Molecular Cell
Short Review

The Coming of Age for Piwi Proteins

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DOI 10.1016/j.molcel.2007.05.021

Piwi proteins, a subfamily of Argonaute (Ago) proteins, have recently been shown to bind endogenous small RNAs. However, differences between Ago proteins (which bind microRNAs and small interfering RNAs) and Piwi proteins and Piwi-interacting RNAs (piRNAs) suggest novel functions for Piwi proteins. Here, we highlight the recent progress in understanding Piwi function and the implications for germline and stem cell development.

Argonaute (Ago) proteins comprise the central protein component of RNA-mediated gene silencing complexes and utilize short guide RNAs to direct function toward a specific nucleic acid target. The best-characterized small RNA partners of Ago proteins are microRNAs (miRNAs), which predominantly silence genes posttranscriptionally, and small interfering RNAs (siRNAs) that participate in transcriptional and posttranscriptional mechanisms. Agos are defined by two major protein motifs: the PAZ and PIWI domains. These motifs point to Agos' function as an RNA interactor: the PAZ domain is a single-stranded nucleic acid binding motif and the PIWI domain has an RNase H fold (reviewed in Tolia and Joshua-Tor [2007]).

Phylogenetic analysis indicates that known Agos can be divided into two subclades: the Ago subclade based on *Arabidopsis thaliana* Ago1 and the Piwi subclade based on *Drosophila melanogaster* Piwi (see Figure 1). Much progress has been made to elucidate the biological function and mechanism of action of the Ago subclade in RNA interference pathways. The accompanying review in this issue of *Molecular Cell* covers recent advances in this area (Peters and Meister, 2007). Meanwhile, functional analysis of the Piwi subclade has lagged behind that of the Ago subclade, in part due to the lack of definition concerning RNAs that interact with Piwi. This has changed recently, as Piwi proteins have now been found to interact with a new class of small RNAs that has intriguing differences from miRNAs and siRNAs. Recent reviews have addressed advances concerning the role for Piwi proteins in the biogenesis of piRNAs (Lin, 2007; O'Donnell and Boeke, 2007). The goal here is to summarize Piwi and piRNA biology and to focus on their possible roles in transcriptional gene silencing and germline/stem cell maintenance.

Piwi Proteins as Small RNA Interactors

Given the conservation of the major protein domains between the Ago and Piwi subclades, it may not have been surprising to expect Piwi proteins to bind RNA. Indeed, the genetic studies of abrogated transposon and repeat-element silencing observed in fly Piwi-family mutants were linked to RNA-dependent mechanisms. Transgene silencing that was affected by mutations in the *piwi* gene also seemed to depend on small RNAs that are complementary to the transgenes (Pal-Bhadra et al., 2004). Additionally, mouse Piwi proteins exhibited RNA binding activity and appeared responsible for the stability of a subset of mRNAs (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). The surprise, however, came from the unusual qualities of endogenous RNAs that interact with Piwi proteins.

In 2006, six groups converged upon the landmark finding that Piwi proteins in flies and mammals bind a class of small RNAs distinct from miRNAs and typical siRNAs. Two groups (Saito et al., 2006; Vagin et al., 2006) determined that Piwi and Aubergine bind repeat-associated siRNAs (rasiRNAs). The rasiRNAs were first characterized in small RNA cloning studies from flies at different stages of development, which revealed that the rasiRNAs were longer in length than canonical small RNAs (24–27 nucleotides, as opposed to 21–22), were enriched in the testes and early in development, and derived from retrotransposons and other repetitive elements (Aravin et al., 2003). In addition to their longer length, rasiRNAs can be distinguished from miRNAs by a modification to the 3′ terminal nucleotide (Vagin et al., 2006). rasiRNAs copurify with Piwi, but not with DmAgo1, which binds miRNAs. Consistent with the Piwi protein and the rasiRNAs working together, an endonuclease activity on a target RNA complementary to a short guide RNA in the protein could be detected (Gunawardane et al., 2007; Saito et al., 2006).

