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PIWI proteins and their interactors in piRNA biogenesis, germline development and gene expression

Hsueh-Yen Ku and Haifan Lin*

ABSTRACT

PIWI-interacting RNAs (piRNAs) are a complex class of small non-coding RNAs that are mostly 24–32 nucleotides in length and composed of at least hundreds of thousands of species that specifically interact with the PIWI protein subfamily of the ARGONAUTE family. Recent studies revealed that PIWI proteins interact with a number of proteins, especially the TUDOR-domain-containing proteins, to regulate piRNA biogenesis and regulatory function. Current research also provides evidence that PIWI proteins and piRNAs are not only crucial for transposon silencing in the germline, but also mediate novel mechanisms of epigenetic programming, DNA rearrangements, mRNA turnover, and translational control both in the germline and in the soma. These new discoveries begin to reveal an exciting new dimension of gene regulation in the cell.

Keywords: PIWI, piRNA, TDRD, epigenetic, RNA decay, translational regulation

INTRODUCTION

Study of non-coding RNAs represents one of the most exciting frontiers of current biomedical research. Non-coding RNAs are commonly classified as long non-coding RNAs (lncRNAs) that are generally longer than 200 nucleotides (nt) and small non-coding RNAs (sncRNAs) that are mostly 20–35 nt [1]. Among sncRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs) are generally 21 nt in length and have been extensively studied, whereas PIWI-interacting RNAs (piRNAs), mostly 24–32 nt in length, and other types of sncRNAs were discovered more recently and are thus less well characterized. Among all types of coding and non-coding RNAs, piRNAs are by far the most numerous, existing in at least hundreds of thousands of species in a multicellular organism, mostly in its germline. This number far exceeds the total number of all other types of known RNAs altogether. Furthermore, piRNAs correspond to all types of genomic sequences (see below). These two salient features of piRNAs imply their potential function in diverse forms of gene regulation.

piRNAs are defined by their specific binding to the PIWI subfamily of ARGONAUTE (AGO)/PIWI family proteins. This is in contrast to miRNAs and siRNAs, both of which specifically bind to the AGO subfamily of the AGO/PIWI family proteins. PIWI proteins are required for piRNA biogenesis and function, and play a central role in germline development and gametogenesis (reviewed in [2]). Piwi mutants in Drosophila, C. elegans, mice, and zebrafish cause gametogenic defects such as failure in germline establishment, loss of germline stem cells (GSCs) meiotic arrest, and blockage in spermiogenesis, leading to sterility (reviewed in [3]). Because piRNAs are derived from the RNA transcripts of transposons, protein-coding genes, and specific intergenic loci [4–8], piRNAs are thought to act as sequence-specific guides of PIWI proteins to regulate the expression of genes and transposons at both transcriptional and post-transcriptional levels [9–11]. Most recently, PIWI proteins and piRNAs have also been found in somatic tissues [12]. Reviews on the PIWI–piRNA pathway have mostly been focused on its biogenesis and function in transposon silencing in the germline.
Here, we will first give a brief update on these two topics with focus on PIWI proteins and their main interactors, TUDOR-domain-containing proteins. We will then review the regulatory function of the PIWI–piRNA pathway beyond transposon silencing both in germline and somatic cells.

**AGO PROTEINS AND THEIR INTERACTING PROTEINS—AN OVERVIEW**

The AGO protein family was first discovered as a gene family with stem cell function well conserved in both animal and plant kingdoms [13]. The AGO family proteins are defined by the presence of the highly conserved Piwi–Argonaute–Zwille (PAZ) and PIWI domains, as well as the less conserved N-terminal (N) and Middle (Mid) domains [14]. Structural studies revealed that the PAZ domain comprises an oligonucleotide-binding fold which anchors the two-nucleotide 3′-overhang of siRNAs resulting from digestion by RNase III (a step in the processing of siRNAs) [15–17], whereas the PIWI domain shows extensive homology to RNase H. In many AGO/PIWI proteins, such as the *Drosophila* Piwi protein (herein and hereafter, all-capitalized protein names, such as AGO and PIWI, are used as family and subfamily names, whereas first-letter-capitalized names are for individual proteins, such as Ago 1 and Piwi), the PIWI domain exhibits endonuclease activity, also known as the slicer activity [4]. However, this activity is not required for some AGO proteins, such as the *Drosophila* Piwi protein, for their function [18,19]. Based on phylogenetic analysis, the AGO/PIWI family is divided into two subfamilies: the AGO subfamily and the PIWI subfamily. In most organisms, PIWI subfamily proteins are mainly expressed in the germline, in contrast to AGO subfamily proteins that are ubiquitously expressed in all tissues (reviewed in [3]).

