Chapter 5
Piwi Proteins and piRNAs Step onto the Systems Biology Stage

Josef P. Clark and Nelson C. Lau

Abstract Animal germ cells are totipotent because they maintain a highly unique and specialized epigenetic state for its genome. To accomplish this, germ cells express a rich repertoire of specialized RNA-binding protein complexes such as the Piwi proteins and Piwi-interacting RNAs (piRNAs): a germ-cell branch of the RNA interference (RNAi) phenomenon which includes microRNA and endogenous small interfering RNA pathways. Piwi proteins and piRNAs are deeply conserved in animal evolution and play essential roles in fertility and regeneration. Molecular mechanisms for how these ribonucleoproteins act upon the transcriptome and genome are only now coming to light with the application of systems-wide approaches in both invertebrates and vertebrates. Systems biology studies on invertebrates have revealed that transcriptional and heritable silencing is a main mechanism driven by Piwi proteins and piRNA complexes. In vertebrates, Piwi-targeting mechanisms and piRNA biogenesis have progressed, while the discovery that the nuclease activity of Piwi protein is essential for vertebrate germ cell development but not completely required in invertebrates highlights the many complexities of this pathway in different animals. This review recounts how recent systems-wide approaches have rapidly accelerated our appreciation for the broad reach of the Piwi pathway on germline genome regulation and what questions facing the field await to be unraveled.

Keywords Piwi pathway • Small regulatory RNA • Germ cell development

1 Introduction: RNA Interference (RNAi) Pathways in Animal Germ Cells

Gene expression control is the sum of both gene activation and gene repression, and in nearly all animal cells, RNAi is a central pathway for cells to execute broad and rapid gene silencing at the transcriptional and posttranscriptional level.

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G.W. Yeo (ed.), *Systems Biology of RNA Binding Proteins*, Advances in Experimental Medicine and Biology 825, DOI 10.1007/978-1-4939-1221-6_5

Each cell type expresses specific repertoires of genome-encoded small regulatory RNAs that become incorporated into ribonucleoprotein (RNP) complexes. These small RNAs then serve as guides to direct the RNP complexes to search out target transcripts and genomic loci, thereby providing a dynamic closed circuit for gene regulation. In essence, the activation of a small RNA-producing gene leads to the repression of a target gene with base-pairing homology to the small RNA.

In animal cells, the most ubiquitous arm of RNAi is the microRNA (miRNA) pathway. The 20–23-nt-long miRNAs are incorporated into Argonaute (AGO) proteins and have evolved to search for messenger RNA (mRNA) targets using the complementarity of the first 2–9 base pairs in the 5′ end of the miRNA to "seed" an interaction before locking the interaction in through a combination of mismatches and pairings with the rest of the miRNA (Bartel 2009). The AGO-miRNA RNP forms the core of a larger, less defined RNA-induced silencing complex (RISC) that typically seeks the 3′ untranslated regions of target mRNAs and can induce inhibition of mRNA translation as well as mRNA destabilization. Although animal genomes encode several hundreds of different individual miRNA sequences, different cell types can express specific sets of miRNAs because each miRNA derives from a single small hairpin-structured precursor (~60–100 bp) that can sit in the middle of an intron or a longer noncoding transcript made by RNA Polymerase II (Pol II) (Carthew and Sontheimer 2009). Despite being short, some miRNAs have deep conservation through their entire mature miRNA sequence, such as miR-1 and miR-Let-7, which may be attributed to the broad number of mRNA targets regulated by these miRNAs (Ambros 2011).

A second arm of RNAi is the endogenous small interfering RNA (endo-siRNA) pathway which is found in invertebrate somatic cells and only mammalian oocytes, cells which do not express vertebrate innate immunity factors that drive cellular shutdown in the presence of long double-stranded RNA (dsRNA) (Okamura and Lai 2008). Although endo-siRNAs are generally ~21 nt long, they are different from miRNAs because they are thought to derive from longer (>100 bp) dsRNA precursors forming from very long fold-back structures, from two RNAs from different loci interacting in *trans*, or from the direct conversion of an mRNA into dsRNA by an RNA-dependent RNA Polymerase (RdRP). In flies, endo-siRNAs preferentially load into Ago2 as opposed to miRNAs tending to load into Ago1. In nematodes, endo-siRNAs partner with a myriad of AGO homologs; however in mammals, the distinction between miRNA- and endo-siRNA-AGOs is unclear. The target selection mechanisms for endo-siRNAs are presumed to entail mainly complete complementarity towards genes, repetitive elements such as transposable elements (TEs), and viral transcripts (Ghildiyal and Zamore 2009). The physiological role for endo-siRNAs in animal development remains unclear because mutants that specifically disrupt endo-siRNA accumulation in *Drosophila* have subtle phenotypes, whereas in mammals there is only one Dicer enzyme that processes both miRNA and endo-siRNAs, thus complicating the analysis of endo-siRNAs alone. However, endo-siRNAs generated via RdRPs are likely involved in important gene regulatory pathways like nematode dauer formation (Hall et al. 2013), and antiviral responses in flies (Goic et al. 2013).

2 The Piwi Pathway: A Germ Cell Innovation

The third arm of RNAi is the Piwi-interacting RNA (piRNA) pathway, which is distinct from miRNAs and endo-siRNAs because piRNA biogenesis does not depend on Dicer, the key enzyme that matures miRNAs and endo-siRNAs (Ishizu et al. 2012). Although Piwi proteins form a distinct subclade of the AGO protein family, Piwi proteins and piRNAs are mainly enriched in animal germ cells while miRNAs bound by AGO proteins are ubiquitous in somatic cells. Furthermore, piRNAs appear to derive from single-stranded transcript precursors that are noncoding with no annotated features, are transcripts that correspond to the 3′UTR of certain protein-coding genes, or are transcripts that bear an unusually high concentration of transposable element (TE) sequences. Somehow, these transcripts are selected and processed into a diverse array of piRNAs for which the pattern of piRNAs sometimes appears erratic yet nonrandom (Betel et al. 2007), such as some piRNAs that repeatedly accumulate more abundantly, and many preferentially begin with a 5′ uridine or having an adenine at position 10 (see below about the "ping-pong" cycle, and Siomi et al. 2011).

Despite the characteristics mentioned above, our understanding of piRNA biogenesis is still in its infancy. We know that piRNAs are longer in length (25–31 nt) than miRNAs and endo-siRNAs (20–23 nt), the 3′ terminal 2′ oxygen of piRNAs is methylated by the Hen1 enzyme, and there are many putative helicases, putative endonucleases, and tudor-domain-containing proteins genetically required to generate and/or stabilize piRNAs (Ishizu et al. 2012). However, our mechanistic picture of the Piwi pathway is still not fully defined: the only in vitro piRNA biogenesis activity known is a "Trimmer" activity observed in *Bombyx* gonadal cell extracts which can trim a long 5′ phosphorylated transcript bound by SIWI into a mature piRNA (Kawaoka et al. 2012). Although genetic studies that placed artificial sequences like GFP into a piRNA precursor demonstrated artificial piRNA production (Kawaoka et al. 2012; Muerdter et al. 2012), we still cannot predict exactly which piRNAs would be generated most abundantly from these piRNA-generating transgenes.

However, piRNAs are clearly essential for the proper development of germ cells and fertility, because mutations that ablate Piwi protein function or piRNA biogenesis in both invertebrates and vertebrates result in germ cell death, gonadal atrophy, and ultimately sterility. One molecular consequence of losing piRNAs is the volatile expression of TE transcripts severalfold above the negligible level in wild-type animals, and TE mobilization is thought to be damaging germ cell DNA and to cause de novo mutations that result in germ cell apoptosis or failed fertilization. Since a major proportion of metazoan piRNAs are perfectly complementary to the coding sequence of TEs, the role of piRNAs in taming TEs is obvious. However, many animals have large complements of piRNAs that lack homology to TEs, and given the extraordinary diversity of piRNA populations in germ cells compared to miRNAs, the challenge is to determine the full breadth of targets that are regulated by piRNAs.

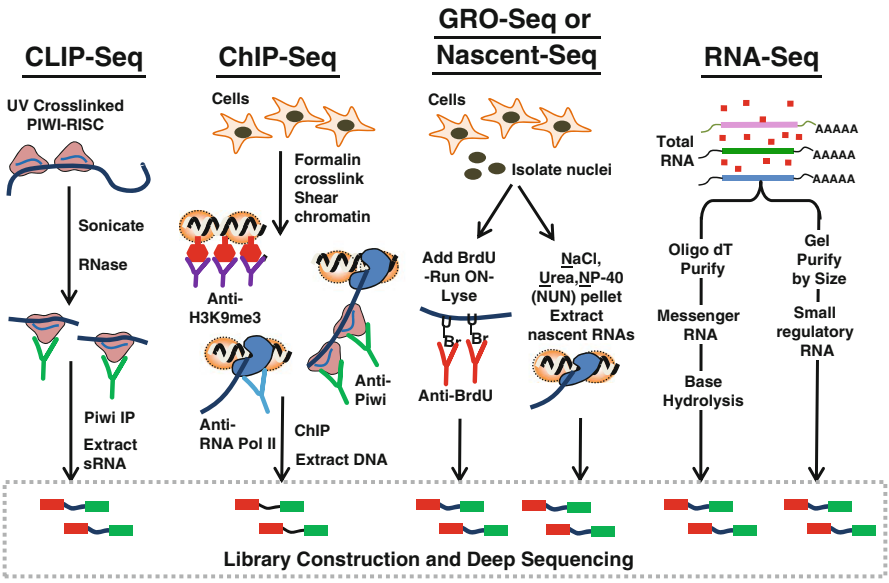

Fig. 5.1 Systems biology approaches applied to study Piwi pathways. Four main biochemical techniques now enable systems-wide analyses of the Piwi-piRNA pathway. Cross-linking immunoprecipitation (CLIP) enriches transcripts associated with Piwi proteins. Chromatin immunoprecipitation (ChIP) enriches genomic DNA regulated by Piwi complexes. Global run on (GRO) and nascent RNA analyses reinforce a transcriptional gene silencing role by Piwi, while messenger RNA and piRNA analyses were facilitated by high-throughput deep sequencing of cDNA libraries

In this chapter, we cover the monumental progress in recent years towards dissecting the functions and mechanisms of Piwi protein complexes and piRNA biogenesis factors, due in large part to the application of systems-wide approaches to this biomedically and biologically important RNA-binding protein pathway. These systems-wide approaches have been built around the extensions of biochemical techniques that recover RNAs and genomic DNAs and characterizing the nucleic acids extremely thoroughly with high-throughput deep sequencing technologies (Fig. 5.1). Supporting these approaches have been heavy dependence on available sequenced and assembled animal genomes and the application of new bioinformatics tools and infrastructure to handle large datasets.

