NON-CODING RNA

PIWI-interacting RNAs: small RNAs with big functions

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Abstract | In animals, PIWI-interacting RNAs (piRNAs) of 21–35 nucleotides in length silence transposable elements, regulate gene expression and fight viral infection. piRNAs guide PIWI proteins to cleave target RNA, promote heterochromatin assembly and methylate DNA. The architecture of the piRNA pathway allows it both to provide adaptive, sequence-based immunity to rapidly evolving viruses and transposons and to regulate conserved host genes. piRNAs silence transposons in the germ line of most animals, whereas somatic piRNA functions have been lost, gained and lost again across evolution. Moreover, most piRNA pathway proteins are deeply conserved, but different animals employ remarkably divergent strategies to produce piRNA precursor transcripts. Here, we discuss how a common piRNA pathway allows animals to recognize diverse targets, ranging from selfish genetic elements to genes essential for gametogenesis.

PIWI-interacting RNAs (piRNAs) are an animal-specific class of small silencing RNAs, distinct from microRNAs (miRNAs) and small interfering RNAs (siRNAs). piRNAs bear 2′-*O*-methyl-modified 3′ termini and guide PIWI-clade Argonautes (PIWI proteins) rather than the AGO-clade proteins, which function in the miRNA and siRNA pathways[1–19] (BOX 1).

miRNAs and siRNAs derive from double-stranded RNA precursors, but piRNAs are processed from long single-stranded precursor transcripts[1–3,7,20]. The exception is nematodes, whose piRNAs (21U-RNAs) are made one at a time from single-stranded precursors of 25–27 nucleotides in length, each of which is transcribed from its own mini-gene[21–23]. piRNA precursors are transcribed from genomic loci known as piRNA clusters. In many arthropods, piRNA clusters correspond to large graveyards of transposon remnants[20,24–26]; in birds and mammals, piRNA clusters give rise to long non-coding RNAs (lncRNAs), which are processed into piRNAs[1,2,27]. piRNA sequences are immensely diverse and rarely conserved among species (FIG. 1a).

In most animals, at least a subset of piRNAs defend the germline genome against transposon mobilization[3,7,20,28–30]. How the piRNA pathway discriminates between self transcripts and non-self transcripts remains a central question in piRNA research. This Review discusses current models for piRNA cluster transcription, piRNA biogenesis and piRNA functions in the context of the developmental challenges faced by different animals.

Discovery of piRNAs

piRNAs were first identified in the fly testis as a novel class of 'long siRNAs' that silence *Stellate*, a multi-copy gene on the *Drosophila melanogaster* X chromosome[31]. Unchecked, the Stellate protein crystalizes in spermatocytes, impairing male fertility[32–37]. Consequently, the Y chromosome has amassed many copies of *Suppressor of Stellate*, a piRNA-producing gene derived from *Stellate* itself[31,38]. The subsequent discovery that *flamenco* — a gene long known to repress *gypsy* family transposons[39] — produced piRNAs rather than encoding a protein united piRNAs with earlier genetic studies of transposon silencing and implicated the protein Piwi as central to this process[40].

piRNAs guide PIWI proteins in gonads of insects[3,6], mammals[1,2,4,5], nematodes[8,9] and fish[7]. To date, piRNAs and PIWI proteins have been found in the vast majority of animals, except for several species including most nematodes[41–43].

Challenges of transposon silencing in animals

Every animal genome fights an endless war against parasitic transposable elements. Over evolutionary time, battles are won and new conflicts begin. The war is fought in the genome of the germ line: transposons must integrate into the germ cell DNA to survive. Once transposons are silenced, mutations ultimately inactivate transposon-encoded proteins, leading to the demise of the transposon. The saga of host–transposon conflict is best understood for the *D. melanogaster* and mouse piRNA pathways, which highlight the common

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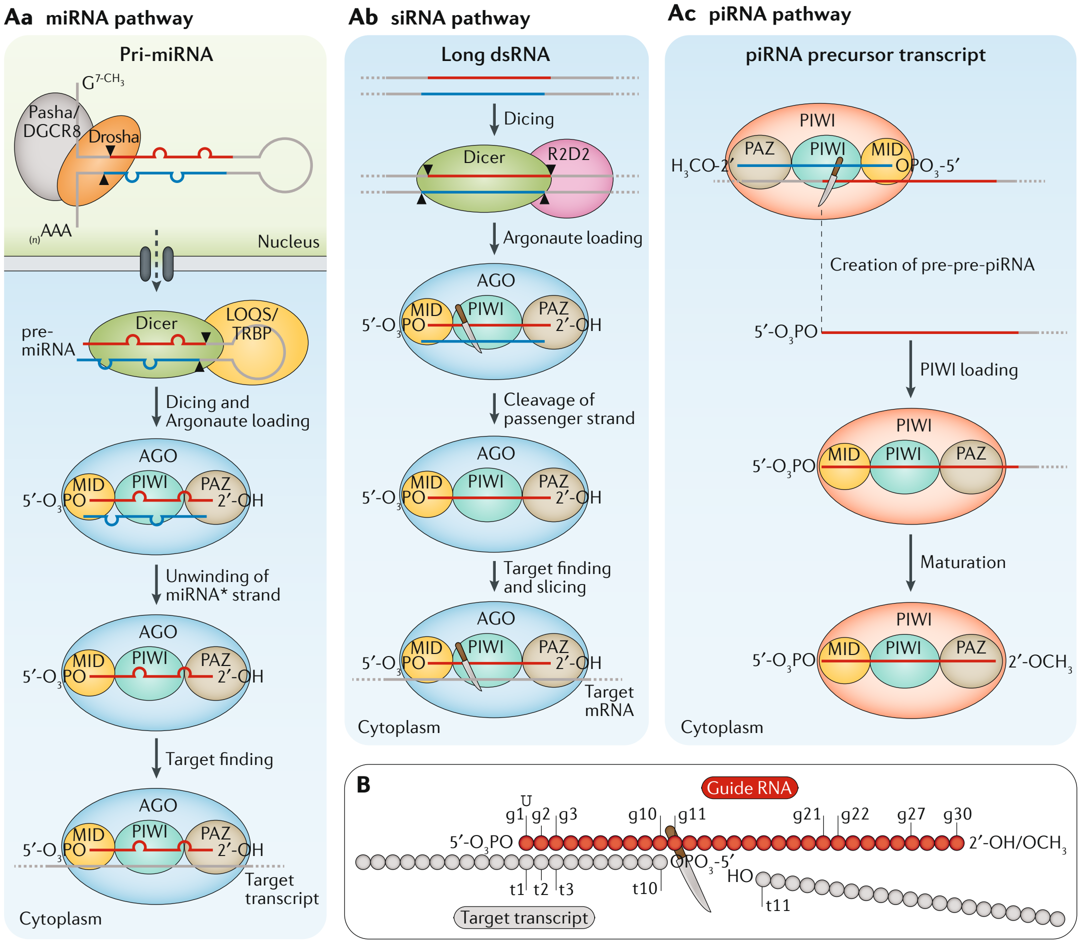
Box 1 | **Argonaute family proteins**

Small silencing RNAs, 21–35 nucleotides in length, bind to Argonaute family proteins and silence complementary transcripts either transcriptionally or post-transcriptionally. Argonaute family proteins are classified into the AGO and PIWI clades (reviewed previously[239,240]). Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are cleaved from double-stranded RNA (dsRNA) precursors by ribonuclease III family endonucleases and guide the ubiquitously expressed AGO proteins[241–246]. Drosha generates pre-miRNAs from primary miRNA (pri-miRNA) transcripts[247], whereas Dicer converts pre-miRNAs into mature miRNAs and long double-stranded RNA into siRNAs[244,248–250]. The resulting small duplex RNAs bear 5′ monophosphate and 2′,3′ hydroxyl, 2-nucleotide overhanging 3′ ends, the hallmarks of ribonuclease III enzyme products. Once an miRNA or an siRNA duplex is loaded into an Argonaute protein, the choice of guide strand reflects the relative thermodynamic stability[251–254] and first nucleotide composition of its 5′ ends[255]. The passenger strand is eliminated by passive unwinding or is cleaved by Argonaute itself; the guide strand, whose 5′ end is less tightly paired, is retained[256–258] (see the figure, part **A**). Many animal siRNAs and all animal miRNAs bear 2′,3′ hydroxy termini, although some arthropod siRNAs are 2′-*O*-methylated[26,259] like PIWI-interacting RNAs (piRNAs).

PIWI-clade proteins are often restricted to gonadal cells and are loaded with piRNAs of 21–35 nucleotides in length. Unlike siRNAs and miRNAs, piRNAs are processed from single-stranded RNA precursors; their processing does not require Dicer[3,7]. Typically, piRNAs begin with uridine and possess 5′ monophosphate and 2′-*O*-methyl 3′ termini[3,10,11,13,14,21,98,260]. Both AGO and PIWI proteins contain three characteristic domains: PAZ, MID and PIWI. The PAZ domain, residing at the amino terminus, provides a binding pocket for the 3′ end of guide RNAs[261,262]. The PAZ domain differs between AGO and PIWI proteins. For example, human AGO1 binds less well to an RNA duplex containing a 3′ terminal 2′-*O*-methyl group[263], whereas the PAZ domains of PIWI proteins better accommodate the bulky 2′-*O*-methyl modification[94,133–135]. The 5′ phosphate of the guide RNA is anchored in the MID domain[104,105]. The MID domain presents the seed sequence of the guide as a helix, pre-paying the entropic cost of binding to its target[264]. Target cleavage occurs in the PIWI domain, whose RNase H-like fold presents a catalytic triad, aspartate–aspartate–glutamate (DDE), that positions a divalent cation, typically Mg^{2+}, to hydrolyse the phosphodiester bond linking target nucleotides t10 and t11 (REFS[265–269]). Argonaute cleavage leaves a 3′ hydroxyl and 5′ monophosphate[268], allowing the use of chemically selective high-throughput sequencing methods to identify AGO and PIWI protein cleavage products[270–273] (see the figure, part **B**; g numbers represent nucleotide positions of the guide RNA and t numbers represent the equivalent paired positions on the target RNA).



and distinct challenges faced by germline genomes in different animals.

How the germ line is specified defines the first challenge for piRNA-based transposon defence. In many animals, including most arthropods and many chordates, maternally deposited factors define primordial germline cells from which the entire germ lineage descends (reviewed previously[44]). Maternal specification of the germ line provides direct continuity of germ cells across generations and the opportunity for mothers to transmit information — in the form of RNA and protein — about the transposons present in the maternal genome. Indeed, PIWI proteins and piRNAs are maternally deposited in insect oocytes, thus providing progeny with immunity to transposable elements[45–50]. However, maternally inherited piRNAs cannot protect progeny from novel transposons present only in the father. In flies, for example, when naive mothers mate with fathers bearing genomic insertions of the P element transposon, the offspring are sterile because they cannot silence P elements in their own germ cells[45,51–53].