PIWI proteins interact with a number of proteins, such as Armitage (Armi) and Zucchini (Zuc) in *Drosophila*, to regulate the piRNA biogenesis (Table 1). Among these factors, the most predominant class is Tudor-domain-containing proteins (a.k.a. Tudor domain-related proteins, TDRDs). Their function and interaction with PIWI protein is becoming increasingly studied, whereas little is known about how other proteins interact with PIWI proteins. Therefore, this review highlights TDRD proteins as PIWI interactors.

TDRD proteins belong to the TUDOR protein family, which received the name from the *Drosophila* tudor gene. Tudor was discovered in a genetic screen for maternal effect mutations that cause lethality or sterility in the progeny [20]. Subsequently, the Tudor domain has been found in proteins from a broad range of eukaryotes, including fission yeast, fungi, plants, and animals. Based on the sequence and structural similarity, Tudor domain, together with chromatin-binding (Chromo), malignant brain tumor (MBT), PWWP (conserved Proline and Tryptophan), and plant Agenet domains, comprises the Tudor domain ‘Royal Family’ [21]. Tudor domain has a ~60 amino acid core structure composed of four antiparallel β-strands that form a barrel-like structure with an aromatic binding pocket at the surface to accommodate methylated lysine/arginine ligands [21]. Tudor domain exists singly or in multiple copies, in the absence or in conjunction with other types of domains. The versatile protein domain architecture confers a diverse protein–protein/RNA interacting network and endows a wide variety of functions including RNA metabolism, spliceosome assembly, piRNA biogenesis, histone modification, and germline development to the TUDOR family members [20,22–32].

Recent studies have revealed that PIWI subfamily proteins, but not AGO proteins, are arginine-methylated by protein methyltransferase 5 (dPRMT5, also known as Capsuleen/Dart5) to form symmetrically dimethylated arginines (sDMAs) at their N-termini [29,33–35]. The sDMAs of PIWI proteins serve as binding motifs for TDRDs and play crucial roles in PIWI function [29,30,33–38]. Several TDRDs, such as Spindle-E (Spn-E), Tudor (Tud), Krimper (Krimp), and Tejas (Tej) in *Drosophila* [29,30,35,39–41], as well as Tdrd1, Tdrd2, Tdrd4, Tdrd5, Tdrd6, Tdrd7, Tdrd9, and Tdrd12 in mice [33,36–38,42–45], have recently been shown to be required for PIWI function.

**PIWI5, TUDORS, AND OTHER PROTEINS IN piRNA BIOGENESIS**

piRNAs was discovered and defined as a novel class of sncRNAs that bind to PIWI subfamily proteins in mammalian testes in 2006 [5–8]. In fact, the piRNA-like RNAs were identified even earlier in plants [46] and trypanosomes [47]. These RNAs contain sequences matching repetitive intergenic elements and thus are termed repeat-associated small RNAs (rasiRNAs). Subsequent studies in *Drosophila* ovaries further reported that PIWI proteins are associated with small RNAs identified as rasiRNAs [4,39,48]. Therefore, rasiRNAs are essentially piRNAs [48,49]. Genetic studies have demonstrated that the RNase III-like enzyme Dicer, which
Table 1. List of PIWI-interacting proteins involved in the piRNA pathway.*

| TUDOR family proteins Flyhomolog | Mammalianhomolog | Proteindomain(s) | Interactingprotein(s) | Reference(s) |
|----------------------------------|------------------|------------------|-----------------------|--------------|
| CG9684/CG9925                   | Tdrd1/Mtr-1      | TUDOR(x2/x4), MYND | Miwi, Miλi, Miwi2, Tdrd12 | [36,38,42,44,106,109] |
| PAPI                            | Tdrd2/Tdrdh      | TUDOR, KH(x2)     | Piwi, Ago3, Tra1, Me31B, TER94, Miwi, Miλi, Miwi2 | [33,35,42,108] |
| Kumohi/Qin                      | Tdrd4/RNF17      | TUDOR(x5), RING, B-box | Piwi, Aub, Ago3, Vas, Spn-E, HP1, Miwi | [69,70,105] |
| Tej                             | Tdrd5            | TUDOR, Lotus      | Aub, Vas, Spn-E        | [41,45] |
| Ttd                            | Tdrd6            | TUDOR (x11)       | Aub, Ago3, Vls         | [29,42,67,107,110,153] |
| CG5820                          | Tdrd7/TRAP       | TUDOR (x3), Lotus (x2) | Miwi, Tdrd1, Tdrd6, VASA | [42,43,110] |
| Spn-E                           | Tdrd9            | TUDOR, DEXDc, HELICc, HA2 | Miwi, Miλi, Miwi2 | [37,40,42] |
| Yb(Femalesterile(1)Yb)          | Tdrd12/ECAT8     | TUDOR, DEAD, Helicase, Helicase-like, ZnF | Piwi, Armj, Vret, Mili, Tdrd1 | [44,52–55] |
| BoYb                            | Tdrd12/ECAT8     | TUDOR, DEAD, Helicase, ZnF | Mili, Tdrd1 | [44,57] |
| SoYb                            | Tdrd12/ECAT8     | TUDOR (x2), DEAD, Helicase, ZnF | Mili, Tdrd1 | [44,57] |
| Vret                            | None             | TUDOR (x2), RRM, MYND | Piwi, Aub, Ago3, Yb, Armj | [56,57] |
| Other known PIWI-interacting proteins | VASA/MVH        | DEAD, HELICc      | Piwi, Aub, Tej, Spn-E, Miwi, Miλi, Tdrd6 | [40,86,90,107,154] |
| Armj                            | MOV10,MOV10L1    | Helicase          | Piwi, Yb, Vret, Miwi, Miλi, Miwi2, Tdrd1 | [44,52–55,155,156] |
| Zac                             | mitoPLD          | PLD-family nuclease | Aub | [58,157–159] |
| Sgu (Squash)                    | None             | RnaseHIII-like    | Aub | [58] |
| Mael                            | Maelstrom        | HMG-box           | MTOC proteins, Miwi, Miλi, VASA | [68,125,160] |
| Hen1                            | HEN1             | d-RBD3, SAM-methyltransferase | Piwi, Aub, Ago3 | [62,161] |
| Hsp83                           | Hsp90            | Hsp90, ATPase     | Piwi, Hop, Fkbp6 | [59,72,73,162] |
| Shu                             | Fkbp6            | FKBP, TPR         | Piwi, Armj, Hsp90 | [59,60,162] |
| dPRMT5                          | PRMT5/Capsuleen(Cusl) | SAM-methyltransferase | Vls, Miwi, Miwi2 | [29,42,153] |
| Vls (Valois)                    | MEP50            | WD(x4)           | dPRMT5, Tsd, Miwi, Miλi, Miwi2 | [42,153] |

