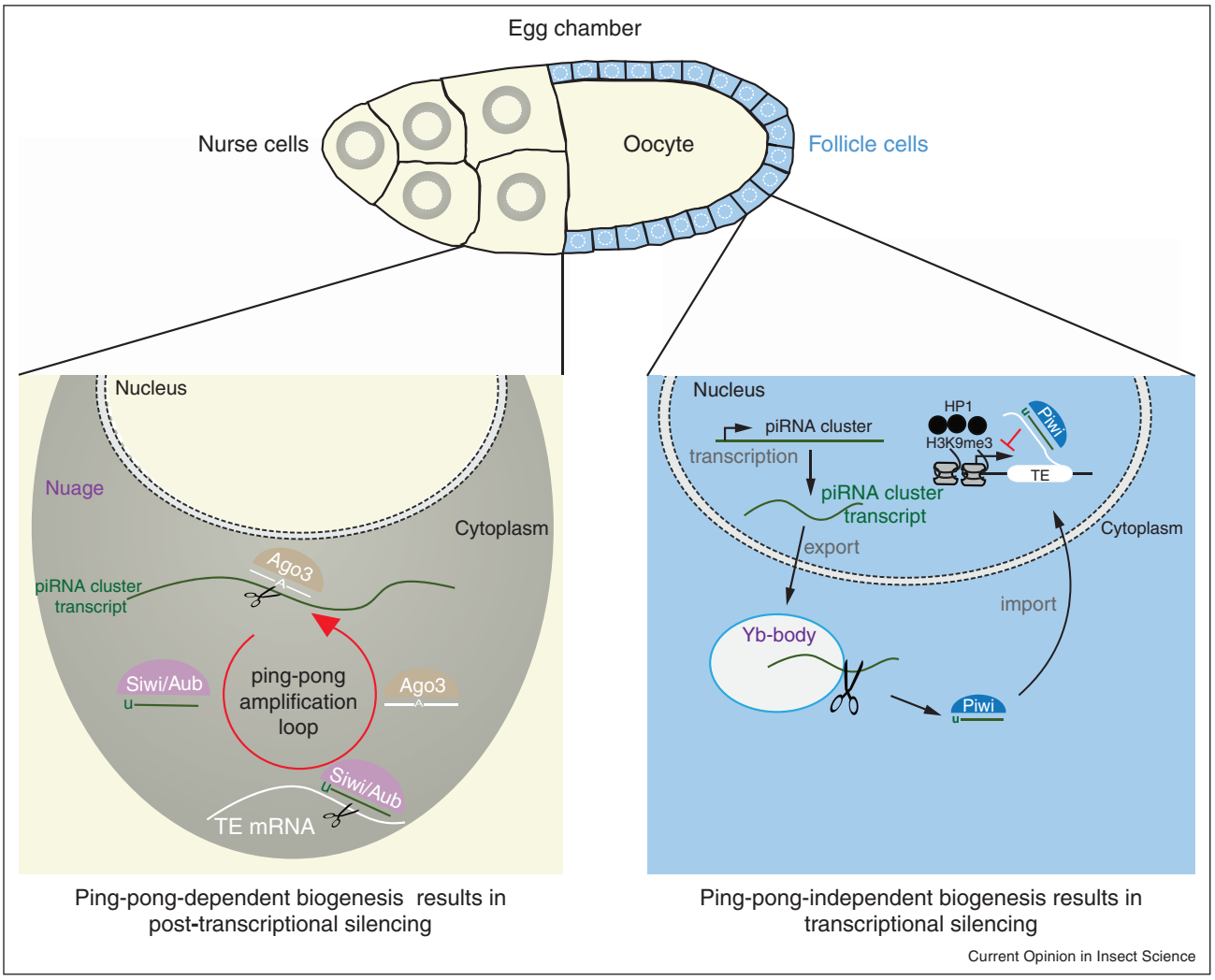
Figure 1



Biogenesis of piRNAs. Bottom left: the canonical piRNA biogenesis involves the ping-pong amplification loop between Ago3-loaded piRNAs and Aub-loaded (in *Drosophila*) or Siwi-loaded (in silkworm) piRNAs [17,19,37]. That process takes place in the cytoplasmic region surrounding the nucleus, called 'nuage' (here in grey). In mosquitoes, piRNA sequence biases typical of the ping-pong loop have been observed, suggesting that mosquito piRNAs are generated by the same mechanism [40–42]. Top and bottom right: in *Drosophila*, follicle cells produce piRNAs by an alternative mechanism, involving a cytoplasmic maturation in Yb bodies followed by nuclear re-import [45–47]. These piRNAs are loaded on Piwi and they direct heterochromatinization of target genomic loci.

Cnidarian *Nematostella vectensis* [39] whose last common ancestor with insects occurred more than 600 million years ago. Not surprisingly, these piRNA sequence biases are also observed in *Bombyx* [19] and in mosquitoes [40–42], and an ortholog for Ago3 is easily identifiable in most insect species ('Ago3 clade' and 'Putative divergent members of Ago3 clade', in Figure 2).

The ortholog for Aub underwent a more complex evolutive history. Most insects possess a single Aub protein (e.g. the Siwi protein in *B. mori*), but *Drosophila* expresses a protein related to Aub, named Piwi (it is the founding member of the Piwi subfamily, whose name was given to the whole protein subfamily). In *Drosophila* ovaries, the Piwi protein is expressed not only in the germ cells, but also in follicle cells (somatic cells flanking the oocyte). Follicle cells, which do not express either Aub or Ago3, produce piRNAs by a mechanism that does not appear to involve the ping-pong amplification loop [34,38,43,44]. Rather, maturation of Piwi-loaded piRNAs in follicle cells requires a transit through Yb bodies (these are cytoplasmic foci where several proteins involved in piRNA biogenesis are concentrated), before the mature complex is re-imported to the nucleus [45–47].

The duplication of an ancestral Aub/Piwi protein into Aub and Piwi is recent: only the Brachycera suborder