Many animals, including amniotes other than birds, employ a different, probably ancestral mode of germline specification: somatic cells are induced to become germ cell progenitors late in development. This strategy eliminates the generational continuity of the germ line, requiring the piRNA pathway to recognize transposon sequences without prior information. Moreover, the acquisition of primordial germ cells from the soma requires germline reprogramming to reset the epigenome and erase genomic imprinting[54]. In mice, germ cell reprogramming erases the DNA methylation that silences transposons, causing a burst of transposon transcription to which the piRNA pathway must respond.

Gonad anatomy and transposon life cycle also create specific challenges for germ cells. In *D. melanogaster*, both germline stem cells and differentiating germ cells contact supporting somatic cells. Several endogenous retroviruses in these somatic cells can produce infectious virions that are able to infect adjacent germ cells[55]. *D. melanogaster* has evolved an abridged piRNA pathway in somatic follicle cells to counteract this threat[40]. Mouse spermatogonial stem cells do not face such a challenge, as the active transposons in mice cannot produce infectious particles[56,57].

Finally, changes in chromatin during gametogenesis pose unique challenges for the restriction of transposons. For example, in mice, meiosis in males includes a period of transcriptional quiescence and loss of repressive chromatin marks that is followed by resumption of transcription and concomitant derepression of many transposon promoters[58]. The loss of transcriptional repression necessitates continuous post-transcriptional silencing of transposon mRNAs by piRNAs throughout mouse spermatogenesis[59,60]. The piRNA pathway provides both innate and adaptive solutions to these challenges.

piRNA biogenesis

Genomic sources of piRNAs. What defines a piRNA-producing gene and what marks its transcripts for piRNA production remain central unsolved questions in the field. Historically, piRNA-producing loci have been called clusters because they were initially defined by the high density of piRNAs mapping to them[1,2,4,5]. In flies, piRNA precursors come from heterochromatic loci (FIG. 1b,c), whereas in mammals, piRNA clusters appear to be indistinguishable from canonical euchromatic RNA polymerase II (RNA Pol II) transcription units (FIG. 1d). ‘Uni-strand’ clusters generate piRNA precursors by conventional, unidirectional transcription and have been found in all piRNA-producing animals examined to date (FIG. 1c,d). ‘Dual-strand’ clusters, which are convergently transcribed from both DNA strands, have been identified in dipterans[61,62] and lepidopterans[24] and are likely to be present in other arthropods (FIG. 1b).

In flies, piRNA clusters record the history of transposon invasion in a species, allowing piRNAs to silence the large number of active transposon families present in the *D. melanogaster* genome. In the germ line, dual-strand clusters produce the majority of fly piRNAs[20]. Fly dual-strand clusters lack the hallmarks of canonical transcription, such as the active promoter mark histone H3 lysine 4 trimethylation (H3K4me3) and the use of standard RNA signal sequences to remove introns and terminate transcription. Dual-strand clusters, by their nature, produce sense and antisense piRNAs regardless of transposon orientation[63,64]. The current model for piRNA production from dual-strand clusters seeks to explain the findings that their transcription requires both the transcriptionally repressive chromatin mark H3K9me3 and the piRNA-guided, transcriptional silencing protein Piwi[65,66].

Dual-strand clusters make piRNA precursor RNAs via non-canonical transcription facilitated by the germline-specific, H3K9me3-binding protein Rhino, a variant of heterochromatin protein 1 (HP1)[61,62,67–69]. Together with Deadlock (Del) and Cutoff (Cuff), Rhino bypasses the need for promoter sequences. Binding of Rhino to H3K9me3 tethers the germline-specific transcription initiation factor IIA subunit 1 (TFIIAL; also known as GTF2A1) paralogue, Moonshiner, along both strands of the piRNA cluster DNA. Moonshiner, in turn, forms an alternative TFIIA pre-initiation complex with TATA box-binding protein-related factor 2 (Trf2), allowing RNA Pol II to initiate dual-strand cluster transcription incoherently, that is, from many sites and on both DNA strands[69]. Thus, dual-strand piRNA cluster transcription reflects the occupancy of chromatin by Rhino rather than specific DNA regulatory sequences such as promoters (FIG. 1b).

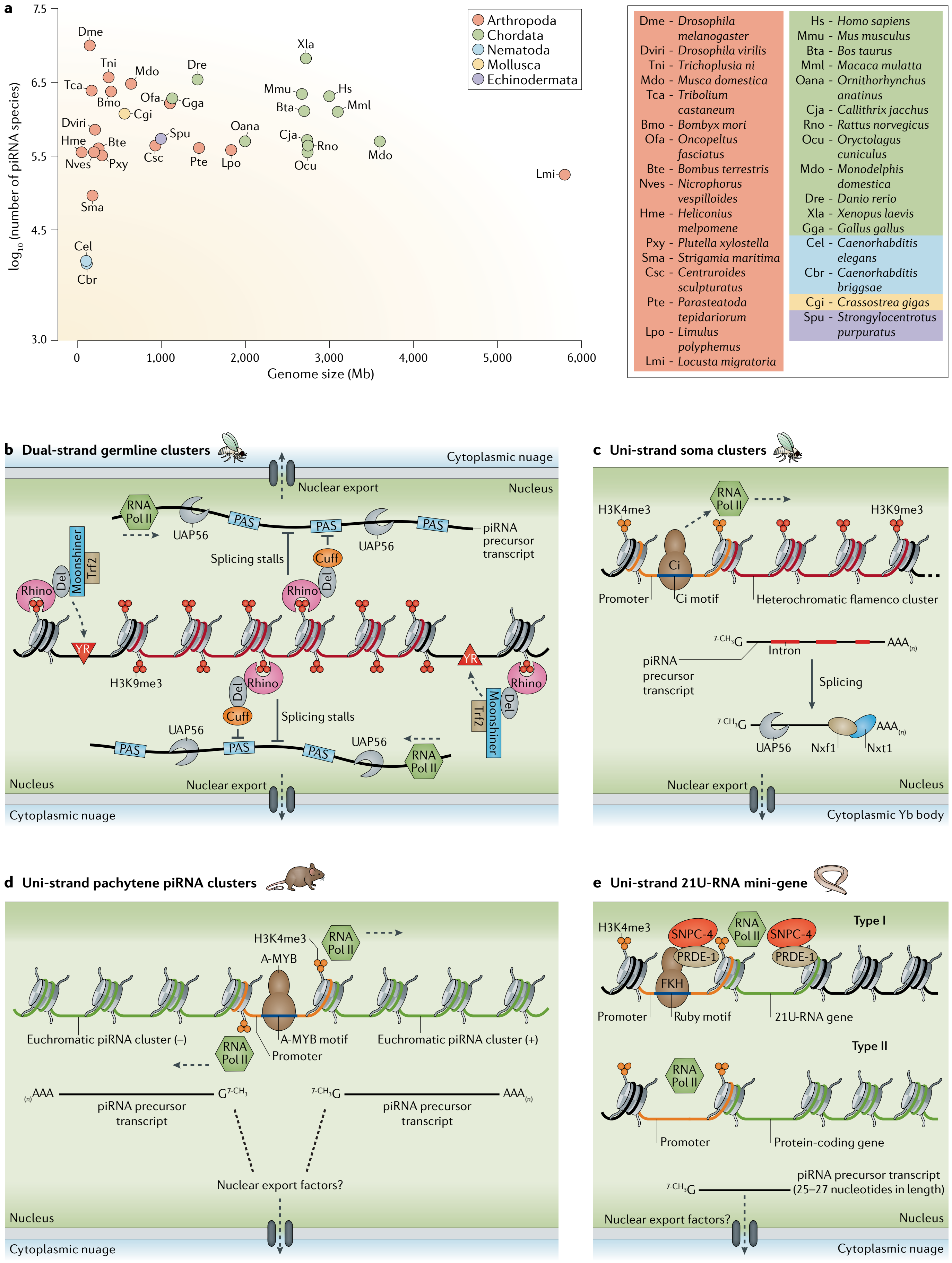
With Cuff and Del, Rhino also represses the splicing of dual-strand cluster transcripts and the use of canonical cleavage and polyadenylation sequence motifs[67,70] (FIG. 1b). Cuff has been proposed to compete with cap-binding proteins that promote splicing, and, together with UAP56 (also known as Hel25E) and THO-complex proteins, to send cluster transcripts to the piRNA-producing machinery present in nuage, a specialized perinuclear structure unique to germ cells[71,72] (BOX 2). Alas, Rhino, Cuff, Del and Moonshiner, so central to the identity of fly dual-strand piRNA clusters, are rapidly evolving and not found outside of drosophilids[24,73,74].

In the somatic follicle cells that support fly oogenesis, uni-strand clusters alone prevent endogenous

Spermatogonial
Related to spermatogonia, which are the undifferentiated germ cells located at the periphery of seminiferous tubules. They undergo mitosis and later give rise to developing spermatocytes.

Heterochromatic
Consisting of heterochromatin, the tightly packed form of DNA whose histones are heavily modified with repressive marks, typically histone H3 lysine 9 trimethylation (H3K9me3).

