

Uniting Germline and Stem Cells: The Function of Piwi Proteins and the piRNA Pathway in Diverse Organisms

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Abstract

The topipotency of the germline is the full manifestation of the pluripotency and multipotency of embryonic and adult stem cells, thus the germline and stem cells must share common mechanisms that guarantee their multipotentials in development. One of the few such known shared mechanisms is represented by Piwi proteins, which constitute one of the two subfamilies of the Argonaute protein family. Piwi proteins bind to Piwi-interacting RNAs (piRNAs) that are generally 26 to 31 nucleotides in length. Both Piwi proteins and piRNAs are most abundantly expressed in the germline. Moreover, Piwi proteins are expressed broadly in certain types of somatic stem/progenitor cells and other somatic cells across animal phylogeny. Recent studies indicate that the Piwi-piRNA pathway mediates epigenetic programming and posttranscriptional regulation, which may be responsible for its function in germline specification, gametogenesis, stem cell maintenance, transposon silencing, and genome integrity in diverse organisms.

GSC: germline stem cell

Piwi: P-element induced wimpy testis

miRNAs: microRNAs

siRNAs: small-interfering RNAs

piRNAs: Piwi-interacting RNAs

INTRODUCTION

Stem cells possess the unique ability to undergo self-renewing divisions to self-maintain and to provide a continual supply of differentiated cells for their resident tissues (67). Depending on how many types of differentiated daughter cells a specific type of stem cell can produce, stem cells are classified as pluripotent, multipotent, or unipotent. Pluripotent stem cells, known to exist as mammalian embryonic stem cells, can give rise to all types of tissues in an individual but cannot give rise to extraembryonic tissues. Multipotent stem cells, such as hematopoietic stem cells, are able to give rise to many differentiated cell types. Germline stem cells (GSCs), which give rise to a constant supply of gametes, are unipotent. However, germ cells have the intrinsic capability of producing a totipotent zygote that ultimately gives rise to all cell types. Therefore, although highly specialized, germ cells retain totipotency and likely use the same or similar mechanisms that regulate stem cells. Stem cells possess great therapeutic potentials in regenerative medicine. In order to effectively harness this power, extensive efforts have been invested in exploring regulatory mechanisms that underlie their maintenance and differentiation. These research efforts have revealed small RNA pathways as key regulators of diverse types of stem cells, which are best known to repress the translation of specific mRNAs in stem cells and differentiated daughter cells (35).

Small RNAs often regulate gene expression by guiding Argonaute (Ago) protein-containing complexes in a nucleotide sequence-specific manner to different sites for molecular actions (31). Ago proteins have a variable N-terminal domain followed by a highly conserved PAZ domain (PAZ is the acronym for Piwi and its homologs in *Arabidopsis*, Argonaute, and Zwillie), which together with the Mid (middle) domain binds a small RNA (111). The C-terminal domain, called the Piwi domain, resembles RNase H and is often capable of cleaving target RNAs (41, 83). The Ago protein family is divided into the Ago and Piwi subfamilies based on phylogenetic analysis. Ago subfamily proteins bind

microRNAs (miRNA) and small-interfering RNAs (siRNA), which are both made from double-stranded precursors. In animal cells, precursors are provided either endogenously (miRNAs and endo-siRNAs) or exogenously (siRNAs), and both are ultimately processed by Dicer into 20–22-nucleotide small RNAs (49). Both miRNAs and siRNAs bind to Ago subfamily proteins, which are ubiquitously expressed in animal tissues to repress gene expression by promoting heterochromatin formation, mRNA turnover, and/or translational repression (37, 128). In contrast, Piwi protein expression is mostly restricted to germ cells and stem cells (116). Piwi proteins bind to Piwi-interacting RNAs (piRNAs, generally 26–31 nucleotides in length), which are processed in a Dicer-independent manner from long single-stranded precursors (3, 39, 44, 105). In this review, we discuss the biological functions of Piwi proteins in animal germ cells and stem cells. Piwi proteins are required to maintain fertility and repress transposons in the germline and regulate gene expression at the epigenetic, posttranscriptional, and translational levels. Studies in animals from diverse taxa also demonstrate that Piwi proteins have a conserved stem cell function. Therefore, understanding how Piwi proteins regulate gene expression is key to understanding the regulation of both germline and stem cell identity, maintenance, and differentiation.

THE EXPRESSION AND FUNCTION OF PIWI PROTEINS IN THE GERMLINE

Piwi Proteins Are Required for Fertility

The expression of Piwi proteins is enriched in the germline of many animals. In *Drosophila*, there are three Piwi proteins: Piwi, Aubergine (Aub), and Ago3, all of which are expressed in the male and female germline (9, 22, 42, 65) (Table 1, Figure 3, below).

In the *Drosophila* embryo Piwi and Aub proteins are enriched in the pole cells, which give

Table 1 Expression and function of Piwi proteins in animals

| Expression* | Species | Name | Mutant phenotype | References |
|--------------------------------------|--|---|----------------------------|----------------|
| Germline | <i>Drosophila melanogaster</i> | P-element induced wimpy testes (Piwi) | ♂♀ sterile | (22, 23) |
| | | Aubergine (Aub) | ♂♀ sterile | (42, 107, 126) |
| | | Argonaute3 (Ago3) | ♀ sterile ♂ semifertile | (65) |
| | <i>Mus musculus</i> | Mouse Piwi (Miwi, PiwiL1) | ♂ sterile | (27) |
| | | Miwi-like (Mili, PiwiL2) | ♂ sterile | (57) |
| | | Mouse Piwi 2 (Miwi2; PiwiL4) | ♂ sterile | (13) |
| | <i>Danio rerio</i> | Zebrafish Piwi (Ziwi) | ♂♀ Sterile | (44) |
| | | Ziwi-like (Zili) | ♂♀ sterile | (43) |
| | <i>Caenorhabditis elegans</i> | Piwi related gene 1 (Prg-1) | Sterile at 25°C | (123) |
| Piwi related gene (Prg-2) | | No obvious defect | (123) | |
| Totipotent stem cells | <i>Ephydatia fluviatilis</i> (sponge) | <i>Ephydatia fluviatilis</i> Piwi A (EfPiwiA) | N/A | (34) |
| | | <i>Ephydatia fluviatilis</i> Piwi B (EfPiwiB) | N/A | |
| Tentacle stem cells | <i>Clytia hemisphaerica</i> (Hydrozoan) | <i>Clytia hemisphaerica</i> Piwi (ChePiwi) | N/A | (28) |
| Somatic stem cells, germ cells | <i>Pleurobrachia pileus</i> (Ctenophore) | <i>Pleurobrachia pileus</i> Piwi 1 (PpiPiwi1) | N/A | (2) |
| | | <i>Pleurobrachia pileus</i> (PpiPiwi2) | | |
| Totipotent stem cells | <i>Botrylloides leachi</i> (colonial Ascidian) | <i>Botrylloides leachi</i> Piwi (Bl-Piwi) | Unable to regenerate | (99) |
| Totipotent stem cells | <i>Schmidtea mediterranea</i> (Planaria) | <i>Schmidtea mediterranea</i> Piwi (Smedwi-1) | No phenotype | (85, 95) |
| | | <i>Schmidtea mediterranea</i> Piwi (Smedwi-2) | Unable to regenerate | |
| | | <i>Schmidtea mediterranea</i> Piwi (Smedwi-3) | | |
| Totipotent stem cells, germ cells | <i>Macrostomum lignano</i> (marine flat worm) | <i>Macrostomum</i> Piwi (MacPiwi) | Loss of stem cells | (25) |
| Hematopoietic stem cells, germ cells | <i>Homo sapiens</i> | Human Piwi (Hiwi) | N/A | (94, 109) |

*If germline expression is not listed this does not necessarily mean Piwi expression is absent from the germline, but rather that it was not experimentally tested.

rise to the germ cells (42, 75). In the adult ovary and testis, Piwi is enriched in the nucleus of both the germ and somatic cells of the gonads (23). Aub and Ago3, however, are selectively expressed in the germ cells of adult ovaries and are enriched in the cytoplasmic polar granules or germlasm, which is presumably the precursor of the nuage, a structure common to all animal germ cells (9). In mouse, there are also three Piwi homologs, Miwi (murine Piwi,

Piwi-like protein 1), Mili (Piwi-like protein 2), and Miwi2 (Piwi-like protein 4) (**Table 1, Figure 3**, below), which display distinct expression patterns and functions during male germ-cell specification and differentiation (13, 27, 57). Mili and Miwi proteins are mostly, if not exclusively, localized to the cytoplasm. Miwi expression is found in spermatocytes to elongating spermatids and Mili is expressed in spermatogonia to round spermatids (27, 117).