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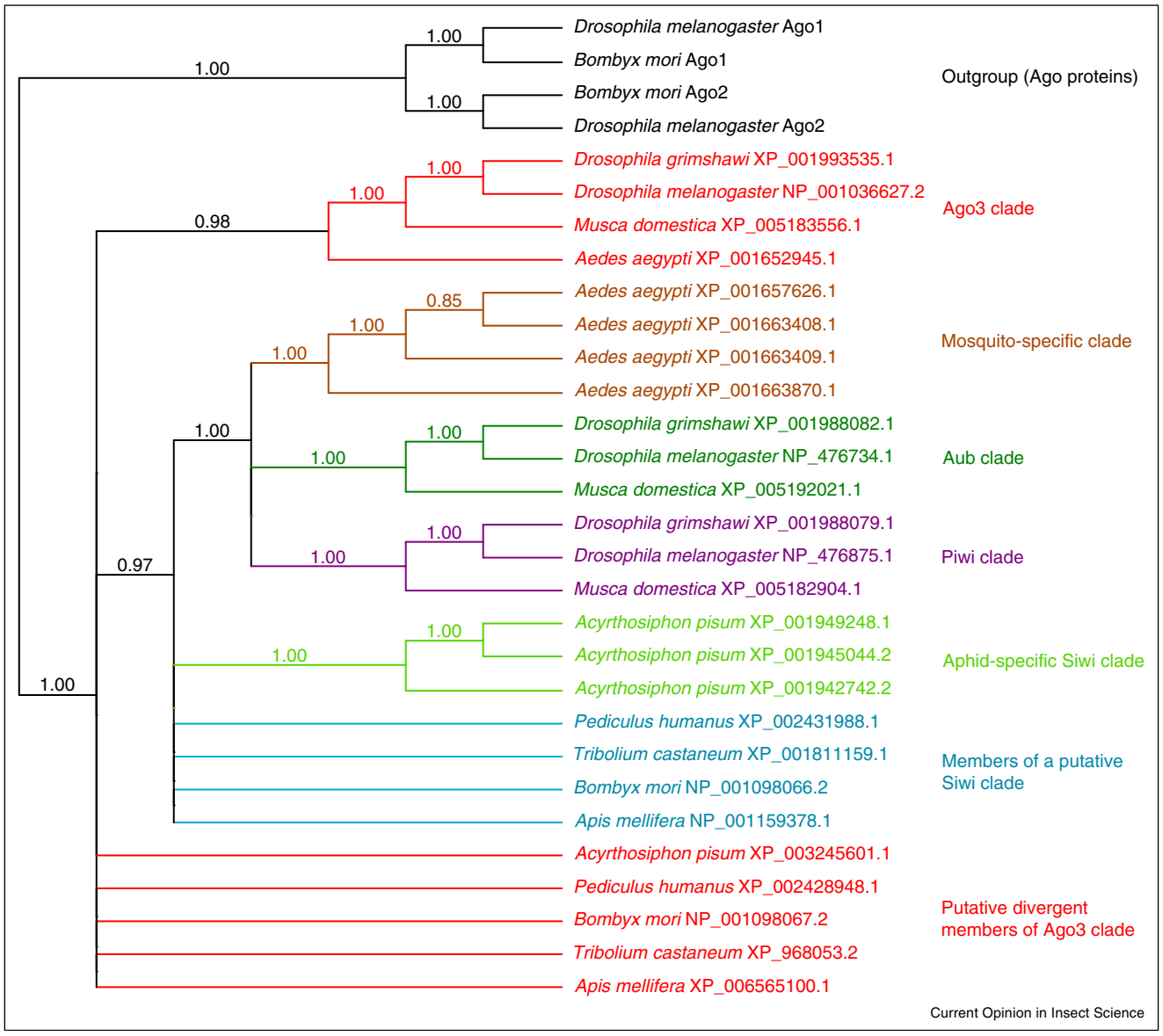
Figure 2



Piwi protein subfamilies in insects. Piwi subfamily protein sequences were searched in the RefSeq database using *HMMer*, then aligned using *clustalw* and the tree was computed using *RAxML* (using *D. melanogaster* and *B. mori* Ago protein sequences as an outgroup). Numeric values indicate the bootstrap score of each branch; branches with bootstrap scores lower than 0.7 were dissociated (e.g. the putative Siwi clade). Only nine insect species were retained for graphical clarity.

(*Drosophila* and related fly species, such as *Musca domestica* and *Ceratitis capitata*) possess recognizable forms of both Aub and Piwi. Other Diptera (e.g. mosquitoes) and other insects possess Aub/Piwi orthologs, which are as similar to *Drosophila* Aub as to *Drosophila* Piwi. Mosquito and aphid orthologs for Aub and Piwi were also duplicated, but after the divergence with Brachycera. Nothing indicates that any of their Aub/Piwi proteins plays a role similar to that of the *Drosophila* Piwi. Hence the role of *Drosophila* Piwi in guiding chromatin changes (see below) may only be shared with Brachycera species.

Mature piRNAs are methylated on the 2′ oxygen of their 3′-most nucleotide, by a methyl-transferase named Hen1 [48,49]. In *Drosophila*, that chemical modification is not unique to piRNAs, since Ago2-loaded small RNAs also bear a 2′-*O*-methyl group on their 3′ end. *Drosophila* Ago2-loaded RNAs consist of siRNAs [50] as well as some miRNAs [51] and miRNA*s[3] [52–54].

[3] miRNA*s are small RNAs generated by the maturation of miRNA precursors, they are sometimes as abundant as miRNAs and they can also be loaded on Ago proteins: they are now considered to be as functional as miRNAs.

