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PIWI-Interacting RNA in *Drosophila*: Biogenesis, Transposon Regulation, and Beyond

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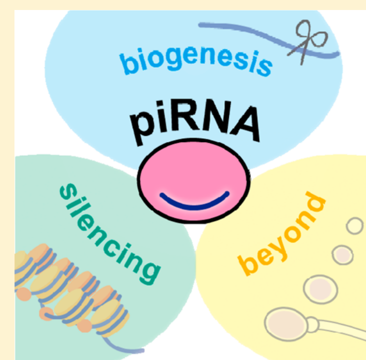
ABSTRACT: PIWI-interacting RNAs (piRNAs) are germline-enriched small RNAs that control transposons to maintain genome integrity. To achieve this, upon being processed from piRNA precursors, most of which are transcripts of intergenic piRNA clusters, piRNAs bind PIWI proteins, germline-specific Argonaute proteins, to form effector complexes. The mechanism of this piRNA-mediated transposon silencing pathway is fundamentally similar to that of siRNA/miRNA-dependent gene silencing in that a small RNA guides its partner Argonaute protein to target gene transcripts for repression via RNA−RNA base pairing. However, the uniqueness of this piRNA pathway has emerged through intensive genetic, biochemical, bioinformatic, and structural investigations. Here, we review the studies that elucidated the piRNA pathway, mainly in *Drosophila*, by describing both historical and recent progress. Studies in other species that have made important contributions to the field are also described.



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1. INTRODUCTION

A wide range of organisms has permitted symbiosis between genomes and transposons, non-self DNA elements, over a long evolutionary period.[1,2] The best known characteristic of transposons is that they can move around (i.e., transpose) the genome in a random fashion.[3,4] Transposons can be subdivided into DNA and RNA transposons, the latter of which are also known as retrotransposons. DNA transposons have the ability to extract themselves from, and insert themselves into, the genome, and they are considered "cut and paste" type transposons. RNA transposons are also considered "copy and paste" transposons because RNA transcripts are reverse-transcribed by their own enzymes and the resultant DNA fragments are inserted into the genome. Living organisms have allowed transposon symbiosis, because the transposition of transposons is often advantageous. For example, transposons act as mutagens to enhance various abilities, allowing the organism to adapt and survive severe, life-threatening environments. However, in parallel, their unrestrained, selfish movement across a genome can cause DNA damage by insertion mutations. If this happens in the germline genome, gonadal development deteriorates, leading to infertil-

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ity.[5−12] Therefore, organisms have acquired an elaborative system for controlling transposons, particularly in the germline, namely the PIWI-interacting RNA (piRNA) dependent transposon silencing mechanism.

piRNA-dependent transposon silencing is one form of small RNA-mediated gene silencing (also known as RNA silencing), a regulatory mechanism for controlling gene expression.[13−15] In this pathway, a small noncoding RNA of 21−33 nucleotides (nt) guides the Argonaute proteins to target gene transcripts by means of RNA−RNA base pairings. The Argonaute−small RNA complex is known as the RNA-induced silencing complex (RISC). RNA silencing occurs either transcriptionally or post-transcriptionally depending on subcellular localization of the RISC. Nuclear RISC represses target genes transcriptionally by remodeling chromatins through repressive histone modification and/or DNA methylation. Cytoplasmic RISC, on the other hand, represses target genes post-transcriptionally, which mainly depends on target RNA cleavage via the endonuclease activity of Argonaute. This activity, known as Slicer activity, slices the target RNAs at a site facing the center of the small guide RNA.[13,14] Some Argonaute members do not possess Slicer activity. In these cases, the proteins trigger RNA silencing by inducing translational repression and/or mRNA decay by associating with cofactors such as GW182.[16,17]

While bacteria express one Argonaute protein, multicellular organisms express multiple Argonaute family members, which are divided into two subclades, AGO and PIWI.[18,19] AGO subclade members (hereinafter referred to as AGO proteins) are ubiquitously expressed and form RISCs with microRNAs (miRNAs) or small interfering RNAs (siRNAs), which are also ubiquitous. In contrast, PIWI subclade proteins (PIWI proteins) expressed in the germline only form RISCs with piRNAs. This PIWI-piRNA complex is referred to as piRISC to distinguish it from ubiquitous RISCs.

RNA interference (RNAi) was discovered in 1998 in a developmental study of the nematode *Caenorhabditis elegans* (*C. elegans*), in which RNAi was artificially induced by injecting living *C. elegans* with double-stranded (ds) RNAs, which were designed to be completely complementary to target genes.[20] Intrinsically, RNAi is a self-defense mechanism against viral infection and/or other non-self dsRNAs. dsRNAs that trigger the RNAi pathway are processed into siRNAs by Dicer RNase III endonuclease, loaded onto AGO proteins to form RISCs, which then induce specific and efficient repression of complementary gene targets[21−23] (Figure 1). Since the initial study, many research studies have used RNAi as an experimental tool to investigate the functions of genes in a variety of organisms as well as cultured cells. Technically, RNAi is inducible in any cell of any organism as long as the RNAi machinery is conserved.

Exogenous dsRNAs are not, however, essential for organisms to operate RNAi, because most organisms, if not all, express their own siRNAs, known as endogenous siRNAs (endo-siRNAs). Endo-siRNAs have been relatively well documented in mice and fruit flies, where endogenously formed dsRNAs and/or single-stranded (ss) RNAs form stem−loop structures that serve as a source of endo-siRNAs[24,25] (Figure 1). For instance, in mice, dsRNAs composed of RNA transcripts from pseudogenes and active parental genes often generate endo-siRNAs, which regulate the expression of parental genes.[25,26]

miRNAs are transcribed from their own genes by RNA polymerase II (Pol II), and RNA products are processed to mature miRNAs[27,28] (Figure 1). This processing requires Drosha and Dicer, both of which are RNase III endonucleases. Drosha is

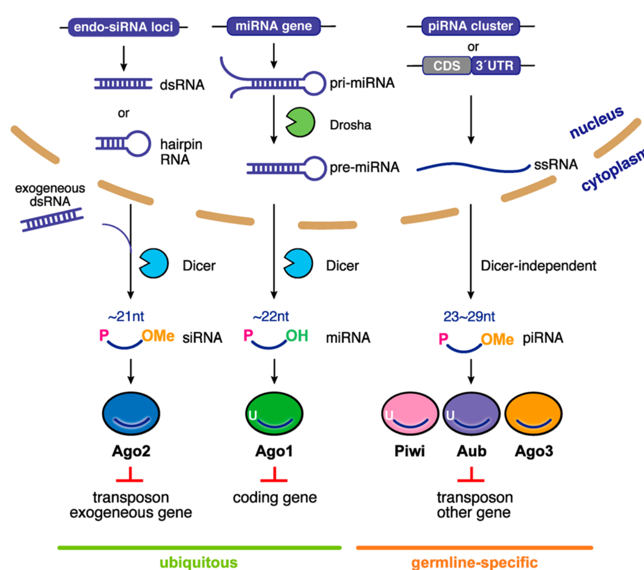

Figure 1. Small RNA-mediated silencing pathways in *Drosophila*. siRNAs (∼21 nt long), both endogenous (known as endo-siRNAs) and exogenous, are produced from precursors by Dicer, and form RISC with Ago2. RISC represses genes that are alomost 100% complementary to the siRNAs. Endo-siRNAs target transposons. miRNAs (∼22 nt long) are produced from their own precursors transcribed from miRNA genes depending on two RNase III ribonucleases, Drosha and Dicer, in a sequential manner. miRNAs form RISC (often referred to as miRISCs) with Ago1. miRNAs target protein-coding genes. piRNAs (23−29 nt long) arise from RNA transcripts transcribed from piRNA clusters via complex piRNA biogenesis machinery. Some protein-coding mRNAs, particularly their 3′-untranslated regions (UTR), produce genic piRNAs[38,41] (see section 3.4), although their target genes remain unknown. piRNAs form piRISCs with PIWI proteins, Aub, Ago3, and Piwi, and repress transposons. piRISCs also repress protein-coding genes if the RNA transcripts show high complementarity with piRNAs. Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; CDS, coding sequence; UTR, untranslated region; OMe, 2′-*O*-methylation; P, phosphate group.

localized in the nucleus, and processes primary miRNA precursors (pri-miRNAs) to secondary miRNA precursors (pre-miRNAs). Dicer is localized in the cytoplasm and processes pre-miRNAs to mature miRNAs. Nuclear export of pre-miRNAs after Drosha processing is governed by Exportin 5. miRNAs control the expression of protein-coding genes by blocking translation or inducing mRNA instability. Plant miRNAs are unique in the sense that their processing is Drosha-independent and that they often repress target genes via RNA cleavage, similar to siRNAs in RNAi.[29] This is possible because miRNAs in plants are highly complementary to target RNAs.

piRNAs, unlike miRNAs, do not have their own genes but originate from intergenic DNA elements known as piRNA clusters, which are rich in transposon sequences, particularly in *Drosophila*. These sequences correspond to a variety of transposons but are fragmented and lack the ability to transpose in the genome. Therefore, piRNA clusters are often considered transposon graveyards. piRNAs are expressed in *dicer* mutants, indicating that piRNA processing does not require Dicer endonuclease activity, a unique feature of piRNAs[30−32] (Figure 1). In accordance with this, while siRNA/miRNA precursors are dsRNAs, piRNA precursors are ssRNAs.