In this review, we first focus on piRNAs from the nematode *Caenorhabditis elegans*, which are quite distinct from other metazoan piRNAs, but appear to influence epigenetic inheritance. Next, we discuss new insight into piRNA biogenesis and targeting mechanisms uncovered in *Drosophila* mutants, ovarian cell line studies, and genome-wide knockdown screens. Finally, we examine the latest findings of vertebrate Piwi protein function and biogenesis that highlight what may be specific distinctions of the Piwi pathway that differ between vertebrates and invertebrates. Because we will be covering mainly the latest progress on how systems biology approaches have propelled the Piwi pathway field, we refer readers to several recent

reviews that provide additional detailed and historical coverage of the Piwi field, from early genetics and cell biology to the advent of deep sequencing technology that have provided comprehensive views of piRNA diversity (Lau 2010; Juliano et al. 2011; Siomi et al. 2011; Ishizu et al. 2012).

3 Astounding Argonaute Diversity in *C. elegans*

The nematode *C. elegans* has the distinction of being the first animal where RNAi was described (Fire et al. 1998), where the first two miRNAs were cloned (Lee et al. 1993; Reinhart et al. 2000), and where its small RNAs were first deeply sequenced by next-generation sequencing technology (Ruby et al. 2006). The conservation of Piwi protein function in animal germline development was also first demonstrated in *C. elegans* via RNAi knockdown more than a decade ago (Cox et al. 1998). However, thorough understanding of *C. elegans* Piwi protein mutations has now progressed significantly due to the investigations of the expanded family of other AGO genes in *C. elegans*: 21 worm-specific AGOs (WAGOs and others) accompany the metazoan AGO-related *alg-1 and -2* and the Piwi-related genes *prg-1*, *prg-2*, and *ergo-1*. As such, *C. elegans* has a smorgasbord of different small RNAs: miRNAs, exogenous siRNAs, endogenous siRNAs that come in two flavors (22G-RNAs and 26G-RNAs named by their length and most common first nucleotide), and piRNAs that are also known as 21U-RNAs (Fischer 2010; Ketting 2011). Although miRNAs partition into ALG-1 and ALG-2 complexes, and 21U-RNAs partition into PRG-1 complexes, the line is blurry amongst which assorted worm AGO proteins load which particular 22G- and 26G-RNAs (Lau 2010; Claycomb 2012).

Before the systems biology revolution swept over *C. elegans*, it was assumed at first that the miRNA, piRNA, and endo-siRNA pathways were somewhat distinct pathways with just a few shared enzyme factors. Although Dicer is required for the biogenesis of miRNAs, primary exogenous siRNAs that feed into RDE-1, and the 26G-RNAs, there seems to be a separated generation of secondary endo-siRNAs by different RdRPs. RRF-3 is required for 26G-RNA biogenesis whereas RRF-1 and EGO-1 are required for 22G-RNA biogenesis. Mutations in *prg-1/prg-2* that affect 21U-RNA biogenesis do not affect overall endo-siRNA function (Batista et al. 2008; Das et al. 2008), while mutations that affect 22G-RNA and 26G-RNA accumulations do not impact 21U-RNA production (Han et al. 2009; Pavelec et al. 2009).

In fact, most mutations that affect endo-siRNA formation have not displayed overt phenotypes other than an enhanced RNAi (ERI) response when exogenous dsRNAs are used to target endogenous genes. However, notable exceptions are mutations that perturb 22G-RNA production in the germline (*csr-1*, *ego-1, drh-3*, *ekl-1*, and *cde-1*), and *eri-3* and *alg-3/alg-4* mutations that affect sperm-dependent 26G-RNAs (Pavelec et al. 2009; Conine et al. 2010). The *csr-1* and *ego-1* mutant embryos have severely compromised cell division cycles so that they arrest early in embryonic development (Claycomb et al. 2009; She et al. 2009), whereas *eri-3* and *alg3/4* mutants display decreased sperm count at elevated temperatures compared to

wild type (Pavelec et al. 2009; Conine et al. 2010). Gonadal development defects were also observed in *prg-1* mutants particularly at elevated temperatures, but at below the standard rearing temperature of 20 °C, these mutants can sustain gametogenesis despite lacking 21U- and 26G-RNAs (Batista et al. 2008; Das et al. 2008). In particular, *ergo-1* is a PIWI-subclade AGO gene whose mutants fail to generate 26G-RNAs yet appear nearly normal in development (Vasale et al. 2010). These mild phenotypes under laboratory conditions seen in mutants lacking most endo-siRNAs and piRNAs populations underscore the mystery of how nematodes can compensate so well when other metazoan germ cells are so dependent on the Piwi pathway.

4 *C. elegans* piRNAs Are Different from Other Metazoan piRNAs

Although the ERGO-1 and PRG-1/PRG-2 proteins have sequence homology to other metazoan Piwi proteins, we believe that *C. elegans* piRNAs are highly distinct from metazoan piRNAs, contrary to the impressions of similarity given from phylogenetic trees. First, the 21U-RNAs bound by PRG-1/PRG-2 are quite sharply restricted to 21 nt long and shorter than the 24–31 nt length of other animal piRNAs. Second, although the 26G-RNAs bound by ERGO-1 are more similar in length to other animal piRNAs, they require Dicer and the RdRP RRF-3 for biogenesis (Vasale et al. 2010), whereas general animal piRNA biogenesis in germ cells is independent of Dicer and RdRP activity (Vagin et al. 2006; Houwing et al. 2007). Third, many other animal piRNAs mature as a complex cluster of overlapping small RNAs derived from a long intergenic transcript (see sections below), but 21U-RNA and 26G-RNA genomic loci are also strikingly different in their configuration.

Rather than a single long transcript that gives rise to thousands of overlapping piRNAs in other animals, 21U-RNAs arise from thousands of individual miniature loci that consist of a small GTTTC-containing motif and other signatures existing upstream of the sequence that is putatively transcribed as a short transcript (Ruby et al. 2006). Transcription of 21U-RNAs may be triggered by worm-specific Forkhead box transcription factors binding at or near this motif to recruit RNA Pol II (Cecere et al. 2012), and then an ~26-nt-long capped transcript is then somehow processed at both the 5′ and 3′ ends to yield a mono-phosphorylated 21U-RNA (Gu et al. 2012). In the genomes of *C. elegans* and the related species *C. briggsae*, the 21U-RNA loci are all clustered together in two main regions of chromosome IV and chromosome I, suggesting an evolutionary requirement of this arrangement perhaps to facilitate epigenetic control (Ruby et al. 2006; Shi et al. 2013). However, the close proximity of each 21U-RNA locus to each other in *C. elegans* genome is not required for 21U-RNA biogenesis, because an autonomous transgene with a single 21U RNA locus can efficiently produce an exogenous 21U-RNA (Billi et al. 2012). This indicates that we do not fully understand the transcription termination control and the genetic requirements for the concentration of 21U-RNA loci into clusters.

Comparative genomics of small RNA populations between the soil nematodes of the *Caenorhabditis* genus (Shi et al. 2013) and other parasitic nematodes such as *Ascaris* further highlight the conundrum of the functional role of nematode piRNAs (21U-RNAs). The total number of 21U-RNAs in *C. elegans* may still be in flux between ~10,000 and ~24,000 individual sequences as different approaches to library construction, greater sequencing depth, and bioinformatics predictions sort out the final tally (Gu et al. 2012; Shi et al. 2013). However, a recent study reported that the 21U-RNA gene number may be doubled in *C. remanei* and *C. brenneri* compared to *C. elegans* and *C. briggsae*. The Shi et al. study suggested that 21U-RNA diversification may be more important for germline development in gonochoristic nematodes (male and female sexes, *C. remanei* and *C. brenneri*) compared to androdioecious nematodes (male and hermaphrodite sexes, *C. elegans* and *C. briggsae*). However, the parasitic nematode *Ascaris suum* is also gonochoristic yet appears to completely lack PRG-1/PRG-2 homologs and 21U-RNAs all together, even though *Ascaris* produce miRNAs and 22G- and 26G-RNAs (Wang et al. 2011). *Ascaris* gonocyte production is extremely prolific, and there is also a chromosome diminution process in somatic cells of the embryos which eliminates DNA sequences that are typically only expressed in the germline. Although chromosome diminution and maintenance of the germline in protozoans are dependent on small RNA pathways, it currently seems that *Ascaris* small RNAs are not involved in chromosome diminution. Notwithstanding that the miRNA pathway is conserved in *Ascaris*, we conjecture that nematode versions of the piRNA and endo-siRNA pathways are much more extensively evolved to suit a soil or a gut environment, such that their RNAi pathways are quite distinct from their counterparts in other metazoans.

5 Worm Piwi Pathways: More Interconnected than We Thought

Despite the differences between *C. elegans* piRNAs and other animal piRNAs, the field has also searched for commonality amongst these piRNAs. Since the Piwi pathway clearly suppresses TEs in fly and mammalian germ cells, TEs were examined in the *prg-1* and *prg-2* null mutants, and only a limited number of TEs such as Tc3 showed a robust up-regulation of its transcript in mutants despite little evidence of TE mobilization (Batista et al. 2008; Das et al. 2008). Another shared feature with general animal piRNAs is that 21U- and a subset of the 26G-RNAs are specifically methylated at the 3′ terminal 2′ hydroxyl by the HENN1 RNA methylase, similar to fly, fish, and mammalian piRNAs and endo-siRNAs being methylated by HEN1 orthologs (Billi et al. 2012; Kamminga et al. 2012; Montgomery et al. 2012). Despite its evolutionary conservation, the functional importance of Hen-1 for general animal piRNAs and endo-siRNAs is not yet clear because in the fly *hen-1* mutant there is only a modest decrease in piRNA and endo-siRNA levels and few obvious developmental defects (Horwich et al. 2007; Saito et al. 2007). However, *C. elegans* 21U-RNA and 26G-RNA levels are clearly perturbed and diminished,

respectively, in *henn-1* null mutants (Billi et al. 2012; Kamminga et al. 2012; Montgomery et al. 2012). Although there are indications that secondary endo-siRNAs like 22G-RNAs are not modified with a 2′-O-methyl mark (Montgomery et al. 2012), the gene-silencing function of the 22G-RNAs is affected in *henn-1* mutants due to the genetic connection between 26G-RNAs and 22G-RNAs.

The genetic connection between 22G- and 26G-RNAs has been previously appreciated with the isolation and characterization of several mutants that display consistent loss of 22G-RNA accumulation and function whenever 26G-RNAs were also affected (Conine et al. 2010; Gent et al. 2010; Vasale et al. 2010; Zhang et al. 2011a). As mentioned earlier, the expanded variety of worm AGO proteins mirrors the diversification of 26G- and 22G-RNAs: some small RNAs are germline or soma specific (Yigit et al. 2006; Conine et al. 2010; Vasale et al. 2010), sperm or oocyte specific (Han et al. 2009; Gent et al. 2010), perhaps even specific to developmental stages and environmental responses (Hall et al. 2013). The emerging model is that 26G-RNAs are the primary endo-siRNA trigger that initiates pairing to a target transcript, which then subsequently stimulates 22G-RNA production and amplification of the silencing process through worm-specific RdRPs and AGO proteins (Fig. 5.2).

