PIWI-interacting small RNAs: the vanguard of genome defence

Mikiko C. Siomi[*‡], *Kaoru Sato*[*], *Dubravka Pezic*[§] *and Alexei A. Aravin*[§]

Abstract | PIWI-interacting RNAs (piRNAs) are a distinct class of small non-coding RNAs that form the piRNA-induced silencing complex (piRISC) in the germ line of many animal species. The piRISC protects the integrity of the genome from invasion by 'genomic parasites' — transposable elements — by silencing them. Owing to their limited expression in gonads and their sequence diversity, piRNAs have been the most mysterious class of small non-coding RNAs regulating RNA silencing. Now, much progress is being made into our understanding of their biogenesis and molecular functions, including the specific subcellular compartmentalization of the piRNA pathway in granular cytoplasmic bodies.

Gene expression in individual cells of multicellular organisms is elaborately controlled by various mechanisms to initiate and maintain cellular differentiation, resulting in distinct gene expression profiles in each cell. One of the regulatory mechanisms present in almost all eukaryotic organisms is RNA silencing, which provides highly specific inhibition of gene expression through complementary recognition of RNA targets by small (20–30 nucleotide (nt)) RNA molecules. The RNA-induced silencing complex (RISC) forms the core of the RNA silencing machinery, and consists of a protein from the Argonaute (AGO) family and a small RNA that acts to guide RISC to its targets (a 'guide' RNA)[1–5]. Once loaded with a small RNA, AGO proteins inhibit the expression of their targets, either by cleavage using Slicer endonuclease activity, or by attracting additional proteins that can affect translation, RNA stability or chromatin structure. The majority of eukaryotic organisms possess more than one AGO protein, and the functions of individual members of the family are often non-redundant.

Small silencing RNAs in metazoa can be divided into three classes: small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs)[1–3]. Compared with siRNAs and miRNAs, piRNAs are the least investigated class of small RNA molecules. However, early on in the study of RNA silencing, an analysis of how *Stellate* protein-coding gene repeats are silenced in the *Drosophila melanogaster* male germ line provided a hint that piRNAs existed[6]. A small RNA profiling study in the *D. melanogaster* testes and early embryos later revealed an abundance of endogenous 23–29 nt small RNAs that were derived from repetitive genomic elements and were originally termed "repeat-associated small interfering RNAs"[7].

Different classes of small RNAs arise by varying biogenesis mechanisms. The processing of both miRNAs and siRNAs from their precursors requires cleavage by the Dicer endonuclease before loading onto an AGO protein (FIG. 1). piRNAs, by contrast, are processed through Dicer-independent mechanisms[8–13] (FIG. 1), and this finding led to the realization that they are distinct from siRNAs and miRNAs. piRNAs specifically associate with PIWI proteins, which are germline-specific members of the AGO protein family, whereas siRNAs and miRNAs associate with ubiquitously expressed AGO proteins. In this regard, piRNAs can also be distinguished from siRNAs and miRNAs.

In this Review, we summarize our understanding of the biogenesis and function of piRNAs, mainly focusing on studies done in two model systems, *D. melanogaster* and mice. We also review recent progress in our understanding of how the piRNA pathway is spatially controlled in cytoplasmic bodies that appear during gonadal development.

Protective functions of piRNAs

The main function of piRNAs is to silence transposable elements (TEs) in the germ line, and this role is highly conserved across animal species. TEs are genomic parasites that threaten the genomic integrity of the host genome; they can move to new sites by insertion or transposition and thereby disrupt genes and alter the genome[14] (BOX 1). In animals, endogenous siRNAs also silence TEs[15], but the piRNA pathway is at the forefront of defence against transposons in germ cells. To achieve this goal, piRNAs associate with PIWI proteins to form an active piRNA-induced silencing

*Keio University School of Medicine, Tokyo 160-8582, Japan.
‡Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Saitama 332-0012, Japan.
§Division of Biology, California Institute of Technology, 1200 E. California Boulevard, Pasadena, California 91125, USA.
Correspondence to M.C.S. and A.A.A.
e-mails: siomim@sc.itc.keio.ac.jp; aaa@caltech.edu
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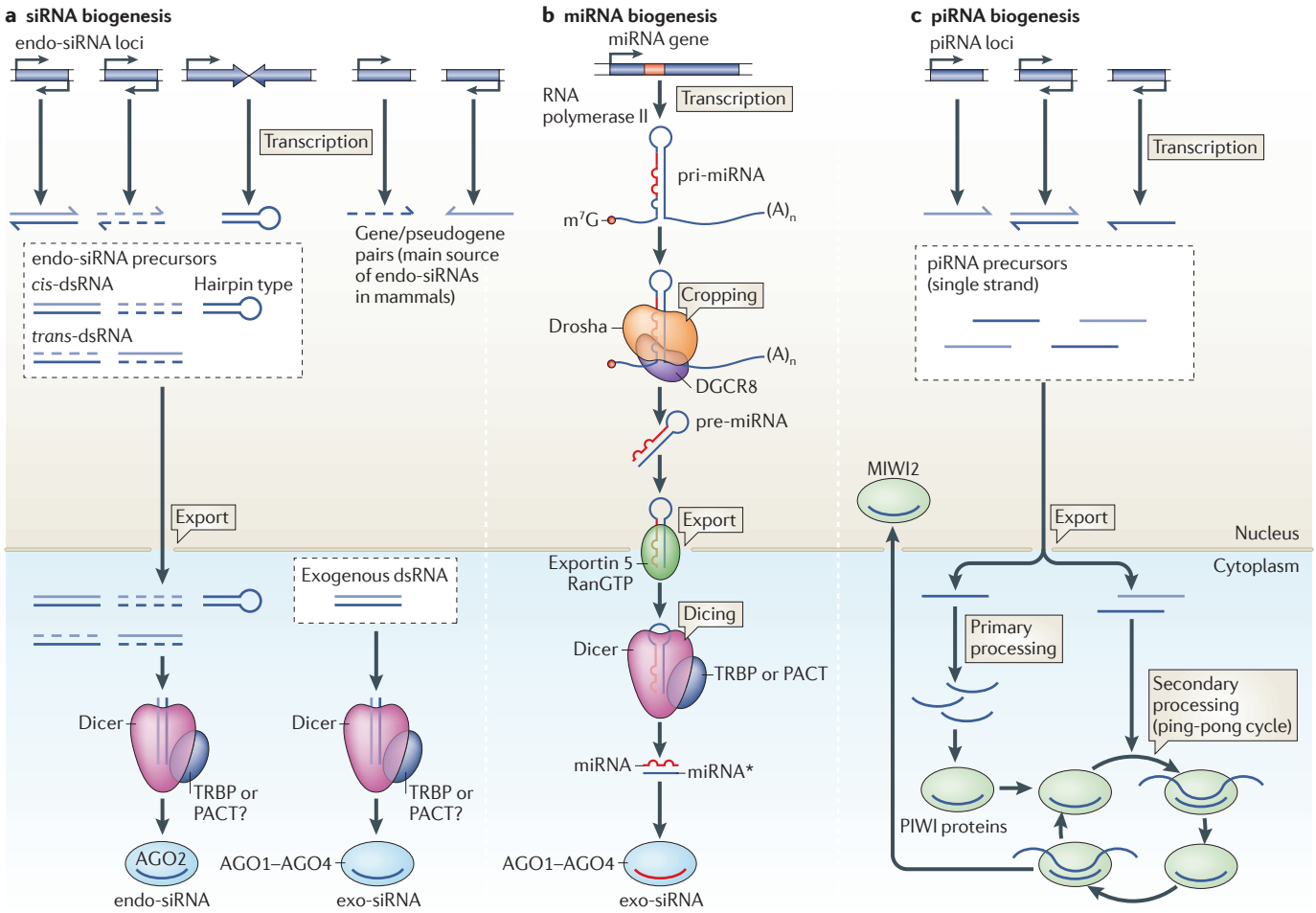