Working in mammals, four other groups revealed that Piwi proteins interact with novel RNAs derived from intergenic regions but were not as dominated with matches to repeat elements as the rasiRNAs from flies (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a; Lau et al., 2006). Mammalian Piwi-interacting RNAs (piRNAs), like the rasiRNAs, were longer than miRNAs, and the pool of piRNA sequences was extremely complex. These piRNAs coimmunoprecipitated with two mouse Piwi subclade members, Miwi and Mili (Aravin et al., 2006; Girard et al.,

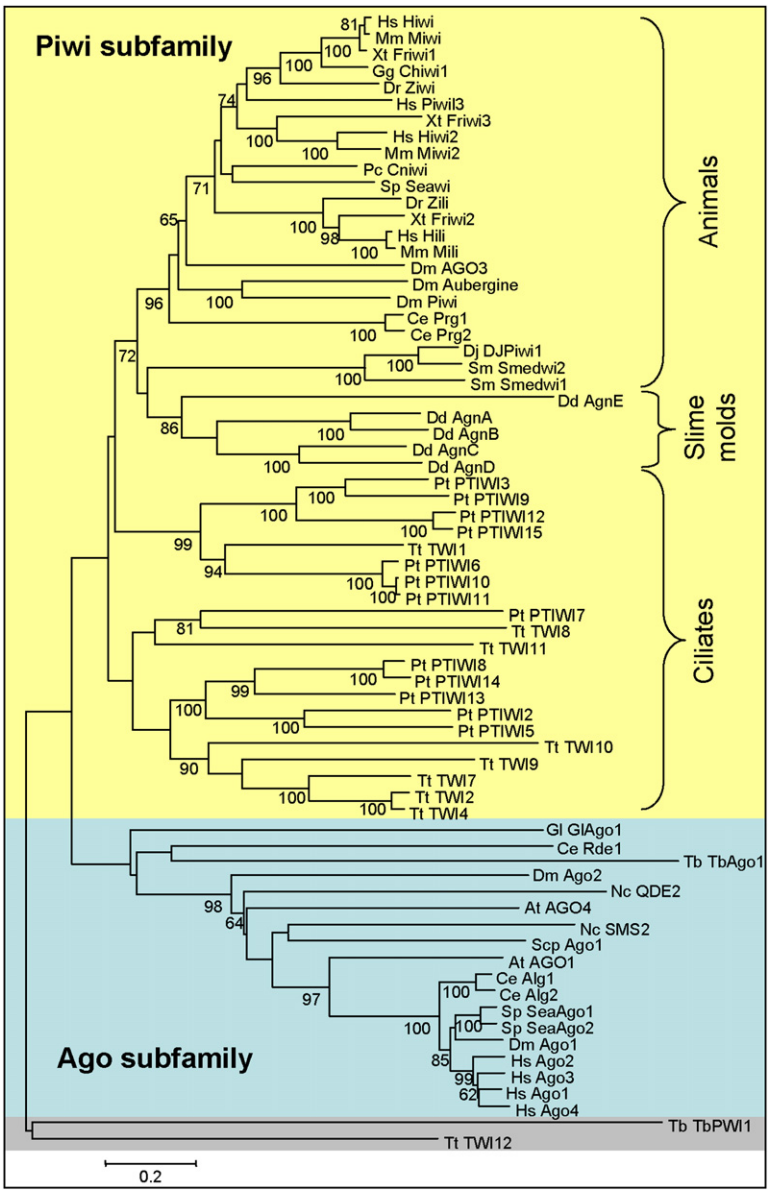

Figure 1. A Piwi-Focused Phylogenetic Tree of Argonaute-Family Proteins

Alignments, tree construction, and bootstrap analysis were performed with the MEGA v3.1 software. Only bootstrap values greater than 60 are shown for this neighbor-joining tree. Protein sequences and accession numbers are available upon request. Abbreviations: At: *Arabidopsis thaliana*; Ce: *Caenorhabditis elegans*; Dd: *Dictyostelium discodium*; Dj: *Dugesia japonica*; Dm: *Drosophila melanogaster*; Dr: *Danio rerio*; Gl: *Giardia lambia*; Gg: *Gallus gallus;* Hs: *Homo sapiens*; Mm: *Mus musculus*; Nc: *Neurospora crassa;* Pc: *Podocoryne carnea*; Pt: *Paramecium tetraurelia*; Scp: *Schizosaccaromyces pombe*; Sm: *Schmidtea mediterranea*; Sp: *Strongylocentrotus purpuratus*; Tb: *Trypanosoma brucei*; Tt: *Tetrahymena thermophila*; and Xt: *Xenopus tropicalis*.

