PIWI-Interacting RNA: Its Biogenesis and Functions

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Abstract

PIWI-interacting RNAs (piRNAs) are a class of small RNAs that are 24–31 nucleotides in length. They associate with PIWI proteins, which constitute a germline-specific subclade of the Argonaute family, to form effector complexes known as piRNA-induced silencing complexes, which repress transposons via transcriptional or posttranscriptional mechanisms and maintain germline genome integrity. In addition to having a role in transposon silencing, piRNAs in diverse organisms function in the regulation of cellular genes. In some cases, piRNAs have shown transgenerational inheritance to pass on the memory of "self" and "nonself," suggesting a contribution to various cellular processes over generations. Many piRNA factors have been identified; however, both the molecular mechanisms leading to the production of mature piRNAs and the effector phases of gene silencing are still enigmatic. Here, we summarize the current state of our knowledge on the biogenesis of piRNA, its biological functions, and the underlying mechanisms.

Contents

INTRODUCTION

Our genome encodes thousands of genes responsible for various cellular functions, and the regulation of the expression levels and patterns of these genes is crucial for development and homeostasis. This regulation is performed by a collection of intramolecular and intermolecular events. RNA silencing, also referred to as RNA interference (RNAi), has emerged as one of the key gene regulatory pathways in most eukaryotes (1, 2). Central to RNA silencing pathways is the generation of small RNAs of 20 to 31 nucleotides (nt). These form an RNA-induced silencing complex (RISC) with Argonaute proteins and recognize their targets via Watson–Crick base pairing. The Slicer endonuclease activity of Argonaute proteins cleaves target transcripts to accomplish gene silencing; posttranscriptional gene silencing, such as translational repression, or transcriptional gene silencing via specific chromatin modifications is performed through recruitment of other proteins (3).

Argonaute proteins are divided into two subfamilies, the Argonaute (AGO) and PIWI families (4). AGO subfamily proteins are ubiquitously expressed and can bind to microRNAs (miRNAs) and small interfering RNAs (siRNAs), both of which are processed from double-stranded precursors into mature small RNAs of 20 to 22 nt in length in a Dicer-dependent manner (**Figure 1**) (3). PIWI subfamily proteins (PIWI proteins) are expressed mainly in germline cells and form specific RISCs with a small RNA population known as PIWI-interacting RNAs (piRNAs); these RISCs are termed piRISCs.

piRNAs are slightly longer (24–31 nt) than miRNAs and siRNAs, possess 2′-*O*-methyl modification sites at the 3′ terminus, and are processed from single-stranded precursor transcripts expressed from intergenic regions termed piRNA clusters via a Dicer-independent mechanism (5, 6). piRNA clusters harbor a large number of and various types of transposons; therefore,

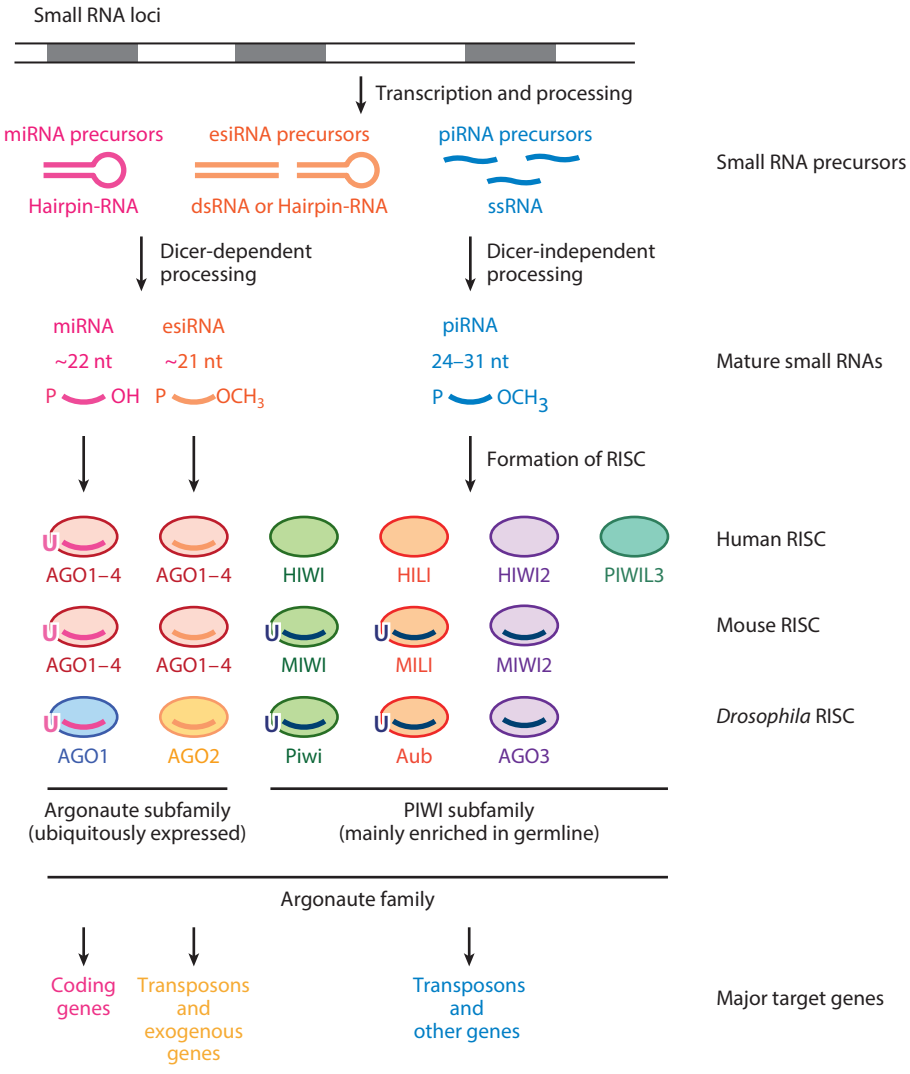


Figure 1

RNA silencing by small RNAs and their partner Argonaute family proteins in human, mouse, and *Drosophila*. Expression of a fourth PIWI protein (PIWIL3) has been detected specifically in humans. Characteristics of piRNA precursors, mature sequences, RISC formation, and target genes are summarized for miRNAs, esiRNAs, and piRNAs. An association between piRNAs and human PIWI proteins has not yet been identified. Abbreviations: dsRNA, double-stranded RNA; esiRNA, endogenous small interfering RNA (siRNA); miRNA, microRNA; nt, nucleotide; piRNA, PIWI-interacting RNA; RISC, RNA-induced silencing complex; ssRNA, single-stranded RNA.

piRNAs regulate mainly the activity of transposons, namely their expression and transposition within the genome. Because transposition of transposons has a high risk of damaging the genome intracellularly, the piRNA-mediated regulation of transposons is essential, especially for preserving normal gametogenesis and reproduction. Owing to their processing mechanisms and the variety of source transposons, piRNA sequences are much more diverse than those of any other known

class of cellular RNAs and constitute the largest class of noncoding RNAs (6, 7). Transposon regulation by piRNAs is conceptually similar to that in immune systems, which can achieve "self" and "nonself" recognition. As with our immune systems, piRNAs use a complex mechanism to effectively select and regulate the nonself genes for regulation (8).

Recent studies have revealed a large number of cytoplasmic factors that support and maintain piRNA biogenesis, as well as some nuclear factors that either recognize and transcribe piRNA clusters to produce piRNA precursors or function in piRNA-mediated transcriptional silencing. Additionally, analyses of various eukaryotes have identified piRNAs that target protein-coding genes and piRNAs that are passed through generations to transmit a memory of past transposon activity (9–11). Here, we discuss how piRNAs are generated and how they function to recognize and regulate their target genes. We focus mostly on recent research in the model animals *Drosophila melanogaster* and mouse, but we also discuss important findings in other model animals.

PIWI PROTEINS AND piRNAs FOR MAINTAINING GENOME INTEGRITY

PIWI proteins and piRNAs are conserved in a wide range of eukaryotes, from sponges to humans, and they are expressed mainly in the gonads (6, 12). The prototype of PIWI proteins is encoded by the *Drosophila piwi* (P-element-induced wimpy testes) gene, which was originally identified as an essential gene for germline development (13, 14). In *Drosophila*, which contains three distinct *PIWI* genes [*ago3*, *aubergine* (*aub*), and *piwi*], *piwi* and *aub* are required for both male and female fertility, whereas *ago3* is essential for female fertility (14–17). Derepression of transposons is observed in each of these *PIWI* mutant ovaries, indicating that all three PIWI proteins have nonredundant roles in gonad development and transposon silencing. Aub and AGO3 cleave their target transposon transcripts in the cytoplasm, whereas Piwi can regulate its target transposons at the transcriptional level in the nucleus (15, 18–22). Interestingly, the *Drosophila* piRNA pathway also regulates transposon activity to maintain the telomeres. Unlike most other eukaryotes, the transposition of a distinct set of transposons to the chromosomal ends maintains the telomeres of *Drosophila* chromosomes, whereas defects of the piRNA pathway in gonads reduce expression of telomere-specific piRNAs and disrupt assembly of the telomere protection complex. Meanwhile, piRNA pathway defects do not affect transposon expression or telomere structure in somatic tissues (23–25).

Mice also express three PIWIs (MIWI, MIWI2, and MILI). All three PIWI proteins are expressed at different stages during spermatogenesis (**Figure 2**), but only MILI is expressed, albeit weakly, in female germ cells. Mutations in mouse *PIWI* genes affect the male germline but not the female germline (26–29). Deficiency in MILI or MIWI2 leads to the activation of long interspersed nuclear element and long terminal repeat (LTR) retrotransposons including L1 and IAP elements, and spermatogenic stem cell arrest is observed. During MIWI depletion, the L1 transposon is also dysregulated, and spermatogenesis is arrested at the early spermatid stage (26, 28, 30–32).

