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Review Article

piRNA pathway and the potential processing site, the nuage, in the *Drosophila* germline

Jun Wei Pek,[1†] Veena S. Patil[2†] and Toshie Kai[1*]

[1]*Department of Biological Sciences and Temasek Life Sciences Laboratory, 1 Research Link, The National University of Singapore, Singapore 117604, Singapore; and* [2]*Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA*

The accurate transfer of genetic material in germline cells during the formation of gametes is important for the continuity of the species. However, animal germline cells face challenges from transposons, which seek to spread themselves in the genome. This review focuses on studies in *Drosophila melanogaster* on how the genome protects itself from such a mutational burden via a class of gonad-specific small interfering RNAs, known as piRNAs (Piwi-interacting RNAs). In addition to silencing transposons, piRNAs also regulate other processes, such as chromosome segregation, mRNA degradation and germline differentiation. Recent studies revealed two modes of piRNA processing – primary processing and secondary processing (also known as ping-pong amplification). The primary processing pathway functions in both germline and somatic cells in the *Drosophila* ovaries by processing precursor piRNAs into 23–29 nt piRNAs. In contrast, the secondary processing pathway functions only in the germline cells where piRNAs are amplified in a feed-forward loop and require the Piwi-family proteins Aubergine and Argonaute3. Aubergine and Argonaute3 localize to a unique structure found in animal germline cells, the nuage, which has been proposed to function as a compartmentalized site for the ping-pong cycle. The nuage and the localized proteins are well-conserved, implying the importance of the piRNA amplification loop in animal germline cells. Nuage components include various types of proteins that are known to interact both physically and genetically, and therefore appear to be assembled in a sequential order to exert their function, resulting in a macromolecular RNA-protein complex dedicated to the silencing of transposons.

Key words: germline, nuage, Piwi-interacting RNA.

Transposons and their harmful effects

The germline cells of an organism form a very crucial cell lineage as they confer species continuity. Hence, it is essential for germline cells to maintain the stability and fidelity of their genomes throughout generations. To achieve this, the germline genome employs several regulatory mechanisms that are both temporally and spatially controlled. Indeed, the genome of the germline is challenged by various factors that target both its stability and integrity. One such risk factor is the occurrence and the activity of deleterious transposons/selfish genetic elements. A wide variety of transposons is found to populate eukaryotic genomes and are known to propagate by populating the germline genome. These parasitic genetic elements can cause various defects when mobilized, including the disruption of protein-coding genes, chromosomal breakage, genome rearrangements and an alteration in the transcription network (McClintock 1951; Goodier & Kazazian 2008). Transposons/transposable elements can be categorized into two classes on the basis of their structure and their mode of propagation: class I comprises retrotransposons (also called retroelements) that mobilize by a ''copy and paste'' mechanism, and class II consists of DNA transposons that move by a ''cut and paste'' mechanism (reviewed in Goodier & Kazazian 2008; Slotkin & Martienssen 2007). As a consequence, class I retroelements generate an additional copy with every transposition, whereas the transposition of class II DNA transposons is conservative, where one site loses the transposon while another gains it. Retroelements are further classified into Long Terminal Repeats (LTR) and non-LTR subtypes, on the basis of whether the

*Author to whom all correspondence should be addressed.
Email: toshie@tll.org.sg
†Those authors contributed equally to this work.
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elements are bound by long terminal repeats or not, respectively. Depending on the size and origin, the non-LTRs retroelements are grouped into Long Interspersed Nucleotide Elements (LINEs) or Short Interspersed Nucleotide Elements (SINEs) (reviewed in Goodier & Kazazian 2008).

While transposons constitute approximately 40% of the mouse genome, they constitute only 7–8% of the *Drosophila* genome (Smith *et al.* 2007). Not only the extent but the type of the transposons that populate the eukaryotic genome is also variable. For example, the *Saccharomyces cerevisiae* genome contains only the members of a single LTR transposon family whereas the *Arabidopsis thaliana* genome harbors a wide variety of transposons. The genome of *Drosophila melanogaster* contains approximately 202 different elements consisting of a variety of LTR and non-LTR retrotransposons, and each of these is present in limited amounts in the genome. Although the mouse genome is also dominated by retrotransposons, only a few elements from each group, such as IAP (LTR), and SINE B1 and LINE (non-LTR), dominate the genome (reviewed in Malone & Hannon 2009). In general, the germline genomes in many organisms are populated with retrotransposons.