Canonical transcription
This standard transcription requires a promoter (typically marked by histone H3 lysine 4 trimethylation (H3K4me3)) and generates RNA with a 5′ 7-methylguanosine cap and a 3′ poly(A) tail.


a
7.5
6.5
5.5
4.5
3.0
log$_{10}$ (number of piRNA species)
0
1,000
2,000
3,000
4,000
5,000
6,000
Genome size (Mb)
Dme
Tni
Mdo
Dre
Xla
Tca
Bmo
Ofa
Gga
Cgi
Mmu
Hs
Bta
Mml
Dviri
Hme
Bte
Spu
Oana
Cja
Rno
Mdo
Nves
Pxy
Csc
Pte
Lpo
Ocu
Lmi
Sma
Cel
Cbr
Arthropoda
Chordata
Nematoda
Mollusca
Echinodermata
Dme - Drosophila melanogaster
Dviri - Drosophila virilis
Tni - Trichoplusia ni
Mdo - Musca domestica
Tca - Tribolium castaneum
Bmo - Bombyx mori
Ofa - Oncopeltus fasciatus
Bte - Bombus terrestris
Nves - Nicrophorus vespilloides
Hme - Heliconius melpomene
Pxy - Plutella xylostella
Sma - Strigamia maritima
Csc - Centruroides sculpturatus
Pte - Parasteatoda tepidariorum
Lpo - Limulus polyphemus
Lmi - Locusta migratoria
Hs - Homo sapiens
Mmu - Mus musculus
Bta - Bos taurus
Mml - Macaca mulatta
Oana - Ornithorhynchus anatinus
Cja - Callithrix jacchus
Rno - Rattus norvegicus
Ocu - Oryctolagus cuniculus
Mdo - Monodelphis domestica
Dre - Danio rerio
Xla - Xenopus laevis
Gga - Gallus gallus
Cel - Caenorhabditis elegans
Cbr - Caenorhabditis briggsae
Cgi - Crassostrea gigas
Spu - Strongylocentrotus purpuratus
b Dual-strand germline clusters
Cytoplasmic nuage
Nuclear export
Nucleus
RNA Pol II
UAP56
PAS
PAS
PAS
UAP56
piRNA precursor transcript
Splicing stalls
Cuff
Moonshiner
Trf2
Del
Rhino
Rhino
Del
YR
YR
H3K9me3
Rhino
Del
Cuff
Splicing stalls
Rhino
Del
Moonshiner
Trf2
UAP56
PAS
PAS
PAS
UAP56
RNA Pol II
Nucleus
Nuclear export
Cytoplasmic nuage
c Uni-strand soma clusters
RNA Pol II
H3K4me3
H3K9me3
Ci
Promoter
Ci motif
Heterochromatic flamenco cluster
$^{7\text{-}CH_3}$G
AAA$_{(n)}$
Intron
piRNA precursor transcript
Splicing
$^{7\text{-}CH_3}$G
AAA$_{(n)}$
UAP56
Nxf1
Nxt1
Nuclear export
Nucleus
Cytoplasmic Yb body
d Uni-strand pachytene piRNA clusters
H3K4me3
RNA Pol II
A-MYB
Euchromatic piRNA cluster (–)
A-MYB motif
Promoter
Euchromatic piRNA cluster (+)
RNA Pol II
$_{(n)}$AAA
G$^{7\text{-}CH_3}$
piRNA precursor transcript
$^{7\text{-}CH_3}$G
AAA$_{(n)}$
piRNA precursor transcript
Nuclear export factors?
Nucleus
Cytoplasmic nuage
e Uni-strand 21U-RNA mini-gene
H3K4me3
SNPC-4
RNA Pol II
SNPC-4
Type I
PRDE-1
PRDE-1
FKH
Promoter
Ruby motif
21U-RNA gene
Type II
RNA Pol II
Promoter
Protein-coding gene
piRNA precursor transcript (25–27 nucleotides in length)
$^{7\text{-}CH_3}$G
Nuclear export factors?
Nucleus
Cytoplasmic nuage

◀ Fig. 1 | **Genomic sources of piRNAs. a** | Genome size and the number of PIWI-interacting RNA (piRNA) species do not correlate. **b** | Fly germline dual-strand clusters exhibit 'incoherent' transcription where the histone H3 lysine 9 trimethylation (H3K9me3) repressive mark is recognized by Rhino. Rhino forms a complex with Deadlock (Del) and Cutoff (Cuff). Rhino–Del recruits Moonshiner and TATA box-binding protein-related factor 2 (Trf2) to the pyrimidine–purine (YR) elements to initiate promoter-independent transcription. Rhino–Del–Cuff ensures transcriptional elongation by repressing splicing and termination at polyadenylation signal sequences (PASs) within the clusters. Thereafter, piRNA precursor transcripts are routed to nuage by UAP56. **c** | The uni-strand *flamenco* cluster resides in heterochromatin but is conventionally transcribed from a promoter element recognized by the protein Cubitus interruptus (Ci). Splicing of piRNA precursor transcripts generates several isoforms that are shuttled to cytoplasmic Yb bodies by UAP56 and the exportins Nxf1 and Nxt1. **d** | Mouse pachytene piRNA clusters are transcribed from canonical promoters. A-MYB drives their transcription bidirectionally or unidirectionally at the onset of meiosis. **e** | *Caenorhabditis elegans* type I 21U-RNAs are individually transcribed from mini-genes by the protein Forkhead family transcription factor (FKH), which binds the *Ruby* motif upstream of each piRNA precursor. The A-MYB-like transcription factor SNPC-4 — recruited by PRDE-1 — enhances transcription. Transcription of type II 21U-RNAs initiates at the promoters of full-length protein-coding genes. RNA Pol II, RNA polymerase II.

Pachytene
The stage of meiotic prophase I when homologous recombination occurs.

retroviruses from infecting adjacent germ cells[20,63,64]. The largest somatic piRNA cluster, *flamenco*, resides in the pericentromeric heterochromatin of the X chromosome yet is conventionally transcribed to produce a long precursor transcript that generates piRNAs[20,39,40,63,64,75]. *flamenco* contains many antisense transposon sequences, allowing it to directly produce piRNAs that target transposon mRNAs. The transcription factor Cubitus interruptus (Ci) drives *flamenco* transcription[76] (FIG. 1c). Conservation of *flamenco* and its Ci-binding site across drosophilids suggests that the cluster arose recently in arthropod evolution but before drosophilid speciation (≥50 million years ago)[49,64,76,77]. Other arthropods are likely to possess uni-strand piRNA clusters that are evolutionarily unrelated to *flamenco*[24], and we do not yet know whether they share common strategies for designating the transcripts of uni-strand clusters as piRNA precursors.

What features distinguish *flamenco* from other conventional lncRNAs that do not produce piRNAs? Alternative splicing of *flamenco* has been proposed to promote the binding of UAP56 and exportins, which are proteins that help to transport *flamenco* RNA from the nucleus to cytoplasmic piRNA processing sites[78–80] (FIG. 1c). However, UAP56 and exportins also transport transcripts that produce no piRNAs. A recent study reported that the protein Yb binds *cis*-acting RNA elements in the *flamenco* transcript in the cytoplasm, triggering its processing into piRNAs[81–83]. Given that *flamenco* is an evolutionarily young innovation[24,64,76], the mechanism in which an RNA-binding protein recognizes specific sequences in a piRNA precursor transcript may be unique among drosophilids.

Although dual-strand piRNA clusters have not been identified outside arthropods, uni-strand clusters may play a role in mammalian transposon silencing during fetal spermatogenesis. In the mouse testis, PIWI proteins appear around the thirteenth day of embryonic development[29,30,84]. Thereafter, piRNA production persists throughout spermatogenesis[1,2,4,27–29], except in the leptotene and zygotene stages of meiosis, during which PIWI proteins have not been detected[60]. Among the loci that produce transposon-silencing piRNAs in the fetal mouse testes are two uni-strand clusters that, like *flamenco* in flies, produce piRNAs that are mainly antisense to transposon mRNAs[29].

In adult male mice, piRNAs in germ cells at stages before pachytene (pre-pachytene piRNAs) include transposon-silencing piRNAs, but most piRNAs derive from the coding and 3′ untranslated regions (3′ UTRs) of hundreds of mRNAs[27,85]. Such 3′ UTR piRNAs have been found in the somatic and germline tissues of just a few arthropods, including the follicle cells of the fly ovary[26,86]. Why some mRNAs make piRNAs and what purpose these sense piRNAs serve remain mysterious. Perhaps 3′ UTR piRNAs act in *trans* to regulate partially complementary mRNAs, or maybe they simply reflect

Box 2 | **Cytoplasmic foci and piRNA biogenesis**

'Nuage', French for cloud, collectively describes membraneless electron-dense structures found in animal germ cells: intermitochondrial cement in the oocytes and early spermatogenic cells of mammals[153,274], chromatoid bodies in the late spermatogenic cells of mammals[275], perinuclear nuage in fly nurse cells and zebrafish germ cells[276,277] and P granules in worm germ cells[278,279]. Nuage proteins depend on each other for their proper localization. In mice and flies, Vasa sits at the top of the hierarchy and is essential for the localization of all other components[63,64,136–139,141,142]. In *Caenorhabditis elegans*, the proteins PGL-1 and PGL-3 are indispensable for the formation of P granules[280,281].

Yb bodies are electron-dense perinuclear spots in fly ovarian somatic follicle cells[152]. In addition to the protein Yb, these structures contain Armitage[147,282] and Vreteno[236].

Accumulating evidence suggests that the properties of nuage follow the concept of liquid–liquid phase separation (reviewed previously[283,284]). Worm P granules behave like liquid droplets with a viscosity higher than that of the surrounding cytoplasm[285]. The human homologue of Vasa, DDX4, can assemble into phase-separated membraneless bodies both in vitro and when expressed in HeLa cells[286]. Compellingly, the domains shown or proposed to promote phase separation are also present in nuage proteins: an intrinsically disordered region in Vasa[286] and tandem Tudor domains enabling multivalent interactions in many nuage proteins (reviewed previously[287]). Like other membraneless organelles, nuage contains RNA as an essential component: for example, the perinuclear nuage of fly nurse cells is lost in the absence of PIWI-interacting RNA (piRNA) cluster transcription[61]. Although fluorescence recovery after photobleaching (FRAP) experiments show that protein components of worm P granules and fly nurse cell nuage are mobile and exchange with the cytoplasm[165,285], liquid–liquid phase separation is hypothesized to slow this exchange[283]. The higher viscosity of these structures coupled with specific protein–protein interactions may maintain the distinct content of granules by retaining some biomolecules and slowing the entry of others. Future studies should help to understand exactly how membraneless structures contribute to piRNA biogenesis and function.

Initiator piRNA
A PIWI-interacting RNA (piRNA) that guides a PIWI protein to slice a piRNA precursor transcript, triggering production of responder and trailing piRNAs from it.

Pre-pre-piRNA
A 5′ monophosphorylated long RNA created by an initiator PIWI-interacting RNA (piRNA)-guided PIWI-catalysed slicing of a piRNA precursor transcript.

Responder piRNA
A PIWI-interacting RNA (piRNA) whose 5′ end is generated by initiator piRNA-guided PIWI-catalysed slicing of a piRNA precursor transcript.

co-option of the piRNA pathway to target some mRNAs for destruction.