Although this 26G- to 22G-RNA link is now apparent, earlier Northern blots indicated that 21U-RNA accumulation was not linked to bulk 26G-RNA nor 22G-RNA biogenesis, which predated our understanding that there are two overarching cohorts of 22G-RNAs: the CSR-1-specific 22G-RNAs versus the WAGO-specific 22G-RNAs (Claycomb et al. 2009; Gu et al. 2009). By constructing *C. elegans* strains containing integrated transgene reporters that could be targeted and silenced by a 21U-RNA and PRG-1, three groups performing small RNA deep sequencing discovered that exogenous, transgene-specific 22G-RNAs were generated in a *prg-1-dependent* manner (Ashe et al. 2012; Bagijn et al. 2012; Buckley et al. 2012; Lee et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012). The most abundant sets of these exogenous 22G-RNAs accumulated proximally from the 21U-RNA-binding site. Interestingly, exogenous 22G-RNAs and transgene silencing were maintained even with two mismatches between the 21U-RNA and the binding site (Bagijn et al. 2012). Since few perfectly complementary targets to 21U-RNAs had been detected, the search was broadened to 21U-RNA-binding site with up to three mismatches and a strong correlation could now be seen between some populations of endogenous 22G-RNAs and predicted 21U-RNA-binding sites on the genome (Bagijn et al. 2012; Lee et al. 2012). Two genes detected as being strongly mis-regulated in the *prg-1* mutant were *bath-45* and *F54F2.2b* which lost almost all 22G-RNAs in the *prg-1* mutant. However, other genes dependent on *prg-1* to produce antisense 22G-RNAs were rather modestly up-regulated while many other genes and TEs that have associated 22G-RNAs retain them regardless of *prg-1*. Why did *prg-1* have such a strong influence on a subset of 22G-RNAs against certain genes and not have much effect on other 22G-RNAs, sometimes against the same gene or elements like Tc3?

To resolve this conundrum, two groups tracked derepression of their 21U-RNA fluorescent reporter transgene in various mutant backgrounds, and discovered that

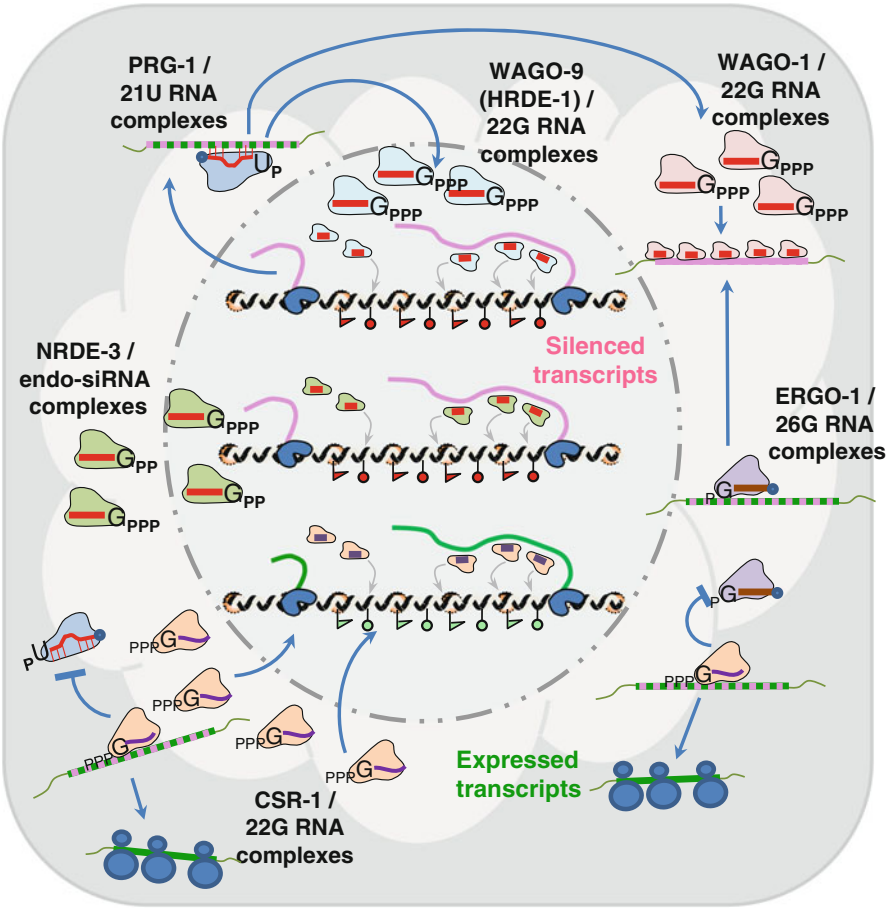

Fig. 5.2 The *C. elegans* Piwi pathway is genetically connected to other endogenous RNAi pathways. This diagram represents a developing germ cell in *C. elegans* with several small RNA pathways operating concurrently, some in the nucleus like the PRG-1 and WAGO-9 complexes and the NRDE-3 and CSR-1 complexes, while ERGO-1 and WAGO-1 are speculated to work in the cytoplasm. These current models depict these small RNA complexes in either silencing or maintaining gene expression in the *C. elegans* germline. Many important biogenesis factors are omitted (i.e., RdRPs, Dicer, helicases) because this diagram has been simplified to focus on the AGO-small RNA complexes

prg-1-mediated silencing in the germline was mainly linked genetically with WAGO-9/HRDE-1, a nuclear-localized worm-specific AGO (WAGO) protein that binds 22G-RNAs generated from upstream factors like *rrf-1*, *mut-7*, *drh-3*, and *rde-2* (Bagijn et al. 2012; Lee et al. 2012). These WAGO-bound 22G-RNAs are distinct from CSR-1-bound 22G-RNAs even though biogenesis factors like *drh-3* may be shared and both CSR-1 and WAGO-9/HRDE-1 are abundantly expressed in the germline. Whereas WAGO-9/HRDE-1 is primarily nuclear, CSR-1 is mostly concentrated in the cytoplasm and perinuclear organelles called P-granules where some

RNA processing events are speculated to occur. Nevertheless, CSR-1 may also be exerting effects in the nucleus because during mitosis CSR-1 can be seen on the metaphase plate and has been shown by chromatin immunoprecipitation (ChIP) to associate with target genomic loci (Claycomb et al. 2009). Additionally in the *C. elegans* germline, there are ERGO-1 and ALG-3/ALG-4 AGO proteins binding 26G-RNAs that trigger downstream 22G-RNAs which load into other worm AGO proteins like WAGO-1 (Conine et al. 2010; Vasale et al. 2010). Thus, the PRG-1/21U-RNA complex specifies mainly the downstream generation of WAGO-9/HRDE-1/22G-RNA complex in an analogous fashion to how ERGO-1 and ALG-3/ALG-4 direct downstream formation of the WAGO-1/22GRNA and other WAGO complexes. The interplay of the WAGO-9/HRDE-1/22GRNA complex with the CSR-1/22GRNA complex is discussed next.

6 Germline "Everlasting": Small RNAs and the Chromatin Connection.

For a simple animal with a highly streamlined genome, the *C. elegans* germline has a staggering number of different small regulatory RNAs and AGO proteins (Fig. 5.2). In the oocyte alone, the current tally is at least four small RNA pathways: the NRDE-3 pathway, the CSR-1 pathway, the PRG-1::WAGO-9/HRDE-1 pathway, and the ERGO-1::WAGO-1 pathway (which can be impacted upstream by the PRG-1::WAGO-9 pathway). Other articles have reviewed gene expression control and gene silencing in the *C. elegans* germline, which also includes the mechanism of silencing unpaired chromosomes during meiosis (Fischer 2010; Lau 2010; Ketting 2011; Claycomb 2012). The salient points for this review are that endogenous RNAi pathways are essential for proper germline development to generate gametes that form the totipotent zygote, and *C. elegans* embryogenesis is extracorporeal, so maternal stores of mRNAs, proteins, and lipids must be deposited and organized in the oocytes.

To highlight one essential maternal factor, the CSR-1 complex is absolutely required for proper embryonic cell division and chromosome segregation events, and molecular studies suggest that 22G-RNAs guide CSR-1 to specific chromatin domains in order to help establish the boundaries needed to segregate the holocentric chromosomes of *C. elegans* (Claycomb et al. 2009; van Wolfswinkel et al. 2009). Thus, CSR-1 represents one important layer of epigenetic control in the germline.

However, the future challenge will be to understand why there are so many additional concurrent small RNA-AGO complexes in the *C. elegans* germ cell that are acting either redundantly or antagonistically. For example, one group has proposed that CSR-1 may serve an anti-silencing role in antagonizing the gene-silencing activities of the WAGO complexes, but the details are obscure for how these two complexes bind similar 22G-RNAs yet partition and regulate different genes ((Shirayama et al. 2012), Fig. 5.2). Furthermore, in addition to the nucleus-directed

PRG-1::WAGO-9/HRDE-1 pathway, there is also the NRDE-3 pathway that is triggered by exogenous siRNAs and also leads to gene silencing at the chromatin level in the nucleus (Guang et al. 2010). Although the *nrde-1*, *nrde-2*, and *nrde-4* genes are required for gene silencing by the NRDE-3/endo-siRNA complex and the WAGO-9/HRDE-1/22G-RNA complex, NRDE-3 is not required for the inherited silencing by WAGO-9/HRDE-1 (Ashe et al. 2012; Buckley et al. 2012). We summarize that there is a cross talk in common genetic factor as well as partitioning of targeting functions and primary triggers in *C. elegans* germline RNAi pathways, and much further study is needed to sort all these pathways out.

A new paradigm proposed in these recent studies is that these multiple germline RNAi processes establish a mechanism for controlling and discriminating nonself versus self transcripts (Ashe et al. 2012; Shirayama et al. 2012). This paradigm argues that when a complex containing PRG-1 and a 21U-RNA fortuitously binds to a foreign transgene, a TE, or a virus (via base mismatches analogous to miRNA recognition), this event licenses WAGO-9/HRDE-1 to mount a seemingly permanent silencing of the nonself locus. This model is compelling for artificial transgenes, but is less able to explain why most *C. elegans* TEs and viruses are not massively mis-regulated in *prg-1* and *wago-9/hrde-1* mutants. Perhaps these nonself elements are redundantly controlled by other WAGO/endo-siRNA complexes like NRDE-3 or WAGO-1.

While *prg-1* and *wago-9/hrde-1* mutant germlines appear as fertile as wild type, perhaps reflecting the modest expression changes in limited numbers of genes (Batista et al. 2008; Das et al. 2008; Bagijn et al. 2012; Lee et al. 2012), a possible explanation to this conundrum is that the *wago-9/hrde-1* mutants lose germline immortality. In other words, although the brood sizes of these mutants are similar to wild type at the F1 progeny (the first descendants of a mutant in-bred cross), the F2 brood size begins to drop and reaches near complete sterility by the F5 progeny (Buckley et al. 2012). This outcome suggests that the germline may be highly sensitive to subtle yet compounding gene expression perturbations from multiple generations.

Perhaps germline immortality requires redundant levels of small RNAs for regulating key protein expression to levels that we cannot yet pinpoint as important for fertility. In fact, *prg-1* mutants only display their brood size deficiencies at elevated temperatures (the phenotype is masked at cooler temperatures), where perhaps germline transcriptional enzyme activity or other gene-regulatory processes may be more unbridled. Therefore, we propose that another natural function of the *prg-1/wago-9/hrde-1* pathway may be to keep the self transcriptome and proteome in the germline in check, perhaps to regulate endogenous soma-expressed genes that may be promiscuously expressed in the totipotent epigenetic state of the germline. This idea may also be related to the fact that the general Piwi/piRNA pathways in all other animals from flies to humans are primarily enriched in rapidly dividing germ cells, which display the widest diversity in its transcriptome (Yeo et al. 2004; Pao et al. 2006; Ravasi et al. 2006).