The piRNA pathway

Recently, a class of gonad-specific small interfering RNAs, known as the piRNAs (PIWI-interacting RNA), have been identified in many animals (reviewed in Klattenhoff & Theurkauf 2008). piRNAs were first detected against the Stellate repeats in the *Drosophila* testes by the Gvozdev's group (Aravin *et al.* 2001). Later, more piRNAs were reported by Tuschl and his co-workers, while they were profiling the small RNAs (mainly miRNAs) from various developmental stages of *Drosophila* (Aravin *et al.* 2003). These authors observed a distinct fraction of small RNAs that were 23–29 nt long, in addition to the 22-nt-long miRNA (Aravin *et al.* 2003). These longer species lacked the complementary star strand nearby that is generally found in the miRNA/miRNA precursor structure, suggesting a novel biogenesis pathway (Aravin *et al.* 2003). The majority of these RNAs were mapped to genomic repeats or transposon loci in *Drosophila.* Hence, they were initially termed as repeat-associated small interfering RNAs (rasiRNAs) and, subsequently, renamed as piRNA when they were found to be associated with the Piwi family proteins (Aravin *et al.* 2006; Girard *et al.* 2006; Grivna *et al.* 2006; Lau *et al.* 2006; Saito *et al.* 2006; Brennecke *et al.* 2007; Gunawardane *et al.* 2007). In *Drosophila*, more than 90% of the piRNAs mapping to genome arise from discrete regions in the genome, which mostly are pericentromeric and sub-telomeric in positions (Brennecke *et al.* 2007). These regions mostly comprise fragmented and imperfect copies of the retroelements that are incapable of mobilization. piRNAs are believed to arise as longer precursor transcripts from these discrete genomic regions, which are called piRNA clusters. Precursor piRNAs are then cleaved into mature piRNAs by a primary processing machinery and are amplified to produce both sense and antisense piRNAs by a feed-forward amplification loop in the germline cells (see next section).

The *Drosophila* Piwi family proteins, Piwi, Aubergine (Aub) and Argonaute3 (Ago3), have been shown to participate in the production of piRNAs. piRNAs are produced in a Dicer-independent manner, unlike the other two classes of small RNAs, microRNA (miRNA) and small interfering RNA (siRNA) (Vagin *et al.* 2006; Brennecke *et al.* 2007; Gunawardane *et al.* 2007; Houwing *et al.* 2007; Li *et al.* 2009; Malone *et al.* 2009), and were initially shown to be involved in retrotransposon silencing in *Drosophila* (Vagin *et al.* 2006). Importantly, piRNAs were also found to perform similar functions in many other organisms, such as mouse, rat and zebrafish (Lau *et al.* 2006; Saito *et al.* 2006; Vagin *et al.* 2006; Aravin *et al.* 2007; Brennecke *et al.* 2007; Gunawardane *et al.* 2007; Houwing *et al.* 2007; Li *et al.* 2009; Malone *et al.* 2009).

Recent studies in *Drosophila* have identified two distinct groups of piRNAs that function in the ovary: one in the ovarian somatic follicle cells and the other in the ovarian germline cells. Each group of piRNAs are derived from distinct clusters and shared clusters in the *Drosophila* genome, such as the somatic *flamenco* cluster, 42AB germline piRNA cluster, and shared 20A cluster. Among the three Piwi-family proteins, the ovarian somatic cells express only Piwi, and indeed, the somatic piRNAs are generated in a Piwi-dependent but Aub- and Ago3-independent manner (Lau *et al.* 2006; Li *et al.* 2009; Malone *et al.* 2009). Deep sequencing analysis revealed that most of the Piwi-bound transposon piRNAs are antisense and map exclusively to the anti-sense strand of the *flamenco* cluster. However, the piRNAs functioning in the germline cells are generated in an Aub- and Ago3-dependent manner in the ping-pong cycle, where sense and antisense piRNAs are generated (see next section for details) (Saito *et al.* 2006; Gunawardane *et al.* 2007; Nishida *et al.* 2007; Brennecke *et al.* 2008; Li *et al.* 2009; Malone *et al.* 2009). The resulting piRNAs associate with Piwi family proteins to form the piRISC, thereby targeting complementary transposon transcripts, causing post-transcriptional silencing. Despite the abundant evidence for the piRNAs in promoting post-transcriptional regulation, the piRNA pathway has also been shown to

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repress the expression of telomeric transposons by promoting heterochromatin formation (Klenov *et al.* 2007; Shpiz *et al.* 2011). This process of transcriptional silencing involves the piRNA pathway components, *piwi* and *spn-E*. Other studies have also suggested the role of Piwi on heterochromatin formation or Polycomb group-mediated silencing outside the germline (Pal-Bhadra *et al.* 2004; Grimaud *et al.* 2006; Brower-Toland *et al.* 2007; Kavi & Birchler 2009). However, the mechanism of how piRNAs promote heterochromatin formation in the germline requires further clarification.

Biogenesis and compartmentalization

The biogenesis of piRNAs involves precursor transcription, primary processing and ping-pong amplification (only in the germline). Early studies have shown that piRNAs are produced in a Dicer-independent manner (Vagin *et al.* 2006; Houwing *et al.* 2007), and recent studies have provided important insights into their biogenesis.