Pachytene piRNAs begin to accumulate in spermatocytes at the pachytene stage of meiosis, representing ~95% of all piRNAs in the adult mouse testis[1,2]. Pachytene piRNA precursors are transcribed from ~100 standard lncRNA genes that are depleted of transposons compared with the rest of the genome[27]. The transcription factor A-MYB (also known as MYBL1) coordinately initiates transcription of these loci as well as numerous piRNA biogenesis components, including MIWI (also known as PIWIL1), MILI (also known as PIWIL2) and VASA (also known as DDX4)[27,87] (FIG. 1d). Pachytene piRNA loci are often divergently transcribed from a central promoter, and some of these produce a piRNA precursor transcript from one arm and an mRNA or lncRNA from the other. Again, why pachytene piRNA precursor transcripts make piRNAs, while other A-MYB-regulated mRNAs and lncRNAs do not, is currently unknown.

Caenorhabditis elegans, unlike most nematodes, retains a piRNA pathway, albeit highly evolutionarily derived[41]. For historical reasons, *C. elegans* piRNAs are called 21U-RNAs, reflecting their length and first nucleotide bias, and many aspects of *C. elegans* piRNA production and function have not yet been observed outside of roundworms. *C. elegans* presents an exception to the general mechanism of piRNA production from long precursor RNAs. *C. elegans* type I 21U-RNAs are produced from ~12,000 dedicated mini-genes controlled by the same set of proteins, including the Forkhead family transcription factor FKH and a MYB-like transcription factor SNPC-4, which is assisted by the nuclear protein PRDE-1 (REFS[22,88,89]) (FIG. 1e). Each type I mini-gene generates a 7-methylguanosine-capped piRNA precursor transcript that is just 25–27 nucleotides in length[21,23,88] (FIG. 1e). By contrast, the type II 21U-RNAs are generated at the transcription start sites of conventional protein-coding genes and other RNA Pol II transcripts[23]. RNA Pol II pausing or premature termination is hypothesized to produce both types of 21U-RNAs[23,90], which are initially 25–27 nucleotides in length and subsequently processed by an as yet unknown mechanism that removes the 7-methylguanosine cap and the first two nucleotides of the precursor, establishing U as the first nucleotide (FIG. 2).

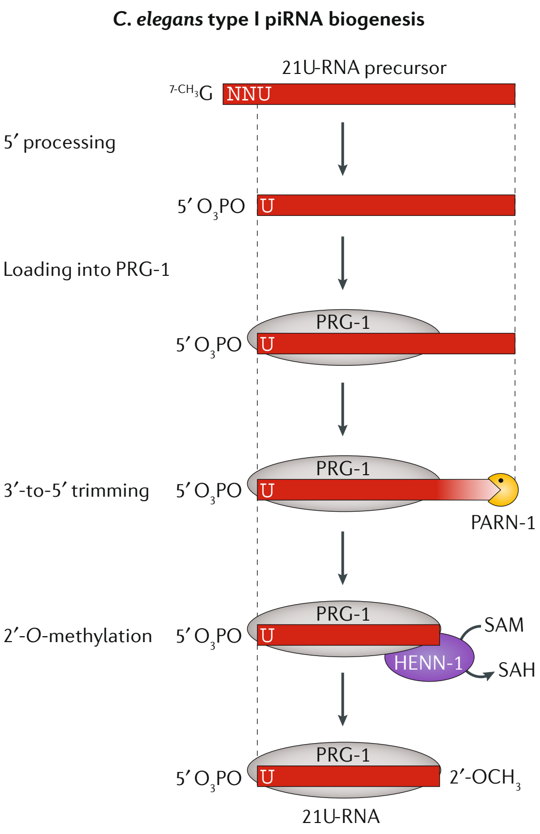


Fig. 2 | **Type I piRNA biogenesis in *Caenorhabditis elegans*.** The initial precursor PIWI-interacting RNA (piRNA) must be processed at both ends. Processing at the 5′ end creates a monophosphorylated 5′ end that can bind the PIWI protein PRG-1. The 3′ end of the PIWI-bound pre-piRNA is then trimmed by PARN-1, followed by 2′-*O*-methylation by HENN-1 to produce a mature worm piRNA (because worm piRNAs are 21 nucleotides long with a 5′ U bias, they are also known as 21U-RNAs). SAM and SAH are the methyl donor *S*-adenosylmethionine, and the product of methyl donation, *S*-adenosylhomocysteine, respectively.

Making piRNA 5′ ends. From flies to mice to worms, piRNA precursor transcripts begin with a 7-methylguanosine cap, yet piRNAs start with a 5′ monophosphate. The first step in committing an RNA to produce piRNAs appears to be endonucleolytic cleavage that generates the monophosphorylated end (FIGS 2,3) that is required for PIWI protein binding to RNA[91–94]. The requirement for a 5′ monophosphate licenses piRNA precursors: only long, single-stranded, 5′ monophosphorylated RNAs can enter the piRNA pathway[93,95–97].

In most animals, current evidence suggests that two pathways make piRNA 5′ ends. In the first, slicing of long precursor transcripts by piRNA-guided PIWI proteins initiates the production of piRNAs via a process known as the ping-pong cycle[20,98] (FIG. 3). The ping-pong pathway begins when a PIWI protein, guided by an initiator piRNA, cleaves a complementary target transcript to generate a pre-pre-piRNA with a monophosphorylated 5′ end. In many animals, initiator piRNAs are maternally inherited. Binding of a PIWI protein to the pre-pre-piRNA commits the RNA to produce a responder piRNA from its 5′ end. The responder piRNA 3′ end is established by an endonuclease in the second pathway (see below). In many animals, the intermediate product of this process — a pre-piRNA bound to the PIWI protein — is longer than a piRNA and must be trimmed to generate the mature responder piRNA. Because all Argonaute proteins, including PIWI proteins, slice their targets between nucleotides 10 and 11 of their guide, the first 10 nucleotides of the responder piRNA are complementary to the first 10 nucleotides of the initiator piRNA directing the cut (FIG. 3). The new responder piRNA can itself act as an initiator piRNA, producing a new responder piRNA that is identical to the original initiator piRNA. Thus, the ping-pong pathway functions as an amplification loop that is limited only by the availability of piRNA precursor substrates (FIG. 3).

In the second pathway, a piRNA-independent endonuclease in a complex of proteins on the mitochondrial

Pre-piRNA
The intermediate product of PIWI-interacting RNA (piRNA) biogenesis loaded into a PIWI protein. Pre-piRNAs are 3′-to-5′ trimmed and 2′-*O*-methylated at their 3′ termini to yield mature piRNAs.

Trailing pre-piRNAs
A string of tail-to-head, phased trailing pre-piRNAs follows a responder piRNA. Both 5′ and 3′ ends of trailing piRNAs are made by the stepwise endonucleolytic fragmentation of a piRNA precursor transcript.

outer membrane establishes the 3′ end of the responder pre-piRNA. The same complex of proteins fragments the remaining 3′ section of the pre-pre-piRNA into a string of tail-to-head, phased trailing pre-piRNAs[95,96]. The two pathways collaborate: the ping-pong pathway slices long piRNA precursor transcripts, creating 5′ monophosphorylated pre-pre-piRNAs that provide entry points for the production of responder and trailing piRNAs by the phased piRNA pathway (FIG. 3).

Before the discovery that initiator piRNAs act to generate responder and trailing piRNAs[95,96], the terms primary for trailing piRNAs and secondary for initiator and responder piRNAs were used. We propose to replace these historical terms with the more intuitive names initiator or responder for ping-pong piRNAs and trailing for phased piRNAs.

Which pathway creates a piRNA influences its nucleotide sequence. In the phased piRNA pathway, trailing pre-piRNAs often start with uridine (1U)[1,20] (FIG. 3). This 1U bias is likely to reflect the specificity of the endonuclease — thought to be Zucchini (called PLD6 in mammals) — that generates the ends of phased trailing pre-piRNAs[99–101]. By contrast, piRNAs generated by the ping-pong pathway bear a characteristic adenine at position 10 (10A)[20,25,98,102]. Although base pairing between a 1U initiator piRNA and its target is a logical explanation for the 10A in the responder piRNAs, it is not the actual source of the 10A signature. In fact, the structure of Argonaute does not allow the first nucleotide of a guide RNA (g1) to base pair with the corresponding target nucleotide (t1)[91,92,94,103–111] (FIG. 4). Instead, PIWI proteins possess an intrinsic affinity for a t1A in the target, regardless of the identity of the g1 nucleotide of the piRNA[93,112]. When an initiator piRNA directs a PIWI protein (for example, fly Aubergine (Aub); FIG. 4) to bind to and slice a target, its preference for t1A targets generates a responder piRNA with g10A. That is, the t1A of the target RNA becomes g10A of the responder piRNA. Subsequently, when the g10A responder piRNA guides PIWI-catalysed slicing of targets, it selects a complementary t10U. Slicing converts the t10U to g1U in the resulting responder piRNA. Consequently, the preference of PIWI proteins for t1A targets is one of the sources of the g1U bias of its piRNA guides (FIG. 4). The preference of PIWI proteins for t1A parallels that of miRNAs, which repress targets more efficiently when they bear t1A because miRNA-binding Argonautes contain a t1A pocket that reads the identity of the target nucleotide[113–118].