*Blue and red words indicate Drosophila and mammalian proteins, respectively. B-box, B-box zinc finger domain; DEAD, DEAD-box helicase; DEXDc, DEAD-like helicase domain; d-RBD3, double-stranded RNA-binding domain; FKBP, FK506-binding protein domain; HA2, helicase-associated domain; HELICc, helicase C-terminal domain; HMG-box, high mobility group box; KH, K homology domain; MBD, methyl-CpG-binding domain; MYND, zinc-finger myeloid-nervy-DEAF-1 domain; N.D., not determined; PAZ, PIWI/Argonaute/Zwille; PIWI, P-element-induced wimpy testes; PIWIL1–4, Piwi-like protein 1–4; PLD, phospholipase D; Pre-SET, pre-SET domain; RING, really interesting new gene finger domain; RRM, RNA recognition motif domain; SAM, S-Adenosyl methionine; SET, Su(var)3-9/Enhancer-of-zeste/Trithorax domain; TPR, tetratricopeptide-repeat domain; WD, WD-repeat domain; ZnF, zinc finger.

is necessary for processing miRNAs and siRNAs, is dispensable for piRNA biogenesis [39,48], and that piRNAs precursors are single-stranded RNAs. Furthermore, deep sequencing of small RNAs associated with Drosophila Piwi, Aubergine (Aub), and Argonaute 3 (Ago3) revealed a number of characteristics of piRNAs [4,9,48,50]. For example, in Drosophila, piRNAs are mainly derived from intergenic repetitive sequences in the genome, including transposable elements (TEs) and their
Figure 1. piRNA biogenesis pathways in Drosophila. In the primary processing pathway in the germline, the antisense piRNA precursor transcripts produced from piRNA clusters are possibly processed by Zuc, a putative endonuclease [53,58]. The resulting piRNA intermediates with a U at the first nucleotide position are preferentially selected and loaded onto Piwi or Aub (in ovarian somatic cells, only Piwi is expressed and involved in the primary piRNA pathway). Hsp83 and its hypothesized co-chaperone Shuar are required for the piRNA loading [59,60]. The 3′ end of piRNA intermediates is further trimmed by an unknown enzyme [61], and then methylated by the methyltransferase Hen1 [62,63] to mature the piRNAs and complete primary processing pathway. Aub and its bound mature antisense piRNAs can then enter the secondary processing pathway (the ping-pong cycle). piRNAs guide Aub to cleave the transposon transcripts and sense piRNA precursors produced from piRNA clusters [48,50]. The resulting products are then loaded onto Ago3 with the help of Shu and Hsp83 [59,60], and undergo 3′ end trimming and methylation to mature the sense piRNAs [61–63]. In turn, Ago3 and its associated mature sense piRNAs further cleave the target antisense piRNA precursors based on the sequence complementarity [48,50]. The resulting antisense piRNA intermediates are loaded onto Aub, with the assistance of Shu and Hsp83 [59,60], and further trimmed and methylated at the 3′ end as in the primary processing pathway [61–63]. The piRNA biogenesis pathways are well conserved across species such as C. elegans, fish, and mice (reviewed in [3]). Figure modified from [163].