7 Flies Push Piwi/piRNA Insights into New Heights

Drosophila germline genetic screens pioneered the discovery of the Piwi pathway (Schupbach and Wieschaus 1989; Schupbach and Wieschaus 1991; Wilson et al. 1996; Lin and Spradling 1997) and the *Drosophila* female germline has continued to be the most fruitful system to study this pathway. Basic features of *Drosophila* piRNA biogenesis steps have been introduced here and in other reviews (Juliano et al. 2011; Siomi et al. 2011; Ishizu et al. 2012); therefore this section discusses how recent advances in our recent understanding of piRNA biogenesis and Piwi regulation mechanisms have benefited from systems biology approaches now being applied on *Drosophila* Piwi pathway mutants and *Drosophila* gonadal cell lines.

Starting with the beginning of the pathway, there has been recent progress in understanding the regulation of piRNA precursor transcription from the large intergenic piRNA clusters within pericentromeric heterochromatin. The two prototypical major piRNA clusters in *Drosophila* are the *Flamenco* locus on the X chromosome and the *42AB* piRNA cluster locus on chromosome 2R (Fig. 5.3). In addition

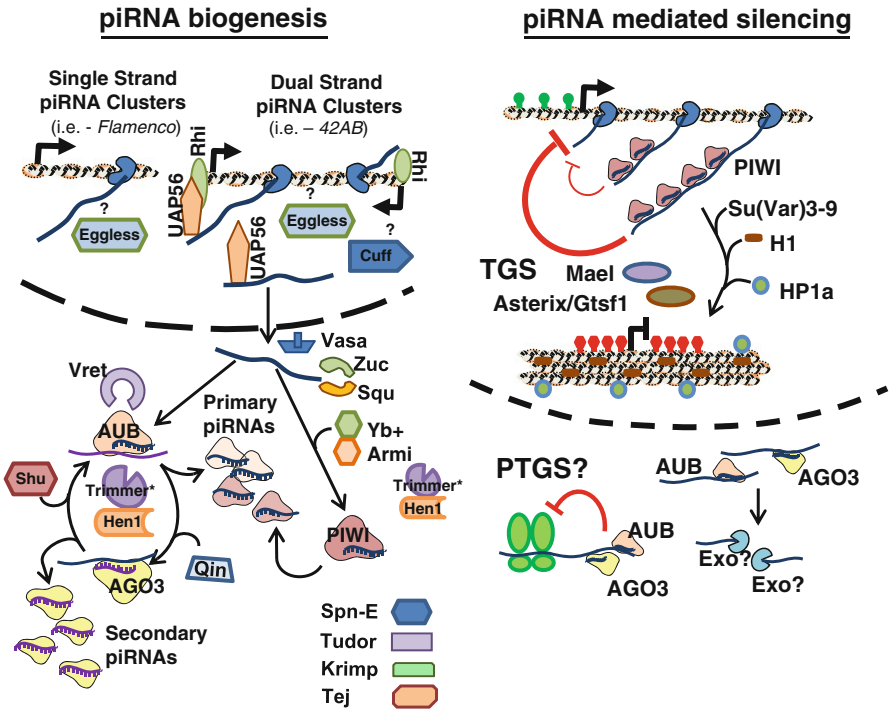

Fig. 5.3 Current models of piRNA biogenesis and piRNA-mediated gene silencing mechanisms in *Drosophila*. (*Left*) Depiction of known roles for factors genetically implicated in *Drosophila* piRNA biogenesis. More factors are known to regulate dual-strand than single-strand piRNA clusters. The piRNA precursor transcripts either are directly processed into primary piRNAs bound by AUB and

to location, these clusters are distinct because *Flamenco* generates transcripts from one genomic strand and is mainly expressed in the somatic follicle cells that surround the egg chamber, whereas *42AB* generates transcripts from both genomic strands and is mainly expressed in the nurse cells of the female germline and these transcripts are then deposited into the oocyte (Juliano et al. 2011; Siomi et al. 2011). Both clusters, however, are highly concentrated in genomic sequences corresponding to TE relics, mutated copies of TEs that have landed in these clusters to become templates for the piRNAs which can then silence the active TE copies elsewhere in the genome.

For piRNAs to be expressed abundantly in gonadal cells there is exquisite regulation of the piRNA cluster loci chromatin. The histone methyltransferase *Eggless* (also referred to as *Egg* and dSETDB1) may broadly impact transcription of both *42AB* and *Flamenco* clusters by specifically tri-methylating histone H3 on lysine 9 (leaving a H3K9me3 mark) (Rangan et al. 2011). One protein that binds the H3K9me3 mark is heterochromatin protein-1 (HP1, also known as *Su(Var)2-05*) which is genetically linked to the Piwi pathway (see below). However, a gonad-specific HP1 homolog called *Rhino* (*Rhi*) is enriched at and is required for transcription of the *42AB* piRNA cluster (Klattenhoff et al. 2009). Associated with *Rhi* at the *42AB* cluster is *Cutoff* (*Cuff*), a germ cell-specific protein homologous to the Rai1 nuclease, and *Cuff* is required for expression of both *42AB* cluster expression and a single-strand piRNA cluster called the *20A* cluster (Pane et al. 2011). Despite appearing as general chromatin-associated factors, *Rhi* or *Cuff* is required only for *42AB* piRNA expression but not *Flamenco* piRNA expression, and questions remain as to what specifies RHI and CUFF to only localize at one piRNA cluster rather than being a general factor like EGG.

After transcription, RNA processing and transport are likely to play a special role in handling piRNA precursor transcripts. Vasa is one well-known germ cell-specific RNA-binding protein with putative helicase activity because it contains a DEAD-box domain, and mutations in this gene abrogate *42AB* piRNA expression (Malone et al. 2009). Recently, a second DEAD-box containing protein, UAP56, was found to be required for piRNA expression, is localized near Rhi foci on nurse cell chromatin, and appears to bind newly transcribed piRNA-precursor transcripts from the *42AB* locus (Zhang et al. 2012). The model from this study is that UAP56 may shuttle a piRNA precursor transcript to the nuclear membrane for a hand off to Vasa for further processing at the nuclear periphery (Zhang et al. 2012).

Fig. 5.3 (continued) PIWI or engage in a subsequent secondary piRNA "ping-pong" amplification by AUB and AGO3. *The identity of "Trimmer" has not been genetically determined. (*Right*) Since AUB and AGO3 are in perinuclear nuage and cytoplasm, they may mediate posttranscriptional gene silencing. The nuclear PIWI binds to nascent TE transcripts, where it can recruit chromatin-modifying factors to induce a repressive chromatin state. Many other factors not shown in this diagram that may affect TE silencing have been identified in knockdown screens, but placement into the model is unclear, as is the role for tudor-domain-containing factors like Spn-E, Tudor, Krimp, and Tej

8 Pulling Back the Layers of piRNA Biogenesis

Some hints to the puzzle of piRNA biogenesis have come from understanding how the front and back termini of piRNAs are formulated. Despite the immense diversity of piRNA sequences, two common features define the termini of piRNAs—a preference for uridine at the 5′ end, and a highly conserved 2′O-methylation modification added to the 3′ end of piRNAs by Hen1 (Horwich et al. 2007; Saito et al. 2007). Recently, a biochemical extract from silkworm cells overexpressing the Piwi protein SIWI was able to recapitulate the activity of piRNA 3′ end formation and SIWI loading in vitro when given an ~50 bp 5′ radioactive phosphate-labeled RNA (Kawaoka et al. 2011). The Tomari group also demonstrated that the 3′ end trimming was coordinated with Hen-1-directed 2′O-methylation; however efforts to purify and identify the putative ribonuclease for "Trimmer" were stymied by the Trimmer activity being restricted to the insoluble fraction (Kawaoka et al. 2011).

Nevertheless, one factor, Zucchini (Zuc), is a tempting candidate for being a Trimmer enzyme because it is absolutely required for piRNA biogenesis and has homology to bacterial endonucleases (Pane et al. 2007). In addition, Zuc's metazoan homologs are mitochondrial phospholipase D (also known as *Pld6* in mouse) (Huang et al. 2011a; Watanabe et al. 2011; Anand and Kai 2012) and are localized to the surface of mitochondrial membranes that might exhibit an insoluble characteristic in extracts. Two recent studies solved the structures of recombinant forms of Zuc and have confirmed that Zuc has endoribonuclease activity in vitro, but the nature of the phospholipase D function remains unclear (Ipsaro et al. 2012; Nishimasu et al. 2012). Structure and biochemical data indicate that Zuc cleaves single-stranded DNA and RNA, and does so without requiring magnesium, a typical ion required by many ribonucleoprotein enzymatic activities including Argonaute's Slicer activity. The catalytic activity of Zuc was not enhanced against a 5′ uridine (5′U), the preferred starting base of many piRNAs; however its RNase activity was only enhanced with the absence of sodium chloride, which is an interesting reflection of perhaps lower ionic strengths in a membraneous, less soluble microenvironment where Zuc may act to process piRNA precursors.

The "ping-pong" model of piRNA biogenesis is one potential mechanism for specifying the 5′ end of many piRNAs. In brief, one Piwi-family protein like aubergine (AUB) prefers to bind 5′U-starting piRNAs derived from piRNA cluster loci and are antisense to the coding strand of TEs, while Argonaute-3 (AGO3) prefers to bind piRNAs that have a preference of adenine at position 10 and are derived from TE-coding strands (Brennecke et al. 2007; Gunawardane et al. 2007). In this model, AUB and its piRNA bind perfectly complementary to a TE transcript and slice the transcript to yield a cleavage product with a free 5′ phosphate. The 5′ end of this cleavage product is then bound by AGO3 and "trimming" of this transcript leads to mature piRNAs. Vice versa in fashion, AGO3 and its piRNA specify the 5′ end and loading the next AUB and piRNA complex (Fig. 5.3). While the "ping-pong" model

is not yet proven biochemically, there is genetic support from *ago3* mutations that cause the collapse of AUB piRNAs (Li et al. 2009), and signatures of the ping-pong cycle appear to be conserved in vertebrates as well (Houwing et al. 2007; Aravin et al. 2008; Lau et al. 2009). Recently, a newly identified factor called Qin, which has E3 ligase and TUDOR domains, was shown to modulate the biased loading of specific ping-pong piRNAs into AUB or AGO3. Although *qin* mutants (also known as *kumo* (Anand and Kai 2012)) still produce piRNAs, *qin* mutations perturb normal AGO3 piRNA loading and affect the protein-protein interactions between AUB and AGO3 (Zhang et al. 2011b).