Precursor transcription

Little information is available on the regulation of piRNA precursor transcription. piRNA clusters are located at pericentromeric and sub-telomeric regions, which consist of mainly heterochromatin. Therefore, the transcription of precursor piRNA requires an active transcription machinery. Recently, it has been reported that Rhino, an HP1 homologue, localized to the pericentromeric piRNA clusters in the germline cells and was required for the transcription of precursor piRNAs (Klattenhoff *et al.* 2009); however, the exact mechanism by which Rhino promotes the transcription of precursor piRNAs is not known. Piwi promotes 3R-TAS piRNA transcription by the formation of euchromatin at the 3R-TAS piRNA locus in somatic cells, as there is a simultaneous increase in the HP1a occupancy at the 3R-TAS piRNA locus and a decrease in the 3R-TAS piRNA production in *piwi* mutants (Yin & Lin 2007). Similarly, HP1a occupancy also increases at the piRNA loci in the somatic cells of other piRNA pathway mutants (Moshkovich & Lei 2010). Recently, the importance of heterochromatin was again highlighted in a report describing *eggless*, which encodes a *Drosophila* H3K9 methyltransferase that functions in H3K9 trimethylation in the germarium and early stage egg chambers for the maintenance of robust piRNA precursor transcription (Rangan *et al.* 2011). Taken together, emerging evidence suggests that the transcription of piRNA precursors requires an intricate balance of heterochromatin formation at the piRNA clusters.

Primary processing

The long piRNA precursors are processed into mature piRNAs and loaded onto the Piwi family proteins (Piwi in gonadal somatic cells and Piwi, Aub and Ago3 in germline cells). Although this primary processing pathway for piRNAs exists in both somatic and germline cells in the *Drosophila* ovaries, the mechanism has been better studied in the somatic follicle cells where only Piwi, but not Aub or Ago3, is expressed. In the somatic follicle cells, primary processing appears to be the only known mechanism that produces piRNAs. Conversely, the predominant mode of piRNA production in germline cells arises from secondary amplification, the ping-pong cycle. The exact mechanism of the primary processing in germline cells remains elusive.

Unlike germline cells, transposon activity is lower in somatic cells and is suppressed by somatic piRNAs associated with Piwi. In addition to Piwi, genetic and biochemical analysis combined with RNAi studies have identified Zucchini (Zuc), Armitage (Armi), Yb and Vreteno (Vret) as factors that are required for primary processing in follicle cells (Szakmary *et al.* 2009; Haase *et al.* 2010; Olivieri *et al.* 2010; Saito *et al.* 2010; Handler *et al.* 2011; Qi *et al.* 2011; Zamparini *et al.* 2011). Armi, Yb and Vret localize to discrete cytoplasmic bodies known as Yb bodies, whereas Zuc localizes in close proximity to the mitochondria. The Yb body is believed to be the site where the Piwi-piRNA complex becomes loaded, after which, the piRISC then shuttles into the nucleus (Fig. 1). Unlike Zuc, Armi and Vret, which are also expressed in germline cells, Yb is expressed only in the somatic follicle cells. However, Yb homologues, CG11133 (Brother of Yb) and CG31755 (Sister of Yb), may function as germline counterparts of Yb in primary processing in the germline cells (Handler *et al.* 2011). The resulting germline piRNAs from the primary processing, in turn, may initiate the ping-pong amplification cycle.

Ping-pong amplification loop

The ping-pong amplification loop in the germline has been well-reviewed (Khurana & Theurkauf 2010; Saito & Siomi 2010; Senti & Brennecke 2010; Siomi *et al.* 2011). Briefly, following the primary processing that produces the mature piRNAs, these piRNAs (mainly anti-sense) are loaded onto Aub in the cytoplasm. Aub-bound anti-sense piRNAs target the sense retroelement transcripts and slice them to produce the 5′ ends of the sense piRNAs. The 3′ ends of the sense piRNAs are then processed by an unknown mechanism. Sense piRNAs are then loaded onto Ago3. Ago3-bound sense piRNAs then target the precursor

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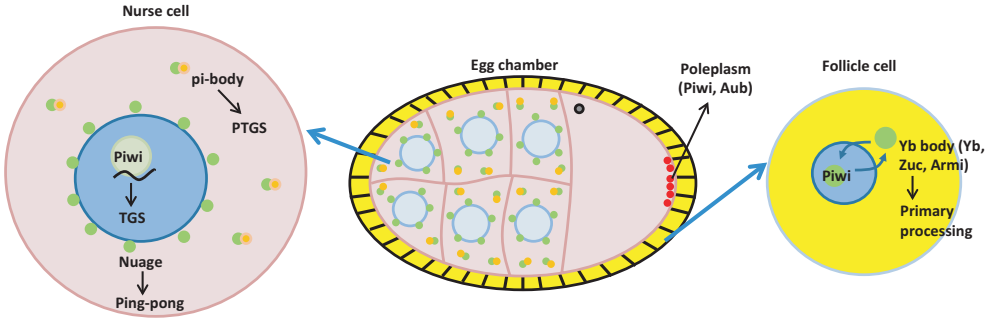

Fig. 1. Mechanisms of silencing in the germline and somatic cells in the *Drosophila* egg chamber. The germline cells are pink in color, the somatic follicle cells are yellow, the nuclei are blue, and the P-body components are orange.

