piRNAs, transposon silencing, and *Drosophila* germline development

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Transposons are prominent features of most eukaryotic genomes and mobilization of these elements triggers genetic instability. Transposon silencing is particularly critical in the germline, which maintains the heritable genetic complement. Piwi-interacting RNAs (piRNAs) have emerged as central players in transposon silencing and genome maintenance during germline development. In particular, research on *Drosophila* oogenesis has provided critical insights into piRNA biogenesis and transposon silencing. In this system, the ability to place piRNA mutant phenotypes within a well-defined developmental framework has been instrumental in elucidating the molecular mechanisms underlying the connection between piRNAs and transposon control.

Introduction

Transposons are major structural elements of essentially all eukaryotic genomes, and mobilization of these elements can lead to genetic instability and cause deleterious mutations (McClintock, 1953). Mobile genetic elements also carry transcriptional enhancers and insulators, thus transposition can alter expression of nearby genes and potentially large chromatin domains, triggering coordinated changes in gene transcription that could disrupt development or drive evolution (Feschotte, 2008). Transposon silencing is particularly important in the germline, which maintains the genetic information that will be inherited by future generations. Recent studies indicate that transposon silencing during germline development is imposed by Piwi-interacting RNAs (piRNAs), which guide a small RNA-based immune response related to RNA interference (RNAi; Malone and Hannon, 2009). Here we review piRNA biogenesis and function during *Drosophila* female germline development, where recent molecular and biochemical observations have provided significant insight into the mechanism of piRNA production and transposon silencing, and where the developmental defects associated with piRNA mutations can be evaluated within a well-established genetic, cellular, and developmental framework (Spradling, 1993).

Gene silencing by microRNAs (miRNAs) and small interfering RNAs (siRNAs) is well established (Filipowicz et al., 2005; Ghildiyal and Zamore, 2009), and studies on these small regulatory RNAs have guided work on the more recently identified piRNAs. The 21- and 22-nucleotide siRNAs and miRNAs are generated from double-stranded precursors by the RNase III enzyme Dicer and bind to Argonaute proteins (Ghildiyal and Zamore, 2009). The Argonaute–miRNA complexes direct sequence-specific translational silencing or target destruction. siRNAs in animals, in contrast, appear to primarily induce target destruction. However, endogenous siRNAs (endo-siRNAs) direct chromatin assembly and transcriptional silencing in the fission yeast *Schizosaccharomyces pombe,* and endo-siRNAs have been implicated in repressing transposons and other repetitive sequences during somatic development in flies (Volpe et al., 2002; Verdel et al., 2004; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008; Hartig et al., 2009). miRNAs and siRNAs, in complexes with Argonautes, can therefore silence transcription, trigger target destruction, or inhibit translation. The piRNAs are less well understood, but may be equally versatile.

piRNA identification and genomic origins

piRNAs were first identified through studies on the *Drosophila Stellate* locus, which is composed of repeated copies of a gene encoding a casein kinase II β-subunit homologue (Livak, 1990). The *Drosophila Stellate* protein has no known biological function, but mutations in the *suppressor of stellate [su(ste)]* locus lead to Stellate protein overexpression during spermatogenesis, which leads to Stellate crystal formation and reduced fertility (Livak, 1990). It is now clear that *su(ste)* encodes piRNAs that are homologous to *ste* and silence this locus in trans (Aravin et al., 2001). Small RNA cloning and sequencing studies subsequently showed that related 22–30-nucleotide-long RNAs, derived largely from retrotransposons and other repetitive sequence elements, are abundant in the male and female germline (Aravin et al., 2003). These novel small RNAs were therefore

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Abbreviations used in this paper: Ago3, Argonaute3; Aub, Aubergine; HP1, heterochromatin protein 1; miRNA, microRNA; MTOC, microtubule-organizing center; piRNA, Piwi-interacting RNA; *rhi*, *rhino*.

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initially named repeat-associated siRNAs (rasiRNAs; Aravin et al., 2003). In some other systems, however, the majority of small RNAs in this class are not enriched in transposon sequences. In addition, these RNAs bind a germline-enriched PIWI clade of Argonaute proteins that are distinct from the Argonautes that bind miRNAs and siRNAs (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a; Lau et al., 2006). As a result, this new small RNA family was subsequently renamed Piwi-interacting RNAs (piRNAs; Brennecke et al., 2007; Yin and Lin, 2007).

Many of the piRNAs expressed in *Drosophila* ovaries are derived from transposons and other repeats, and thus cannot be assigned to specific chromosomal loci (Brennecke et al., 2007; Gunawardane et al., 2007; Yin and Lin, 2007). piRNAs that map to unique sites, however, are clustered in large pericentromeric or subtelomeric domains of up to 240 kb that are rich in transposon fragments (Brennecke et al., 2007). Most of these clusters produce piRNAs from both genomic strands, but a subset of clusters produce unique piRNAs almost exclusively from one strand (Aravin et al., 2006; Girard et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007; Houwing et al., 2007). The *Drosophila flamenco* locus falls into this second class, and genetic and molecular studies on *flamenco* have provided important insights into piRNA function (Brennecke et al., 2007; Malone et al., 2009). Single P-element insertion mutations in the telomere-proximal side of *flamenco* disrupt piRNA production and down-regulate expression of longer transcripts from across the entire 60-kb locus, suggesting that transposition has disrupted a transcriptional promoter for this cluster (Brennecke et al., 2007). *flamenco* contains fragments of active transposons that are located throughout the genome; therefore, mutations in this locus lead to overexpression of these dispersed elements (Brennecke et al., 2007; Mével-Ninio et al., 2007). These observations strongly suggest that piRNAs derived from *flamenco* silence transposon expression in trans.