The piRNA "ping-pong" cycle was originally hypothesized to be hierarchical such that primary piRNAs primed the amplification loop for secondary piRNAs (Brennecke et al. 2007; Gunawardane et al. 2007). This connection is supported by mutations in protein factors that abolish primary piRNAs from *Flamenco* which also ablate the secondary piRNAs such as the ones from the *42AB* cluster. The mutations of processing factors that destroy primary and secondary piRNA biogenesis include *armi*, *zuc*, *vert*, *shu*, and *gasz* (Saito et al. 2009; Haase et al. 2010; Zamparini et al. 2011; Olivieri et al. 2012; Preall et al. 2012) (Handler et al. 2011; Handler et al. 2013). In contrast are mutations that only affect *42AB* piRNAs but leave *Flamenco* piRNAs relatively unaffected, such as *spn-E*, *krim*, *vasa*, *rhi*, *UAP56*, *cuff*, *kumo*, and *tejas* (Lim and Kai 2007; Klattenhoff et al. 2009; Malone et al. 2009; Olivieri et al. 2010; Patil and Kai 2010; Anand and Kai 2012; Zhang et al. 2012). *Yb* is an exceptional factor because its loss of function mainly affects only the *Flamenco* piRNAs without much effect on *42AB* piRNAs, analogous to the *Flamenco* promoter mutations that only affect *Flamenco* piRNAs (Malone et al. 2009; Olivieri et al. 2010; Handler et al. 2011). Although this list of factors can be divided based on their roles in either primary or secondary piRNA biogenesis, it is still unclear how 5′ ends of primary piRNAs are defined and why the 3′UTR of certain genic transcripts are selected for primary piRNA biogenesis. Perhaps this gap is attributed to an incompleteness of the list of genes that do impinge upon the Piwi pathway.

Addressing this issue are recent reports by the Hannon and Brennecke labs on genome-wide knockdown screens in flies and cultured follicle cell lines that have now greatly expanded this gene list (Czech et al. 2013; Handler et al. 2013; Muerdter et al. 2013). Many previously identified genetic factors implicated in piRNA biogenesis were recovered and validated in these powerful screens, and gratifyingly several new factors have been identified to impact piRNA biogenesis, such as the *Drosophila* GasZ homolog CG2183, nuclear pore component Nxt1, and the sumolyation E1 ligase Uba2, just to name a few. These screens provided between ~50 and ~90 strongly validated new factors that impact the piRNA pathway in the *Drosophila* female germline, either at the biogenesis or the gene silencing effector level. Many future studies will ensue to place each of these factors in more discreet positions in the pathway.

9 Multi-talented Piwi Proteins Do TGS (and PTGS?)

Insights into how Piwi proteins carry out their effecter role in gene silencing have also dramatically increased recently due to systems approaches in *Drosophila.* Besides the *Drosophila* Piwi proteins themselves (PIWI, AUB, AGO3), a couple of Piwi pathway factors have been defined as effectors of Piwi-mediated gene silencing because their loss of function did not affect piRNA accumulation but did allow for TE transcript up-regulation. Squash (*squ*) and Maelstrom (*mael*) were two of the first known effectors because mutations or knockdowns greatly impacted fertility yet most piRNAs were sustained in these animals or gonadal cells (Pane et al. 2007; Malone et al. 2009; Haase et al. 2010). Squ has some homology to an RNase HII protein, but its biochemical activity is still not well understood, whereas Mael can associate with the microtubule-organizing center and serves a role in specifying the polarity of *Drosophila* oocytes (Sato et al. 2011). A third effecter named Asterix/Gstf1 was recently recovered in the genome-wide RNAi screens as crucial for TE repression by the Piwi pathway despite little change in piRNA production (Donertas et al. 2013; Muerdter et al. 2013; Ohtani et al. 2013). We can expect more effecters to emerge as the scrutiny turns to how the PIWI complex with piRNAs regulates targets for silencing.

With *Drosophila* PIWI being the founding member of this protein sub-clade, earlier studies had pointed to a potential transcriptional gene silencing (TGS) role for PIWI. PIWI was known to be nuclear, genetically and biochemically interacted with HP1, and was localized on polytene chromosomes in proximity to HP1 (Cox et al. 2000; Pal Bhadra et al. 2006; Brower-Toland et al. 2007). In addition, Piwi pathway mutations genetically interacted with Polycomb complex genes (Grimaud et al. 2006) and have been shown to modulate heterochromatin formation on transgenic loci harboring TE sequences as well as telomeres and insulated elements (Haynes et al. 2006; Yin and Lin 2007; Moshkovich and Lei 2010; Sentmanat and Elgin 2012). But the question remained as to how directly PIWI was involved in instigating TGS because these studies examined somatic cell phenotypes that were thought to be established by *piwi* in the germline.

Several recent systems-wide studies interrogating chromatin and nascent RNAs following perturbation of *piwi* function now clearly indicate that PIWI intimately directs TGS in gonadal cells. These studies corroborate that the loss of PIWI results in strong reduction of HP1 deposition, histone H3, and lysine 9 tri-methylation (H3K9me3), and concomitant increases in RNA Pol II occupancy at TE loci (Sienski et al. 2012; Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013). These signatures were consistent with the response of increased levels of steady-state and nascent TE transcripts across the *Drosophila* genome (Sienski et al. 2012; Sytnikova et al. 2014). These data suggest that PIWI association at TE chromatin helps recruit H3K9me3 and HP1 marks that prevent the transcription machinery from engaging. However, the answer to whether H3K9me3 and HP1 marks are the cause or the consequence of gene silencing may be complicated because the Brennecke group observed that the loss of the effecter, Mael, which allows dramatic TE up-regulation

like the loss of PIWI, had very little reduction in H3K9me3 and HP1 (Sienski et al. 2012). Future studies may clarify other notable exceptions to the link between HP1 and PIWI, such as continued HP1 deposition at certain piRNA-targeted loci in *piwi* mutants (Moshkovich and Lei 2010), and PIWI transgenes that delete putative HP1-binding motifs yet can rescue TE silencing (Wang and Elgin 2011).

Another open question regarding PIWI triggering TGS is whether PIWI binds chromatin directly or through nascent transcripts. Although one study was able to achieve a chromatin immunoprecipitation of PIWI (Huang et al. 2013), another study that performed CLIP-Seq on PIWI instead revealed that TE transcripts are the most heavily associated transcripts bound by PIWI, dominating a smaller fraction of genic transcripts (Sytnikova et al. 2014). Although coding gene expression changes have not been detected in fly ovaries deficient of *piwi* (Le Thomas et al. 2013), several genes did become strongly up-regulated in OSS and OSC cells upon *piwi* knockdown on the account of a de novo TE insertion in close proximity to the gene (Sienski et al. 2012; Sytnikova et al. 2014). By using the NUN-pellet protocol to obtain nascent transcripts, the Lau group was able to detect nascent TE transcripts up-regulated during PIWI knockdown that were independently transcribed from the nearby affected gene, and this TE transcript was complementary to the TE piRNAs. Furthermore, they showed with crafted reporter genes that PIWI and piRNAs must pair with the nascent transcript and not the DNA, and a threshold number of perfectly complementary piRNAs to the target transcript are required in order to initiate the TGS mechanism (Post et al. 2014).

Defining how far PIWI-mediated TGS can spread and what chromatin modulators are required for this spread will be an important next question to address. The reporter gene data point to a nascent transcript association requirement and is in agreement with mass spectrometry analysis of alternative splicing and nuclear RNA processing factors associated with PIWI (Le Thomas et al. 2013), indicating that PIWI/piRNA complex can rapidly interact with a TE nascent transcript and promote a transcriptionally repressed state that can spread at least a few kilobases away. As the scrutiny turns to other chromatin factors, one recent report suggests that depletion of the linker histone H1 can alter H3K9 methylation and in turn unleash TE expression as well as strangely increase the accumulation of piRNA-like species (Lu et al. 2013).

Although the recent attention of TGS mechanisms has been focused on PIWI, other diverse mechanisms of gene regulation may exist for the other Piwi family members. For instance, *Aub* can regulate *Nanos* (also known as *Nos*) mRNA expression by genetically determining its localization to the posterior pole of the *Drosophila* oocyte where it helps direct the formation of the pole plasm in the embryo (Harris and Macdonald 2001; Megosh et al. 2006). The biased transport of *Nanos* mRNA is required for anterior-posterior patterning and primordial germ cell specification during embryogenesis (Lehmann and Nusslein-Volhard 1991; Wang et al. 1994). To achieve this, AUB can bind the 3′UTR of *Nanos* mRNA directly with possible help from the Rump protein (Becalska et al. 2011). However, 4 h after fertilization, *Nanos* mRNA is also degraded; and recently *Aub* was implicated by associating with the general mRNA degradation factors like Smg and the CCR4 de-adenylase

complex (Rouget et al. 2010). Furthermore, it was suggested that the Nanos 3′UTR contained imperfect complementary to some TE piRNAs to specifically recruit AUB (Rouget et al. 2010); however this targeting model does not yet resolve how *Nanos* gets discriminately selected over other transcripts.

Interestingly, *Nanos* mRNA posterior pole localization also depends on the function of protein chaperones like Hsp90 (Song et al. 2007), and genetic phenomenon like canalization has been proposed to connect the role of Hsp90 chaperones and the piRNA pathway (Sato and Siomi 2010). The term canalization describes how organisms maintain developmental robustness despite so much variability from environmental pressures and genetic variation. Hsp90 chaperones are thought to be one level of this canalization because they help mutated or misfolded proteins still fold into functional enzymes, thereby "buffering" phenotypes to remain similar across a population of varying genotypes. However, to explain how Hsp90 perturbations allowed genetic variation to magnify the connection to piRNAs was only recently made in a study showing that genetic and chemical disruptions of Hsp90 activity can negatively affect piRNA populations and therefore yield derepression of TEs (Specchia et al. 2010). This was further supported by data showing that Hsp90 may foster posttranslational modifications of PIWI necessary for TE repression (Gangaraju et al. 2011), and a new linking of the Piwi pathway to Shu/FKBP6 as additional chaperones that may have functions related to Hsp90 (Olivieri et al. 2012; Preall et al. 2012; Xiol et al. 2012). These findings reflect how beautifully complex and intertwined these genetic and biochemical pathways are to ensure proper animal germ cell development.

10 The Role piRNAs Play in Hybrid Dysgenesis and Species Evolution

A key evolutionary pressure for the Piwi and piRNA pathway is to ensure fertility within a species, but it seems that the Piwi pathway is also a potent licensing system that opposes the formation of certain hybrids. In the hybrid dysgenesis phenomenon, daughter animals resulting from a mating of two hybrids seem to suffer from poorer health compared to parents, such as sterility despite normal development to adulthood. For intraspecies hybrid dysgenesis of *D. melanogaster* strains (Shpiz and Kalmykova 2009), dysgenesis has a pattern that suggests that a maternally deposited epigenetic factor in the embryo is a key determinant to ensure fertility, while the lack of the maternal factor means that the embryo cannot respond to a harmful paternal factor. A breakthrough in understanding hybrid dysgenesis systems was placing piRNAs as the maternally deposited factor required to respond to a particular TE imparted by the paternal chromosomes (Brennecke et al. 2008; Malone et al. 2009). Compellingly, in a *Drosophila* mother with an entirely intact Piwi pathway and diverse piRNA populations, lacking just the small complement of piRNAs against a novel TE that invades the father's germline can spell trouble in the progeny.