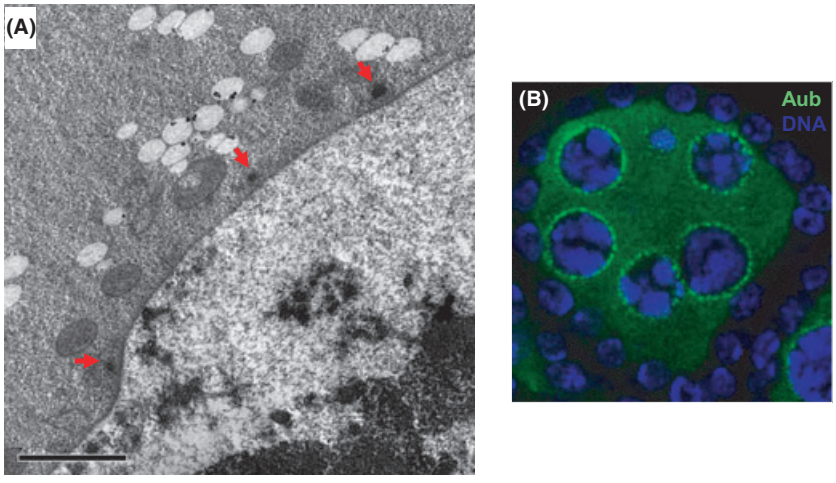

Fig. 2. The nuage in *Drosophila* ovaries. (A) An electron micrograph showing the perinuclear electron-dense nuage structures (arrows) on the cytoplasmic face of the nuclear envelope in *Drosophila* ovaries. (B) Immunostaining using an antibody against Aubergine to visualize the perinuclear nuage (green) in the *Drosophila* egg chamber. The DNA (blue) is labeled by 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI).

anti-sense piRNAs and process them into the mature piRNAs that are loaded onto Aub. This creates a feed-forward loop between Aub and Ago3 that is known as the ping-pong amplification loop and generates piRNAs that possess 10 nt overlaps at their 5′ ends, a characteristic known as the ping-pong signature (Brennecke *et al.* 2007; Gunawardane *et al.* 2007).

The nuage: a potential processing site of the ping-pong amplification loop of germline piRNAs

The Piwi sub-family of proteins involved in the ping-pong amplification loop, Aub and Ago3, localize to a unique perinuclear structure, the nuage, in the germline cells (Harris & Macdonald 2001; Brennecke *et al.* 2007; Gunawardane *et al.* 2007; Li *et al.* 2009; Fig. 2). By electron micrograph, the nuage is observed as a fibrillar, electron-dense, amorphous and membraneless organelle often found on the outer surface of the nuclear envelope of animal germline cells. These structures were first described in rat spermatids more than 100 years ago and have intrigued researchers over the past few decades. Similar structures are also known by various names in different animals, such as the inter-mitochondrial cement and chromatoid body in mouse and P granules in *Caenorhabditis elegans* and zebrafish (reviewed by Eddy 1975), indicating its conservation across species in the animal kingdom and suggesting a pivotal role in the germline. In *Drosophila*, the nuage is first detectable when the primordial germ cells (PGCs) form and persist throughout oogenesis in the adult germline cells (except for the oocyte) (Saffman & Lasko 1999). Experiments involving RNase and protease treatments have shown that the nuage is enriched with both RNAs and proteins (Mahowald 1968, 1971). In *Drosophila*, many of the proteins that localize to the nuage have been reported to be involved in the production of germline piRNAs,

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including the following: Vasa (Vas), a conserved DEAD box RNA helicase (Liang *et al.* 1994); Aub and Ago3, two Piwi-family proteins (Harris & Macdonald 2001; Gunawardane *et al.* 2007; Li *et al.* 2009; Fig. 2B); Krimper (Krimp), a tudor domain-containing protein (Lim & Kai 2007); Maelstrom (Mael), an HMG box-containing protein (Findley *et al.* 2003; Pek *et al.* 2009); Spindle-E (Spn-E), a DExH box putative RNA helicase (Gillespie & Berg 1995; Patil & Kai 2010), Tejas (Tej), a tudor domain protein (Patil & Kai 2010), and Vreteno (Vret), another tudor domain protein (Handler *et al.* 2011; Zamparini *et al.* 2011). In the ovaries of their respective mutants, retroelements become derepressed as a result of insufficient piRNA production. Indeed, the deep sequencing analysis of piRNAs in many of these mutants revealed the loss of primary piRNA production and collapse of the ping-pong cycle (Malone *et al.* 2009).

Interestingly, the *Drosophila* nuage components involved in the ping-pong amplification of piRNAs exhibit a hierarchical genetic interaction for the localization of their encoded proteins to the nuage (Findley *et al.* 2003; Lim & Kai 2007; Patil & Kai 2010). In the hierarchical assembly, *vas* appears to be the most upstream, followed by *tej/spn-E*, *aub/ago3*, *krimp* and *mael*. In the absence of *vas*, all of the components participating in this interaction are mislocalized from the perinuclear nuage, and Vas remains localized to nuage in all of the other mutant ovaries (Findley *et al.* 2003; Lim & Kai 2007; Patil & Kai 2010). In addition, Aub and Ago3, which are the main components of the ping-pong amplification loop, are interdependent for their perinuclear localization and *aub* is required for the proper localizations of the remaining downstream components, Krimp and Mael (Lim & Kai 2007; Li *et al.* 2009). These hierarchical relationships among the piRNA components could possibly be an indication of their sequential order of function in the biogenesis of piRNAs and in nuage assembly.