The *flamenco* locus appears to function primarily in ovarian somatic cells, while the major piRNA-producing dual-strand cluster at cytological position 42AB appears to be germline specific. Mutations in 42AB and other dual-strand clusters have not been reported, but mutations in the *rhino* (*rhi*) locus lead to both dramatic reductions in piRNAs from these clusters and to 10–150-fold overexpression of ∼20% of transposon families (Klattenhoff et al., 2009). piRNAs derived from dual-strand clusters thus appear to act in the germline to silence target transposons in trans.

piRNA clusters represent ∼1% of the genome, and it is unclear how these limited chromatin domains are specified. Most clusters are located in heterochromatin and contain complex arrays of transposon fragments, but only a subset of transposon-rich heterochromatic regions produce piRNAs. These observations suggest that piRNA clusters are epigenetically defined. However, single P-element insertions disrupt *flamenco* locus function, suggesting that, at a minimum, cluster promoters are hard-wired. The *rhi* locus is required for accumulation of putative piRNA precursor RNAs from the 42AB cluster, and the heterochromatin protein 1 (HP1) homologue encoded by this locus binds to this cluster (Vermaak et al., 2005; Klattenhoff et al., 2009). HP1a, the founding member of the HP1 family, binds to methylated lysine 9 on histone H3 (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). HP1 then recruits histone methyltransferase, which methylates neighboring H3 to extend an epigenetic structure that is generally associated with transcriptional silencing (Nakayama et al., 2001). Rhi binding may therefore promote histone modifications that differentiate piRNA clusters from surrounding chromatin.

Transposons and other repetitive elements are among the most divergent components in the genome. This calls for a selection for advantageous changes in host genes involved in transposon targeting. Thus, the host and parasite are in a constant genetic conflict inside the cell and coevolve with each other. Intriguingly, *rhi* is rapidly evolving and appears to be under strong positive selection, which is a hallmark of genes involved in host–pathogen interactions. This led Vermaak et al. (2005) to speculate that *rhi* evolution is driven by a germline-specific genomic conflict. The role for Rhino in piRNA biogenesis strongly suggests that the conflict between transposons and the host genome drives *rhi* evolution (Klattenhoff et al., 2009). Brennecke et al. (2007) speculated that piRNA clusters actively attract transposons, which would presumably lead to production of homologous piRNAs capable of trans-silencing active elements throughout the genome. Within this appealing model, Rhino protein could interact directly with transposon-encoded integration proteins, and thus drive adaptive silencing by promoting transposition into clusters.

piRNA biogenesis

Deep sequencing and genetic studies suggest that two spatially and mechanistically distinct processes drive piRNA biogenesis (for review see Siomi et al., 2010). As noted above, the majority of unique piRNAs are derived from transposon-rich heterochromatic clusters (Brennecke et al., 2007; Yin and Lin, 2007). The most abundant piRNAs are antisense to mRNAs from active transposons, and these antisense RNAs preferentially associate with Piwi and Aubergine (Aub), two PIWI clade Argonautes (Brennecke et al., 2007; Gunawardane et al., 2007; Yin and Lin, 2007). Sense-strand piRNAs, in contrast, preferentially associate with Argonaute 3 (Ago3; Brennecke et al., 2007; Gunawardane et al., 2007). In vitro, all three *Drosophila* PIWI proteins, when programmed with piRNAs, cleave target RNAs between positions 10 and 11 of the guide strand (Saito et al., 2006; Gunawardane et al., 2007; Nishida et al., 2007). Significantly, *Drosophila* piRNAs from opposite strands tend to have a 10-nt 5′-end overlap, and antisense piRNAs bound to Piwi and Aub show a strong bias toward a Uracil (U) at the 5′ end, whereas sense-strand piRNAs bound to Ago3 tend to have an Adenine (A) at position 10 (Brennecke et al., 2007; Gunawardane et al., 2007). These findings suggest that antisense piRNAs derived from piRNA clusters bind to Aub and Piwi and direct cleavage of sense-strand transcripts from active transposons, generating RNA fragments with an A 10 nt from the 5′ terminus (Fig. 1 A). These sense-strand cleavage products are proposed to associate with Ago3, after 3′ trimming by an undefined mechanism producing mature sense-strand piRNAs. The resulting piRNA–Ago3 complexes then cleave antisense piRNA precursors from clusters to produce