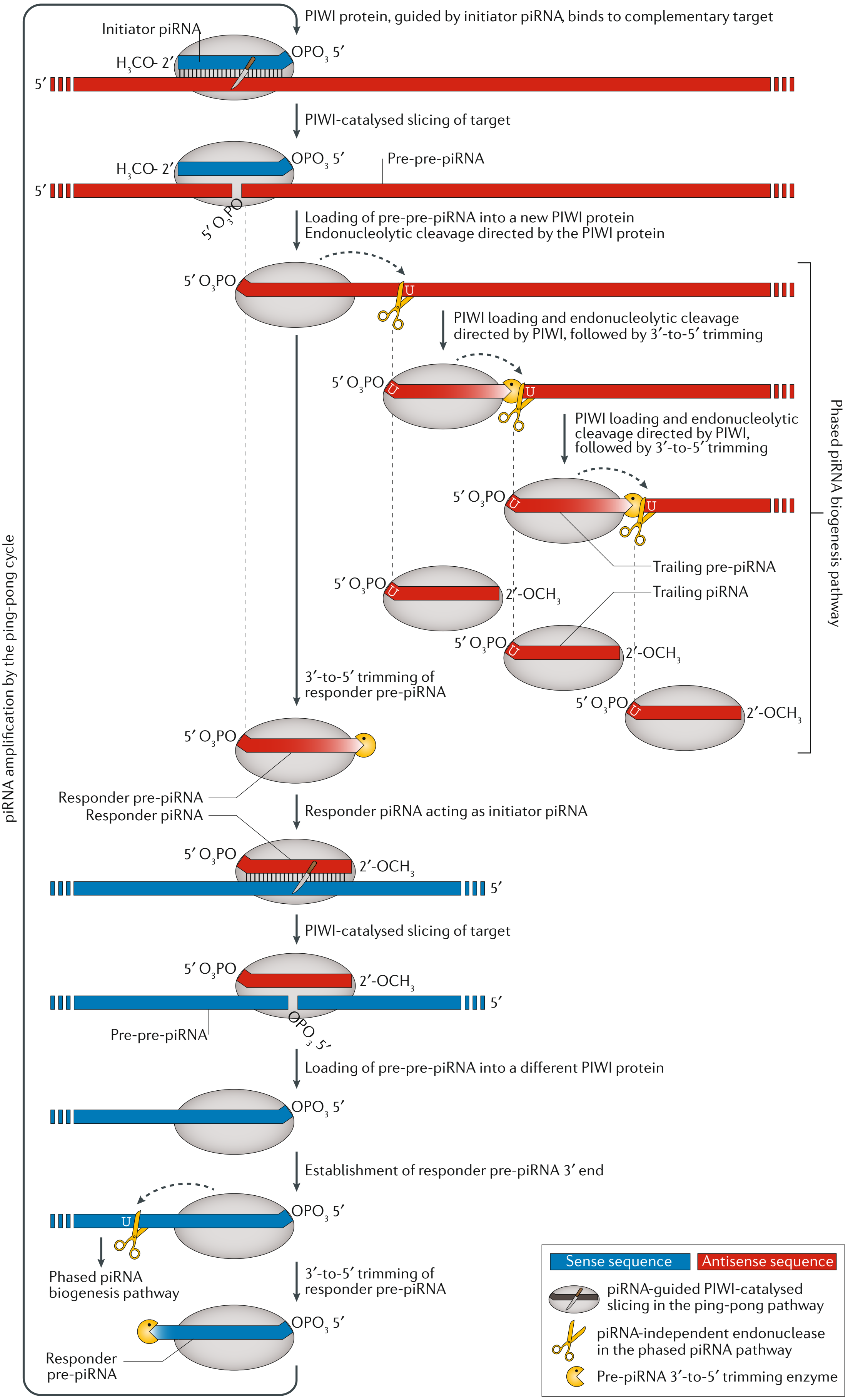
Ping-pong amplification increases the abundance of pre-existing piRNAs, while the phased piRNA pathway expands the diversity of piRNA sequences by spreading piRNA production 5′-to-3′ downstream of the cut directed by the initiator piRNA. Recent studies of flies and mice[82,95–97,119,120], and data from an evolutionarily broad range of non-model species[85], suggest that in most animals the ping-pong and phased piRNA pathways collaborate to make complex populations of piRNAs.

Polishing piRNA 3′ ends. Trimming and 2′-*O*-methylation of pre-piRNA 3′ ends concludes piRNA biogenesis[10,11,15,16,91,121–123]. piRNA 3′ terminal 2′-*O*-methylation has been hypothesized to improve small RNA stability by protecting the piRNA from non-templated nucleotide addition and 3′-to-5′ exonucleases[16,19]. How piRNA trimming supports piRNA function is not known. In many animals, the pre-piRNAs of 25–50 nucleotides in length require extensive 3′ trimming to generate functional piRNAs[85,121,122,124–126]. Studies in silkmoth and mouse identified the exonuclease (Trimmer in silkmoth; poly(A)-specific ribonuclease-like domain-containing 1 (PNLDC1) in mouse) that trims pre-piRNAs[122,124–126]; its orthologue PARN-1 trims *C. elegans* pre-piRNAs[121]. In mice and worms, this trimming activity is required for fertility, but in flies, trimming is largely dispensable, probably because fly pre-piRNAs are often no longer than mature piRNAs[96]. Thus, it is not surprising that the mechanism by which flies shorten their pre-piRNA 3′ ends is evolutionarily atypical. *D. melanogaster* and the rest of the Brachycera suborder of Diptera lost orthologues of both PNLDC1 and PARN-1 ~270 million years ago[123,127] and use the miRNA-trimming exonuclease Nibbler to resect piRNAs[123,128–130].

piRNA 3′ ends are 2′-*O*-methylated by an *S*-adenosylmethionine (SAM)-dependent methyltransferase (Hen1 in flies; HENMT1 in mice; HENN-1 in worms)[3,7,10–19]. HEN1 was first discovered in plants, where it modifies siRNAs and miRNAs[131,132]. Consistent with a role for 2′-*O*-methylation in stabilizing piRNAs, PIWI proteins bind more tightly to 2′-*O*-methyl-modified 3′ ends[94,133–135].

piRNAs are made in specialized cytoplasmic compartments. Most piRNA pathway proteins localize to specific cytoplasmic compartments, including nuage in animal germ cells, Yb bodies in the somatic ovarian follicle cells of flies and the mitochondrial outer membrane in all phased piRNA-producing cells (BOX 2). The enrichment of the piRNA machinery in these subcellular structures may serve to increase the local concentration of specific proteins or protect piRNA precursors from housekeeping nucleases. Compartmentalization may also prevent mRNAs and lncRNAs from entering the piRNA pathway. Supporting the idea that piRNA precursor transcripts are shunted to sites of piRNA production, nuclear-localized piRNA pathway proteins can be found opposite nuage-localized piRNA pathway proteins across the channel of a single nuclear pore in fly germline cells[72], suggesting that piRNA precursor transcripts are exported from the nucleus directly into nuage, unlike other cytoplasmic RNAs.

Factors that initiate piRNA biogenesis by generating pre-pre-piRNAs are found in nuage (BOX 2; TABLE 1), for example, fly Aub, Argonaute3 (Ago3) and Vasa[20,136–140]. Male mouse fetal germ cells contain two types of nuage. MILI and TDRD1 are found in nuage localized between clusters of mitochondria[141,142], that is, the classical intermitochondrial cement[143], whereas MIWI2 (also known as PIWIL4), MAEL and TDRD9 are in perinuclear nuage granules[142,144]. By contrast, factors such as Zucchini, Gasz and Papi in flies (PLD6, GASZ (also known as ASZ1) and TDRKH, respectively, in mice), which are required to generate responder pre-piRNA 3′ ends or produce both ends of trailing pre-piRNAs, localize to the outer membrane of mitochondria[79,145–151]. The enzyme PNLDC1/Trimmer, which carries out 3′-to-5′


PIWI protein, guided by initiator piRNA, binds to complementary target
Initiator piRNA
H_3CO- 2′
OPO_3 5′
5′
PIWI-catalysed slicing of target
H_3CO- 2′
OPO_3 5′
Pre-pre-piRNA
5′
5′ O_3PO
Loading of pre-pre-piRNA into a new PIWI protein
Endonucleolytic cleavage directed by the PIWI protein
5′ O_3PO
U
PIWI loading and endonucleolytic cleavage directed by PIWI, followed by 3′-to-5′ trimming
5′ O_3PO
U
U
PIWI loading and endonucleolytic cleavage directed by PIWI, followed by 3′-to-5′ trimming
5′ O_3PO
U
U
Trailing pre-piRNA
Trailing piRNA
5′ O_3PO
2′-OCH_3
5′ O_3PO
2′-OCH_3
5′ O_3PO
2′-OCH_3
Phased piRNA biogenesis pathway
3′-to-5′ trimming of responder pre-piRNA
5′ O_3PO
Responder pre-piRNA
Responder piRNA
Responder piRNA acting as initiator piRNA
5′ O_3PO
2′-OCH_3
5′
PIWI-catalysed slicing of target
5′ O_3PO
2′-OCH_3
5′
Pre-pre-piRNA
OPO_3 5′
Loading of pre-pre-piRNA into a different PIWI protein
OPO_3 5′
Establishment of responder pre-piRNA 3′ end
OPO_3 5′
U
Phased piRNA biogenesis pathway
3′-to-5′ trimming of responder pre-piRNA
OPO_3 5′
Responder pre-piRNA
piRNA amplification by the ping-pong cycle
Sense sequence
Antisense sequence
piRNA-guided PIWI-catalysed slicing in the ping-pong pathway
piRNA-independent endonuclease in the phased piRNA pathway
Pre-piRNA 3′-to-5′ trimming enzyme

◀ Fig. 3 | **piRNA biogenesis in most animals.** PIWI-interacting RNA (piRNA)-guided PIWI slicing (ping-pong pathway) generates a responder piRNA and initiates the production of phased trailing piRNAs (phased piRNA pathway). In flies, the establishment of the responder piRNA 3′ end is initiated by either a piRNA-independent endonuclease or piRNA-guided PIWI-catalysed slicing[123].

trimming of responder and trailing pre-piRNAs, is also believed to be located at the same site where these piRNAs are produced, that is, on the outer membrane of mitochondria[122]. Conversely, in the evolutionarily derived architecture of the fly piRNA pathway, the piRNA-trimming enzyme Nibbler is found in the perinuclear nuage and does not trim trailing piRNAs[123]. In fly ovarian somatic follicle cells, granules called Yb bodies have also been implicated in non-ping-pong piRNA biogenesis[147,152]. Rigorous proof that sites with high concentrations of piRNA pathway proteins participate in piRNA production continues to be elusive, but it is striking that cellular compartments containing proteins that act sequentially in piRNA biogenesis — for example, nuage and mitochondria — are frequently near one another[142–144,147,152,153]. In support of nuage serving as a piRNA factory, artificially tethering nuage proteins to an RNA triggers its processing into piRNAs[83,154]

Tudor domains — four-stranded β-barrels — scaffold the assembly of complex cellular machines by binding symmetrically dimethylated arginines (sDMAs), a modification found on PIWI proteins. Tudor domain proteins have been proposed to coordinate nuage

