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Small interfering RNAs and microRNAs are generated from double-stranded RNA precursors by the Dicer endonucleases, and function with Argonaute-family proteins to target transcript destruction or to silence translation. A distinct class of 24- to 30-nucleotide-long RNAs, produced by a Dicer-independent mechanism, associates with Piwi-class Argonaute proteins. Studies in flies, fish and mice implicate these Piwi-associated RNAs (piRNAs) in germline development, silencing of selfish DNA elements, and in maintaining germline DNA integrity. However, whether piRNAs primarily control chromatin organization, gene transcription, RNA stability or RNA translation is not well understood, neither is piRNA biogenesis. Here, we review recent studies of piRNA production and function, and discuss unanswered questions about this intriguing new class of small RNAs.

Introduction

In 1993, Ambros and colleagues showed that the *C. elegans lin-4* gene encodes a small regulatory RNA with complementarity to the *lin-14* transcription unit, which it negatively regulates (Lee et al., 1993). These pioneering studies thus identified the first microRNA (miRNA). Small non-coding RNAs have subsequently emerged as powerful experimental tools and as crucial developmental regulators in animals and plants (Baulcombe, 2004; Hannon, 2002; Kloosterman and Plasterk, 2006; Mello and Conte, 2004). 21-nucleotide (nt) small interfering RNAs (siRNAs), now ubiquitously used to experimentally manipulate gene expression, are processed from long, double-stranded RNA (dsRNA) precursors by the Dicer endonucleases. The resulting 21-nt dsRNAs are incorporated into an intermediate RNA-protein complex. Displacement of one of the RNA strands (referred to as the 'passenger strand') then produces the mature RNA-induced silencing complex (RISC), which contains a single 'guide strand' bound to a member of the Argonaute protein family. When the guide-strand siRNA is perfectly complementary to a target RNA, the Argonaute protein catalyzes sequence-specific endonucleolytic cleavage (for reviews, see Hannon, 2002; Meister and Tuschl, 2004). In vivo, the siRNA pathway destabilizes RNA intermediates generated during the viral life cycle, and thus plays an important role in limiting virus infectivity (Wang et al., 2006). By contrast, miRNAs are derived from stem-loop transcripts encoded by chromosomal genes. Primary stem-loop RNAs (priRNA) are processed in the nucleus by the ribonuclease Drosha, producing pre-miRNAs that are exported from the nucleus and cleaved in the cytoplasm by a Dicer endonuclease to yield ~22-nt mature miRNAs. These miRNAs associate with Argonaute proteins and induce the homology-dependent downregulation of target gene activity. Imperfect miRNA base-pairing to target transcripts appears to induce translational silencing, whereas perfect base-pairing triggers RNA destruction. Mutations in the miRNA pathway disrupt development and often lead to embryonic lethality (reviewed by Du and Zamore, 2005; Kloosterman and Plasterk, 2006).

Recent studies have revealed a new class of 24- to 30-nt RNAs that are generated by a Dicer-independent mechanism and that interact with a subset of Argonaute proteins related to Piwi (Aravin et al., 2006; Brennecke et al., 2007; Girard et al., 2006; Grivna et al., 2006a; Gunawardane et al., 2007; Houwing et al., 2007; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2006), which is required for female and male fertility in *Drosophila* (Lin and Spradling, 1997). In some systems, these Piwi-interacting RNAs (piRNAs) are primarily derived from transposons and other repeated sequence elements (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2006), leading to their alternative designation as repeat-associated small interfering RNAs (rasiRNAs) (Aravin et al., 2003). It is now clear that piRNAs can be derived from either repeated or complex DNA sequence elements (Aravin et al., 2007; Brennecke et al., 2007; Houwing et al., 2007), and that rasiRNAs are a subset of piRNAs. We therefore use the more generic term piRNA in the following discussions. Genetic studies in mice, *Drosophila* and zebrafish indicate that piRNAs are crucial to germline development (Carmell et al., 2007; Chen et al., 2007; Cook et al., 2004; Cox et al., 1998; Cox et al., 2000; Deng and Lin, 2002; Gillespie and Berg, 1995; Houwing et al., 2007; Kuramochi-Miyagawa et al., 2004; Pane et al., 2007; Schupbach and Wieschaus, 1991). However, proteins involved in piRNA production have also been implicated in the control of gene expression in somatic cells (Grimaud et al., 2006; Pal-Bhadra et al., 2002; Pal-Bhadra et al., 2004) and in learning and memory (Ashraf et al., 2006), suggesting that piRNAs might have an impact on a broad range of biological processes.

piRNA production

The 24- to 30-nt length of piRNAs is an indication that they are not generated by a Dicer, which produce 21- to 22-nt products from double-stranded precursors (Bernstein et al., 2001). Recent genetic studies are consistent with the conclusion that piRNA production is a Dicer-independent process (Houwing et al., 2007; Vagin et al., 2006). Insight into the mechanism of piRNA production has come from studies of their genomic origin and of Argonaute binding in *Drosophila* (Aravin et al., 2006; Brennecke et al., 2007; Girard et al., 2006; Grivna et al., 2006a; Gunawardane et al., 2007; Houwing et al., 2007; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006). In *Drosophila* ovaries, the vast majority of piRNAs appear to be derived from a limited number of pericentromeric and telomeric sites that are enriched for retrotransposon sequences (Brennecke et al., 2007). The most abundant piRNAs derive from the antisense strand of retrotransposon sequences, and these RNAs preferentially associate with the Argonaute proteins Piwi and Aubergine (Aub). Sense-strand piRNAs, by contrast, preferentially associate with Argonaute 3 (Ago3) (Brennecke et al., 2007; Gunawardane et al., 2007). Piwi, Aub and Ago3, in complex with piRNAs, can cleave target RNAs between positions 10 and 11 of the guide strand (Gunawardane et al., 2007; Saito et al., 2006). Significantly,