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Piwi-dependent transcriptional silencing

While the *Drosophila* post-transcriptional repressors Aub and Ago3 are cytoplasmic, the third *Drosophila* Piwi protein (which is named Piwi itself) is predominantly nuclear. Piwi-mediated repression can be assessed in follicle cells, that do not express Aub and Ago3: the role of Piwi is not shadowed by these two subfamily members. Consequently, most mechanistic studies regarding the role of Piwi were performed in follicle cells, or a cultured cell line derived from follicle cells. However, it should be kept in mind that Piwi may exert a different molecular function in the germ line [55••].

The RNase activity of Piwi is not required for TE repression [46,56], suggesting that it does not repress its target post-transcriptionally by RNA cleavage. Rather, Piwi appears to be guided towards TE loci and to promote the deposition of heterochromatic marks (e.g. histone 3 lysine 9 trimethylation, H3K9me3) on the neighbouring chromatin, resulting in transcriptional silencing [55••,57••,58••] (see bottom right panel of Figure 1). These studies showed that the loss of Piwi results in increased levels of nascent TE transcripts, of PolII occupancy on TE genes, and a decrease in H3K9me3 marks on TE genes. Piwi and its associated piRNAs also affect the expression of genes surrounding euchromatic TE copies by the spreading of the H3K9me3 repressive mark on flanking sequences [57••]. It is hypothesized that piRNAs guide the Piwi protein to target loci by base-pairing to the nascent transcript (rather than to a DNA strand), since piRNAs can trigger heterochromatinization only when they are antisense to an artificial target transcription unit [57••] and Piwi interacts indirectly (in an RNase-sensitive manner) with several proteins that bind nascent transcripts [58••]. As the nascent transcript is physically bound to chromatin, recruitment of Piwi on these RNAs could trigger heterochromatinization locally, on the target gene and neighbouring genes.

So far, a single cofactor (Gtsf1, also known as Asterix) has been reported to be involved in Piwi-dependent heterochromatinization [59•,60•,61•]. The murine Gtsf1 is also known to be essential for retrotransposon control, but only in the male germ line [62]. Another *Drosophila* cofactor, Maelstrom, is implicated in Piwi-dependent transcriptional silencing, but not for heterochromatin formation [57••]. Biochemical approaches will now be needed to clarify the molecular mechanism of Piwi-dependent TE transcriptional repression and the exact role of each of these cofactors.

piRNAs and epigenetic inheritance

In addition to their role in heterochromatinization, *Drosophila* piRNAs participate in another form of epigenetic control, based on the inheritance of piRNAs across generations. piRNAs, loaded on Piwi proteins, are deposited in the embryo by the mother [63,64]. The pool of inherited piRNAs accumulates mainly in the posterior pole of the embryo, which will give rise to germinal cell progenitors, but it can also be detected in the bulk of the embryo that will give rise to both gonadal and non-gonadal somatic tissues (Figure 3). Maternal deposition of piRNAs is important for the production of piRNAs in both germinal and somatic gonadal tissues in the adult fly following development of the embryo [63,64]. While the maternal deposition of Aub and Piwi has been demonstrated, deposition of *Drosophila* Ago3 has not yet been observed. Ago3 may also be deposited though, and its low intracellular abundance may explain why it has escaped detection. However, Ago3 deposition has been described in *B. mori* [65].

In the germ line, two possible mechanisms have been proposed to explain the role of maternal deposition in the accumulation of piRNAs in the next generation. These two models are not mutually exclusive.

The first proposed mechanism is based on the transcriptional effects of Piwi. Owing to its ability to affect chromatin marks through its binding to nascent RNAs, Piwi might guide the deposition of special, piRNA cluster-specific marks on loci recognized by its piRNAs. Such molecular flags could then convert these loci into piRNA factories, directing their transcripts to the piRNA biogenesis pathway. Such a phenomenon might explain the awakening of a transgenic piRNA cluster by maternally deposited piRNAs [66••]. Indeed, a repeated genomic region that does not generate piRNAs becomes a germinal piRNA producer in the presence of maternally inherited homologous piRNAs. Once acquired, the ability to produce piRNAs is stably transmitted over generations.