Genome-wide gene screening in fruit flies has identified a large number of piRNA factors.[33−35] When the functions of these factors are disturbed by mutations, severe defects in transposon

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silencing occur in the germline, resulting in infertility. Many factors are dedicated to piRNA biogenesis, while others play important roles in the effector (silencing) step of the pathway. Such a large number of necessary factors (Table 1) complicates the study of piRNAs. Moreover, although evidence has supported the functions of PIWI/piRNA in cancerous cells or even in nongonadal somatic cells,[36] the piRNA pathway is intrinsically germline-specific; therefore its investigation requires high numbers of ovaries and testes, the collection of which is time-consuming and tends to hinder investigation. However, the recent use of cultured cells derived from the flies and silkworm ovaries,[37−39] which retain the ability to produce piRNAs and to perform piRNA-dependent transposon silencing, has contributed to our knowledge of the molecular mechanism underlying the piRNA pathway.[40] Deep-sequencing and computational analysis of piRNAs and their precursors in various piRNA mutants have also helped in dissecting the piRNA biogenesis pathway, revealing a systematic mechanistic model of the pathway.

2. KEY PLAYERS IN THE piRNA PATHWAY: piRNAs AND PIWI PROTEINS

2.1. Characteristics of piRNAs

Drosophila piRNAs normally range from 23 to 29 nt in length, so they are slightly longer than siRNAs and miRNAs, which are normally 21−22 nt long (Figure 1). The 5′-end of piRNAs is rich in uridine, known as the 1U bias, and has a monophosphate, whereas the 3′-end does not show any typical nucleotide bias but is 2′-*O*-methylated.[19,42−45] Both miRNAs and siRNAs have a monophosphate at the 5′-end, indicating this modification is not unique to piRNAs (Figure 1). In *Drosophila*, 2′-*O*-methylation occurs in siRNAs but not in miRNAs[18,46] (Figure 1). This modification, performed by the methyltransferase Hen1/Pimet (Table 1), increases the stability of small RNAs in vivo.[47,48] Why 2′-*O*-methylation is not necessary in fly miRNAs remains unclear. miRNAs in plants are 2′-*O*-methylated by Hen1/Pimet.[48,49]

2.2. Characteristics of PIWI Proteins

Drosophila expresses five Argonaute proteins, two of which, Ago1 and Ago2, are ubiquitously expressed (i.e., AGO proteins) while three PIWI members, Piwi, Aubergine (Aub), and Argonaute3 (Ago3), are expressed specifically in ovaries and testes[18,45,50,51] (i.e., PIWI proteins) (Table 1). *Drosophila* mutants lacking any one of the three PIWI members cause a derepression of transposons,[6−10] suggesting no functional redundancy in the PIWI members. In germ cells, Aub and Ago3 are localized in the cytoplasm and they repress transposons by cleaving target RNA transcripts using their Slicer activity (Figure 2). Slicer-dependent cleavage occurs on target RNAs between the 10 and 11 nt relative to piRNAs, similar to AGO protein activity.[42−44]

Gonadal somatic cells in both the ovaries and testes lack the expression of Aub and Ago3 (Figure 2). In contrast, Piwi is expressed in both germ and gonadal somatic cells, where Piwi is localized in the nucleus to silence transposons transcriptionally by inducing heterochromatinization through histone H3K9 trimethylation (H3K9me3) at genomic loci where active transposons are present[10,42−44,52−54] (Figure 2). Piwi lacking the N-terminal end failed to be translocated into the nucleus.[38,55] Therefore, a nuclear localization signal (NLS) is implicated in this region. piRNA-free Piwi is localized in the cytoplasm where piRNA biogenesis occurs,[56,57] even though the protein contains an NLS, suggesting that both an NLS and piRNA loading are required for Piwi nuclear localization. This piRNA-loading-dependent regulatory system is required because nuclear Piwi without piRNAs lacks a target, which might cause dominant negative effects in the pathway.

PIWI proteins have arginine-glycine-rich regions toward the N-terminal end, which is not observed in AGO proteins.[58−61] Mass spectrometric analysis showed that certain arginines in the PIWI N-terminal regions are symmetrically dimethylated, which converts the arginine resides to symmetrically dimethylated arginines (sDMAs). The enzyme responsible for this post-translational modification is PRMT5/Capsuleen/DART5.[58,60] sDMAs in Aub and Ago3 are required for binding with Tudor domain-containing piRNA factors, such as Tudor (Table 1), and this protein−protein interaction is usually required for piRNA biogenesis.[58,62]

Drosophila ovaries and testes express both AGO and PIWI proteins. However, piRNAs are specifically funneled onto PIWI proteins. Likewise, siRNAs and miRNAs are loaded onto AGO but not onto PIWI proteins. PIWI- and AGO-mediated RNA silencing are independent of each other.

2.3. Crystal Structure of PIWI-piRISC

The crystal structure of Siwi protein, one of two PIWI members in *Bombyx mori*, has been solved[63] (Figure 3A). To date, this is the only solved full-length PIWI protein crystal structure in any organism, albeit missing the N-terminal 130 amino acids. A remarkable feature of this study is that endogenous Siwi-piRISC immunopurified from silkworm ovary-derived cultured BmN4 cells[39] was employed for crystallization. The structure showed that Siwi (a representative PIWI protein) consists of four domains, N, PAZ, MID, and PIWI, and three linkers connecting the adjacent domains, L0, L1, and L2, similar to AGO proteins (Figure 3B). The crystal structures of PAZ and PIWI domains of mammalian PIWI were previously solved using recombinant peptides corresponding to the domains.[64−67]

Comparison of the Siwi-piRISC structure with that of human Ago2 (hAgo2)[68−70] loaded with a 21-nt guide RNA (Figure 3A) revealed that the structures of their domains were highly similar, although each domain at the amino acid sequence level shows only 13−32% identity.[63] A notable difference was observed in the spatial arrangement of the lobe containing the N and PAZ domains. This might be because hydrophobic residues that form a β-barrel-like structure at the L1−PAZ−L2 interface in Siwi (Figure 3C) are exchanged to other residues in hAgo2, which appear to prevent barrel formation.[63] These hydrophobic residues are highly conserved in PIWI members. Both N and PAZ domains of AGO proteins play an important role in RISC formation:[71−74] AGO first binds to a small guide RNA duplex (either siRNA or miRNA duplex) and later releases one strand of the duplex, resulting in active RISC formation.[75,76] However, during the piRISC formation process, piRNA precursors and intermediates are single-stranded (Figure 1). This difference in the configuration of small RNA intermediates and RISC/piRISC assembly might be related to the spatial arrangement difference between AGO and PIWI proteins, although, currently, no evidence supporting this notion is available.

The 5′-end of a piRNA inserts into the 5′-binding pocket located at the interface of MID and PIWI domains in the Siwi-piRISC structure. The 5′-binding pocket contains a Mg^{2+} ion (Figure 3D), similar to bacterial RISC,[77] although that of hAgo2-RISC contains no metal ion.[68−70] In this regard, PIWI resembles bacterial AGO more than mammalian AGO. The Mg^{2+} ion in the Siwi pocket coordinates with the 5′-phosphate of piRNA

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Table 1. *Drosophila* piRNA Factors[a]

