H O OR S F S T O OPI I- O RNA S?

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PI I (P-OO O ; O S Θ) O O O A O O OO Θ O O S, O O Θ O S *Drosophila* . PI I O - O O S PI I- O RNA (RNA), RNA O O Θ O O S O Θ, O O OONA OO O O O Θ O , Θ O Θ O O O RNA. T O O - O O S O O O O Θ O O O OΘ. PI I O O O OO OO S S O O S Θ (DMA), O O S O O S O O PRMT5 (D 5 C OO). I O O O O O OT (T) S O O Θ S O O Θ O O PI I O O S DMA A T T S O O O O DMA- Θ, O O OT S O O ΘO O RNA S S OO S O .

Argonaute (AGO) proteins associate with small noncoding RNAs of 20–30 nucleotides (nt) to negatively regulate the expression of genes targeted by the Argonaute–small RNA complexes (Siomi and Siomi 2009). In this mechanism, termed RNA silencing, genes silenced by the catalytic activities of Argonaute proteins are involved in fundamental cellular processes, such as development, differentiation, metabolism, and apoptosis (Kim et al. 2009). Thus, Argonaute proteins are essential for many, if not all, living organisms (Bartel 2009; Malone and Hannon 2009; Voinnet 2009).

The number of Argonaute family members in a species differs; for example, *Schizosaccharomyces pombe* has only one Argonaute, while *Drosophila* and humans possess five and eight members, respectively (Hutvagner and Simard 2008). Each Argonaute member falls into one of two subgroups: the AGO and PIWI (P-element-induced wimpy testis) subfamilies (Farazi et al. 2008). Expression of AGO members is ubiquitous, whereas PIWI proteins are detected predominantly in germline cells (Farazi et al. 2008). Depletion of AGO functions often causes developmental defects; for example, *Ago2*-null mice show embryonic lethality (Liu et al. 2004), while PIWI mutants show defects in gametogenesis, but otherwise develop normally (Cox et al. 1998; Harris and Macdonald 2001; Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007; Li et al. 2009).

AGO proteins associate with microRNAs (miRNAs), ubiquitously expressed small RNAs that function in RNA silencing (Kim et al. 2009). In *Drosophila*, endogenous siRNAs (endo-siRNAs) are also ubiquitous, and associate with AGO proteins (predominantly with AGO2). Furthermore, exo-siRNAs (siRNAs exogenously introduced into cells to artificially induce RNAi) also associate with AGO in *Drosophila* and mice (Siomi and Siomi 2009). PIWI proteins associate specifically with PIWI-interacting RNAs (piRNAs) in germline cells, although endo-siRNAs and/or miRNAs are coexpressed with piRNAs in these cells (Kim et al. 2009). Thus, loading of different kinds of small RNAs onto individual Argonaute proteins is considered to be a "molecule-specific" event.

piRNAs have been studied extensively with regard to their biogenesis, characteristics, and functions, especially in *Drosophila*, fish, nematodes, and mice (in *Caenorhabditis elegans,* piRNAs are known as 21U RNAs) (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Ruby et al. 2006; Saito et al. 2006; Vagin et al. 2006; Watanabe et al. 2006; Brennecke et al. 2007; Gunawardane et al. 2007; Houwing et al. 2007; Batista et al. 2008; Das et al. 2008). piRNAs are longer than miRNAs and endo-siRNAs by several bases; for example, in *Drosophila*, piRNAs range between 24 and 30 nt, while miRNAs and endo-siRNAs are ~20–23 nt long. In addition, piRNAs contain 2′-*O*-methyl groups at their 3′ ends, unlike miRNAs (except in plants) (Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007; Ohara et al. 2007; Saito et al. 2007). piRNAs are derived mostly from repetitive intergenic DNA elements, including transposons, and these loci are collectively called "piRNA clusters" (Aravin et al. 2007a). Protein-coding genes such as *traffic jam* (*tj*) could also account for piRNA

[*Keywords*: PIWI; PRMT5; RNA silencing; Tudor; piRNA; sDMA]

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production (Robine et al. 2009; Saito et al. 2009). Because of these features, and considering their limited expression in germlines and their specific associations with PIWI proteins, piRNAs are considered to be a unique set of endogenous small RNAs.