The primary pathway

In Drosophila gonadal somatic cells, where Piwi, but not Aub or Ago3, is expressed [51], the single-stranded piRNA precursors are transcribed and then processed by a Dicer-independent machinery to generate piRNA intermediates [39,48]. These piRNA intermediates are delivered from the nucleus to the Yb body, the cytoplasmic ribonuclease-protein (RNP) complex comprising Piwi, RNA helicase Armi, and TDRD proteins Yb, Vreteno (Vret), and Sister of Yb (SoYb) [52–57]. The intermediate piRNAs are subsequently processed at the 5′ end possibly by Zuc, a putative endoribonuclease that
localizes to mitochondria [53,58]. The intermediate piRNAs are loaded onto Piwi with the help of Shutdown (Shu) co-chaperone protein and Heat shock protein 83 (Hsp83) chaperone protein [59,60]. After loading onto the Piwi protein, the 3′ end of piRNAs is trimmed by an unknown enzyme [61] and then methylated by the methyltransferase Hen1 to form mature piRNAs [62,63]. The Piwi–piRNA complex is then transported into the nucleus to exert transposon silencing and gene regulation functions. Without piRNA loading, Piwi is not localized to the nucleus. These findings suggest that the Yb body not only generate mature primary piRNAs for Piwi, but also scrutinize the functional Piwi–piRNA complex before it enters the nucleus [52–55]. Similarly to gonadal somatic cells, germ cells also produce primary piRNAs that are loaded onto both Piwi and Aub. The primary piRNAs can further initiate the ping-pong cycle of piRNA biogenesis [48,64].

The secondary pathway—the ping-pong cycle

In Drosophila, the ping-pong cycle is primed by both maternally deposited piRNAs through germline transmission and zygotic primary piRNAs that are antisense to retrotransposon coding strands and loaded onto Aub [57,64–66]. In germ cells, Piwi shows steady-state localization to the nucleus [51], whereas Aub and Ago3 localize to the nuage and associate with Tudor [29,67]. Tudor might serve as a scaffold protein to recruit other piRNA biogenesis factors and initiate the ping-pong cycle. During the ping-pong amplification cycle, Ago3 binds to sense-strand piRNAs and catalyzes antisense-strand cleavage at an A:U bp that generates the 5′ end of antisense piRNAs [48,50]. The 5′ ends of the resulting cleavage products are loaded onto Aub with the help of Shu and Hsp83 [59] and then further trimmed by an unknown enzyme and methylated by Hen1 at the 3′ end to generate mature antisense piRNAs [62,63]. Shu and Hsp83 have been hypothesized but not been demonstrated to directly interact with each other for this function [59,60]. The antisense piRNAs then guide Aub to cleave sense-strand RNAs and generate the 5′ end of sense piRNA precursors that associate with Ago3 [48,50], again with the help of Shu and Hsp83 [59]. Further 3′ end processing by an unknown nuclease and methylation by Hen1 produce mature sense-strand piRNAs and complete the ping-pong cycle [62,63].

In addition to Aub, Ago3, and Tudor, recent studies have shown that many of the ping-pong cycle components localize to the nuage, such as the RNA helicase Armi, the DEAD-box helicase Vasa (Vas), and the HMG protein Maelstrom (Mael), as well as the TDRD proteins Spn-E, Kumo, Krimp, Vret, and Tej [40,41,67–70]. Their nuage localization suggests that the nuage is the cytoplasmic site where the ping-pong cycle takes place. Mutants of the nuage components such as vas, mael, armi, zuc, squ, krimp, and tej exhibit defects in piRNA production [40,41,58,71], supporting that the nuage is involved in the piRNA pathway. Despite these findings, the exact molecular functions of nuage and most of the nuage components remain largely to be investigated.

Although there are some distinguishing factors between the primary pathway and the ping-pong cycle, many proteins are involved in both. The common factors include the methyltransferase Hen1, the chaperone Hsp83, the co-chaperone Shu, and the TDRD protein Vret. While the knowledge about the precise roles of these proteins is still limited, mutations of any of them disrupt the normal function of PIWI proteins and piRNAs decrease dramatically [52–57,59,60,62,63,72,73].

Features of the Drosophila piRNA system have been identified in a number of other organisms, such as human, mice, rat, platypus, marsupials, zebrafish, planaria, silkworm, C. elegans, and Hydra. For example, signatures of the ping-pong cycle also exist in mice [5,6,74,75]. Moreover, piRNAs are also found in human and rat, with major clusters occurring in syntenic locations [6]. Zebrafish has also been demonstrated to have the piRNA ping-pong cycle [76,77]. Additionally, planaria piRNAs are in part organized in genomic clusters and share characteristic features with mammalian and fly piRNAs [78]. In C. elegans, 21U-RNAs, the piRNAs of nematodes, display 5′ U bias and their precursors are not dsRNAs [79]. Furthermore, these 21U-RNAs complex with PRG-1, one of two C. elegans PIWI proteins, to regulate spermatogenesis [80,81]. In Hydra, two PIWI proteins, Hywi and Hyli, and piRNAs have been identified. Hywi- and Hyli-associated piRNAs show strong ping-pong signature. Furthermore, hywi function is shown to be essential in the somatic epithelial lineages [82]. This is the lowest eukaryote in which PIWI proteins and piRNAs have been found so far. Given that only AGO protein is present in fission yeast S. pombe but not baker’s yeast S. cerevisiae, it is clear that the separation between AGO and PIWI must occur in multicellular eukaryotes with germ cells.