Mouse PIWI proteins are bound to piRNAs expressed in two phases: prepachytene piRNAs and pachytene piRNAs. Prepachytene piRNAs are derived mostly from transposable elements and are associated with MILI and MIWI2 in the gonocyte stage, whereas pachytene piRNAs originate from piRNA clusters located in various regions of the genome and bind to both MILI and MIWI in pachytene spermatocytes to the round spermatid stage. Although a fraction of pachytene piRNAs originates from transposons, the largest fraction comprises those originating from an unannotated region (27–29, 31, 33). MIWI and MILI are necessary to maintain the Slicer-dependent silencing of the L1 transposon in the mouse testis after birth, indicating that Slicer activity directly cleaves

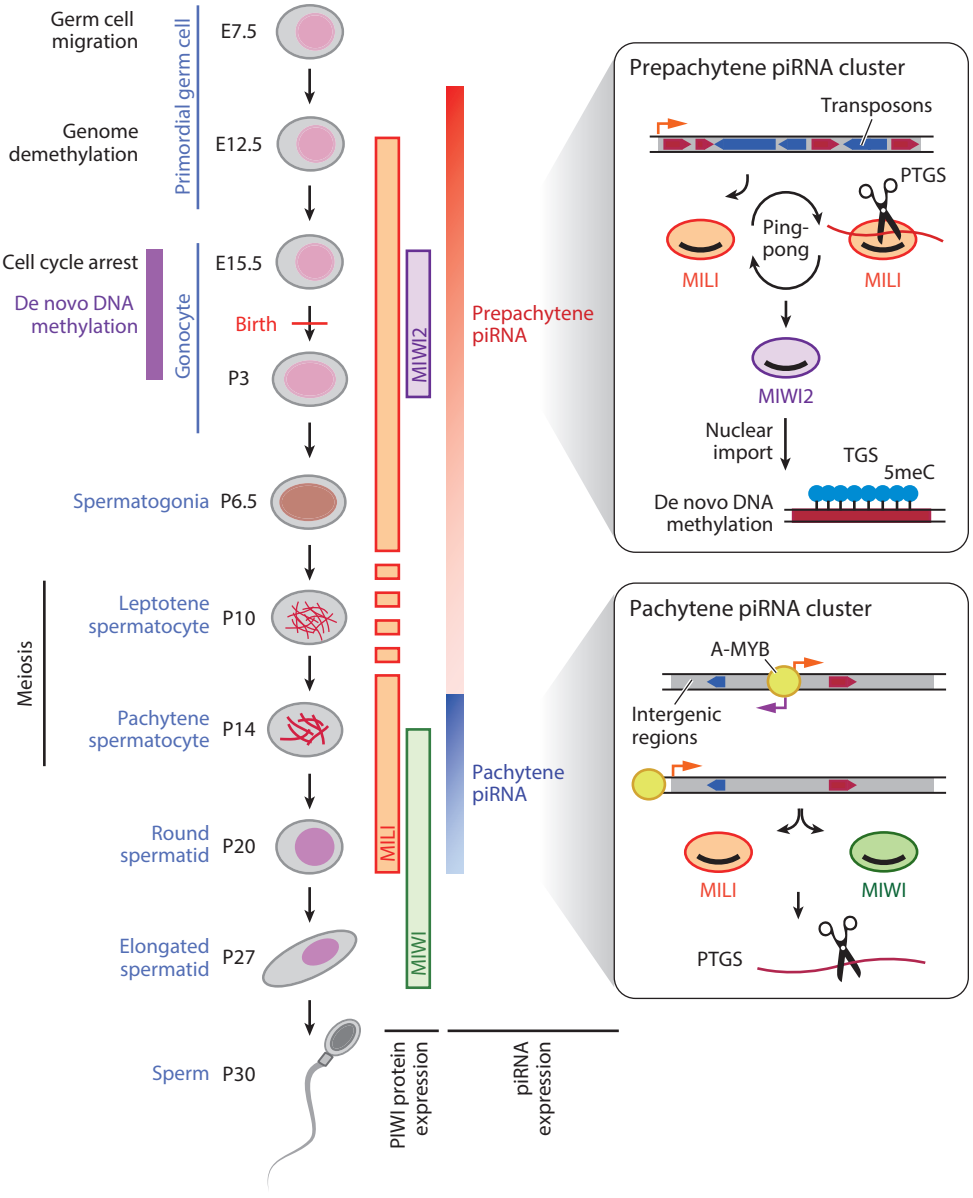


Figure 2

Expression patterns of MILI, MIWI, MIWI2, and mouse piRNAs during spermatogenesis. piRNAs are classified into pachytene piRNAs and prepachytene piRNAs on the basis of the stage in spermatogenesis when they are expressed. In gonocytes, MILI and MIWI2 bind to piRNAs from a prepachytene piRNA cluster, which consists mainly of transposons. MILI performs the homotypic ping-pong cycle to silence targets by PTGS and produce piRNAs that associate with MIWI2. MIWI2 localizes to the nucleus upon piRNA loading to accomplish nuclear silencing by de novo DNA methylation. Beyond the pachytene stage, MIWI and MILI are bound to piRNAs from pachytene piRNA clusters, a large fraction of which consists of intergenic regions. Pachytene piRNAs regulate their target genes by PTGS in the cytoplasm. Abbreviations: piRNA, PIWI-interacting RNA; PTGS, posttranscriptional gene silencing; TGS, transcriptional gene silencing.

transposon messenger RNAs (mRNAs) (**Figure 2**) (34, 35). Moreover, mouse PIWI proteins function not only in posttranscriptional gene silencing by cleaving transposon transcripts, but also in transcriptional silencing by directing CpG DNA methylation on transposon loci. Indeed, silencing and de novo DNA methylation of L1 and IAP elements are decreased in male germlines that are defective for the activity of the *MILI* or *MIWI2* gene (27–29). This finding indicates that mouse piRNAs guide specific de novo DNA methylation to silence their target transposons. Because mouse PIWI proteins are expressed in a developmental stage–specific manner (29), the proper removal of PIWI proteins is essential for male germ-cell development. It was recently reported that ubiquitination of piRNA-associated MIWI triggers degradation of the MIWI–piRNA complex via the APC/C–26S proteasome pathway (36).

Although transposons are the major targets of PIWI–piRNA complexes, how regulation of transposons is connected to defects in gametogenesis remains unknown. Activation of transposons in PIWI protein mutants may lead to the generation of double-stranded DNA breaks during abortive or successful transposition that activate a DNA damage checkpoint, resulting in a sterile phenotype (37). Additionally, loss of the piRNA biogenesis factor Maelstrom (Mael) in mouse results in acrosome and flagellum defects that lead to spermiogenic arrest (38). Thus, tissue-specific and developmental timing–specific expression of PIWI proteins and piRNAs may play major roles in maintaining the integrity of the genome and fertility of the organism. However, a mutation in *Drosophila piwi* that leads to transposon activation does not directly affect germline development (39). Also, derepression of L1 transposons by deletion of a part of a pachytene piRNA cluster does not affect spermatogenesis in mouse (40), suggesting that transposon silencing and germline development can be separated.

BIOGENESIS OF piRNAs

Two major pathways generate piRNAs: the primary processing pathway and the ping-pong cycle that amplifies secondary piRNAs (**Figure 3**). Primary piRNAs have a bias toward having uridine (U) at their 5′ nucleic acid (1U bias), whereas secondary piRNAs show 10-nt complementarity with primary piRNAs at their 5′ ends and possess a sense bias with adenosine at the tenth nucleotide (10A bias) (5, 21, 22, 29–31).

Primary piRNA Biogenesis

In *Drosophila* ovaries, the primary pathway operates in both germline and surrounding somatic cells, whereas the ping-pong cycle operates only in germline cells. Within the primary pathway in *Drosophila* ovarian somatic cells, only Piwi is expressed among PIWI subfamily members, and piRNA precursors are transcribed from piRNA clusters such as the *flamenco* (*flam*) locus as long unidirectional single-stranded transcripts and processed in multiple steps (**Figure 4*a***) (21). The *flam* locus harbors a large number of truncated transposons, most of which are antisense oriented relative to transposon coding strands (21, 41, 42). Long primary transcripts from the piRNA clusters are exported to the cytoplasm and possibly processed into intermediates, but the detailed processes are still largely unknown. Although an endonuclease, Zucchini (Zuc), which is located on the surface of mitochondria, is required for processing precursor RNAs (43–46), whether Zuc preferentially produces precursor RNAs with U at the 5′ end remains to be addressed. The loading and subsequent maturation steps probably occur within perinuclear granules termed Yb bodies, which are also formed on the surfaces of mitochondria (**Figure 4*b***); moreover, components of Yb bodies, including fs(1)Yb (Yb), Armitage (Armi), Vreteno (Vret), Shutdown, and Sister of Yb (SoYb), are essential for producing mature piRNAs (**Table 1**) (47–52). Additionally, Minotaur

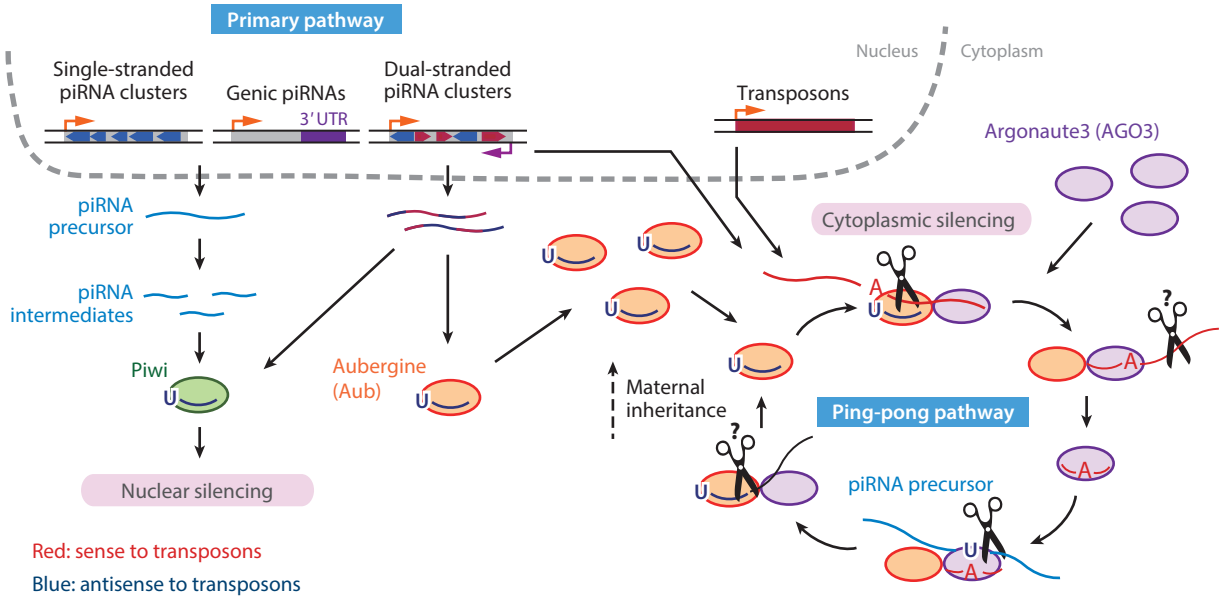


Figure 3

Biogenesis pathway of *Drosophila* piRNAs, consisting of the primary and ping-pong pathways. In the primary pathway, piRNAs are transcribed from genomic regions called piRNA clusters, processed, and loaded onto Piwi or Aub. 3′-UTR sequences of some protein-coding genes can also serve as a source of primary piRNAs. Silencing takes place both in the cytoplasm and nucleus (also see **Figure 4**). Piwi performs transcriptional gene silencing in the nucleus. Together with AGO3, the Aub–piRNA complex serves as a trigger to start the ping-pong amplification pathway. The ping-pong pathway silences the target transposon sequence and amplifies the piRNA sequence at the same time. Note that some Aub–piRNA complexes are also maternally inherited. Abbreviations: piRNA, PIWI-interacting RNA; UTR, untranslated region.