2006; Grivna et al., 2006a), whereas a conventional chromatographic purification of proteins associated with piRNAs from rat testes followed by protein sequencing identified piRC (piRNA complex), containing the rat Piwi protein, Riwi, and a RecQ1 helicase that has genetic links to known RNA silencing pathways (Lau et al., 2006). Most recently, a new subset of prepachytene piRNAs was discovered to be bound by Mili (Aravin et al., 2007). These are less abundant and are expressed earlier than the major complement of adult piRNAs, which are expressed starting at the pachytene stage of meiosis. Although prepachytene piRNAs are more enriched in repetitive sequences than pachytene piRNAs, the majority of both prepachytene and pachytene piRNAs each map as a single copy within the mouse genome (Aravin et al., 2007; Girard et al., 2006; Lau et al., 2006).

Genomic analysis of mammalian piRNAs indicates that they cluster in primarily unannotated regions. Pachytene piRNA clusters range in size from 1 to 100 kb in length (for example see Figure 2A), comprise less than 0.1% of rodent genomes, and distribute nonuniformly as about 100 different loci across mainly autosomes (Girard et al., 2006; Lau et al., 2006). Prepachytene piRNAs, however, are more broadly distributed, with over 900 smaller clusters present, and appear not to overlap with pachytene piRNA clusters (Aravin et al., 2007). A distinguishing feature of clusters of uniquely mapping piRNAs is the pronounced strand bias of the sequences, thereby leading to the proposal that the biogenesis of piRNAs might involve a long single-stranded precursor. However, detectable secondary structures or pronounced double-stranded RNA precursors have not been systematically