TE: transposable element

Both proteins are enriched in nuage and its derivative, the chromatoid body in spermatids (57, 117, 124). Miwi2, which is both nuclear and localized to the perinuclear nuage, has a relatively narrow expression window during spermatogenesis, which is detected from 18 days postcoitum (dpc) in the gonocytes to three days postpartum (dpp) in spermatogonia stem cells (13). Two homologs of Piwi were identified in zebrafish: Ziwi (zebrafish Piwi), and Zili (zebrafish Piwi-like) (43, 44) (Table 1, Figure 3, below). Ziwi is expressed in the nuage of early embryos and in both female and male germ cells of the adult fish, with the highest expression seen during the mitotic and early meiotic stages of germ-cell differentiation (44). Zili expression is first detected in primordial germ cells from three days postfertilization (dpf), and after 21 dpf, Zili is found in cytoplasmic granules. In the adult, Zili is present throughout oogenesis and in both mitotic and meiotic male germ cells, but expression is not detected in mature sperm (43). The *Caenorhabditis elegans* genome encodes two Piwi orthologs: *prg-1* and *prg-2* (*prg* stands for *piwi*-related gene) (Table 1, Figure 3, below). *Prg-1* is expressed in perinuclear P-granules of the male germ cells (123). Therefore, the enrichment of Piwi protein in the nuage of germ cells is evolutionarily conserved.

Drosophila Piwi is required for the self-renewal of GSCs. In *piwi* mutant flies, GSCs are depleted and the gonads contain only a few egg chambers or sperm bundles, which causes infertility in females and males, respectively (22, 68). In *ago3* mutant male flies, GSCs are not properly maintained either, causing reduced fertility (65). Miwi2 and Mili mutant mice show arrest of spermatogonia and the complete loss of male germ cells at three and six months, respectively, strongly suggesting a role for these proteins in GSC maintenance (13, 117). Finally, RNAi knockdown of both *prg-1* and *prg-2* in *C. elegans* leads to decreased germ-cell proliferation (22). Therefore, Piwi proteins have a conserved role in maintaining GSCs during gametogenesis in animals.

In addition to maintenance of the GSCs, Piwi proteins are required for other aspects of gametogenesis. In *mili* mutants, spermatogenesis is blocked at the midpachytene stage; whereas in *miwi* mutant males, spermatogenesis is arrested at the early stages of spermiogenesis—a cellular morphogenic process through which round spermatids become mature sperm (27, 57). Similarly, in *C. elegans*, *prg-1* mutant adults are sterile at 25°C because of defective spermatogenesis (123). In zebrafish *ziwi* and *zili* mutants, germ cells are lost in both males and females because of elevated levels of apoptosis at the various stages of germ-cell development (43, 44), which might be an indirect consequence of arrested meiosis and failure in transposon silencing.

Piwi proteins are also involved in embryogenesis and germline specification. In *Drosophila*, Piwi proteins are required early in embryogenesis for patterning and pole cell formation. Both Piwi and Aub are essential for the formation of pole cells in *Drosophila* (42, 75). In addition, all three *Drosophila* Piwi proteins are required for proper body-axis patterning during early embryogenesis (22, 65, 107). Because Piwi proteins are required for formation of the germline and/or patterning of the embryo, techniques such as clonal analysis are required to further interrogate the functions of these proteins in the adult germline. From the above review, it is clear that Piwi proteins have diverse functions in gametogenesis, many of which are conserved in animals.

Piwi Proteins Repress Transposon Activation in the Germline

Transposable elements (TE) are sequences of DNA first discovered in maize by Barbara McClintock in the 1940s that can transpose themselves to new positions in the genome. Transposons can be assigned to Class I and Class II based on the mechanism of transposition. Class I (copy and paste) transposons, also called retrotransposons, copy themselves through transcription, then reverse transcribe RNA into DNA, and integrate the DNA into

the genome. There are two types of retrotransposons: the long terminal repeats (LTR) and the non-LTR retrotransposons. Non-LTR retrotransposons consist of long interspersed nuclear elements, which encode reverse transcriptase by RNA polymerase II, and short interspersed nuclear elements encoding reverse transcriptase by RNA polymerase III. In contrast, Class 2 TEs consist of DNA fragments only and integrate into the genome through a cut and paste mechanism in which no mRNA intermediates are involved. This transposition is catalyzed by various transposases.

Significant percentages of piRNAs in *Drosophila*, *C. elegans*, mice, and zebrafish are derived from intergenic regions containing TEs and repetitive sequences. Expectedly, Piwi proteins are required to repress transposons in the germline (9, 44, 123). In *Drosophila*, approximately 75% of piRNAs are derived from repetitive sequences; mutations in Piwi, Aub, and Ago3 result in transposon derepression in the germline (54, 65, 118). In the mouse, loss of Mili, Miwi, and Miwi2 also leads to transposon desilencing in the male germline (5, 13, 39, 125). In prenatal testis, Mili and Miwi2 associate with piRNAs enriched in transposon sequences (~47%) (4, 58). After birth, 35% of Mili-bound prepachytene piRNAs are derived from transposons (5). However, Miwi-bound adult pachytene piRNAs are not significantly enriched in transposon sequences (~15%), suggesting an additional function for Miwi in the mouse male germline (39, 125). In zebrafish and *Xenopus*, piRNAs are significantly enriched in transposon sequences, suggesting a conserved function in transposon repression for Piwi proteins in vertebrates (43, 60).

The Piwi-piRNA Complex Is Required for Genome Integrity in the Germline

Loss of Piwi proteins leads to germline-specific apoptosis, which may be triggered by DNA damage, including, but not limited to, elevated transposition (13, 44, 52, 57). In the *miwi2* mutant mouse, the level of phosphorylated

histone H2AX (γ -H2AX), which marks the sites of DNA double-stranded breaks that naturally occur during the leptotene stage of meiosis, is increased in zygotene-stage spermatocytes as compared to the wild-type cells (13). The failure to repair the double-stranded breaks and/or defective synapsis prevents germ cells from entering the pachytene stage of spermatogenesis and leads to apoptosis (13). However, despite increased levels of apoptosis, there is no significant difference in γ -H2AX staining between *mili* mutant and wild-type animals, suggesting an alternate pathway leading to apoptosis (57). In *zirvi* mutant zebrafish, increased levels of apoptosis are observed in the testes, but the prevalence of DNA double-stranded breaks has not been investigated (44). RIWI (rat Piwi) comigrates with rRecQ1, an enzyme regulating DNA homologous recombination, which provides additional evidence that the Piwi-piRNA complex is involved in regulating genome integrity (45, 62).

In *Drosophila*, *Aub* and other piRNA pathway mutants lead to embryonic patterning defects and accumulation of γ -H2Av (equivalent to γ -H2Ax) foci in the germline (52). The embryonic patterning defects of piRNA pathway mutants, but not transposon derepression, can be rescued by mutations in the Chk2 and ATR [ATM (Ataxia Telangiectasia Mutated) and Rad3-related] kinase components of the DNA damage-signaling pathway (52). The nature of the relationship between DNA damage and transposon mobility is currently unclear. One possibility is that transposon mobilization directly leads to DNA double-stranded breaks. However, it is also possible that double-stranded breaks induce transposon mobilization. In support of this, DNA damage can lead to transposon desilencing in cell culture, and transposon mobilization can lead to double-stranded break repair (32, 78, 102). In addition, *Aub* is required for the production of telomere-specific piRNAs that may guide the telomere protection complex, which prevents chromosome fusion during mitosis and meiosis (48). Therefore, the piRNA pathway has functions in maintaining genome integrity in the

P-body: processing body

germline that are separable from transposon repression.