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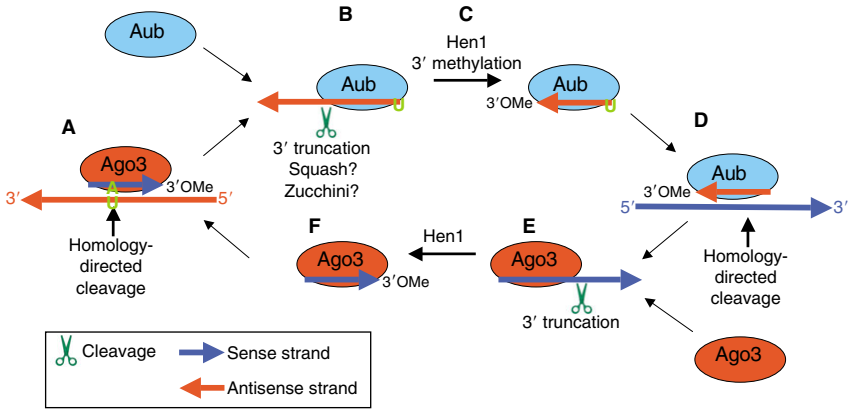

Fig. 1. Ping-pong model for piRNA production. (**A**) The Piwi-class Argonaute protein Argonaute 3 (Ago3) binds to sense-strand piRNAs (blue) and directs the cleavage of target antisense-strand transcripts (red), producing the 5′ end of antisense-strand piRNAs. (**B**) Aubergine (Aub) and Piwi (not shown) bind to the resulting piRNA precursor, which is trimmed to its final length. This might be catalyzed by the putative nucleases Squash and Zucchini. (**C**) *Drosophila* Hen1 methylates the 3′ ends of piRNAs (3′OMe). (**D**) Aub–antisense-strand piRNA complexes catalyze the cleavage of sense transcripts (blue), producing the 5′ end of sense piRNAs. (**E**) Ago3 binds the resulting sense piRNA precursors, which are trimmed and (**F**) methylated, as described for antisense-strand piRNAs. Based on the model proposed by Brennecke et al. (Brennecke et al., 2007).

Drosophila piRNAs from opposite strands tend to have a 10-nt overlap. Furthermore, antisense piRNAs bound to Piwi and Aub show a strong bias toward a U at the 5′ end, whereas sense-strand piRNAs bound to Ago3 tend to have an A residue at position 10 (Brennecke et al., 2007; Gunawardane et al., 2007). Based on these observations, two groups concurrently proposed a ‘ping-pong’ model of piRNA production, in which Ago3 bound to sense-strand piRNAs catalyzes antisense-strand cleavage at an A:U base-pair that generates the 5′ end of antisense piRNAs (Fig. 1) (Brennecke et al., 2007; Gunawardane et al., 2007). The 5′ ends of the resulting cleavage products are proposed to associate with Aub or Piwi, with nucleolytic processing of the 3′ overhangs generating mature 23- to 30-nt antisense piRNAs (Fig. 1B). The mature antisense piRNA-Argonaute complexes are then proposed to bind and cleave sense-strand RNAs, silencing gene expression and generating the 5′ end of sense-strand piRNA precursors that associate with Ago3 (Fig. 1D). Processing of the 3′ overhang produces mature sense-strand piRNAs, completing the cycle (Fig. 1E). This model is based on studies in *Drosophila*, but recent findings suggest that a similar mechanism might operate in mouse (Aravin et al., 2007).

The proteins that mediate a number of the proposed steps in the ‘ping-pong’ model of piRNA production have not been identified, but the results of recent studies might help fill some of these gaps. For example, mutations in the *Drosophila zucchini* and *squash* genes disrupt piRNA production and lead to a loss of retrotransposon silencing, and both of these genes encode putative nucleases (Pane et al., 2007). Zucchini and Squash may therefore process the 3′ end extensions to generate mature piRNAs (Fig. 1). In addition, the 3′ end of mature piRNAs are methylated (Kirino and Mourelatos, 2007; Ohara et al., 2007). In *Drosophila*, this reaction is carried out by the Hen1 RNA methyltransferase (also known as DmHen1 and Pimet), and methylation appears to take place after piRNAs bind to Argonautes (Horwich et al., 2007; Saito et al., 2007). A mutation in *Drosophila hen1* reduces the length and steady-state level of piRNAs, suggesting that methylation limits the extent of 3′ processing and increases the stability of piRNAs (Horwich et al., 2007). Methylation could also influence interactions between piRNAs and additional components of the piRNA pathway. However, *Drosophila hen1* mutants are viable and fertile, indicating that this modification is not essential to piRNA function (Saito et al., 2007).

Genetic screens in *Drosophila* have identified several additional factors that are required for piRNA production or function. For example, the *armitage* and *spindle E* genes encode putative helicases that are required for piRNA production and for retrotransposon silencing (Aravin et al., 2004; Vagin et al., 2006). These proteins could unwind duplex intermediates formed during piRNA production, target recognition, or cleavage. By contrast, the *cutoff* gene is required for retrotransposon silencing, but is not needed for piRNA production (Chen et al., 2007). Yeast homologs of Cutoff have been implicated in RNA decay (Kim et al., 2004; Xue et al., 2000). Cutoff might therefore facilitate gene silencing by enhancing the activity of piRNA-Argonaute complexes.

Compartmentalization of the piRNA pathway

Recently, the *Drosophila* Tudor-domain protein Krimper has been implicated in both retrotransposon repression and piRNA production (Lim and Kai, 2007). This protein is a component of nuage, a germline-specific perinuclear structure that has been implicated in RNA processing, and *krimper* mutations block nuage assembly. Intriguingly, many piRNA-pathway-related proteins accumulate in nuage, which is prominent in nurse cells. The *Drosophila* oocyte develops in a cyst with 15 nurse cells, which synthesize RNAs and proteins that are transported through ring canals to the oocyte (Spradling, 1993). Nuage was first identified in electron micrographs as an amorphous electron-dense cloud that surrounds the nurse cell nuclei (Allis et al., 1979; Mahowald, 1971). Nuage is enriched for the Piwi-class Argonautes Aub and Ago3 (Fig. 2A) (Brennecke et al., 2007; Harris and Macdonald, 2001), the helicases Armitage and Spindle E (Cook et al., 2004; Lim and Kai, 2007), the nucleases Zucchini and Squash (Pane et al., 2007), Maelstrom and Cutoff (Chen et al., 2007; Findley et al., 2003). In contrast to most piRNA-pathway proteins, *Drosophila* Piwi localizes almost exclusively to nurse cell nuclei (Fig. 2A) (Brennecke et al., 2007; Cox et al., 2000; Saito et al., 2006). These observations suggest that piRNA production and function might be compartmentalized (Lim and Kai, 2007).

piRNA-Argonaute complexes appear to be the catalytically active effectors of the pathway, and these localization studies thus suggest that Piwi mediates nuclear functions for the piRNA pathway, whereas Ago3 and Aub drive cytoplasmic functions (Fig. 2B). We speculate that piRNA biogenesis, which is proposed to require