Loss of PIWI proteins in *Drosophila* and mice causes derepression of transposons and results in severe defects in gametogenesis (Vagin et al. 2006; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008; Li et al. 2009). As a result, the homozygous mutant lines cannot be maintained. Deletion of a *Drosophila* piRNA cluster, *flamenco* (*flam*), located on the X chromosome, also causes derepression of particular transposons, such as *gypsy*, *ZAM*, and *Idefix* (Prud'homme et al. 1995; Desset et al. 2003; Mével-Ninio et al. 2007), because *flam* gives rise to piRNAs that show strong complementarities to transcripts from these transposons (Brennecke et al. 2007). These studies have made it very clear that both PIWI proteins and piRNAs are required for transposon silencing. Targets of PIWI–piRNA complexes are not limited to transposons. In fact, a subset of piRNAs in *Drosophila* has been shown to function in silencing protein-coding genes. The best examples are piRNAs derived from *suppressor of stellate* [*su*(*ste*)] and *tj*, which down-regulate protein-coding *stellate* (*ste*) and *fasciclin III* (*fas III*) genes, respectively (Livak 1984; Aravin et al. 2001, 2004; Vagin et al. 2006; Nishida et al. 2007; Saito et al. 2009).

In *Drosophila*, AGO3 (Argonaute3), Piwi , and Aub (Aubergine) belong to the PIWI protein family, while in mice, MILI, MIWI, and MIWI2 belong to the mouse PIWI family (Siomi and Siomi 2009). Bioinformatic analyses of piRNAs that associate with PIWI proteins in both *Drosophila* and mice germlines have led to two models for piRNA biogenesis: the amplification loop pathway (also termed the Ping-Pong pathway) and the primary processing pathway (Aravin et al. 2007a; Siomi and Siomi 2009). Observations in *Drosophila* support the concepts that, in the amplification loop pathway, Aub (mainly associated with antisense piRNAs, which show a preference for a uracil [U] at the 5′ end) and AGO3 (mainly associated with sense piRNAs, which show a preference for an adenine [A] at position 10) reciprocally cleave target RNAs in sense and antisense orientations, respectively, and that this reciprocal target RNA cleavage by Aub and AGO3 constantly gives rise to abundant piRNAs in germline cells (Brennecke et al. 2007; Gunawardane et al. 2007). The vast majority of their RNA targets are transposon transcripts; thus, transposon silencing occurs in germlines in parallel with piRNA production through the amplification loop. In mice, of the three PIWI proteins, MILI and MIWI2 function in the amplification loop system (Aravin et al. 2007b).

Involvement of Aub and AGO3 in the primary processing pathway, however, is unlikely—at least in somatic cells of *Drosophila* ovaries. Evidence supporting this notion was obtained from studies on ovarian somatic cells (OSCs) and ovarian somatic sheets (OSSs). In *Drosophila* OSCs/OSSs, levels of Aub and AGO3 are below detection, but piRNAs are abundantly expressed and loaded onto Piwi (Lau et al. 2009; Saito et al. 2009). Deep sequencing analyses of small RNAs in piRNA-related mutants also provided strong evidence for an Aub–AGO3-independent piRNA pathway in somatic cells in ovaries (Li et al. 2009; Malone et al. 2009). The requirement for MILI and MIWI2 in the mouse somatic primary processing pathway remains undetermined. Factors necessary for primary piRNA processing may include Zucchini (Zuc), a putative cytoplasmic nuclease, because loss of *Zuc* function caused a severe reduction in the expression levels of primary piRNAs, such as *flam*-originating piRNAs (in ovaries) (Malone et al. 2009) and *tj*-originating piRNAs (in OSCs) (Saito et al. 2009). *dicer* mutant ovaries accumulate piRNAs, similarly to wild-type ovaries (Vagin et al. 2006). *Dicer*-independent piRNA production was also observed in zebrafish (Houwing et al. 2007). Thus, it seems that both the amplification loop and primary processing pathways for piRNA production do not require *Dicer*. Other genes—such as *Armitage*, *Spindle E* (*Spn-E*), *Maelstrom*, *Krimper*, *Vasa*, and *Squash*—might be involved in piRNA biogenesis (Vagin et al. 2006; Lim and Kai 2007; Pane et al. 2007). However, the molecular details of the requirement of these genes in piRNA biogenesis remain unclear.