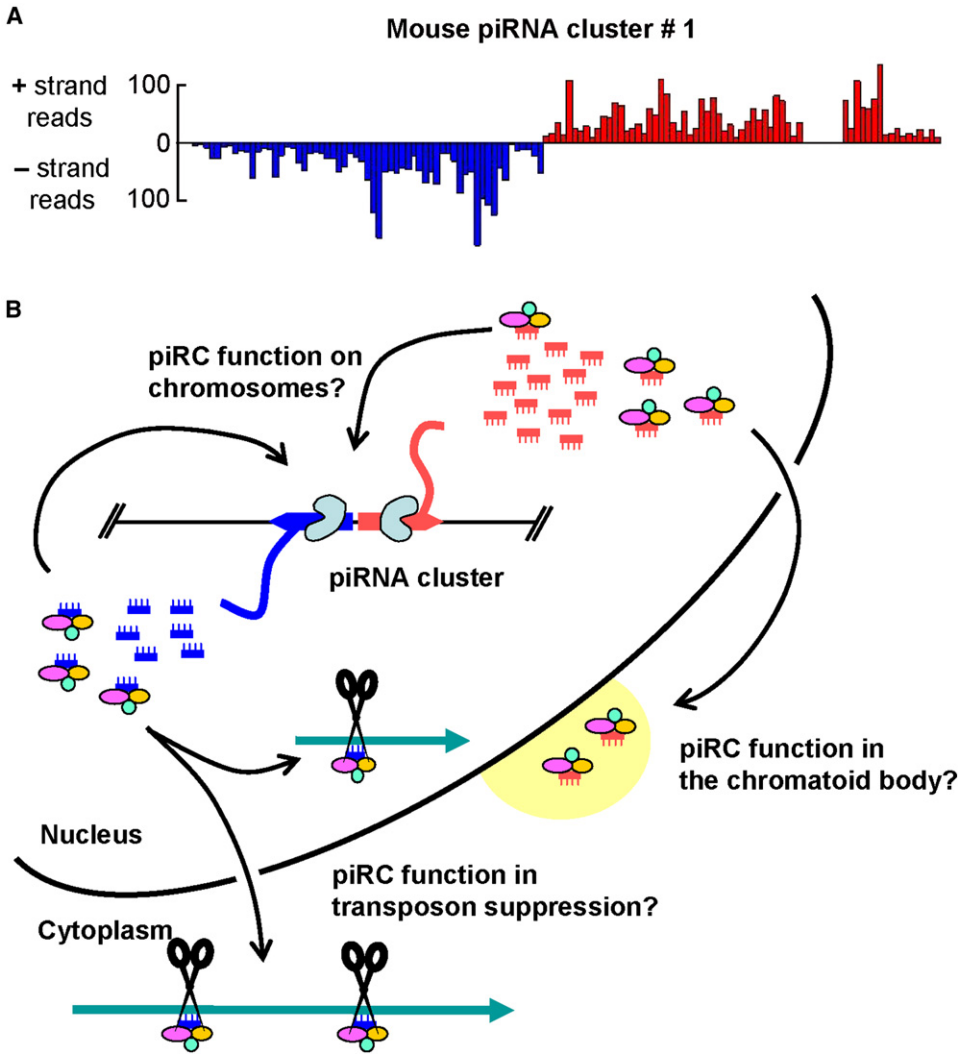

Figure 2. Potential Sites and Modes of Action for piRC

(A) Clusters of piRNAs derive from discrete 20–100 kb loci, several of which are bidirectionally transcribed (i.e., piRNA cluster #1, which is 80 kb). Bars indicate number of individual piRNAs identified in each interval (red, top strand; blue, bottom strand.).

(B) piRC may serve a function in the nucleus, such as marking important genomic loci through an epigenetic mechanism (similar to that in ciliates) or through an RNA cleavage event (similar to *S. pombe*). piRC could also act in the cytoplasm, to suppress transposon activity (similar to the rasiRNA complex in *Drosophila*), or it might act at the chromatoid body, an organelle postulated as an RNA-processing center.

detected for the putative piRNA precursors, thus the biogenesis of mammalian piRNAs remains a mystery.

Nevertheless, recent characterization of a third Piwi member in flies, Ago3, has yielded insight into the biogenesis of rasiRNAs. Piwi, Aub, and Ago3 each bind a different set of rasiRNAs, and all three proteins produced in *E. coli* exhibit RNA cleavage activity in vitro (Brennecke et al., 2007; Gunawardane et al., 2007). Aub- and Piwi- associated rasiRNAs derive mainly from the antisense strand of retrotransposons, whereas Ago3 rasiRNAs derive mainly from the sense strand. The finding that many Aub-associated rasiRNAs are complementary to Ago3-bound rasiRNAs at only the first ten base pairs led to a model that Ago3 is involved in rasiRNA biogenesis by cleaving across a dsRNA precursor, thus specifying the 5′ end of rasiRNAs that are bound to Aub (Brennecke et al., 2007; Gunawardane et al., 2007). The base-paired configuration is supported by a strong preference for adenine at position 10 of Ago3 rasiRNAs, which would be opposite the biased presence of uridine at the 5′ end of Aub rasiRNAs. Thus, the endonuclease activities of Piwi proteins might serve a dual capacity: to degrade sense transposon transcripts and to produce the antisense rasiRNAs.

This biogenesis mechanism might extend to mammals. The population of repetitive piRNAs that bind to Mili during the prepachytene stage of spermatogenesis contains sense and antisense piRNAs derived from transposable elements that have the same base-paired configuration as the Ago3/Aub rasiRNA pairing (Aravin et al., 2007). The biochemically purified endogenous piRC exhibits

RNA cleavage activity, which presumably comes from the Riwi protein (Lau et al., 2006). However, whether recombinant mammalian Piwi proteins have the same biochemical attributes as the *Drosophila* proteins remains to be seen. In addition, piRNAs associated with Miwi/Riwi appear to derive from a single strand, not both strands as most simply expected if this biogenesis model is general.

A model invoking Piwi-family proteins as the sole cleavage agents would explain how rasiRNA biogenesis can proceed without either Dcr-1 or Dcr-2, the RNase III-type enzymes that produce miRNAs and siRNAs, respectively, in flies (Vagin et al., 2006). However, this model does not address the mechanism for the 3′ end modification of rasiRNAs and piRNAs that is not seen for miRNAs (Houwing et al., 2007; Vagin et al., 2006). Thus, there are open questions. How are *Drosophila* Ago3, Piwi, and Aub able to specifically recognize the respective sense and antisense strands of repetitive elements? Which Piwi proteins are the mammalian counterparts to the *Drosophila* proteins?