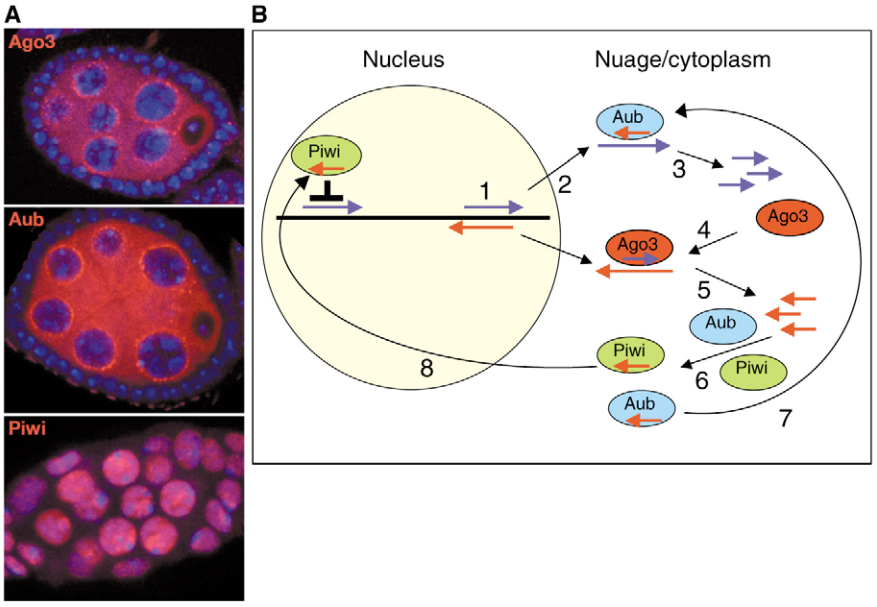

Fig. 2. Compartmentalization of piRNA production and function. (**A**) Localization of Piwi-class Argonautes in the *Drosophila* ovary. Argonautes (red), DNA (blue). Argonaute 3 (Ago3, top) and Aubergine (Aub, middle) localize to the cytoplasm and nuage, which is a perinuclear structure rich in RNA-processing enzymes. Piwi (bottom) localizes predominantly to germline nuclei. (**B**) Model for compartmentalized production and function of piRNAs. (1) Sense (blue) and antisense (red) strands of piRNA 'master control' regions are transcribed and (2) exported from the nucleus. (3) In the nuage, Aub-piRNA complexes cleave sense transcripts, leading to the production of sense-strand piRNAs that (4) associate with Ago3. (5) Ago3-piRNA complexes cleave antisense transcripts, producing piRNAs that (6) associate with Aub and Piwi. (7) Aub complexes remain in the nuage and cleave sense-strand complexes. (8) Piwi-piRNA complexes are imported into the nucleus, where they silence homologous genes in euchromatin and heterochromatin.

sense- and antisense-strand Argonaute complexes, takes place in the nuage. In this model, the sense-strand piRNA precursor transcripts are exported to the nuage, where they are cleaved by Aub-antisense piRNA complexes, silencing target gene expression and generating precursors of sense-strand piRNAs. These sense-strand precursors associate with Ago3 and are trimmed to mature length. Ago3-sense-strand complexes then catalyze the cleavage of the antisense transcripts, producing piRNA precursors that associate with Aub and Piwi. Mature Aub complexes then remain in the nuage and function in piRNA production and sense-strand transcript destruction, whereas mature Piwi complexes are imported into the nucleus and mediate heterochromatin assembly and transcriptional silencing, or co-transcriptional RNA destruction. This model is highly speculative, but makes a number of clear predictions and might therefore serve as a useful framework for further studies on piRNA biogenesis.

Genetic studies in *Drosophila*, mice and zebrafish have provided insight into the biological functions of piRNAs and highlight the significance of these RNAs in germline development. The phenotypes of piRNA-pathway mutations in each of these systems are outlined below.

Stem cell division and axis specification in *Drosophila* females

Mutations in piRNA-pathway genes were first identified in *Drosophila* through screens for mutations that disrupt oogenesis and embryonic axis specification (Gillespie and Berg, 1995; Schupbach and Wieschaus, 1991). *Drosophila* oogenesis begins with a germline stem cell division that produces a cystoblast, which divides four times with incomplete cytokinesis to produce 16 interconnected cells that form a single oocyte and 15 nurse cells (for a review, see Spradling, 1993). The nurse cells provide most of the RNA and protein components of the oocyte, which remains transcriptionally silent through most of oogenesis. Embryonic axis specification in *Drosophila* depends on the asymmetric localization of a small number of morphogenetic RNAs in the oocyte. These RNAs are transferred from the nurse cells to the oocyte, where localization is driven by interactions with a polarized microtubule cytoskeleton. Microtubule polarization is controlled by a cascade of germline-to-soma and soma-to-germline signaling events (reviewed by Grunert and St Johnston, 1996). Mutations in piRNA-pathway genes disrupt both stem cell maintenance and oocyte production, and the localization of morphogenetic RNAs in the oocyte during axis specification (Chen et al., 2007; Cook et al., 2004; Cox et al., 1998; Cox et al., 2000; Gillespie and Berg, 1995; Lin and Spradling, 1997; Pane et al., 2007; Schupbach and Wieschaus, 1991).

The *piwi* gene encodes the founding member of the Piwi class of Argonautes (Cox et al., 1998). Mutations in *piwi* lead to severe defects in oogenesis, including loss of germline stem cells (Fig. 3A) (Cox et al., 1998; Cox et al., 2000; Lin and Spradling, 1997). Clonal studies indicate that stem cell maintenance and division require *piwi* expression in the somatic cells that form the stem cell niche. Loss of *piwi* in the germline, by contrast, reduces stem cell division rates, but does not lead to a loss of stem cells or to a block in oogenesis (Cox et al., 2000). Mutations in other piRNA-pathway genes, including *zucchini* and *squash*, also lead to germline stem cell loss (Chen et al., 2007; Pane et al., 2007). It is unclear whether these genes are required in the germline, soma, or both. The molecular functions of Piwi and the piRNA pathway in germline stem cell division and maintenance have not been defined.