THE PIWI–piRNA PATHWAY IN GERMLINE DEVELOPMENT

The PIWI–piRNA pathway has been intensively studied for their function in the germline. Several
Known gene regulation mechanisms mediated by the PIWI–piRNA pathway. PIWI proteins and piRNAs regulate the expression of genes and transposon at both transcriptional and post-transcriptional levels. (1) Sense and antisense piRNA precursor transcripts are produced in the nucleus. (2) Antisense piRNA precursor transcripts are transported to the cytoplasm and processed by the primary biogenesis pathway to generate mature sense piRNAs that associate with PIWI proteins. (3) The PIWI–antisense piRNA complexes mediate cleavage of sense piRNA precursors and transposon (and protein-coding) transcripts, which silences transposon and gene expression at the post-transcriptional level. The resulting sense transcripts are taken up by PIWI proteins responsible for sense piRNA binding. The PIWI–sense piRNA complexes then cleave antisense piRNA precursor transcripts to amplify the piRNA biogenesis cycle [48,50]. (4) The PIWI–piRNA complexes are involved in translational regulations by interacting with polysomes, mRNA cap-binding complex (CBC, in mice) [7,74,94], and mRNA deadenylase (DeA, in Drosophila) [133]. In addition, the PIWI–piRNA complexes are associated with P-body components [37,109,136,137] and piRNAs are mapped to the 3′UTR of mRNAs [131,132]. (5) The PIWI–piRNA complexes can enter the nucleus and regulate gene transcription through epigenetic mechanisms including heterochromatin formation [9,11,114–117] and DNA methylation [10,120] in the promoter region of target genes. 3′UTR, 3′ untranslated region; CBC, cap-binding complex; DeA, deadenylase; DNMT, DNA methyltransferase; HMT, histone methyltransferase; HP1, heterochromatin protein 1; Me, methylation.

studies have shown that the PIWI–piRNA pathway is implicated in germline development, epigenetic regulation, transposon silencing, mRNA turnover, and translational control (Fig. 2). Importantly, these functions of PIWI proteins and the piRNA pathway have been demonstrated in a number of organisms, suggesting that this mechanism is evolutionarily conserved [4,8,34,39,48,50,64,76,83].

In Drosophila, there are five AGO/PIWI proteins: Ago 1 and Ago 2 belong to the AGO subfamily, whereas Piwi, Aub, and Ago3 are in the PIWI subfamily [84]. Piwi, the founding member of the Drosophila AGO/PIWI family, was initially identified in a genetic screen for genes affecting the GSC maintenance [85]. piwi mutant males and females fail to self-renew their GSCs, resulting in germline degeneration and thus sterility [13,85]. Importantly, clonal analyses in adult fly ovaries have revealed that the function of Piwi for GSC maintenance resides in the somatic niche composed of terminal filaments and cap cells, while germline expression of Piwi controls the division of germ cells [13]. Furthermore, maternal Piwi is shown to be required for PGC formation [86]. In addition to Piwi, the other two Drosophila PIWI subfamily members, Aub and Ago3, are also involved in germline development. Genetic studies have demonstrated that Aub is essential for establishing PGCs [86,87]. Besides, the
mutations of *aub* cause transposon derepression and DNA damage accumulation in germ cells that lead to sterility [39,50,88,89]. Similarly, *ago3* mutant female flies are reported to be sterile. Homozygous *ago3* mutant females lay fewer eggs than the wild-type or heterozygote sibling controls, and these eggs fail to hatch. Moreover, *Ago3* may be necessary for GSC maintenance as *ago3* mutant males do not contain Vas-positive germ cells adjacent to the hub [34].

