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 et al., 2008; Kawamura et al., 2008; Okamura et al., 2008; Hartig et al., 2009). 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., 2004; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008; Hartig et al., 2009).

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...
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 germ-line-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 stra...
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. 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.
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 splicimg.

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 spin-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...
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GERMLINE-SPECIFIC DEAD BOX PROTEIN REQUIRED FOR PI RNA PRODUCTION

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 gerarium (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 show 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 mutants, the earliest phenotype is an increase in DNA damage in germline cells of the gerarium (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 gerarium 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 gerarium 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-1 (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...
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 findings 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 (Khranra 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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