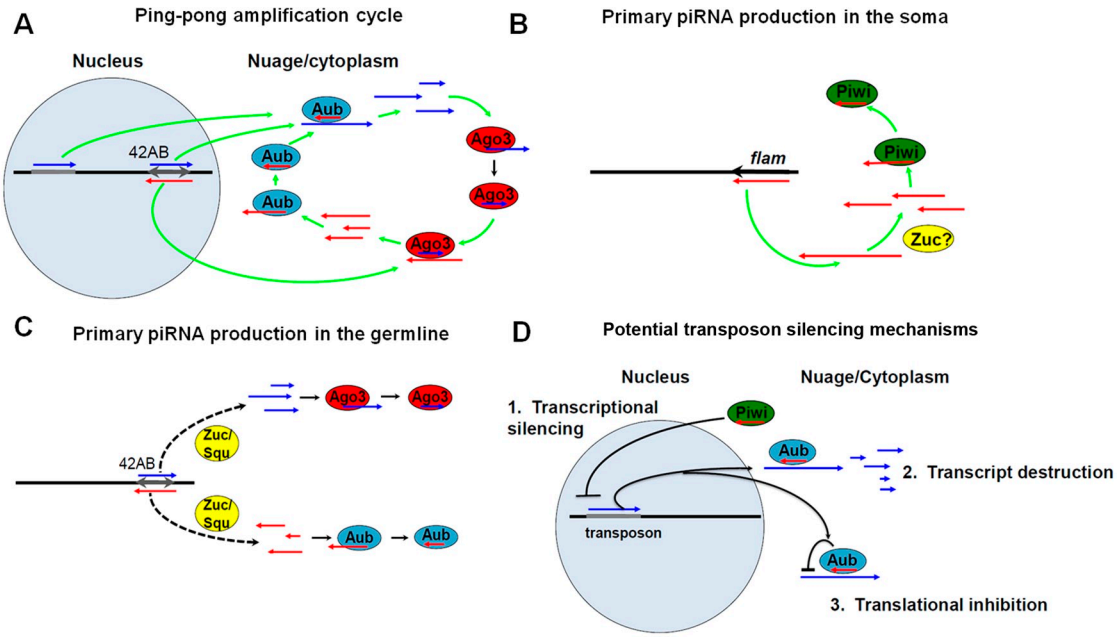

Figure 1. **piRNA biogenesis and transposon silencing in the germline and soma.** The mechanisms that drive piRNA biogenesis and transposon silencing are not well understood. Here we summarize speculative models based on the available data. (A) Ping-pong amplification in the germline. Transcripts from functional transposons (blue) and piRNA clusters (blue and red) are exported from the nucleus. Aub, preprogrammed with piRNAs generated through the primary biogenesis pathway, cleaves complementary transposon and cluster transcripts (blue), yielding randomly sized RNA fragments that bind Ago3. 3′-end trimming produces mature Ago3-sense strand piRNA complexes, which cleave anti-sense cluster transcripts (red). The resulting fragments bind to Aub and 3′-end processing generates anti-sense piRNAs, completing the amplification cycle. (B) Primary piRNA biogenesis in the soma. Anti-sense precursor transcripts (red) from *flam* and other uni-strand clusters are cleaved by Zuc to produce intermediate species that bind to Piwi. 3′ processing generates mature anti-sense piRNAs. (C) Primary piRNA biogenesis in the germline. Long-sense (blue) and anti-sense (red) precursor transcripts from piRNA clusters are cleaved by sequence-independent nucleases, which could include Zucchini (Zuc) and/or Squash (Squ), producing intermediates that bind Ago3 and Aub. Processing and modification of the 3′ ends generates mature piRNA complexes that drive that ping-pong amplification loop. (D) Potential modes of piRNA-mediated transposon silencing. (1) Transcriptional silencing of target transposons. piRNAs bound to Piwi, which accumulates in the nucleus, direct heterochromatin assembly at target elements. (2) Post-transcriptional target destruction. Transposon transcripts are recognized by Aub–piRNA complexes in the nuage, which catalyze homology-dependent cleavage. (3) Aub–piRNA complexes bind transposon transcripts and repress translation.

RNA fragments that associate with Aub and Piwi (Fig. 1 A). Trimming generates mature antisense piRNAs, completing the cycle. In this model, reciprocal cycles of PIWI-mediated cleavage thus amplify the pool of sense and antisense piRNAs. This "ping-pong" amplification cycle thus obviates the need for an RNA-dependent RNA polymerase (RdRp), which is needed to amplify siRNA triggers in plants, nematodes, and yeast (Verdel et al., 2009). The ping-pong model was developed from observations in *Drosophila,* but a similar mechanism appears to function in other animal groups (Aravin et al., 2007; Houwing et al., 2007; Grimson et al., 2008; Palakodeti et al., 2008; Lau et al., 2009a).

The ping-pong model requires preexisting "primary" piRNAs, presumably derived from clusters, to initiate the amplification cycle. How these primary piRNAs are produced remains to be determined, but piRNA production from the *flamenco* cluster has been proposed as a model for this process. piRNAs from this locus appear to be expressed primarily in the somatic follicle cells, which express only one PIWI Argonaute, Piwi. In addition, this locus produces unique piRNAs from only one genomic strand and complementary piRNAs drive biogenesis in the ping-pong model (Brennecke et al., 2007). Somatic piRNA production by *flamenco* may provide a model for primary piRNA biogenesis. However, somatic follicle cells surround the germline cells in the ovary, and the mixture of germline and somatic tissue complicates interpretation of studies on intact tissue. Recently, homogenous cell lines derived from the ovarian somatic sheets (OSSs) and ovarian somatic cells (OSCs) have been used to circumvent this limitation (Niki et al., 2006; Lau et al., 2009b; Robine et al., 2009; Saito et al., 2009). These cells express Piwi but do not express Ago3 or Aub, and produce piRNAs from one strand of the *flamenco* cluster (Lau et al., 2009b; Saito et al., 2009). Piwi thus appears to drive ping-pong–independent piRNA production in somatic cells. The putative nuclease encoded by the *zucchini* locus is also required for piRNA production in the soma (Malone et al., 2009; Robine et al., 2009; Saito et al., 2009). Transcripts encoded by *flamenco* could be cleaved by Zucchini, producing RNA fragments that bind to Piwi (Fig. 1 B). Each of the PIWI-clade proteins binds piRNAs with a unique length distribution, suggesting that processing takes place after binding (Brennecke et al., 2007). Precursor RNA fragments bound by Piwi could be trimmed to produce mature primary piRNAs (Fig. 1 B).