Piwi Proteins Interact with Tudor Domain-Containing Proteins in the Germline

To understand the molecular mechanisms of Piwi protein functions, many recent studies have focused on identifying and characterizing Piwi-interacting proteins. The N termini of Piwi proteins contain conserved RG/RA (arginine-glycine/arginine-alanine) motifs with dimethylated arginines that are required for Piwi binding to Tudor domains (119). Structural analysis of Tudor domain-containing protein 2 (Tdrd2; a.k.a. Tdrkh) and the ancestral staphylococcal nuclease domain-containing 1 (SND1) protein provides a binding mechanism of the Tudor domain to Piwi proteins (17, 69, 71). Piwi proteins contain multiple RG/RA repeats, which are potential methylation sites and may recruit different Tudor proteins. Proteins containing multiple Tudor domains can potentially bind to different Piwi proteins simultaneously to serve different molecular functions.

In the mouse, there are 28 Tudor family proteins (based on a search result from <http://pfam.sanger.ac.uk>). Several labs have demonstrated that different mouse Tudor proteins interact with Piwi proteins in various but specific combinations (17, 55, 97, 110, 119, 124) (Table 2). For example, Tdrd1 interacts with Mili, Miwi, and Miwi2, but the Mili/Tdrd1 interaction is the strongest (17, 119, 124). Tdrd1, which contains four Tudor domains and one MYND domain, is essential for spermatogenesis (86). Similar to mouse Piwi proteins, Tdrd1 is predominantly localized to the nuage in spermatogonia and spermatocytes, as well as to a nuage-derived structure, the chromatoid body, in late spermatocytes and round spermatids (20). In the *tdrd1* mutant, expression and localization of Mili are not affected; however, Miwi2 localization is disrupted with no change in expression levels (97, 110, 119), indicating that Tdrd1 is specifically required for the

proper localization of Miwi2. In the *tdrd1* mutant mouse, piRNAs from exonic reads are overrepresented (48%) compared to wild type (16%) (97). This suggests that Tdrd1 may perform quality control in the selection of certain transcripts to undergo piRNA biogenesis.

Tdrd9, which associates with Miw2, is required for meiosis during spermatogenesis. The loss-of-function mutation of *tdrd9* results in spermatogenic arrest due to chromosome synapses failure (110). In fetal spermatogonia, Tdrd9 and Miwi2 colocalize in granules with the processing body (P-body) markers DDX6 and GWB (6). Miwi2/Tdrd9 granules are distinct from Mili/Tdrd1 granules, which are finer and more numerous than Miwi2/Tdrd9 granules (6, 110). Tdrd9 is not required for the localization of Mili, Miwi, and Miwi2, and Miwi2 is not required for the localization of Tdrd9 and Tdrd1 (119). In *mili* mutants, Tdrd9 nuage localization is lost, and in *tdrd1* mutants both Tdrd9 and Miwi2 are dispersed in the cytoplasm, whereas Mili is not affected (110). Taken together, these data demonstrate the interdependence of Tudor family and Piwi family proteins and suggests that the Miwi2/Tdrd9 complex is downstream of the Mili/Tdrd1 complex.

The interaction between Piwi and Tudor proteins also exists in *Drosophila*. The germline gene *Tudor* contains 11 Tudor domain repeats and is required for polar granule formation (8, 91, 115). Aub and Ago3 bind to Tudor in an sDMA (symetrically dimethylated arginine)-dependent manner, and this binding is required for germline assembly and primordial germ cell specification (24, 50, 51, 82) (Table 2). In *Drosophila*, PRMT5 is required for arginine methylation of Piwi proteins (50, 72). Tudor appears to form various heteromeric complexes with Aub and Ago3, which are lost in *prmt5* mutants (82). In the fly *tudor* mutant, the expression levels of Aub and Ago3 are not affected, but similar to mice, the identity of bound piRNAs is significantly changed (82). A novel Tudor-containing protein in *Drosophila*, Papi (partner of Piwis), binds to the N terminus of Ago3 in the nuage. Ago3 is mislocalized from nuage in *papi*

Table 2 Interactions between Piwi protein and Tudor domain-containing protein in *Drosophila* and the mouse

| species | Piwi protein | Tudor protein | References |
|--------------------------------|--------------|---|-----------------------------|
| <i>Mus musculus</i> | Miwi | Tdrd1/MTR1; Tdrd2/Tdrkh; Tdrd4/RNF17; Tdrd6; Tdrd7; Tdrd8; Tdrd9 | (17, 51, 119, 120) |
| | Mili | Tdrd1/MTR1; Tdrd2/Tdrkh; Tdrd6 | (17, 55, 97, 110, 119, 124) |
| | Miwi2 | Tdrd1/MTR1; Tdrd2/Tdrkh; Tdrd9 | (55, 110, 119, 124) |
| <i>Drosophila melanogaster</i> | Piwi | Papi; Yb | (72, 93, 104) |
| | Aubergine | Tudor; Papi; Papi | (24, 50, 51, 72, 82) |
| | Argonaute 3 | Tudor; Papi | (72, 82) |

or *prmt5* mutants, whereas Papi localization to nuage is not affected in *ago3* or *prmt5* mutant flies. This suggests that Papi lies upstream of a pathway regulating Ago3 function (72).

Similar to the Piwi family, loss of Tudor proteins in *Drosophila* and mouse leads to transposon desilencing. In mouse, loss of Tdrd1 and Tdrd9 leads to transposon overexpression and altered piRNA profiles (97, 110). However, in *tldr6* mutant mice, upregulation of transposon transposition activities is not observed (120). In *Drosophila*, Tudor domain-containing proteins, such as Spindle E, Krimper, Tajas, PAPI, and Yb, function in silencing transposons (66, 88, 93, 104, 118). Therefore, Tudor proteins have a conserved role in the Piwi-piRNA pathway and silencing transposons in the germline.

THE PIWI-piRNA COMPLEX MEDIATES EPIGENETIC FUNCTION

Epigenetic mechanisms ensure heritable patterns of gene expression by regulating chromatin structure through histone modification and/or DNA methylation (113). Small RNAs are required for epigenetic processes, such as heterochromatin formation and maintenance by the RNA interference (RNAi) pathway. For example, in *Schizosaccharomyces pombe*, the RNAi machinery produces siRNAs from transcripts arising from centromeric repeat regions.

Subsequently, the RNA induced transcriptional silencing complex, which contains Ago1 bound to these siRNAs, is targeted to nascent transcripts at the centromeres, leading to the formation and maintenance of heterochromatin via the recruitment of chromatin-modifying and-binding proteins. These proteins include the histone H3 lysine 9 (H3K9) methyltransferase, Clr4, and heterochromatin protein 1 (HP1, also called SWI6) (12, 121, 122).

Recent evidence suggests that the Piwi-piRNA complex may regulate epigenetic states via a similar mechanism in multicellular organisms. *Drosophila* Piwi protein is predominately located in the nucleus of both germline and somatic cells. Piwi proteins bind to chromatin, and its mutation suppresses position effect variegation (PEV) in somatic cells (10, 84, 129). In somatic cells, Piwi binds to polytene chromosomes in an RNA-dependent manner as well as directly binds to HP1a, a central player in heterochromatic gene silencing (10). In Piwi and Aub mutants, HP1 and HP2 are delocalized from polytene chromosomes, and the levels of H3K9 methylation are reduced (84). Taken together, these studies suggest that Piwi acts upstream of HP1 and H3K9 methylation (Figure 1a) (10, 84). The HP1 homolog Rhino in *Drosophila* is required to silence transposons through a distinct unknown pathway in which Rhino functions upstream of Ago3 and Aub, but may have a less critical role in Piwi-dependent heterochromatin formation (53). In contrast to

