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STATE-OF-THE-ART REVIEW

piRNA processing within non-membrane structures is governed by constituent proteins and their functional motifs

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Discovered two decades ago, PIWI-interacting RNAs (piRNAs) are crucial for silencing transposable elements (TEs) in animal gonads, thereby protecting the germline genome from harmful transposition, and ensuring species continuity. Silencing of TEs is achieved through transcriptional and post-transcriptional suppression by piRNAs and the PIWI clade of Argonaute proteins within non-membrane structured organelle. These structures are composed of proteins involved in piRNA processing, including PIWIs and other proteins by distinct functional motifs such as the Tudor domain, LOTUS, and intrinsic disordered regions (IDRs). This review highlights recent advances in understanding the roles of these conserved proteins and structural motifs in piRNA biogenesis. We explore the molecular mechanisms of piRNA biogenesis, with a primary focus on *Drosophila* as a model organism, identifying common themes and species-specific variations. Additionally, we extend the discussion to the roles of these components in non-gonadal tissues.

Introduction

The silencing of transposable elements (TEs) via piRNA pathway is predominantly observed in metazoans, specifically in animal gonads, which are essential for sexual reproduction. This RNA silencing mechanism serves as a critical defense against deleterious genetic traits, including repetitive sequences and TEs, by producing piRNAs—short non-coding RNAs that maintain the genome integrity of the germline [1,2]. In addition to piRNAs, siRNA and miRNA pathways are other significant RNA interference mechanisms that function in gene silencing across various tissues. Unlike siRNAs and miRNAs, piRNAs are generated in a Dicer-independent manner, range from 24 to 29 nucleotides (nt) in length, and were first discovered in *Drosophila* testes. Here, piRNAs play a critical role in suppressing Stellate (Ste), which originates from the repetitive loci. These piRNAs, along with other repeat-associated small interfering RNAs (rasiRNAs), were subsequently discovered in *Drosophila* ovaries and embryos [3,4]. Later, they were also identified

Abbreviations
Armi, Armitage; CPSF, cleavage and polyadenylation specificity factor; Cuff, Cutoff; Daed, Daedalus; eLOTUS, extended LOTUS; G4, G-quadruplex; HP1d, Heterochromatin protein 1d; IDRs, intrinsically disordered regions; Krimp, Krimper; L(3)mbt, Lethal(3)malignant brain tumor; LINEs, long interspersed nuclear elements; LLPS, liquid–liquid phase separation; Mael, Maelstrom; MBT, Malignant brain tumor; mLOTUS, minimal LOTUS; non-LTRs, non-long-terminal repeats; nt, nucleotides; Panx, Panoramix; piRNAs, PIWI-interacting RNAs; poly(A), polyadenylated; rasiRNAs, repeat-associated small interfering RNAs; RDC, Rhino–Deadlock–Cutoff; Rhi, Rhino; RNPs, ribonucleoproteins; Shu, Shutdown; SINEs, short interspersed nuclear elements; snRNPs, small nuclear ribonucleoproteins; SoYb, Sisters of Yb; Spn-E, Spindle-E; Ste, Stellate; Su(Ste), Suppressor of Stellate; TDRDs, Tudor domaincontaining proteins; Tej, Tejas; TEs, transposable elements; Tj, traffic jam; TREX, TRanscription-EXport; Tud, Tudor; Vas, Vasa; Vret, Vreteno; Zuc, Zucchini.

in mice and found to associate with PIWI family proteins, a subclade of the Argonaute proteins that are expressed exclusively in gonads [1,5], leading to their classification as PIWI-interacting RNAs (piRNAs).

The prevalence of TEs within an organism's genome is loosely correlated with its genome size, although it varies significantly across eukaryotes. TEs constitute over half of the human genome, while they account for around 20% of the *Drosophila melanogaster* genome, which itself represents about 10% of the size of the human genome [6–8]. TEs are categorized into two main classes, distinguished by their transposition mechanisms. Class I TEs, or retrotransposons, include long interspersed nuclear elements (LINEs), non-long-terminal repeats (non-LTRs), and short interspersed nuclear elements (SINEs). They move through a reverse-transcribed RNA intermediate, following a "copy-and-paste" mechanism. In contrast, Class II TEs, or DNA transposons, move directly between genomic locations using a "cut-and-paste" mechanism, excising and reinserting themselves without replication [9]. Their mobilization across the host genome poses a major threat to genome integrity, potentially leading to instability through their invasion.

Despite their harmful effects, TEs have co-evolved with their hosts and play pivotal roles in enhancing genetic diversity, either by cis-regulating DNA or producing proteins that are co-opted for physiological or developmental functions [10–12]. For example, *Drosophila* harbors three non-LTR retrotransposons, TAHRE, HeT-A, and TART, at the ends of chromosomes, thereby forming protective telomere arrays [13]. The processes of protecting against transposon invasion and maintaining the integrity of the host genome have also evolved under selective pressure. In this context, piRNAs have emerged as key guardians of genome integrity across metazoans, along with their associated partner proteins [14]. piRNAs and PIWI proteins are widely conserved in metazoans, from basal species to higher mammalians, such as sponges, mice, rats, and humans, playing a crucial role in safeguarding the gonadal genome from transposon activity (Fig. 1) [5,15–18].

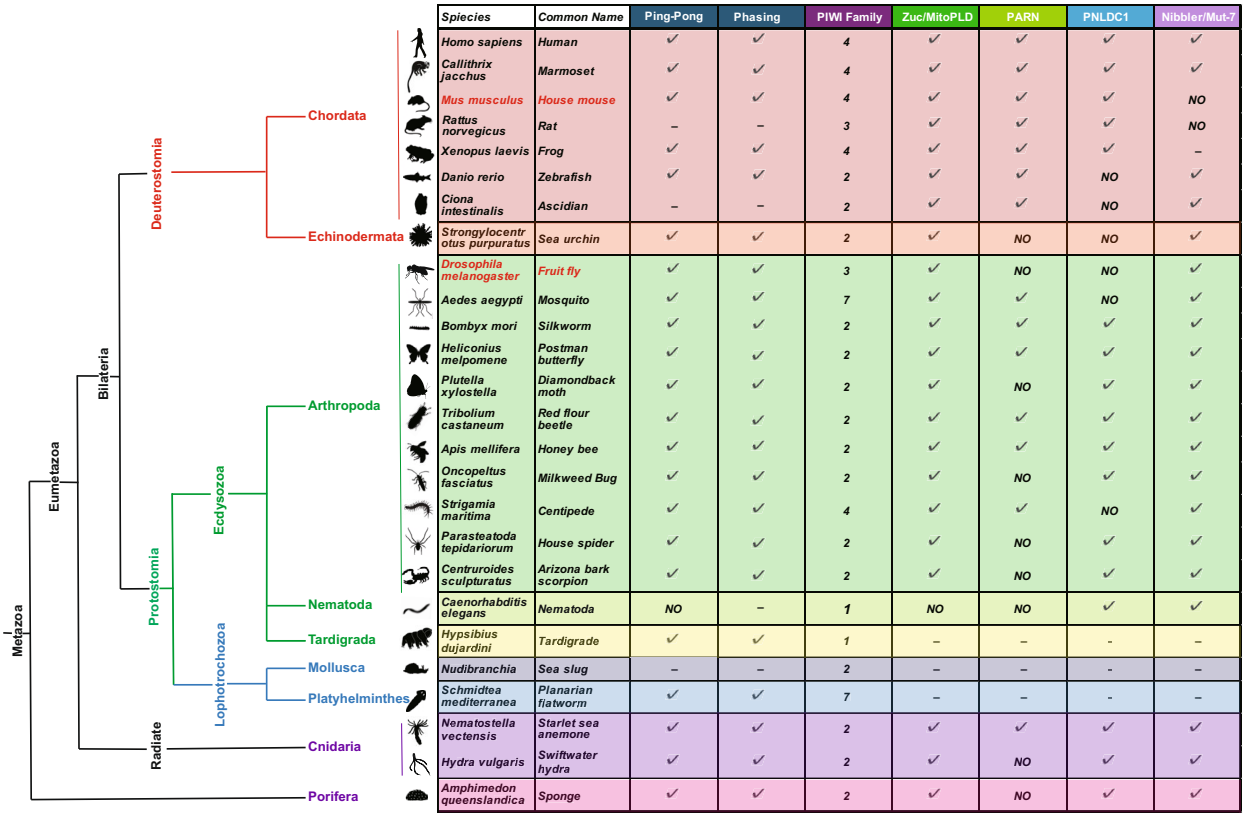
In this review article, we focus on the detailed molecular mechanisms orchestrating the piRNA biogenesis and the organization of their processing sites in model organisms, predominantly *Drosophila*, with a few examples from other animals. We also explore advances in understanding of the molecular machinery involved in piRNA biogenesis in non-membrane organelles, such as nuage and Yb bodies. In addition, we discuss the fundamental roles of conserved proteins and motifs, such as Tudor, LOTUS, or intrinsically disordered regions (IDRs) for the TE silencing by piRNAs. Finally, we examine the implications of these mechanisms in somatic cells, particularly their association with cancers, developmental defects and other diseases.