(Mino) and GasZ are localized to mitochondria and function in primary piRNA processing (53, 54). Although these connections between piRNA biogenesis and mitochondria have emerged, whether some mitochondrial activity is required for piRNA biogenesis is unclear. Within the process, however, the precursors are further trimmed to the mature piRNA size by the activity of an unknown 3′–5′ exonuclease (55). The DmHen1/Pimet methyltransferase then 2′-*O*-methylates the 3′ ends of piRNAs to produce mature Piwi–piRNA complexes or Piwi–piRISCs (56, 57). Piwi–piRNA complexes are then imported into the nucleus to transcriptionally regulate target genes (58). piRNA-free Piwi stays in the cytoplasm, suggesting that the Yb body is where functional piRISC formation is assessed: Only functional complexes can be imported into the nucleus (47, 48). Interestingly, transcripts (or processed intermediates of them) derived from the *flam* locus accumulate at perinuclear foci adjacent to the Yb body, termed Flam bodies (59). The formation of a Flam body depends on the RNA-binding activity of Yb, indicating that Yb integrates primary piRNA transcripts or their intermediates into the Flam body.

In *Drosophila* germline cells, primary piRNAs are also derived from dual-stranded piRNA clusters such as the *42AB* locus and loaded onto both Aub and Piwi to form piRISCs (**Figure 1**). The amount of germline piRNAs is reduced in *Drosophila* mutants upon Armi depletion (60), indicating that some of the somatic factors described above are also required for germline piRNA biogenesis. However, the germline counterparts for components of Yb bodies remain to be identified. Thus, the primary piRNA pathway partly differs between somatic and germline cells, and the details of how the primary pathway operates in *Drosophila* germline cells are still unknown.

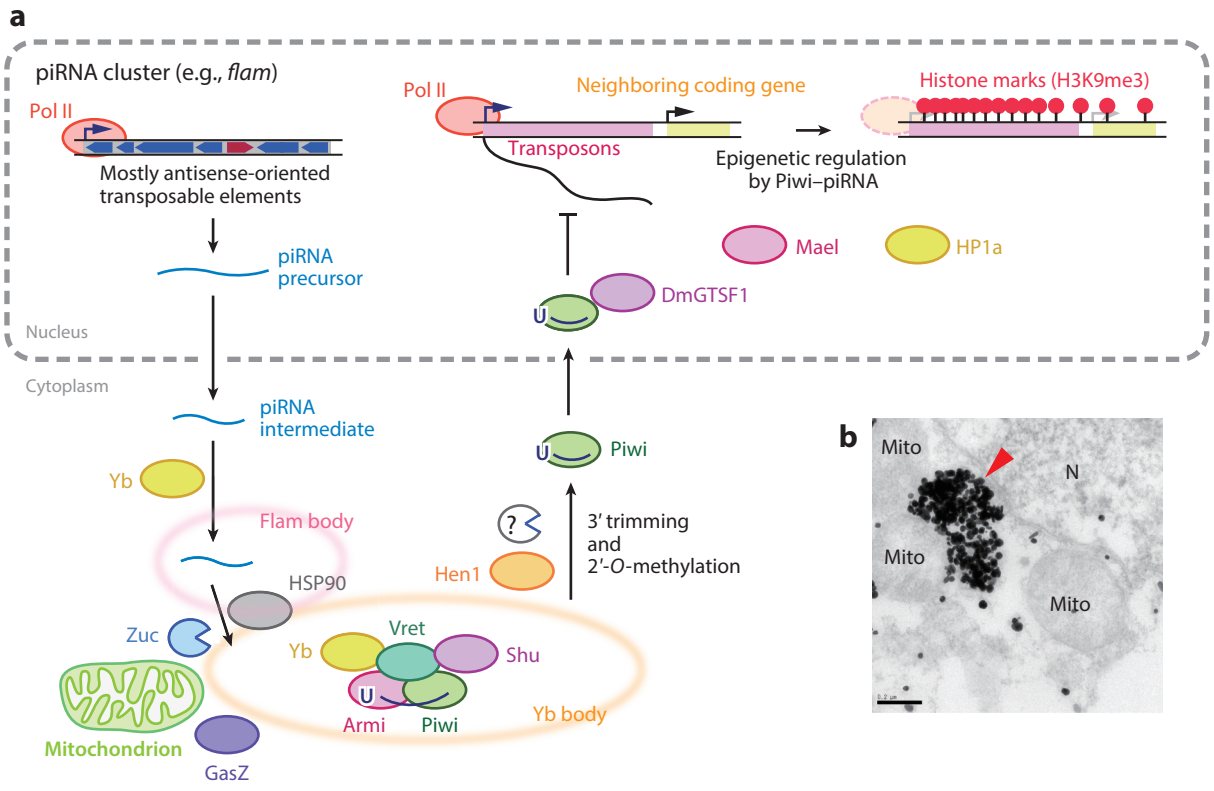


Figure 4

Epigenetic silencing by Piwi–piRNA in *Drosophila*. (*a*) Piwi, a *Drosophila* PIWI protein, is localized to the nucleus and can epigenetically silence target genes. Transcripts of piRNA clusters, which contain numerous sequences complementary to transposons, serve as precursors of piRNAs. piRNA precursors are processed into piRNA intermediates and exported to the cytoplasm. Intermediates are processed by the endonuclease Zuc near the mitochondria, localized to granules termed Flam bodies, and then to Yb bodies, where factors such as Yb, Armi, Vret, and Shu are localized. Armi is recruited to mitochondria by Gasz. Here, piRNAs are processed and loaded onto Piwi. Then, piRNAs are 3′ trimmed and 2′-*O*-methylated by Hen1 and transferred into the nucleus. Within the nucleus, Piwi–piRNA complexes regulate their target genes by modifying histone marks and association of Pol II with target genes. Several factors, such as DmGTSF1, Mael, and HP1a, are involved in this process, but the regulatory mechanism remains to be revealed. (*b*) Immunoelectron microscopy using an anti-Yb antibody shows a perinuclear Yb body in an ovarian somatic cell (*red arrowhead*). Abbreviations: Armi, Armitage; Mael, Maelstrom; Mito, mitochondria; N, nucleus; piRNA, PIWI-interacting RNA; Pol II, RNA polymerase II; Shu, Shutdown; TE, transposable element; Vret, Vreteno; Yb, fs(1)Yb; Zuc, Zucchini. Scale bar, 0.2 μm.

Mammalian orthologs of *Drosophila* somatic primary piRNA biogenesis factors have been identified (**Table 1**) (61–65). For example, a mouse ortholog of Zuc is MitoPLD, which is also a mitochondrial protein involved in piRNA production (63, 65). Additionally, MOV10L1, a mouse ortholog of Armi, is an RNA helicase that is essential for piRNA biogenesis (62, 66). Thus, primary piRNAs in mammals may be produced via pathways that are similar to the *Drosophila* somatic primary pathway, although the details have not been explored.

Epigenetic Regulation of Transposable Elements by piRNAs

After Piwi–piRISCs are imported to the nucleus, they direct methylation of histone 3 lysine 9 (H3K9me3) on chromatin at target transposon loci to induce heterochromatin formation,

Table 1 Factors involved in the piRNA pathway

Protein name	Domain	Interacting PIWI protein	Expression	Homolog in mouse	Reference(s)
Piwi	Paz, Mid, Piwi	ND	Gonad	MIWI	83, 91, 128
Aub	Paz, Mid, Piwi	Ago3	Gonad	MILI	15, 128
Ago3	Paz, Mid, Piwi	Aub	Gonad	MIWI2	15, 47, 48
Tud	Tudor	Aub, Ago3	Ubiquitous	TDRD6	85
PAPI	Tudor, KH	Ago3, Piwi	Ubiquitous	TDRD2	88
Spn-E	Tudor, DEAD, Hel-C, HA2	Aub	Gonad	TDRD9	76, 91
Qin/Kumo	Tudor, RING, B-box	Piwi, Aub	Gonad	TDRD4	89, 90
Tejas	Tudor, Lotus	Aub	Gonad	TDRD5	91
Yb	Tudor, Hel-C, DEAD	Piwi	Gonad	ND	47–50
BoYb	Tudor, DEAD, Hel-C	NA	Gonad	ND	50
SoYb	Tudor, DEAD, Hel-C	NA	Gonad	TDRD12	50
Vret	Tudor, RRM, MYND	Piwi, Aub, Ago3	Gonad	ND	49, 50
Krimp	Tudor	NA	Gonad	ND	76, 92
Egg/SetDB1	Tudor, MBD, preSET, SET, postSET	NA	Ubiquitous	SETDB1/ SETDB2	74
Vasa	DEAD, Hel-C	Aub	Gonad	MVH	91
Armi	P-loop NTPase, Uvr_D_C_2	Piwi	Gonad	MOV10	47–50
Shu	PPlase, TPR	Piwi	Gonad	FKBP6	87, 51
Zuc	PLD-like	Piwi, Aub	Gonad	MitoPLD	43–47
Squ	RNase HII-like	Piwi, Aub	Gonad	ND	45, 46
Mino	GPAT	NA	Ubiquitous	GPAT1, GPAT2	54
GasZ	Ankyrin-rpt, SAM,	NA	Ubiquitous	GASZ	53
Mael	Mael	NA	Gonad	Mael	58
DmGTSF1/Arx	UPF0224_CHHC_Znf	Piwi	Gonad	GTSF1	70–72
Rhi	Chromo	NA	Gonad	Cbx5	95–97
Cuff	RAI1	NA	Ubiquitous	Dom3z	95, 96
Del	NA	NA	Gonad	ND	95, 96

Abbreviations: NA, not available; ND, not detected; piRNA, PIWI-interacting RNA.

thereby transcriptionally silencing transposons (**Figure 4**) (39, 58, 67–69). A nuclear protein termed Asterix/DmGTSF1 associates with Piwi–piRISCs to mediate the addition of this silencing histone mark (70–72). Mael is also involved in Piwi-piRISC-mediated transposon silencing in the nucleus, but it is not required for the establishment of H3K9me3 on silenced transposon loci (58).