Interestingly, with time dysgenic daughters can begin to regain some fertility, but by what mechanism? By carefully tracking piRNAs and re-sequencing progeny genomes in the P-element system of hybrid dysgenesis, the Theurkauf group showed that P-element TEs were indeed mobilizing in the dysgenic progeny, and likely causing genomic damage in the germ cells that result in sterility (Khurana et al. 2011). But when the mobile TE lands back into a piRNA cluster, new P-element piRNAs begin to emerge in aging dysgenic progeny that also begin to regain some (but not all) fertility. This observation was bolstered by other studies demonstrating an analogous recovery from complete infertility in dysgenic progeny from an I-R element hybrid system (Grentzinger et al. 2012) and in a paramutation-based genetic system for P-elements (de Vanssay et al. 2012). Together, these studies indicate that primary piRNA biogenesis systems are insufficient to handle novel TEs contributed by the paternal genome. Instead, the gain of TEs landing into specific piRNA clusters that yield maternally deposited piRNAs is needed to stimulate some aspect of the "ping-pong cycle" for sustaining piRNA function through embryogenesis and into larval gonad development.

Although the study of the Piwi pathway in dysgenic strains of *D. melanogaster* has been extremely insightful, these strains are nonnatural products of careful laboratory rearing practices, whereas in the wild the selection pressures are stronger and more rapid on the Piwi pathway to maintain fertility. One evolutionary study has found that the piRNA compositions in *D. melanogaster* populations certainly provide evidence for a positive selection signal (Lu and Clark 2010); however there also seems to be a tolerance for *Drosophila* to allow TEs more leeway to expand in the genome, perhaps due to the canalization roles that Piwi proteins and piRNAs play. The role of piRNA incompatibility with general species barriers has also been proposed (Shpiz and Kalmykova 2009), but the first study to examine piRNAs in an actual interspecies hybrid was in a special cross of a *D. melanogaster Hmr* mutant with the closest relative *D. simulans*, in which hybrid progeny development can proceed to adulthood (Kelleher et al. 2012). Although these interspecies hybrid progeny eventually share similar phenotypic deficiencies with intraspecies dysgenic hybrids, such as distorted populations of maternally deposited piRNAs, sterility, and loss of repression for a broad number of TEs, the Barbash group could not detect a clear correlation between TEs derepressed highly in the hybrid and decreases or divergences in sequences between parental piRNAs and hybrid progeny piRNAs. Furthermore, by showing that a transgene bearing the *D. simulans Aub* was unable to rescue the *D. melanogaster aub* mutant, the authors suggest that there may be underlying deficiencies or incompatibilities between the Piwi pathway protein components of different species rather than just differences in piRNAs may account for dysgenesis in interspecies hybrids (Kelleher et al. 2012). Another evolutionary study of Piwi pathway gene codon bias may support this notion amongst *Drosophilids* (Castillo et al. 2011). These fly experiments are initial forays of evolutionary studies applied to the Piwi pathway. However, a greater diversity of model systems like vertebrates must be brought to bear to see how pervasive these genetic and evolutionary phenomena extend into animal evolution.

11 Regulating the Vertebrate piRNA Pathway

Similar to flies, mammalian genomes encode at least three clear Piwi homologs, and nomenclature for vertebrate Piwi homologs is unfortunately complicated. The first letter of the animal denotes the Piwi homologs' origin; hence the mouse encodes Miwi, Miwi2, and Mili (Miwi-like), while humans encode Hiwi, Hiwi2, and Hili. A Miwi knockout mouse was one of the first mutants demonstrating the conserved function of Piwi pathway in vertebrates for germ cell development (Deng and Lin 2002). However, as the Piwi pathway in mammals and other model vertebrates comes into better focus (Fig. 5.4), it is apparent that there are important differences between vertebrates and flies in the function and requirement of Piwi pathway components. For example, although knockout mutations in any of the loci of Miwi, Mili, or Miwi2 render male mice completely sterile because of spermatogenic arrest and atrophy of the testes, all female homozygous mutants are superficially as fertile as heterozygous littermates. This contrasts with flies where severe Piwi pathway mutations impair fertility in males and females (Cox et al. 2000), and contrast with zebrafish where Ziwi and Zili null mutants in both genders cannot form rudimentary gonads and become sterile masculinized fish (Houwing et al. 2007; Houwing et al. 2008). Although Mili and some piRNAs are expressed in mouse oocytes, the proportion of piRNAs is comparably lower with respect to abundant endo-siRNAs in mouse eggs (Tam et al. 2008; Watanabe et al. 2008), and mammalian oogenesis is typically restricted to very few mitotic divisions after birth while fish and fly ovaries continuously engage in mitotic cell divisions of oogonia. We speculate that the Piwi pathway may be most essential in germ cells that must engage in continuous rounds of mitosis during gametogenesis, but formally testing this hypothesis is challenging.

Mouse Piwi protein expression is temporally regulated during spermatogenesis, such that Miwi2 is expressed earliest and only temporarily in the embryonic gonad, whereas Miwi is expressed latest after 10 days postpartum (dpp) when the first synchronized wave of spermatogenesis approaches the pachytene stage of meiosis. Mili's expression is most pervasive, starting at 12.5 days postcoitus in male primordial germ cells and extending through adulthood. The temporal regulation of the Piwi proteins themselves coincides with three rough categories of piRNA expression: pre-natal piRNAs, pre-pachytene piRNAs, and post-pachytene piRNAs, which differ on the level of how many TE sequences and how strong a ping-pong cycle signature is present. In prenatal piRNAs, a ping-pong cycle signature exists between Miwi2 and Mili piRNAs which tend to be more enriched in TE sequences such as LINE1, the most prevalent TE class in mammalian genomes. After birth but before 12 dpp, when only Mili dominates, these pre-pachytene piRNAs have some enrichment of TE sequences suggesting that Mili can perform the ping-pong cycle with itself as well as a strong enrichment of 3′UTR genic piRNAs (Robine et al. 2009). Finally, post-pachytene piRNAs are incorporated into both Miwi and Mili, are depleted in TE sequences, almost devoid of ping-pong cycle signatures (Beyret et al. 2012), and derive from over a hundred intergenic and genic clusters that presumably express the piRNA precursor as single-stranded RNA (Robine et al. 2009).

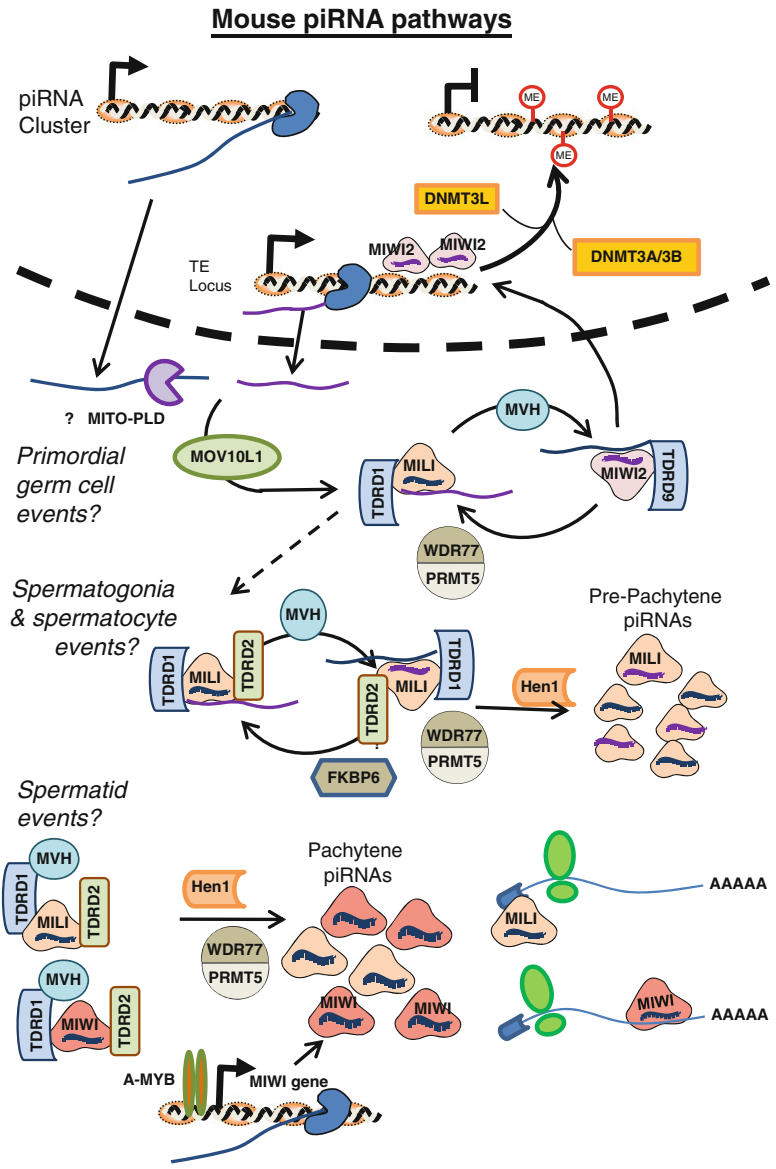

Fig. 5.4 The mouse piRNA pathway is tightly regulated throughout spermatogenic development. During early spermatogenesis, primary pre-pachytene piRNAs are produced from TE and genic transcripts. These MILI-bound, sense-oriented piRNAs can then direct "ping-pong" cycle production of MIWI2-bound or MILI-bound antisense piRNAs in primordial germ cells and spermatogonia, respectively. MIWI2-piRNA complexes are then able to translocate to the nucleus to initiate transcriptional gene silencing by promoting de novo DNA methylation. After meiosis and during spermatid maturation, non-repetitive pachytene piRNAs begin to dominate and load into both MILI and MIWI with the assistance of tudor-domain-containing proteins. The post-pachytene MIWI and MILI complexes are hypothesized to regulate target transcripts posttranscriptionally

Although reasons are still unknown for the temporal expression of mammalian Piwi pathway genes, an important transcription factor has recently been discovered to play a role in this temporal expression of Miwi and post-pachytene piRNAs. A mouse mutagenesis screen had previously pinpointed the transcription factor A-MYB being required for spermatogenesis as well as breast tissue development (Toscani et al. 1997). Recently, A-MYB was implicated in promoting pachytene piRNA and Miwi transcript expression in spermatocytes and spermatids (Li et al. 2013). ChIP-Seq of A-MYB found it at the promoters of Miwi and other piRNA biogenesis factor genes like MitoPLD, Mov10L, and Tdrd6, the promoters of pachytene piRNA clusters, and its own promoter. Therefore, A-MYB may engage in a feed-forward circuit that allows it to ramp up transcription of both piRNA precursors and piRNA biogenesis machinery. However, the presence of A-MYB in breast tissue, which lacks mouse Piwi protein and piRNA expression, also suggests that A-MYB itself is not sufficient to drive the expression of the entire Piwi pathway. Perhaps the Piwi pathway demands a combination of transcription factors, such as the USF and NF-Y factors recently suggested to also affect Miwi expression (Hou et al. 2012). Finally, although Miwi and piRNAs are most highly expressed in round spermatids, they are absent in mature sperm, and this turnover may either be part of the standard process of cytoplasmic shedding during the final stages of spermatogenesis, or it may be part of an active process of turnover by the proteasome (Zhao et al. 2013).