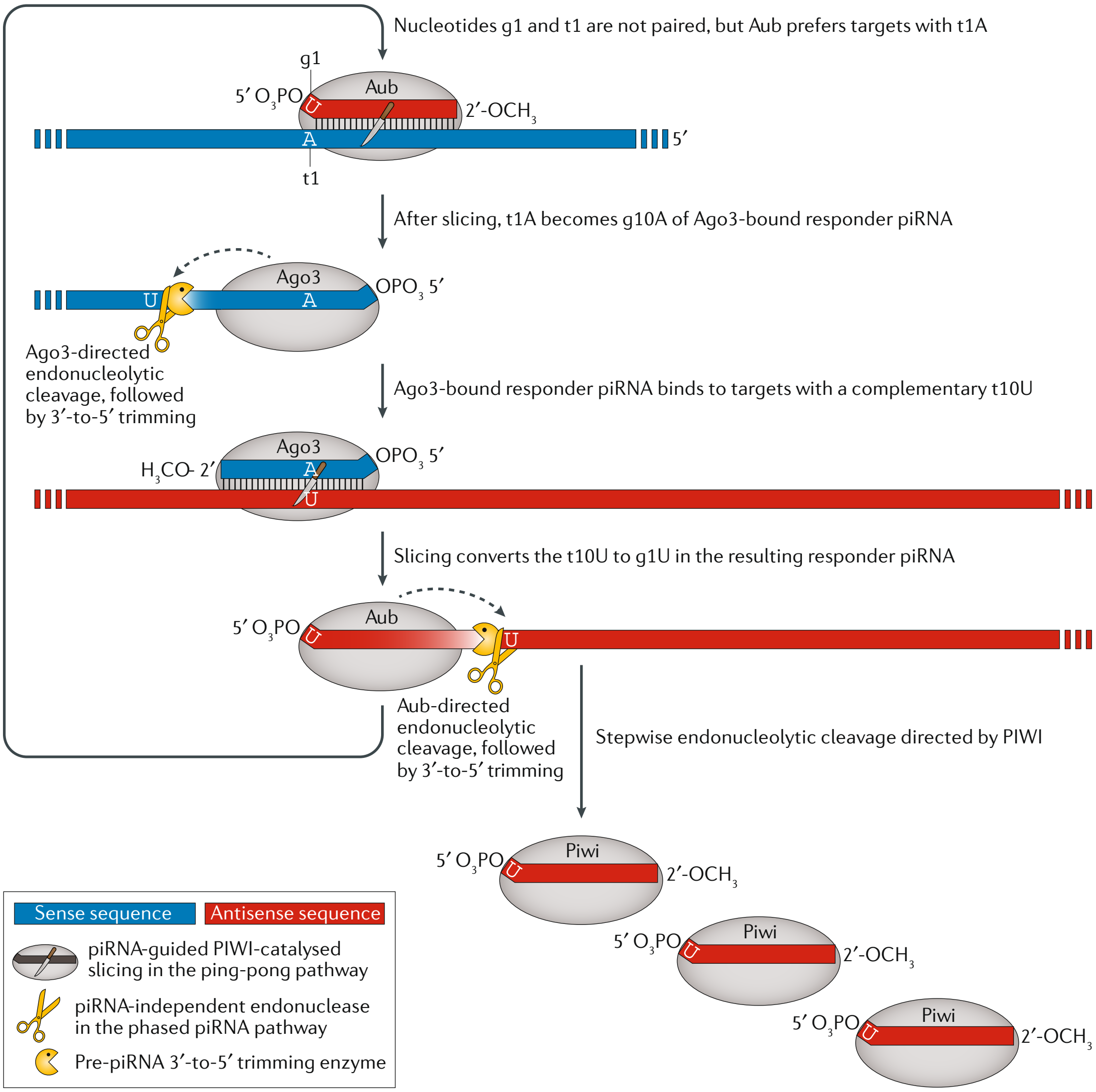


Fig. 4 | **The preference of PIWI proteins for t1A targets is one of the sources of the g1U bias of piRNAs.** The structure of Argonaute proteins precludes pairing between the first nucleotide of the guide (g1) and the opposing nucleotide of the target (t1) (shown for fly Aubergine (Aub) and Argonaute3 (Ago3)). However, targets of Aub often have adenine at the t1 position because the protein has an intrinsic preference for t1A. Aub slicing converts t1A to g10A of the resulting sense responder PIWI-interacting RNA (piRNA) loaded into Ago3. Targets of Ago3 often bear a t10U because of complementary pairing with g10A. Slicing by Ago3 converts t10U to g1U in the resulting antisense responder piRNA. Heterotypic Aub–Ago3 ping-pong in flies ensures the antisense bias of Aub-bound ping-pong piRNAs and Piwi-bound trailing piRNAs.

Table 1 | **Proteins implicated in the piRNA pathway**

Drosophila melanogaster	***Mus musculus***	**Lepidoptera**	**Function**	**Conservation**	**Refs**
Rhino (Rhi)	–	–	Promotes non-canonical transcription and suppresses splicing in dual-strand piRNA clusters	Fly-specific	61,62,67,69,70
Deadlock (Del)	–	–	Collaborates with Rhino to promote transcription in dual-strand piRNA clusters	Fly-specific	62,67,69
Cutoff (Cuff)	–	–	Collaborates with Rhino to promote transcription and suppress termination in dual-strand piRNA clusters	Fly-specific	62,67,68
Moonshiner (Moon)	–	–	Collaborates with Rhino and Del to promote transcription in dual-strand piRNA clusters	Fly-specific	69
Cubitus interruptus (Ci)	GLI1, GLI2 and GLI3	Ci	Promotes transcription of uni-strand clusters in somatic follicle cells of fly gonads	Most animals	76
–	A-MYB (also known as MYBL1)	–	Promotes transcription of pachytene piRNA clusters	Birds and mammals	27
UAP56 (also known as Hel25E)	DDX39B	Uap56	Enables nuclear export of piRNA precursor transcripts to sites of piRNA production	Most animals	71,72
Piwi	MIWI2 (also known as PIWIL4)	–	Nuclear PIWI protein that directs transcriptional silencing	Most animals	192,199,230,288
Asterix (Arx)	GTSF1 (also known as CUE110)	Gtsf1	Required for transcriptional silencing (in flies) and ping-pong piRNA production (in mice)	Most animals	80,195,289–291
Panoramix (Panx)	–	–	Required for transcriptional repression (in flies)	Fly-specific	193,194
Maelstrom (Mael)	MAEL	Mael	Required for transcriptional repression and piRNA production	Most animals	112,138,142,188,292,293
Aubergine (Aub)	MIWI (also known as PIWIL1) and MILI (also known as PIWIL2)	TnPiwi and Siwi	Cytoplasmic PIWI protein required for ping-pong piRNA production	Most animals	25,38,94,294–298
Argonaute3 (Ago3)	–	TnAgo3 and BmAgo3	Cytoplasmic PIWI protein required to initiate ping-pong and phased piRNA production	Insect-specific	20,25,63,98,299
Zucchini (Zuc)	PLD6	Zuc	Outer mitochondrial membrane protein and putative endonuclease required to establish the 3′ ends of responder pre-piRNAs and both ends of phased, trailing pre-piRNAs	Most animals	99–101,148,149,294,300
Minotaur (Mino)	GPAT2	Mino	Required for phased piRNA biogenesis	Most animals	301,302
Gasz	GASZ	Gasz	Outer mitochondrial membrane protein required for phased piRNA biogenesis	Most animals	79,303,304

Table 1 (cont.) | **Proteins implicated in the piRNA pathway**

Drosophila melanogaster	***Mus musculus***	**Lepidoptera**	**Function**	**Conservation**	**Refs**
Armitage (Armi)	MOV10L1	Armi	Helicase motif protein required for phased piRNA biogenesis	Most animals	83,305–310
Squash (Squ)	–	–	Piwi-interacting protein	Fly-specific	99,300
Vasa (Vas)	MVH	Vasa and BmVasa	Nuage component, RNA-dependent ATPase required for ping-pong piRNA biogenesis	Most animals	24,311–314
Spindle-E (Spn-E)	TDRD9	Spn-E and BmSpn-E	Nuage component, Tudor domain-containing protein, required for ping-pong piRNA biogenesis	Most animals	3,31,139,142,144,312,314
Krimper (Krimp)	–	Krimper	Nuage component, Tudor domain-containing protein that promotes heterotypic ping-pong (in flies)	Insect-specific	139,165,166
Qin	RNF17	Qin and BmQin	Nuage component, Tudor domain-containing protein that antagonizes homotypic ping-pong (flies)	Most animals	140,164,312,315–317
Tejas (Tej)	TDRD5	Tejas	Nuage component, Tudor domain-containing protein	Most animals	163,318–320
Tapas	TDRD7	–	Nuage component, Tudor domain-containing protein	Most animals	321–323
Vreteno (Vret)	TDRD1	Vreteno	Nuage and Yb body component, Tudor domain-containing protein	Most animals	141,158,159,236,324,325
Tudor	TDRD6	Tudor	Tudor domain-containing protein	Most animals	157,326
Sister of Yb (SoYb)	–	–	Tudor domain-containing protein required for piRNA biogenesis (in flies)	Fly-specific	236
Brother of Yb (BoYb)	TDRD12	Tdrd12	Tudor domain-containing protein required for ping-pong piRNA biogenesis	Most animals	120,236,327
Yb (also known as fs(1)Yb)	–	–	Yb body component, Tudor domain-containing protein required for piRNA production in somatic follicle cells	Fly-specific	147,152,282
Shutdown (Shu)	FKBP6	Shu	Co-chaperone required to load guide into PIWI protein	Most animals	328–330
Hsp83	HSP90	Hsp90	Co-chaperone required to load guide into PIWI protein	Most animals	328,330,331
Nibbler (Nbr)	–	–	Pre-piRNA 3′ trimming exonuclease	Fly-specific	123,128–130
Papi	TDRKH (also known as TDRD2)	Papi	Pre-piRNA 3′ trimming cofactor	Most animals	150,151,332
–	PNLDC1	Pnldc1	Pre-piRNA 3′ trimming exonuclease	Most animals	121,122,124,125
Hen1	HENMT1	Hen1	SAM-dependent methyltransferase required to modify 2′ hydroxyl at piRNA 3′ end	Most animals	10,11,15,16

piRNA, PIWI-interacting RNA; PNLDC1, poly(A)-specific ribonuclease-like domain-containing 1; SAM, *S*-adenosylmethionine.

k_{cat}
In Michaelis–Menten enzyme kinetics, the catalytic constant k_{cat} represents the maximum number of substrate molecules converted to product per active site per unit time.

PIWI slicer activity
Endonucleolytic cleavage of the target RNA catalysed by PIWI-interacting RNA (piRNA)-guided PIWI proteins.

assembly and tether PIWI proteins to the outer face of mitochondria[122,155–167].