However, the available data on primary piRNA production are very limited and the proposed model is therefore highly speculative. In addition, several observations suggest that primary piRNA production in the germline may be independent of Piwi. For example, mutations that disrupt piRNA production in

the germline lead to severe defects in axis specification and oocyte nuclear organization (Chen et al., 2007; Klattenhoff et al., 2007, 2009; Pane et al., 2007), but germline depletion of Piwi does not disrupt egg chamber development or axial patterning (Cox et al., 2000). In addition, *piwi* mutations reduce, but do not eliminate piRNAs mapping to the major germline-specific 42AB cluster (Malone et al., 2009). Because a loss of primary piRNAs should lead to a collapse of the entire piRNA biogenesis cycle, these findings suggest that primary piRNA production in the germline does not require Piwi. The mechanism of primary piRNA production in the germline thus remains to be explored, and could be distinct from piRNA production in ovarian somatic tissue.

The majority of germline piRNAs appear to be produced by the ping-pong amplification cycle, and a simple modification of this cycle could explain primary piRNA biogenesis during germline development (Fig. 1 C). During ping-pong amplification, primary piRNAs are generated by Ago3 or Piwi-mediated cleavage of piRNA precursor transcripts derived from clusters, which produces longer fragments that bind to Aub and are subsequently trimmed to final length (Fig. 1 C). During primary piRNA biogenesis, piRNA cluster transcripts could be cleaved by sequence-independent endonuclease producing long RNA fragments that enter the biogenesis cycle by binding to Aub or Ago3. Subsequent processing by the same mechanisms employed using the ping-pong cycle could then generate the mature primary piRNAs that initiate the amplification loop (Fig. 1 A).

Mutations that eliminate primary piRNAs are predicted to lead to a collapse of the ping-pong cycle. However, mutations that only reduce primary piRNA production should allow reduced piRNA production by the ping-pong cycle. Intriguingly, mutations in *squash* and *zucchini,* which encode putative nucleases that localize to the perinuclear nuage, reduce piRNA levels without blocking ping-pong bias (Malone et al., 2009). As noted above, Zucchini has been implicated in ping-pong–independent piRNA biogenesis in somatic cells (Robine et al., 2009; Saito et al., 2009). Zucchini and/or Squash could therefore cleave cluster transcripts to produce RNAs that bind to PIWI-clade proteins and generate the primary piRNAs that initiate the germline amplification loop (Fig. 1, B and C).

Modification of piRNAs and Piwi proteins

Like siRNAs, the 3′ ends of most mature piRNAs are 2′-*O*-methylated, whereas the 5′ end carries a phosphate group (Girard et al., 2006; Grivna et al., 2006a; Vagin et al., 2006; Horwich et al., 2007; Houwing et al., 2007; Saito et al., 2007). The 2′-*O*-methylation is performed by DmPimet (piRNA methyltransferase)/DmHEN1, the *Drosophila* homologue of Arabidopsis HEN1 (Horwich et al., 2007; Saito et al., 2007). *Dmhen1* mutants eliminate 2′-*O*-methylation and reduce average piRNA size and abundance, suggesting that this modification protects mature piRNA from degradation (Horwich et al., 2007; Saito et al., 2007). These mutations also lead to a modest loss of transposon silencing, although mutants are viable and fertile (Horwich et al., 2007; Saito et al., 2007). These findings suggest that 3′-end modification is not essential to piRNA function, but existing *Dmhen1* alleles may not be null.

The Piwi proteins Aub and Ago3 have recently been shown to be modified by the methyltransferase PRMT5, which generates symmetrical dimethyl arginines (sDMAs), which creates a binding site for Tudor domains (Kirino et al., 2009). There are 23 Tudor domain proteins in *Drosophila*, including the founding member of the family, Tudor (Tud), which is required for assembly of germ plasm and Aub localization in the germline (Boswell and Mahowald, 1985; Nishida et al., 2009). In addition, the Tudor domain proteins Krimper, Spindle-E, and Tejas have been implicated in PIWI localization, piRNA production, and transposon silencing (Vagin et al., 2004; Lim and Kai, 2007; Malone et al., 2009; Patil and Kai, 2010). These findings suggest that Piwi family protein dimethylation leads to assembly of higher order complexes that promote piRNA biogenesis and transposon silencing.