PEV: position effect variegation

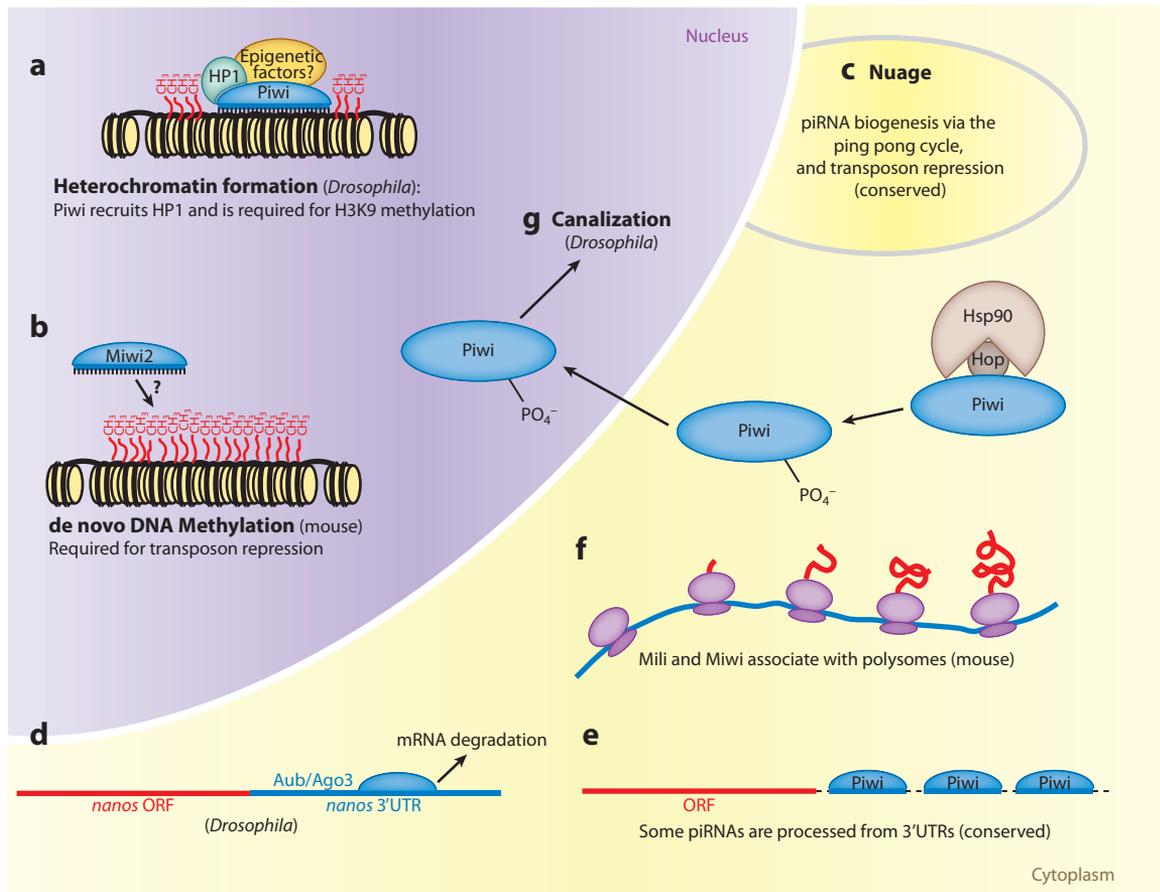


Figure 1

Molecular functions of Piwi proteins. (a) In *Drosophila*, Piwi is a nuclear protein that binds heterochromatin protein 1a (HP1a) and is required for H3K9 (Lysine 9 of Histone 3) methylation and heterochromatin formation (10, 84). (b) In the mouse, Miwi2 protein is enriched in the nucleus and is required for de novo DNA methylation in male germ cells (4). (c) Piwi proteins in the nuage participate in a ping-pong mechanism that simultaneously produces new piRNAs and degrades active transposon mRNAs; see **Figure 2** for details (9, 41). (d) The *Drosophila* Piwi proteins Aub and Ago3 are required for the deadenylation and degradation of maternal mRNAs during the maternal-to-zygotic transition (101). (e) In *Drosophila*, mouse, and *Xenopus*, 10% to 35% of piRNAs are processed from the 3' UTR of select mRNAs. This may represent a mechanism for regulating these mRNAs in *cis*. In addition, the resulting piRNAs may regulate gene expression in *trans* (100). (f) Mili and Miwi associate with polysomes in the mouse male germline to positively regulate gene expression (40, 117). (g) In *Drosophila*, Hsp90 and Hop form a complex with Piwi, which leads to Piwi phosphorylation and is required for Piwi-dependent canalization in the nucleus (36).

the silencing role of Piwi in certain regions of the genome, Piwi is required for transcriptional activation of the telomere-associated sequence of the right arm of chromosome 3 (3R-TAS) (129).

Altogether, these studies show that Piwi can either activate or silence gene expression, and this may be dependent upon the local

chromatin environment and/or its cofactors. It has also been shown that HP1 recruitment to heterochromatin does not depend on Ago2 or Piwi and that loss of a single piRNA cluster leads to HP1 redistribution on the whole genome (79). Thus, the relationship between heterochromatin formation and the piRNA complex may be multifaceted.

In the *mili* and *miwi2* mutant mouse, loss of DNA methylation in TEs is observed, thus the epigenetic functions of Piwi proteins appear to be conserved in mammals (**Figure 1b**) (4, 58). Analysis of fetal germ cells revealed that Mili and Miwi2 play important roles in the establishment of de novo DNA methylation to silence transposons (58). DNA methylation is catalyzed by Dnmt3L in the germline. However, piRNA production is not affected in the *dnmt3l* mutant, which indicates that the piRNA pathway functions upstream of DNA methylation (4). Future work is required to elucidate the molecular function of the piRNA pathway in transposon DNA methylation in the mouse male germline.

PIWI PROTEINS IN REGULATING mRNA TURNOVER

Several Piwi proteins are expressed predominantly in the cytoplasm, including Aub and Ago3 in *Drosophila*, Mili and Miwi in mice, and Piwi in early *Drosophila* embryos (9, 27, 42, 57, 71, 75). This suggests that similar to miRNAs and Ago family proteins, Piwi-piRNA complexes also have a role in posttranscriptional regulation. Aub, Piwi, and Ago3 all associate with germplasm, and Mili and Miwi associate with the nuage and chromatoid body in mice round spermatids (9, 40, 42, 56, 57, 71, 75). Both germplasm and the chromatoid body are electron-dense cytoplasmic perinuclear organelles containing both proteins and RNAs. These analogous structures, collectively called the nuage (cloud in French), contain many RNAs and RNA-binding proteins (103). The nuage is common to animal germ cells, but its function remains enigmatic. It is thought to be a site of RNA storage and/or degradation. Accumulating evidence suggests that Piwi proteins are integral parts of the nuage where they function in posttranscriptional gene regulation.

Piwi Proteins Regulate the Degradation of Transposon mRNAs

Piwi proteins are required to repress transposition in the *Drosophila* germline, because in

piwi, *aub*, or *ago3* mutant flies the expression of transposon RNAs increases significantly (54, 65, 118). Deep sequencing and characterization of subpopulations of piRNAs bound to each *Drosophila* Piwi protein led to the ping-pong hypothesis of piRNA biogenesis and transposon repression (**Figure 2**) (9, 41). This hypothesis is based on two key differences observed when comparing the piRNAs that bind Piwi and Aub versus those that bind Ago3. First, the majority of the piRNAs that bind Piwi and Aub are antisense to transposons, whereas the majority of Ago3-bound piRNAs are sense. Second, Piwi/Aub piRNAs demonstrate a strong 5' uridine bias, whereas the majority of Ago3 piRNAs have an adenine at the tenth position. Piwi/Aub piRNAs and Ago3 piRNAs are often complementary in the first 10 bases of their 5' ends; in particular 48% of sequenced Ago3 piRNAs had complementary partners among the sequenced Aub piRNAs (9). These characteristics, collectively called the ping-pong signature, support a model in which Piwi proteins generate the 5' ends of new piRNAs from both piRNA primary transcripts and active mRNA transposons (**Figure 2**). In *ago3* mutant flies, the number of Aub-bound piRNAs was significantly reduced and the antisense bias of these piRNAs is lost, thus providing experimental evidence for the ping-pong model (65).