symbol	FlyBase gene name	synonym(s)	protein family	domain(s)	expression (based on FlyBase)	subcellular localization in ovaries: nurse cell (germ)	subcellular localization in ovaries: follicle cell (soma)	ref
Piwi	P-element induced wimpy testis	N.A.	Argonaute	Paz, Mid, Piwi	ovary	nucleus	nucleus	8, 19, 42
Aub	Aubergine	sting	Argonaute	Paz, Mid, Piwi	ovary	nuage	N.A.	42, 43, 51
Ago3	Argonaute3	N.A.	Argonaute	Paz, Mid, Piwi	ovary	nuage	N.A.	42, 43
Rhi	Rhino	HP1D	N.A.	Chromo, Chromoshadow	ovary	nuclear foci	N.A.	93, 94, 103, 193
Del	Deadlock	N.A.	N.A.	N.A.	ovary	nuclear foci	N.A.	35, 93
Cuff	Cutoff	N.A.	DXO/Dom3Z	RAI1-like	midgut, ovary, testis	nuclear foci	N.A.	93, 95, 193
UAP56	Helicase at 25E	N.A.	DEAD-box helicase	DEAD box, Hel-C	ovary	nuclear foci	nucleus	103, 107
Moon	Moonshiner	N.A.	N.A.	N.A.	ovary	nuclear foci	N.A.	100
Yb	female sterile (1) Yb	fs(1)Yb	N.A.	Hel-C, DEAD box, Hel-C, Tudor	testis, ovary	N.A.	Yb body	54, 57, 116, 120
BoYb	Brother of Yb	N.A.	N.A.	DEAD box, Hel-C, Tudor, CS	ovary	nuage	N.A.	116
SoYb	Sister of Yb	N.A.	N.A.	Tudor, DEAD box, Hel-C, Tudor, CS	ovary	cytoplasm (enriched at Armi-positive region)	Yb body	116
Armi	Armitage	N.A.	DNA2/NAM7 helicase	DEAD box, Hel-C	ovary	cytoplasm (enriched at nuage, mitochondrion)	Yb body	33, 35, 54, 57, 116, 119
Vret	Vreteno	N.A.	N.A.	RRM, MYND, Tudor, Tudor	ovary	nuage	Yb body	115, 116
Zuc	Zucchini	N.A.	phospholipase D	CHCC Znf, PLD-like	ovary	mitochondrion	mitochondrion	33, 54, 124, 125, 127
Gasz	GASZ ortholog	N.A.	N.A.	Ankyrin repeat-containing, Sterile alpha motif	ovary, thoracic-abdominal ganglion, brain	mitochondrion	mitochondrion	33, 35
Mino	Minotaur	GPAT1	GPAT	GPAT	spermatheca, fat body, crop, heart, ovary	mitochondria, ER, ring canals	N.D.	130
Shu	Shutdown	N.A.	FKBP6	PPIase, TPR	ovary	nuage	Yb body[b]	117, 118
Hsp83	Heat shock protein 83	Hsp90, Hsp82	heat shock protein 90	HATPase-C, ribosomal protein S5 domain 2-type fold	ubiquitous	nuage	cytoplasm	118
Hen1	Hen1 methyltransferase	Pimet	HEN1	*S*-adenosyl-L-methionine-dependent methyltransferase	testis, ovary, accessory glands	N.D.	N.D.	46, 47
Nbr	Nibbler	N.A.	mut-7	3′−5′ exonuclease, ribonuclease H-like	ovary, ganglion, brain	cytoplasm	cytoplasm	135, 137, 138
Vas	Vasa	N.A.	DEAD-box helicase	DEAD box, Hel-C	ovary	nuage	N.A.	44, 110
Krimp	Krimper	N.A.	N.A.	CC, Znf, Tudor	ovary	nuage	Krimp body	118, 133, 144, 145
Qin	Qin	kumo	N.A.	RING Znf, B-box Znf, Tudor, Tudor, Tudor, Tudor, Tudor	ovary	nuage	N.A.	139, 147
Spn-E	Spindle-E	hls	DEAD-box helicase	DEAD box, Hel-C, HA2, Tudor	ovary	nuage	N.A.	18, 140, 141
Tud	Tudor	N.A.	N.A.	Tudor	ubiquitous	nuage	cytoplasm	58
Arx	Asterix	Gtsf1	UPF0224	CHHC Znf, CHHC Znf	ovary	nucleus	nucleus	34, 155, 156
Panx	Panoramix	Silencio	N.A.	N.A.	ovary	nucleus	nucleus	33, 157, 158
Egg	Eggless	dSETDB1, dEset	histone-lysine methyltransferase	Tudor, Tudor, MBD, preSET, SET, postSET	ovary, brain, thoracicabdominal ganglion	nucleus	nucleus	159, 160, 161
Wde	Windei	N.A.	N.A.	CC, FN III	ovary, brain, ganglion	nucleus	nucleus	157, 162
Lsd1	Suppressor of variegation 3-3	Su(var.)3-3	flavin monoamine oxidase	SWIRM, amino-oxidase	ovary, brain	nucleus	nucleus	164, 165
Mael	Maelstrom	N.A.	maelstrom	HMG box, Mael	ovary, testis	nuage, nucleus	cytoplasm, nucleus	148, 175

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Table 1. continued

[a]Abbreviations: N.A., not available; N.D., not determined. [b]Localization in cultured cell line (ovarian somatic cell).

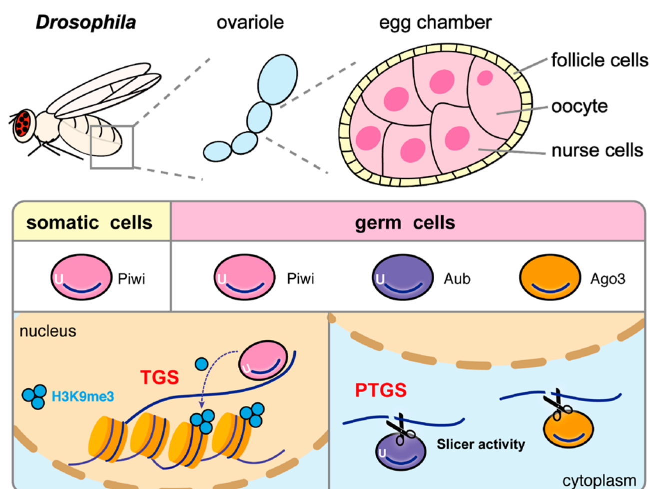

Figure 2. PIWI proteins in *Drosophila* ovaries: expression and transposon control mechanism. *Drosophila* ovaries contain egg chambers at different stages of development. Egg chambers are composed of germ cells (an oocyte and 15 nurse cells; pink) and somatic follicle cells (yellow) that surround them. The expression patterns of PIWI proteins and the mechanisms of transposon silencing mediated by PIWI-piRISC are distinct in somatic and germ cells. Abbreviations: TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing.

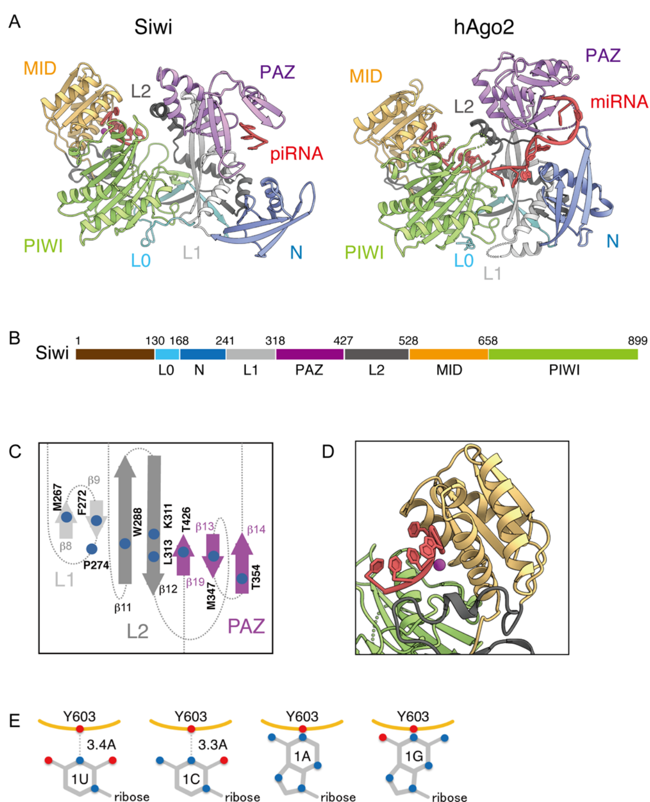

Figure 3. Structure of Siwi, a PIWI member in *Bombyx mori.* (A) Comparison of Siwi-piRNA and hAgo2-miRNA structures. (B) Siwi consists of four domains, N, PAZ, MID, and PIWI, and three linkers, L0, L1, and L2, that connect the domains. (C) Topology of the b-barrel structure found in Siwi-piRISC (based on PDB: 5GUH). This unique structure was not observed in hAgo2-RISC. (D) Mg^{2+} ion (pink) in the 5′-binding pocket in Siwi. (E) Virtual exchange of 1U to 1A, 1C, and 1G. Steric hindrance or charge repulsion with Tyr603 was observed when 1U was altered to 1A or 1G.

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together with glutamine 645 and leucine 899 of Siwi, both of which are highly conserved among PIWI members,[63] suggesting that the spatial arrangement coordinated by the phosphate, Mg^{2+} ion, and two amino acids in Siwi is a conserved feature among PIWI proteins.

1U of piRNA within the Siwi-piRISC structure forms a hydrogen bond with tyrosine 603 in the MID domain.[63] Virtual exchange of 1U to another base (i.e., 1A, 1C, or 1G) is predicted to cause steric hindrance or charge repulsion (Figure 3E). This might be one reason why PIWI proteins prefer to bind 1U-piRNAs.

The 2′-*O*-methyl group at the piRNA-3′ end inserts into the 3′-binding pocket residing in the PAZ domain, which is well coordinated with hydrophobic residues.[63] Alteration of amino acids recognizing the piRNA 5′- and 3′-ends markedly diminishes the Siwi-piRNA binding activity, confirming their importance in piRISC formation. These findings in the Siwi structural study regarding the recognition of the 5′-phosphate and 3′-end of piRNA are consistent with those from previous mammalian studies.[65−67,78]

Studies revealing the structures of other PIWI proteins in the presence or absence of piRNA are awaited. In particular, the structure of *Drosophila* or *Bombyx* Ago3 is of great interest because Ago3-bound piRNAs show no obvious nucleotide bias at the 5′-end.[19,42−45]

3. piRNA BIOGENESIS

3.1. piRNA Clusters

piRNA clusters, which are the primary origin of piRNAs in *Drosophila*, are often located in pericentromeric and telomeric heterochromatin regions, particularly at the boundaries with euchromatin (Figure 4). However, piRNA clusters are transcribed by Pol II.