In *Drosophila*, the nuage can be observed only in the nurse cells but not in the oocytes. Oocyte differentiation starts with cyst formation, where a germline stem cell divides asymmetrically to give rise to two daughter cells – a stem cell and a differentiating cystoblast. The cystoblast undergoes four rounds of mitosis and generates a 16-cell cyst in which all of the resulting germline cells are interconnected, and only one of those 16 cells eventually becomes an oocyte, whereas the others differentiate into nurse cells. Once the oocyte becomes committed, it becomes transcriptionally and translationally quiescent and is nursed by the nurse cells through cytoplasmic bridges. The nurse cells provide abundant RNAs, which are deposited into the oocyte. Transposons are actively transcribed in the nurse cells, and their transcripts could, thus, be delivered to the oocyte; such deleterious transcripts must be cleared when they leave the nurse cell nucleus. Consistent with the high transcriptional activity of transposons in nurse cells, the nuage can be seen only in the nurse cells but not in the oocyte. The piRISCs appear to target such transposon transcripts and piRNA precursors as they are being shuttled from the nucleus to the cytoplasm of the nurse cells.

Whereas the molecular functions of the nuage components have not been fully elucidated, we hypothesize that their functions in the ping-pong amplification at the perinuclear nuage based on their genetic interactions for their localizations and predicted domain functions (Fig. 3). Among those known nuage components, Spn-E, Krimp, Tej, Papi and Vret harbor tudor domains. The structural data from many of the tudor domain proteins, expressed both in the germline and non-germline cells, have revealed that these proteins contain an aromatic cage that has been shown to interact with methylated amino acids, either symmetrically dimethylated arginines (sDMA) or methylated lysines, on their binding partners (reviewed in Arkov & Ramos 2010). Indeed, interactions between some of the tudor domain proteins and Piwi-family proteins are mediated by the sDMAs of the Piwi family of proteins (Kirino *et al.* 2009; Nishida *et al.*, 2009; Vagin *et al.* 2009). Tej contains a tudor domain and a novel domain, the tejas domain (also known as Lotus/OST-HTH), a predicted dsRNA-binding domain at the N-terminus (Anantharaman *et al.* 2010; Callebaut & Mornon 2010). Tej interacts with the RNA helicases, Vas and Spn-E, suggesting that they may form a sub-complex that binds and unwinds potential retroelement transcripts that exit the nucleus (Patil & Kai 2010). Furthermore, Tej and Vas associate with Aub and are required for Aub localization, suggesting that they may initiate the ping-pong cycle by recruiting the Aub-piRNA complex to the retroelement transcripts (Thomson *et al.* 2008; Patil & Kai 2010). The retroelement transcript is then sliced by the Aub-piRNA complex to produce the 5′ end and then loaded onto Ago3. Krimp, a tudor domain protein downstream of Aub, but is required for Ago3 localization (Lim & Kai 2007); thus, Ago3 and Krimp may exist in another sub-complex in the ping-pong cycle. The retroelement transcript may be further processed by a yet-unknown 3′–5′ exo-ribonuclease activity. Mael, which contains a unique domain homologous to the DnaQ-H 3′-5′ exonuclease (Zhang *et al.* 2008), may function to produce the 3′ end of the mature piRNA. The Ago3-piRNA complex then targets the anti-sense piRNA precursor that exits from the nucleus. The Tej-Vas-Spn-E complex may bind such precursors in the vicinity of the nuclear

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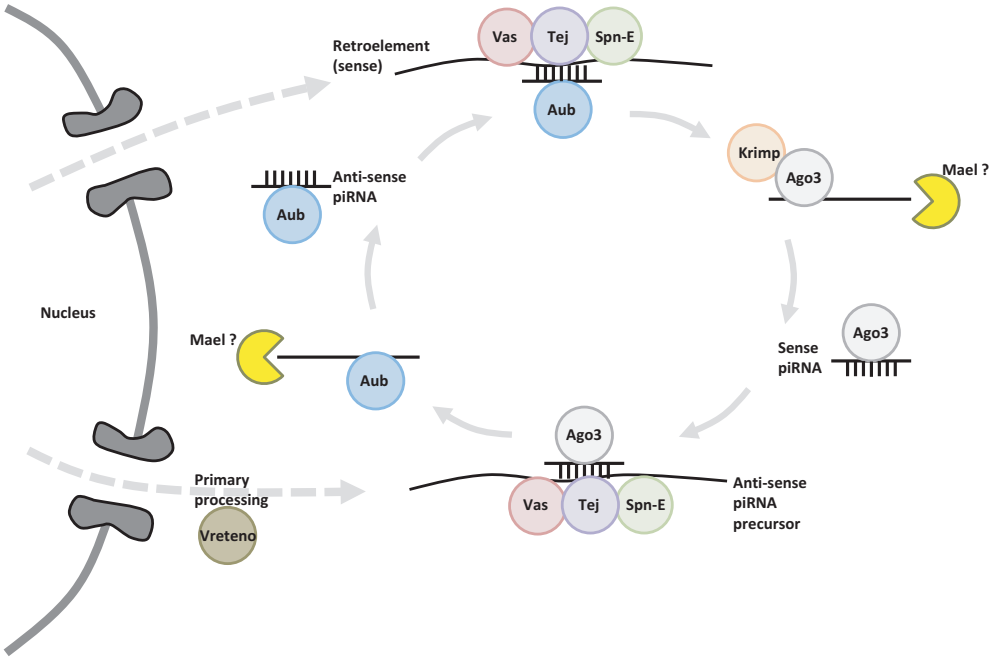