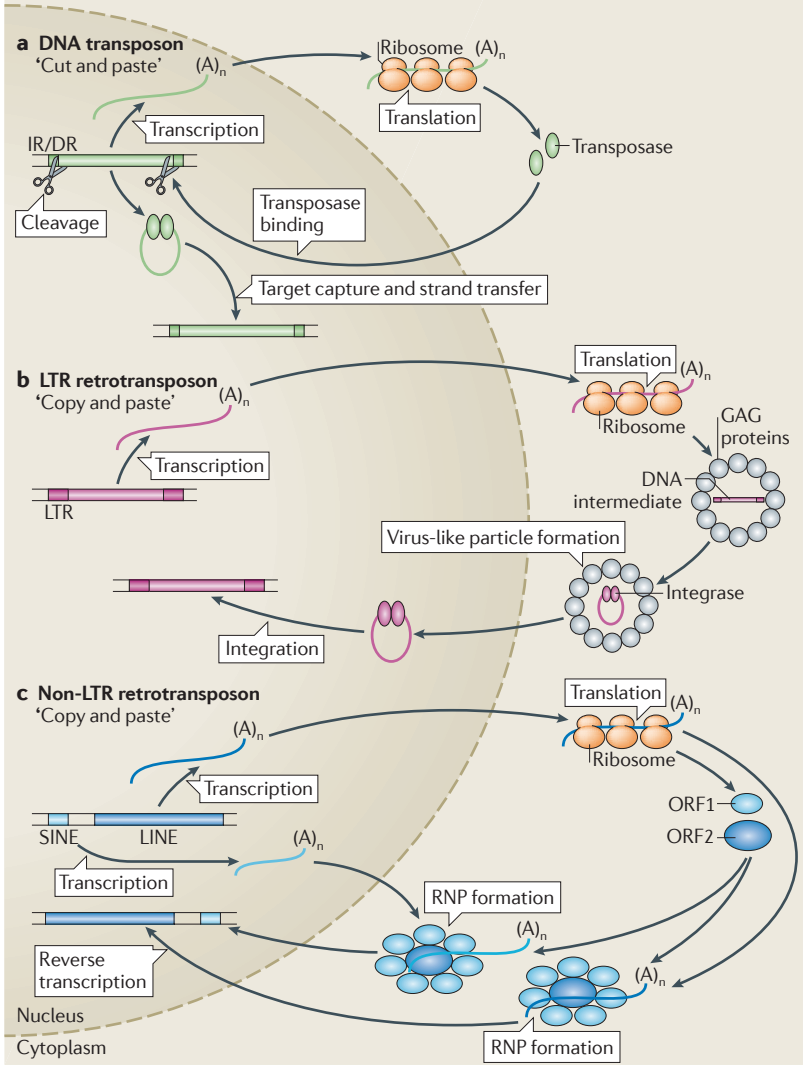
Figure 1 | **Biogenesis of small RNAs in mammals. a** | Small interfering RNA (siRNA) biogenesis. Transcripts that are able to form double-stranded RNA (dsRNA) or long stem–loop structures serve as endogenous siRNA (endo-siRNA) precursors. Pseudogenes transcribed in an antisense orientation produce RNA that pairs with cognate gene mRNAs as well as with transcripts that are derived from intergenic repetitive sequences on the genome, including transposons. The processing of endo-siRNA requires Dicer, whereas the role of Dicer-binding proteins — such as TAR RNA-binding protein (TRBP; also known as TARBP2) and PACT (also known as PRKRA) — remains undetermined. After maturation, endo-siRNAs (which are ~21 nucleotides (nt) in length) are loaded onto Argonaute 2 (AGO2). Whether endo-siRNAs are loaded onto other AGO members, such as AGO1, AGO3 and AGO4, remains to be determined. Exogenous siRNAs (exo-siRNAs) are derived from exogenous dsRNAs by Dicer–TRBP (or Dicer–PACT). exo-siRNAs are loaded onto AGO1, AGO2, AGO3 and AGO4; however, only the AGO2–siRNA complex functions in RNA interference, as other AGO members lack Slicer activity. **b** | MicroRNA (miRNA) biogenesis. The primary transcripts of miRNAs (pri-miRNAs) are transcribed by RNA polymerase II from miRNA genes on the genome. pri-miRNAs form hairpin structures and are processed to ~60–70 nt miRNA precursors (pre-miRNAs) by the microprocessor Drosha–DGCR8 (DiGeorge syndrome critical region 8) complex in the nucleus. After being exported by exportin 5 and RanGTP, pre-miRNAs are further processed to ~22 nt miRNA–miRNA* duplexes (in which miRNA* is the passenger strand that is degraded) by the Dicer–TRBP (or Dicer–PACT) complex. Mature miRNAs are then loaded onto AGO1, AGO2, AGO3 and AGO4. **c** | PIWI-interacting RNA (piRNA) biogenesis. piRNAs (which are 24–32 nt in length) are processed from single-stranded RNA precursors that are transcribed largely from particular intergenic repetitive elements known as piRNA clusters. Unlike miRNAs and siRNAs, piRNAs do not require Dicer for their processing. The requirement for Drosha has not been formally tested. First, primary piRNAs are produced through the primary processing pathway and are amplified through the ping-pong pathway, which requires Slicer activity of PIWI proteins. Primary piRNA processing and loading onto mouse PIWI proteins might occur in the cytoplasm. MIWI2 (also known as PIWI-like protein 4) specifically associates with secondary piRNAs that are processed through the amplification loop, and is localized in the nucleus to exert its silencing function. $(A)_n$ indicates the poly(A) tail formed during translation, and m^7G indicates the 5′-terminal cap of the mRNA.

complex (piRISC) that can recognize and silence complementary RNA targets. Indeed, genetic evidence has shown that, in addition to piRNAs, PIWI proteins are necessary for TE silencing and have crucial roles in gonadal development.

Silencing of transposable elements. In *D. melanogaster*, mutations in each of the three members of the PIWI family — PIWI, Aubergine (AUB) and AGO3 — lead to transposon derepression in the germ line, indicating that they act non-redundantly during TE silencing[16–19].

Box 1 | **Life cycles of transposable elements**

DNA transposons (see the figure, part **a** (green bar)) move around the genome mostly by a 'cut and paste' mechanism. DNA transposons encode a transposase enzyme (green ovals) that catalyses the movement of DNA transposons. Transposases enter the nucleus and bind the ends of DNA transposons at inverted/direct repeats (IR/DRs) to excise them from the genome. An excised DNA element is then integrated into a new site on the genome through 'target capture'. Long terminal repeat (LTR) retrotransposons (see the figure, part **b** (pink bar)) move around the genome by a 'copy and paste' mechanism. LTR retrotransposons contain LTRs at both ends and encode integrases (pink ovals) and glycosaminoglycan (GAG) proteins (grey circles). GAG proteins form virus-like particles, in which retrotransposon mRNAs are captured. Reverse transcription of the viral mRNAs occurs in the virus-like particles to generate the DNA intermediates. This reaction takes place in the cytoplasm. The DNA intermediates associated with integrases emerge from the particles, and are imported into the nucleus and integrated into the genome. Non-LTR retrotransposons (see the figure, part **c** (blue bar)) also move around the genome by a 'copy and paste' mechanism. Non-LTR retrotransposons do not have LTRs and can be divided into two subtypes, long interspersed nuclear elements (LINEs; dark blue) and short interspersed nuclear elements (SINEs; light blue). LINEs encode two proteins, ORF1 (light blue ovals) and ORF2 (dark blue ovals), both of which have a reverse transcriptase domain. ORF1 and ORF2 form ribonucleoprotein (RNP) complexes with their own mRNAs in the cytoplasm. The RNP complexes are transported to the nucleus and the mRNAs are integrated into new sites on the genome by target-primed reverse transcription. SINEs do not encode a functional reverse transcriptase and therefore use the enzyme of LINEs, or other transposable elements, for their transposition[14]. Orange ovals represent ribosomes and $(A)_n$ indicates the poly(A) tail formed during translation.



Indeed, all PIWI proteins have crucial roles in gonad development: both *piwi* and *aub* are required for male and female fertility[20–22] and *Ago3* is required for female fertility, but only partially for male fertility[19]. The non-redundant function of PIWI can be at least partly explained by the fact that, unlike AUB and AGO3, it is expressed in follicular cells that have somatic origins and surround the developing germline cells[20,23,24].

Deficiency in two members of the mouse PIWI protein family, MILI (also known as PIWI-like protein 2) and MIWI2 (also known as PIWI-like protein 4), but not the third member, MIWI (also known as PIWI-like protein 1), leads to the activation of long interspersed nuclear element (LINE) and long terminal repeat (LTR) retrotransposons in the male germ line (BOX 1), as well as arrest of gametogenesis and complete sterility in males[25–27]. However, contrary to the situation in *D. melanogaster*, *piwi* mutations do not affect the female germ line in mice, despite the fact that at least one member, *Mili*, is expressed in female germ cells together with transposon-derived piRNAs[28,29].

Severe defects in gametogenesis observed in *piwi*-deficient flies and *Mili*- and *Miwi2*-knockout mice are probably directly caused by TE activation. The derepression of TEs that occurs as a result of loss of *piwi* functions leads to the generation of double-stranded DNA breaks during abortive or successful transposition, and this activates a DNA-damage checkpoint. In fact, mutations in components of the DNA-damage checkpoint suppress some gametogenesis defects observed in flies deficient in piRNA-mediated TE silencing, although they cannot prevent formation of double-stranded breaks and the eventual sterility of *piwi* mutants[30].

Consistent with their function in TE repression, all members of the PIWI family in *D. melanogaster*, and MILI and MIWI2 in mice, are associated with transposon-derived piRNAs. PIWI proteins loaded with piRNAs that are antisense to TE mRNAs can specifically recognize and cleave transposon transcripts[31,32]. Similarly to AGO proteins, recombinant fly PIWI proteins exhibit Slicer endonuclease activity that cleaves target RNAs that are complementary to the guide small RNAs[32,33]. In mice, the role of PIWI proteins and piRNAs seems to extend beyond post-transcriptional silencing. CpG DNA methylation, which is required for efficient transcriptional silencing of LINE and LTR retrotransposons in the genome, is decreased in the male germ line of *Mili* and *Miwi2* mutants. Genetic evidence suggests that both mutants fail to establish *de novo* methylation of TE sequences during spermatogenesis, leading to the hypothesis that the piRISC can also guide the *de novo* methylation machinery to TE loci[26,27]. MIWI2, loaded with anti-transposon piRNAs, is present in the prospermatogonia nuclei (FIG. 1c) during the short period in embryogenesis when *de novo* DNA methylation takes place in the male germ line[26,27]. piRNAs may present a perfect guide for discriminating TE sequences from normal protein-coding genes and marking them for DNA methylation; however, the biochemical details of how these two mechanisms of piRNA action might be linked have not yet been revealed.