Drosophila piRNA clusters are categorized into two subgroups: unistrand and dual-strand clusters[79,80] (Figure 4). Unistrand clusters are transcribed unidirectionally from a promoter located at one side of the cluster. The products of unistrand clusters

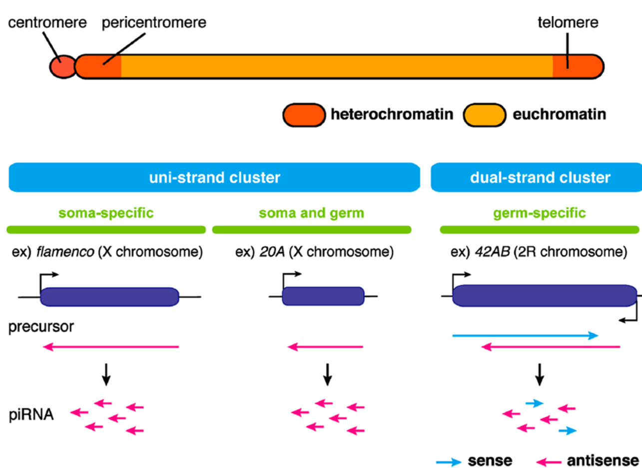

Figure 4. piRNA clusters: distribution on chromosomes and classification. (upper) Simplified diagram showing the distribution of heterochromatin, euchromatin, centromere, pericentromere, and telomere on a chromosome. piRNAs arise from piRNA clusters located at heterochromatin regions, in particular, boundaries of heterochromatin with euchromatin. (lower) Some unistrand clusters are used in both somatic and germ cells (e.g., *20A*), while others are soma-specific (e.g., *flamenco*). Dual-strand clusters (e.g., *42AB*) are germ-specific.

undergo RNA processing in the nucleus similar to that of mRNA precursors, i.e., 5′ capping, splicing, and poly(A) addition, before being exported to the cytoplasm to produce mature piRNAs (for the details, see section 3.2.1). On the other hand, dual-strand clusters lack their own promoters but are transcribed on both sides; therefore, a proportion of the products are complementary[42,44] (for the details, see section 3.2.2).

piRNA clusters are also studied in other species.[81−85] Mammalian piRNA clusters are classified as unidirectional and bidirectional in terms of how they are transcribed.[81,82,86]

3.2. Nuclear Regulatory Mechanisms in piRNA Biogenesis

3.2.1. Unistrand piRNA Clusters. Ovarian somatic cells rely almost exclusively on unistrand clusters to produce piRNAs.[44] A representative cluster is *flamenco* (*flam*) (Figure 4), located near the centromeric region on the X chromosome. The *flam* locus contains fragments of various transposons, such as *gypsy, idefix*, and *ZAM*, most of which are antisense oriented relative to the transposon coding strands. piRNAs originating from the *flam* locus (*flam*-piRNAs) are thus capable of targeting *gypsy, idefix*, and *ZAM* transposons for repression.[44,87−91] Other transposons or even any other genes could be targeted by piRNAs if, by chance, they show high complementarity to piRNAs. The promoter of the *flam* cluster is located between *flam* and the neighboring protein-coding gene *DIP-1*.[42] Transcription of the *flam* cluster requires the transcriptional factor Cubitus interruptus, which is not specific to the piRNA cluster.[92] The *flam* RNA transcripts are then processed to have the CAP structure and poly(A) at the 5′- and 3′-ends, respectively, and even spliced.[92] In this regard, the *flam* RNA transcripts are indistinguishable from mRNAs.

3.2.2. Dual-Strand piRNA Clusters. *Drosophila* germ cells use mostly dual-strand piRNA clusters for piRNA production. A representative is the *42AB* cluster, located in the pericentromeric region of chromosome 2R (Figure 4). The dual-strand clusters are not found in mammals,[81,82,86] and it is likely that they are unique to *Drosophila*.

Dual-strand piRNA clusters do not possess their own promotors.[42,93] How then is the production of piRNAs from these clusters driven in germ cells? The answer to this question was provided by studies showing that three nuclear piRNA factors, Rhino (Rhi), Deadlock (Del), and Cutoff (Cuff), play key roles in piRNA biogenesis from dual-strand clusters[93−97] (Figure 5A, Table 1). First, Rhi, a homologue of heterochromatin protein 1 (HP1), specifically interacts with H3K9me3 accumulated on the dual-strand piRNA clusters. Cuff and Del associate with Rhi on H3K9me3 to form a complex termed the RDC complex.[93,96,97]

In general, transcription of protein-coding genes continues beyond the poly(A) signal (PAS), but once the cleavage-polyadenylation specificity factor (CPSF) complex binds the PAS of a nascent mRNA precursor (pre-mRNA), it cleaves the pre-mRNA at a site several nucleotides downstream of the PAS, poly(A) site, forming its 3′-end (Figure 5B). Subsequently, the 3′-end is polyadenylated to increase RNA stability.[98] However, during piRNA production, Cuff, which contains an RAI1-like domain and belongs to the DXO family of proteins, blocks CPSF binding to the PAS of the mRNAs transcribed from the upstream protein-coding gene, so no 3′-end processing occurs at the poly(A) site[99] (Figure 5B). A proposed model suggested that transcription then reads through the piRNA cluster region, enabling the generation of piRNAs from the cluster.

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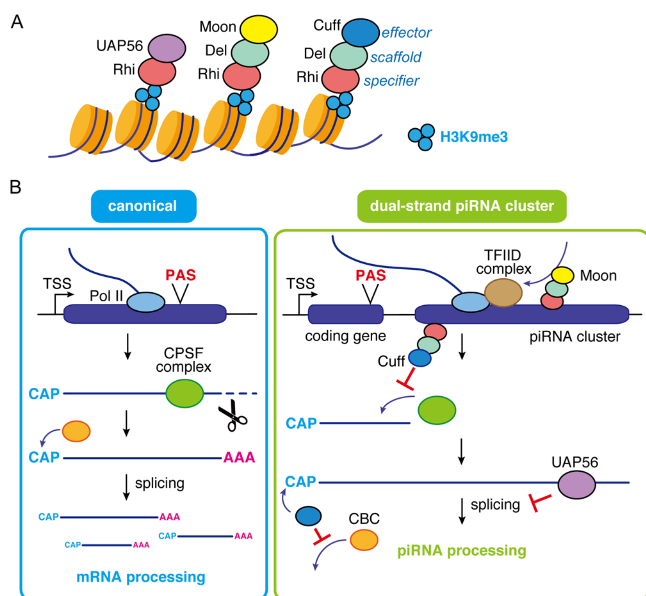

Figure 5. Dual-strand piRNA clusters. (A) Rhi binds to H3K9me3 located on dual-strand clusters and then recruits Del and Cuff by binding with Del to form the RDC complex. In this complex, Del acts as a scaffold while Cuff and Rhi act as the effector and the locus specifier, respectively. Rhi also recruits UAP56 to the dual-strand locus and the Rhi−Del heterodimer interacts with Moon to recruit transcription machinery. (B) (left) In general, the CPSF complex binds the PAS in a nascent mRNA transcript and forms the 3′-end by cleaving several nucleotides downstream of the PAS. Then, the cleavage site is polyadenylated. CBC binds the CAP structure to promote splicing. (right) Cuff interferes with the CPSF complex to bind the PAS of transcripts of a gene located upstream of the dual-strand cluster. As a result, transcription reads through the cluster, enabling piRNA production from the cluster. The Rhi−Del−Moon complex also facilitates the transcription of the cluster by recruiting the TFIID complex to the dual-strand locus. After transcription, Cuff may inhibit splicing by binding to the CAP structure. UAP56 interacts with the transcripts to keep them unspliced. Abbreviations: TSS, transcriptional start site; PAS, poly(A) signal; Pol II, RNA polymerase II; CPSF complex, cleavage−polyadenylation specificity factor complex; TFIID complex, basal transcription factor IID complex; CBC, CAP binding complex.

Dual-strand piRNA clusters can also be transcribed by mechanisms other than the read-through system.[100] Dual-strand clusters contain motifs termed "YR" (pyrimidine/purine) dinucleotides on both strands, to which a basal transcription factor IID (TFIID) complex binds to initiate transcription.[101,102] One such factor responsible for this unique transcription was identified as a paralogue of TFIIA-L, a large subunit of the TFIIA complex, which was termed Moonshiner (Moon).[100] Moon binds Rhi and TATA-box-binding protein-related factor TRF2 to promote transcription of dual-strand clusters by recruiting the TFIID complex (Figure 5B).

The RDC complex may have functions other than cluster transcription. Cuff facilitates piRNA production by protecting piRNA precursors from being spliced by splicing machinery (Figure 5B). This idea was based on the experimental observations that the loss of Cuff resulted in the splicing of piRNA precursors[103] and that a Cuff homologue in mice, DXO, binds to capped RNAs.[104] The CAP binding complex (CBC) normally binds to the CAP structure of pre-mRNAs to facilitate splicing.[105] Cuff may interact with the 5′-CAP structure, interfering with its binding to CBC, leading to the inhibition of splicing. However, direct evidence to support this notion has not been provided. Cuff also binds Thoc5, a component of the THO complex, and recruits the THO/TREX complex to the dual-strand clusters.[106] Normally, the THO/TREX complex binds nascent RNAs in a splicing-dependent manner. However, the complex also binds the RNA transcripts of the dual-strand clusters via Cuff, although the RNA transcripts do not undergo splicing.

A DEAD-box protein, UAP56, originally identified as an mRNA export factor, may also play an important role in keeping piRNA precursors unspliced, because the loss of UAP56 led to the splicing of RNA transcripts arising from dual-strand piRNA clusters[99] (Table 1). UAP56 is recruited to dual-strand piRNA precursors via Rhi.[107] It seems that *Drosophila* has acquired an elaborate method, depending on the RDC complex and UAP56, to secure high expression levels of piRNAs from dual-strand piRNA clusters in germ cells.