The second mechanism involves the post-transcriptional ping-pong loop between Aub and Ago3. Germinal piRNA biogenesis not only requires the production of functional RNA precursor transcripts by competent piRNA clusters, but also some initial, mature piRNAs that can initiate the auto-amplifying loop [64]. The probability of initiation of any given pair of Ago3-loaded and Aub-loaded piRNAs should then determine the final abundance of that piRNA pair in the ovary. Indeed, the amount of piRNAs in the ovary correlates positively with the amount that was deposited by the mother [67••]. The same study also showed that the amount of deposited maternal piRNAs depends on an environmental factor (temperature) as well as on the age of the mother, providing a rationale for the effect of these non-genetic factors [68]. A ping-pong loop only requiring defective heterochromatic transcripts is sufficient for the transmission of epigenetic silencing over generations in the absence of functional TE transcripts. Increased piRNA production is progressively lost over five or more generations.

Ovarian somatic piRNA production also appears to be affected by extrachromosomal inheritance. In *Drosophila*

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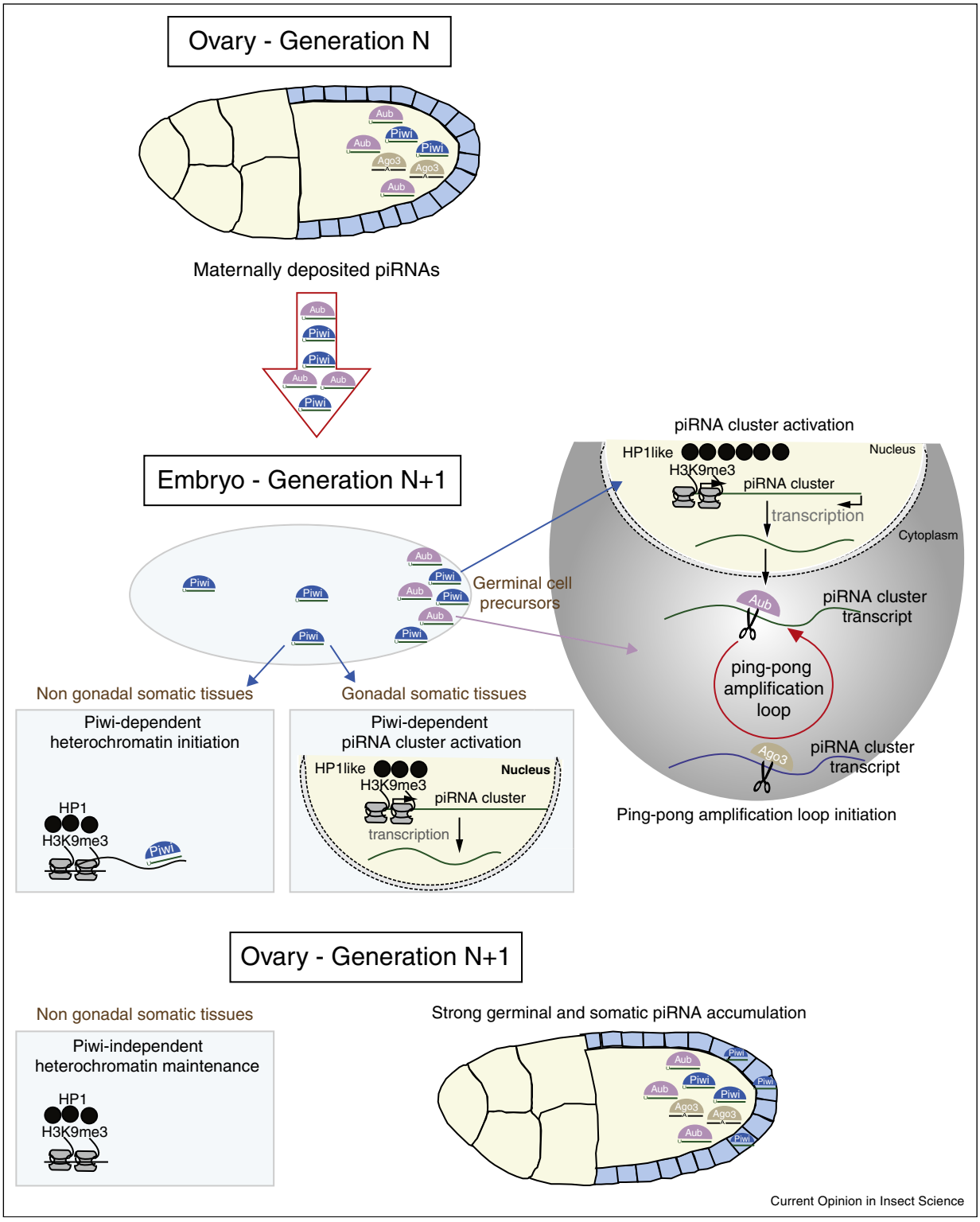
Figure 3