The coupling of piRNA biogenesis to the degradation of transposon mRNAs may allow the piRNA pathway to react specifically to active transposon expression. Although the details of this model are best understood in *Drosophila*, it appears to be a conserved mechanism for controlling transposon expression in animals. In the mouse, evidence for the ping-pong mechanism is seen in prenatal male germ cells when both Mili and Miwi2 are expressed. Mili-bound primary piRNAs (5' U bias) are predominantly sense to transposons, whereas Miwi2-bound secondary piRNAs (tenth position A bias) are mostly antisense (4). Therefore, a reverse ping-pong is occurring in mice where primary piRNAs are made from transposon transcripts and secondary piRNAs are made from piRNA cluster transcripts. Deep sequencing of total

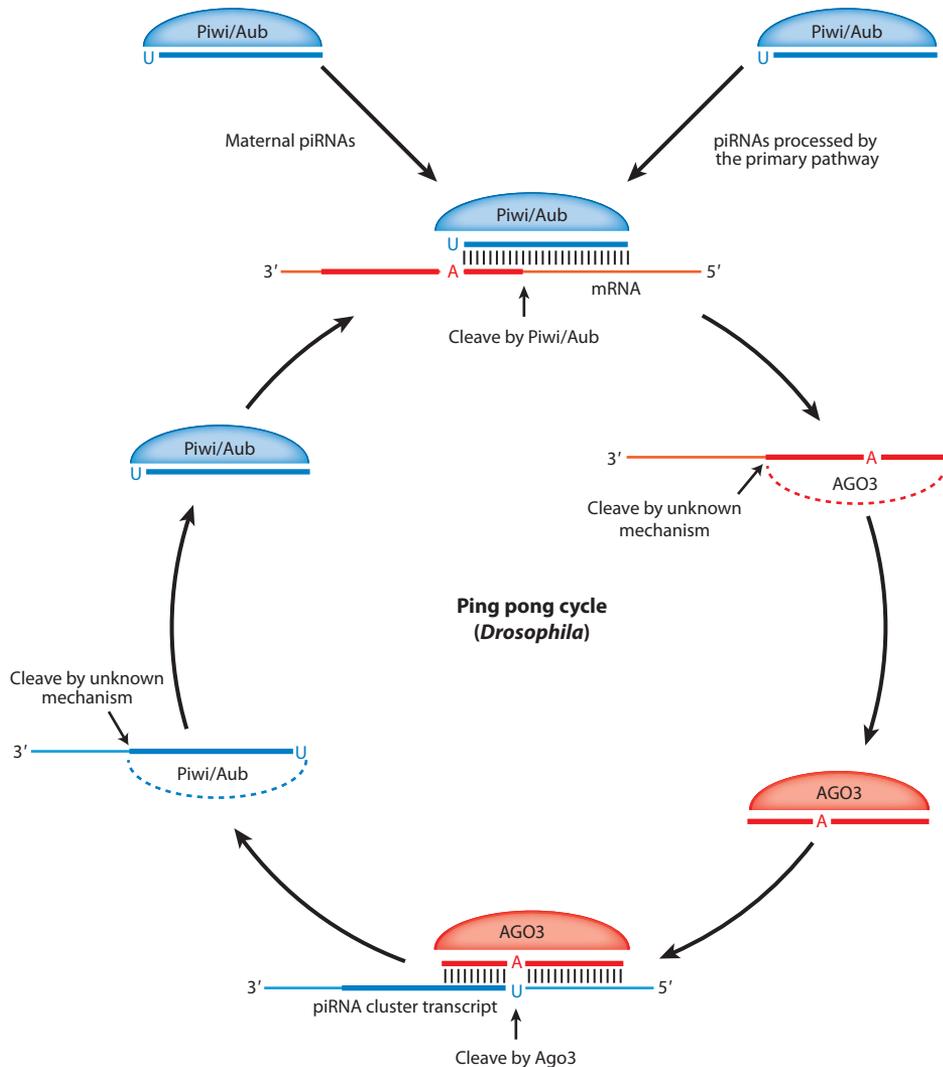


Figure 2

The ping-pong model of piRNA biogenesis and transposon repression in *Drosophila* (9, 41). Piwi proteins generate the 5' ends of new piRNAs that are derived from either piRNA cluster transcripts (primary piRNAs) or active mRNA transposons (secondary piRNAs). Piwi- and Aub-bound primary piRNAs are either maternally loaded or processed by the primary pathway. Next, Piwi and Aub, guided by their bound antisense piRNAs, bind to active transposon mRNAs and antisense the new 5' ends of sense secondary piRNAs. Cleavage of the transposon mRNA occurs 10 nucleotides upstream of the adenine that is complementary to the 5' uridine of the primary piRNA. Ago3 proteins, bound to secondary piRNAs, are then capable of producing the 5' end of new primary piRNAs made from piRNA cluster transcripts. It is not known how the 3' end of piRNAs is created in the ping-pong mechanism.

putative piRNA populations in several animals, including zebrafish and planarians, has revealed ping-pong signatures (33, 44, 85). Posttranscriptional silencing of selfish genetic elements

is likely conserved in the animal germline, but the mechanistic details have yet to be elucidated beyond *Drosophila* and mice. Given the differences observed between these two animals, it

will be informative to uncover how the ping-pong pathway operates in other animals.

A commonality between mouse and *Drosophila* is the localization of the piRNA pathway proteins to the nuage, suggesting that this organelle is at least involved in piRNA biogenesis and transposon repression (Figure 1c). Protein localization data in mouse prenatal male germ cells revealed that granules accumulating Mili and Tdrd1 (pi-bodies) are distinct from granules accumulating Miwi2, Tdrd9, and Maelstrom (piP-bodies) (6). piP-Bodies are so named because of the coincident coaccumulation with known components of the P-body, a well-characterized site of mRNA translational repression and degradation (87). pi-Bodies and piP-bodies are often found immediately adjacent to each other, but not overlapping, thus demonstrating compartmentalization of the piRNA pathway components (6). Mili, which binds primary piRNAs, is required for the formation of piP-bodies, as assessed by Miwi2 and Mael localization, which demonstrates the potential interplay between these two cytoplasmic structures (6, 13). Similar to mice, in the *Drosophila* ovary, several piRNA pathway components are dependent upon Aub and Ago3 expression for their localization to the germlasm (65, 66). Taken together, these data suggest that the piRNA pathway may be one of the central functions of the germ-cell nuage.

Piwi Proteins Regulate the Degradation of Nontransposon mRNAs

Piwi proteins have a prominent role in protecting the germline from selfish genetic elements. However, it is not clear if the myriad of phenotypes observed in various *piwi* mutant animals is due entirely to the derepression of transposons. The piRNA pathway may also regulate the expression of nontransposon genes either transcriptionally (as discussed above) or posttranscriptionally. In support of this possibility, Rouget and coworkers demonstrate that Aub and Ago3 are required for the clearing of

maternal mRNAs in preparation for the *Drosophila* maternal-to-zygotic transition (MZT) (Figure 1d) (101). Aub and Ago3 are in a complex with the CCR4-NOT deadenylation complex and the localization of these proteins to small granules in the bulk cytoplasm is dependent upon the expression of piRNA pathway components. Several transposon-derived piRNAs are complementary to sequences in the *nanos* 3' UTR, and these putative binding sites are necessary for deadenylation and degradation of the mRNA. Injection of sequences antisense to these particular piRNAs results in the same head defects seen in piRNA pathway mutants, which is likely due to a failure to clear *nanos* mRNA, a posterior determinant, from the anterior (22, 64, 101). These data support a model in which Piwi proteins with their bound piRNAs recruit the deadenylation machinery to the *nanos* 3' UTR in the bulk cytoplasm, thus destabilizing the mRNA. This phenomenon is not specific to *nanos*, as several other mRNAs that are degraded at the MZT have potential transposon-derived binding sites in their 3' UTR and depend on the piRNA pathway for their deadenylation (101). Therefore, piRNAs derived from transposon sequences are being used to regulate early development, thus demonstrating potential coevolution between transposon sequences and the host genome (101). The posttranscriptional regulation by the Piwi/piRNA pathway, as found thus far, involves degradation of the message either by the production of piRNAs, as is seen for transposon mRNAs, or by deadenylation and destabilization of the mRNA in the early *Drosophila* embryo. This is in contrast to miRNA-mediated posttranscriptional regulation of mRNAs in animals, which largely occurs via recruitment of the argonaute-containing RNA-induced silencing complex, leading to translational repression of the target mRNA rather than degradation. However, miRNAs with perfect complementarity to their targets direct mRNA degradation, a phenomenon that is more common in plants than animals.

It remains to be determined if piRNA pathway-mediated mRNA degradation of

MZT: maternal-to-zygotic transition

nontransposons is a prevalent paradigm for gene regulation in animals. Large numbers of piRNAs are not derived from transposon sequences, but rather map to diverse genomic regions, suggesting that Piwi proteins have functions in controlling gene expression that go beyond transposon control. For example, 10% of small RNAs in *Drosophila* ovary somatic sheet cell lines are piRNAs derived from the 3' UTRs of select genes (**Figure 1e**) (100). Only the primary piRNA pathway operates in these cells, and accordingly these piRNAs have a 5' U bias (61, 100). Furthermore, the abundance of these piRNAs is not reduced in *ago3* mutant flies, but is dramatically reduced in *piwi* mutant flies (100). In mouse 10-dpp testes, 35% of total small RNAs are derived from the 3' UTR of select genes. These are also likely produced by the primary pathway, as evidenced by their 5' U bias and dependency on *mili* expression (4, 100). There appears to be a selection mechanism in place to choose which 3' UTRs will be processed into piRNAs. For example, genes involved in developmental and regulatory processes are more highly represented than abundant housekeeping genes (100). These piRNAs could be acting in *cis* to destabilize their mRNAs of origin, or in *trans* to regulate other genes either transcriptionally or posttranscriptionally (100). The significance of this discovery is not yet understood, but it does appear to be a conserved phenomenon with potential developmental importance.