While the functional contribution of Rhi and Cuff to piRNA production from dual-strand clusters has gradually emerged, the function of Del in this pathway remains largely unknown. Del has no conserved functional domains, but was shown to bind to Rhi and Cuff in two hybrid experiments.[93] It should be noted that the piRNA transcriptional system governed by the RDC complex is unique to *Drosophila*, and is not conserved in other species including mice. This may be related to the recent finding that Rhino has undergone a rapid evolution.[108]

3.3. Nuclear Export of piRNA Precursors

piRNA biogenesis is considered to occur in the cytoplasm, because all piRNA biogenesis factors have been detected there (for the details, see sections 3.4 and 3.5). This indicates that piRNA precursors transcribed by Pol II in the nucleus require export to the cytoplasm.

Nxf1 and Nxt1 are known as general mRNA export factors. Knockdown of Nxf1 and Nxt1 in fly ovaries caused the derepression of transposons[33−35] and aberrant accumulation of *flam* RNA transcripts in the nucleus of ovarian somatic cells.[109] These findings strongly suggest that Nxf1 and Nxt1 also act as export factors of piRNA precursors (Figure 6). In situ hybridization showed that the nuclear foci where *flam* DNA exists did not colocalize with *flam* RNAs in wild-type ovaries. However, they colocalized in the *nxf1* and *nxt1* mutant ovaries.[109] Nxf1 and Nxt1 might efficiently displace nascent piRNA precursors from their genomic loci upon transcription. The exon junction complex (EJC), a hallmark of splicing, may also be involved in displacing piRNA precursors from their transcription sites.[33−35] Mutations introduced in Mago nashi, a core EJC factor, or an EJC accessory protein, RnpS1, caused *flam* RNA accumulation at the *flam* genomic loci.[109]

Nuclear export of RNA transcripts arising from dual-stranded piRNA clusters is mediated by UAP56 (Figure 7 in section 3.5). UAP56 colocalizes with Rhi in the nucleus and both UAP56 and Rhi are localized in close proximity to Vasa, a cytoplasmic piRNA biogenesis factor,[44,110] across nuclear pores (Table 1). piRNA precursors associated with UAP56 are also bound with Vasa.[107] Thus, UAP56 plays key roles in the nuclear transport of piRNA precursors. First, UAP56 selectively binds to transcripts of dual-strand piRNA clusters in the nucleus, and second, it transfers the RNAs to cytoplasmic Vasa through nuclear pores.[107,111]

3.4. piRNA Biogenesis in Ovarian Somatic Cells

piRNAs are produced through an intricate biogenesis pathway composed of three mechanisms: the primary piRNA pathway, piRNA amplification loop (also known as the ping-pong cycle),

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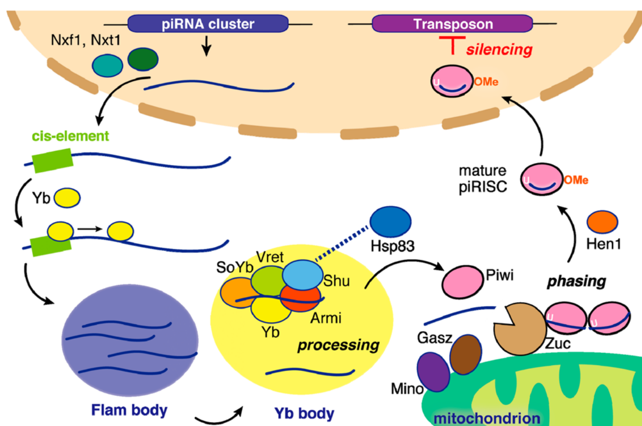

Figure 6. piRNA biogenesis in *Drosophila* somatic cells. Nxf1 and Nxt1 export piRNA precursors transcribed from the piRNA clusters. In the cytoplasm, Yb binds to piRNA precursors through the *cis*-element and sequesters them to the Flam body. Then, the precursors are processed to mature piRNAs adjacent to the Yb body. The Yb body is composed of Yb, Armi, Vret, SoYb, and Shu. Zuc located on the surface of mitochondria cleaves piRNA precursors/intermediates to mature piRNAs. This Zuc-dependent cleavage forms both the 5′- and 3′-ends of piRNAs. The 5′-end is rich in uridine (1U). piRNA biogenesis factors, Gasz and Mino, also localize on the surface of mitochondria, but their functions in piRNA biogenesis are unknown. HSP83 binds to Shu and facilitates piRNA loading onto Piwi. Hen1/Pimet is involved in the 2′-*O*-methylation of the 3′-end of piRNAs. Piwi-piRISC is then translocated to the nucleus, where it represses transposons epigenetically. Abbreviations: OMe, 2′-*O*-methylation.

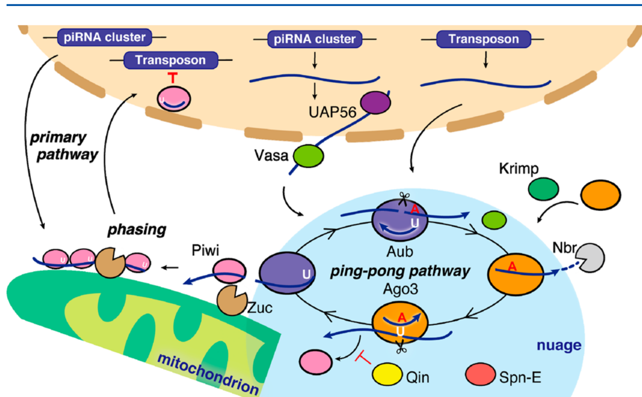

Figure 7. piRNA biogenesis in *Drosophila* germ cells. UAP56 functions in the nuclear export of piRNA precursors transcribed from dual-strand clusters. At the nuclear pores, UAP56 transfers the RNA transcripts to Vasa localized at the cytoplasmic side of the pores. Then, Vasa accumulates piRNA precursors to nuage. In nuage, Aub and Ago3 amplify piRNAs by reciprocally cleaving transposons and cluster transcripts through the ping-pong pathway. Aub-bound piRNAs are rich in 1U, while Ago3-bound piRNAs show a 10A bias. The 5′-end of piRNAs is determined by the Slicer activity of Aub/Ago3. Nbr and Zuc are involved in the 3′-end formation. Vasa facilitates the ping-pong cycle by efficiently displacing cleaved target RNAs from Aub-piRISC. Zuc functions by producing phased piRNAs for Piwi as observed in somatic cells. Krimp, Qin, and Spn-E also play important roles in the ping-pong pathway.

and ping-pong-cycle-dependent phased piRNA biogenesis.[112,113] In *Drosophila* ovarian somatic cells, only the primary piRNA pathway is available, and the piRNA products are loaded onto Piwi (Figure 6), but not onto Aub or Ago3, because they are not expressed in these cells (Figure 2). The resultant Piwi-piRISC is translocated to the nucleus, in which it transcriptionally represses transposons (for the details, see section 4).

Drosophila ovarian somatic cells contain a perinuclear cytoplasmic structure termed Yb body[56,57,114] (Figure 6). The Tudor-domain containing protein, female sterile (1) Yb (Yb), is the core component of the Yb body (Table 1). Other Yb body components include two Tudor-domain proteins, Vreteno (Vret) and Sister of Yb (SoYb), the DEAD-box protein, Armitage (Armi), and Shutdown (Shu), all of which are necessary for piRNA biogenesis[56,57,115−119] (Table 1). Shu is a cochaperone interacting with HSP83 that is important for PIWI-piRNA loading[117,118] (Table 1). Therefore, it is thought that the Yb body is the site for piRNA biogenesis in ovarian somatic cells.

Besides a Tud domain, Yb protein contains a DEAD-box RNA helicase domain, so Yb is expected to bind to RNAs. High-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) revealed that Yb binds to piRNA precursors and that the binding sites overlap with regions where mature piRNAs were mapped.[120] It can be speculated that this Yb binding specifies the piRNA-bearing sequences in the precursors. Armi-bound RNAs may contain a 2′−3′ cyclic phosphate,[56] which is not observed in mature piRNAs; therefore, Armi-bound RNAs are considered to be piRNA intermediates. The functions of Vret, SoYb, and Shu at Yb body remain unknown.

Mapping of Piwi-bound piRNAs in cultured ovarian somatic cells (OSCs) showed that piRNAs arise from piRNA clusters such as *flam* as well as some protein-coding genes, a representative of which is *traffic jam* (*tj*).[38] Interestingly, these genic piRNAs are derived from UTRs particularly the 3′-UTR, but not from the coding region of the mRNAs.[38] Determination of the *cis*-element in the *tj* mRNA revealed that a 100 nt sequence within the *tj* 3′-UTR near the stop codon was sufficient and necessary to produce piRNAs from the downstream element.[121] Yb preferably binds the *tj cis*-element. Indeed, without Yb, piRNA production is abolished. The *cis*-elements were also found in *flam* transcripts.[122] Thus, Yb is a trans-acting protein that selectively binds the *cis*-element embedded in piRNA precursors and translocates it to the Yb body, the piRNA processing site (Figure 6). Another 100 nt RNA fragment downstream of the *tj cis*-element can bind to Yb, but it failed to induce piRNA production from the downstream element.[121] Thus, Yb−RNA binding has two functions, to induce piRNA production and to determine the region from which piRNAs are produced. The former corresponds to the initial, direct binding of Yb to the *cis*-element, while the latter is the secondary binding of Yb (spreading) depending on the initial Yb−*cis*-element binding.

flam-piRNA precursors accumulate perinuclear structures located immediately next to the Yb body, termed Flam body[120] (Figure 6). No known piRNA factors localize to the Flam body. Thus, the Flam body may act as a storage site for piRNA precursors (or intermediates) until these RNAs are processed to mature piRNAs at the Yb body. The *flam*-piRNA precursors might also accumulate at nuclear bodies, termed DotCom,[123] and act as nuclear storage sites of piRNA precursors until they are ready for nuclear export. However, the biological significance of precursor storage in distinct bodies remains unknown.