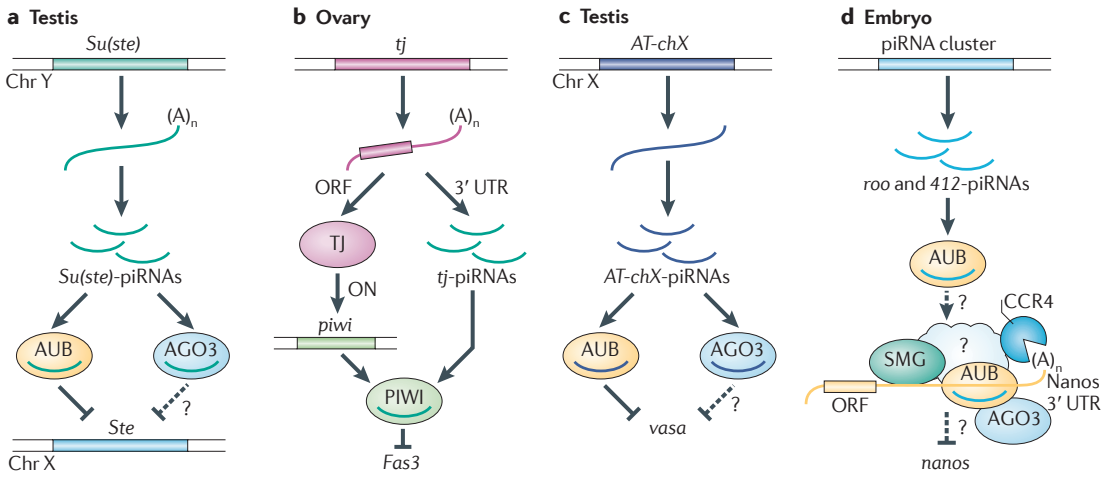


Figure 2 | **Putative roles for piRNAs during silencing of protein-coding genes. a** | *Stellate* (*Ste*) silencing by *Suppressor of Stellate* PIWI-interacting RNAs (*Su(ste)*-piRNAs) in *Drosophila melanogaster* testis. *Su(ste)*-piRNAs are derived from the *Su(ste)* locus on the Y chromosome[36]. *Su(ste)* shows a strong homology to *Ste* on the X chromosome at the nucleotide sequence level. *Su(ste)*-piRNAs bind both Aubergine (AUB) and Argonaute 3 (AGO3) and have a role in *Ste* silencing[33,61]. Without *Su(ste)*-piRNAs, *Ste* is derepressed, which results in the aggregation of STE, or STE crystals, which consequently causes male infertility. **b** | *Fasciclin 3* (*Fas3*) silencing by *traffic jam* (*tj*)-piRNAs in *D. melanogaster* ovary. PIWI expression is driven by TJ, a large MAF transcription factor encoded by the *tj* gene[44]. piRNAs derived from the 3′ untranslated region (UTR) of *tj* mRNAs are loaded onto PIWI, and the resultant complex functions in silencing *Fas3* (REF. 44). The mRNA level of *Fas3* is upregulated in *piwi* mutants, as in *tj* mutant clones, resulting in an SGP (somatic gonadal precursor)-only cell cluster and a failure to intermingle with PGCs (primordial germ cells)[44]. **c** | *vasa* silencing by *AT-chX*-piRNAs in *D. melanogaster* testis. *AT-chX*-piRNAs are derived from the *AT-chX* locus on the X chromosome (Chr X) and associate with both AUB and AGO3 (REFS 33,61). *AT-chX*-piRNAs direct AUB to *vasa* mRNAs to downregulate *vasa*. Increased levels of Vasa protein result in oversized apexes in testes, where spermatogonial cells and spermatocytes are located[33]. Mutations in *Ago3* cause little or no change in the expression level of Vasa[61]. This may be because the expression level of AGO3 in testes is much lower than that of AUB[61]. **d** | *nanos* (*nos*) mRNA decay driven by *roo* and *412*-derived piRNAs in *D. melanogaster* embryos[45]. A failure in piRNA-induced *nos* mRNA deadenylation and translational repression results in head development defects[45]. $(A)_n$ indicates the poly(A) tail formed during translation. CCR4, C-C chemokine receptor 4 (also known as Twin); ORF, open reading frame; SMG, Smaug.

PIWI proteins and piRNAs in *D. melanogaster* may also affect transcriptional silencing through effects on chromatin[5]. In particular, PIWI is present in the nucleus in the steady state[9,20], and its nuclear localization is crucial for its function in TE silencing[34]. A Slicer-deficient PIWI mutant, however, rescued TE silencing[34], indicating that PIWI-mediated silencing is independent of Slicer. Some results suggested that PIWI influences position-effect variegation in somatic cells, wherein gene silencing depends on the genomic loci, by directing Heterochromatin protein 1A (HP1A) localization to heterochromatin[35,36]. It was also proposed that PIWI can activate expression of telomeric sequences, which is dependent on the chromatin context[37]. Other evidence has shown, however, that HP1 recruitment to heterochromatin does not depend on PIWI[38], leaving this issue open to further investigation.

Endogenous siRNAs (endo-siRNAs) also participate in transposon silencing, as TEs are prone to producing double-stranded RNAs that are channelled towards Dicer for processing[15]. In fact, endo-siRNAs repress TEs in *D. melanogaster* somatic cells that lack the piRNA pathway[39,40] and, in mice, endo-siRNAs also target TEs in the female germ line[41,42]. This might explain why *piwi* deficiency in females has no phenotypic consequences.

Control of non-transposable elements. Some piRNAs may target non-transposable elements (non-TEs). Indeed, the first piRNAs that were described in *D. melanogaster* were found to target repetitive, but protein-coding, *Stellate* genes[6] (FIG. 2a). Several studies later suggested that a specific population of *D. melanogaster* piRNAs might also be involved in the silencing of non-repetitive, protein-coding genes. For example, *Fasciclin 3* (*Fas3*), a gene encoding an immunoglobulin-like cell adhesion molecule[43], might be targeted by PIWI-associated piRNAs in follicle cells of fly ovaries[44] (FIG. 2b). The expression of *Fas3* increases in *piwi* mutant gonads. Overexpression of *Fas3* prevents the intermingling of germline and somatic cells in the ovaries, resulting in oogenesis defects[43]. Germline and somatic cells also failed to intermingle in a *piwi* mutant[44]. Thus, it was suggested that the PIWI–piRNA complex represses *Fas3* transcripts[44]. Similarly, AUB-associated piRNAs in the fly testes might target the essential maternal-effect gene *vasa*[33] (FIG. 2c). Some piRNAs may induce degradation of maternally deposited mRNAs, such as Nanos, the *D. melanogaster* posterior morphogen, during the maternal–zygotic transition in the *D. melanogaster* embryo[45] (FIG. 2d). These results suggest that piRNA function extends beyond transposon silencing. However, the complementarities between these piRNAs and their potential targets are marginal. Furthermore, the number

Morphogen
A signalling molecule that provides positional information to cells by forming a concentration gradient across the developmental field, and elicits multiple different cellular responses to specify cell fates in a concentration-dependent manner.

Maternal–zygotic transition
A developmental switch during embryogenesis from control by maternally provided gene products to control by zygotically provided gene products. During this transition, embryonic transcription is initiated and many maternal products are degraded.

of piRNAs for which effects on protein-coding genes have been shown *in vivo* is rather low. Therefore, additional studies are necessary to conclude that piRNAs can indeed target non-TEs. Protein-coding genes that are embedded in heterochromatin regions of the genome often contain TE remnants in their introns and therefore have the potential to be targeted by nuclear piRNAs. However, their expression is maintained in mutants that show a decrease in accumulation of piRNAs, suggesting that these genes may have a mechanism to prevent piRNA-mediated silencing[46].

The first piRNAs described in mammals — which were named pachytene piRNAs because they are first expressed during the pachytene stage of meiosis — do not target transposons[47]. Accordingly, deficiency in MIWI, which associates with pachytene piRNAs, does not lead to TE derepression. The targets of mammalian pachytene piRNAs and MIWI are still unknown.

piRNA biogenesis

Extensive analyses of piRNAs associated with PIWI proteins in flies and mice have identified the genomic origins of piRNAs and led to the proposal of two biogenesis pathways: the primary processing pathway and the ping-pong amplification loop (FIG. 3). Both mechanisms are important for mounting an effective defence against transposons. First, the primary piRNA biogenesis pathway provides an initial pool of piRNAs that target multiple TEs. Next, the ping-pong cycle further shapes the piRNA population by amplifying sequences that target active transposons. These two pathways are conserved in many animal species, including rats (*Rattus norvegicus*), zebrafish (*Danio rerio*), frogs (*Xenopus laevis*) and silkworms (*Bombyx mori*)[48–51].