How H3K9me3 marks are deposited at piRNA target regions is still being discussed, but the involvement of the methyltransferases Eggless (Egg)/SetDB1 and Su(var)3–9, as well as the heterochromatin protein HP1a, has been suggested (71, 73–75). However, it is not clear whether Piwi directly associates with HP1a, which triggers recruitment of methyltransferases, or if Piwi first recruits methyltransferase or some other factor to deposit the H3K9me3 mark, resulting in an association between Piwi and HP1a (10). Therefore, the precise mechanisms have yet to be revealed.

Secondary piRNA Biogenesis

Aub–piRISCs initiate the ping-pong cycle in the cytoplasm together with AGO3 to produce secondary piRNAs (**Figure 3**). The ping-pong pathway is believed to take place at an electron-dense, nonmembranous perinuclear structure known as the nuage because both PIWI proteins and many other factors involved in piRNA biogenesis accumulate in the structure (76). In the ping-pong pathway, AGO3 and Aub act in a complementary fashion to cleave sense and antisense transposon transcripts via their Slicer activities. piRNA generation in a feed-forward mechanism results in the consumption of transposon transcripts, thereby silencing transposons (21, 22). piRNAs produced in the ovary are also deposited in the embryo as Aub–piRISCs, which additionally boost piRNA production by initiating the ping-pong cycle in the ovaries of the offspring (77).

Slicer activity directs cleavage of its cognate target RNAs across the position between 10 and 11 nt, measured from the 5′ end of the associated small RNA (78). Thus, reciprocal cleavage of transposon transcripts by AGO3–piRISCs and Aub–piRISCs determines the 5′ end of secondary piRNAs, although how their 3′ ends are formed is still unknown. As Aub-bound primary piRNAs show an antisense and 1U bias, secondary piRNAs loaded onto AGO3 show 10-nt complementarity at their 5′ ends with Aub-bound piRNAs and possess a 10A bias. The characteristic features of piRNAs with 1U/10A partners and a 10-nt 5′ overlap in the pathway are often referred to as the ping-pong signature (21, 22). Although the precise mechanism remains unknown, recent studies (79, 80) have begun to address how Slicer-mediated cleaved products are handed to a ping-pong partner PIWI: Vasa, a conserved RNA helicase and a component of the nuage, facilitates release of 5′ sliced piRNA precursors from piRISCs via an ATP-dependent mechanism, suggesting that Vasa promotes transfer of cleaved piRNA intermediates between ping-pong partner PIWIs.

In mouse testis, MILI associates with primary piRNAs and hands secondary piRNAs to MIWI2. However, heterotypic MILI–MIWI2 ping-pong may not operate, because, once loaded with secondary piRNAs, MIWI2 is imported into the nucleus to direct specific DNA methylation on transposon loci (28, 29, 34, 35). Therefore, it would be difficult to provoke another round of ping-pong with MILI, which is localized to the cytoplasm. This finding suggests that mouse ping-pong may be a one-way process (**Figure 2**). However, some evidence supports the possibility that MILI–piRNA complexes perform homotypic ping-pong to amplify piRNAs (35). Deviations from the primary and ping-pong paradigms have also been observed in several organisms including nematodes and cilia (see below) (9, 10).

Other Factors Involved in piRNA Biogenesis

In addition to the factors described above, the functions of various proteins, such as Tudor domain–containing (TDRD) proteins, are indispensable for the piRNA biogenesis pathway (**Table 1**). TDRD proteins interact with proteins with symmetrical dimethyl arginine (sDMA) or asymmetrical dimethyl arginine (aDMA) modifications. Moreover, some TDRD proteins also function in RNA metabolism (81). PIWI proteins harbor sDMA modifications in their N-terminal regions, and the modifications are likely to be substrates for TDRD proteins (82–86). TDRD proteins that are involved in the *Drosophila* piRNA pathway include Tudor, Partner of PIWIs (PAPI), Qin/Kumo, Tejas, Spindle-E (Spn-E), Yb, SoYb, Brother of Yb (BoYb), Krimper (Krimp), Egg/SetDB1, and Vret (47–51, 64, 76, 85, 87–92). As described above, some of these proteins function in the primary pathway, and the others function to perpetuate the ping-pong pathway. For example, Tudor, a large TDRD protein containing 11 Tudor domains, interacts with Aub and AGO3 in an sDMA-dependent manner to provide a platform for ping-pong amplification.

Although piRNAs are still loaded onto Aub and AGO3 in *tudor* mutants, the identity of piRNA sequences is significantly changed from that in the wild type, suggesting that Tudor is essential for quality control of piRNAs produced from the ping-pong cycle (85). Qin/Kumo, another TDRD protein containing five Tudor domains, also functions to maintain the ping-pong cycle. In *qin/kumo* mutants, Aub and AGO3 cannot localize to the nuage (90), and Aub starts to perform the homotypic ping-pong cycle, which is weaker than the ping-pong cycle of Aub and AGO3 (89). This finding suggests that Qin/Kumo plays an essential role in maintaining the heterotypic ping-pong of Aub and AGO3 (89, 90). Interestingly, in silkworms, Qin/Kumo, together with a silkworm homolog of Spn-E, functions in primary piRNA processing (80). Mutations in genes encoding TDRD proteins have relatively mild effects on piRNA production compared with mutations in other piRNA biogenesis factors, probably because some TDRD proteins cooperatively function to maintain piRNA biogenesis. Therefore, it would be important to understand the precise function of each TDRD protein and reveal links between them to fully understand piRNA biogenesis.

To gain comprehensive knowledge of the factors involved in piRNA biogenesis, several large-scale screenings of factors involved in the *Drosophila* piRNA pathway have been performed (**Figure 5**). Hannon's group (71, 75) has screened for germline piRNA pathway factors using transgenic RNAi flies and for somatic piRNA pathway factors using double-stranded RNA library transfection into an ovarian somatic sheet cell line. These screenings were performed independently, but many common factors were identified. The genes identified as being involved in the germline piRNA pathways included those related to transcriptional elongation, RNA export, protein nuclear import, and chromatin modification. The somatic piRNA factors identified included those related to functions such as exon junction complex formation, general RNA metabolism factors, and those involved in the SUMOylation machinery.

Brennecke's group (53) has identified ~50 factors involved in piRNA biogenesis. For each line of transgenic RNAi flies, these authors performed primary screening by analyzing a β-galactosidase reporter system constructed using the LTR from the *gypsy* transposon, which serves as a source of piRNAs in both the soma and germline piRNA pathways. The results suggest connections between the piRNA pathway and mitochondrial metabolism, nuclear pore complex factors, transcriptional elongation factors, and chromatin biology factors, among others. Using the *burdock* transposon, an element that is involved only in the germline piRNA pathway, as a reporter, these researchers also identified factors specifically involved in the germline piRNA pathway. They revealed factors that are involved in ping-pong amplification by Aub/AGO3 and processing of dual-stranded piRNA clusters, such as Rhino (Rhi) (see below). As a result of these reports, we now have a good blueprint for what types of genes and machinery are involved in piRNA pathways. These factors must now be analyzed in greater depth if we are to understand how they are involved in piRNA production and to elucidate the mechanisms by which piRNAs silence transposons.

SOURCE OF piRNA PRODUCTION

piRNA Clusters

piRNA clusters have been identified as genomic regions where a large number of piRNA reads are uniquely mapped (**Figure 6**). They often reside within or close to heterochromatin. The length of piRNA clusters ranges from a few kilobases to hundreds of kilobases (21, 60). For example, the *flam* locus, a major somatic primary piRNA cluster in the *Drosophila* X chromosome, is transcribed into an ~180-kb-long single-stranded transcript. As described above, most of the *flam* transcript sequences correspond to transposons, including *gypsy*, *idefix*, and *ZAM*, in an antisense orientation (**Figure 6*a***) (42). Thus, piRNAs derived from the cluster can target sense transcripts

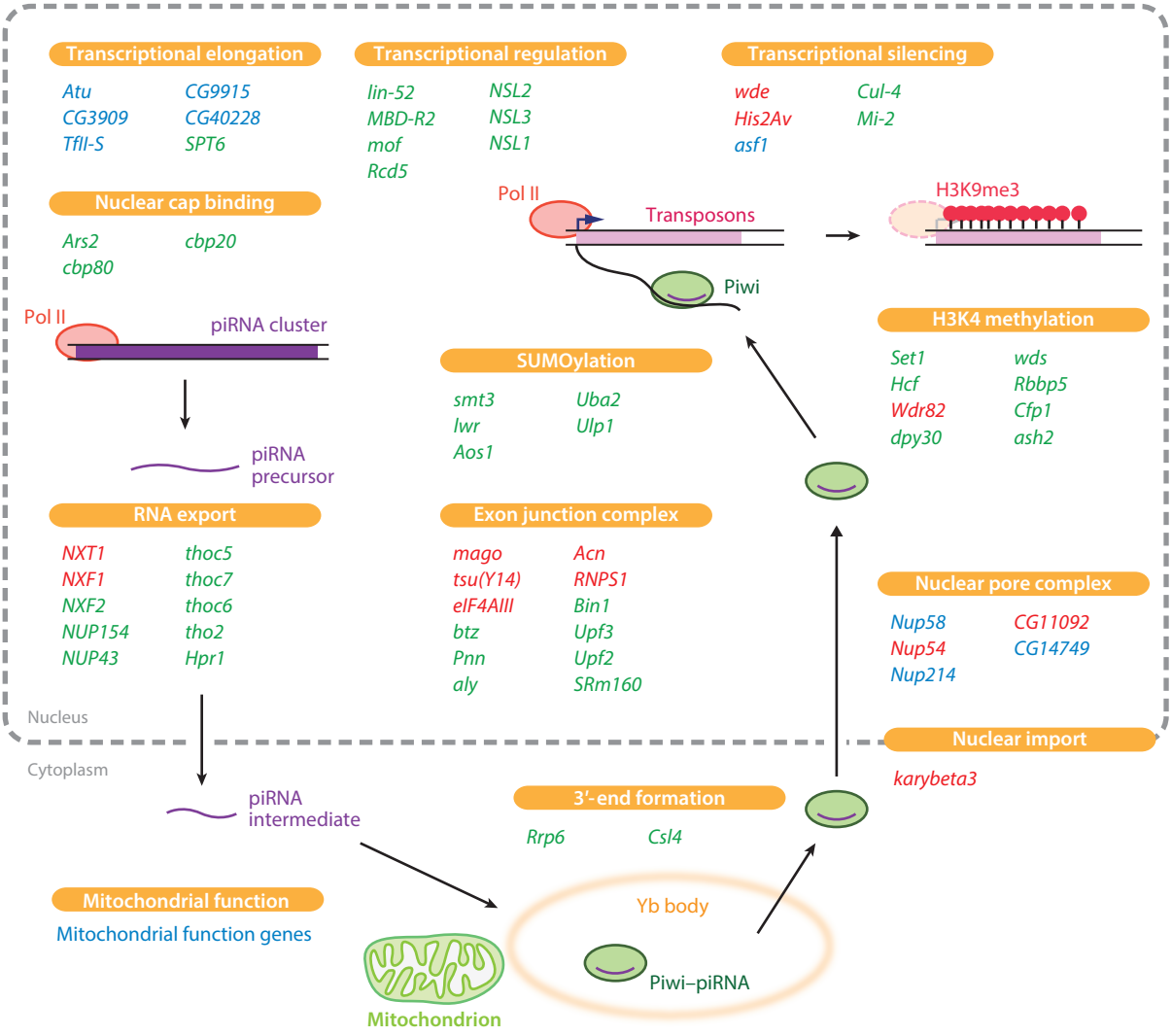


Figure 5

Examples of biological functions and factors newly identified by genome-wide screening to be involved in the piRNA pathway. Factors identified by screenings performed by Hannon's group (*green*) (71, 75), Brennecke's group (*blue*) (53), and both groups (*red*) are listed along with their annotated functions. Abbreviations: piRNA, PIWI-interacting RNA; Pol II, RNA polymerase II; Yb, fs(1)Yb.