piRNA biogenesis in ovarian somatic cells requires Zucchini (Zuc) endonuclease[38,44,124] (Table 1). Zuc is located on the surface of mitochondria through its N-terminal mitochondrial targeting signal (MTS)[56,57] and is responsible for processing piRNA intermediates to mature piRNAs (Figure 6). RNA

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products of Zuc endonucleolytic cleavage contain a monophosphate at the 5′-end, as do mature piRNAs,[125] supporting the idea that Zuc forms the piRNA 5′-end. A previous study of mouse Zuc (also known as MitoPLD) reported that Zuc/MitoPLD might be a phospholipid-digesting enzyme.[126] However, the crystal structure of *Drosophila* Zuc showed that Zuc assembles as a homodimer and that the dimer interface composed of two HKD motifs (one motif per monomer) forms a groove that fits ssRNA, but not dsRNA nor phospholipids.[125,127] This strongly suggests that Zuc is an ssRNA-specific nuclease and not a phospholipid. Indeed, in vitro assays have shown Zuc cleaved ssRNAs but not dsRNAs. However, Zuc showed little nucleotide bias for RNA cleavage, while Piwi-bound piRNAs in vivo have a strong 1U bias.[125] Moreover, primary piRNAs in ovarian somatic cells showed a phasing pattern similar to phased piRNAs produced in a ping-pong-cycle-dependent manner in germ cells[128,129] (for the details, see section 3.6). At present, the molecular mechanism of how Zuc produces 1U-biased, phased piRNAs in ovarian somatic cells remains elusive.

Two other mitochondrial proteins, Gasz and Minotaur (Mino), are also necessary for primary piRNA biogenesis in ovarian somatic cells[33,34,130] (Table 1, Figure 6). However, their functional involvement in piRNA processing remains undetermined. Gasz may play a role in mitochondrial fusion;[131] however, its relationship with piRNA biogenesis is unclear.

3.5. piRNA Biogenesis in Germ Cells

Yb, the core piRNA factor in ovarian somatic cells, is not expressed in germ cells, and therefore germ cells are devoid of the Yb body. An alternative structure formed in germ cells is nuage (named after the French word for “cloud”), which is also found in the perinuclear region[42,43,119,132,133] (Figure 7). The number of Yb bodies per ovarian somatic cell can be determined; however, nuage cannot be easily quantified in germ cells. Although germ cells lack Yb, a Yb homologue, Brother of YB (BoYb), is expressed (Table 1). BoYb is necessary for piRNA biogenesis in germ cells,[116] although its function remains elusive.

piRNAs expressed in germ cells are amplified through the ping-pong cycle (Figure 7), which, in *Drosophila*, depends on the Slicer activity of two PIWI proteins, Aub and Ago3. A proportion of Aub-bound piRNAs may arise from piRNA clusters through the primary piRNA pathway, as in ovarian somatic cells. Aub-bound piRNAs are mostly antisense to transposon mRNAs, while Ago3-bound piRNAs are rich in sense piRNAs.[42,44] This indicates that Aub-piRISC, but not Ago3-piRISC, mediates the silencing of transposons by cleaving mRNAs. In RNAi, RNAs cleaved by Ago2-RISC (in *Drosophila*, Ago2, but not Ago1, specifically binds siRNAs and mediates RNAi, see Figure 1) are totally degraded in the cellular environment. However, RNAs cleaved by Aub-piRISC serve as substrates to produce a new set of piRNAs, which are subsequently loaded onto Ago3 in the ping-pong cycle (Figure 7). This explains why Ago3-bound piRNAs are mostly in the sense orientation with respect to transposon mRNAs. In turn, Ago3-piRISC targets antisense transcripts of transposons, including transcripts of piRNA clusters, giving rise to another new set of antisense piRNAs. These are eventually loaded onto Aub. In this cycle, Aub and Ago3 continue the reciprocal cleavage of transposon transcripts, leading to an efficient post-transcriptional silencing of the mobile DNA elements as well as piRNA amplification, which are, therefore a coupled event.

Aub-bound piRNAs show strong 1U bias, while Ago3-bound piRNAs show 10A bias partly because Ago3-bound piRNAs are products of Aub-Slicer. Moreover, Aub- and Ago3-bound piRNAs are often complementary to each other in the 10 nt from their 5′-end.[42,43] The 10A bias that Ago3-bound piRNAs show might be explained by the binding pocket of Aub, which prefers to accommodate target RNA containing adenine at the position facing the Aub-piRNA 5′-end (i.e., 1U).[134] These specific traits of Aub- and Ago3-bound piRNAs are known as ping-pong signatures and are conserved in a wide variety of species.[85]

In the ping-pong cycle, Slicer activity of Aub and Ago3 form the 5′-end of piRNAs. However, immediately after this reaction, the products may still be intermediates and if the 3′-ends contain extra bases, these should be removed to form mature piRNAs.[78,135] A factor required for this processing is Zuc and/or 3′-to-5′ Nibbler (Nbr) exonuclease[128,129,136−138] (Table 1, Figure 7). Loss of these nucleases causes piRNAs to be longer, but only the 3′-end is extended by several nucleotides.

The ping-pong cycle requires other piRNA factors, including Vasa, and three Tudor-domain containing proteins, Krimper (Krimp), Qin, and Spindle-E (Spn-E)[33−35,44,139−141] (Table 1, Figure 7). Investigation of the ping-pong cycle in cultured silkworm ovary-derived BmN4 germ cells showed that Vasa facilitates the ping-pong cycle by efficiently displacing cleaved target RNAs from Siwi-piRISC.[142,143] Siwi is a silkworm (*Bombyx*) PIWI protein (see section 2.3). Without Vasa function, cleaved RNAs are stuck on the piRISC, and interfere with the ping-pong cycle.[142,143] Krimp binds sDMA-free Ago3 (see section 2.2) to accomplish two functions in the ping-pong cycle:[144,145] First, it promote sDMA modification and nuage localization of Ago3, without which the ping-pong cycle does not proceed properly. Second, it sequesters Ago3 to the cytoplasmic Krimp-rich body when the Aub function is compromised, for example, by introducing mutations into the *Aub* gene. This is to avoid Ago3 being incorrectly loaded with piRNAs that are normally funneled onto Aub/Piwi. This idea is supported by the observation that Krimp knockdown in fly ovaries causes Ago3 to be loaded with Aub/Piwi-piRNAs.[144] Through this control governed by Krimp, the heterotypic ping-pong between Aub and Ago3 is maintained.

Qin functions in maintaining heterotypic ping-pong by ensuring that Ago3-dependently cleaved RNAs are properly loaded onto Aub, but not Piwi.[146] Spn-E contains Tudor domains as well as a DEAD-box helicase. Spn-E interacts with Aub, Ago3, and Qin, and their interaction mutually influences their own localization to nuage.[44,133,147] The molecular function of Spn-E in piRNA biogenesis remains unclear.

3.6. Ping-Pong-Cycle-Dependent Phased piRNA Biogenesis

Piwi-bound piRNAs show a clear “phasing” pattern, similar to siRNAs derived from dsRNAs that are dependent on Dicer activity, although the size of phased piRNAs is still 23−29 nt long, the typical size of piRNAs. This unique pattern disappears in *zuc* mutants.[128,129] Further analysis in germ cells suggested that a proportion of piRNAs generated by Ago3-piRISC trigger the production of phased piRNAs. The “very first” piRNA generated from a target RNA cleaved by Ago3-piRISC is loaded onto Aub (known as responder piRNA) as is usually observed in the ping-pong cycle; however, successive piRNAs arising from the same target RNA in a stepwise manner are loaded onto Piwi (known as trailer piRNAs) (Figure 7). Zuc is responsible for the production of these phased piRNAs.[128,129,146] Therefore, both the 5′- and 3′-ends of phased piRNAs are defined by Zuc endonuclease activity.

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4. TRANSCRIPTIONAL SILENCING OF TRANSPOSONS

4.1. Piwi-piRISC Drives Transcriptional Silencing

Piwi, but not Aub or Ago3, silences transposons at the transcriptional level. Although Piwi-Slicer is unnecessary in the piRNA pathway,[38] Piwi induces the heterochromatinization of target loci by promoting the trimethylation of H3K9.[55,148−153] Recent studies have provided strong evidence to support this notion as well as suggesting the hypothesis that H3K4me2 and chromatin remodeling are involved in the pathway, highlighting the great complexity of Piwi-mediated transposon silencing (Figure 8). Piwi targets nascent RNAs, but not DNAs, for silencing.[149,154] This indicates that transposons to be silenced by Piwi have to be active and transcribed at least in the initial phase of silencing (Figure 8).