Fig. 3. Hypothetical model of the assembly and functions of the various nuage components in the piRNA biogenesis pathway.

membrane and unwind and process them further. The slicer activity on the precursor piRNA by the Ago3-piRNA complex occurs to produce the 5′ end of the anti-sense piRNA. Further trimming by Mael possibly generates the 3′ end of the mature piRNA, which is then loaded onto Aub to complete the cycle. In contrast, Vret is involved in the primary processing of piRNAs (Handler *et al.* 2011; Zamparini *et al.* 2011), while the requirement for Papi in piRNA production is currently unknown (Liu *et al.* 2011).

Similar observations were also reported in mice, whereby the Piwi proteins also interact with TDRD (Tudor domain-containing) proteins and RNA helicases, thus forming a complex at the nuage or inter-mitochondrial cement (reviewed in Siomi *et al.* 2011). Such genetic and physical interactions among the nuage components in mice for their localizations imply a conservation of the mechanism underlying the assembly of those protein complexes or subcomplexes.

The P-body

In addition to the perinuclear nuage, the cytoplasmic foci of Aub, Ago3 and Krimp become discernible in the later stages of the egg chambers (Lim *et al.* 2009; Fig. 1). These cytoplasmic foci become progressively prominent from stage 4 onward during oogenesis and are ubiquitously distributed as discrete puncta throughout the nurse cell cytoplasm. The foci contain retroelement transcripts, anti-sense piRNAs, and proteins involved in mRNA degradation, such as decapping enzymes, dDcp1, dDcp2, and Me31B (a homologue of yeast decapping activator Dhh1p), and the *Drosophila* homologue of yeast Xrn1p, Pcm. In somatic cells, the proteins involved in RNA degradation localize to cytoplasmic foci, called processing bodies (P-bodies) (Parker & Sheth 2007). The co-localization of the nuage/piRNA pathway components with P-bodies requires the sufficient production of germline piRNA; in *spn-E* and *aub* mutants, piRNA pathway proteins no longer overlap with mRNA degradation proteins. Concomitantly, the mutant ovaries show an accumulation and prolonged stabilization of retroelement transcripts. Recently, a new piRNA pathway component, Papi, was also shown to interact physically with the P-body components, Trailer hitch (Tral) and Me31B (Liu *et al.* 2011). These results suggest that the mRNA degradation machinery mediates the post-transcriptional removal of transposon transcripts or decay intermediates, possibly upon piRNA-mediated cleavage. Similarly, the co-localization of piRNA pathway components with the P-body was reported in mouse (Aravin *et al.* 2009), suggesting a conservation of the posttranscriptional silencing machinery involving piRNAs and P-body components in animal germline cells.

Additional functions of the piRNA pathway

As discussed above, the primary function of the piRNA pathway is to regulate the expression of retroelements. However, recent studies have uncovered a myriad of

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processes that are also regulated by the piRNA pathway, suggesting that the piRNA pathway functions at multiple levels during germline development (Fig. 4).

Hybrid dysgenesis

piRNAs serve as vectors for epigenetic information and are responsible for a phenomenon called hybrid dysgenesis in *Drosophila virilis* and *Drosophila melanogaster* (Blumenstiel & Hartl 2005; Brennecke *et al.* 2008). Hybrid dysgenesis can be caused when wild female *Drosophila* strains are crossed to laboratory-kept male strains, producing sterile F1 progeny (Kidwell *et al.* 1977; Bregliano *et al.* 1980; Bucheton 1990). One such dysgenetic cross, the paternal-maternal (P-M) type, led to the discovery of the P-element, which serves as a binary insertional mutation system (Spradling & Rubin 1982). Among the *Drosophila* hybrid dysgeneses, both the P-M and inducer-reactive (I-R) types were molecularly studied, and the involvement of piRNAs was implicated (Brennecke *et al.* 2008). Transposons, such as the *I-element* and *P-element* from which piRNAs are derived, are absent in the laboratory-kept reactive strains (R or M type) but present in the wild inducer strains (I or P type). Hence, the F1 progeny arising from the crosses between laboratory-kept females and wild males lack the inheritance of the maternal piRNAs (Brennecke *et al.* 2008). The lack of the maternal piRNAs deposition reduces the input into the ping-pong amplification loop, thereby, compromising piRNA biogenesis in the F1 generation. As a consequence, the *I-element* and *P-element* become derepressed in F1 gonads, leading to female sterility (Brennecke *et al.* 2008).

Karyosome formation and oocyte polarity

It has been suggested that the activation of retroelements may result in an increase in DNA double strand break (DSB) formation, thereby triggering the meiotic checkpoint and resulting in polarity and karyosome compaction defects (Klattenhoff *et al.* 2007; Fig. 4). However, it has not been experimentally shown that the activation of retroelements in the piRNA pathway mutants directly causes double strand break formation.