Mutations in most piRNA genes in *Drosophila*, including *aubergine*, *spindle E*, *armitage*, *maelstrom*, *krimper*, *zucchini* and *squash*, disrupt the localization of dorsal and posterior RNAs (Cook et al., 2004; Gillespie and Berg, 1995; Pane et al., 2007; Schupbach and Wieschaus, 1991). These mutations do not disrupt anterior localization of *bicoid* mRNA or block oocyte development. Therefore, these genes were initially assumed to control the expression of a specific subset of genes required for anterior-posterior and dorsal-ventral patterning (Cook et al., 2004). Subsequent studies, however, have demonstrated that the dramatic axis-specification defects that are associated with piRNA mutations are a secondary consequence of DNA damage signaling (Chen et al., 2007; Klattenhoff et al., 2007; Pane et al., 2007). These studies suggest that piRNAs have a primary function in maintaining germline DNA integrity.

The link between piRNAs and DNA damage signaling was suggested by studies of *Drosophila* meiotic DNA-repair genes. Meiotic recombination requires DNA break formation by the Spo11 nuclease (Cao et al., 1990), and Schupbach and colleagues have

shown that mutations that disrupt meiotic DNA break repair also disrupt anterior-posterior and dorsal-ventral axis specification (Abdu et al., 2002; Ghabrial et al., 1998). Significantly, the *Drosophila* embryonic patterning defects that are linked to repair mutations are dramatically suppressed by mutations in *mei-41* and *mnk* (also known as *loki* – FlyBase), which encode the ATR and Chk2 kinases, respectively, which function in DNA damage signaling, and by mutations in *mei-W68* (Abdu et al., 2002; Ghabrial et al., 1998), which encodes the fly Spo11 homolog (McKim and Hayashi-Hagihara, 1998). Unrepaired meiotic breaks thus appear to activate the ATR and Chk2 kinases, which in turn trigger the observed axis-specification defects. Recent studies show that the axis-specification defects in *armitage*, *aubergine*, *cutoff* and *squash* are also suppressed by *mei-41* and/or *mnk* (Chen et al., 2007; Klattenhoff et al., 2007; Pane et al., 2007). Furthermore, *armitage*, *aubergine* and *spindle E* mutations lead to a dramatic accumulation of phosphorylated histone H2Av (γ-H2Av) foci in germline nuclei (Fig. 3B); these foci are generally linked to DNA double-strand breaks (Modesti and Kanaar, 2001). Significantly, *mei-W68* (Spo11) does not suppress the patterning defects associated with *armitage*, or the formation of γ-H2Av foci (Klattenhoff et al., 2007). piRNA mutations, like DNA-repair mutations, thus disrupt axis specification through activation of the ATR/Chk2 pathway. However, unlike DNA-repair-pathway mutations, meiotic breaks are not the source of damage.

All of the *Drosophila* piRNA-pathway mutations lead to a significant overexpression of retrotransposons (Aravin et al., 2001; Chen et al., 2007; Kalmykova et al., 2005; Pane et al., 2007; Sarot et al., 2004; Vagin et al., 2006), and *piwi* mutants have been shown to mobilize at least one class of transposon in the male germline (Kalmykova et al., 2005). Retrotransposon mobilization can induce DNA damage (Belgnaoui et al., 2006; Gasior et al., 2006), and high rates of transposon insertion in piRNA mutants could overwhelm the DNA-repair machinery, leading to the breaks that activate ATR and Chk2. However, there is no direct evidence for transposon mobilization in the female germline when piRNA-pathway components are disrupted, and this pathway could have a more direct role in DNA repair, or in establishing chromatin structures that resist damage. *Drosophila* telomeres are composed of retrotransposon repeats, and piRNA mutations increase the number of these repeats (Savitsky et al., 2006). These findings suggest that piRNA mutations could lead to a loss of telomere protection, leading to the recognition of chromosome ends as DNA breaks. However, it is currently unclear whether the break sites in piRNA-pathway mutations are random, linked to transposon insertions, or restricted to specific chromatin domains, and the precise functions of these RNAs in maintaining germline genome integrity remain to be determined.

The Oskar protein is essential for pole plasm assembly and for embryonic patterning, and piRNA-pathway mutations disrupt *osk* mRNA and protein localization (Cook et al., 2004). The translation of *osk* mRNA is tightly linked to its posterior localization, which begins during stage 9 of oogenesis (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Mutations in a number of piRNA-pathway mutations lead to Oskar protein expression during earlier stages of oogenesis, and the *mnk* (Chk2) mutation does not suppress premature *osk* mRNA translation (Cook et al., 2004; Pane et al., 2007). Therefore, premature Osk protein