The primary piRNA processing pathway. Initial analysis of piRNA sequences revealed their extremely high complexity: whereas individual miRNA species number in their hundreds, there are hundreds of thousands, if not millions, of individual piRNA sequences[10,11,31]. Furthermore, there are no specific sequence motifs in piRNAs, except for a strong bias for uridine at position 1 (1U bias). Despite their diversity, most piRNAs can be mapped to a relatively small number of genomic regions called piRNA clusters[10,11,31]. piRNA clusters extend from several to more than 200 kilobases, and each cluster contains multiple sequences that generate piRNAs. Unlike siRNAs, piRNAs do not show any phasing within a cluster sequence and can overlap with each other. Furthermore, no significant secondary structures, reminiscent of the stem–loop structures in miRNA precursors, have been detected in regions surrounding mature piRNAs. In some clusters in both *D. melanogaster* and mice, piRNAs map to both genomic strands, suggesting bidirectional transcription[29,31]. However, other clusters, such as *flamenco* (*flam*) in *D. melanogaster* and pachytene piRNA clusters in mice, produce piRNA from a single genomic strand (although some pachytene clusters are bidirectionally transcribed in opposite directions from a central promoter)[11,31]. Accordingly, it is likely that piRNA precursors are single-stranded and therefore do not require Dicer for their processing. Some evidence from reverse transcription PCR and expressed sequence tag databases supports the notion that long, single-stranded RNAs are indeed transcribed from piRNA clusters[10,11,26,31]. Genetic data from *D. melanogaster* suggest that long RNA molecules, spanning the whole piRNA cluster, are processed into mature piRNAs: P-element insertion at the 5′ end of *flam* that disrupts transcription of the long RNA also leads to elimination of all piRNAs from this cluster, easily more than 100 kilobases from the site of insertion[24,31]. The cause of 1U bias in piRNA is currently unknown. One possibility is that the 1U bias arises during processing of cluster transcripts to mature piRNAs. Another possibility, at least in follicle cells, is that initial piRNA processing occurs in a completely random fashion, but that PIWI, the only recipient of mature *flam*-piRNAs in follicle cells (where AUB and AGO3 are not present), preferentially associates with 1U piRNAs. This would be in accordance with findings from the plant *Arabidopsis thaliana*, in which each AGO protein shows a unique 5′ nucleotide bias in the small RNAs with which it associates[52].

It is currently unclear how primary piRNAs are produced from piRNA clusters (FIG. 3a). Deficiency in PIWI proteins, for example PIWI in *D. melanogaster* and MILI and MIWI in mice, eliminates primary piRNAs. However, it is unlikely that PIWI proteins are directly involved in primary processing. Interestingly, piRNAs that associate with each member of the PIWI protein family have a distinct size (~26 nt, ~28 nt and ~ 30 nt for MILI, MIWI2 and MIWI, respectively, in mice and ~25 nt, ~24 nt and ~23 nt for PIWI, AUB and AGO3, respectively, in *D. melanogaster*), suggesting that PIWI proteins can act as 'rulers' that define the size of mature piRNAs. Recent studies have identified several additional proteins that are involved in primary piRNA biogenesis in *D. melanogaster*, including the putative RNA helicase Armitage (ARMI), the putative nuclease Zucchini (ZUC), and the Tudor domain and helicase domain protein Female sterile (1) Yb (FS(1)YB; also known as Yb). Mutations in and/or depletion of any of these three proteins eliminates primary piRNAs associated with PIWI from somatic follicle cells of the ovaries, and from the ovarian somatic sheet cells or the ovarian somatic cell (OSC) cultured cell line derived from ovarian somatic sheet cells[34,53–55]. ARMI and ZUC are also required for piRNA accumulation in germline cells in ovaries[51], whereas FS(1)YB is exclusively expressed in follicle cells[56].

piRNA clusters work as repositories of information, enabling production of many mature piRNAs that target diverse TEs. piRNA clusters in *D. melanogaster*, as well as clusters expressed during mouse embryogenesis — the 'pre-pachytene piRNA clusters' — are extremely rich in full-length and truncated copies of TEs[29,31,47]. As a result, up to 90% of piRNAs originating from such clusters match transposon sequences. Furthermore, the orientation of piRNAs exclusively derived from the uni-stranded clusters (the piRNA clusters transcribed in only one direction) is usually antisense to that of TE mRNAs[24,29,31]. These piRNAs can recognize and destroy TE transcripts derived from multiple sites spread throughout the genome. The best-characterized example of this is the *flam* locus in

Pachytene stage
A stage in meiotic prophase when homologous chromosomes are completely paired and chromosomal crossover occurs.

Phasing
An endonucleolytic processing pattern with an equal periodicity. Small interfering RNAs are endonucleolytically processed (or 'diced') by Dicer from individual precursors at certain intervals to produce a typical size of ~21 nucleotides. Dicer acts as a 'ruler' during processing.

Tudor domain
A conserved protein motif typically consisting of ~50 amino-acid residues. It is commonly found in proteins, such as RNA-binding proteins. Some Tudor domains specifically recognize and bind symmetrically dimethylated arginines.

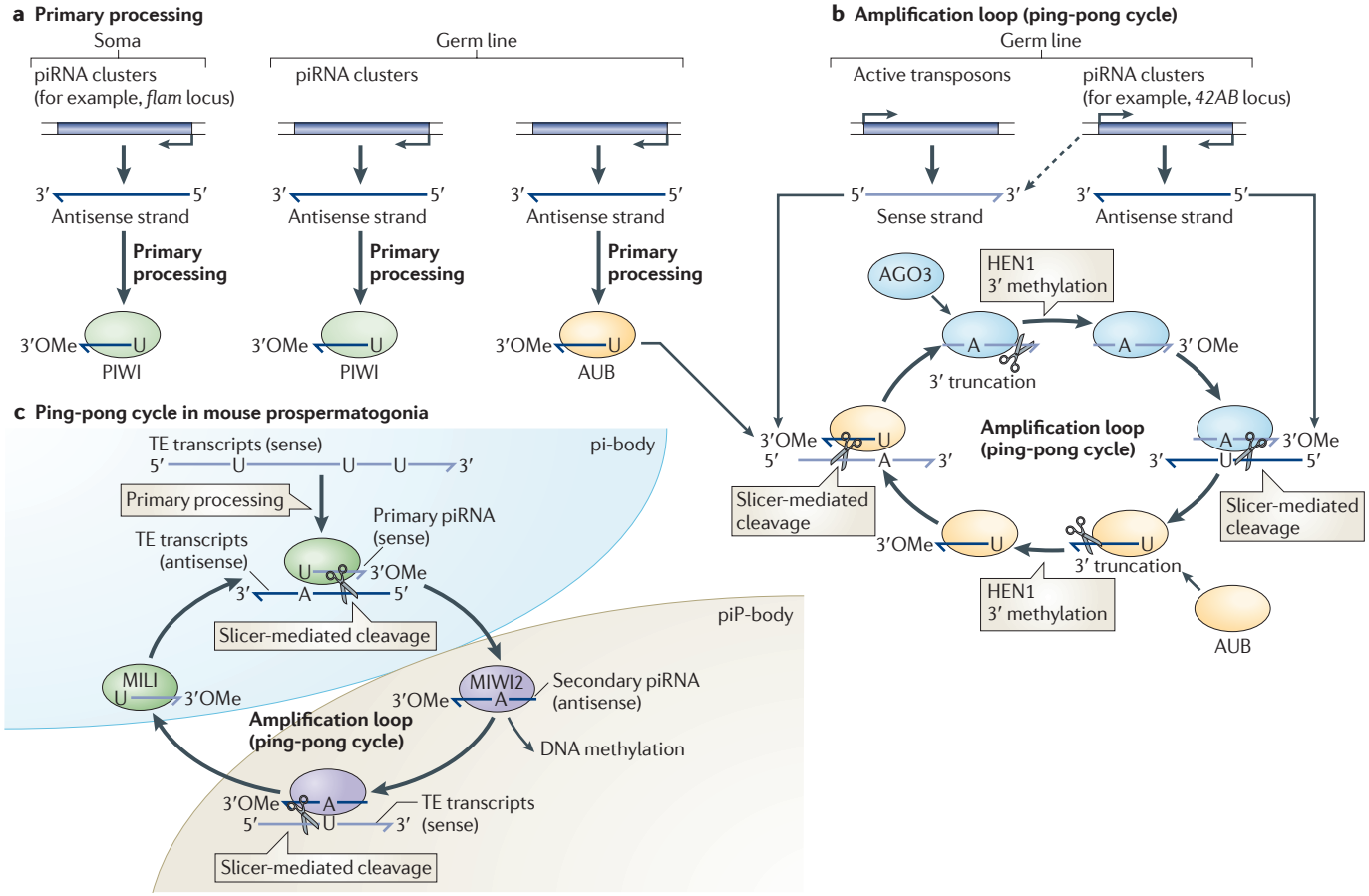


Figure 3 | **Two pathways for piRNA biogenesis. a** | In the *Drosophila melanogaster* primary PIWI-interacting RNA (piRNA) processing pathway, the primary antisense transcripts transcribed from transposons and/or the piRNA clusters are processed to piRNAs by unknown mechanisms and are loaded onto Aubergine (AUB) or PIWI[19,24,45]. piRNAs derived from the *flamingo* (*flam*) locus are exclusively loaded onto PIWI because *flam* is active only in ovarian somas where only PIWI is expressed. piRNA-induced silencing complexes (piRISCs) produced through this mechanism act as a 'trigger' of the amplification loop. **b** | This amplification mechanism (also known as the ping-pong cycle) is most likely to involve Slicer activity of AUB and Argonaute 3 (AGO3)[31,32], but not PIWI itself[24]. AUB associated with antisense piRNA cleaves piRNA precursors in the sense strand. This reaction determines and forms the 5′ end of piRNAs that are loaded onto AGO3. AGO3 associated with sense piRNA cleaves antisense piRNA precursors, generating the 5′ end of antisense piRNAs that subsequently are loaded onto AUB. The 3′ end of piRNA is formed by an unknown nuclease (or nucleases), which is followed by 2′-*O*-methylation mediated by HEN1. piRNAs that induce the amplification loop may also be deposited from the mother. **c** | During the ping-pong cycle in mouse prospermatogonia, primary piRNAs are processed from transposable elements (TEs) and cluster transcripts, and associate primarily with MILI (also known as PIWI-like protein 2), which resides in discrete cytoplasmic granules termed pi-bodies. MILI loaded with sense piRNA cleaves antisense transcripts, generating 5′ ends of secondary piRNAs that are loaded into MIWI2 (also known as PIWI-like protein 4). MIWI2 in turn cleaves sense transcripts, generating new sense piRNAs that are loaded into MILI. MIWI2 resides in cellular bodies that also contain components of processing bodies (P-bodies), and are therefore called piP-bodies. MIWI2 and its partners in turn translocate into the nucleus, where they are involved in *de novo* methylation of TE sequences. 3′OMe, 2′-*O*-methylation of the piRNA 3′ terminus.