Piwi Proteins as Positive Translational Regulators

Given that Mili and Miwi are associated with polysomes in the mouse male germline and that the association is correlated with the translational activity of the cells, Piwi proteins may also positively regulate translation (**Figure 1f**) (39, 117). In support of this notion, Mili interacts with eukaryotic initiation factor 3a (eIF3a) in an RNA-dependent manner. In *mili* mutant testes, the level of protein expression is decreased by 60% without a significant change in mRNA levels (117). This suggests that Mili

acts as a general positive regulator in mouse testes. Miwi interacts generally with mRNAs in polysomes, which was shown both with a ribonucleoprotein-capture assay and with a cap-binding assay (40). *Miwi* mutant mice have decreased levels of specific mRNAs, which are targets of the spermatogenic transcriptional activator CREM (cAMP-responsive element upmodulator) (27). This suggests that Miwi binding to these messages may be required to stabilize and/or protect these mRNAs from degradation for translation. In this way, Miwi may also act as a positive translational regulator in the mouse male germline. It is possible that the putative role of Piwi proteins as positive translational regulators is conserved beyond the mouse germline. In *Drosophila*, Aub is required to enhance Oskar translation (126). The *Xenopus* Piwi protein, Xiwi, interacts with poly-A-binding protein (ePAB) and small ribosomal proteins, but no role for Xiwi in translational regulation has yet been identified (60). These data suggest that further investigation of Piwi function in translational activation is warranted and may uncover a conserved and critical function of Piwi proteins.

THE piRNA PATHWAY AND DEVELOPMENTAL ROBUSTNESS

Several lines of evidence suggest that Piwi proteins act as epigenetic regulators, but little is understood about the function of this epigenetic regulation during development. Recent studies in our lab show that in *Drosophila* Piwi and Aub are required for canalization, which is an organism's ability to produce the same phenotype despite inherent genetic variations and environmental influences (36). Piwi forms a multiprotein complex with Hsp90 and Hop, which directs phosphorylation of Piwi in the cytoplasm. The phosphorylated Piwi then enters the nucleus to suppress the expression of hidden genetic variation and transposon activity (**Figure 1g**) (36, 112). Hsp90 and Hop may regulate the posttranslational modifications of Piwi proteins to function in epigenetic silencing and mediate canalization (36). This ensures

developmental robustness of individual organisms for natural selection but also may allow an increase in genetic and epigenetic variability during times of stress to promote phenotypic diversity for selection.

EVOLUTIONARY PERSPECTIVE: AN ANCIENT FUNCTION FOR PIWI PROTEINS IN GERMLINE AND STEM CELLS

Fungi, plants, and green algae contain only Ago subfamily proteins, whereas animals have both Ago and Piwi subfamily, proteins (14). Some unicellular organisms such as *Amoebozoa* and the ciliated protist *Tetrahymena thermophila* have Piwi subfamily, but not Ago subfamily, proteins (14). This suggests that Piwi proteins are not an invention of multicellular animals but rather have a more ancient origin. Studies in a wide variety of both unicellular organisms and multicellular animals at key phylogenetic positions allow us to ascertain the conserved and fundamental functions of Piwi proteins.

The Function Piwi Proteins During Tetrahymena Sexual Reproduction

The genome of *Tetrahymena thermophila* contains 12 putative Piwi proteins, *twi1-twi12*. Eight of these are known to be expressed and to bind to diverse populations of small RNAs with distinct sizes, genomic origins, genetic requirements for accumulation, and timing of expression during the life cycle (21). *Tetrahymena* undergoes both sexual (conjugation) and asexual (vegetative growth) reproduction (15). During vegetative growth two nuclei are maintained: a silent diploid germline micronucleus, which contains the complete genome, and an active polyploidy somatic macronucleus, which has undergone region-specific DNA elimination. During conjugation, the germline nuclei undergo meiosis, gamete exchange, and fusion. The resulting zygotic nucleus undergoes mitosis and somatic differentiation. This process requires TWI1, which binds to a class of small RNAs called scanRNAs (76). It is pro-

posed that scanRNAs are made in the germline nucleus and then bind to TWI1 protein in the cytoplasm. The TWI1–scan RNA complex subsequently moves to the new somatic nucleus and marks DNA for elimination with an H3K9 methylation (74). As discussed above, *Drosophila* Piwi is required for H3K9 methylation and heterochromatin formation, thus suggesting a conserved function for Piwi family proteins in directing histone modifications (10). In support of this, scanRNAs are similar to piRNAs in size (27 to 30 nucleotides), and they have a 2'-O-methylation (59, 76). On the other hand, scan RNA production requires the Dicer ortholog *dcl1*, whereas animal piRNA production is Dicer-independent (44, 77). Regardless, it is clear that *Tetrahymena* requires Piwi proteins to complete its sexual cycle, which offers an interesting comparison with animal Piwi proteins.

Piwi Genes as Stem Cell Genes in Nonbilaterians

Piwi genes have been identified in representative species of several bilaterian sister groups, which suggests that Piwi proteins were present in the last common ancestor of all bilaterians (**Figure 3**) (2, 28, 34, 108). Nonbilateria phyla include *Porifera* (sponges), *Ctenophora* (comb jellies), and *Cnidaria* (jelly fish, sea anemones, and corals). The evolutionary relationships between these species is controversial, but the cnidarians are the well-accepted sister group to the bilaterians (30, 89, 90). In nonbilaterian metazoans, *piwi* expression is not limited to the germline but instead functions more broadly in stem cells. For example, in the sponge *Ephydatia fluviatilis*, two *piwi* orthologs are selectively expressed in the archeocytes, adult stem cells that give rise to both differentiated somatic and germ cells (34). In the cnidarian *Podocoryne carnea*, a *piwi* homolog is expressed in the germline, as well as in transdifferentiating tissues (108). In the cnidarian *Clytia hemisphaerica*, a *piwi* homolog is expressed in stem cells of the medusa tentacle bulb that give rise to nematocytes (cnidarian stinging cells)