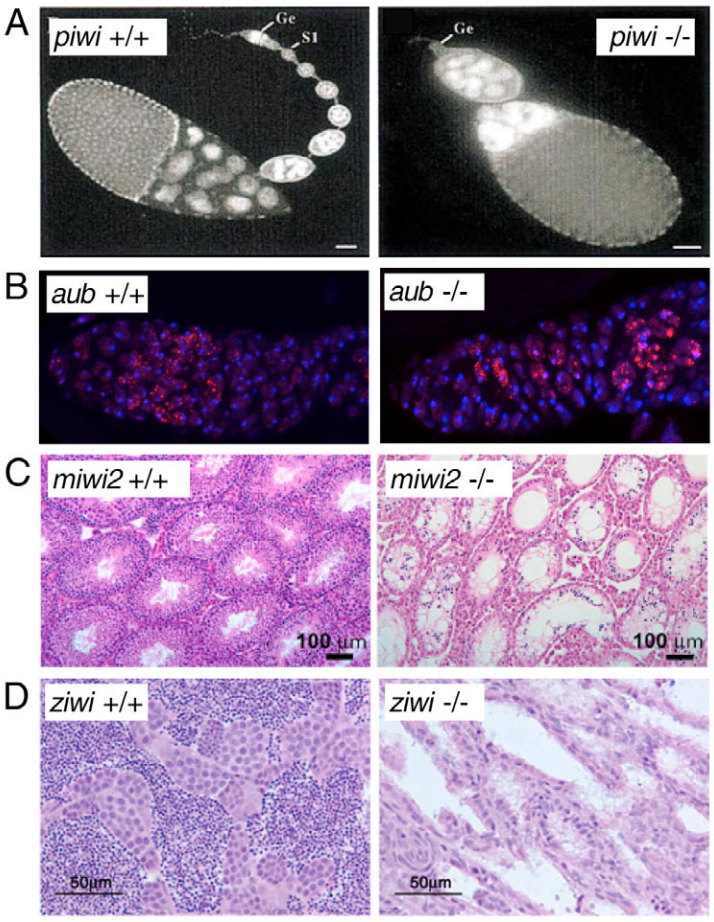

Fig. 3. The piRNA pathway is required for germline development. (**A**) *piwi* is required for the self-renewing division of germline stem cells during oogenesis. DAPI-stained images of 0- to 1-day-old adult ovarioles from wild-type (WT; +/+) and *piwi2 Drosophila*. WT ovarioles contain a long string of developing egg chambers produced through continuous stem cell division. By contrast, *piwi* mutant ovarioles typically contain only two egg chambers, derived through stem cell differentiation and loss. Ge, germarium; S1, stage 1 egg chamber [from Cox et al. (Cox et al., 1998)]. (**B**) Increased DNA damage in the germline cells of *aub* mutant ovaries. *Drosophila* ovaries immunostained to reveal the phosphorylated form of histone H2Av (γ-H2Av, red) and DNA (blue). γ-H2Av accumulates near double-strand break (DSB) sites. In WT ovaries, γ-H2Av foci are restricted to region 2 of the germarium, where meiotic DSBs form. In *aub* mutant ovaries, γ-H2Av foci accumulate in cells within the germarium and persist and increase in intensity only in the germline as cysts bud from the germarium to form egg chambers [from Klattenhoff et al. (Klattenhoff et al., 2007)]. (**C**) *Miwi2* mutation depletes germ cell lineages in mouse testes. Hematoxylin and Eosin (HE) staining of WT and *Miwi2* mutant adult testes shows germline degeneration in *Miwi2* mutant mouse testes [from Carmell et al. (Carmell et al., 2007)]. (**D**) Ziwi is necessary for the maintenance of the zebrafish germline. HE staining shows that *ziwi* mutants have germ-cell-depleted testes, as compared with WT [from Houwing et al. (Houwing et al., 2007)]. (A) Reprinted with permission from Cold Spring Harbor Laboratory Press; (B,C,D) reprinted with permission from Elsevier.

accumulation is not a consequence of DNA damage signaling. Piwi-class Argonaute-piRNA complexes, like siRNA or miRNA-Argonaute complexes, can cleave perfectly matched RNA targets in vitro (Gunawardane et al., 2007; Lau et al., 2006; Saito et al., 2006). As noted above, miRNA-Argonaute complexes that imperfectly pair with target mRNAs induce translational silencing (Valencia-Sanchez et al., 2006). It is therefore possible that piRNA–Piwi-class-Argonaute complexes also trigger the translational silencing of imperfectly matched targets, including mRNAs from single-copy genes such as *oskar*. Consistent with this speculation, a subset of piRNAs associates with polysomes in the mouse (Grivna et al., 2006b).

Fertility and *Stellate* silencing in *Drosophila* males

Most *Drosophila* piRNA-pathway mutations reduce male fertility (Cox et al., 1998; Schmidt et al., 1999; Stapleton et al., 2001; Tomari et al., 2004), and this is linked to the overexpression of Stellate protein. The function of Stellate is not known, but it is encoded by repeated genes on the X chromosome that are suppressed by the Y-linked *Suppressor of Stellate* [*Su(Ste)*] locus (Aravin et al., 2001; Livak, 1990). *Su(Ste)* consists of bi-directionally transcribed repeats that are highly homologous to *Stellate*, and deletion of the *Su(Ste)* locus leads to the massive overexpression of Stellate protein, which assembles into crystals in the testes (Bozzetti et al., 1995; Livak, 1984; Palumbo et al., 1994). piRNAs of 25 to 27 nt are produced from the *Su*(*Ste*) locus, and mutations in piRNA-pathway genes lead to Stellate crystal formation (Aravin et al., 2001; Pane et al., 2007; Stapleton et al., 2001; Tomari et al., 2004). piRNAs from the *Su(Ste)* locus thus silence expression of *Stellate* in trans. It is currently unclear whether this reflects transcriptional or post-transcriptional silencing. Stellate overexpression alone could induce sterility, but defects in silencing of other genes could also impact male fertility. piRNA-pathway mutations lead to mobilization of at least a subset of transposons in the male germline (Kalmykova et al., 2005), and insertional mutations associated with transposon mobilization might also reduce male fertility.

Male germline development in the mouse

The mouse genome encodes three Piwi homologs, Miwi, Miwi2 and Mili (also known as Piwil1, Piwil4 and Piwil2, respectively – Mouse Genome Informatics), and all three are expressed at high levels in testes and are required for male fertility (Aravin et al., 2006; Deng and Lin, 2002; Girard et al., 2006; Grivna et al., 2006a; Kuramochi-Miyagawa et al., 2001; Sasaki et al., 2003). Both Mili and Miwi bind piRNAs, and knockout mutations in the *Mili* and *Miwi* genes block piRNA production (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a). Single null-mutations in each of the three genes lead to male sterility (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Spermatogenesis in the mouse is a coordinated process that can be divided into three phases: mitosis, meiosis and spermiogenesis (de Rooij and Grootegoed, 1998). In the first phase, stem cells localized in the basal layer of the epithelium divide mitotically to self-renew and generate a population of primary spermatocytes. In the second phase, the primary spermatocytes progress through meiosis to generate haploid round-spermatids. During leptotene of meiotic prophase I, duplicated chromosomes condense and begin to pair. Pairing is completed and the synaptonemal complex forms during zygotene, and crossing over occurs in pachytene. The homologs begin to separate in diplotene and finally resolve in diakinesis. During the third phase, round spermatids mature and elongate and are then released into the lumen of the tubule. In *Mili*, *Miwi* and *Miwi2* mutants, the testes appear normal until about 2 weeks post-partum, which roughly corresponds with the first round of meiosis. However, post-meiotic cells do not form (Fig. 3C). Mutations in *Mili* and *Miwi2* block progression through pachytene, whereas *Miwi* mutant spermatocytes develop to the round-spermatid stage but do not complete spermiogenesis (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). The timing of developmental arrest correlates with the temporal expression of Mili and Miwi proteins. Mili is first detected in male primordial germ cells and is present throughout pachytene, whereas Miwi is expressed only from mid-pachytene to the round-spermatid stage (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). The temporal expression pattern of Miwi2 during spermatogenesis has not been reported.