D. melanogaster: *flam* is expressed in follicle cells of the ovaries and contains copies of *gypsy*, *Idefix* and *ZAM* TEs. Accordingly, specific disruption of this cluster activates these three transposons, as well as other elements that can be targeted by *flam*, in follicle cells[57–60]. The sequences of piRNA clusters are likely to be in constant flux, allowing adjustments that are tailored to new active transposons invading the species. Indeed, analysis of piRNA clusters in different *Drosophila* species has shown that, although the clusters' locations are conserved, their sequence content has evolved very quickly[24]. An unexplored question is whether the sequence content of piRNA clusters is updated by random integration of new TEs or by active mechanisms that target these new insertions specifically to the piRNA loci.

piRNAs are also generated from mRNAs of active TEs as well as some protein-coding genes[29,31,51]. This processing probably occurs using the same machinery that processes piRNA cluster transcripts. For protein-coding genes, such as the *traffic jam* (*tj*) locus in *D. melanogaster*, most piRNAs are derived from the 3′ untranslated region[31,51], suggesting that the translational

machinery affects processing (FIG. 2b). As only a small fraction of protein-coding mRNAs generates piRNAs, there must be a specific process that discriminates these transcripts. However, the rules that govern such selection are not clear. The function, if any, of piRNAs originating from gene loci is also mysterious: it is unlikely that they regulate expression of host genes, and neither do they have perfectly complementary targets. Some gene-derived piRNAs, such as those derived from *tj*, seem to make use of their imperfect complementarity and repress other protein-coding genes, including *Fas3* as described above[44] (FIG. 2b).

In some cell types, primary piRNA biogenesis is the only mechanism that generates piRNAs. In particular, all piRNAs in somatic follicle cells of the *D. melanogaster* ovary and pachytene piRNAs expressed during meiosis in mouse spermatocytes are generated by primary processing[10,11,24,44,48]. However, in other cell types, such as germline cells of the *D. melanogaster* ovary and pre-meiotic spermatogonia in mice, there is another mechanism called the ping-pong cycle that amplifies specific sequences generated by the primary biogenesis pathway.

The ping-pong amplification loop. In *D. melanogaster*, primary piRNAs are associated with PIWI and AUB, and are mostly antisense to transposon mRNAs[9,31–33,37]. Surprisingly, cloning of piRNAs associated with AGO3 in *D. melanogaster* revealed that these sequences do not have a 1U bias. Instead, they have a strong bias for adenosine at the tenth nucleotide from their 5′ end (10A) and are in the sense orientation. Furthermore, significant fractions of AGO3- and AUB-associated piRNAs are able to pair through their first ten nucleotides[31,32]. In addition, recombinant PIWI proteins exhibit Slicer activity, which cleaves target RNAs between their tenth and eleventh nucleotides relative to the 'guide' small RNAs[32]. These observations have led to a 'ping-pong' model of piRNA biogenesis, in which PIWI proteins amplify piRNAs in a Slicer-dependent amplification cycle (FIG. 3b). This model suggests that cleavage of complementary transcripts targeted by AUB-bound 1U primary piRNA leads to the generation of the 5′ end of new secondary piRNAs that have a 10A bias, and accordingly can be loaded onto AGO3 (REFS 31,32). The enzymatic activity that generates the 3′ end of these secondary piRNAs is unknown. AGO3, loaded with secondary piRNAs, is able to then recognize and cleave complementary transcripts. This results in the generation of further piRNAs that exactly correspond to the original primary piRNA sequences and are loaded onto AUB. The ping-pong pathway also amplifies piRNAs in *D. melanogaster* testes, especially those originating from TEs. Non-TE-derived piRNAs — such as *Suppressor of Stellate* (*Su(ste)*)-derived piRNAs, which silence *Stellate* — seem to be barely amplified by the amplification loop[61].

The distinct functions of AUB and AGO3 in the ping-pong cycle of *D. melanogaster* explain their non-redundant roles in the silencing of the same set of transposons[19,31]. MILI and MIWI2 in mice also have non-redundant roles in the ping-pong cycle and transposon repression, with MILI preferentially associating with primary piRNAs and MIWI2 with secondary piRNAs[29] (FIG. 3c). However, the orientation of piRNAs in mice is reversed compared with fruitflies, with the majority of mouse primary piRNAs being sense and secondary piRNAs being antisense relative to transposons. The operation of an amplification loop that requires two PIWI members is supported by genetic data: in *Mili*-deficient mice that fail to generate primary piRNAs, MIWI2 also remains unloaded[19,29]; and in fly *Ago3* mutants, the number of AUB-bound piRNAs was markedly decreased and their antisense strand bias was weakened[19]. However, it is also possible that a single PIWI protein might have dual roles in the amplification loop by accepting both primary and secondary piRNAs, as has been shown for MILI expressed in postnatal spermatogonia without its MIWI2 partner[29].

It has been proposed that the biological role of the ping-pong cycle is to fine-tune the piRNA population so that active TEs can be targeted[29,31]. Indeed, the cycle amplifies piRNAs only if corresponding sense and antisense transcripts are present within the cell. Whereas antisense transcripts are usually derived from piRNA clusters, sense transcripts often arise from mRNAs of active transposons[29]. The two steps of piRNA biogenesis — the primary processing pathway and the amplification loop — and their function in mounting defence against TEs can be compared with the function of the adaptive immune system in protecting against pathogens. The primary piRNA biogenesis pathway resembles the initial generation of the hypervariable antibody repertoire, whereas the amplification loop is analogous to antigen-directed clonal expansion of antibody-producing lymphocytes during the acute immune response.

The importance of the amplification loop in defence against transposons is underlined by studies of hybrid dysgenesis in *D. melanogaster*[62] (BOX 2). This phenomenon is observed in crosses between two different fly stocks, which result in sterile progeny with severe gonadal dystrophy and activation of specific transposons, such as P- and I-elements. The peculiarity of hybrid dysgenesis is that it is observed only when males of one stock are crossed to females of the other, and no abnormalities are observed for the reciprocal cross. As the genotypes of the progeny of both crosses are identical, epigenetic factors must be responsible for the hybrid dysgenesis phenotype. It was revealed that the transposon activation that results is caused by a failure to maternally deposit piRNAs that target specific, paternally deposited transposons[62,63]. Maternal deposition of piRISCs into the egg provides the embryo with an initial piRNA pool to initiate the amplification cycle and build up resistance to TEs.

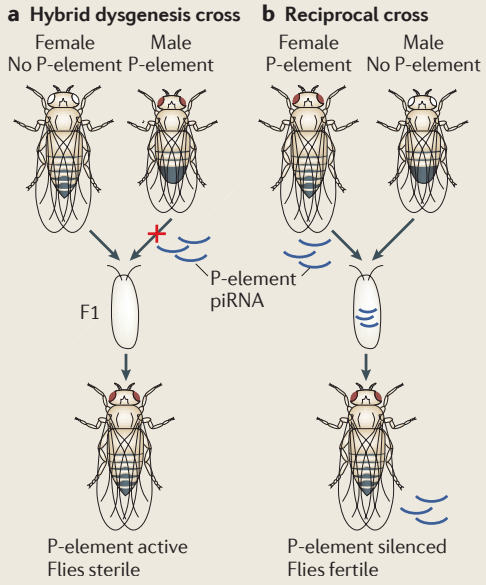
Regulation by PIWI-interacting proteins

piRNAs are tightly bound to PIWI proteins that can repress complementary target loci and generate secondary piRNAs using their Slicer endonuclease activity. However, genetic studies in both *D. melanogaster* and mice have revealed several other proteins that are required for efficient piRNA-mediated silencing. Furthermore, some of these proteins interact directly with PIWI proteins. Analysis of the post-translational modification of PIWI proteins has revealed that they

Hypervariable antibody repertoire
The antibody specificities that can potentially be produced by an individual. A huge repertoire of different antibodies is generated in a single individual.