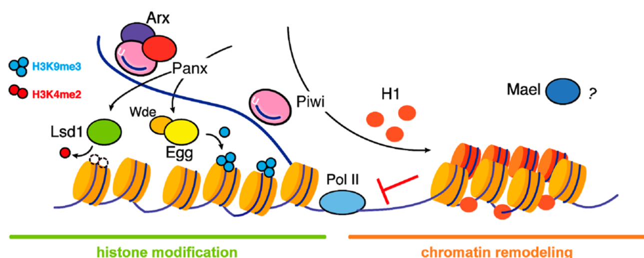

Figure 8. Transcriptional silencing of transposons in the nucleus. Piwi-piRISC in the nucleus targets nascent RNA transcripts transcribed from the transposon loci. Piwi interacts with Panx, which then recruits Egg and its cofactor Wde, and other factors required for heterochromatinization, resulting in the repression of transposons. Arx also interacts with Piwi, but probably mediates its functions upstream of Panx. Panx also induces transposon repression by recruiting Lsd1 and its cofactor CoRest (not shown) to the target loci to facilitate the demethylation of H3K4me2. Linker histone H1 maintains a high-dimensional structure of chromatin at the Piwi-targeted loci. Mael is also required for Piwi-mediated transcriptional silencing, but its role remains unclear.

4.2. Histone Modifications

Piwi requires several cofactors to complete silencing (Figure 8). One such factor is Asterix/DmGtsf1 (Arx) (Table 1), which contains two zinc-finger domains. Arx interacts with Piwi, although its functional contribution to the pathway remains unclear.[34,155,156] Another Piwi cofactor is Panoramix/Silencio (Panx) (Table 1), which interacts directly with Piwi[157,158] but has no known functional domains.[33,35] Knockdown of Panx in *Drosophila* ovaries resulted in the derepression of transposons. Interestingly, tethering of Panx to nascent RNAs of an arbitrary gene induces gene repression.[157,158] This reaction can bypass Piwi and Arx but requires the H3K9 methyltransferase Eggless (Egg),[159−161] its cofactor, Windei (Wde),[33,35,162] and HP1a[148,150−152,157,158] (Table 1). Based on these findings, a model has been proposed (Figure 8), in which Piwi binds RNA transcripts of active transposons through piRNAs, and then Panx, while binding to Piwi, recruits the necessary histone modification enzymes, leading to heterochromatinization at the transposon locus. Arx may function upstream of Panx.

Loci under nuclear piRNA control can be analyzed by the bioinformatic analysis of chromatin immunoprecipitation (ChIP) data. Interestingly, Piwi-piRNA-enriched loci do not always overlap with those rich in the repressive histone mark, H3K9me3, and HP1a. Piwi knockdown in fly ovaries caused a marked reduction in the level of H3K9me3 at some transposon loci, while the level of H3K4me2 was increased at other transposon loci.[149] Lysine-specific demethylase 1 (Lsd1), which converts H3K4me2 to H3K4me, might be involved in the latter phenomenon[35,163−165] (Table 1). Tethering of Panx to the genome via an artificial fusion technique using a λN protein on nascent transcripts induced efficient gene repression; however, this outcome was blocked by the knockdown of Lsd1 and its cofactor, CoRest.[157,163,164] This indicates the hierarchical involvement of Lsd1/CoRest in the pathway: Piwi first recruits Panx and then Lsd1 is recruited to the target locus (Figure 8). *Drosophila* appears to acquire transposon repression that controls both repressive (i.e., H3K9me3) and activating (i.e., H3K4me2) histone marks; yet how these events, which are apparently diametrically opposed, are used for the same purpose remains unknown.

4.3. Chromatin Remodeling

Linker histone H1 maintains the three-dimensional structure of chromatins by binding nucleosomes.[166] This interaction is, however, not stable but quite dynamic because H1 cycles between attachment to and detachment from nucleosomes.[167,168] H1 is present in genomic loci that are rich in transposons,[169] indicating it might control transposon transcription (Figure 8). Piwi physically binds to H1, and this interaction leads H1 to target transposon loci for silencing.[170] A technique known as ATAC-sequencing, which is used to analyze open chromatin structures by artificially inserting a particular DNA transposon into the genome in vivo, showed that repressive chromatin structures induced by Piwi-H1 largely depend on H1. Depletion of H1 had little impact on H3K9me3 accumulation at repressed chromatin regions and vice versa.[170] It is likely that piRNA-dependent transcriptional silencing involves both histone modification and chromatin remodeling, but the two events might occur independently.

4.4. piRNA Factors Function in the Biogenesis and Effector Steps of the piRNA Pathway

Maelstrom (Mael), which has a high-mobility group (HMG) and a MAEL domain, is a unique piRNA factor because it is required for piRNA biogenesis and effector steps in the piRNA pathway (Table 1). The HMG domain is an ssDNA- and a dsDNA-binding domain that might also contribute to protein−protein interactions.[171] The MAEL domain was named based on the high conservation of the domain among its homologues in various species.[172] Mael function in piRNA biogenesis was required in germ cells,[133] while in ovarian somatic cells, the loss of Mael had little impact on piRNA biogenesis,[56,155] although it derepressed transposons.[148] However, Mael also had little effect on the H3K9me3 accumulation at target loci,[148] suggesting it is required downstream of H3K9 trimethylation. Another possibility is that Mael functions in piRNA-mediated transposon silencing that is independent of H3K9me3.

The crystal structures of *Drosophila* and *Bombyx* Mael revealed that the MAEL domain forms an RNaseH-like structure.[173,174] The amino acid residues necessary for exhibiting the RNaseH activity are not conserved in Mael, indicating it might bind to ssRNA although it lacks RNase activity. However, *Drosophila* Mael showed ssRNA-specific, endonuclease activity in in vitro assays,[173] although this ssRNase activity was not required for transposon silencing. Mael may function as a scaffold via its RNA-binding activity in nuclear transposon silencing.

In germ cells, Mael is localized to nuage in a Vasa-dependent manner.[175] Other studies also showed that Mael shuttles between the nucleus and the cytoplasm.[176,177] Therefore, Mael functions might not be dedicated to nuclear silencing but may

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also be necessary for piRNA biogenesis or post-transcriptional silencing.

5. PIWI/piRNA FUNCTIONS BEYOND TRANSPOSON SILENCING

5.1. Protein-Coding Gene Repression by piRNAs

Previous studies reported that piRNAs function in repressing protein-coding genes. One such example is the piRNA-mediated repression of *Stellate* (*Ste*) in fly testes. The *Ste* gene encodes a protein similar to the β-subunit of casein kinase II and is repetitively located on chromosome X. Once the protein is generated in the testes, it forms needle-shaped aggregates and causes a defect in spermatogenesis, leading to infertility.[178] Interestingly, chromosome Y contains a locus rich in *Ste* fragments, which gives rise to RNA transcripts composed of many sequences that are antisense to *Ste* mRNAs. This unique locus was named *Suppressor of Stellate* [*Su*(*Ste*)] because mutations introduced into this locus that abolished its expression lead to the aberrant expression of Ste protein and the formation of needle-shaped aggregates in mutant testes, resulting in infertility. Later, it was found that small RNAs were produced from the *Su*(*Ste*) transcripts, which were loaded onto Aub in the testes.[9,18,132] These needle-shaped aggregates were also found in *aub* male mutants, which are infertile, as predicted. The small RNAs are referred to as *Su*(*Ste*)-piRNAs, which are necessary to repress the complementary *Ste* gene. Likewise, the *Su(Ste)* locus is considered a piRNA cluster dedicated to *Ste* (protein-coding) silencing. The *Vasa* gene may also be a target of Aub-bound piRNAs in fly testes, because a proportion of Aub-bound piRNAs showed high complementarity to *Vasa* mRNAs and the Vasa protein was upregulated in *aub* mutant testes.[132,179] Interestingly, this is a male-specific phenomenon; *Su*(*Ste*)-piRNAs and piRNAs complementary to *Vasa* are not detected in ovaries.

5.2. Regulation of Maternal mRNA Localization by piRNAs

Subsets of mRNAs maternally deposited in embryos accumulate at the posterior pole (Figure 9), which is required for pole cell development.[180−182] Recent studies demonstrated that Aub-piRISC, which is also maternally deposited in embryos, plays important roles in the maternal mRNA localization in embryos.[183−185] The functions of PIWI-piRISC beyond gametogenesis have been clarified.

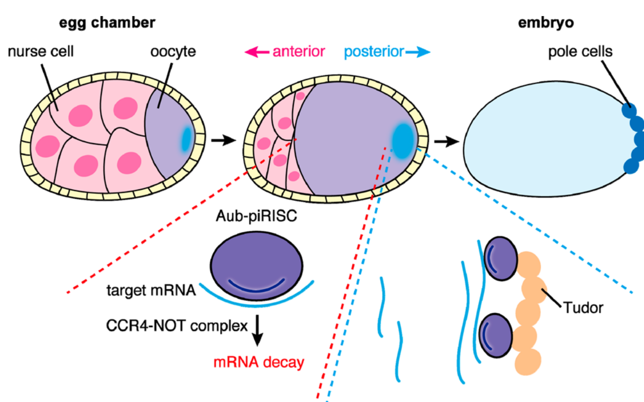

Figure 9. mRNA localization regulated by Aub-piRISC. In oocyte, a proportion of maternally deposited mRNAs accumulate at the posterior pole in an Aub-piRISC dependent fashion. mRNAs and other colocalization factors are necessary for pole cell formation. Target mRNAs associated with Aub-piRISC tend to be longer than other mRNAs. Aub-piRISC anchoring to the posterior pole requires Tudor. At the anterior region, Aub-piRISC induces the degradation of target mRNAs by recruiting the CCR-NOT complex.