The discovery of piRNAs and rasiRNAs in mammals and flies implied that other organisms that express Piwi proteins might express piRNAs. In fact, the first Piwi protein shown to bind small RNAs was TWI1, a *Tetrahymena thermophila* homolog. TWI1 binds ~30 nt long scan RNAs (scnRNAs), which derive from the micronucleus (reviewed in Mochizuki and Gorovsky [2004]). During conjugative replication, the complex of TWI1 and scnRNAs serves to interrogate the genome of the daughter macronucleus, where the complex directs elimination of DNA sequences that are homologous to the scnRNAs. In other vertebrates, the zebrafish Piwi protein, Ziwi, has recently been shown to bind fish piRNAs that derive from single-stranded clusters as well as repetitive elements (Houwing et al., 2007). Because the interaction of Piwi proteins with small RNAs is clearly a conserved feature, key aspects of the molecular function for Piwi proteins might also be conserved.

A Function for Piwi in Transcriptional Gene Silencing Processes?

It is apparent from their conservation and distinct characteristics that elucidating Piwi and piRNA function in mammals will be important; however, at present, we must look toward model genetic organisms for clues about function. Work in flies indicated a role for Piwi proteins and rasiRNAs in silencing of repetitive elements. In the fission yeast *Schizosaccharomyces pombe*, small RNAs and the RNAi pathway are involved in transcriptional gene silencing (TGS). By analogy, mammalian piRNAs might also play a role in TGS (Figure 2B).

The genetics of Piwi proteins in flies have suggested interplay with TGS mechanisms. *Piwi*, *aubergine*, and *spindle-E* (a helicase) mutants, which have compromised levels of fly rasiRNAs (Vagin et al., 2006), can suppress position effect variegation of a *white* reporter and display loss of histone H3-lysine 9 methyl marks (H3K9-Me) and heterochromatin protein 1 (HP1) binding at normally silenced loci (Pal-Bhadra et al., 2004). Additionally, the Piwi gene interacts genetically with the regulation of a Polycomb response element and Polycomb protein function, suggesting a role in influencing cellular identity during embryonic development, through a chromatin-dependent mechanism (Grimaud et al., 2006).

Because yeast and plants lack Piwi subfamily members, small RNA-dependent mechanisms in these organisms rely entirely on Argonaute subclade proteins. Certain Ago proteins in yeast and plants function in TGS (reviewed in Zaratiegui et al. [2007]). *S. pombe* Ago1 binds heterochromatic siRNAs and constitutes part of the RNA-initiated transcriptional silencing (RITS) complex, which directs the establishment of H3K9-Me marks and the subsequent recruitment of the repressive *S. pombe* HP1 protein. Genetic studies suggested that RITS acts in *cis* by base pairing to nascent transcripts formed at these loci and is necessary for proper chromosome segregation. In *A. thaliana*, Ago4 is involved in the silencing of inverted repeats, whereby heterochromatic siRNAs guide Ago4 to specify H3K9-Me marks and DNA methylation to silent repetitive regions of the genome. Like piRNAs and rasiRNAs, plant heterochromatic siRNAs ($\geq$24 nt) are longer than miRNAs and siRNAs. Additionally, both fungal and plant siRNAs map as clusters at heterochromatic loci and lack the sequence conservation seen for plant and animal miRNAs.

Despite the precedents established in yeast and plants, a direct mechanistic link for fly and mammalian Piwi proteins acting on chromatin remains to be demonstrated. There is some support for this notion: piRNA features are clearly distinct from those of miRNAs (piRNAs have poor primary sequence conservation and immense complexity), and piRNA sequences cluster to discrete originating loci. Thus, piRNA clusters resemble plant and fungal loci that produce clusters of heterochromatic siRNAs and the scnRNA elements in ciliates. A major proportion of fly and fish piRNAs also map to transposons, repetitive elements, and heterochromatic regions, which parallels the repetitive nature of heterochromatic siRNA loci in lower eukaryotes (Aravin et al., 2003; Brennecke et al., 2007; Houwing et al., 2007). Various epigenetic mechanisms have been described for controlling repetitive elements (Zaratiegui et al., 2007). Although the biogenesis model proposed above for fly piRNAs suggests a PTGS mechanism for transposon control, it does not rule out TGS processes.