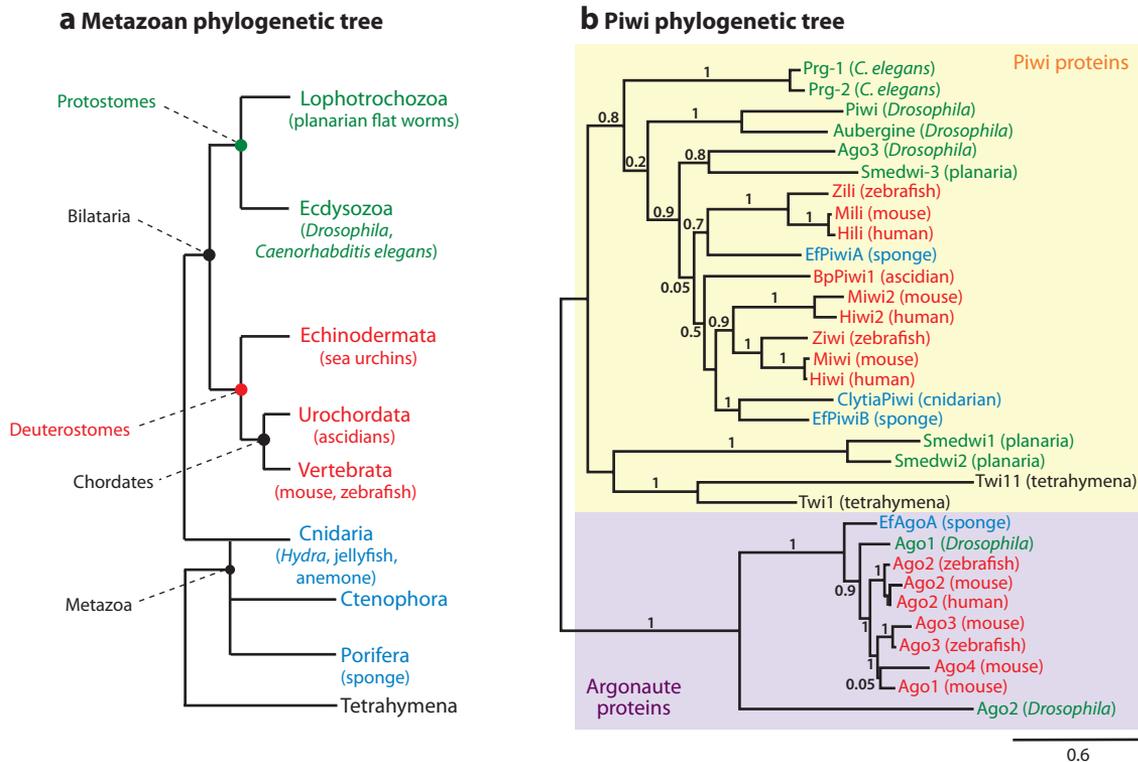


Figure 3

Piwi proteins are conserved in metazoans. (a) A metazoan phylogenetic tree. Bilaterians are classified as either protostomes (green) or deuterostomes (red) based on differences in embryo development. Nonbilaterian phyla (blue) include cnidarians, ctenophores, and sponges. *Tetrahymena* is a ciliated protist that also expresses Piwi proteins. (b) Phylogenetic analysis of Argonaute proteins demonstrates a clear distinction between the Piwi (yellow) and Argonaute (purple) subfamilies. Phylogram generated using Phylogeny.fr (29). Numbers on the tree demonstrate branch supports. National Center for Biotechnology Information accession numbers from top to bottom are as follows: Prg-1 (NM_059720), Prg-2 (NM_068593), Piwi (NM_057527), Aubergine (NM_001103674), Ago3 (NM_001043162), Smedwi-2 (EU586258), Zili (NM001080199), Mili (NM_021308), Hili (NM_001135721), EfPiwiA (AB533505), BpPiwi1 (AB455103), Miwi2 (NM_177905), Hiwi2 (NM_152431), Ziwi (NM_18338), Miwi (NM_021311), Hiwi (NM_004764.4), ClytiaPiwi (EU199802), EfPiwiB (AB533506.1), Smedwi1 (DQ186985), Smedwi2 (DQ186986), Twi11 (EU183125), Twi1 (AB084111), EfAgoA (AB533507), *Drosophila* Ago1 (NM_166020), Zebrafish Ago2 (XM_694134), Mouse Ago2 (AY135688), Human Ago2 (NM_001164623), Mouse Ago3 (AY135689), Zebrafish Ago3 (XM_690923), Mouse Ago4 (AY135690), Mouse Ago1 (AY135687), *Drosophila* Ago2 (NM_140518).

(28). In the ctenophore *Pleurobrachia pileus*, two *piwi* homologs are expressed in the germline as well as in several somatic domains (2). These somatic domains are sites of active cell proliferation and are likely stem cells that give rise to various differentiated adult tissues (2).

To determine if bilaterian-type piRNAs exist in animals such as the cnidarians or sponges, total small RNA populations were sequenced from the sponge *Amphimedon queenslandica* and the starlet sea anemone *Nematostella vectensis*

(38). A large fraction of the small RNAs found in both animals were approximately 27 nucleotides long and were resistant to periodate oxidation implying the existence of a 2'-O-methylation. These small RNAs have a 5' uridine bias and a fraction of them come from clustered genomic loci (45% in *Nematostella* and 10% in *Amphimedon*) (38). Therefore, these are likely primary piRNAs, although true classification of piRNAs requires a demonstrated association with Piwi proteins. In addition, they

found that candidate piRNAs that were sense to the mRNAs (~35% corresponding to transposons) had a bias for a tenth position adenine, thus providing evidence for ping-pong piRNA synthesis in these nonbilaterian animals (38).

As described in this review, the majority of our understanding of Piwi protein function comes from *Drosophila* and mouse. These studies demonstrate diverse roles for Piwi in controlling gene expression at several levels, both transcriptional and posttranscriptional. However, it remains unclear how these molecular functions contribute to the maintenance of GSCs. Nonbilaterian phyla offer an intriguing position on the phylogenetic tree for interrogating Piwi function. Given the common suite of genes (e.g., *vasa*, *nanos*, and *piwi*), the molecular functions of Piwi proteins in the stem cells of nonbilaterian metazoans will likely be conserved in, for example, the germline of *Drosophila* and mice (47). Furthermore, understanding the function of Piwi proteins in nonbilaterian animals may uncover the most conserved functions of these proteins in stem cells, thus shedding light on the unifying mechanisms common to all animals. Thus far, studies of Piwi proteins in early branching metazoans have been restricted to expression analyses. However, in two cnidarian model systems, *Hydra* and *N. vectensis*, modern molecular tools are now being applied to study gene function, including gene knockdown by RNAi or morpholinos, the generation of transgenic animals, and genome sequencing. Thus, researchers are now in a position to interrogate Piwi function in these animals (16, 19, 92, 96, 127).

The Role of Piwi Genes in Bilaterian Adult Stem Cells

Bilaterians are split into two major groups based on the development of their embryos, the deuterostomes and protostomes (Figure 3). The deuterostomes include the vertebrates as well as some invertebrate groups (e.g., echinoderms and tunicates). The tunicates are a subphylum of the chordates and are the sister group to vertebrates (26, 30). In one tunicate group,

the *Botrylloides* colonial ascidians, a *piwi* homolog is expressed in stem cells capable of giving rise to all three germ layers during whole body regeneration (11, 99). In *Botrylloides leachi*, RNAi knockdown demonstrates the requirement of *piwi* for regeneration in this animal (99). Thus, *piwi* may have an ancient association with stemness in the deuterostome lineage. The protostome branch of the *Bilateria* is split into two groups: the ecdysozoans, molting animals, and the lophotrochozoans, which are loosely grouped together based in part on embryological and larval similarities (Figure 3) (30). Two very successful model systems, *Drosophila* and *C. elegans*, are ecdysozoans, and therefore a large amount of functional data on Piwi proteins comes from this group. Functional data from the lophotrochozoan clade are largely missing, with one exception being the flatworms, where RNAi is routinely used to knock-down gene function (80). Flatworms contain totipotent adult stem cells, the neoblasts, which give rise to all somatic and germline cell types, thus giving flatworms remarkable regeneration capabilities (7, 81). In the planarian flatworm *Schmidtea mediterranea* two *piwi* homologs, *Smedwi-2* and *Smedwi-3*, are expressed in the neoblasts and are required for neoblast function in regeneration (85, 95). Deep-sequencing studies in planaria revealed the existence of both primary and secondary putative piRNAs (33, 85). In the marine flatworm *Macrostomum lignano*, a *piwi* homolog is required for the maintenance of neoblasts, reminiscent of its function in maintaining GSCs in *Drosophila* (22, 25). Taking the evidence from both traditional and nontraditional model organisms together suggests that Piwi proteins have an ancient function in regulating stem cells (47).

THE IMPACT OF PIWI PROTEINS ON HUMAN HEALTH

The broad phylogenetic survey of the expression patterns of *piwi* genes described above supports a conserved function for Piwi proteins in the stem cells of both somatic and germline tissues. A human *piwi* ortholog, *hiwi*, is expressed

in hematopoietic stem and progenitor cells but not in the differentiated products of these cells, thus suggesting a role for Piwi proteins in at least one type of human somatic stem cell (109). In addition to the potential function for Piwi proteins in normal somatic stem cells, several studies demonstrate a correlation of ectopic *hiwi* or *hili* expression with the occurrence of several cancer types, including, but not limited to, testicular seminomas, breast cancer, soft tissue sarcomas, gastric cancer, and ovarian cancer (63, 70, 73, 94, 114).

Stem cells and cancer cells share several characteristics, including the ability to self-renew and relative resistance to drugs and radiation. It is hypothesized that in some cases, a deregulation of normal stem cells transforms them into cancer stem cells (CSCs) and that the creation of CSCs is required for transformation from nonmalignancy to malignancy (98). Thus, the identification of stem cell-associated genes in cancerous tissue may reflect the existence of CSCs. Several studies demonstrate that Piwi proteins are required for the continued proliferation of cancer cells, which is similar to their requirement for GSC proliferation in *Drosophila* (18, 23, 63, 73). This supports the hypothesis that cancerous cells appropriate the stem cell machinery to promote their continued survival. In *Drosophila*, brain tumors caused by temperature-sensitive mutations in the *lethal(3) malignant brain tumor* [*l(3)mbt*] gene ectopically express a significant number of genes required for germline formation and maintenance, including Piwi and Aub (46). The average brain tumor size is significantly reduced when *piwi* or *aub* mutations are introduced. Therefore, future studies should focus on the functions of stem cell genes such as the Piwi family in CSCs, as interfering with these functions may be a strategy to develop therapies.