Pathway of piRNA biogenesis

Transcription of piRNA precursors as a source of piRNAs

piRNA biogenesis begins with the transcription of piRNA precursors, primarily from genomic regions known as piRNA clusters. These clusters, which consist of remnants of multiple copies of both sense and antisense TEs, are located in the pericentromeric and telomeric regions [19–21]. In *Drosophila* ovaries, piRNA clusters are actively transcribed not only in the germline but also in somatic follicle cells, where piRNAs play a crucial role in suppressing endogenous retroviral elements to prevent their invasion into the germline genomes [2,22,23]. piRNA clusters can be classified into two groups based on their transcriptional mechanisms: dual-strand clusters such as *38C* and *42AB*, which refer to the cytogenetic position in the *Drosophila* genome and are active in germline cells. These clusters are transcribed bidirectionally, while the *20A* cluster and the *flamenco* gene are transcribed uni-directionally in germline and somatic cells, respectively [24,25].

Since dual-strand clusters in *Drosophila* germline cells are marked by histone H3K9 trimethylation, which is typically associated with transcriptional repression [26], they lack conventional transcriptional features such as active promoter marks (H3K4me3) and produce transcripts devoid of a 5′ cap and a polyadenylated (poly(A)) tail [27,28]. Therefore, a noncanonical transcriptional system is required for their activation. The H3K9me3-binding protein Rhino (Rhi), a variant of Heterochromatin protein 1d (HP1d), recruits Cutoff (Cuff) and Deadlock to form the Rhino–Deadlock–Cutoff (RDC) complex, which controls transcription of dual-strand clusters [26,28,29]. Moonshiner, a germline paralog of the transcription initiation factor II A subunit 1, is recruited to the RDC complex by Deadlock and engages the TATA box-binding protein-associated factor TRF2 to initiate Pol II-mediated transcription on piRNA clusters [30]. Cuff, together with Rhino and the transcription/export (TREX) complex—consisting of UAP56, DEAD-box helicase, and the THO complex—blocks the binding of the cleavage/polyadenylation specificity factor (CPSF) to poly(A) sites, thereby



Species	Common Name	Ping-Pong	Phasing	PIWI Family	Zuc/MitoPLD	PARN	PNLDC1	Nibbler/Mut-7
Homo sapiens	Human	✓	✓	4	✓	✓	✓	✓
Callithrix jacchus	Marmoset	✓	✓	4	✓	✓	✓	✓
Mus musculus	House mouse	✓	✓	4	✓	✓	✓	NO
Rattus norvegicus	Rat	–	–	3	✓	✓	✓	NO
Xenopus laevis	Frog	✓	✓	4	✓	✓	✓	–
Danio rerio	Zebrafish	✓	✓	2	✓	✓	NO	✓
Ciona intestinalis	Ascidian	–	–	2	✓	✓	NO	✓
Strongylocentrotus purpuratus	Sea urchin	✓	✓	2	✓	NO	NO	✓
Drosophila melanogaster	Fruit fly	✓	✓	3	✓	NO	NO	✓
Aedes aegypti	Mosquito	✓	✓	7	✓	✓	NO	✓
Bombyx mori	Silkworm	✓	✓	2	✓	✓	✓	✓
Heliconius melpomene	Postman butterfly	✓	✓	2	✓	✓	✓	✓
Plutella xylostella	Diamondback moth	✓	✓	2	✓	NO	✓	✓
Tribolium castaneum	Red flour beetle	✓	✓	2	✓	✓	✓	✓
Apis mellifera	Honey bee	✓	✓	2	✓	✓	✓	✓
Oncopeltus fasciatus	Milkweed Bug	✓	✓	2	✓	NO	✓	✓
Strigamia maritima	Centipede	✓	✓	4	✓	✓	NO	✓
Parasteatoda tepidariorum	House spider	✓	✓	2	✓	NO	✓	✓
Centruroides sculpturatus	Arizona bark scorpion	✓	✓	2	✓	NO	✓	✓
Caenorhabditis elegans	Nematoda	NO	–	1	NO	NO	✓	✓
Hypsibius dujardini	Tardigrade	✓	✓	1	–	–	-	–
Nudibranchia	Sea slug	–	–	2	–	–	-	–
Schmidtea mediterranea	Planarian flatworm	✓	✓	7	–	–	-	–
Nematostella vectensis	Starlet sea anemone	✓	✓	2	✓	✓	✓	✓
Hydra vulgaris	Swiftwater hydra	✓	✓	2	✓	NO	✓	✓
Amphimedon queenslandica	Sponge	✓	✓	2	✓	NO	✓	✓

Fig. 1. Conservation of ping-pong and phasing pathways along with proteins involved in these pathways across metazoans. The presence or absence of the ping-pong and phasing pathways, along with associated proteins Zucchini/MitoPLD, PARN, PNLDC1, and Nibbler/Mut-7, are shown. 'Ticks', 'NO' and '–' denote the presence of the protein, the absence of the protein, and unavailability of the data, respectively. The numbers in the PIWI column indicate the number of PIWI proteins in each species. Evolutionary relationships among animal phyla are presented in a phylogenetic tree; however, the branch lengths do not represent evolutionary distances. Taxonomic groups mentioned in the text are highlighted in red.

inhibiting cleavage and polyadenylation and suppressing splicing of piRNA precursors [26,29,31–33]. The transcripts are transported by the export protein, CRM1, together with Nxf3, Nxf1, Bootlegger, and UAP56 [34,35] for further processing in the cytoplasm [26,30,34–38]. Once captured by the DEAD-box RNA helicase Vasa (Vas) in the nuage, the cluster transcripts are processed into mature piRNAs, either by conserved endonuclease Zucchini (Zuc)/mitoPLD or through the alternative ping-pong mechanism, producing distinct 5′ and 3′ ends (Fig. 2A) [2,25,39].

In contrast, in the somatic follicle cells, uni-strand piRNA clusters lacking the H3K9 trimethylation mark are transcribed in an RDC-independent manner. These are capped at the 5′-end, polyadenylated, and subjected to splicing [20,37,40]. The export complex of Nxf1–Nxt1 with the exon junction complex, along with Nup54 and Nup58, two nucleoporins that function exclusively in this pathway, facilitate the nuclear export of transcripts [41–46]. After being transported to the cytoplasm, the precursor transcripts are then processed into mature piRNAs via the Zuc-dependent piRNA biogenesis pathway in the Yb bodies (Fig. 2B) [2,25,47].

piRNA processing in the cytoplasm

In both germline cells and somatic cells, the processing of piRNA precursors necessitates specific non-membrane perinuclear structures that house the requisite proteins, mature piRNAs, and precursor piRNAs. These structures include the nuage in germline cells [48,49] and the Yb bodies in somatic cell [50,51]. They serve as robust molecular platforms that facilitate piRNA biogenesis and the silencing of TEs in the cytoplasm through distinct strategies.