12 Not Quite the Same: Piwi Pathway Nuances in Vertebrates

With so many genetic factors implicated in the Piwi pathways in *Drosophila*, we might expect that the larger mammalian genomes would encode as many if not more Piwi pathway factors as possible. However, there are only as many mouse Piwi protein effectors as there are in *Drosophila*, but it remains a challenge to designate which mouse Piwi gene is the true fly ortholog. MIWI2 has the same nuclear localization and chromatin regulation characteristics with PIWI, while MILI and MIWI are cytoplasmic like AUB and AGO3. Furthermore, PIWI and MIWI mainly engage in primary piRNA biogenesis and not the ping-pong cycle, while MILI and MIWI2 can engage in the cycle in the mouse. Finally, MILI and MIWI2 have been genetically connected to directing de novo DNA methylation of TE elements during embryogenesis (see below), whereas in flies there is only minor evidence that DNA methylation has an influence on *Drosophila* development and no evidence yet for a link to PIWI, AUB, or AGO3.

Amongst the mammalian Piwi pathway factors that can be clearly assigned to orthologs in *Drosophila*, there appears to be a difference in the degree that mutations in mouse genes affect piRNA biogenesis and gametogenesis compared to the fly genes. Many mutations in the *Drosophila* Piwi pathways are extremely severe, causing complete sterility and major piRNA loss. However, other than the mouse

Piwi genes themselves, the only other gene mutation to cause complete loss of mouse piRNAs in the testes is the putative helicase Mov10-Like (Mov10L), which is distinct from the Mov10 paralog that is required for miRNA maturation into the RISC (Meister et al. 2005; Frost et al. 2010). Mov10L is the mammalian ortholog of *Drosophila armi*, and the *Mov10L* mutant male is infertile with shrunken testes that are devoid of functional sperm due to changes in spermatogonia and spermatocyte organelle morphology and an increased buildup of mitochondria (Zheng et al. 2010).

In contrast are several mouse Piwi factors where the mutations still cause spermatogenic arrest and male infertility, but their effect on global piRNA production during the initial waves of spermatogenesis is surprisingly mild. For example, whereas *Drosophila* vasa mutant ovaries are deficient of most piRNAs (Malone et al. 2009), mutations in Mouse Vasa Homolog (MVH) seem to only cause loss of Miwi2 piRNAs in the fetal testes, while Mili piRNAs can still accumulate (Kuramochi-Miyagawa et al. 2010). Mouse Maelstrom (ortholog of *Drosophila mael*) resides most frequently in the piP (piRNA Processing) bodies, associates with the Tdrd9 and Tdrd1 factors, and influences Miwi2 piRNAs but not Mili piRNAs (Soper et al. 2008; Aravin et al. 2009). Since the suppression of mouse TEs is affected in mouse *mael* mutants without loss of piRNAs, the effector role of silencing rather than piRNA biogenesis may be conserved between mouse and fly *mael* orthologs. Although mutations and knockdowns of fly Zuc severely compromise *Drosophila* piRNAs, knockout mutants in the mouse ortholog MitoPLD seem to affect the production of some piRNAs, but not others, particularly SINE element piRNAs bound by Mili (Huang et al. 2011a; Watanabe et al. 2011). Mouse GasZ knockout mutants (Ma et al. 2009) were created before knockdowns of fly GasZ (CG2183) were studied (Czech et al. 2013; Handler et al. 2013), and whereas TEs are derepressed in both fly and mouse GasZ mutants, piRNA biogenesis is much more severely impacted in the fly GasZ mutant. Knockdowns and mutations of fly Shutdown (Shu) strongly impair piRNA biogenesis (Olivieri et al. 2012; Preall et al. 2012), but knockouts of the mouse ortholog, FKBP6, only cause some loss of Miwi2 piRNAs but effects are quite modest against Mili piRNAs (Xiol et al. 2012). Finally, knockdown of the glycerol-3-phosphate acyltransferase 2 (GPAT2), a mitochondrial outer membrane protein, in a germinal stem cell mouse line was shown to affect piRNA loading into Mili (Shiromoto et al. 2013) and its fly homolog CG5508 also recovered in one of the RNAi screens for being required in TE silencing (Czech et al. 2013) but role of CG5508 in piRNA biogenesis is not yet known.

A large group of genes linked to the Piwi pathway and also conserved throughout animal evolution are the tudor-domain-containing proteins which are specifically expressed in germ cells. The tudor domain was named after the founding member, the *Drosophila Tudor* (*Tud*) gene whose closest mammalian homolog is TuDoR-Domain 6 (TDRD6). Tud and TDRD6 are the largest Tudor proteins with 11 and 8 repeated tudor domain in the fly and mouse genes, respectively. Tud mutants display the grandchildless phenotype where the daughters develop infertility, similar to hybrid dysgenesis and to the PRMT5 arginine methylase mutants that deposit symmetrical dimethyl arginines onto Aub and AGO3 to facilitate its interaction with Tud (Kirino et al. 2009; Nishida et al. 2009). Some amount of TE derepression and

piRNA decrease is observed in these mutants, but these phenotypes are mild compared to more severe phenotypes of Piwi protein mutations themselves or other biogenesis factors. Like Tud in flies, Tdrd6 is also required for spermatogenesis in mouse and associates with Miwi and Mili (Vasileva et al. 2009). The tudor domain is hypothesized to foster protein-protein docking interactions, so the presence of just multiple tudor domains in Tud and Tdrd6 suggests that their main function is to act as scaffolds.

Connected to the Piwi pathway are three other tudor-domain-containing genes with clear orthologs between flies and mice: *papi*, whose mouse homolog is Tdrd2 (also called Tdrkh); *tejas* (tej), whose mouse homolog is Tdrd5; and *spindle-E* (Spn-E), whose mouse homolog is Tdrd9. These genes differ from Tud because they only contain a single tudor domain, but Papi has transmembrane domains, Tej has a LOTUS domain, and Spn-E has helicase and RNA-binding domains (Handler et al. 2011). Papi associates with Ago3 and its mutant may display higher TE expression (Liu et al. 2011), but RNAi knockdown of Papi has not replicated this phenotype (Czech et al. 2013; Handler et al. 2013; Muerdter et al. 2013), and Papi's influence on piRNA production is not clear. Tdrd2 interacts strongly with Miwi (Chen et al. 2009), and a recent knockout mouse of Tdrd2 shows LINE1 derepression, spermatogenic arrest before pachytene is reached, and a very interesting lengthening of Mili-bound piRNAs to ~6–8 nt longer (Saxe et al. 2013). Although Tejas and Tdrd5 are essential for female and male germline development, respectively, only Tejas has been shown to be important for piRNA biogenesis and TE silencing (Patil and Kai 2010; Yabuta et al. 2011). Classic female sterile fly mutations of Spn-E suffer severe loss of piRNAs (Vagin et al. 2006; Malone et al. 2009), but while TDRD9 knockout mice are male sterile, they have overall similar levels of piRNAs in pre-pachytene stage testis to wild type, with just minor differences in a few specific piRNA sequences (Chuma et al. 2006; Shoji et al. 2009). Although TDRD9 associates with Miwi2 and its localization in spermatogonia is influenced by Mili (Shoji et al. 2009), it is enigmatic why in contrast to Spn-E that mouse Tdrd9 is not critical for piRNA biogenesis.

For other tudor-domain-containing genes in fly and mouse, the homologs have diverged such that either the obvious ortholog is not apparent or designating the ortholog bioinformatically may not be obvious. For example, Tdrd1 has been proposed to the closest homolog to *Drosophila* Qin in one study (Siomi et al. 2010) or to CG9925 in another study which suggested that Qin was more related to Tdrd4 (also known as RNF17) (Pan et al. 2005; Handler et al. 2011). Tdrd1 is essential for male germ cell development and TE silencing via promoting efficient piRNA biogenesis in Mili, but whether Tdrd1 has a role in the ping-pong cycle like Qin remains open because there are still significant piRNAs present in the Tdrd1 −/− mutant (Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009). A purification of zebrafish Tdrd1 identified longer transcripts which may be putative piRNA precursors (Huang et al. 2011b), perhaps analogous to the putative piRNA precursors found associated in an ARMI purification from *Drosophila* OSC cells (Saito et al. 2010). Although the Tdrd4 knockout mouse has not been reported yet, knockdown of CG9925 did not elicit TE derepression (Handler et al. 2011); thus it remains open which is the

clear *Drosophila* ortholog of Tdrd1. Then there is the example of *Drosophila* Krimper, which lacks any mammalian ortholog (Siomi et al. 2010; Handler et al. 2011), but clearly has a strong effect on the Piwi pathway in flies when mutated or knocked down (Lim and Kai 2007; Malone et al. 2009; Handler et al. 2011).

13 Mammalian Piwi Proteins Need "Slicing" Activity to Do Their Jobs

Despite the progress in dissecting the Piwi pathway in flies and mice, there are still open questions regarding the silencing mechanisms of the Piwi proteins. Like certain AGO proteins, the Piwi-group proteins retain the key amino acid residues required for endonucleolytic slicing activity that occurs upon a target transcript substrate paired against a guide small RNA in the protein. The slicing activity is central to the ping-pong cycle model (Brennecke et al. 2007; Gunawardane et al. 2007; Li et al. 2009); however it has not been reported whether AUB and AGO3 transgenes mutated in catalytic residues can rescue the *aub* and *ago3* mutants. Although PIWI has slicing activity in vitro (Saito et al. 2006), two studies using a Piwi transgene mutated in the catalytic residues indicate that slicing activity is dispensable for TE silencing in *Drosophila* OSC cells and in the female germline (Saito et al. 2010; Darricarrere et al. 2013). With the revelation that *Drosophila* Piwi mediates TGS, the dispensability of the slicing activity is surprising because Ago1 from fission yeast requires slicing activity in order to manifest its TGS on heterochromatic loci (Irvine et al. 2006).

Two mouse Piwi proteins, Mili and Miwi2, have also been implicated to promote TGS upon TEs in the early development phases of fetal and prepubertal sperm. In Mili and Miwi2 knockout mice, there is a substantial loss of DNA methylation upon chromatin containing LINE1 sequences (Aravin et al. 2007; Carmell et al. 2007; Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008), mirroring the phenotype of *dnmt3l* mutants, the gene responsible for de novo establishment of this silencing mark on TEs during mammalian embryogenesis, after parental epigenetic imprints are erased (Lane et al. 2003). The genetic function of Mili and Miwi2 in specifying DNA methylation of TEs is compelling, and may even extend to imprinted loci like *Rasgrf1* (Watanabe et al. 2011). However, the biochemical mechanism and questions of how directly do Mili and Miwi2 direct DNA methylation remain obscure because *dnmt3l* mutants have no effect on piRNA biogenesis (Aravin et al. 2008), and proteomic analysis of Mili and Miwi2 complexes has not identified associated DNA methyltransferases (Vagin et al. 2009). In addition, not all TEs are equally repressed at the TGS level, such as the IAP TE which is not affected in Miwi2 mutants (Kuramochi-Miyagawa et al. 2008).