Mutations in each of the three genes lead to the degeneration of the male germline, whereas somatic cells appear to remain relatively unaffected (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004) (Fig. 3C). Similar spermatogenesis-arrest phenotypes have been observed in mutants that disrupt synapsis or DNA repair (Baarends et al., 2001; Barchi et al., 2005; Xu et al., 2003). Additionally, high levels of γ-H2AX staining, indicative of DNA break formation, have been observed in *Miwi2* mutants (Carmell et al., 2007). All of the above suggest that mutations in *piwi* homologs in mice, as with piRNA-pathway mutations in *Drosophila*, lead to DNA damage and to the activation of a DNA damage response, including apoptotic degeneration of germline cells.

In piRNA-pathway *Drosophila* mutants, germline DNA damage is associated with the massive overexpression of retrotransposons, and most of the piRNAs are linked to retrotransposon and repeated sequences (Brennecke et al., 2007; Klattenhoff et al., 2007; Vagin et al., 2006). These observations suggest that germline DNA damage is caused by transposon mobilization, although this has not been demonstrated (Klattenhoff et al., 2007). By contrast, the piRNAs from adult mouse testes are depleted of repeated and retrotransposon sequences (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a). However, a recent study has identified a pre-pachytene cluster of Mili-interacting piRNAs that include a substantial number of repeat and retrotransposon sequences (Aravin et al., 2007). Moreover, *Mili* and *Miwi2* mutations in mice lead to the derepression of retrotransposon transcripts (Aravin et al., 2007; Carmell et al., 2007). The piRNA pathway might therefore have a conserved function in silencing retrotransposons and preventing DNA damage in the germline. However, in contrast to flies, the female germline is not affected by single mutations in mouse Piwi-class Argonaute genes (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). This could indicate that a distinct pathway fulfils this role in the mammalian female germline. However, the Piwi-class Argonautes could also act redundantly during oogenesis, and double or triple mutants might therefore reveal a role for piRNAs in the mouse female germline.

Sex determination and germline development in zebrafish

The zebrafish genome encodes two clear Piwi homologs, *ziwi* (also known as *piwil1* – ZFIN) and *zili*. Ziwi appears to be an ortholog of the mouse Miwi protein, whereas Zili is more similar to mouse Mili. Only Ziwi, which is expressed specifically in the male and female germline cells, has been characterized (Houwing et al., 2007). Ziwi, like the *Drosophila* Ago3 and Aub proteins, is primarily cytoplasmic and localizes to perinuclear nuage. Strikingly, *ziwi*-null mutations also result in the apoptotic loss of germ cells from the testes (Fig. 3D). Reduced levels of *ziwi* function permit the survival of male germ cells to the adult stage, but lead to elevated levels of apoptosis

in adult germ cells and to varying levels of infertility. piRNAs isolated from zebrafish testes and ovaries show the same molecular properties as piRNAs from other organisms, and many are derived from repetitive sequences. Mutations in *ziwi* also affect sex determination, and all surviving mutant animals are male (Houwing et al., 2007). As a result, the role of *ziwi* in the female germline could not be assessed. Other mutations that reduce germ cell number also lead to male development, suggesting that the sex-determination phenotype is secondary to the loss of germ cells (Slanchev et al., 2005). However, a more direct role for piRNAs in sex determination cannot be excluded.

Conclusions and open questions

In flies, fish and mice, piRNA-pathway mutations lead to germline-specific defects, and studies in *Drosophila* indicate that some of these defects result from DNA damage signaling. piRNAs might therefore have a conserved function in preserving germline genome integrity. In flies, piRNA mutations lead to the overexpression of retrotransposons, and retrotransposon mobilization could cause the DNA lesions that lead to germline DNA damage. However, piRNA-pathway mutations have been linked to the mobilization of a single transposon in the *Drosophila* male germline (Kalmykova et al., 2005), and there is no direct evidence that the breaks that accumulate in piRNA-pathway mutations in the female germline are associated with transposition events. piRNAs could therefore directly promote repair, induce the assembly of damage-resistant chromosome structures, or suppress the expression of euchromatic genes that induce DNA breaks.

The mechanism of piRNA-based gene silencing also remains to be determined. Mutations in genes involved in the piRNA pathway in *Drosophila* have been reported to disrupt position-effect variegation (PEV), a form of transcriptional silencing that is caused by the spreading of heterochromatin from peri-centromeric and telomeric regions (Pal-Bhadra et al., 2002; Pal-Bhadra et al., 2004). piRNAs could therefore silence gene expression by promoting heterochromatin assembly, which could directly suppress transcription. Alternatively, piRNA-Argonaute complexes could associate with heterochromatin and catalyze the co-transcriptional destruction of nascent transcripts. The latter possibility is suggested by studies in fission yeast that indicate that siRNA-containing Argonaute proteins are recruited to heterochromatic regions, where they degrade transcripts as they are produced (Verdel and Moazed, 2005). However, mouse and *Drosophila* Piwi-class Argonautes are also present in the cytoplasm, and piRNA–Piwi-class-Argonaute complexes could silence gene expression by targeting the destruction of mature mRNA following exit from the nucleus. It is also possible that piRNA-Argonaute complexes function in both the nucleus and cytoplasm during the development of complex multi-cellular organisms.

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References

Abdu, U., Brodsky, M. and Schupbach, T. (2002). Activation of a meiotic checkpoint during Drosophila oogenesis regulates the translation of Gurken through Chk2/Mnk. *Curr. Biol.* **12**, 1645-1651.

Allis, C. D., Underwood, E. M., Caulton, J. H. and Mahowald, A. P. (1979). Pole cells of Drosophila melanogaster in culture. Normal metabolism, ultrastructure, and functional capabilities. *Dev. Biol.* **69**, 451-465.

Aravin, A. A., Naumova, N. M., Tulin, A. V., Vagin, V. V., Rozovsky, Y. M. and Gvozdev, V. A. (2001). Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. *Curr. Biol.* **11**, 1017-1027.

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. and Tuschl, T. (2003). The small RNA profile during Drosophila melanogaster development. *Dev. Cell* **5**, 337-350.

Aravin, A. A., Klenov, M. S., Vagin, V. V., Bantignies, F., Cavalli, G. and Gvozdev, V. A. (2004). Dissection of a natural RNA silencing process in the Drosophila melanogaster germ line. *Mol. Cell. Biol.* **24**, 6742-6750.

Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M. J., Kuramochi-Miyagawa, S., Nakano, T. et al. (2006). A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **442**, 203-207.

Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K. and Hannon, G. J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **316**, 744-747.

Ashraf, S. I., McLoon, A. L., Sclarsic, S. M. and Kunes, S. (2006). Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. *Cell* **124**, 191-205.

Baarends, W. M., van der Laan, R. and Grootegoed, J. A. (2001). DNA repair mechanisms and gametogenesis. *Reproduction* **121**, 31-39.

Barchi, M., Mahadevaiah, S., Di Giacomo, M., Baudat, F., de Rooij, D. G., Burgoyne, P. S., Jasin, M. and Keeney, S. (2005). Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage. *Mol. Cell. Biol.* **25**, 7203-7215.

Baulcombe, D. (2004). RNA silencing in plants. *Nature* **431**, 356-363.

Belgnaoui, S. M., Gosden, R. G., Semmes, O. J. and Haoudi, A. (2006). Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell Int.* **6**, 13.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Bozzetti, M. P., Massari, S., Finelli, P., Meggio, F., Pinna, L. A., Boldyreff, B., Issinger, O. G., Palumbo, G., Ciriaco, C., Bonaccorsi, S. et al. (1995). The Ste locus, a component of the parasitic cry-Ste system of Drosophila melanogaster, encodes a protein that forms crystals in primary spermatocytes and mimics properties of the beta subunit of casein kinase 2. *Proc. Natl. Acad. Sci. USA* **92**, 6067-6071.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. and Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**, 1089-1103.

Cao, L., Alani, E. and Kleckner, N. (1990). A pathway for generation and processing of double-strand breaks during meiotic recombination in S. cerevisiae. *Cell* **61**, 1089-1101.

Carmell, M. A., Girard, A., van de Kant, H. J., Bourc'his, D., Bestor, T. H., de Rooij, D. G. and Hannon, G. J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev. Cell* **12**, 503-514.

Chen, Y., Pane, A. and Schupbach, T. (2007). Cutoff and aubergine mutations result in retrotransposon upregulation and checkpoint activation in Drosophila. *Curr. Biol.* **17**, 637-642.

Cook, H. A., Koppetsch, B. S., Wu, J. and Theurkauf, W. E. (2004). The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* **116**, 817-829.

Cox, D. N., Chao, A., Baker, J., Chang, L., Qiao, D. and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev.* **12**, 3715-3727.

Cox, D. N., Chao, A. and Lin, H. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514.

de Rooij, D. G. and Grootegoed, J. A. (1998). Spermatogonial stem cells. *Curr. Opin. Cell Biol.* **10**, 694-701.

Deng, W. and Lin, H. (2002). miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev. Cell* **2**, 819-830.

Du, T. and Zamore, P. D. (2005). microPrimer: the biogenesis and function of microRNA. *Development* **132**, 4645-4652.

Findley, S. D., Tamanaha, M., Clegg, N. J. and Ruohola-Baker, H. (2003). Maelstrom, a Drosophila spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. *Development* **130**, 859-871.

Gasior, S. L., Wakeman, T. P., Xu, B. and Deininger, P. L. (2006). The human LINE-1 retrotransposon creates DNA double-strand breaks. *J. Mol. Biol.* **357**, 1383-1393.

Ghabrial, A., Ray, R. P. and Schupbach, T. (1998). okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in Drosophila oogenesis. *Genes Dev.* **12**, 2711-2723.

Gillespie, D. E. and Berg, C. A. (1995). Homeless is required for RNA localization in Drosophila oogenesis and encodes a new member of the DE-H family of RNA-dependent ATPases. *Genes Dev.* **9**, 2495-2508.

Girard, A., Sachidanandam, R., Hannon, G. J. and Carmell, M. A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* **442**, 199-202.