PIWI proteins in other organisms are functionally conserved in germline development. There are three murine PIWI proteins, Miwi, Mili, and Miwi2, all of which are expressed during spermatogenesis [90–93]. *Mili* and *miwi2* knockout mice show spermatogenic stem cell arrest [90,93,94]. Only few stem cells in the *mili* mutant can escape the arrest and produce spermatocytes that are then arrested at prophase I of meiosis [94]. In addition, the conditional inactivation of *miwi2* has revealed that *miwi2* is essential for male PGC reprogramming but is dispensable for postnatal male germine development and testicular function in mice [95]. In contrast, global knockout of *miwi* causes post-meiotic arrest of spermatogenic cells [92]. Additionally, Miwi forms RNP complexes that stabilize mRNAs essential for spermiogenesis [96]. PIWI proteins have also been characterized in other vertebrates. For example, zebrafish expresses two PIWI proteins: Zwiwi (zebrafish *Piwi*) and Zili (*ziwi-like*). Although there is no clear block of germ cell formation in *ziwi* mutant fish, germ cells undergo increased apoptosis that results in loss of all germ cells [76]. Strikingly, in *zili* mutant fishes, the number of germ cells is severely decreased and germ cell differentiation is abolished, suggesting that *zili* is required for GSC maintenance [77]. In *C. elegans*, depletion of two *piwi* orthologs, *prg-1* and *prg-2*, leads to meiotic defects [80]. Moreover, PRG-1 has been demonstrated to be essential for fertility [81]. Similar to PIWI proteins in *Drosophila*, mice, zebrafish, and *C. elegans*, PIWI proteins in jellyfish and *Xenopus* have also been reported to participate in germline development, indicating an evolutionarily conserved role of PIWI proteins in the germline [77,97–99].

Recent studies have identified a large group of TUDOR family members expressed in the germline, where they interact with sDMA-modified PIWI proteins to modulate PIWI functions. For example, the *Drosophila* Tudor, the founding member of the TUDOR protein family, contains 11 Tudor domains [100]. It is a component of the polar granule and is involved in germ plasm assembly and PGC formation [20,26,101]. In addition, Tudor associates with *Aub* and *Ago3*, specifically through the Tudor domains and the sDMAs, and regulates the piRNA pathway [29]. In *tudor* mutants, *Aub-* and *Ago3*-associated piRNAs are different from those in the wild-type control, suggesting that Tudor scrutinizes piRNAs and loads them onto *Aub* and *Ago3* [29]. In addition to the Tudor protein, several other TDRDs, including Partner of PIWI1s (PAPI), Kumo/Qin, Tej, Spn-E, and Vret, also interact with PIWI proteins [35,40,41,52,53,55–57,69,70]. Additionally, a group of TDRDs including Brother of Yb (BoYb), SoYb, Krimp, and dSETDB1 have been demonstrated to participate in piRNA biogenesis [40,57,67,102], but their interacting PIWI partners have not yet been identified.

The crucial roles of TUDOR family proteins in germline development and PIWI functions are evolutionarily conserved. In mice, it has been reported that Tdrd1, Tdrd2, Tdrd4, Tdrd5, Tdrd6, Tdrd7, Tdrd8, Tdrd9, Tdrd10, SND1/Tdrd11, Tdrd12, and SetDB1 are expressed in the germline [33,44,103,104]. Among them, Tdrd1, Tdrd2, Tdrd4, Tdrd5, Tdrd6, Tdrd7, Tdrd9, and Tdrd12 have been demonstrated to be involved in nuage formation, spermatogenesis, and the piRNA pathway [33,38,43–45,105–110].

Recent advances in uncovering PIWI–TDRD interactions have demonstrated an elaborate interplay between these proteins in regulating transposon silencing, germ granule formation, and germ cell development (Table 1). Although more and more TUDOR family proteins have been discovered as PIWI protein interactors or the piRNA pathway components, the precise molecular functions of these proteins in the PIWI–piRNA pathway and how these proteins achieve optimal specificity for natural ligands await systematic investigation.

In *Drosophila*, many of the piRNA pathway genes such as *Piwi*, *Aub*, *Armi*, *Zuc*, and *Squ* were initially identified by screening for mutations that cause GSC loss, abnormal axis specification, defective gametogenesis, and sterility [13,34,51,58,71,87,111,112]. In mice, *mili* and *miwi2* mutants also show defects in spermatogenic stem cell maintenance and a reduction of germ cells in the adult stage [93,94]. Subsequent studies further reported that the mutations of genes involved in DNA damage signaling are sufficient to repress some germ line defects caused by the mutations of the piRNA pathway genes. These data suggest that the germline development defects in the piRNA pathway gene mutants may arise from DNA damage [88,93,112,113]. Additionally, upregulation of phosphorylated H2Ax (also called γ-H2Av in *Drosophila*), a marker for unrepaired DNA double-stranded breaks, has been observed in *aub* mutant fly ovaries and *miwi2* mutant mouse testes [58,93,112,113]. These observations further support the idea that the mutations of the piRNA pathway genes cause DNA damage, which
activates the DNA damage response and thus results in germline development defects.