Transposon silencing

The majority of *Drosophila* piRNAs map to transposons and other repetitive elements, and piRNA mutations lead to massive transposon overexpression. piRNA–PIWI complexes are therefore assumed to directly control transposon activity. piRNAs bound to PIWI proteins direct homology-dependent target cleavage in vitro, suggesting that transposons are silenced through post-transcriptional transcript destruction (Saito et al., 2006; Gunawardane et al., 2007; Nishida et al., 2007). Intriguingly, a number of the piRNA pathway components, including Aub and Ago3, localize to Nuage, an evolutionarily conserved perinuclear structure associated with germline RNA processing (Eddy, 1974; Ikenishi, 1998; Saito et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007; Nishida et al., 2007). In addition, protein-coding genes with transposon insertions within introns escape silencing by the piRNA pathway. These observations suggest that piRNAs bound to Aub and Ago3 direct homology-dependent cleavage of mature transposon transcripts after export from the nucleus (Fig. 1 D). In this model, protein-coding genes containing intronic transposon insertions are not silenced because piRNA homology is removed by splicing.

However, several lines of evidence raise the possibility that piRNAs act at several levels. Piwi, the founding member of the PIWI clade, localizes to the nucleus, binds HP1a, and has been implicated in heterochromatin assembly in the soma (Pal-Bhadra et al., 2004; Brower-Toland et al., 2007). In addition, mutations in *spn-E,* which encodes a putative helicase required for piRNA production, reduce HP1a binding to the telomere-specific transposon *TART* (Klenov et al., 2007). These findings suggest that piRNA bound to Piwi guide heterochromatin assembly, and thus impose transcriptional silencing. Consistent with this speculation, piRNA mutations reduce DNA methylation in mouse testes. However, piRNAs have also been found in polysome fractions (Grivna et al., 2006b) and the mouse Piwi protein Mili associates with translation initiation factors and may positively regulate translation (Unhavaithaya et al., 2009). These findings raise the possibility that piRNAs also control translation (Fig. 1 D).

piRNA control of gene expression

In many organisms, including poriferans, cnidarians, *Caenorhabditis elegans*, and mouse, the majority of piRNAs map to

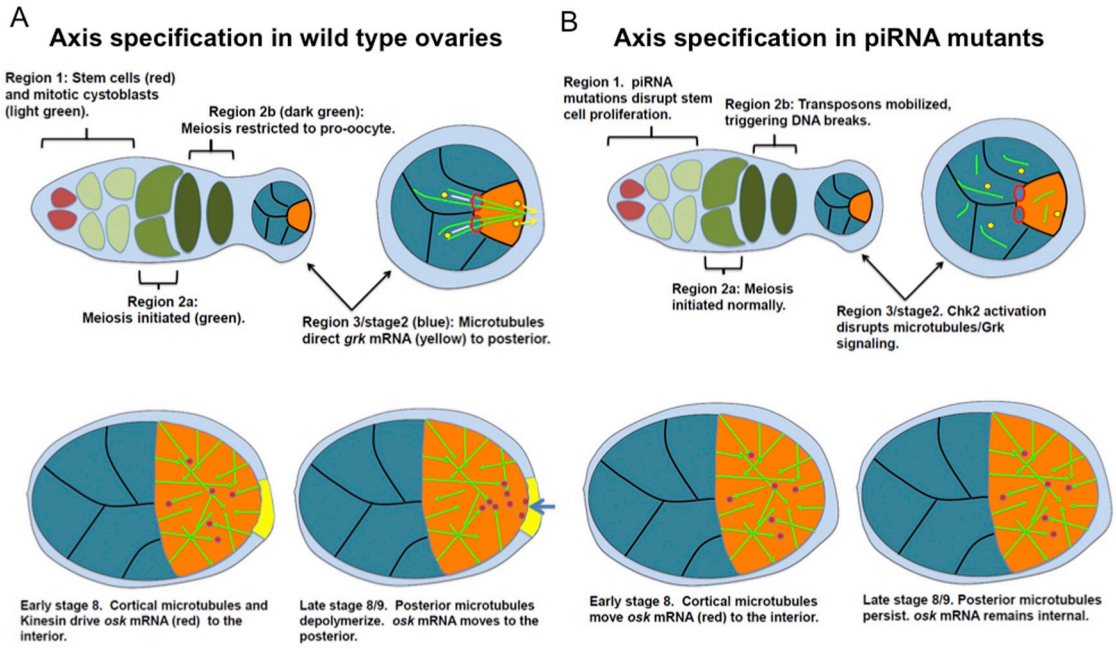

Figure 2. **Microtubule polarity and axis specification in wild-type and piRNA mutant oocytes.** A pair of germline stem cells (red) in region 1 of the germarium divide to produce cystoblasts (light green), which undergo four divisions with incomplete cytokinesis to generate inter-connected 16-cell cysts. Meiotic recombination initiates in region 2a (green) and DSBs are formed. Meiosis is restricted to a single pro-oocyte in the center of the cyst in region 2b (dark green). DSBs are repaired by region3/stage 2 (blue) of oogenesis. A microtubule-organizing center (MTOC) forms in the oocyte where microtubules direct *osk* mRNA (yellow) to the posterior pole. In piRNA mutants, meiosis is initiated normally in region 2a (B). However, transposons are overexpressed and DSBs accumulate in region 2b. DSBs persist in region 3, activating Chk2 signaling, which blocks MTOC formation and *grk* mRNA localization. Bottom panel shows early and late stage 8 oocytes in wt (A) and piRNA mutants (B). The oocyte cortex nucleates microtubules (green, arrowheads indicate plus end). Kinesin moves osk mRNA (red) to the interior. In the wild type, posterior follicle cells (yellow) signal to the oocyte (blue arrow), triggering depolymerization of cortical microtubules. Osk mRNA moves to the posterior by kinesin-dependent random walk. In piRNA mutants (B, bottom panel), *osk* mRNA moves to the interior, but posterior follicle cell signaling fails, posterior microtubules persist, and *osk* mRNA is trapped in the interior.