In flies, Tudor domain proteins are also required for heterotypic ping-pong between the fly PIWI proteins Aub and Ago3 (REFS[140,165]). Heterotypic Aub–Ago3 ping-pong drives the production of antisense piRNAs that direct Aub to bind transposon mRNAs in the cytoplasm and direct Piwi — the third fly PIWI protein — to bind to nascent transposon transcripts in the nucleus (FIG. 4). The antisense bias of fly piRNAs suggests that for the k_{cat} catalytic constants, k_{cat} *(Ago3)* >> k_{cat} *(Aub)*. In an animal inheriting antisense, Aub-bound piRNAs, a greater catalytic efficiency for Ago3 would ensure that heterotypic Aub–Ago3 ping-pong generates an excess of both antisense responder and trailing piRNAs. The Tudor domain protein Krimper promotes heterotypic Aub–Ago3 ping-pong[165], while the Tudor domain protein Qin thwarts futile homotypic Aub–Aub ping-pong[140]; together, Krimper and Qin ensure that the ping-pong cycle favours the production of piRNAs antisense to transposon mRNAs. By contrast, mouse piRNAs are amplified by homotypic MILI–MILI ping-pong[168], which appears to suffice for post-transcriptional control of transposons.

piRNA functions

Studies of animals ranging from humans to hydra suggest that silencing transposons in the germ line is the ancestral function of piRNAs[26,29,169–172]. Mosquitoes also use piRNAs to fight viruses in the soma[173–175]. However, many piRNAs, particularly in the mammalian testis, correspond to unique genomic sequences unrelated to transposable elements[1,2]. Although understanding the function of these evolutionarily younger, non-transposon piRNAs remains technically and intellectually challenging, accumulating evidence suggests that they regulate expression of host mRNAs. Below, we discuss our latest understanding of classic roles of piRNAs in silencing repetitive elements, as well as emerging roles in host mRNA regulation.

Transposon silencing. Transposons pose multiple threats to the genome. Their presence at multiple genomic sites promotes illegitimate recombination, their replication can generate double-stranded DNA breaks, their insertion in new sites can disrupt coding sequences, and their promoters can drive aberrant expression of neighbouring genes[58,176–178]. Nearly all animals rely on piRNAs to defend the germline genome from transposon expression. Arthropods and molluscs also use piRNAs to repress transposons in the soma[26,179], suggesting that the last common ancestor of Protostomia and probably all other animals produced both germline and somatic piRNAs. In the evolutionarily exceptional instance of the *Drosophila* genus, piRNAs also help maintain telomeres[180–182]. Flies lack telomerase and instead use telomeric retrotransposons that recursively integrate into telomeric regions to maintain chromosome ends (reviewed previously[183,184]). piRNA-mediated silencing of these telomeric retrotransposons sustains the heterochromatin environment that is required to maintain a stable telomere length[182,185].

In worms, piRNA-directed silencing of transposons is achieved by initiating a secondary siRNA response (see the section ‘Worm piRNAs distinguish self from non-self’). In other animals, piRNAs silence transposons either by repressing their transcription or by slicing (cleaving) their mRNAs (FIG. 5a). The cytoplasmic PIWI proteins Aub and Ago3 in flies, Siwi and BmAgo3 in silkmoth and MILI and MIWI in mice mediate post-transcriptional transposon silencing[20,25,28,59,60,98,168]. The nuclear proteins Piwi in flies and MIWI2 in mice repress transposons transcriptionally[29,66,186–190].

Transcriptional silencing is thought to occur when piRNA-guided PIWI proteins bind to nascent transposon transcripts (FIG. 5a), a model that is based on siRNA-directed heterochromatin formation in *Schizosaccharomyces pombe*[191]. Transcriptional repression does not require PIWI slicer activity[168,192]. The specific mechanism of transcriptional repression differs between flies and mice. In flies, Piwi promotes H3K9 methylation, a repressive chromatin mark, through recruitment of Eggless (also known as dSetdb1) by the Piwi-interacting mediator proteins Asterix and Panoramix[66,80,186–190,193–196]. At dual-strand piRNA clusters and potentially at transposons themselves, the presence of H3K9me3 promotes Rhino-dependent non-canonical transcription but blocks the production of functional, spliced transposon mRNA[62,69,70,197]. Thus, Piwi-dependent repression of transposons in flies is not formally an example of transcriptional silencing but rather reflects a change in the mode of transcription from producing protein-coding mRNAs to generating multiple, unspliced piRNA precursor RNAs from both genomic strands[69,197]. Such precursor RNAs are funnelled into the piRNA biogenesis machinery, which turns stand-alone transposon insertions into piRNA-producing loci[62,198]. Whether the lessons learned from *D. melanogaster* reveal evolutionarily conserved principles for transcriptional silencing is not known. Many piRNA pathway proteins — including Rhino, Cuff, Del and Moonshiner — are poorly conserved, lacking identifiable homologues in most other arthropods, let alone mice (TABLE 1).

By contrast, piRNA-dependent transcriptional silencing in mouse fetal gonocytes directs both DNA and H3K9me3 histone methylation[29,30,84,199–205]. Both DNA and histone H3K9me3 methylation is targeted by the piRNA pathway to evolutionarily young copies of transposons[84,201]. In muroid rodents, the promoters of young transposons are methylated by a dedicated DNA methyltransferase, DNMT3C, encoded by a Muroidea-specific duplication of *Dnmt3b*[206,207]. How the mouse nuclear PIWI protein MIWI2 recruits the chromatin and DNA methylation machinery to transposon genomic sequences is unknown.

In mouse male germ cells, the dramatic changes in the chromatin and transcriptional landscape during meiotic and post-meiotic stages make piRNA-guided post-transcriptional control of transposon mRNAs indispensable[59,60]. In addition to the piRNA pathway, other repressive mechanisms silence transposons at some stages of mouse spermatogenesis[60,208]. For example, piRNA-independent histone H3 lysine 9 dimethylation (H3K9me2) is necessary and sufficient to silence long interspersed nuclear element 1 (LINE-1) transposons before the onset of meiosis[60,208].

Adaptive and innate features of piRNA-directed transposon silencing. The piRNA pathway provides features of both innate and adaptive immunity against transposons. For example, maternally deposited initiator piRNAs in *D. melanogaster* act as pattern recognition receptors that recognize transposon sequences and respond by amplifying piRNAs that are specific to the threat. Indeed, a lack of innate immunity — protective maternal piRNAs — explains hybrid dysgenesis[45], a phenomenon in which a transposon-carrying male mated to a naive female produces sterile offspring because the female cannot deposit the relevant piRNAs in her oocytes. These offspring remain sterile for most of their adult life, until adaptive piRNA-mediated immunity is re-established in germline stem cells when the invading transposon integrates into a piRNA-producing locus[53,209]. These novel transposon insertions provide a record of the invasion by updating the piRNA cluster, and this new information immunizes future generations to the new threat.

The mouse germ line is induced from somatic cells, and maternal deposition is unlikely to supply initiator piRNAs to jump-start piRNA production. However, *flamenco*-like, uni-strand piRNA-producing loci, which are rich in antisense transposon insertions, may provide innate memory. Broadly speaking, these uni-strand piRNA clusters may allow the piRNA machinery to recognize both known and — probably through partial complementarity — novel invaders. Such innate piRNAs could then trigger amplification of relevant antisense piRNAs[29,168]. Moreover, the production of phased trailing piRNAs from sequences downstream of the initiator and responder piRNAs could provide adaptive immunity by favouring piRNA production from those sequences most closely related to the novel transposon. It is conceivable that these fetal, uni-strand piRNA-producing loci can accumulate transposon insertions to expand the innate memory of possible threats. Whether the A-MYB-regulated, pachytene piRNA-producing loci expressed in adult mice play a similar role remains unknown. However, pachytene piRNA-producing loci abide by conventional transcriptional rules, suggesting that, unlike fly dual-strand piRNA clusters, they are likely to be disrupted by new transposon insertions[27]. This may explain why A-MYB-regulated, pachytene piRNA clusters are depleted of transposons compared with the rest of the genome[1].

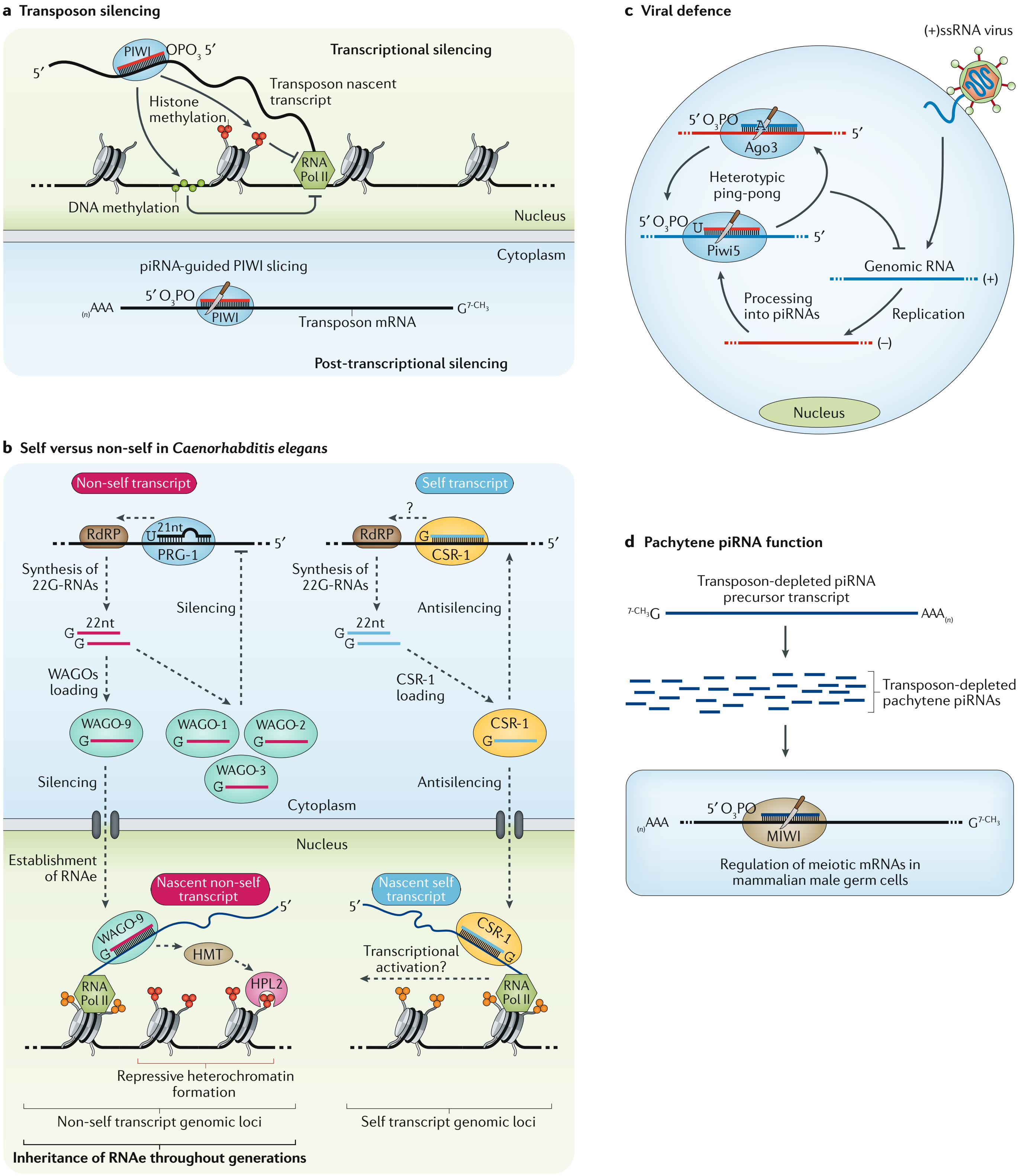
Worm piRNAs distinguish self from non-self. *C. elegans* possesses a complex system of small RNA pathways. Several studies have proposed that worm piRNAs possess broad targeting capacity, potentially recognizing any transcript present in the germ line[210–213]. This targeting flexibility may allow piRNAs to recognize and silence non-self transcripts such as transgenes and new transposon insertions. Two models explain how self transcripts can be spared silencing (FIG. 5b): first, Argonaute CSR-1 may maintain both the transcription and stability of endogenous mRNA[214–217]; second, germline-expressed self transcripts may contain specific sequences conferring resistance to piRNA silencing[213].