**THE PIWI–piRNA PATHWAY IN EPIGENETIC REGULATION**

Recent studies have suggested that the PIWI–piRNA pathway is involved in epigenetic regulation through histone modification and DNA methylation (Fig. 2). In *Drosophila*, Piwi and Aub are found to act as regulators of position-effect variegation (PEV), in which the expression of an euchromatic gene is silenced to variable extent in different cells within the same tissue when it is localized in proximity to a heterochromatic region. This indicates that the piRNA pathway silences gene expression by promoting heterochromatin assembly [114]. Moreover, Piwi is a nuclear protein that physically interacts with Heterochromatin Protein 1a (HP1a) and colocalizes with HP1a on many sites along the chromosomes [9,11,115]. Flies lacking this interaction exhibit ineffective PEV that is caused by a loss of heterochromatin, suggesting that the Piwi–HP1a interaction is required for heterochromatin formation [115]. Besides the role in epigenetic silencing, the PIWI–piRNA complex can also function as an epigenetic activator at the subtelomeric region 3R-TAS1. It has been shown that in *piwi* mutants, 3R-TAS1 becomes heterochromatic, which indicates the role of Piwi in promoting the euchromatic state of this chromatin locus [9]. Additionally, the insertion of piRNA-complementary sequences into an ectopic site of *Drosophila* genome causes the recruitment of Piwi, HP1a, and Su(var)3–9 as well as H3K9me enrichment and reduced RNA polymerase II association at this ectopic site [11]. This evidence further supports the notion that piRNA is both necessary and sufficient to recruit Piwi and epigenetic factors to specific genomic loci. The interplay between Piwi, HP1, and H3K9me has also been demonstrated in *C. elegans*. PRG-1, the Piwi ortholog in *C. elegans*, can initiate transgenerational gene silencing in the germline by regulating H3K9me, HP1, and histone methyltransferases [116,117]. Importantly, in addition to histone modifications, the epigenetic functions of PIWI proteins can act through DNA methylations. In *mili* or *miwi2* knockout mouse testes, the Cpg methylation level in PGCs is reduced, with concordant transposon upregulation [83,118,119]. In addition, piRNAs are shown to direct DNA methylation on a non-transposon locus to regulate genomic imprinting in the mouse male germline [10]. Recent studies have also suggested that piRNAs influence synaptic plasticity of neuronal cells in *Aplysia* through DNA methylation on non-transposon genomic regions [120].

**THE PIWI–piRNA PATHWAY IN TRANSPOSON SILENCING**

Several genetic studies have reported that the PIWI–piRNA pathway genes play a critical role in transposon repression. For example, *Piwi* is required for silencing transposons within the *flamenco* (*flam*) locus which is active in gonadal somatic cells [121]. In addition, the mutations of *aub* cause increased transposon activity [89,112,122–124]. Moreover, the localization of Aub and Ago3 to the nuage is mutually dependent, and mutations of either gene lead to defects in piRNA production and elevated levels of transposon transcripts [34]. Several piRNA pathway genes have also been reported to be involved in transposon regulation. For example, Mael, an HMG-box-containing protein localized to nuage, is required for Piwi-mediated transposon silencing [125,126]. Rhino (Rhi), an HP1 subfamily member, interacts with Cutoff (Cuff) to regulate the transcription of 1/42AB piRNA cluster. The mutations of either *cuff* or *rhi* cause defects in piRNAs biogenesis and transposon derepression [127,128]. Importantly, a large number of piRNAs have been mapped to the transposon-containing genomic loci [129]. These observations further support the model that PIWI proteins bind to piRNAs that direct them to silence harmful TE in the germline [40,41,48,50,58,71]. Similar transposon silencing functions of PIWI proteins and piRNAs have also been demonstrated in other species. For example, mouse pre-pachytene piRNAs associated with Mili or Miwi2 that are expressed at the prenatal stage are mostly derived from repeated elements and transposons [83]. Furthermore, in the germ cells of *mili* or *miwi2* knockout mice, transposons are dramatically upregulated [83,93]. In zebrafish, piRNAs are mainly derived from transposon sequences [76]. In *Hydra*, piRNA profiling analyses have suggested that transposons are the targets of the piRNA pathway [82,130]. These findings indicate that the piRNA pathway has a conserved function in silencing transposons across species.