Interestingly, localization studies of some of the retroelements in *Drosophila* suggest that retroelements may have some direct impact on karyosome formation. Retroelements TART, HeT-A and I-element have been found to localize to the oocyte in some of the piRNA pathway mutants in *Drosophila* (Klattenhoff *et al.* 2007; Brennecke *et al.* 2008; Chambeyron *et al.* 2008), suggesting that activated retroelements in the

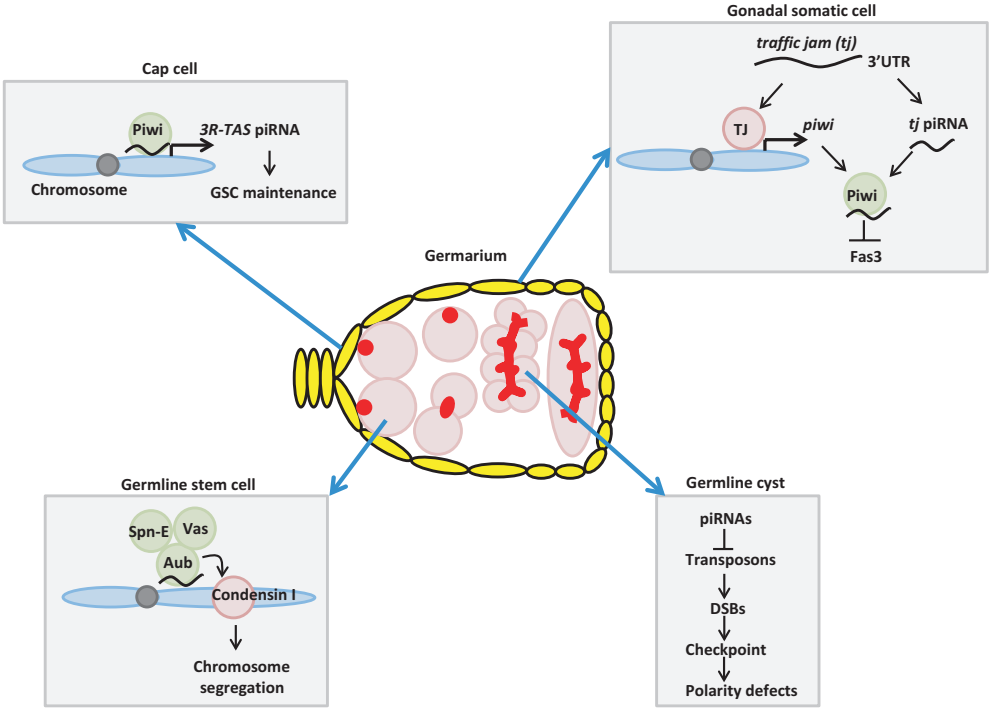

Fig. 4. Various functions of the piRNA pathway in the somatic and germline cells of the *Drosophila* germarium. The germline cells are pink in color, the somatic cells are yellow, and the spectrosomes/fusomes are red.

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oocyte may lead to the formation of DSBs and further impact karyosome compaction.

In addition to reduced piRNA production, most of the known piRNA pathway mutants show defects in oocyte polarity formation, implicating a probable link between the two phenotypes. Interestingly, the analysis of the *tej* mutant contradicts this: unlike other piRNA pathway mutants, the *tej* mutants establish proper polarity but show reduced germline piRNA levels, indicating that these two processes can be uncoupled. These reduced levels could be due to piRNA-independent functions of other components in the formation of polarity, which do not involve *tej*. Alternatively, the establishment of polarity may depend on specific piRNA(s) that require other piRNA/nuage components, but not the *tej* function, for their production. Comparing the piRNA profiles from *tej* and other piRNA pathway mutants may provide some insights to address this issue.

Germline stem cell maintenance

Piwi was originally identified as a factor that is important in the somatic cap cells to maintain the self-renewal of germline stem cells (GSCs) (Cox *et al.* 2000). The exact function of Piwi in the GSC niche is still not well understood. The discovery of piRNAs led to some insights of how Piwi may function in the somatic cap cells. It was proposed that the Piwi-3R-TAS piRNA complex activates the transcription of the 3R-TAS piRNA. Piwi does this by promoting the euchromatin status of the 3R-TAS promoter region, a function that is different from the commonly known role of Piwi, which is to promote heterochromatin formation (Yin & Lin 2007). Furthermore, this somatic function of Piwi and the 3R-TAS piRNA appears to be important for GSC maintenance (Yin & Lin 2007; Fig. 4). Additionally, other mutants that are defective in somatic piRNAs, such as *vret* and *flamenco*, exhibit germline differentiation defects (Zamparini *et al.* 2011). Such germline defects can be rescued by the somatic expression of *piwi* or *vret* in their respective mutants (Wang & Lin 2005; Zamparini *et al.* 2011), suggesting an essential requirement of the somatic piRNA pathway for the proper differentiation of germline stem cells.