produced from cognate transposons dispersed throughout the genome. Another type of piRNA cluster, namely the *42AB* cluster in germline cells, is transcribed from both strands as a dual-stranded transcript. piRNAs generated from this type of cluster are integrated into both the primary pathway and the ping-pong cycle (21). Unlike the *flam* locus, dual-stranded piRNA clusters contain transposons that are sense and antisense oriented, mostly in a random manner, relative to the polarity of the locus transcription; therefore, it is unclear how the antisense bias observed for the germline primary piRNAs is generated initially. Perhaps Aub may associate with both sense and antisense primary piRNAs. Because the ping-pong cycle requires ongoing expression

of the cluster and target transposons, the amplification loop steers piRNA production toward transcriptionally active and highly expressed transposons (**Figure 3**). Therefore, as long as there is an input of active transposon transcripts, antisense piRNAs are preferentially produced for Aub and the bias can be maintained. However, how the antisense bias for Piwi-bound piRNAs is enforced is enigmatic because, as in MIWI2, once loaded with piRNAs, Piwi is imported into the nucleus (47, 48); thus, it is unlikely to participate in the ping-pong cycle with AGO3, which is localized to the cytoplasm.

In mammals, the genomic location or synteny of piRNA clusters is highly conserved, although their primary sequences are not conserved (27, 30, 31, 93). Two types of piRNA clusters exist in mouse: unidirectional piRNA clusters and bidirectional piRNA clusters (**Figure 2**) (27, 29, 31, 33). As in *Drosophila*, some mouse piRNA clusters are transcribed as a single strand, frequently spanning a long region of the genome; these are termed unidirectional piRNA clusters. Bidirectional piRNA clusters are transcribed both from sense and antisense strands, but unlike dual-stranded piRNA clusters in *Drosophila*, they are transcribed from a single central promoter. Transcription then switches to the opposite direction. A MYB-related protein underlies transcription of these piRNA clusters in mouse (94).

Molecular Process to Define piRNA Clusters

It was unknown for a long time how transcripts of the piRNA clusters can produce piRNAs and how they are protected against degradation to be relatively long transcripts. Dual-stranded piRNA cluster transcripts have unique characteristics: They lack a clear promoter, 5′ methylguanosine caps, and clear transcription termination (95). Also, they seem not to be alternatively spliced, although they harbor intron-like sequences (96). These transcripts somehow escape from transcription termination and RNA decay and are processed into mature piRNAs.

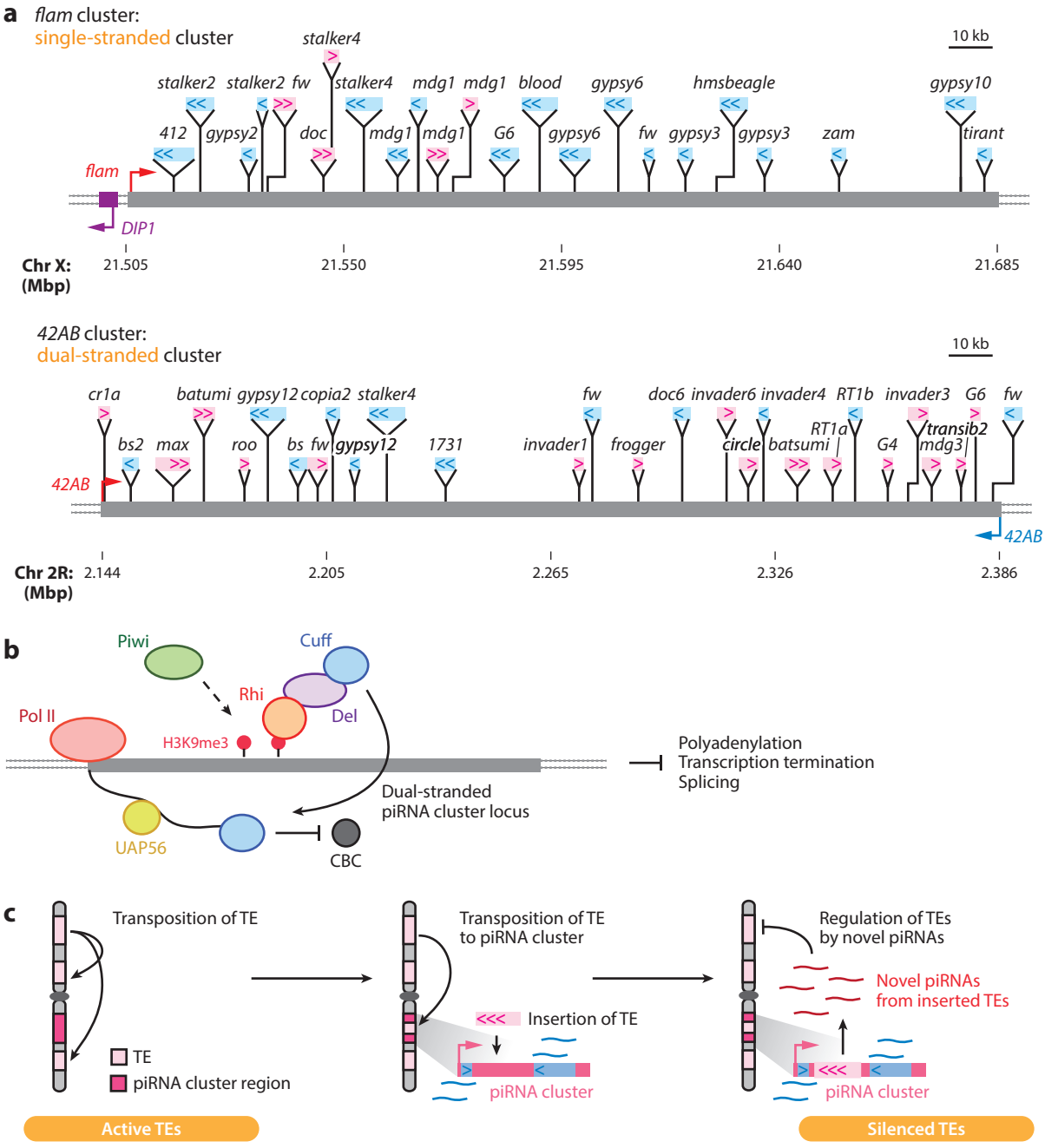
Recent studies have shed some light on how piRNA generation from dual-stranded piRNA clusters in *Drosophila* is initiated (**Figure 6*b***). *Rhi*, an HP1a family gene, plays a major role in the identification of dual-stranded piRNA clusters in germline cells (97). Furthermore, Rhi forms a complex together with Deadlock (Del) and Cutoff (Cuff), and this complex is anchored to H3K9me3-marked chromatin, where it would be defined as a piRNA cluster (95). Cuff protects the 5′ ends of piRNA precursor transcripts from the cap-binding complex. This results in inhibition of alternative splicing as well as polyadenylation and transcription termination, leading to continuous transcription of the piRNA cluster transcript. Importantly, depletion of Piwi results in the loss of the Rhi–Del–Cuff complex at a subset of piRNA clusters. Although it seems counterintuitive, the competence of genomic regions to generate piRNAs appears to depend on the presence of a high level of H3K9me3 marks on the cluster. Association of the Rhi–Del–Cuff complex would allow piRNA clusters to be transcribed and would also protect the transcripts from transcription termination. Therefore, Piwi can specify piRNA clusters by guiding H3K9me3 marks to recruit the Rhi–Del–Cuff complex. Rhi also functions together with Cuff and UAP56 to suppress alternative splicing of piRNA clusters (96). The suppression of alternative splicing would distinguish piRNA clusters from mRNAs, although the underlying mechanism is not yet clear.

Single-stranded primary piRNA clusters, such as *flam*, seem to be independent of this transcription mechanism, because the loss of Rhi leads to loss of piRNAs only from dual-stranded piRNA clusters. Indeed, *Rhi* is not expressed within ovarian somatic cells, where only single-stranded piRNA clusters are active. The molecular mechanism underlying transcription of single-stranded piRNA clusters remains unknown. Additionally, how piRNA clusters in different species are transcribed and whether they also have specific factors for the identification of piRNA clusters are issues that have only just begun to be addressed (98).

RECOGNITION OF SELF AND NONSELF BY piRNAs

Acquisition of Novel piRNAs for Regulation of Transposons

Regulation of transposons by piRNAs can be described as a recognition system of "self" genes (coding and essential genes) and "nonself" sequences (transposons and repeat sequences). Because



transposons do not have structural or sequence characteristics in common, the PIWI–piRNA pathway must somehow recognize self and nonself sequences to selectively silence the latter. Transposons fall into several types, such as DNA transposons, LTR transposons, and non-LTR transposons, and they require different strategies to spread their copies around the genome (99). Even within the same types, they can be subdivided into many different families/subfamilies. To discriminate among these sequences, the *Drosophila* PIWI–piRNA pathway makes use of an essential characteristic of transposons: the mobility of the elements. As described above, piRNAs arise from piRNA clusters encoding long piRNA precursors. Because transposons move into different regions in the genome, they will eventually jump into a piRNA cluster, which serves as a transposon trap (8, 100). By falling into a piRNA cluster region, novel piRNAs targeting original copies of transposons will be produced and possibly amplified by the ping-pong pathway (77, 101). In this way, piRISCs can selectively and effectively target harmful transposons with higher mobility (**Figure 6*c***). Numerous transposons are found within piRNA cluster regions in nested form (42), indicating that piRNA clusters have successfully trapped a number of mobile elements at different time points. A DNA structural characteristic or epigenetic status of piRNA cluster regions may be necessary to effectively attract transposons (98), but this remains to be determined. The fact that mammalian species also have piRNA clusters with large numbers of embedded transposons leads us to speculate that this system is conserved in a wide range of species. In fact, a recent finding has suggested that it may also be conserved in adult primates, which express mainly pachytene-piRNA-like clusters (93).