The first study showed that Aub-piRISC functions in destabilizing maternally deposited mRNAs, including *nanos* and *oskar* mRNAs encoding the embryonic posterior morphogen, at anterior embryos.[183,184] To accomplish this, Aub-piRISC recruits the CCR4-NOT complex, which possesses deadenylase activity. Deadenylated mRNAs are further degraded by 3′-to-5′ exonucleases, resulting in mRNA decay. The piRISC may use its own Slicer activity depending on the complementarity between piRNAs and target RNAs. At the posterior pole, these mRNAs should be stabilized, allowing Aub-piRISC to avoid the CCR4-NOT complex at the posterior pole. However, how this is mechanistically accomplished remains unclear.

Later larger ultraviolet cross-linking followed by stringent immunoprecipitation (lgCLIP) was performed, which revealed that Aub-piRISC binds to numerous mRNAs in embryos.[185] The PIWI-piRISC binds to posterior mRNAs more abundantly than ubiquitous mRNAs. Posterior mRNAs tend to be longer than ubiquitous mRNAs, and therefore have a higher capacity to be bound with Aub-piRISC, which might explain why posterior mRNAs accumulate at the posterior pole in an Aub-piRISC-dependent fashion. In *tudor* mutants, Aub and its target mRNAs were distributed throughout embryos, suggesting that the subcellular localization of maternally deposited mRNAs depends on Tudor.[186,187] Therefore, it is very important to determine which factors are required for Tudor localization.

One of the important issues in this research field is that Aub-piRISC is maternally deposited as a ternary complex with target RNAs as a stable complex without inducing cleavage. If complementarity between piRNAs and target RNAs is high, it may allow Aub to cleave the targets, leading to a failure in mRNA maternal deposition and/or posterior localization; however, if complementarity is low, RNA−RNA base pairing between piRNAs and target RNAs is not maintained. The Slicer activity of Aub-piRISC during this process may be inhibited by cofactor(s) binding to the complex, but such factors have not been identified to date. This PIWI-piRISC function in embryogenesis is not unique to *Drosophila*, but is also studied in mice.[135,188,189]

5.3. Maternal piRNAs Specify the Use of piRNA Clusters in Adult Ovaries

PIWI and piRNAs are maternally deposited in *Drosophila* early embryos.[190,191] These maternal piRISCs have important roles in specifying the piRNA clusters used later in adult ovaries. This claim is supported by the observation that the zygotic knockdown of Piwi reduces H3K9me3 accumulation at the piRNA clusters in adult ovaries, leading to a reduced piRNA expression and transposon derepression.[192] Interestingly, this defect was not induced when *Piwi* was knocked down in the larval-to-adult stages. This indicates that H3K9me3 deposition on the piRNA cluster must occur at an earlier embryonic stage, but once it occurs at that stage, its effect lasts until embryos develop into adults, even in the absence of Piwi.[96,192,193]

5.4. Control of PIWI-Dependent, piRNA-Independent Spermatogenesis in Mice

A recent mouse study discovered a new PIWI function in spermatogenesis. Mammalian studies have mostly been excluded from this review; however, we have included this finding for two reasons. First, this PIWI function is independent of piRNAs and is, therefore, intriguing, and second, the discovery originated from investigating a human condition, azoospermia.[194]

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Strands of genomic DNA normally wrap around nucleosomes composed of core histones. However, these histones are replaced with protamines late in the haploid phase of spermatogenesis.[195−197] Protamines are arginine-rich proteins similar to, but smaller than, histones. The aim of this molecular replacement is nucleosome compaction and condensation, which enables the genome to fit into sperm heads. The trigger for histone/protamine replacement is the ubiquitination of histones H2A and H2B by ubiquitin ligase, RNF8.[198] A recent study revealed PIWI involvement in this event, but it was independent of piRNA.

Miwi, a mouse PIWI, starts to be expressed at the pachytene stage of spermatogenesis during meiosis, and its expression lasts until the elongated spermatid stage.[5,86,199,200] During this development, Miwi is localized to the cytoplasm.[201] Miwi has a unique domain, the Destruction box (D-box) at the N-terminal end, which serves as the substrate for APC/C ubiquitin E3 ligase.[202] The D-box is conserved in human PIWI proteins, such as Hiwi, but not in *Drosophila* PIWI proteins.[203] Miwi is ubiquitinated through the D-box, and degraded by ubiquitination in late spermatogenesis.[202] In normal cases when Miwi is expressed, RNF8 is localized to the cytoplasm so histones are not ubiquitinated.[198,204] However, when Miwi is absent because of ubiquitin-dependent degradation or other reasons, RNF8 is translocated to the nucleus, where it ubiquitinates and destabilizes histones, allowing replacement with protamines to occur. However, Miwi D-box mutant mice fail to replace histones with protamines through spermatogenesis, because the Miwi D-box mutant is not ubiquitinated and thus remains stable and sequesters RNF8 in the cytoplasm. Under such conditions, histones are not ubiquitinated, which prevents replacement between histones and protamines, causing azoospermia (Figure 10). This new function of Miwi is independent of piRNA, because the *miwi* mutant that lacks the ability to bind piRNA

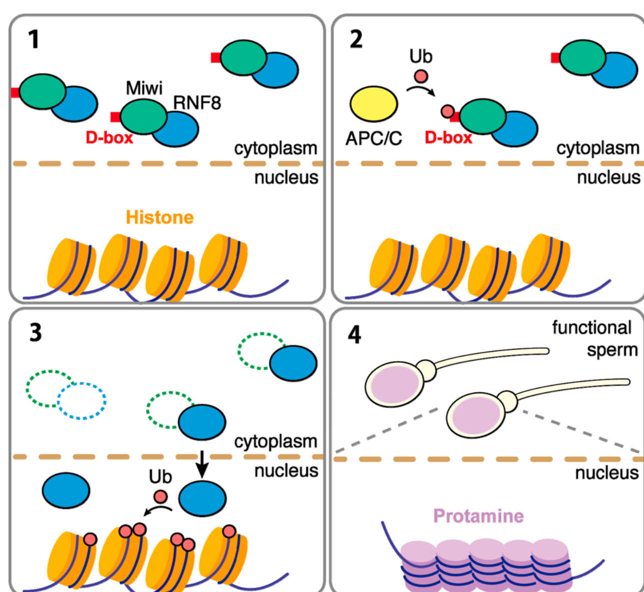

Figure 10. Miwi controls spermatogenesis piRNA-independently. Miwi binds to RNF8 and sequesters it to the cytoplasm. In late spermatogenesis, Miwi is ubiquitinated through the D-box by APC/C ubiquitin E3 ligase and degraded by ubiquitination. As a result, RNF8 is translocated to the nucleus, where it ubiquitinates H2A and H2B and destabilizes them, leading to histone exchange with protamines. The aim of this molecular replacement is nucleosome compaction and condensation. Abbreviations: D-box, Destruction box; Ub, ubiquitin.

retains this function.[194] This study was prompted by the authors' discovery that some patients with azoospermia have mutations in the *Hiwi* gene, particularly in the area corresponding to the D-box. This is the first example connecting a lack of PIWI function to a human medical condition. In this sense, this study is worthy of discussion here, although an analogous event might not occur in *Drosophila*.

6. OUTLOOK

Although there were indications of their existence before 2000, the study of piRNAs essentially started in 2003 when genome-wide small RNAs in *Drosophila* embryos and testes were sequenced and computationally analyzed.[205] This led to the discovery that 24−30 nt long small RNAs could be mapped on various transposons in tissues. Since then, piRNA studies, mainly using fruit flies and mice as experimental models, have successfully identified numerous piRNA factors and dissected the piRNA pathway to provide a comprehensive understanding of the underlying mechanism and to lay out a systematic mechanistic model of the pathway.

Progress greatly depends on cutting-edge techniques and powerful technological devices, such as HITS/i-CLIP, CRISPR-Cas9, next-generation sequencing, and high performance computational transcriptome profiling. However, commonly used, classical lab techniques, such as genetics-based gene screening, in vitro assays, chromatographic protein purification, and immunoprecipitation, have also been crucial to progress in the piRNA field. The use of ovary-derived, cultured cell lines, whose genomes are still safeguarded by piRNA function, has also been essential for dissecting the piRNA pathway from a biochemical point of view.[38,39,206,207]

Rarely used organisms such as planarians and *Aplysia* have shed light on new aspects of piRNA function. piRNAs in planarians are crucial for maintaining the endless stemness of neoblasts during development and degeneration.[208−210] piRNAs in the nervous system of *Aplysia* play important roles in developing long-term memory.[211,212] piRNA studies in mosquitos are now popular because of the aim of exterminating vectors that carry infectious viruses, such as Zika virus.[213−215] This can be achieved by bioengineered infertile adults in which the piRNA pathway is genetically damaged. It would be of interest to summarize these piRNA studies in unusual model organisms.

As discussed in this review, a recent study has finally connected PIWI functions to a human medical condition, although this function of PIWI involves protein stability/instability issues but not piRNAs.[194] We predict that further associations of piRNA functions with human diseases, including infertility, will be discovered. Such progress in the piRNA field will hopefully lead to a new understanding of the mechanism underlying the piRNA pathway and also to novel insights into human disease.

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The authors declare no competing financial interest.

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Biographies

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Mikiko C. Siomi received her doctoral degrees in agricultural chemistry and medical sciences from Kyoto University and the University of Tokushima, respectively. Since 2012, she has been full professor at the Graduate School of Biological Sciences at the University of Tokyo. Her study lately mainly focuses on piRNA-mediated gene silencing occurring in *Drosophila* gonads. Currently she serves as the President of the RNA Society of Japan (2014−), and is a member of the Science Council of Japan (2015−).

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