Transgenerational inheritance of epigenetic piRNA-based repression in *Drosophila*. Maternally deposited piRNAs (loaded on their cognate Piwi proteins) accumulate mostly in the posterior pole of the embryo, that will give rise to germinal cells. They could prime the ping-pong amplification loop in the future germinal cells of the embryo, and activate the production of piRNAs from piRNA clusters by directing the deposition of specific chromatin marks. Piwi-loaded piRNAs are also detected, but less abundantly, in the bulk of the embryo, where they are believed to guide heterochromatinization in future somatic cells (gonadal and non-gonadal).

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simulans, maternally deposited piRNAs are essential for the transcriptional repression of the *tirant* TE in ovarian soma [69•]. This study suggests that maternally deposited piRNAs might initiate the production of Piwi-dependent piRNAs by inducing changes on the chromatin state of piRNA clusters in gonadal somatic tissues, similarly to what was proposed in the germ line (see above).

Finally, Piwi also appears to affect the chromatin of a reporter gene in non-gonadal somatic tissues. Once deposited, heterochromatin marks are maintained throughout development in a Piwi-independent manner [70]. Hence, Piwi-dependent heterochromatinization seems to also take place in the bulk of the embryo, thus controlling transcriptional activity in adult extra-gonadal tissues.

Conclusion

Among the three classes of small regulatory RNAs in insects, piRNAs have a clear effect on the acquisition and propagation of epigenetic changes. In the best studied species, *D. melanogaster*, piRNAs exert two types of epigenetic controls. On the one hand, Piwi-loaded piRNAs can guide heterochromatinization of homologous loci, apparently triggered by the recruitment of piRNAs through complementary nascent transcripts. This process could be unique to the Brachycera suborder (Drosophilids and related fly species, such as *Musca domestica* and *Ceratitis capitata*). Other insects possess orthologs for the other two Piwi subfamily proteins (Aub and Ago3), but nothing indicates that they express a functional ortholog of *Drosophila* Piwi. Nevertheless, a conserved role for piRNAs in heterochromatin formation cannot be ruled out, as mammalian piRNAs seem to be able to direct DNA methylation [71]. A recent study indeed suggests that the two *Bombyx* Piwi proteins, named Siwi and Ago3, play a transcriptional role in addition to their post-transcriptional role [72]. Further studies are needed to determine what fraction of Siwi and Ago3 is involved in these nuclear processes.

On the other hand, maternally deposited piRNAs, loaded onto Piwi, Aub or Ago3, are responsible for an extrachromosomal form of epigenetic inheritance, where maternally inherited piRNAs prime the production of secondary piRNAs in the embryo, thus allowing the continuous repression of piRNA targets.

These two modes of action for piRNAs ensure a stable silencing of repeated sequences across generations. The peculiar biogenesis of piRNAs, expressing a large diversity of small RNAs from fast evolving genomic clusters, provides the basis for a dynamic genome protection system, which can adapt rapidly to emerging TE invasions.

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- •• of outstanding interest

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