Box 2 | **Hybrid dysgenesis and piRNA silencing**

When two *Drosophila melanogaster* stocks are crossed together, the genome of one stock (shown with red eyes in the figure) contains P-element transposons, the activity of which is suppressed by corresponding PIWI-interacting RNAs (piRNAs). Another stock (shown with white eyes in the figure) is devoid of P-elements and cognate piRNAs. In a 'hybrid dysgenesis' cross (part **a** of the figure), piRNAs cannot be deposited into the embryo through the paternal germ line, which results in transposon activation and sterility. By contrast, in a 'reciprocal' cross (part **b** of the figure), maternal deposition of piRNAs into the embryo through the female germ line initiates the ping-pong cycle of piRNA amplification, which provides resistance against P-elements in the progeny and results in flies that are fertile[62].



contain conserved symmetrical dimethyl arginine (sDMA) residues that provide a binding platform for Tudor-domain-containing proteins[64,65] (FIG. 4).

Tudor-domain proteins. Biochemical analysis of proteins that co-purify with PIWI proteins in *D. melanogaster* and mice has revealed that PIWIs associate with protein arginine N-methyltransferase 5 (PRMT5; also known as DART5 or Capsuleen in *D. melanogaster*)[66–68]. In conjunction with methylosome protein 50 (MEP50; also known as Valois in *D. melanogaster*), PRMT5 symmetrically methylates conserved arginine residues present at the amino termini of PIWI proteins in multiple animal species[67–70]. Interestingly, these residues are absent in AGO proteins, suggesting that arginine dimethylation is a molecular signature of the piRNA pathway. Proteomic studies have revealed that mouse PIWI complexes contain several proteins that have single or multiple Tudor domains that are known to interact with sDMAs[68–70]. The three mouse PIWI members associate with several Tudor-domain proteins that have varying expression patterns during gametogenesis[69–72] (FIG. 4a). Furthermore, Tudor and PIWI proteins interact when expressed in a heterologous cell culture system, and this interaction requires the presence of sDMAs[68]. Biochemical studies have suggested the existence of a complex recognition code between members of the PIWI and Tudor families[68,73–75] (FIG. 4b). In *D. melanogaster*, Tudor (TUD), a factor that is necessary for gametogenesis and contains 11 Tudor domains[76–79] (FIG. 4c), associates with AUB and AGO3 (REFS 66,67). *D. melanogaster* PIWI also contains sDMAs; however, it is unknown which Tudor-domain protein binds to these[66]. The structural basis for the sDMA-dependent interaction between PIWI proteins and Tudor domains was recently determined, which revealed that the five carboxy-terminal tandem Tudor domains of TUD (TUD7 to TUD11) are necessary and sufficient for AUB binding and germ cell formation, and that the binding characteristics of eTUD11 (a fragment encompassing the eleventh TUD domain of Tudor) resembles those of TUD7 to TUD11. Four aromatic residues that are located in eTUD11 — Phe2403, Tyr2410, Phe2427 and Phe2430 — were determined to form the sDMA binding cage[80,81]. Several other proteins that are required for piRNA-mediated silencing in flies, such as Spindle-E (SPN-E), FS(1)YB, Krimper (KRIMP) and Tejas, have a single Tudor domain, although their direct binding partners are not yet known (FIG. 4c). The interaction between Tejas and AUB is sDMA-independent[75], suggesting another mechanism by which PIWI proteins can interact with Tudor-domain-containing proteins.

Proteins that contain multiple Tudor domains have the potential to interact simultaneously with several proteins through sDMAs and function as a scaffolding platform for the assembly of multi-protein complexes. This might be particularly important for the ping-pong mechanism, which requires close association between two PIWI proteins. Indeed, an estimation based on structural studies suggested that TUD might bind several AUB molecules simultaneously[80]. Accordingly, analysis of the piRNA pathway in Tudor domain containing 1 (*Tdrd1*)-deficient mice demonstrated that although piRNAs are still present, the ping-pong cycle for generating secondary piRNAs is impaired[68]. Another study produced different results indicating that, in *Tdrd1*-deficient mice, MILI starts to accept small RNAs originating from ribosomal RNAs and protein-coding RNAs, both exonic and intronic, although the underlying mechanism remains unclear[70]. Similarly, loss of *Tud* function in *Drosophila* ovaries did not eliminate piRNAs; however, the population of transposon-derived piRNAs was substantially changed[67]. Similarly to deficiencies in MILI and MIWI2, deficiencies in the Tudor proteins TDRD1 and TDRD9 lead to a failure in *de novo* methylation of TEs in mouse germline cells, and consequent TE overexpression[70,71]. Overall, it seems that deficiencies in individual Tudor-domain proteins significantly impair the piRNA pathway, especially its ping-pong components, but do not abolish it. It is plausible that several Tudor-domain-containing proteins have partially redundant roles in the piRNA pathway, explaining the relatively mild phenotypes of the individual mutants. Some Tudor-domain-containing proteins, such as SPN-E and FS(1)YB, are crucial for the piRNA pathway[24,34,54,56,82]. These proteins also contain a DEAH-type RNA helicase domain, but their molecular functions in the PIWI complex are unclear.

Additional regulators of piRNA-mediated silencing. There are several proteins that do not contain Tudor domains but are required for piRNA-mediated silencing. In particular, the putative RNA helicase ARMI is crucial for primary piRNA biogenesis in *D. melanogaster,* and its mammalian homologue MOV10L1 seems to have a similar role in mice[54,82–84]. It is unknown,

however, whether the RNA helicase domain exhibits genuine enzymatic activity and how this affects piRNA biogenesis. *Armi* may also be necessary for secondary piRNA amplification, because mutations in *Armi* caused a reduction in PIWI- and AUB-associating piRNAs; this was also observed for mutations in *rhino*, which encodes an HP1 homologue that associates with DNA of the *42AB* piRNA cluster (a dual strand cluster), and Rhino is required for its transcription and secondary piRNA accumulation[46].

Vasa and its murine homologue, MVH (also known as DDX4), are conserved components of germline granules and have been implicated in the piRNA pathway[61,72,82,85–87]. Vasa contains an RNA helicase domain, which has RNA helicase activity[88], but the importance of this domain in the piRNA pathway is still unknown. Similarly to PIWI proteins, Vasa has sDMAs at its N terminus that can be recognized by Tudor domains[74]. However, the proteins that interact with Vasa specifically through its sDMAs remain undetermined.

Maelstrom (MAEL), a high mobility group box (HMG-box) protein, is involved in the piRNA pathway in both *D. melanogaster* and mice[82,89–92], although its molecular functions during piRNA biogenesis are not yet fully understood. In mice, the MILI-interacting protein GASZ (also known as ASZ1), a structural protein that is crucial for MILI stability and mediates formation of the cellular bodies occupied by MILI, has also been implicated in the biogenesis of pachytene piRNAs[93]. Squash (SQU), a putative nuclease in flies, might also be involved in the piRNA pathway, particularly at the 'effector' (silencing) step; this is in contrast to ARMI and ZUC, both of which function in the 'initiation' (piRNA biogenesis) phase of the piRNA pathway[53]. This is because mutations in *squ* show little impact on piRNA populations[24] but display an effect on TE silencing[53] in mutant ovaries. The precise molecular functions of many PIWI-interacting proteins in the piRNA pathway await further investigation.