Unlike cytoplasmic PIWI proteins in other animals, the slicer activity of the worm PIWI protein PRG-1 is dispensable for target silencing[210,211]. Instead, piRNAs induce the synthesis of secondary siRNAs on the target transcript by RNA-dependent RNA polymerase (RdRP)[210,211,215,218–221] (FIG. 5b). RdRP-mediated amplification of the silencing signal is conceptually analogous to ping-pong amplification in other animals. The secondary siRNA response, RNA-induced epigenetic silencing (RNAe), can be inherited. piRNA-guided PRG-1 initiates RNAe, but other factors maintain the silencing for generations[210,211,215,218–221].

Viral defence. Antiviral defence in somatic tissues is typically ascribed to siRNAs. However, some invertebrates use piRNAs to tackle viral infection in the soma[26,173–175]. Mosquitoes appear to fight RNA viruses using the ping-pong pathway: two mosquito PIWI proteins — Piwi5 and Ago3 — participate in heterotypic ping-pong, consuming viral (+) and (−) strand RNAs to produce piRNAs[175] (FIG. 5c). Genomic viral integrations acting as piRNA-producing loci probably allow the mosquito piRNA pathway to recognize viral RNA by initiating the ping-pong cycle[222,223]. How the piRNA pathway recognizes and tackles viral RNAs in other animals is currently unknown, as virus-derived piRNAs in other invertebrates show no signs of ping-pong[26].

Why do some animals mount piRNA-based antiviral responses while others rely entirely on the siRNA-driven RNA interference (RNAi) pathway for antiviral defence? The use of two different classes of small silencing RNAs to fight viruses may reflect the distinct precursors that can enter the RNAi and piRNA pathways: RNAi is triggered by double-stranded RNA, whereas piRNAs are produced from single-stranded RNA. The two pathways may target RNA from different types of viruses or stages of viral infection, boosting the overall antiviral response. Testing these ideas remains an important challenge for the small RNA field.

Mammalian pachytene piRNAs: regulating gene expression? In mammals, fetal piRNAs silence transposons in male germ cells. By contrast, the most abundant piRNA population in mammals, the pachytene piRNAs, are depleted of transposon sequences[1,2]. Each spermatocyte cell contains >5 million pachytene piRNA molecules[85]. Until recently, pachytene piRNAs had not been formally proved to have a function, but a recent study reports compromised sperm function in mice lacking a major piRNA-producing locus on chromosome 6 (REF. 224). However, the regulatory targets of pachytene piRNAs are not obvious, as >80% of pachytene piRNAs map only to the loci producing them[1,2,27]. Thus, no consensus model for how pachytene piRNAs ensure normal spermatogenesis has been established. One study reported that pachytene piRNAs guide PIWI proteins to destabilize their mRNA targets via an miRNA-like mechanism[225], whereas another proposed that PIWI proteins do not use pachytene piRNAs as conventional guides and instead bind and stabilize mRNAs in a sequence-independent manner[226]. Two recent reports show that pachytene piRNAs regulate gene expression by guiding conventional, PIWI-dependent cleavage of targets[227,228] (FIG. 5d). Nevertheless, the minimal overlap among the targets identified in these studies suggests that we are



still far from understanding what rules govern target recognition by pachytene piRNAs.

Other functions of the piRNA pathway. When discovered, the fly PIWI protein Piwi was reported to be essential for germ stem cell regeneration[229,230]. Whether piRNAs participate in this function of Piwi has not been directly tested. In fact, the transposon-silencing function of Piwi can be genetically separated from its role in germ stem cell maintenance by removing an amino-terminal region of the protein[187]. Roles for PIWI proteins in sustaining stem cell populations have been described outside of flies, but the molecular mechanism of these pathways also remains unknown (reviewed previously[231]).

Fig. 5 | **Diverse functions of piRNAs. a** | PIWI-interacting RNAs (piRNAs) silence transposons transcriptionally and post-transcriptionally. Nuclear PIWI proteins are guided by piRNAs to nascent transposon transcripts and generate heterochromatin via DNA or histone methylation, thus silencing transcription. In the cytoplasm, piRNAs elicit post-transcriptional silencing by directing PIWI proteins to slice target transcripts. **b** | *Caenorhabditis elegans* piRNAs distinguish self transcripts from non-self transcripts. When a PRG-1-bound worm piRNA (21U-RNA) finds its target, it recruits an RNA-dependent RNA polymerase (RdRP) to synthesize secondary small interfering RNAs (siRNAs) (22G-RNAs) using the target as a transcription template. (21U and 22G indicate the length of the small RNA guides — 21 or 22 nucleotides — and the 5′ nucleotide bias, U or G). The 22G-RNAs are loaded into the worm-specific Argonautes, WAGOs, which silence non-self transcripts. In the nucleus, WAGO-9 silences non-self transcription by recruiting histone methyltransferases (HMTs) and the heterochromatin protein 1 (HP1) homologue HPL2 to the target locus. Such RNA-induced epigenetic silencing (RNAe) persists over generations. The Argonaute protein CSR-1 counteracts WAGO silencing, protecting self transcripts. The nuclear localization of CSR-1 suggests that CSR-1 may also license transcription of self transcripts. **c** | In some animals, somatic piRNAs fight viruses. When infected by a positive strand, single-stranded RNA (ssRNA) virus, mosquitoes mount an antiviral piRNA-based response. Upon viral replication, Piwi5 (loaded with 1U antisense piRNAs) and Argonaute3 (Ago3) (loaded with 10 A sense piRNAs) participate in heterotypic ping-pong, consuming viral RNAs. 1U and 10 A indicate piRNA guide strands with a U at position 1 or an A at position 10, respectively. **d** | In mouse male spermatocytes, pachytene piRNAs are first made as cells enter the pachytene stage of meiosis. Recent studies suggest a role for pachytene piRNAs in regulating gene expression during meiosis and late spermiogenesis by directing PIWI proteins to cleave target mRNAs. nt, nucleotides; RNA Pol II, RNA polymerase II.

In *D. melanogaster*, the PIWI protein Aub has been reported to play a piRNA-directed role in embryonic patterning[232,233]. Transposon-derived piRNAs were identified that can pair with partially complementary target sites in the 3′ UTR of *nanos* mRNA and induce its decay outside the posterior pole of the embryo, where Nanos protein acts to repress the anterior–posterior determinant *hunchback*[232,233]. However, earlier experiments identified *cis*-acting RNA elements responsible for *nanos* translational repression that do not overlap the two piRNA binding sites[234,235]. Whether the two regulatory mechanisms act redundantly or additively and whether piRNA-dependent mechanisms regulate *nanos* in other Diptera remain to be determined.

Conclusions

Despite 17 years of study, the central questions posed when piRNAs were first discovered remain unanswered for most animals.

First, what defines a piRNA cluster? That is, what marks a specific genomic region to produce piRNAs? Why do fly uni-strand clusters and mouse pachytene piRNA loci produce piRNAs while other mRNA and lncRNA genes do not? In flies, discrete RNA sequence elements have been suggested to funnel conventional RNA Pol II *flamenco* transcripts into the somatic piRNA pathway[81–83], but these sequences are not deeply conserved. piRNAs direct H3K9me3 marks to fly dual-strand piRNA clusters and dispersed transposon copies. In turn, H3K9me3 binds Rhino, silencing transposons and enabling cluster transcription. However, many regions of the genome replete with H3K9me3 marks neither bind Rhino nor make piRNAs. What distinguishes heterochromatic piRNA clusters from other regions of heterochromatin? Moreover, Rhino homologues have not been identified outside of drosophilids[24,73,74], suggesting that yet undiscovered mechanisms promote piRNA production from dual-strand clusters in other arthropods[24]. The divergence of proteins involved in piRNA precursor transcription contrasts sharply with the deep conservation of the downstream piRNA-producing machinery[22,42,61,69,123,236]. The rapid evolution of some piRNA pathway components may reflect an 'evolutionary arms race' between the host genome and the rapidly evolving targets of the piRNA pathway, for example, transposons[74,237,238].

Second, in animals that induce the germ line from somatic cells and therefore do not deposit piRNAs maternally, what enables the piRNA pathway to specifically recognize transposon sequences? Are piRNAs derived from the *flamenco*-like fetal clusters in mice[29] sufficient to start the ping-pong cycle by cleaving transposon mRNAs, thus triggering subsequent transcriptional and post-transcriptional repression? Or is the piRNA pathway instructed by a yet to be discovered transposon-sensing system?

Third, why have the ancestral somatic functions of piRNAs been lost in many animal lineages? What drives the repeated repurposing of the piRNA pathway across different animal phyla[26,41]? Because the miRNA biogenesis machinery produces small RNA guides that are highly conserved among animals and the RNAi response targets only transcripts that are homologous to a double-stranded RNA trigger, the target repertoires of the two pathways are limited. By contrast, the piRNA pathway makes guides from single-stranded RNA, a substrate that is abundant in cells. Moreover, the intrinsic imprecision of piRNA biogenesis machinery produces enormously diverse piRNA guide sequences. This may allow the target repertoire to drift during evolution, enabling the fortuitous acquisition of new targets, whose regulation by piRNAs becomes fixed when it confers a selective advantage, driving the evolution of new piRNA functions. The recurrent emergence of piRNA functions unrelated to transposon repression suggests that novel, unexpected roles for piRNAs remain to be discovered.

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