the unannotated regions of the genome and only a limited set match transposons and other repeats (Aravin et al., 2006; Girard et al., 2006; Ruby et al., 2006; Batista et al., 2008; Grimson et al., 2008). *Drosophila* also express piRNAs derived from the 3′-UTRs of a subset of mRNAs (Aravin et al., 2006; Robine et al., 2009; Saito et al., 2009). These observations suggest that piRNAs may control gene expression. Several recent studies support this hypothesis. The most abundant genic piRNAs in *Drosophila* somatic cells are linked to the 3′-UTR of a transcription factor, *traffic jam* (*tj*) (Robine et al., 2009; Saito et al., 2009). In cultured somatic cells, *tj* piRNAs coimmunoprecipitate with Piwi protein, and in ovaries their levels are reduced in *zucchini* mutants, but not in ovaries mutant for several other genes implicated in secondary piRNA amplification (Saito et al., 2009). Mutations in *tj* appear to reduce Piwi protein levels in somatic follicle cells, suggesting that this locus controls Piwi expression and is the source of piRNAs that bind to it. Mutations in *tj* and *piwi* produce similar defects in oogenesis and lead to two- to fourfold overexpression of *FasIII*, a cell adhesion molecule necessary for oogenesis. These changes are modest compared with the 100–200-fold increases in transposon expression observed in several piRNA pathway mutants. Nonetheless, these findings suggest that piRNAs from the *tj* locus down-regulate *fasIII* in the somatic follicle cells (Saito et al., 2009). In fly testes, the *vasa* and *stellate* (*ste*) genes also appear to be targeted by the piRNA pathway (Aravin et al., 2001; Vagin et al., 2006; Nishida et al., 2007). The *vasa* gene encodes a germline-specific DEAD box protein required for piRNA production (Schüpbach and Wieschaus, 1991; Malone et al., 2009), piRNAs derived from the AT-chX-1 and AT-chX-2 loci are homologous to the *vasa* gene, and mutations in *aub* and *ago3* that disrupt production of these piRNAs lead to Vasa overexpression (Nishida et al., 2007; Li et al., 2009). During early embryogenesis, maternally deposited mRNAs are destroyed as transcription is activated, leading to a switch from maternal to zygotic control of development. Recent studies suggest that the piRNA pathway may have a role in this developmental switch (Rouget et al., 2010). However, genome-wide tiling array analyses show that mutations in the piRNA pathway genes *aub*, *ago3*, *rhi*, and *armi* do not significantly alter expression of protein-coding genes during oogenesis (Klattenhoff et al., 2009; Li et al., 2009). piRNA control of gene expression may therefore be restricted to specific tissues or developmental stages.

piRNA function and *Drosophila* germline development

In every system studied to date, mutations in piRNA pathway genes disrupt germline development, often producing complex and poorly understood phenotypes that are difficult to directly associate with transposon targets of the pathway. Analyses of the ovarian phenotypes in *Drosophila* piRNA mutants, however, have helped link transposon mobilization to germline development and may provide a paradigm for phenotypic analysis of piRNA mutants in other systems.

Drosophila oogenesis is initiated by the division of a germline stem cell within a somatic cell niche at the tip of the germarium (Fig. 2; Spradling, 1993). Signaling between the niche and the stem cell controls stem cell division and is likely to orient division plane (Deng and Lin, 1997; Lin and Spradling, 1997). The latter process is critical to asymmetric cleavage, which regenerates the stem cell and produces the cystoblast precursor of the oocyte and nurse cells (Deng and Lin, 1997). Mutations in *piwi*, which encodes a founding member of the PIWI clade of Argonaute proteins, lead to a near complete loss of germline stem cells (Cox et al., 1998). Genetic mosaic studies indicate that Piwi protein is required in both the somatic cells of the niche and in the germline (Cox et al., 1998, 2000). Eliminating *piwi* from the soma disrupts stem cell maintenance, but does not alter the viability of the eggs that are produced (Cox et al., 2000). In contrast, germline clones of *piwi* mutations slow stem cell division and the eggs that are produced do not hatch (Cox et al., 2000). Unlike mutations in many other piRNA pathway genes, however, *piwi* germline clones do not disrupt oocyte patterning, which appears to be a downstream consequence of transposon overexpression (see below). The function for Piwi and piRNAs in stem cell maintenance and divisions are not well understood, and may be distinct from latter functions in transposon control.