CONCLUSION

Current studies of the Piwi-piRNA pathway have revealed its diverse biological and molecular functions, including germline specification, gametogenesis, stem cell maintenance,

epigenetic programming, transposon silencing, genome protection, and posttranscriptional regulation of mRNAs. Among these discoveries, perhaps the most extensively reported is its role in repressing TEs in the germline, both at the transcriptional and posttranscriptional levels (106). Despite these reports, this process remains mysterious in many ways. For example, the production of primary piRNAs, such as the transcriptional control and processing of the precursors, is not understood. In addition, the details of the quality-control mechanism used to select mRNAs (often transposon mRNAs) for the production of secondary piRNAs are not understood. It is also not clear how the desilencing of transposons might be connected to the various developmental phenotypes observed for *piwi* mutants, such as GSC loss and embryonic patterning defects in *Drosophila*, GSC loss and spermatogenic arrest in mice, germ-cell death in zebrafish, and inhibition of stem cell function in planarians (13, 23, 27, 42, 44, 57, 65). It is possible that transposon activation triggers regulatory pathways, which in turn leads to these aberrant phenotypes.

There is evidence that the Piwi-piRNA complex functions in epigenetic control, but many aspects of this mechanism remain elusive. For example, only HP1 has been found to be involved in heterochromatin formation with Piwi proteins. What is the relationship between histone methyltransferase, DNA methyltransferase, and Piwi in epigenetic regulation? If piRNAs target Piwi proteins to the chromosomes, do they bind DNA sequences or nascent transcripts? At the level of epigenetic regulation, how do piRNAs distinguish transposon sequences and protein-coding genes? The answers to these questions are important because the Piwi-piRNA complex may act to select specific chromosomal regions to be targeted by more general epigenetic factors.

There is also evidence that the Piwi-piRNA pathway regulates nontransposon genes posttranscriptionally in *Drosophila*, such as the targeting of maternal genes for deadenylation at the MZT (101). Given that a significant

subpopulation of piRNAs do not map to transposons, future research is likely to uncover new instances of Piwi-piRNA regulation of non-transposon genes. To harness the therapeutic power of stem cells, we need to understand precisely the underlying molecular circuitry governing their cell fate decisions. To accomplish this goal, a number of model organisms have been instrumental in the identification

of genes and pathways involved in stem cell regulatory control. The Piwi-piRNA pathway is required for maintaining stem cells in the animal germline and perhaps in a wider variety of stem cells, including in humans (23, 109). Understanding the full regulatory capabilities of the Piwi-piRNA pathway will be essential for understanding how it regulates stem cells and other developmental functions.

SUMMARY POINTS

1. Piwi proteins bind to piRNAs and function in germ cells and stem cells throughout animal phylogeny.
2. piRNAs are produced via a Dicer-independent mechanism in two steps: Primary piRNAs are made by an unknown mechanism from long single-stranded precursors; and secondary piRNAs are then made by the ping-pong mechanism in which Piwi proteins cleave piRNA cluster transcripts or active mRNAs into piRNAs.
3. Piwi proteins maintain fertility in *Drosophila*, *C. elegans*, and mouse, at least in part through maintenance of GSCs.
4. Piwi proteins repress transposition in the animal germline, presumably by both epigenetic suppression and degradation of transposon mRNA (e.g., via ping-pong mechanism).
5. Piwi proteins bind to Tudor domain-containing proteins in the nuage of germ cells. Tudor domain-containing proteins may function to ensure quality control of the mRNAs selected for piRNA biogenesis by Piwi proteins.
6. Some Piwi proteins are in the nucleus and function in epigenetic mechanisms, including histone modification in *Drosophila*; Piwi proteins are also known to regulate DNA methylation in mice, possibly via a cytoplasmic mechanism.
7. Some Piwi proteins are in the cytoplasm, where they are often localized to the nuage and function in posttranscriptional gene regulation.
8. Piwi function is not limited to animal germlines but instead is required more broadly in stem cells in diverse organisms.

FUTURE ISSUES

1. Many questions remain open regarding Piwi-piRNA control of TEs. For example, how is the transcription of TE piRNA precursors regulated? What is the mechanism of TE primary piRNA production? And how are specific TE mRNAs selected for secondary piRNA biogenesis?
2. The developmental phenotypes of *piwi* mutants are varied and complex. What molecular mechanism mediated by Piwi is related to a specific phenotype?

3. What is the relationship between Piwi proteins and other epigenetic regulators? Do Piwi-piRNA complexes bind to DNA or to nascent RNA transcripts? How do Piwi-piRNA complexes recognize specific genomic sequences?
4. What is the function of Piwi-piRNA complexes that do not target TEs for repression? How prevalent is Piwi-directed regulation of nontransposon genes, and what other processes do Piwi-piRNA complexes regulate?

DISCLOSURE STATEMENT

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Contents

| | |
|--|-----|
| Comparative Genetics and Genomics of Nematodes: Genome Structure, Development, and Lifestyle <i>Ralf J. Sommer and Adrian Streit</i> | 1 |
| Uncovering the Mystery of Gliding Motility in the Myxobacteria <i>Beiyun Nan and David R. Zusman</i> | 21 |
| Genetics and Control of Tomato Fruit Ripening and Quality Attributes <i>Harry J. Klee and James J. Giovannoni</i> | 41 |
| Toxin-Antitoxin Systems in Bacteria and Archaea <i>Yoshihiro Yamaguchi, Jung-Ho Park, and Masayori Inouye</i> | 61 |
| Genetic and Epigenetic Networks in Intellectual Disabilities <i>Hans van Bokhoven</i> | 81 |
| Axis Formation in Hydra <i>Hans Bode</i> | 105 |
| The Rules of Engagement in the Legume-Rhizobial Symbiosis <i>Giles E.D. Oldroyd, Jeremy D. Murray, Philip S. Poole, and J. Allan Downie</i> | 119 |
| A Genetic Approach to the Transcriptional Regulation of <i>Hox</i> Gene Clusters <i>Patrick Tschopp and Denis Duboule</i> | 145 |
| V(D)J Recombination: Mechanisms of Initiation <i>David G. Schatz and Patrick C. Swanson</i> | 167 |
| Human Copy Number Variation and Complex Genetic Disease <i>Santhosh Girirajan, Catarina D. Campbell, and Evan E. Eichler</i> | 203 |
| DNA Elimination in Ciliates: Transposon Domestication and Genome Surveillance <i>Douglas L. Chalker and Meng-Chao Yao</i> | 227 |
| Double-Strand Break End Resection and Repair Pathway Choice <i>Lorraine S. Symington and Jean Gautier</i> | 247 |

| | |
|--|-----|
| CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation <i>Devaki Bhaya, Michelle Davison, and Rodolphe Barrangou</i> | 273 |
| Human Mitochondrial tRNAs: Biogenesis, Function, Structural Aspects, and Diseases <i>Tsutomu Suzuki, Asuteka Nagao, and Takeo Suzuki</i> | 299 |
| The Genetics of Hybrid Incompatibilities <i>Shamoni Maheshwari and Daniel A. Barbash</i> | 331 |
| Maternal and Zygotic Control of Zebrafish Dorsal-Ventral Axial Patterning <i>Yvette G. Langdon and Mary C. Mullins</i> | 357 |
| Genomic Imprinting: A Mammalian Epigenetic Discovery Model <i>Denise P. Barlow</i> | 379 |
| Sex in Fungi <i>Min Ni, Marianna Feretzaki, Sheng Sun, Xuying Wang, and Joseph Heitman</i> | 405 |
| Genomic Analysis at the Single-Cell Level <i>Tomer Kalisky, Paul Blainey, and Stephen R. Quake</i> | 431 |
| Uniting Germline and Stem Cells: The Function of Piwi Proteins and the piRNA Pathway in Diverse Organisms <i>Celina Juliano, Jianquan Wang, and Haifan Lin</i> | 447 |

Errata

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