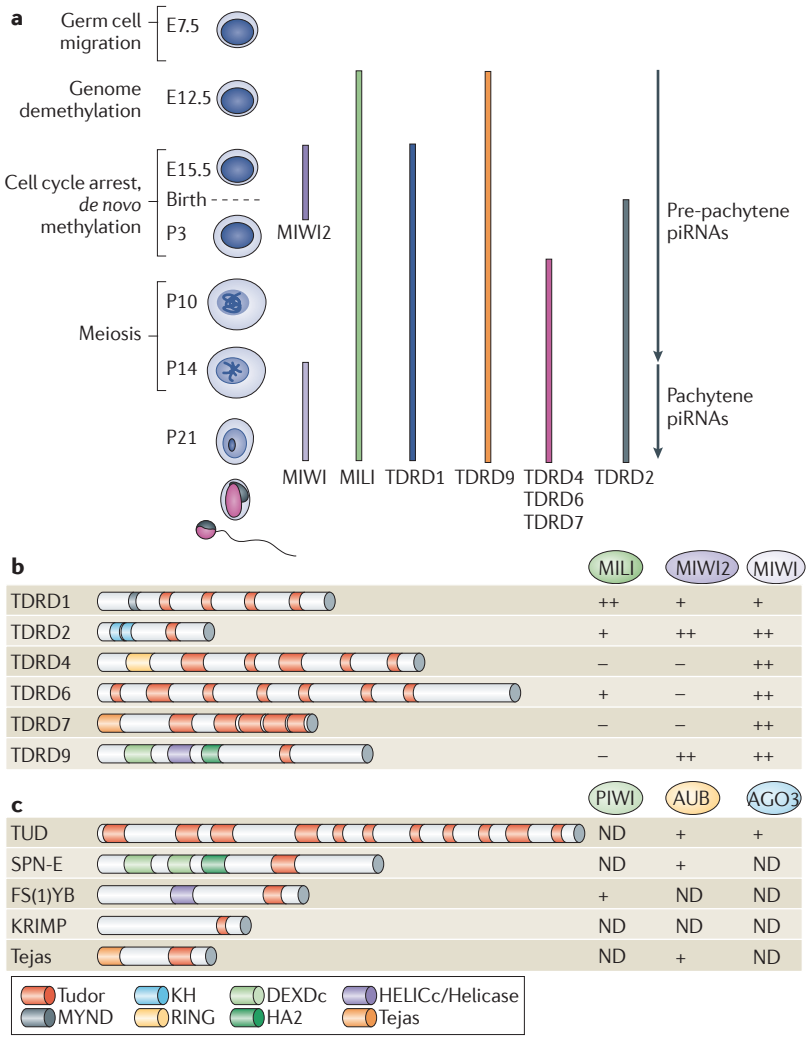


Figure 4 | **PIWI and Tudor proteins regulate the piRNA pathway. a** | Expression of PIWI, Tudor proteins and PIWI-interacting RNAs (piRNAs) during mouse spermatogenesis. MIWI2 (also known as PIWI-like protein 4) is the only mouse PIWI protein that, together with Tudor domain containing 9 (TDRD9), localizes in the nucleus when *de novo* methylation is established (between embryonic day 15.5 (E15.5) and postnatal day 2 (P2)). Pre-pachytene piRNAs are enriched in sequences that match transposable elements (TEs) and associate with MILI (also known as PIWI-like protein 2) and MIWI2. Pachytene piRNAs that are expressed starting at the pachytene stage of meiosis (P14) are not enriched in TE sequences, are expressed from a different set of piRNA clusters and are associated with MIWI (also known as PIWI-like protein 1) and MILI. Expression of different Tudor-domain proteins also starts at different stages of spermatogenesis. **b** | Biochemical interactions between mouse PIWI and Tudor proteins. The domain structure of Tudor proteins is shown on the left[64] and the PIWI proteins that these proteins interact with are shown on the right. **c** | Biochemical interactions between *D. melanogaster* PIWI and Tudor proteins. The domain structures of Tudor proteins are shown on the left and PIWI proteins that interact with these proteins are shown on the right. AGO, Argonaute; AUB, Aubergine; DEXDc, DEXDc helicase domain; FS(1)YB, Female sterile (1) Yb (also known as Yb); HA2, helicase-associated domain; HELICc, helicase superfamily carboxy-terminal domain; KH, K homology RNA-binding domain; KRIMP, Krimper; MYND, MYND (myeloid, Nervy, DEAF-1)-type zinc finger domain; ND, not determined; RING, RING-type zinc finger domain; SPN-E, Spindle-E; TUD, Tudor.

Spatial control of piRNA biogenesis

Different PIWI proteins are localized to either the cytoplasm or the nucleoplasm. In the cytoplasm, PIWI proteins and many of their interactors colocalize in distinct granular cytoplasmic structures that are present in the germline cells of many metazoa species. It should be emphasized that, even for PIWI members that are present in the nucleus, such as PIWI in *D. melanogaster* and MIWI2 in mice, there is accumulating evidence that production and loading of piRNAs occurs in the cytoplasmic granules[15,29,44,54,92]. Germline granules that contain components of the piRNA pathway tend to be located in close proximity to the nucleus[15,33,61,67,71,75,86,90–97], suggesting that they serve as structures that recognize and process piRNA precursors exported from the nucleus to the cytoplasm.

Germline granules for piRNA production. Developing germ cells of the *D. melanogaster* ovary contain nuage, an electron-dense, non-membranous perinuclear structure[98,99] (FIG. 5a). Nuage can be detected in nurse cells but is absent from the transcriptionally quiescent oocyte; thus, the presence of nuage seems to correlate with active transcription. Many nuage components, including PIWIs loaded with piRNAs, are supplied by nurse cells to the oocyte and localize at its posterior end[33,66,94].

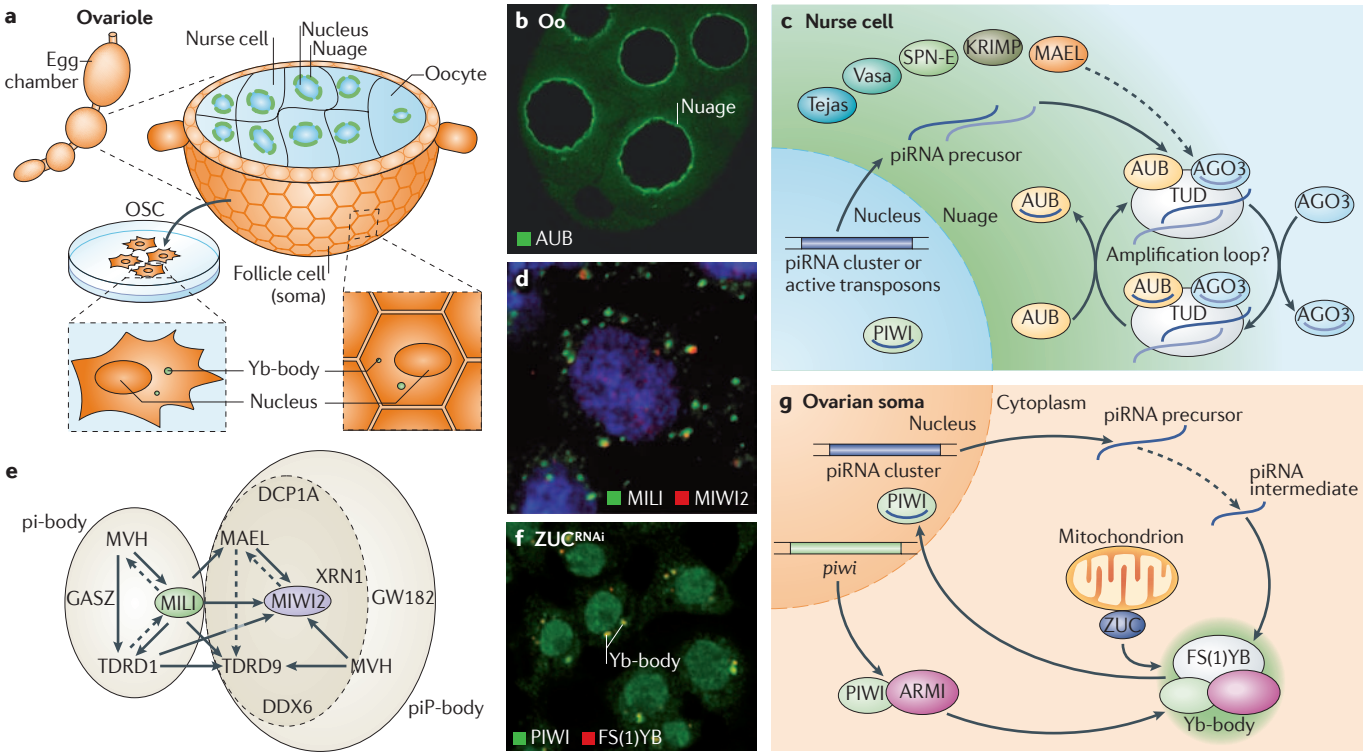


Figure 5 | **piRNA biogenesis takes place in cellular bodies. a** | A schematic representation of *Drosophila melanogaster* egg chambers. At mid-stage oogenesis, the *D. melanogaster* egg chamber consists of one oocyte and 15 nurse cells and somatic follicle cells. Nuage is a non-membranous structure that is located in the perinuclear region of nurse cells. Yb-bodies are cytoplasmic non-membranous structures that are found only in somatic follicle cells, and these can also be observed in ovarian somatic cells (OSCs) derived from follicle cells. **b** | Cellular localization of Aubergine (AUB; shown in green) in a stage-6 egg chamber[33], which clearly shows that AUB accumulates at nuage. **c** | Nuage is probably where the amplification loop takes place. In nurse cells, PIWI-interacting RNA (piRNA) factors such as AUB and Argonaute 3 (AGO3) localize to the nuage. Tudor (TUD) associates with AUB and AGO3 simultaneously in germ cells. Through this association, Tudor might activate the amplification loop. Tudor may also have a role in recruiting piRNA precursors to piRNA-induced silencing complexes (piRISCs). Vasa, Spindle-E (SPN-E), Krimper (KRIMP), Tejas and Maelstrom (MAEL) localize to nuage, which suggests that they are involved in this pathway. However, their functions in piRNA biogenesis still remain unclear. Unlike AUB and AGO3, PIWI shows steady-state localization to the nucleus in nurse cells. **d** | In mouse prospermatogonia, MILI (also known as PIWI-like protein 2) localizes in multiple small granules termed pi-bodies, whereas MIWI2 (also known as PIWI-like protein 4) is present in fewer and bigger granules, known as piP-bodies, that are often in contact with MILI granules. **e** | The composition of pi- and piP-bodies. Strong and weak genetic interactions between different components that guarantee correct localization of corresponding proteins are shown as solid and dashed arrows, respectively. **f** | Immunofluorescence of *zucchini* (*zuc*)-depleted OSCs stained with Female sterile (1) Yb (FS(1)YB; also known as Yb)-specific antibodies shows that PIWI is located in Yb-bodies in OSCs[34]. **g** | Yb-bodies are the sites where primary piRNAs are produced in follicle cells of the ovary, where PIWI, but not AUB or AGO3, is expressed. Armitage (ARMI) associates with PIWI and localizes it to Yb-bodies[34,54]. FS(1)YB is the main component of Yb-bodies. The piRNA intermediates, which are partially processed from the primary precursors, are loaded onto the complex comprising ARMI, PIWI and FS(1)YB at Yb-bodies and processed into mature piRNAs. ZUC, a putative nuclease that localizes to mitochondria, is also required for the primary piRNA processing. The majority of ZUC-positive mitochondria are in fact located close to Yb-bodies[34]. Without piRNA loading, PIWI is not localized to the nucleus. These findings support the idea that Yb-bodies not only generate primary piRNAs for PIWI but also inspect the functional piRNA–PIWI complex before it enters the nucleus. DCP1A, Decapping protein 1A; DDX6, DEAD-box protein 6; GASZ, also known as ASZ1; GW182, also known as TNRC6; MVH, also known as DDX4; Oo, oocyte; TDRD, Tudor domain containing. Part **b** is reproduced, with permission, from REF. 33 © (2007) Cold Spring Harbor Laboratory Press. Part **d** is reproduced, with permission, from REF. 29 © (2008) Elsevier. Part **f** is reproduced, with permission, from REF. 34 © (2010) Cold Spring Harbor Laboratory Press.