In the majority of piRNA pathway mutations, the earliest phenotype is an increase in DNA damage in germline cells of the germarium (Klattenhoff et al., 2007, 2009). After stem cell division, the cystoblast proceeds through four incomplete divisions to produce a cyst of 16 interconnected cells that will differentiate into a single oocyte and the nurse cells (Spradling, 1993). Region 2a of the germarium contains early 16 cell cysts, and all 16 cells begin to accumulate double-strand breaks and initiate synaptonemal complex (SC) assembly (Carpenter, 1975, 1979). The SC is progressively restricted to a single oocyte, located at the posterior pole, as cysts progress to region 3, where they are surrounded by a monolayer of somatic follicle cells and bud from the germarium to form stage 2 egg chambers (Spradling et al., 1997). During the progression, meiotic DNA breaks are first restricted to the pro-oocyte and then repaired in the oocyte (Jang et al., 2003). Reorganization of the microtubule cytoskeleton is coordinated with these nuclear changes. In early region 2a cysts, the microtubule network shows no clear polarity. However, a single microtubule-organizing center (MTOC), focused on the pro-oocyte, begins to dominate as cysts progress through region 2b and into region 3. This polarized microtubule scaffold is required for asymmetric localization of a TGF-β homologue encoded by the *grk* gene, which signals to posterior follicle cells that are in contact with the pro-oocyte. This initiates a reciprocal germline-to-soma signaling cascade that patterns the oocyte and the surrounding egg shell (Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993). In piRNA mutants, double-strand breaks form normally in region 2a cysts, but the breaks persist and appear to increase as egg chambers mature (Klattenhoff et al., 2007). In addition, the microtubule network is not polarized, which disrupts Grk signaling and initiation of oocyte patterning (Chen et al., 2007; Klattenhoff et al., 2007, 2009; Pane et al., 2007).

The first clear oocyte patterning defects associated with piRNA mutations are observed in late stage 8 and early stage 9 (Chen et al., 2007; Klattenhoff et al., 2007; Pane et al., 2007). By early stage 8, most of the oocyte cortex appears to nucleate microtubules, and the microtubule network shows no clear polarity. At this stage, *osk* mRNA, which specifies the posterior pole, is localized to the anterior and lateral cortex (Kim-Ha et al., 1991). By stage 9, however, *osk* mRNA is tightly localized to the posterior cortex. Both fluorescence in situ hybridization and time-lapse studies using molecular beacons show that *osk* mRNA transiently accumulates in the center of the oocyte before moving to the posterior pole (Cha et al., 2002; Bratu et al., 2003). The second step in *osk* mRNA localization temporally correlates with loss of cortical microtubules specifically at the posterior pole, and mutations in *grk*, *pka*, and *par1* trap *osk* mRNA in the interior of the oocyte and block depolymerization of microtubules at the posterior cortex (Lane and Kalderon, 1993; Roth et al., 1995; Cox et al., 2001; Benton et al., 2002). In addition, *osk* mRNA remains uniformly at the cortex in oocytes mutant for *khc,* which encodes the plus end–directed microtubule motor kinesin-I (Brendza et al., 2000; Cha et al., 2002). These findings support a two-step model in which microtubules nucleated at the cortex and randomly projecting into the oocyte support kinesin-dependent movement of *osk* mRNA toward the interior. Depolymerization of posterior microtubules, induced by a signal from the posterior follicle cells and mediated by par-1 and cAMP-dependent protein kinase in the oocyte, eliminates the cortical exclusion force specifically at the posterior pole (Fig. 2). The remaining oocyte microtubules then support a biased random walk toward the posterior (Serbus et al., 2005; Zimyanin et al., 2008). Assembly of a single MTOC in the oocyte during early oogenesis thus leads to polarized Grk signaling to follicle cells (Fig. 2, bottom), which differentiate and signal back to the oocyte during mid-oogenesis, inducing a second microtubule reorganization that allows *osk* mRNA movement to the posterior cortex (Fig. 2 A). At the same time, *grk* mRNA localizes to the anterior–dorsal cortex of the oocyte, leading to Grk/TGF-β signaling to the dorsal follicle cells. It is unclear how *grk* mRNA moves to the dorsal cortex, but this process requires microtubules and the minus-end motor, dynein. Mutations that disrupt *osk* mRNA localization generally disrupt *grk* mRNA localization, suggesting that both processes may be initiated by Grk signaling from the oocyte to the follicle cells during early oogenesis.

In piRNA pathway mutants, *osk* mRNA fails to localize to the posterior pole and *grk* mRNA fails to localize to the dorsal cortex during late stage 9 and early stage 10, and this correlates with persistence of cortical microtubules at the posterior pole (Fig. 2 B; Cook et al., 2004; Chen et al., 2007; Klattenhoff et al., 2007; Pane et al., 2007). These patterning defects during mid-oogenesis lead to production of elongated eggs with reduced or missing dorsal appendages, which are egg shell structures induced by Grk signaling. These findings suggest that piRNA mutations disrupt assembly of the MTOC early in oogenesis, disrupting an early step in oocyte patterning that ultimately leads to production of spindle-shaped eggs.