Grimaud, C., Bantignies, F., Pal-Bhadra, M., Ghana, P., Bhadra, U. and

Cavalli, G. (2006). RNAi components are required for nuclear clustering of Polycomb group response elements. *Cell* **124**, 957-971.
Grivna, S. T., Beyret, E., Wang, Z. and Lin, H. (2006a). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev.* **20**, 1709-1714.
Grivna, S. T., Pyhtila, B. and Lin, H. (2006b). MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc. Natl. Acad. Sci. USA* **103**, 13415-13420.
Grunert, S. and St Johnston, D. (1996). RNA localization and the development of asymmetry during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **6**, 395-402.
Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. and Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. *Science* **315**, 1587-1590.
Hannon, G. J. (2002). RNA interference. *Nature* **418**, 244-251.
Harris, A. N. and Macdonald, P. M. (2001). Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development* **128**, 2823-2832.
Horwich, M. D., Li, C., Matranga, C., Vagin, V., Farley, G., Wang, P. and Zamore, P. D. (2007). The Drosophila RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr. Biol.* **17**, 1265-1272.
Houwing, S., Kamminga, L. M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D. V., Blaser, H., Raz, E., Moens, C. B. et al. (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* **129**, 69-82.
Kalmykova, A. I., Klenov, M. S. and Gvozdev, V. A. (2005). Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline. *Nucleic Acids Res.* **33**, 2052-2059.
Kim, M., Krogan, N. J., Vasiljeva, L., Rando, O. J., Nedea, E., Greenblatt, J. F. and Buratowski, S. (2004). The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature* **432**, 517-522.
Kim-Ha, J., Kerr, K. and Macdonald, P. M. (1995). Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell* **81**, 403-412.
Kirino, Y. and Mourelatos, Z. (2007). Mouse Piwi-interacting RNAs are 2′-O-methylated at their 3′ termini. *Nat. Struct. Mol. Biol.* **14**, 347-348.
Klattenhoff, C., Bratu, D. P., McGinnis-Schultz, N., Koppetsch, B. S., Cook, H. A. and Theurkauf, W. E. (2007). Drosophila rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev. Cell* **12**, 45-55.
Kloosterman, W. P. and Plasterk, R. H. (2006). The diverse functions of microRNAs in animal development and disease. *Dev. Cell* **11**, 441-450.
Kuramochi-Miyagawa, S., Kimura, T., Yomogida, K., Kuroiwa, A., Tadokoro, Y., Fujita, Y., Sato, M., Matsuda, Y. and Nakano, T. (2001). Two mouse piwi-related genes: miwi and mili. *Mech. Dev.* **108**, 121-133.
Kuramochi-Miyagawa, S., Kimura, T., Ijiri, T. W., Isobe, T., Asada, N., Fujita, Y., Ikawa, M., Iwai, N., Okabe, M., Deng, W. et al. (2004). Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. *Development* **131**, 839-849.
Lau, N. C., Seto, A. G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D. P. and Kingston, R. E. (2006). Characterization of the piRNA complex from rat testes. *Science* **313**, 363-367.
Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854.
Lim, A. K. and Kai, T. (2007). Unique germ-line organelle, nuage, functions to repress selfish genetic elements in Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **104**, 6714-6719.
Lin, H. and Spradling, A. C. (1997). A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development* **124**, 2463-2476.
Livak, K. J. (1984). Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. *Genetics* **107**, 611-634.
Livak, K. J. (1990). Detailed structure of the Drosophila melanogaster stellate genes and their transcripts. *Genetics* **124**, 303-316.
Mahowald, A. P. (1971). Polar granules of Drosophila. 3. The continuity of polar granules during the life cycle of Drosophila. *J. Exp. Zool.* **176**, 329-343.
Markussen, F. H., Michon, A. M., Breitwieser, W. and Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. *Development* **121**, 3723-3732.
McKim, K. S. and Hayashi-Hagihara, A. (1998). mei-W68 in Drosophila melanogaster encodes a Spo11 homolog: evidence that the mechanism for initiating meiotic recombination is conserved. *Genes Dev.* **12**, 2932-2942.
Meister, G. and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343-349.
Mello, C. C. and Conte, D., Jr (2004). Revealing the world of RNA interference. *Nature* **431**, 338-342.
Modesti, M. and Kanaar, R. (2001). DNA repair: spot(light)s on chromatin. *Curr. Biol.* **11**, R229-R232.
Ohara, T., Sakaguchi, Y., Suzuki, T., Ueda, H., Miyauchi, K. and Suzuki, T. (2007). The 3′ termini of mouse Piwi-interacting RNAs are 2′-O-methylated. *Nat. Struct. Mol. Biol.* **14**, 349-350.
Pal-Bhadra, M., Bhadra, U. and Birchler, J. A. (2002). RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. *Mol. Cell* **9**, 315-327.
Pal-Bhadra, M., Leibovitch, B. A., Gandhi, S. G., Rao, M., Bhadra, U., Birchler, J. A. and Elgin, S. C. (2004). Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. *Science* **303**, 669-672.
Palumbo, G., Berloco, M., Fanti, L., Bozzetti, M. P., Massari, S., Caizzi, R., Caggese, C., Spinelli, L. and Pimpinelli, S. (1994). Interaction systems between heterochromatin and euchromatin in Drosophila melanogaster. *Genetica* **94**, 267-274.
Pane, A., Wehr, K. and Schupbach, T. (2007). zucchini and squash encode two putative nucleases required for rasiRNA production in the Drosophila germline. *Dev. Cell* **12**, 851-862.
Rongo, C., Gavis, E. R. and Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. *Development* **121**, 2737-2746.
Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. and Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev.* **20**, 2214-2222.
Saito, K., Sakaguchi, Y., Suzuki, T., Suzuki, T., Siomi, H. and Siomi, M. C. (2007). Pimet, the Drosophila homolog of HEN1, mediates 2′-O-methylation of Piwi- interacting RNAs at their 3′ ends. *Genes Dev.* **21**, 1603-1608.
Sarot, E., Payen-Groschene, G., Bucheton, A. and Pelisson, A. (2004). Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the Drosophila melanogaster flamenco gene. *Genetics* **166**, 1313-1321.
Sasaki, T., Shiohama, A., Minoshima, S. and Shimizu, N. (2003). Identification of eight members of the Argonaute family in the human genome small star, filled. *Genomics* **82**, 323-330.
Savitsky, M., Kwon, D., Georgiev, P., Kalmykova, A. and Gvozdev, V. (2006). Telomere elongation is under the control of the RNAi-based mechanism in the Drosophila germline. *Genes Dev.* **20**, 345-354.
Schmidt, A., Palumbo, G., Bozzetti, M. P., Tritto, P., Pimpinelli, S. and Schafer, U. (1999). Genetic and molecular characterization of sting, a gene involved in crystal formation and meiotic drive in the male germ line of Drosophila melanogaster. *Genetics* **151**, 749-760.
Schupbach, T. and Wieschaus, E. (1991). Female sterile mutations on the second chromosome of Drosophila melanogaster. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**, 1119-1136.
Slanchev, K., Stebler, J., de la Cueva-Mendez, G. and Raz, E. (2005). Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proc. Natl. Acad. Sci. USA* **102**, 4074-4079.
Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster*. Vol. I (ed. M. Bate and A. M. Arias), pp. 1-70. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Stapleton, W., Das, S. and McKee, B. D. (2001). A role of the Drosophila homeless gene in repression of Stellate in male meiosis. *Chromosoma* **110**, 228-240.
Tomari, Y., Du, T., Haley, B., Schwarz, D., Bennett, R., Cook, H., Koppetsch, B., Theurkauf, W. and Zamore, P. D. (2004). RISC assembly defects in the Drosophila RNAi mutant armitage. *Cell* **116**, 831-841.
Vagin, V. V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P. D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320-324.
Valencia-Sanchez, M. A., Liu, J., Hannon, G. J. and Parker, R. (2006). Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev.* **20**, 515-524.
Verdel, A. and Moazed, D. (2005). RNAi-directed assembly of heterochromatin in fission yeast. *FEBS Lett.* **579**, 5872-5878.
Wang, X. H., Aliyari, R., Li, W. X., Li, H. W., Kim, K., Carthew, R., Atkinson, P. and Ding, S. W. (2006). RNA interference directs innate immunity against viruses in adult Drosophila. *Science* **312**, 452-454.
Watanabe, T., Takeda, A., Tsukiyama, T., Mise, K., Okuno, T., Sasaki, H., Minami, N. and Imai, H. (2006). Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev.* **20**, 1732-1743.
Xu, X., Aprelikova, O., Moens, P., Deng, C. X. and Furth, P. A. (2003). Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice. *Development* **130**, 2001-2012.
Xue, Y., Bai, X., Lee, I., Kallstrom, G., Ho, J., Brown, J., Stevens, A. and Johnson, A. W. (2000). Saccharomyces cerevisiae RAI1 (YGL246c) is homologous to human DOM3Z and encodes a protein that binds the nuclear exoribonuclease Rat1p. *Mol. Cell. Biol.* **20**, 4006-4015.