Germline granule
A specialized electron-dense structure that comprises particular RNAs and proteins that are indispensable for germline development. Vasa, an RNA helicase, is a well-characterized component of germline granules.

High mobility group box
(HMG-box). A protein domain involved in DNA binding that comprises ~80 amino-acid residues that form three α-helices.

Some of the nuage components give rise to the pole plasm, the structure that is both necessary and sufficient to induce formation of primordial germ cells in the developing embryo[100].

Nuage contains many other proteins that are genetically required for piRNA-mediated silencing, such as SPN-E, MAEL, Vasa, KRIMP, Tejas and TUD[75,82] (FIG. 5b,c). Deficiencies in these proteins eliminate piRNAs or distort the piRNA profile. Furthermore, they can cause other components from nuage to delocalize or even cause the structure to disintegrate. Many nuage components, including TUD, SPN-E, KRIMP and Tejas, have Tudor domains, whereas others, such as PIWI proteins and Vasa, have sDMAs that are recognized by

Tudor domains. Accordingly, protein–protein interactions within nuage are often mediated by Tudor–sDMA associations. It has been proposed that nuage might be a site for piRNA biogenesis and/or for the recognition and destruction of piRNA targets[82].

In mice, PIWI proteins are expressed at different stages of testis development, but all are localized in the cytoplasm of germ cells in electron-dense granular structures that resemble nuage in *D. melanogaster*[28,29,72,92,93,95]. These granules have been termed 'intermitochondrial cement' or 'chromatoid bodies' (CBs), depending on the particular stage of spermatogenesis. Some components of these granules, such as MVH, are present at all stages[96,98]. MILI and MIWI2 are co-expressed during embryonic testis development; however, unlike AUB and AGO3 in *D. melanogaster*, they are localized in two distinct types of granule. MILI granules, which were originally described as intermitochondrial cement, contain the Tudor-domain-containing protein TDRD1 in addition to MVH[70,87,98] and GASZ[93] (FIG. 5d,e). MIWI2 granules, which are often found in a close proximity to MILI granules, include another Tudor protein, TDRD9, and MAEL. MIWI2 granules contain all of the known components of processing bodies (P-bodies), which are cytoplasmic granules that are responsible for mRNA storage and decay in somatic cells (FIG. 5d,e). Accordingly, they have been dubbed 'piP-bodies'. As MILI granules or intermitochondrial cement harbour components that are exclusively involved in the piRNA pathway, they are also called 'pi-bodies'[98]. MILI, but not MIWI or MIWI2, has been reported to be expressed in the female germ line, where it localizes in cytoplasmic granules in both arrested and growing oocytes[29].

The localization of many components of pi- and piP-bodies is co-dependent. For example, pi-bodies do not seem to form in *Mvh* and *Gasz* mutants[87,93]. The presence of MILI in pi-bodies depends on TDRD1 (REF. 68). Furthermore, TDRD1 can attract MILI into the granules when both proteins are co-expressed in a heterologous cell culture system, underlining the pivotal role of the Tudor–sDMA interaction in granule formation[68]. Currently, it is unknown whether there is functional crosstalk between components of the piRNA and P-body pathways within individual piP-bodies, although pi- and piP-bodies do physically and functionally interact with each other. Disruption of MILI-containing pi-bodies also disintegrates MIWI2-containing piP-bodies, but not vice versa[29]. This agrees with the notion that MIWI2 is downstream of MILI in the ping-pong mechanism (FIG. 3c). Disruption of pi-bodies also prevents loading of piRNAs onto MIWI2 and prevents it from entering the nucleus. This suggests that the key steps of piRNA biogenesis occur in these cytoplasmic granules. Disruption of the pi-bodies and piP-bodies also results in overexpression of TEs and loss of *de novo* DNA methylation of their corresponding genomic loci. Thus, the correct structure of these compartments heavily influences the efficiency of TE silencing. After birth, MIWI2 expression ceases, but pi-bodies are still present in spermatogonia and early spermatocytes. Expression of MIWI commences during meiosis, followed by the formation of CBs. A CB is a single large granule present in haploid spermatids and contains MILI, MIWI, MVH, MAEL and multiple Tudor-domain-containing proteins, such as TDRD1 and TDRD6 (REFS 72,95,97). Similarly to piP-bodies, CBs harbour some P-body components, such as AGO proteins[101]. The CB is probably the site where pachytene piRNAs, in complex with MIWI and MILI, exert their function. However, as their targets are unknown, this assumption still awaits experimental verification.

Yb-bodies in the* D. melanogaster *ovary. Primary piRNA biogenesis and silencing of TEs take place in somatic follicle cells of the *D. melanogaster* ovary and in the cultured OSC line derived from *D. melanogaster* follicle cells[24,34,44,54] (FIG. 5a). However, these follicle cells are devoid of typical nuage as they do not express a nuage marker, Vasa, as well as the two PIWI proteins AUB and AGO3. Many proteins that are required for the piRNA pathway in germline cells, such as SPN-E, Vasa, MAEL, TUD and KRIMP, are dispensable for primary piRNA production in somatic cells, suggesting that the piRNA pathway uses different machineries in germline and somatic cells. Interestingly, recent studies have demonstrated that, similarly to germline cells, the piRNA pathway in somatic follicle cells is tightly associated with cytoplasmic granules called Yb-bodies[34,54,55] (FIG. 5a,f,g). Yb-bodies do not overlap with known cytoplasmic granules such as P-bodies or U-bodies (the latter of which are implicated in snRNP (RNA-protein complex) metabolism) and have been previously implicated in RNA metabolism[56].

Genetic and RNA interference-based screens in flies and OSCs, respectively, have identified PIWI and three other proteins, ARMI, ZUC and FS(1)YB, as being required for piRNA-mediated TE silencing and primary piRNA accumulation[34,54,55] (FIG. 5f,g). ARMI and FS(1)YB colocalize at Yb-bodies. However, FS(1)YB seems to be the core factor in Yb-body formation: ARMI localization in Yb-bodies requires FS(1)YB, but FS(1)YB does not require ARMI for localizing to Yb-bodies. Both PIWI and FS(1)YB interact with ARMI. Without FS(1)YB or ARMI, PIWI localizes to neither Yb-bodies nor the nucleus. Thus, PIWI's subcellular localization depends on ARMI. In the absence of ZUC, PIWI accumulates together with ARMI in the Yb-bodies and does not enter the nucleus (FIG. 5f,g). Upon depletion of ARMI or FS(1)YB, PIWI is no longer loaded with piRNAs and does not localize to the nucleus, suggesting that piRNA loading is required for PIWI nuclear entry. It is plausible that cytoplasmic Yb-bodies are the sites at which piRISC function is checked before nuclear entry is permitted (FIG. 5g). Taken together, these data indicate that cytoplasmic granular structures are indispensable for piRNA-related mechanisms in both germline and somatic follicular cells, although the composition of the granules is different in these two cell types.

Conclusions

piRNAs, in complex with PIWI proteins, form a conserved silencing pathway that is related to other small RNA silencing pathways but is clearly distinct in its mechanism and functions. piRNAs provide an elaborate system that protects the germline and gonadal

somatic cells against the harmful expression of TEs. Several reports indicate that piRNAs might also target and regulate expression of normal host protein-coding genes. However, these data should be further verified. piRNAs also mediate *de novo* methylation of TE sequences in the mammalian male germ line. This provides a tantalizing link between two modes of epigenetic regulation of gene expression: post-transcriptional silencing by small RNAs and transcriptional repression by local chromatin structure. Further studies should reveal the molecular mechanism by which piRNAs affect DNA methylation and potentially expand our use of small RNAs as tools in research and therapy.

Compared with other small RNA silencing mechanisms, the piRNA pathway remains poorly investigated, mainly because it primarily operates in germline cells. Biochemical and genetic studies in flies and mice have identified the protein partners of PIWI proteins; however, crucial questions regarding the enzymatic machineries that process piRNAs and the rules that define the recognition of precursor transcripts remain unanswered. Future research should explain how the transcription of piRNA clusters, which occupy a substantial portion of our genomes, is regulated. Furthermore, understanding the molecular control of the piRNA pathway will hopefully allow researchers to direct piRNAs to new artificial targets. Studies in flies and mice have revealed a tight association between the piRNA machinery and germline granules (specific cytoplasmic organelles that are present in animal gonads). These reports have raised the idea, which should be explored further, that nuage is a processing site for piRNAs and/or the place where silencing takes place. Overall, the hope is that future studies will fill in these major gaps in our understanding of how piRNA biogenesis and function is governed and, undoubtedly, yield many exciting discoveries.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Mikiko C. Siomi's homepage: http://web.sc.itc.keio.ac.jp/dmb/sindex.html
Alexei A. Aravin's homepage: http://biology.caltech.edu/

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