The link between repetitive elements and piRNAs is less obvious in mammals, because repeat element sequences are underrepresented in pachytene piRNA sequences (~20%), compared to the ~40% total repetitive elements in the genome (Girard et al., 2006; Lau et al., 2006). However, prepachytene piRNAs are more enriched in repeats (35%), and Mili mutant testis exhibit substantial DNA demethylation of the L1 retrotransposon (Aravin et al., 2007). In addition, the knockout of Miwi2, a third mouse Piwi member, results in transposon expression and loss of DNA methylation at some transposon loci (Carmell et al., 2007). These studies suggest a role for mammalian

Piwi proteins in TGS by regulating transposable elements (TEs) via an RNA-dependent DNA methylation mechanism. The mammalian germline is known to undergo extensive genome-wide reprogramming events during development—perhaps a portion of these events involve Piwi and piRNAs, given the abundant, germline-specific expression of this ribonucleoprotein. However, it remains to be determined whether piRNA complexes target transposable elements throughout the genome, or whether they target the piRNA clusters in *cis*. Determining the chromatin structure at piRNA loci will be important for assessing their role in TGS.

Piwi's Roles in Germline and Stem Cell Maintenance

An epigenetic role for Piwi proteins in animals is an attractive hypothesis, because epigenetic regulation has emerged as an important paradigm for how stem cells and germ cells maintain pluripotency. Such a consideration puts into new perspective the rich biology of germline and stem cell regulation that was previously characterized for animal Piwi proteins. Fly *piwi* and *aubergine* mutants have been known to have defects in gaemetogenesis (reviewed in Carmell et al. [2002]). Female *aubergine* flies produce mostly deformed oocytes, whereas male *aubergine* mutants are sterile because sperm are unable to silence the deleterious and repetitive *Stellate* elements. Male and female *piwi* mutants begin with normal numbers of germ cells at the onset of gametogenesis, but the renewal of the germ stem cells are disrupted and adults become completely depleted of gametes. Knockdowns of both *Caenorhabditis elegans* Piwi homologs, *prg-1* and *prg-2*, produces various penetrance of reduced gonads (Cox et al., 1998); however, only a knockout of *prg-2* results in a temperature-sensitive sperm defect (C.C. Mello and P.J. Batista, personal communication). Because *C. elegans* has an expanded class of worm-specific Ago members that segregate from both the Ago and Piwi subfamilies (Tolia and Joshua-Tor, 2007), assessing function and redundancy of *prg-1* and *prg-2* is complicated.

The germline role for Piwi proteins is conserved in mammals. Homozygous male knockout mice of Miwi, Mili, and Miwi2 become sterile as a result of arrested spermatogenesis and eventual apoptosis of the germ cells, which is evident in the smaller size of the testis (Carmell et al., 2002, 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Despite the similar phenotypic outcomes of apoptotic sperm and depleted germ cells in these knockouts, there may be subtle functional differences between the three proteins because of differential timings of expression and different aspects of spermatogenic arrest. In contrast, homozygous knockout females of Miwi, Mili, and Miwi2 are fertile and produce normal broods, suggesting either a lack of function or redundancy for these proteins in mammalian oogenesis.

Amongst vertebrates, the male-specific germline function of Piwi proteins may be unique to mammals, because other vertebrates like zebrafish depend on Piwi proteins for germ-cell development in both the testis and ovary (Houwing et al., 2007). Perhaps Piwi proteins exert a pronounced role in mammalian males because only the male germ cells undergo continuous mitosis and meiosis during adulthood, like the oocytes of flies and fish. Alternatively, Ziwi may resemble fly Piwi proteins more closely than mammalian Piwi proteins, as reflected in not only the genetic requirement in oogenesis but also the enrichment of repeat-associated piRNAs. This parallel might be extended by the maternal contribution of Piwi proteins and piRNAs to the oocyte, which has also been observed for the sea urchin Piwi homolog, Seawi (Rodriguez et al., 2005).

Maternal contribution of Piwi proteins and piRNAs, as well as the sequences of repetitive piRNA themselves, supports the hypothesis that they have roles in transposon control in the *Drosophila* and zebrafish germline. In fact, inheritance of transposon control may be a necessary component of the female germline. For example, in the genetic phenomenon of hybrid dysgenesis, the cross of a male carrying a repressed transposon to a female lacking the transposon, results in offspring containing the active transposon, which ultimately results in sterility. The reciprocal case—a cross between a female carrying a repressed transposon with a male lacking it—does not result in transmission of the transposon to the offspring. In *Drosophila*, the inheritance of transposon repression is mediated by rasiRNAs and Piwi proteins. rasiRNAs corresponding to the *Penelope* transposon mediate female germline fertility in *D.virilis* (Blumenstiel and Hartl, 2005), whereas the *flamenco* locus, a master controller of the activity of the retroviral *gypsy* element, is a rasiRNA cluster that, when mutated, results in severe reductions in *flamenco*-derived rasiRNAs (Brennecke et al., 2007). Additionally, a *piwi* mutant results in elevated *gypsy* transcription (Brennecke et al., 2007; Pelisson et al., 2007). In mice, the prepachytene piRNAs map to loci that resemble the master transposon control loci in *Drosophila*, and together with Mili, may have a function in transposon suppression (Aravin et al., 2007).