Recent studies have shown that piRNA biogenesis is remarkably conserved across metazoans, from sponges

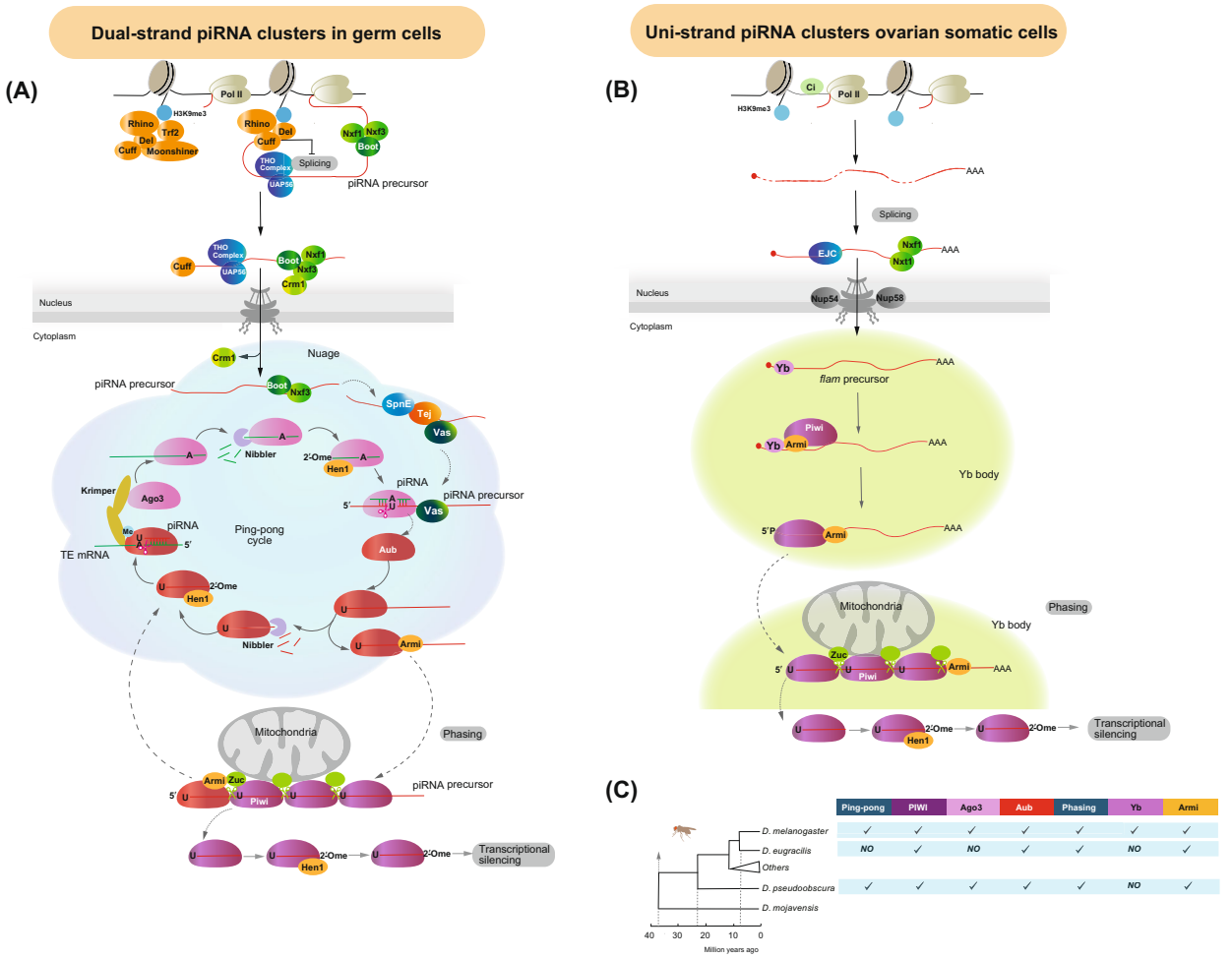


Fig. 2. Transcription and processing machinery for piRNA Precursors in *Drosophila melanogaster*. (A) piRNA biogenesis in germline cells. Rhino recognizes H3K9me3 modifications on dual-strand piRNA clusters and forms the RDC complex with Deadlock and Cutoff. This complex facilitates promoter-independent transcription and contributes to the nuclear export of piRNA precursor transcripts via Bootlegger, which recruits the Nxf3–Nxt1 complex. Nxf3 together with exportin Crm1, then transports the piRNA precursors to the perinuclear nuage on the cytoplasmic side. Within nuage, Aub and Ago3 sequentially cleave TE mRNAs and cluster transcripts, respectively, in turns in the ping-pong cycle. Nibbler trims the 5′ ends of pre-piRNAs, while Hen1 2′-O-methylates their 3′ ends, resulting in mature piRNAs. piRNA precursors can also be processed through phasing. Aub-bound piRNA precursors are transported to the mitochondrial outer membrane by Armi, where they are cleaved by Zuc. Some of these phased piRNAs participate in the ping-pong cycle. Other piRNA precursors, bound by Piwi and cleaved by Zuc, generate Piwi-piRNAs that are subsequently transported into the nucleus. (B) piRNA biogenesis in somatic cells. piRNA precursor transcripts derived from uni-strand piRNA clusters, such as *flamenco*, undergo canonical splicing, 5′ capping and polyadenylation. These processed transcripts are then exported through a complex involving the exon junction complex and Nxf1–Nxt1 complex, which interacts with nucleoporins Nup54 and Nup58 to reach the Yb bodies. Yb binds to these piRNA precursor transcripts and recruits Armi to the Yb bodies, where Piwi binds to the 5′ end of piRNA precursor transcripts after cleavage by a nuclease. Once piRNA precursor transcripts are translocated to the mitochondrial outer membrane, Piwi and Zucchini cleave them to produce Piwi-bound phased piRNAs. These mature piRNAs are imported into the nucleus in complex with Piwi. Figures in A and B are adapted from Ref. [2] with some modification, with permission. (C) Conservation of processing machinery and the related proteins among *Drosophila* species. Both ping-pong-mediated piRNA biogenesis in germline cells and Yb-dependent piRNA biogenesis in somatic cells are generally conserved among *Drosophila* species. However, evolutionary changes have led to the loss of components critical for piRNA biogenesis. For instance, Yb has been independently lost in *Drosophila eugracilis* and the *obscura* group. In addition, *D. eugracilis* has lost Ago3, adopting a ping-pong-independent mechanism for piRNA production, whereas the *obscura* group retains Ago3 and the ping-pong cycle. Figure in C is adapted from Ref. [100] which is copyrighted under a CC-BY-4.0 license, with some modification.

and cnidarians to higher mammalians, including humans (Fig. 1). A unified model of piRNA biogenesis has been proposed encapsulating two interrelated mechanisms: ping-pong amplification, which occur in the perinuclear granule and phasing, which takes place at the mitochondrial outer membrane (Fig. 2) [2,25,52]. While there remains some controversy regarding the presence of the phasing process in silkworm BmN4 cells [52,53], these multifaceted piRNA biogenesis pathways are believed to have co-evolved in the last common ancestor of metazoans, spanning an evolutionary timeline of approximately 800 million years [54].

Ping-pong amplification cycle of piRNAs in germline cells

Following transcription, in *Drosophila* germline cells, piRNA precursors are transported to the nuage, where they are processed into mature piRNAs in a feed-forward amplification mechanism known as the ' ping-pong cycle' (Fig. 2A) [2,25]. This cycle involves the alternating cleavage of precursor antisense strands transcribed from piRNA clusters and sense strand TEs within the nuage [20,49]. This sophisticated mechanism also produces sense-strand piRNAs, which facilitates the post-transcriptional TE repression by amplifying piRNAs [20,49].

In this cycle, Aubergine (Aub) (MIWI in mice) and Argonaute 3 (Ago3) (MILI in mice), two PIWI family proteins in *D. melanogaster*, cooperate with other nuage components to cleave TEs and piRNA precursor transcripts in a complementary manner. Aub initiates ping-pong biogenesis by loading maternal or phasing-derived antisense piRNAs, recognizing and cleaving complementary sense TE transcripts and/or piRNA precursors at a site between the 10th and 11th nucleotides from the 5′ end of the annealed piRNA, thereby generating new 5′ ends (Fig. 2A) [2,19,20,25,55]. During the ping-pong cycle, Krimper (Krimp) mediates the loading of 3′ fragments of sense strand piRNA precursors into Ago3, following their cleavage by Aub, thereby facilitating the production of mature sense piRNAs [56,57]. In addition, Qin/Kumo promotes the heterotypic dimerization between Aub and Ago3, ensuring TE silencing [58,59]. After cleavage by either Aub or Ago3, the 3′ ends of resulting RNAs are further trimmed by the exoribonuclease Nibbler [60–62] and methylated at the 2′-O position by Hen1 (HENMT1 in mice), a modification that protects piRNAs from degradation [63,64]. The resulting mature piRNAs then participate in subsequent rounds of the ping-pong cycle, targeting piRNA precursors with complementary sequences, thus perpetuating a self-sustaining feed-forward loop. This recognition-cleavage loop generates complementary 10-nt sequences at the 5′ ends of Aub-bound piRNAs, which characteristically begin with a uracil (U) at the 1st position of the 5′ end. In contrast, Ago3-bound piRNAs typically have an adenine at the 10th position, reflecting Aub's preference for adenine at the 1st position of the target (t1A preference) [65]. Together, these features—along with their sequence complementarity—constitute the defining 'ping-pong signature' [19,20,29].