Hybrid Dysgenesis

Hybrid dysgenesis is a phenomenon within correlated genetic traits that is spontaneously induced in hybrids between certain mutually interacting strains (102). It includes the sterility syndrome observed between two fly strains of the same species, where a particular transposon is expressed within one but not the other. In this case, silencing of the transposon depends on the direction in which the two fly strains are crossed (**Figure 7**). The transposon is activated if it was inherited from the father, leading to a sterile phenotype (dysgenic cross). Conversely, transposons inherited from the mother are silenced, leading to a fertile phenotype (reciprocal cross), even though the genotypes are common between dysgenic cross and reciprocal cross progeny. This phenomenon was first observed in the early 1970s, but it has taken a while to determine that the activation of transposons in a dysgenic cross correlates with the absence of piRNAs targeting those elements.

Figure 6

Transposons in *Drosophila* piRNA clusters and generation of novel piRNAs. (*a*) The structure of the *flam* piRNA cluster (single-stranded piRNA cluster) and *42AB* piRNA cluster (dual-stranded piRNA cluster) is illustrated with embedded transposons. In the case of *flam*, the transposons are frequently inserted in the antisense direction, resulting in production of piRNAs complementary to the original transposon. In contrast, no significant bias could be observed for *42AB*. Transposons longer than 2 kb are illustrated with approximate length and genomic position. The information on transposons was obtained from the UCSC Genome Browser (**http://genome.ucsc.edu/**). (*b*) Noncanonical transcription of dual-stranded piRNA cluster transcripts. The dual-stranded piRNA cluster locus is H3K9me3 marked by Piwi, and Rhi is recruited together with Del and Cuff to the region. Cuff then binds to the newly formed 5′ end of a nascent piRNA cluster transcript, preventing polyadenylation and termination of Pol II by competing with the CBC. Cuff also inhibits splicing together with UAP56, leading to export and processing of piRNA cluster transcripts. (*c*) Model showing integration of transposons into piRNA clusters. Transposon integration results in acquisition of novel piRNA sequences, which can regulate the original transposon. Abbreviations: CBC, cap-binding complex; Cuff, Cutoff; Del, Deadlock; piRNA, PIWI-interacting RNA; Pol II, RNA polymerase II; Rhi, Rhino; TE, transposable element.

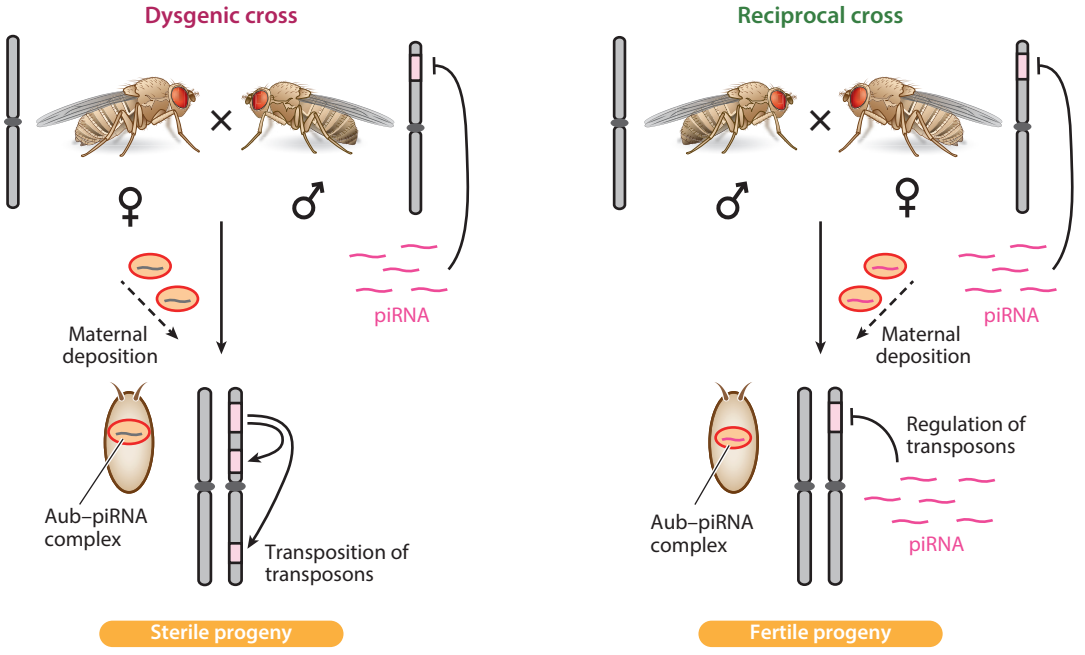


Figure 7

Hybrid dysgenesis. Sterility and abnormality occur from crosses between different strains of the same *Drosophila* species. When female flies lacking a transposon and male flies carrying a transposon are crossed, the progeny becomes sterile owing to activation of the transposon (dysgenic cross). Conversely, crosses between male flies lacking the transposon and female flies carrying the transposon result in fertile progeny (reciprocal cross) because of the maternal transmission of piRNAs, which can target the transposon. The PIWI protein Aub is localized to the posterior pole of the oocyte and embryo, indicating that the Aub–piRNA complex mediates maternal inheritance. Abbreviations: Aub, Aubergine; piRNA, PIWI-interacting RNA.

In a dysgenic cross or horizontal transmission, transposons cannot be regulated by the PIWI–piRNA pathway, resulting in a sterile phenotype (77). Surprisingly, the PIWI–piRNA pathway can establish silencing of newly invading transposons and restore fertility in a single generation (101). This has been made feasible by using piRNA clusters, which can trap active transposons. As described above, newly invading transposons jump into piRNA clusters as they increase their copy numbers in the genome, triggering production of novel piRNAs (**Figure 6*c***). This heritable change in the genome structure results in silencing of transposons over generations. Similarly, maternal inheritance of lacZ piRNAs could convert the lacZ transgene into a piRNA-generating locus, which can transmit the acquired silencing capacity through generations (103). Additionally, inherited piRNAs provide a trigger for piRNA production by enhancing production of homologous transcripts by the ping-pong cycle in the cytoplasm and by inducing installment of H3K9me3 marks on piRNA cluster sequences so that Rhi can bind to those genomic regions in the nucleus (104).

In *Drosophila*, germ-cell fate determinants are inherited in pole cells from the pole plasm at the posterior of the oocyte, and the primordial germ cells of the offspring are produced from these pole cells (105). Aub and Piwi, *Drosophila* PIWI proteins, are detected at the posterior pole of the oocyte and embryo, suggesting that Aub–piRNA and Piwi–piRNA complexes are inherited from the mother (21, 85, 106). Indeed, Aub-bound piRNAs produced in the ovary of the mother are also deposited into offspring embryos (77). Thus, piRNAs are inherited together with transposons from the mother, resulting in inheritance of the silencing machinery for transposons and the fertile

phenotype. This indicates that piRNAs can serve as a molecular memory, which can be transmitted from one generation to the next. Because piRNAs are most likely inherited as a complex associated with Aub, it may be possible to amplify the silencing signal via the ping-pong pathway.

Use of Small RNAs for Genome-Wide Recognition of Self and Nonself

As discussed above, *Drosophila* and mammals use some piRNAs, if not all, to recognize transposons as nonself sequences. In contrast, ciliates and nematodes use piRNAs to recognize both nonself and self sequences in order to identify which sequences to silence. Unlike most other eukaryotes, ciliates have two distinct sorts of nuclei: the germline micronucleus for reproduction and a large somatic macronucleus for general cell regulation (107). These nuclei have discrete functions: Only the micronucleus DNA is passed on during conjugation (i.e., the sexual reproduction process), and only the macronucleus provides nuclear RNA for vegetative growth and determines the phenotypes of individuals. Extensive DNA rearrangement and an amplification process would be necessary to maintain this nuclear dimorphism. Formation of the macronucleus requires the elimination of much of the repetitive content and intergenic sequences found in the micronucleus, and the coding genes need to be rearranged for their expression to occur.

A PIWI ortholog in the ciliate *Tetrahymena* (Twi1p) is essential for proper genome rearrangement (108). Twi1p-bound small RNAs named scan RNAs (scnRNAs) are similar to piRNAs because they are associated with a PIWI-related protein. However, unlike piRNAs, they are produced via a Dicer-dependent mechanism from dual-stranded precursors encoded in the micronucleus genome. These scnRNAs are able to scan through developing macronucleus RNAs to mark homologous sequences for elimination. scnRNAs use genomic information obtained from the micronucleus to recognize unwanted repeated and intergenic sequences within the macronucleus. Interestingly, the silencing of marked genes is likely mediated by histone modifications, such as H3K9me and H3K27me, as in *Drosophila* piRNAs (109). These modifications are required for proper genome rearrangements and marking sequences for elimination in *Tetrahymena* (**Figure 8*a***).

DNA elimination in *Oxytricha trifallax*, another ciliate protozoan species that uses piRNAs for the elimination and reconstruction of DNA in the macronucleus, revealed the possibility of piRNA recognition (110). *Oxytricha* PIWI ortholog (Otiwi-1)-bound piRNAs are ~27 nt long and possess a 1U bias, as in the primary piRNAs in other species, but they lack 3′-terminal modifications. Like *Tetrahymena*, piRNAs are expressed during conjugation to play a role in the selection of self and nonself genes in the macronucleus. However, deep sequencing of Otiwi-1-bound piRNAs revealed a striking characteristic of these piRNA populations: They map to the macronucleus somatic genome, not to the micronucleus somatic genome (110). In other words, these piRNAs recognize self sequences to specify genome retention, but not elimination (**Figure 8*a***).