Insight into the link between piRNA function in transposon silencing and these polarity defects came from studies by

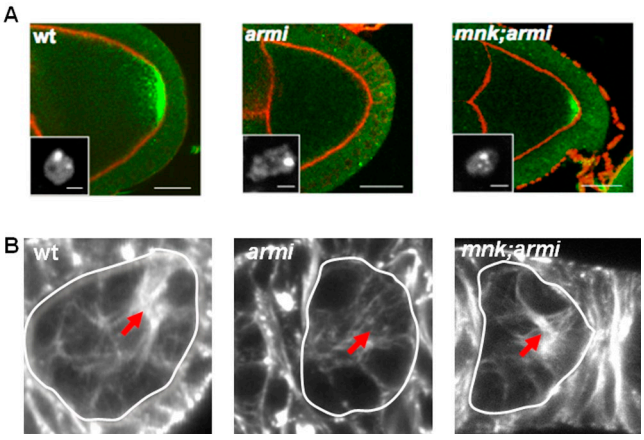

Figure 3. **piRNA mutations trigger Chk2-dependent defects in microtubule polarity.** (A) Osk protein (green) localizes to the posterior of wild-type stage 9 oocytes (wt), but is dispersed in *armi* mutants (*armi*). Posterior localization of Osk protein is restored in oocytes mutant for both *armi* and *mnk,* which encodes the DNA damage signaling kinase Chk2 (*mnk;armi*). F-actin is shown in red. Adapted from Klattenhoff et al. (2007) with permission from Elsevier. The insets show chromatin organization in the oocyte. (B) During early oogenesis in wild-type females (wt), a prominent microtubule-organizing center (MTOC) forms in the pro-oocyte. The resulting microtubule scaffold mediates asymmetric *grk* mRNA localization and Grk signaling to the follicle cells, initiating axis specification. The MTOC fails to form in *armi* mutants. In contrast, a prominent MTOC forms in female mutants for both *armi* and *mnk*.

Ghabrial et al. (1998), who showed that a subset of spindle class genes encodes meiotic DNA break repair enzymes, and that these mutations lead to persistent DNA breaks during early oogenesis. They speculated that these breaks activate damage signaling, which in turn disrupts oocyte patterning. Supporting this hypothesis, they showed that mutations in *mei-41* and *mnk,* which encode ATR and Chk2 kinases that function in DNA damage signaling, suppress the axis specification defects associated with meiotic DNA repair mutations (Ghabrial and Schüpbach, 1999; Abdu et al., 2002). Transposon mobilization, and particularly the excision of DNA elements, can lead to DNA breaks (Belgnaoui et al., 2006; Gasior et al., 2006), and piRNA mutations lead to persistent DNA damage during early oogenesis. Significantly, mutations in *mnk* and *mei-41* dramatically suppress the patterning defects associated with these mutations (Fig. 3 A; Chen et al., 2007; Klattenhoff et al., 2007, 2009; Pane et al., 2007). These observations support a model in which loss of silencing leads to transposon mobilization and DNA break accumulation, which in turn triggers Chk2-dependent defects in axis specification (Klattenhoff and Theurkauf, 2008).

As noted above, posterior patterning of the oocyte appears to require assembly of a single MTOC in the pro-oocyte during oogenesis. This leads to oocyte-specific localization of *grk* mRNA and Grk/TGF-β signaling to the posterior follicle cells. Mutations in the piRNA genes *armi* and *aub* disrupt this MTOC, and the subsequent depolymerization of microtubules at the posterior cortex of stage 9 oocytes (Cook et al., 2004). Significantly, the mutations in *mnk* and *mei-41* that suppress defects in patterning also restore MTOC formation during early oogenesis (Fig. 3 B; Klattenhoff et al., 2007). In early *Drosophila* embryos, Chk2 activation triggers γ-tubulin ring complex dissociation from centrosomes, disrupting mitotic MTOC formation (Takada et al., 2003). Taken together, these finding suggest that piRNA pathway mutations lead to transposon overexpression and mobilization, which triggers Chk2-dependent defects in MTOC formation early in oogenesis, thus preventing an early step in the oocyte patterning cascade (Fig. 3, A and B).

Although this model is appealing, DNA damage in the piRNA pathway mutations has not been directly linked to transposon mobilization, and the mechanism of Chk2-dependent disruption of the oocyte MTOC remains to be determined. In addition, mutations in *mnk* and *mei-41* do not suppress the maternal-effect embryonic lethality associated with piRNA pathway mutation, and the essential embryonic functions for this pathway remain to be explored. Nonetheless, the available data suggest that the axis specification defects produced by many *Drosophila* piRNA mutations are an indirect consequence of transposon overexpression and DNA damage signaling.

Conclusions

Mutations that disrupt the piRNA pathway in mouse and fish lead to germline-specific cell death and sterility, and are also associated with increased transposon expression (Aravin et al., 2007; Carmell et al., 2007; Houwing et al., 2007). Studies in *Drosophila* suggest that transposon mobilization represents the primary biological trigger for these phenotypes, and that mobile elements are the primary targets for the piRNA pathway. However, the vast majority of piRNAs in the mouse germline map to unique sequences in unannotated regions of the genome, a subset of *Drosophila* piRNAs is derived from protein-coding genes, and piRNAs appear to control at least one gene target in *Drosophila* ovarian somatic cells. The biological relevance of genic piRNAs remains to be fully explored. There is also intriguing data implicating the piRNA pathway in learning and memory and chromatin assembly in the soma (Pal-Bhadra et al., 2004; Ashraf et al., 2006; Brower-Toland et al., 2007), and we have recently found that a subset of piRNA pathway mutations disrupt telomere protection and lead to chromosome fusion segregation during meiosis and mitosis (Khurana et al., 2010). The biological function for this novel class of small RNAs may therefore extend well beyond transposons and germline development.

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