Phasing process of generating mature piRNAs

Different species exhibit variations in the number of PIWI family proteins; most have multiple PIWIs that engage in the alternate cleavage of TE sense or antisense piRNA precursors, where their preferences toward either strand affect the alternation (Fig. 1). Although piRNA processing in the germline is well conserved, the trimming by Nibbler in *Drosophila* is evolutionarily atypical. In contrast, other species utilize the poly(A)-specific 3′-to-5′ exoribonucleases, such as PNLDC1 in mice, Trimmer in silkworms, and PARN-1 in *Caenorhabditis elegans* are involved in this process (Fig. 1). All nucleases involved in these processes, including Zuc, PNLDC1, Nibbler/Mut-7 are well conserved from sponges to mammals, albeit not in all, indicating their ancient evolutionary origins (Fig. 1) [62]. Worms lack Zuc and utilize PARN for trimming small RNAs bound to Piwi [66] and Nibbler/Mut-7 is involved in 22G siRNA biogenesis [67], whereas PARN or PNDLC1 are absent in flies (Fig. 1) [62].

The majority of Piwi-bound piRNAs in germline cells possess a U at the first position, yet lack the ping-pong signature, suggesting integration of the phasing process into the ping-pong cycle [60,61,68]. The production of piRNAs in the phasing pathway involves the interaction between nuage components and mitochondria-associated factors [52]. Aub, bound to piRNA precursors, interacts with the RNA helicase Armitage (Armi; MOV10L1 in mice) in the nuage. Armi then transfers the Aub-bound transcripts to the outer mitochondrial membrane where Zuc (mitochondrial phospholipase, or PLD6 in mice) cleaves the transcript, generating the 3′ end of piRNAs (Fig. 2A) [2,25,60,61,68]. The remaining 5′ fragments of piRNA precursors are subsequently bound and cleaved by Piwi (MIWI2 in mice), which are then loaded onto Piwi for nuclear import, facilitating iterative piRNA generation [60,61,69]. This Zuc-mediated processing, known as phasing, strongly biases the initial nucleotide

of piRNAs toward uracil (1U), which aligns with the preference for 5′ end of Piwi-bound mature piRNAs. This specific nucleotide preference enhances the stability of piRNA binding to the MID domain of PIWI [70,71]. In *Drosophila*, phasing not only generates new piRNA sequences but also integrates these phased piRNAs into the ping-pong cycle for further amplification of piRNAs. Depletion of Zuc and Nibbler can induce compensatory mechanisms, including the formation of new ping-pong pairs to maintain the piRNA production, yet no transposon-derived suppressors of the piRNA pathway are observed [62].

The processing of piRNA 3′ end is mediated by either Zuc functions or Nibbler/PNLDC1, which determines whether piRNA precursors are loaded onto Piwi or to Aub/Ago3. In *Drosophila*, Nibbler is enriched in the perinuclear nuage and facilitates the ping-pong pathway, whereas Zuc localizes to the mitochondria and functions in the phasing pathway for piRNA processing. Each pathway engages the resulting piRNAs in post-transcriptional or transcriptional silencing, respectively [62]. Perturbation of Nibbler significantly affects piRNA maturation, highlighting its role in balancing the two pathways of piRNA 3′ ends, similar to PNLDC1 in mice [62,68,72–74]. Zuc compensates for the absence of Nibbler by enhancing the phasing process and using piRNA precursors to reduce biogenesis through the ping-pong pathway. This reveals a competitive relationship between the two piRNA 3′ end formation processes [62].

In the *Drosophila* testes, piRNA biogenesis displays stage-specific variations during spermatogenesis: piRNAs targeting TEs exhibit a more prevalent ping-pong signature in spermatogonia compared to spermatocytes, whereas piRNAs targeting repeats such as *Suppressor of Stellate (Su(Ste))* and *AT-chX* are predominantly generated in spermatocytes rather than spermatogonia [75]. These distinct pathways represent a developmentally programmed intergenerational mechanism. The generation of *Su(Ste)* piRNA is triggered by maternally deposited *1360*/*Hoppel* piRNA through phasing in the spermatogonia, which in turn suppresses the *Ste* expression in spermatocytes [76].

Collectively, piRNA-induced silencing complexes (piRISC), containing Piwi (or MIWI2 in mice) and piRNAs produced through the ping-pong and phasing pathways by processing of cluster transcripts, enable both post-transcriptional and transcriptional TE silencing. Nevertheless, the significance of a large number of piRNAs from clusters in TE silencing in *Drosophila* has recently been questioned, as simultaneous deletion of three major piRNA clusters (*42AB*, *38C* and *20A*) did not cause TE transposition or female sterility [77]. This suggests that piRNAs essential for TE silencing may originate from TE insertions scattered throughout euchromatic regions, which are potentially converted into piRNA-generating loci through yet unidentified mechanisms [26,78].

piRNA pathways in somatic cells

In *Drosophila* somatic cells, Aub and Ago3 that are involved in the germline-specific ping-pong cycle, are not expressed. Instead, piRNA biogenesis relies exclusively on Piwi, Zuc, and several co-factors that localize to the mitochondrial surface adjacent to a perinuclear cytoplasmic structure known as Yb bodies [40]. Composed of various proteins, including Sisters of Yb (SoYb), Vreteno (Vret), Shutdown (Shu) and Armi, Yb bodies serve as the piRNA processing sites [50,51,79–82].

The piRNA precursors are transported to the cytoplasm, where they are recognized by the DEAD-box helicase, Yb, through its binding to piRNA precursors, such as *traffic jam* (*tj*) RNA and *flamenco* cluster transcripts [47,69,83]. Once recognized and processed by Yb in the Yb bodies, the Piwi-bound piRNA precursors binds to Armi and are transported from the Yb bodies to mitochondria, where Zuc initiates the phasing step (Fig. 2B) [2,25,70]. Daedalus (Daed), which interacts with Gasz anchors the piRNA precursors to the mitochondrial outer membrane for processing. Upon binding to piRNAs, Piwi undergoes a conformational change that exposes its nuclear localization signals, facilitating the transport of the piRNA (methylated by Hen1)-Piwi complex into the nucleus through the canonical importin-mediated transport pathway (Fig. 2B) [2,25,64,84].

piRNA biogenesis in mice

Three PIWI family proteins in mice, MILI, MIWI2, and MIWI, are expressed in a developmental stage-dependent manner in the testes, and their absence causes male infertility [85–87]. The 26–31 nt of testicular piRNAs are classified into two populations: pre-pachytene and pachytene piRNAs. Pre-pachytene piRNAs, produced in embryonic and perinatal germ cells, are associated with MILI and MIWI2, while pachytene piRNAs, generated in spermatocytes and round spermatids, are bound to MILI and MIWI [85,88]. Here, we focuses on the well-studied biogenesis of pre-pachytene piRNAs, which involves phasing processing and ping-pong amplification. For other mechanisms of piRNA biogenesis in mice, we direct readers to further specialized literature.

Pre-pachytene piRNAs are generated from transcripts with a cap structure and a poly (A) tail,

originating from the unistrand clusters and TE RNAs through both phasing and ping-pong processing pathways [89,90]. In the phasing pathway, the piRNA cluster transcript is consecutively cleaved by MitoPLD/PLD6 (Zuc in *Drosophila*), supported by MOV10L1 (Armi in *Drosophila*), producing a 5′U RNA fragment [60,61,91]. The 5′U RNA fragment is then incorporated into MILI, which is subsequently recruited to the mitochondria by TDRKH [73]. The 3′–5′ exonuclease PNLDC1 (Trimmer in silkworm and Nibbler in *Drosophila*) then trims the 3′ ends [92–94], and HENMT (Hen1 in *Drosophila*) adds 2′O-methyl to the 3′ end of piRNAs, maturing them [95], for loading into MILI. This complex can then engage in the ping-pong cycle, cleaving complementary antisense transcripts and generating secondary piRNAs bound to either MILI or MIWI2. MIWI2-bound piRNAs translocate to the nucleus and suppresses transcription through CpG methylation at TE loci, such as LINE or IAP [96]. The absence of MILI reduces MIWI-2 bound piRNA and its nuclear localization [88,97], highlighting that MILI play an essential role in the ping-pong cycle and that most piRNAs originate from ping-pong pathways.