There is both supporting and confounding evidence for the hypothesis that Piwi proteins serve an epigenetic role in nuclear surveillance. Piwi is observed in the nucleus as well as the cytoplasm of germline cells (Cox et al., 2000; Megosh et al., 2006; Saito et al., 2006), whereas Ago3 and Aub are mostly detected in the cytoplasm and in germline granules called nuage (Brennecke et al., 2007; Gunawardane et al., 2007; Harris and Macdonald, 2001). Miwi and Ziwi also appear to localize intensely in perinuclear germline granules (Grivna et al., 2006b; Houwing et al., 2007), and in particular, Miwi is a component of the chromatoid body, a dynamic cytoplasmic structure that repeatedly contacts the nuclear periphery (Kotaja and Sassone-Corsi, 2007). Finally, a fraction of Miwi comigrates with polysomes on density gradients, suggestive of a large cytoplasmic piRNA complex (Grivna et al., 2006b). However, the cytology of Piwi will not necessarily reflect the dynamic functions of this protein. For example, Ago1

in *S. pombe* clearly regulates chromosome segregation, yet cytology shows pronounced cytoplasmic staining and modest nuclear localization (Buker et al., 2007). Studies that catalog interacting partners of Piwi proteins should yield insight into these issues. For example, evidence has suggested that Seawi can interact with the spindle apparatus (Rodriguez et al., 2005), whereas *aubergine* ovarioles have disorganized microtubules (Klattenhoff et al., 2007). Thus, it is possible that animal Piwi proteins in the cytoplasm are poised in machinery that impacts chromatin during cell division.

The strongest evidence for a direct epigenetic role for Piwi-like proteins has been described in ciliates, which have expanded the numbers of Piwi members in their genome (and notably do not contain Ago-subclade members, Figure 1). TWI1 is essential for conjugative replication and maturation of the macronucleus, or the "somatic" genome that is actively transcribed and is separate from the micronucleus, or "germline" genome that is transcriptionally inert (Mochizuki and Gorovsky, 2004). TWI1 triggers site-specific elimination of repetitive, transposon-like sequences within the macronucleus by using scnRNAs to direct H3K9-Me marks to these loci. Thus, in contrast to animals, TWI1 seems to directly impact the somatic genome rather than the germline in the ciliate.

Do Piwi proteins have a broader function beyond regulating the germline? Although the data for mammalian Piwi proteins impacting somatic stem cells is still unclear, a remarkable example of Piwi proteins regulating stem cells is Smedwi-2 from the flatworm planaria *Schmidtea mediterranea*. Smedwi-2's expression pattern near-completely coincides with the patterns of the neoblasts, totipotent stem cells that endow planarians the extraordinary capacity to regenerate entire organs and body structures (Reddien et al., 2005). Knockdown of Smedwi-2 significantly impairs planaria from restoring body parts after wounding; however, this does not alter the expansion and migration of neoblasts to the wound. Rather, proper differentiation of the neoblast progeny is perturbed, and renewal of neoblasts at the damage site is not maintained (Reddien et al., 2005). Although planarian piRNAs have not been described, it is tempting to speculate what genetic elements are being regulated by Smedwi-2.

Resolving the function of Piwi proteins and piRNAs has broad implications not only in understanding their essential role in fertility, germline, and stem cell development but also in the basic control and evolution of animal genomes. Because intergenic and repetitive elements comprise the majority of animal genomes, cells must effectively deal with this complex yet poorly understood bulk sequence. Piwi proteins and piRNAs add to our comprehension of genomic elements that previously were not known to transcribe functional gene products.

ACKNOWLEDGMENTS

We thank K. Collins, C.C. Mello, K. Mochizuki, and M. Siomi for unpublished sequencing and other data. We apologize for omitted citations due to space constraints. N.C.L. is supported by a Whitney Foundation Fellowship, and R.E.K. is funded by the National Institutes of Health.

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