Gene regulation via somatic piRNAs derived from the 3′UTR

In addition to transposon or heterochromatin sequences, another class of somatic piRNAs are derived from the 3′UTR of the mRNAs of protein-coding genes (Robine *et al.* 2009; Saito *et al.* 2009). It was discovered that *traffic jam* (*tj*) contributes to the somatic piRNA pathway in two ways: the processing of the mRNA 3′UTR to produce *tj* piRNAs and the promotion of the expression of Piwi, which later is loaded onto the somatic piRNAs (Saito *et al.* 2009; Fig. 4). The functions of these piRNAs remain elusive, but it is believed that they regulate gene expression.

Maternal mRNA degradation

The piRNA pathway also functions outside the germline to regulate maternal mRNA degradation (Fig. 5). In *Drosophila*, piRNAs are maternally deposited in the embryos. During embryogenesis, the piRNA pathway functions to degrade maternally deposited mRNAs, such as *nanos*, by targeting the piRNA target sites in the 3′UTR of the mRNAs (Rouget *et al.* 2010; Fig. 5). As in the case of the ovaries, this process also makes use of the RNA degradation machinery.

Chromosome segregation

In the germline stem cells, the piRNA pathway components, Vasa, Aub and Spn-E, localize to the mitotic chromosomes at the piRNA-generating loci and promote the chromosomal loading of the condensin I complex (Pek & Kai 2011a,b; Fig. 4). This process is important for proper mitotic chromosome condensation and chromosome segregation, suggesting novel roles for the piRNA loci as nucleation sites to facilitate condensin I loading via the piRNA pathway. Interestingly, the role of the small RNA pathway in promoting chromosome segregation appears to be conserved in the somatic cells in *Drosophila* and human (Pek & Kai

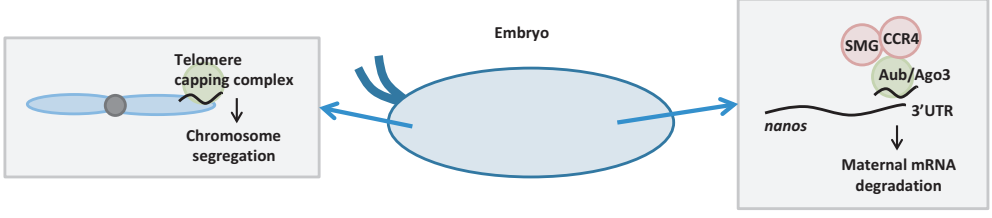

Fig. 5. Functions of the piRNA pathway in the *Drosophila* embryo.

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2011c). Furthermore, studies in *Caenorhabditis elegans* have also shown that P granule components, including Argonaute CSR-1 (and its 22G-RNAs), RNA-dependent RNA polymerase EGO-1, Dicer-related helicase DRH-3, and Tudor-domain protein EKL-1 localize to chromosomes during mitosis and are required for proper chromosome segregation, suggesting a conserved mechanism (Claycomb *et al.* 2009; van Wolfswinkel *et al.* 2009).

Another mechanism in which the piRNA pathway promotes chromosome segregation is through the promotion of the assembly of the telomere-capping complex (Khurana *et al.* 2010; Fig. 5). It was found that *aubergine* and *armitage* are genetically required for the production of a sub-population of 19–22 nt telomere-specific piRNAs. These piRNAs are required for the recruitment of the HOAP telomere-capping protein to the telomeres, which promotes chromosome segregation during meiosis and embryonic cleavage divisions (Khurana *et al.* 2010). Therefore, a certain proportion of piRNAs play a role in protecting the telomeres.

Perspectives and conclusions

Since the discovery of rasiRNAs (repeat-associated siRNAs, now known as piRNAs) in 2001 (Aravin *et al.* 2001), our understanding in the piRNA pathway has been rapidly expanding over the years. Although much is known about the genetic requirements of various proteins in the piRNA biogenesis pathway, little is still known about the molecular roles of each component. The intricate involvement of several different types of proteins such as Argonaute proteins, tudor domain proteins, RNA helicases in the piRNA pathway suggests a more robust and a much more complicated pathway for the biogenesis of piRNAs. The exact mechanism of how these protein functions are coordinated remains a mystery. Furthermore, it would also be important to understand how the piRNA biogenesis is regulated, especially in times of stress, which would confer a selective advantage to the survival of the species.

Amplification of germline piRNAs in the ping-pong cycle especially involves many of the tudor domain proteins, which interact with symmetrically dimethylated arginine residues on Piwi family proteins (reviewed in Siomi *et al.* 2011 and Chen *et al.* 2011). It is tempting to speculate that these proteins function in concert with Piwi family proteins by forming a macromolecular complex (at nuage) for effective processing of piRNAs. Therefore, the nuage may serve as a platform or scaffold for piRNA pathway proteins to assemble the efficient and robust piRNA processing machinery. Further genetic and biochemical analysis should help to resolve this issue.

Equally important, we now are starting to understand the myriad of roles played by piRNAs. Besides the well-known function of transposon silencing, it is now clear that piRNAs function at various levels in regulating germline stem cell differentiation, mitotic chromosome dynamics, and even gene expression. Future studies would certainly shed light on more surprising functions of the piRNA pathway in the germline or even in other tissues.

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