Conservation of TE silencing among arthropods

Transcriptional and post-transcriptional silencing of TEs is highly conserved across nearly all animals descended from the last common ancestor of arthropods. Notably, the genus *Drosophila*, an arthropod, has evolved a unique somatic piRNA biogenesis that operates independently of the ping-pong cycle, highlighting the evolutionary adaptability of the piRNA pathway [98–100]. In *Drosophila* somatic gonadal cells, *gypsy* retrotransposons, which are capable of invading adjacent germline cells, are suppressed by piRNAs processed through phasing, a mechanism that is highly conserved within the genus [23,40,51,83,98,101–103]. However, the *Yb* gene is not conserved in more distant *Drosophila* species such as *Drosophila obscura* and *Drosophila eugracilis* (Fig. 2C) [99,100]. Nevertheless, somatic gonadal cells of the both species contain perinuclear Yb body-like structures with intense localization of Armi and produce TE antisense piRNAs similar to germline cells. This suggests that an alternative protein to Yb may recruit Armi for efficient transcript processing (Fig. 2C) [99,100].

Moreover, in the germline of *D. eugracilis*, Aub exclusively loads piRNAs generated through phasing, and no ping-pong signature is observed (Fig. 2C) [99,100]. This indicates that TE antisense piRNAs are generated through phasing on the mitochondrial surface by Zuc without the involvement of slicing by endonucleases in the ping-pong pathway [99]. Nevertheless *D. eugracilis* still possesses components of the ping-pong pathway such as Spindle-E (Spn-E), Vas, and Qin [40,68,104], implying a similar, but distinct piRNA processing other than the ping-pong cycle. These observations highlight that species within the obscura group and *D. eugracilis* may have evolved a novel mechanism for phasing piRNA biogenesis. This raises questions about the functional diversity of the piRNA pathway and presents biological challenges in understanding how these species distinguish between self and non-self RNA during evolution.

Proteins in non-membrane nuage structures for piRNA processing and others

In eukaryotes, unlike organelles surrounded by a lipid bilayer such as the nucleus, endoplasmic reticulum, or Golgi apparatus, ribonucleoprotein (RNP) complexes form compartments in either the nucleus or cytoplasm and function as non-membrane organelles. Within these biomolecular condensates, RNAs and proteins respond rapidly to stimuli such as stress or environmental changes due to thermodynamic forces, including changes in density [105,106]. Among these non-membrane structures, P-granules in nematodes are a notable example of RNP complexes found in animal germline cells. Pioneering studies have highlighted their dynamic behavior, including fusion, division, and component exchange, demonstrating their organization as phase-separated, RNP-containing non-membrane organelles [24,107–110].

Unlike P-granules, not all components of the non-membrane nuage are regulated by phase separation. However, certain elements exhibit this property, which contributes significantly to RNP granule formation. For example, Vas, an essential component in piRNA biogenesis that recruits piRNA precursors to nuage together with UAP56 [39,111], also functions to form germ granules by being enriched at the posterior pole of *Drosophila* oocytes [112,113]. In silkworm germline culture cells, BmN4, the RNA-binding activity of Vas is crucial for droplet formation, and its ATP activity is required to accelerate the functional assembly of Siwi–Ago3–RNA complexes [104,114]. In the following subsection, we focus on the nuage components, particularly discussing the unique domains that are commonly found among them: Tudor domain, LOTUS domain and the IDR domain, which are most relevant for biomolecular condensates.

Tudor domain containing proteins for piRNA processing and constitution of other cytoplasmic bodies

Tudor domain-containing proteins (TDRDs) are characterized by the presence of Tudor domain(s), comprising approximately 60 amino acids and forming antiparallel beta-barrel structure [115,116]. These proteins are involved in a variety of cellular processes by interacting with a diverse array of molecules, including proteins, DNA, and RNA. Their functions extends to gene silencing, DNA damage response, and regulation of protein synthesis [80]. TDRDs containing multiple Tudor domains facilitate multivalent interactions with other proteins and are abundantly localized in specific non-membrane structures such as nuage and Yb bodies, indicating a crucial role in RNP granule formation [117].

Tudor (Tud), the founding member of the TDRD family, was originally identified as an RNA-binding protein involved in RNA metabolism in *Drosophila*. It contains 11 Tudor domains and interacts with methylated Aub in germ granules, serving as a scaffold for their assembly [118–120]. In *Drosophila*, many TDRDs, Krimp, Qin/Kumo (mouse Tdrd4), Tejas (Tej) (mouse Tdrd5), Tud (mouse Tdrd6), Tapas (Tap) (mouse Tdrd7), Vret, and the ATP-binding helicase Spn-E (mouse Tdrd9), are localized at nuage in a hierarchical manner and are engaged in ping-pong amplification [48,58,79,120–122], suggesting that disruption of upstream steps during the piRNA biogenesis impairs nuage formation, the processing site for piRNAs.

Our recent studies have shown that Tej, a TDRD localized to the nuage, functions as a hub for proper nuage formation by recruiting two RNA helicases, Vas and Spn-E, through distinct domains, along with piRNA precursors [111]. Indeed, Tej has been suggested to function upstream of the ping-pong pathway, as evidenced by the delocalization of other components upon loss of Tej (Fig. 2A) [2,111,121]. In addition, Spn-E interacts with several nuage-piRNA components, such as Qin/Kumo, Aub and Ago3 [123], and Krimp's interaction with Ago3 enhances heterotypic ping-pong amplification [57]. These findings underscore that nuage formation is regulated through interactions between scaffolding proteins such as TDRDs and other transiently interacting proteins. These interactions facilitate the formation of highly organized aggregates akin to phase separation [124].

Interactions between nuage components through post-translational modifications also play a crucial role in the ping-pong cycle, a further processing step of piRNA precursors. TDRDs interact with PIWI family proteins through symmetrically dimethylated or unmethylated arginine residues, playing critical roles for piRNA biogenesis [19,20,80,125–131]. The Tudor domain on Krimp specifically binds to unmethylated arginine residues on piRNA-unloaded Ago3, while recognizing methylated arginine residues on piRNA-loaded Aub.

This dual binding facilitates the efficient transfer of piRNA intermediates between Aub and Ago3, which is crucial for maintaining the antisense bias of Aub-bound piRNAs [56,57,68,132]. Qin/Kumo, on the other hand, prevents homotypic Aub–Aub interactions by correctly loading cleaved piRNA products to Aub, thereby promoting efficient heterotypic Aub–Ago3 ping-pong interactions in *Drosophila* [68]. In addition, silkworm Spn-E suppresses homotypic Siwi-Siwi ping-pong interactions independently of its ATPase activity, while it is required for the heterotypic Siwi–BmAgo3 interaction [133]. Similarly, its mouse orthologue, RNF17, suppresses homotypic ping-pong in meiotic cells and prevents promiscuous piRNA production [68].

TDRD proteins function in the formation of non-membrane structures not only in germline cells but also in somatic cells, specifically in the formation of Yb bodies. Yb, the core component of the Yb bodies, is crucial for Zuc-dependent piRNA biogenesis and is located near the mitochondria. It harbors a helicase-like domain, similar to DEAD/DEAH-box RNA helicases, and a Tudor domain [51,134]. Other Yb body proteins containing Tudor domains, such as BoYb, SoYb and Vret, are also present in Yb bodies and/or nuage [80], but the specific roles of the these Tudor domain proteins in RNP granules remain poorly understood.

TDRDs are indispensable not only for non-membrane bodies involved in piRNA processing but also for other cytoplasmic structures such as Cajal bodies, stress bodies and P-bodies. For example, piRNA components are co-localized with P-bodies, which are sites of mRNA turnover and silencing, forming the structure called Pi-bodies or piP-bodies [135–137]. Collin, a critical component of Cajal bodies, plays essential roles in the assembly and modification of small nuclear ribonucleoproteins (snRNPs) for the maturation of spliceosomal components [138]. Stress granules containing TDRD3 regulate mRNA stability and translation during stress responses [139].