**THE PIWI–piRNA PATHWAY IN mRNA TURNOVER AND TRANSLATIONAL CONTROL**

The slicer activity of Aub and Ago3 not only contributes to the amplification loop of piRNA production, but also silences transposons at the post-transcriptional level (Fig. 2). Furthermore, it has been shown that the 3′ untranslated regions (3′UTRs) of a broad set of protein-coding mRNAs are selected for piRNA production in *Drosophila*.
ovaries, mouse testes, and Xenopus eggs. These findings suggest that piRNAs may function in mRNA turnover for cellular regulation [131]. In support of this idea, the protein level of the Traffic Jam (Tj), whose 3' UTR generates abundant sense piRNAs, is upregulated in piwi mutants [131,132]. Additionally, Aub is in the same complex with mRNA deadenylase CCR4, nanos (nos) mRNA, and piRNAs that target the nos 3'UTR region in the bulk of the embryo. In aub and ago3 mutant embryos, the deadenylation and decay of nos mRNA are defective and the Nanos protein is ectopically accumulated [133]. Vas protein levels are also upregulated in aub and ago3 mutants, which is possibly caused by the depletion of piRNAs derived from the vas mRNA [34,134]. Several lines of evidence have demonstrated that the PIWI–piRNA pathway is closely related to processing bodies (P-bodies), cytoplasmic RNP complexes intensively engaging in translational control (reviewed in [135]). It has been shown that the piRNA pathway proteins such as Aub, Ago3, and Tud colocalize with P-body proteins in germ cells [136,137]. Importantly, the role of the PIWI–piRNA pathway in mRNA turnover and translational control has also been reported in other organisms. In mice, Mili, and piRNAs are associated with mRNAs in polysomes and RNP fractions [7,74]. Furthermore, Mili binds to eIF4E, an mRNA cap-binding protein functions in translational control [74]. Similarly, Mili also forms a complex with eIF3a, and interacts with the eIF4E and eIF4G-containing mRNA S' cap-binding complex [94]. Besides, Mili2, Tdrd9, and Mael are localized to piP-bodies [37,109], a variant of P-bodies (reviewed in [135]). It has also shown that Mili associates and stabilizes mRNAs of genes required in the post-meiotic stages of spermatogenesis [96]. In C. elegans, mRNAs from spermatogenesis genes are downregulated in prg-1 mutants [80]. Together, these observations reveal an evolutionarily conserved role for PIWI proteins and piRNAs in post-transcriptional and translational control, a role that has been underinvestigated.

**SOMATIC FUNCTION OF THE PIWI–piRNA PATHWAY**

Increasing lines of evidence indicate that PIWI protein and piRNAs function not only in the germline, but also in somatic tissues. In Drosophila, maternal PIWI proteins are essential for the maintenance of chromatin structure and cell cycle progression during early embryogenesis—the first phase of somatic development [138]. Moreover, Drosophila Piwi binds to chromosomes in somatic tissues such as salivary glands and is responsible for epigenetic effects at the binding sites [9,11,114,115,139], but these results await more lab to repeat and follow up. In Hydra, piRNAs are mainly mapped to non-transposon genes and Hywi is essential for somatic epithelial lineages [82]. In planaria, the two PIWI proteins, SMEDWI-2 (S. mediterranea Piwi2) and SMEDWI-3 (S. mediterranea Piwi3), are needed for the maintenance of neoblasts, the totipotent adult stem cells for tissue regeneration [140,141]. In Aplysia, piRNAs mediate epigenetic DNA methylation which is required for synaptic plasticity in neuronal cells [120]. A similar mechanism has also been identified in mouse hippocampus, in which piRNAs may target non-transposon genes to control spine shape [142]. Mouse Mili and piRNAs are also found to be expressed in adult mouse mesenchymal stem cells [143]. In human, HIWI (human PIWI protein) is expressed in CD34 (+) hematopoietic progenitor cells [144]. Furthermore, a large number of studies have reported that PIWI proteins are upregulated in cancer cells and tissues, as reviewed in detail by [12]. For example, human Hili is expressed in prostate, breast, gastrointestinal, ovarian, and endometrial cancers [145]. In addition, human Hiwi is expressed in seminomas, and gastric, uterine cervical, breast, ovarian, and endometrial cancers [146–148]. Furthermore, piRNAs are detected in cancer cells [149–151]. These results indicate that cancer development may be linked to PIWI proteins and the piRNA pathway. In Drosophila, a study has demonstrated that ectopic expression of piRNA pathway genes contributes to the growth and development of MBT [152]. Moreover, inactivation of the germline genes vasa, piwi, or aub suppressed malignant tumor growth, demonstrating that germline traits are necessary for tumor growth, at least in Drosophila [152]. Although the functions of PIWI proteins and piRNAs in the somatic tissues remain largely unclear, these findings have pointed out that the PIWI–piRNA pathway exerts a broader-than-expected role beyond the germline.

**CONCLUDING REMARKS**

PIWI proteins and piRNAs have been long known as key players in germline development with a focus on transposon silencing. Recent effort in identifying other piRNA pathway factors leads to the discovery of TUDOR family proteins as a major class of PIWI interactors. Meanwhile the scope of PIWI–piRNA studies has been significantly expanded from transposon silencing to other functions in gene regulation in both the germline and somatic tissues. Yet, there are still many intriguing and fundamental
questions awaiting further investigations. For example, what are molecular mechanisms for each step of pathway of piRNA biogenesis? What are the exact mechanisms by which PIWI–piRNAs mediate epigenetic and post-transcriptional regulation? How do these mechanisms contribute to germline development such as germline specification, GSC maintenance, germ cell proliferation, differentiation, meiosis, and oocyte axis formation? What is the role of PIWI–piRNAs in somatic tissues and cancer cells? When these questions are addressed, we will be able to map the complex PIWI–piRNA regulatory network and apply this knowledge towards clinical applications such as the treatment of cancers.

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