As in ciliates, *Caenorhabditis elegans* recognizes self and nonself sequences not only via piRNAs that can recognize nonself genes for elimination, but also through cooperation with small RNAs in other categories, which can protect and promote self gene expression (**Figure 8*b***). *C. elegans* expresses 21U-RNAs and 22G-RNAs. 21U-RNAs are so named because of their 21-nt length and their 1U bias. RNA polymerase II transcribes these RNAs from a piRNA cluster region as an ~26-nt transcript. These RNAs are then loaded onto the PIWI protein PRG-1 (111–114). The population of 21U-RNAs transcribed from a piRNA cluster region on chromosome IV contains the conserved sequence motif CTGTTTCA ~42 nt upstream of the coding region. Forkhead family transcription factors recognize this sequence motif. As a result, ~26-nt-long precursors are decapped, and 2 nt at the 5′ end are removed (111, 115). Afterwards, the sequences are loaded onto PRG-1, along with 3-nt trimming at the 3′ end, forming the PRG-1–21U-RNA complex. Recently, several factors, such as PRDE-1, PID-1, and TOFUs, were identified as regulators of

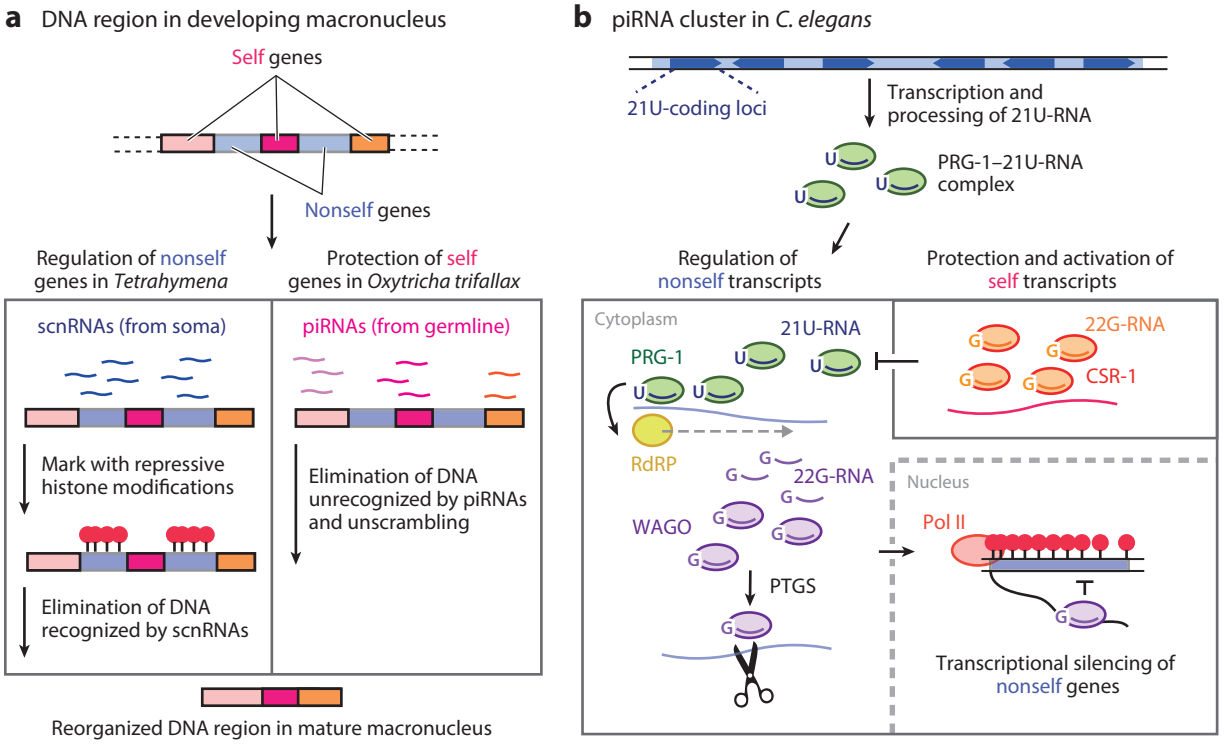


Figure 8

Recognition of self and nonself by piRNAs. Some organisms use small RNAs for recognition of both nonself and self sequences in the genome. (*a*) In the case of *Tetrahymena*, scnRNAs recognize nonself sequences in the macronucleus, which leads to epigenetic regulation of the targeted region. However, in the case of *Oxytricha*, piRNAs recognize the self gene coding sequence in the macronucleus genome. (*b*) *Caenorhabditis elegans* also recognize self and nonself sequences to effectively downregulate nonself genes. 21U-RNA is expressed from a piRNA cluster region where 21U-RNA is tandemly coded. The PRG-1–21U-RNA complex leads to transcription of 22G-RNAs, which bind to WAGO for posttranscriptional and transcriptional silencing of nonself genes. Conversely, 22G-RNA bound to another protein, CSR-1, recognizes self genes and acts to protect the recognized region from regulation. Abbreviations: piRNA, PIWI-interacting RNA; PTGS, posttranscriptional gene silencing; scnRNA, scan RNA.

these steps (116–118). Whether there is a direct target of PID-1-associated 21U-RNAs is still unknown. However, an important indirect silencing mechanism of 21U-RNAs has been revealed. PRG-1–21U-RNA complexes can scan through the genome, recognize foreign RNA sequences, and initiate production of RdRP-dependent siRNAs known as 22G-RNAs (113, 119, 120). 22G-RNAs are loaded onto worm-specific Argonaute members, the WAGO proteins, which mediate transcriptional silencing via histone modifications such as H3K9me3. In this way, PRG-1–21U-RNAs can silence their target sequences via WAGO–22G-RNA complex–mediated gene silencing. Moreover, 22G-RNAs bound to another protein, CSR-1, can recognize and protect mRNAs that should not be silenced. piRNAs sharing a common target with 22G-RNA bound to CSR-1 cannot efficiently downregulate their targets (120–124), perhaps because CSR-1–22G-RNA acts as a dominant negative form of WAGO–22G-RNA. Interestingly, the next generation inherits these phenomena (121–126). Therefore, in *C. elegans*, several types of small RNA–mediated gene regulatory systems have been combined to identify nonself as well as self sequences and to efficiently control the gene expression network over generations.

THE IMPACT OF piRNAs ON PROTEIN-CODING GENES

piRNA-Mediated Control of Nontransposon Genes

In retrospect, the first piRNAs found in *Drosophila* were those derived from the *Suppressor of Stellate* locus on the Y chromosome (127). These piRNAs were suggested to target *Stellate*, a repetitive but protein-coding gene on the X chromosome. Similarly, Aub-associated piRNAs from a repetitive region on the X chromosome target the essential maternal effector gene *Vasa* in *Drosophila* testis (128, 129). Additionally, some piRNAs produced from transposons, *roo* and *412*, induce deadenylation and degradation of maternally deposited mRNAs, such as *nanos*, in *Drosophila* embryos (130). Defects in piRNA-mediated *nanos* regulation result in head-development defects.

Genome-wide mapping of piRNAs has revealed that transposons as well as the 3′ UTRs of coding genes serve as sources of piRNAs, specifically termed genic piRNAs. For example, the *traffic jam* (*tj*) gene in *Drosophila* is one source of genic piRNAs (131, 132). A search for the sequence complements to *tj*-derived piRNAs identified *Fasciclin 3* (*Fas3*), which encodes an immunoglobulin-like cell adhesion molecule, as a potential target gene. Indeed, expression of *Fas3* is increased in *piwi* mutant gonads (132). In mammals, including primates, expression of piRNAs from a certain population of coding genes is also conserved, suggesting that genic piRNAs may have a conserved function, such as regulation of coding genes (93, 131). In piRNAs produced from protein-coding genes, the molecular mechanism underlying their recognition to initiate piRNA production remains to be revealed.

Pachytene piRNAs bound to MIWI are responsible for eliminating the expression of bulk mRNAs in spermatids (34). These piRNAs form a complex with CAF1, a catalytic subunit of the CCR4–CAF1–NOT deadenylase complex; select their target mRNA by partial sequence complementarity to the 3′ UTR; and promote deadenylation and decay of targets, similar to the workings of the miRNA machinery (133). Importantly, ∼5,000 protein-coding genes were significantly upregulated upon MIWI or CAF1 knockdown, indicating that this mechanism may play a critical role in the maintenance of reproductive tissue.

Involvement of the mouse PIWI–piRNA pathway in genomic imprinting has also been suggested (134). Genomic imprinting causes silencing of one of the two alleles by epigenetically marking the region through de novo methylation. The components of the piRNA pathway are required for de novo methylation of the differentially methylated region (DMR) located near the imprinted *Rasgrf1* gene locus in chromosome 9. piRNAs derived from transposons coded in chromosome 7 target noncoding RNA (piRNA-targeted noncoding RNA, also known as pit-RNA) derived from the DMR of *Rasgrf1*. The promoter of pit-RNA is a direct repeat in the DMR, which is required for the de novo methylation and imprinting of *Rasgrf1*, and the *Rasgrf1* gene fails to undergo de novo methylation when piRNAs target pit-RNA. Although the mechanism remains unknown, piRISCs may recruit DNA methyltransferase to the DMR of *Rasgrf1*.

Meanwhile, in *Drosophila*, H3K9me3 modifications to regulate transposons via the Piwi–piRNA pathway can spread around neighboring chromosome regions and epigenetically regulate coding genes in the region (58, 70). Whether regulation of transposon neighboring genes also possesses an important function is still unknown. However, the number of regulated genes can be large given that regulated transposons are spread around the genome. McClintock (135) observed the developmental consequences of transposon activity and referred to transposons as "controlling elements." Similarly, the cases described in this section suggest that transposons can regulate genes from a distance through the PIWI–piRNA pathway.

Sex Determination and Mating-Type Determination by piRNAs

As described thus far, piRNAs can regulate both transposons and protein-coding genes. However, in many cases, the biological significance of this regulation remains elusive. A recent study indicated that sex determination in the silkworm *Bombyx mori* is accomplished by piRNA-mediated protein-coding gene regulation (**Figure 9*a***) (137). *B. mori* makes use of a WZ sex determination mechanism: Males have two Z chromosomes, whereas females have both W and Z chromosomes.