In contrast to Miwi2 and Mili being genetically linked to TGS, Miwi has mainly been thought to regulate target transcripts posttranscriptionally, although demonstrating that this mechanism has not been fully addressed. It is clear that Miwi is mainly cytoplasmic and concentrated in the perinuclear organelle called the

chromatoid body, a dense structure believed to be a transit hub for mRNAs (Kotaja and Sassone-Corsi 2007). Miwi can also bind and perhaps stabilize some mRNAs (Deng and Lin 2002) as well as pachytene piRNA precursor transcripts (Vourekas et al. 2012), but whether Miwi has sequence-binding specificity or how mechanistically Miwi can pair with certain transcripts is unclear (Robine et al. 2009). A translational regulation role of Miwi and Mili has mainly been portrayed from density gradient fractionations of adult testis extracts that show Miwi and Mili co-fractionating with polyribosomes (Grivna et al. 2006; Unhavaithaya et al. 2009). It has been challenging to formally test the translational regulatory activity of Miwi and Mili upon synthetic mRNAs and transgenes in testis extracts competent for in vitro translation. However the recent report of a mouse gonadal cell culture line (Germline Stem Cells, or GSCs) (Shiromoto et al. 2013) with endogenous Mili and piRNAs might possibly open a door to more rigorous biochemistry if these cells can be cultured in bulk.

Mammalian Piwi proteins were first demonstrated to exhibit Slicer activity in vitro (Lau et al. 2006), and this activity was thought to sustain a ping-pong cycle signature between Miwi2 and Mili (Aravin et al. 2008). However, only mammalian Ago2 retains slicer activity to form the RISC capable for target RNA cleavage, while Ago1, -3, and -4 are incapable of slicing activity (Liu et al. 2004) but can regulate targets through binding and recruiting translation-repressing factors. Although Ago2's catalytic residues are critical for mouse hematopoiesis because Ago2 slicing is required to process the essential miR-451 miRNA (Cheloufi et al. 2010; Cifuentes et al. 2010), a mutated Ago2 transgene lacking slicing activity can rescue hematopoiesis in Ago2 –/– mice, suggesting that the Ago2 slicing activity is highly specialized (O'Carroll et al. 2007).

To address the importance of the slicing activity in mouse Piwi proteins, point mutations that disrupted the catalytic residues of Miwi (Reuter et al. 2011), Mili, and Miwi2 (De Fazio et al. 2011) were generated and revealed some surprising and perplexing genetic effects. Slicing-inactive Mili mutant males ($Mili^{DAH}$) were infertile and displayed up-regulated LINE1 TE expression and decreased DNA methylation, but overall piRNA production within Mili was comparable to wild type with mainly a mild reduction of piRNAs complementary to LINE1 sequences (De Fazio et al. 2011). Interestingly, it was Miwi2 piRNA loading that was perturbed in $Mili^{DAH}$ mutants, suggesting that Mili slicing was required for specifying a ping-pong cycle mechanism to load piRNAs into Miwi2. Perplexingly, inactivation of slicing residues in Miwi2 failed to reveal a reciprocal defect in piRNA production neither in Mili nor in Miwi2 itself, with TE silencing also intact (De Fazio et al. 2011). Whereas catalytic residue mutations in Mili and Miwi2 are genetically recessive, the catalytic residue mutation in Miwi ($Miwi^{ADH}$) turned out to be a dominant negative in causing male sterility in the heterozygous state (Reuter et al. 2011). If one speculates that Miwi proteins form dimers as an explanation of the dominant negative phenotype, then it is mysterious that pachytene piRNA accumulation and general testis transcriptome profiles were largely unaffected in the $Miwi^{ADH}$ mutant. Indeed, this study displayed LINE1 TE up-regulation in $Miwi^{ADH}$ mutants and some evidence that Miwi may use certain piRNAs to slice LINE1 TE transcripts

posttranscriptionally as a means to suppress TE mobilization (Reuter et al. 2011). Recently, conditional Mili knockout and conditional slicing Mili^{DAH} mutants were shown to still exhibit mouse male sterility despite functional Mili capable of establishing proper DNA methylation of TE loci (Di Giacomo et al. 2013), thus leading to the model that LINE1 TEs reanimate during pachytene stages of meiosis but require both Mili and Miwi protein to prevent germ cell damage. Future work is required to tease the mechanistic details for primary piRNA biogenesis in mouse spermatocytes, which apparently can proceed even when slicing activity is compromised. These genetic studies have raised more open questions and new conundrums to the Piwi pathway that await future novel approaches and additional model organisms and germ cell systems to fully dissect this pathway.

14 Conclusion: Piwi "on the Brain" and Going Beyond the Germline?

The Piwi pathway genes and piRNAs are most influential and abundantly expressed in animal gonads, but the detection and genetic influence of this pathway in other somatic animal tissues continue to be debated. It is possible that Piwi-mediated chromatin modifications determined in the germ cells and early embryo are later perpetuated in a Piwi-independent fashion via a "cellular memory" to somatic tissues like eye pigmentation or salivary gland polytene chromosomes (Pal-Bhadra et al. 2002; Brower-Toland et al. 2007; Moshkovich and Lei 2010). However, caution should also be heeded in determining whether bona fide piRNAs are indeed expressed in somatic cells, because several abundant and stable structural RNA fragments have been misclassified as potential piRNAs in somatic cells without the confirmation that they are indeed loaded into a Piwi protein complex (Janic et al. 2010; Lee et al. 2011; Yan et al. 2011).

Nevertheless, there is a convergence of data implicating that some invertebrate animal neurons may harbor their own repository of piRNAs and Piwi machinery. The sea slug *Aplysia* is a classic model system for neurophysiology because of its exceptionally large, well-characterized ganglion comprising many cell types including neurons (Bailey et al. 2004). It was recently shown that *Aplysia* ganglions contain putative piRNAs (Rajasethupathy et al. 2012), but characterization of the *Aplysia* piRNAs, whether they are derived from large intergenic clusters or target TEs, is hampered by the rough draft stage of the *Aplysia* genome (Moroz et al. 2009; Rajasethupathy et al. 2012). Injection of mimics and inhibitors against a single putative *Aplysia* piRNA was somehow able to instill neurophysiological responses and DNA methylation changes in these ganglions (Rajasethupathy et al. 2012). A focused scrutiny of specific neurons in the fly brain has also suggested that Piwi proteins may be expressed and playing a role in controlling TE mobilization in the fly brain (Perrat et al. 2013). As a possible mechanism for fly neuroplasticity, this study hypothesized that certain neurons lacking AUB and AGO3 would allow TE activity to yield heterogeneity within the *Drosophila* brain that could be useful for

learning and memory (Perrat et al. 2013). Although biochemical analysis of specific fly neurons is technically challenging because of the small amount of tissue, genetic and chemical ablation of miRNAs and endo-siRNAs has enabled the detection of putative piRNAs in fly heads (Ghildiyal et al. 2008). Finally, there is a recent report suggesting that piRNAs are expressed in human induced pluripotent stem cells (Marchetto et al. 2013). So as technology in this arena advances, including more efficient and cost-effective systems biology approaches, we expect to uncover new avenues where the Piwi pathway and piRNAs may influence animal behavior and other processes beyond the animal germline.

The greatest progress in applying systems approaches to the Piwi pathway has occurred in flies and mice because of the ease of genetically manipulating flies and the large knowledgebase for mice as a model mammal of humans. As such, both organisms have the strongest foundation of genomics resources and largest community of researchers. But understanding the Piwi pathway fully will demand going beyond the fly and mouse and making use of other vertebrate and invertebrate models. For example, to ask if vertebrates are susceptible to hybrid dysgenesis like in flies where a mother lacking just one class of piRNAs will produce sterile daughters, mice may not be an optimal system because mammalian oogenesis does not appear to depend on the Piwi pathway. There is some controversy whether mammalian sperm may transmit piRNAs as epigenetic factors (Krawetz et al. 2011), because there is also Miwi turnover during sperm maturation (Sytnikova and Lau 2013; Zhao et al. 2013). However, the frog *Xenopus tropicalis* has well-characterized piRNA clusters configured in an analogous fashion to mammalian clusters (Armisen et al. 2009; Lau et al. 2009), as well as highly abundant levels of Xiwi in the oocyte that appears to be concentrated asymmetrically in the germplasm (Lau et al. 2009), specialized cytoplasm that can specify the formation of primordial germ cells (Houston and King 2000). We propose that *X. tropicalis* will be a useful vertebrate model to compare and contrast the Piwi pathway with respect to mammals and flies.

In addition to gametogenesis, the Piwi pathway may also be connected to regeneration of lost body parts in certain animals. Planarians and cnidarians contain a large and dispersed population of special stem cells called neoblasts that express Piwi proteins and piRNAs (Reddien et al. 2007; Palakodeti et al. 2008). These neoblasts endow these animals with extraordinary regenerative capabilities such as restoring complete body plans from nearly any piece of severed tissue (Petersen and Reddien 2009). In fact, the major small RNA populations in these animals are not miRNAs but rather piRNAs (Grimson et al. 2008; Palakodeti et al. 2008). Amongst vertebrates, the axolotl salamander is prominently one of a few vertebrates capable of regenerating a severed limb during adulthood, and recently it was suggested that axolotl Piwi transcripts might be triggered for expression in the regenerating blastema (Zhu et al. 2012). Perhaps somatic cells in the blastema are reanimating the Piwi pathway as a form of induced dedifferentiation similar to induced pluripotency, and indeed mutations of a tumor-suppressor gene in flies may possibly be one route to achieve this dedifferentiation state (Janic et al. 2010). We speculate that the majority of other vertebrates where somatic cells lack the expression of the Piwi pathway may explain our limited regenerative potential.

Finally, we discuss lastly (but not the least) that the Piwi pathway is not the sole purview of multicellular organisms: the single-celled protozoans have co-opted the Piwi pathway for reproductive and growth functions. *Tetrahymena* encodes at least eight genes that are more similar to metazoan Piwi genes than Ago genes, and the *Tetrahymen*a Piwi genes (TWI) have very essential roles in growth (Couvillion et al. 2010) and nuclear RNA metabolism (Couvillion et al. 2010). Notably, *Tetrahymena* express scnRNAs that are just one form of protozoan piRNAs, which are required for meiosis, conjugation, and a complex process of DNA elimination from the macronucleus (Mochizuki and Gorovsky 2004a; Schoeberl et al. 2012). The *Tetrahymena* scnRNAs appear to direct chromatin modification events that lead to the removal of large swaths of DNA to yield a slimmed macronucleus perhaps optimized for genic transcription (Mochizuki et al. 2002; Mochizuki and Gorovsky 2004b). But in opposite fashion, another protozoan, *Oxytricha*, uses its piRNAs to instead specify the retention of DNA elements and prevent genes from being lost when its genome rearranges into thousands of tiny chromosomes (Fang et al. 2012; Swart et al. 2013). These quirky protozoan Piwi pathways are a testament to the rich diversity of natural biological processes, and should motivate us to continue casting a wide net of systems biology approaches across multiple model organisms.

Acknowledgments We are grateful to members of the Lau lab, Dianne Schwarz and Julie Claycomb, for discussions and critical reading of this manuscript. This work was supported by the Searle Scholars foundation to NCL and an NIH genetics training grant to JPC.

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