LOTUS domain proteins function for piRNA processing

In addition to TDRDs, proteins containing the highly conserved LOTUS domain (also known as OST-

HTH), which consists of approximately 80–100 amino acid residues, also contribute to the formation of non-membrane structures. While LOTUS domain family proteins are found in metazoans from bacteria to eucaryotes, their function is predominantly in the germline cells [140–143]. The LOTUS domain has been further categorized into extended LOTUS (eLOTUS) and minimal LOTUS (mLOTUS) domains based on structural and functional variations. The eLOTUS domain recruits Vas and stimulates its ATP activity, acting as a DEAD-box RNA helicase regulators, whereas the mLOTUS domain does not [144,145].

In *Drosophila* and mice, LOTUS domain proteins such as Oskar (no mouse homolog), Tej/TDRD5, Tap/TDRD7, and Meiosis arrest female 1 (MARF1) (mouse Limkain B1) play essential roles in various processes during gametogenesis, including germ granule formation, meiosis, and TE repression via the piRNA pathway. At the posterior pole of the *Drosophila* oocyte, Oskar specifically through the eLOTUS domain, interacts with Vas, assembling other proteins and over 200 maternal mRNAs in germ granules, which contributes to embryonic and axial formation [144]. dMarf1, the *Drosophila* homolog of MARF1, is important for oocyte maturation, though the specific role of its LOTUS domain in structure formation remains unclear [146,147]. Tej and Tap, the *Drosophila* homologs of Tdrd5 and Tdrd7 respectively, contain not only Tudor domains but also a LOTUS domain (Fig. 3), which contribute both individually and synergistically to piRNA biogenesis [121,122]. Various LOTUS domains from different species have been shown to strongly interact with the C-terminal RecA-like domain of Vas to activate its ATP hydrolysis. This suggests that activation of Vas is a conserved function of the eLOTUS domain [144]. The eLOTUS domain of Tej interacts with Vas to promote the processing of piRNA precursors (Fig. 2A) [2,111]. In contrast, the mLOTUS domain lacks a C-terminal extension and does not interact with Vas or related DEAD-box proteins [144,146]. Furthermore, the LOTUS domain of Tej and Osk recognizes the guanine-rich RNA forming G-quadruplex (G4) structures, while LOTUS domain of Tap does not bind to them, though the biological significance of these differences in G4 RNA binding remains unclear [148].

Recent studies in *C. elegans* have also highlighted the role of LOTUS or Tudor domain proteins in the assembly of non-membrane P-granules and perinuclear P-granules which are akin to germ granules and nuage in *Drosophila* [149–151]. The LOTUS domain proteins MIP-1 and MIP-2 have been identified as interacting with MEG-3, which is exclusively localized in P-granules. These proteins facilitate the condensation of P-granules and serve as a hub to recruit the Vas homolog GLH-1 though the LOTUS domain in the germline lineage, tethering P-granule to the nuclear periphery. This interaction potentially initiates the formation of extensive networks that scaffold and nucleate core processes within germ granules [149]. The LOTUS and Tudor domain-containing protein LOTR-1 interacts with the ZNFX-1 helicase in a sub-granule known as the Z-granule, which is essential for small RNA generation and piRNA-mediated transposon silencing [151]. Thus, analysis of these domains provides valuable insight into the molecular functions and hierarchical structural organization of granule components.

Function of IDR for RNA processing in non-membrane structure and LLPS

IDR domains constitute approximately one-third of the eukaryotic proteome and play crucial roles in various cellular functions [152]. Unlike folded domains, IDRs exist as conformationally flexible structures. They exhibit structural biases based on their amino acid sequences, influenced by factors such as polarity, hydrophobicity, electrostatic or cation-π interactions between side chains. These interactions generate attractive or repulsive forces between distal regions of IDRs [153–156]. While folded domains benefit from a network of non-covalent interactions within the molecule that determine molecular topology, all residues within IDRs are exposed, at least transiently, due to their disordered nature. As a result, the entire sequence can respond directly and rapidly to changes in the subcellular environment, potentially playing important roles in downstream biological functions [157,158]. Thus, IDR-containing proteins involved in non-membrane structures facilitate rapid adaptation to changes in the subcellular environment with conformational flexibility [153–156]. They are also abundant in various RNA-containing granules such as P-granules, germ granules, and nuage in the germline lineage, in addition to neurodegenerative diseases [159–162]. Notably, IDR-containing proteins such as Osk, Vas, Aub, and Ago3 participate in critical roles within these granules. For example, Osk regulates the interaction of Vas in germ granules through the liquid-like or hydrogel-like properties of its IDR [163]. In addition, mammalian DDX3X, having a long IDR at its N terminus similar to that of Vas, contributes to the formation of granules in the germline by facilitating phase separation associated with RNA binding [106,164,165]. Indeed, other RNA-dependent DEAD-box ATPases are also