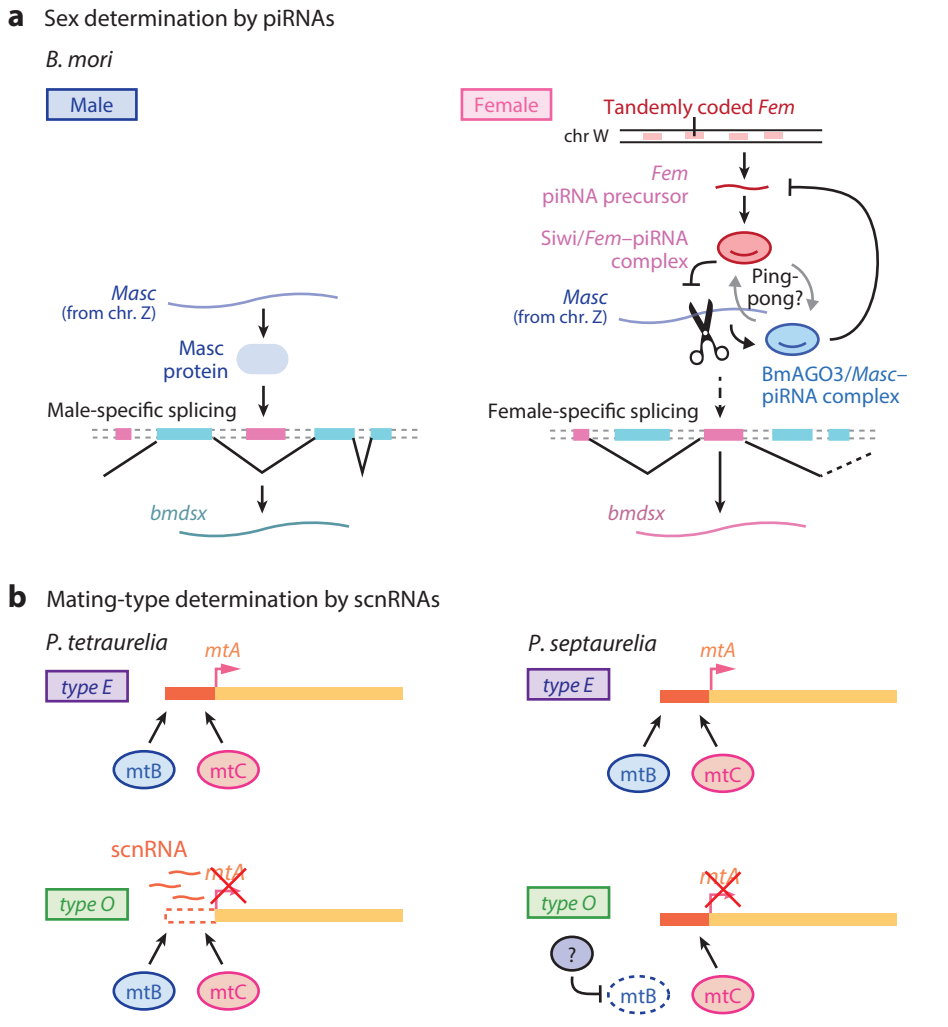


Figure 9

Sex determination and mating-type determination by piRNAs. (*a*) A piRNA-mediated sex determination model in *Bombyx mori*. In the case of *B. mori*, a piRNA transcribed from the *Fem* gene regulates a gene termed *Masc*. This gene is essential for regulation of male-specific splicing of the *Bmdsx* gene, which acts as a determinant gene of sex. (*b*) In the case of the ciliate *Paramecium tetraurelia*, the *mtA* gene serves as a determinant of mating type. This gene is regulated by scnRNAs of *P. tetraurelia* in the mating type O gene. In the case of another ciliate, *Paramecium septaurelia*, the *mtA* gene is regulated not by scnRNAs, but by expression of another coding gene called *mtB*. Abbreviations: *Bmdsx*, *doublesex*; Fem, Feminizer; Masc, Masculinizer; piRNA, PIWI-interacting RNA; scnRNA, scan RNA.

Therefore, W chromosomes are expected to play an important role in sex determination. Interestingly, the W chromosome is largely occupied by transposons, and protein-coding genes have not been identified in the chromosome. *Feminizer* (*Fem*), which is tandemly found on the W chromosome, encodes a noncoding RNA, which was identified as a piRNA precursor. After processing, piRNAs derived from *Fem* are loaded onto the *B. mori* PIWI protein Siwi. Analyses suggest that a target of *Fem*–piRNA is a newly identified gene on the Z chromosome termed *Masculinizer* (*Masc*), a factor necessary for the production of a male-specific splice variant of *B. mori doublesex* (*Bmdsx*). The specific splice variant of *Bmdsx* serves as a determinant of global gene expression in a sex-specific manner. In females, *Masc* is downregulated by *Fem*–piRNAs, resulting in a female-specific splicing pattern of *Bmdsx*. Interestingly, piRNAs are also produced from *Masc*, and *Masc*–piRNAs are loaded onto another PIWI protein, BmAGO3. *Masc*–piRNAs possess a ping-pong signature (10-nt overlap between sense and antisense piRNAs) with *Fem*–piRNAs, suggesting that they may be generated through ping-pong amplification. In this way, female-specific piRNAs can target the coding gene *Masc* and serve as a feminizing factor. This system is somewhat similar to the *Suppressor of Stellate* and *Stellate* system that *Drosophila* uses in testis (127); that is, piRNAs derived from a noncoding RNA–encoding gene on a male-specific or female-specific sex chromosome are loaded onto a particular PIWI protein, and the resultant piRISCs downregulate a homologous protein-coding gene on the other sex chromosome via piRISC-mediated cleavage of the target mRNA.

Another example of clear phenotypic regulation by piRNAs was reported from a study using ciliate *Paramecium tetraurelia*, which showed that piRNAs can serve as an essential factor for epigenetic mating-type inheritance via regulation of a coding gene (**Figure 9*b***). As described above, scnRNAs excise transposons from germline micronuclei to form new somatic macronuclei. During this process, the mating type (type E or type O in *Paramecium*) is determined by expression of the *mtA* gene (136). *mtA* is expressed in type E but repressed in type O. The repression of *mtA* in type O *P. tetraurelia* was due to scnRNAs, which can be maternally inherited by the next generation. Interestingly, in the case of a sibling species, *Paramecium septaurelia*, expression of *mtA* is regulated not by scnRNAs but by another gene, *mtB*. Despite *mtA* serving as a common factor determining E/O expression, the mechanisms used to produce O clones differ even among closely related species. This may indicate the frequent exaptation of the scnRNA pathway for gene silencing. The sequences excised from *mtA* genes are functional parts of coding genes and are not derived from the insertion of transposons, leaving a big mystery as to how this gene got "selected" as a piRNA target. As with *Masc* regulation by *B. mori* piRNAs, this is another good example showing the possible capacity of piRNAs to inactivate single-copy coding genes, leading to phenotypic variation.

Nongonadal Function of PIWI Proteins and piRNAs

Some studies have suggested that the PIWI–piRNA complex possesses regulatory functions in germ cells and somatic cells (9, 10). In *Aplysia*, memory storage in the brain requires PIWI–piRNA function (138). *Aplysia* piRNAs are expressed from piRNA clusters in the genome. These piRNAs are localized to the nucleus and can induce DNA methylation to regulate their target genes. The targets include CREB2, a transcriptional repressor of memory. Analyses suggest that the promoter region of *CREB2* is methylated by piRNAs, leading to its regulation. Interestingly, piRNAs were also observed in mouse hippocampus (139). These piRNAs associate with MIWI and may be required for the development of dendritic spines.

In ascidians, PIWI orthologs (Bl-Piwi and Piwi) are involved in whole-body regeneration, which is a regeneration phenomenon of functional adults from minute vasculature fragments. In

Botrylloides leachi, whole-body regeneration occurs in cells expressing Bl-Piwi, and knockdown of Bl-Piwi results in cell regeneration arrest (140). In another ascidian, *Botryllus schlosseri*, Piwi depletion causes the gradual loss of cycling stem cells, resulting in regeneration arrest (141). Thus, Piwi plays an essential role in maintaining the functionality of stem cells during whole-body regeneration of ascidians. It still remains unclear whether piRNAs are involved in this process.

PIWI function may also be involved in human cancer development. In *Drosophila*, a loss-of-function screening for the factors responsible for malignant brain tumors has demonstrated that Piwi and Aub contribute to tumor growth (142). Human PIWI proteins are expressed in a variety of human cancer cells. The first example of this was the finding of overexpression of HIWI in seminomas, a male germ-cell cancer (143). Since then, ectopic expression of PIWI proteins has been observed within cell lines and tissue samples of a variety of cancer cells, including those associated with breast, cervical, gastric, and liver cancers, among others (reviewed in Reference 144). Additionally, HILI overexpression in NIH3T3 cells, a mouse fibroblast cell line, results in activation of the transcription activator STAT3 and the antiapoptotic factor BCLX, suggesting that HILI may be involved in cell growth, adhesion, and apoptosis (145). However, piRNA expression and piRISC formation, for example, have not been convincingly demonstrated in cancer cells. Therefore, further investigations are awaited to conclude whether the expression of PIWI proteins in cancer cells is biologically significant.

CONCLUSION

We now know that a large number of factors cooperate to generate piRNAs and assist them in selectively regulating nonself genes. Nevertheless, their functional roles are largely unknown, especially with respect to the epigenetic modifications induced by piRNAs within the nucleus. Moreover, high-throughput screening has identified numerous factors that may be involved in piRNA production and/or transposon silencing, and their precise contribution within the piRNA biogenesis pathway remains to be revealed. Fundamentally, we still do not know how regulation of transposons by piRNAs is linked to fertility. It may be necessary to consider the regulation of not only transposons but also coding genes by the PIWI–piRNA pathway to elucidate how defects in piRNA pathways lead to germline development.

A variety of genes are involved in the PIWI–piRNA pathway, and examples show that inheritable PIWI–piRNAs have functions inside and outside the germline. Thus, cross talk may occur between PIWI–piRNA pathways and other biological pathways, a suggestion that awaits further investigation. Exploring novel model organisms may be a useful approach, as in the case of the somatic genome rearrangement discovered by analyses of *Tetrahymena* piRNAs. For example, a naked mole rat can live for more than 30 years without any sign of cancer, and its genome possesses mostly old, inactivated transposons, although it encodes PIWI proteins (146, 147). Therefore, analyzing this species may reveal a novel role other than regulation of transposons for mammalian PIWI proteins and piRNAs.

Additionally, because piRNAs are involved in epigenetic modifications of gene expression, PIWI proteins and piRNAs may have further roles in maintaining genomic structure. It will be interesting to analyze the effect of PIWI protein expression on genomic structure by approaches such as 3C (chromosome conformation capture)-based technologies, which enable us to determine the three-dimensional architecture of a genome (148, 149). Because piRNAs also have a flexible mechanism for identifying self and nonself genes that are transgenerationally inherited, molecular signals that define the epigenetic status of cells can achieve long-lasting effects for transgenerational inheritance by getting integrated into the piRNA pathway. Therefore, we may be able to develop an artificial piRNA-producing system to transgenerationally regulate certain genes or groups of genes.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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