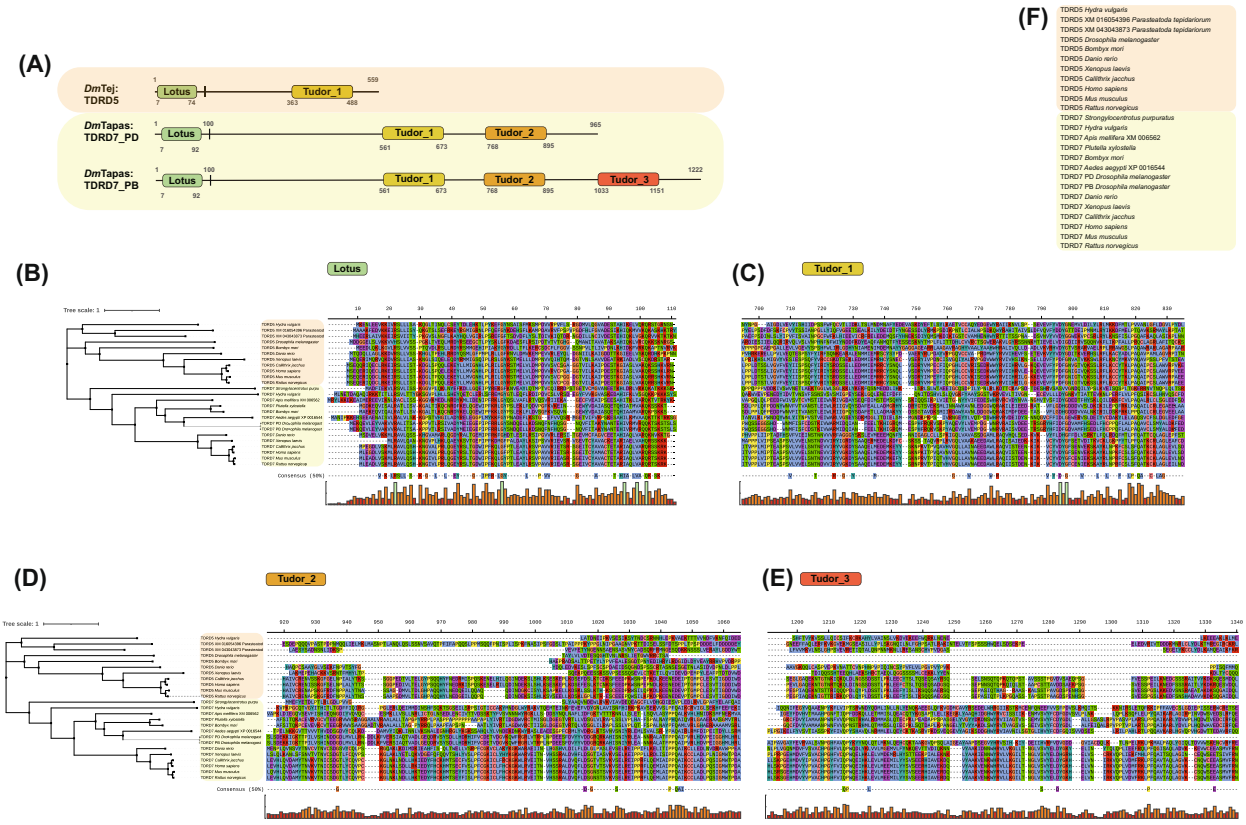


Fig. 3. Conservation of LOTUS and Tudor domains of the TDRD5 and TDRD7. (A) Schematic representations of TDRD 5 and TDRD7 in *Drosophila melanogaster*. (B) Homology alignment of LOTUS domain of TDRD5 and TDRD7 across metazoans. Accession numbers for TDRD5: *Hydra vulgaris*: XM047278614.1, *Parasteatoda tepidariorum*: XM016054396, XM043043873, *D. melanogaster*: FBgn0033921 [A1Z9P1], *Bombyx mori*: A0A8R2M345, *Danio rerio*: A0A8M9PDW1, *Xenopus laevis*: NP001090599.1 [A1L1H3], *Callithrix jacchus*: ENSCJAG00000008098 [F7IN5], *Homo sapiens*: XP054190703.1, *Mus musculus*: NP001264659.1, *Rattus norvegicus*: A0A0H2UHC6. TDRD7: *Strongylocentrotus purpuratus*: XP011669388, *H. vulgaris*: XM047289989.1, *Apis mellifera*: XM006562, *Plutella xylostella*: XP037969050, *B. mori*: A0A8R2C856, *Aedes aegypti*: XP0016544, PD *D. melanogaster*: FBpp0290425, PB *D. melanogaster*: FBpp0085592, *Da. rerio*: NP998270.1 [A6NAF9], *X. laevis*: NP001084569.2 [Q6NU04], *C. jacchus*: XP002743155.1 [F7HUL2], *H. sapiens*: NP001289813.1 [Q8NHU6], *M. musculus*: NP001277404.1 [Q8K1H1], *R. norvegicus*: NP620226 [Q9R1R4]. (C–E) Homology alignment and a phylogenetic tree of the Tudor domains of TDRD5 and TDRD7 across metazoans generated by CLUSTALW along with ITOL. Note that TDRD5 homologs typically contain only one Tudor domain, whereas TDRD7 homologs generally harbor three Tudor domains. This structural variance accounts for the observed gaps and differences in domain conservation among the species analyzed. (F) Names of various species used for homology alignment in TDRD5 and TDRD7.

known to regulate RNA-containing phase-separated organelles [106,165].

In piRNA biogenesis pathways, piRNA-mediated post-transcriptional and transcriptional silencing of TEs leads to the formation of piRNA-containing ribonucleoprotein (RNP) granules. Within these RNP granule, RNA and RNA-binding proteins can segregate from their surroundings by phase separation at a certain concentration threshold. Inhibition of piRNA cluster transcription results in the dissociation of nuage, suggesting that the processing of piRNA precursors itself induces the formation of the RNP condensate [28]. Consistently, catalytic mutants of Siwi (Piwi homolog in silkworm) disrupt the proper distribution of nuage structure [137]. IDRs within PIWIs are thought to facilitate the phase separation necessary for RNP granule formation [70,166,167]. Tej, a *Drosophila* homolog of Tdrd5 that serves as a core component in the proper nuage formation, recruits Vas and Spn-E and contributes to the dynamics of Vas through IDR in the ovaries [111] (Fig. 4A–D). Thus, IDRs of nuage components emphasize their significance across species in contributing to RNP granule formation and protecting the germline genomes.

Liquid–liquid phase separation (LLPS) also contributes to the formation of Yb bodies, which are RNP

granules where phased piRNA processing occurs in *Drosophila* somatic gonadal cells. Yb, a core component of Yb bodies, contains a Tudor domain and an IDR (Fig. 4E,F), and the formation of Yb bodies is sensitive to 1,6-hexanediol, indicating a role of LLPS in their assembly [168]. Importantly, the production of selective phased piRNAs from piRNA precursor transcripts depends on the interaction of Armi with Yb and its subsequent localization to Yb bodies [102,168], indicating that the accumulation of these molecules in these condensates is crucial for proper piRNA processing.

In *D. melanogaster*, Piwi, the founding member of PIWI family proteins, is predominantly localized in the nucleus, where it functions to suppress the transcription of TEs by forming piRISC [169,170]. Piwi's cofactors, Maelstrom (Mael) and Panoramix (Panx) that have also IDRs, play a central role in TE transcriptional silencing, as well as promoting Rhino-dependent cluster transcription and the generation of piRNA precursors, thereby supporting piRNA production [44,45,169,171–176]. This transcriptional TE silencing also depends on the formation of nuclear RNP granules composed of Panx-Cut up/LC8-Nxf2-Nxt1 complexes, interacting with DNA and RNA along with facilitating granule formation (Fig. 4G,H) [177,178]. Future studies on the morphology or dynamics of functional granules will further illuminate the importance of RNP condensates in piRNA biogenesis.

piRNA functions beyond the gonads

piRNA and diseases

Dysregulation of PIWI and piRNA functions has been implicated not only in infertility but also in various diseases, including cancer and neurodegenerative disorders [179]. Recent studies have revealed the role of piRNAs in cancer cell malignancy via RNA modification. For example, piRNAs and/or PIWI proteins are involved in the epigenetic control of gene expression [180], such as the upregulation of METTL14 mRNA via m6A DNA methylation by the specific piRNA-14633 in cervical cancer cell malignancy [181] or the modulation of ZEB1 via m6A RNA modification of obesity-associated protein genes by piRNA-17560 [182]. In addition, TDRDs are more closely linked to the structural aspects of the non-membrane SMN (survival motor neuron) protein, causing severe neurodegenerative disorders involving RNA metabolism [117].

Other studies highlight the involvement of piRNAs and the piRNA–PIWI complex in neural cells and brain [183–185]. In the central nervous system of *Aplysia*, specific piRNAs, piR-F modulates the expression of CREB-2 affecting gene expression either through epigenetic silencing or enhancement [186]. In *Drosophila*, the loss of PIWI family proteins leads to the upregulation of TEs in αβ neurons [187]. Furthermore, several TEs are upregulated in a *Drosophila* model of tauopathy expressing pathogenic Tau, where overexpression of Piwi suppresses dysregulated TEs in the brain [188]. The piRNA pathway also functions in neural crest specification in chicks by modulating the expression of a transposon-derived gene ERNI, which in turn regulates Sox2 expression [189]. Additionally, somatic piRNAs and Piwi expression have been observed in the *Drosophila* fat body [190]. In Piwi mutants, enhanced DNA damage and reduced lipid stores in the fat body indicate a sensitivity to starvation and reduced lifespan, suggesting that the piRNA pathway is essential for metabolism and overall health in flies.

However, the majority of non-gonadal piRNAs identified in mammals are often fragments of non-coding RNAs, such as rRNAs, tRNAs, YRNAs, snRNAs, snoRNAs and intermediates of miRNAs, which are frequently misclassified as piRNAs in various databases [191]. These findings suggest potential contamination unrelated to piRNAs generated by PIWI's function. Furthermore, the functional piRNA pathway may not directly induce carcinogenesis. This is supported by the lack of expression of DDX4/Vas and the absence of functional piRNA silencing complexes in non-germline cancers [192,193]. In addition, the loss of *PIWIL1* does not affect TE expression in a colon cancer cell line [192]. Human PIWIL1 also enhances metastasis in pancreatic ductal adenocarcinoma and promotes gastric cancer by piRNA-independent mechanisms [194]. More detailed studies of piRNA pathways and the piRNA-PIWI complex are needed to clarify whether piRNA expression is merely correlated with, or actively contributes to, carcinogenesis and other diseases.

piRNA functions in other tissues

Beyond germline cells, only a few cases involve piRNAs production via the ping-pong cycle in non-membrane organelles. For example, in *Drosophila* somatic gonadal cells, piRNA processing through ping-pong amplification can be artificially induced by perturbing lethal(3)malignant brain tumor (L(3)mbt), a

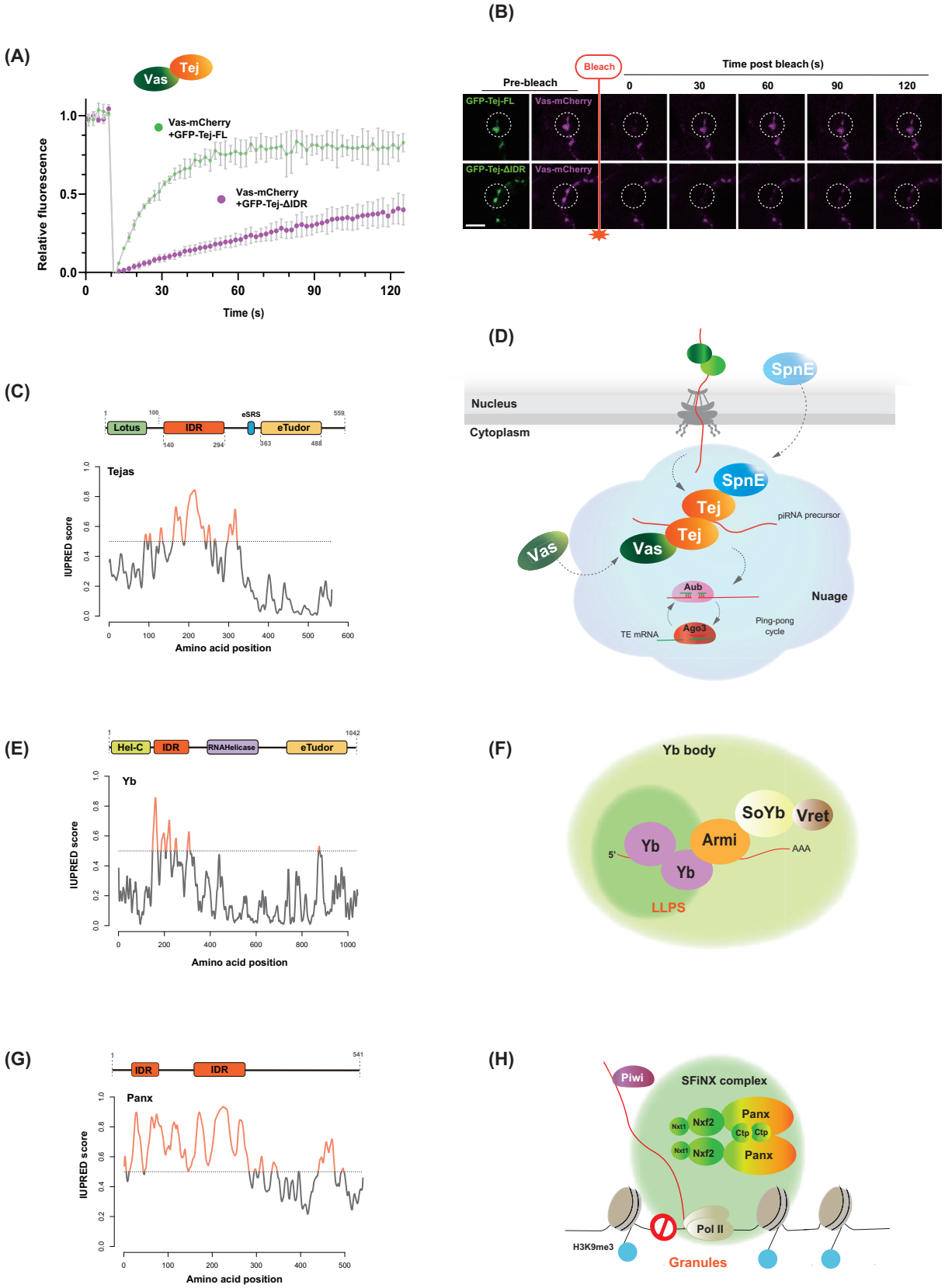


Fig. 4. Function of proteins containing IDR in the piRNA pathways. (A) FRAP analysis demonstrating that IDR of Tej facilitates Vas mobility in *Drosophila* ovaries. The fluorescence intensity of Vas-mCherry, co-expressed with either full-length Tej (Tej-FL) (green) or Tej lacking IDR (Tej-ΔIDR) (magenta), is plotted over time after bleaching. (B) Sequential images of *Drosophila* nuage show the recovery of the Vas-mCherry fluorescence intensity in the presence of Tej-FL or Tej-ΔIDR before and after photobleaching (marked by dotted white circles). Scale bar indicates 5 μm. (C, E, G) Schematic diagrams illustrate the domain structures of Tej, Yb, and Panx proteins (upper, each panel). IUPRED profiles for these proteins are displayed below (lower, each panel), with the *y*-axis representing IUPRED scores. Scores above 0.5 are highlighted in orange. The *x*-axis represents the amino acid positions. (D) A schematic model illustrating the function of Tej in ping-pong piRNA processing. Tej recruits Spn-E to the cytoplasmic perinuclear nuage via its eSRS motif. In addition, Tej recruits Vas through the LOTUS domain and modulates Vas dynamics through its IDR. (F) A schematic model illustrating the interactions between Yb, Armi, SoYb and Vret within the Yb bodies. Yb forms a homodimer and exhibits sensitivity to 1,6-hexanediol. (H) A schematic model of the SFiNX complex involved in TE silencing, composed of Panx, Ctp, Nxf2, and Nxt1. This complex forms a DNA- or RNA-dependent granule structure. Figures in A–D are adapted from Ref. [111], which is copyrighted under a CC-BY-4.0 license.

protein containing three malignant brain tumor (MBT) domains involved in chromatin regulation and transcriptional repression [195,196]. Loss of L(3)mbt in the somatic gonadal OSC cells leads to the ectopic expression of germline genes involved in piRNA processing, resulting in the generation of piRNAs that exhibit a ping-pong signature [195–198]. Coincidentally, proteins associated with ping-pong amplification are found to be enriched in the granular structures resembling nuage in germline cells.

Interestingly, piRNAs exhibiting ping-pong signatures have been detected in stem cells of basal metazoans [199], particularly in multipotent interstitial stem cells in Hydra [200,201] and pluripotent neoblasts in planarians [202,203]. These stem cells, which contain piRNAs and PIWI family proteins enriched in nuage-like non-membrane structures, can differentiate into germline cells. This germline competence may trigger TE activation, leading to the activation of the piRNA pathway for their repression. In Hydra, not only germline competent interstitial stem cells but also perpetually dividing epithelial cells contain piRNAs with a ping-pong signature and harbor nuage-like structures [201,204]. These observations suggest a strong link between the activation of transposons and the defensive response of the organism, possibly mediated by the formation of non-membrane organelles and the generation of piRNAs through the ping-pong amplification cycle.

Conclusions

piRNA biogenesis plays a pivotal role in the transcriptional and post-transcriptional silencing of TEs in animal gonads for safeguarding the integrity of germline genomes. While these mechanisms are broadly conserved across metazoans, evolutionary adaptations have led to the loss or acquisition of specific components. This has resulted in the emergence of alternative proteins or complexes that compensate for or replace the original piRNA production and/or TE silencing function. However, many factors and unique molecular processes, such as protein modifications or transient interactions, remain poorly understood. With the evolution of these components, TEs also adapt in response to various environmental and developmental cues, including viral infection and stress. This highlights the necessity of exploring TE variation not only across deviated animal species but also in response to diverse environmental pressures.

Two fundamental TE silencing pathways operate within specialized non-membrane structures: the ping-pong amplification cycle in nuage within germline cells and phasing processing in Yb bodies within somatic cells. These non-membrane structures in the cytoplasm house individual proteins such as PIWI family proteins or Yb and Armi, ensuring the effective maturation of piRNAs through mechanisms that forms RNP granules plausibly controlled by LLPS.

In addition to PIWI family proteins, other key components of piRNA processing—including those with specific domains such as Tudor, LOTUS, and IDRs—are also essential for the formation and function of these non-membrane structures. Recent biochemical and structural studies have revealed that TDRDs contribute to the assembly and functionality of piRNA processing granules with PIWI family proteins, other RNA-binding modules and DNA/RNA, regulating the dynamics of the granules mediated by specific domains or IDRs. Similarly, proteins with LOTUS domains can enhance the stability and specificity of interactions with other nuage components, thereby facilitating the assembly of the silencing machinery to combat TEs. Further studies of their cellular and molecular mechanisms will provide insights into the dynamics of these granules and the populations of RNAs, including piRNA precursors, that assemble via LLPS or other mechanisms. These studies will also elucidate how

these processes respond to developmental stages and biological cues, thereby advancing our understanding of piRNA-mediated genome defense.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

RS designed the outline and drafted, edited, revised the manuscript and generated the figures. TK drafted, edited and revised the manuscript. Both authors reviewed and approved the final version of the manuscript.

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