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(2009) demonstrated that a protein pool obtained by immunoprecipitation from mouse testes using an sDMA-specific antibody, Y12, contained both MILI and MIWI. PIWI proteins in *Drosophila* ovaries and *Xenopus* oocytes (Xiwi and Xili are the *Xenopus* PIWI) were also shown to contain sDMAs by Western blot analyses using Y12 or SYM11, another sDMA-specific antbody (Kirino et al. 2009; Nishida et al. 2009). Y12 coimmunoprecipitated piRNAs with PIWI proteins (Kirino et al. 2009), indicating that sDMA modification does not abolish the PIWI–piRNA association.

The involvement of PRMT5 in the PIWI-sDMA modification was investigated for two major reasons: (1) Loss of PRMT5 caused complete loss of Sm-sDMA modification in *Drosophila* (Gonsalvez et al. 2006; Anne et al. 2007), and (2) *prmt5* mutants phenocopy *aub* mutants (Harris and Macdonald 2001). No PIWI-sDMA modification was detectable in *prmt5* mutants (Kirino et al. 2009). Thus, it was concluded that PRMT5 is the factor responsible for the sDMA modification.

The binding capacity of PIWI proteins for piRNAs seems unaltered by *prmt5* mutations (Kirino et al. 2009). However, derepression of a transposon, *Het-A*, was observed in *prmt5* mutant ovaries (Kirino et al. 2009). This result made it clear for the first time that *prmt5* is tightly connected to transposon silencing. PRMT5-mediated sDMA modification appears to control the subcellular localization of PIWI proteins in ovaries because, in *prmt5* mutants, Aub does not concentrate at the nuage (Nishida et al. 2009), an electron-dense material associated with nurse cell nuclei. This suggests that nuage localization of Aub is important for silencing. Whether Aub localization at the posterior pole of oocytes (Kirino et al. 2009), where materials required for primordial germ cell (PGC) specification are accumulated, is affected by loss of PRMT5 remains controversial (Kirino et al. 2009; Nishida et al. 2009).

Studies describing mouse *prmt5* mutants have not yet been published. In transgenic mice expressing individual mouse PIWI proteins, the PRMT5 complex containing WDR77 (also known as MEP50), a cofactor of PRMT5, was able to associate with all three PIWI proteins (Vagin et al. 2009). MIWI2 and MILI association with the PRMT5–WDR77 complex, termed the methylosome, was observed in embryonic testes, while MIWI (and also MILI) association with the methylosome was observed in adult testes. This was simply due to the stage-specific expression of individual PIWI proteins during germline development. Whether other members of the PRMT family associate with mouse PIWI proteins remains undetermined.

The *Drosophila* homolog of WDR77/MEP50 is Valois (Vls) (Anne and Mechler 2005; Cavey et al. 2005). Genetic disruption of *vls* results in phenocopies of *tud* mutants; both mutants cause a grandchild-less phenotype (Boswell and Mahowald 1985; Schupbach and Wieschaus 1986). Vls, like Aub and Tud, is a component of the nuage and pole plasm (Bardsley et al. 1993; Harris and Macdonald 2001). Vls interacts with not only PRMT5, but also Tud, and localization of Tud at the nuage and at the posterior pole of oocytes depends on Vls expression (Anne and Mechler 2005). These observations clearly indicate a direct relationship of Vls with Aub and Tud in *Drosophila*.

sDMA motifs in *Drosophila* PIWI proteins

Putative "sDMA motifs" GRG and ARG/GRA (G, glycine; R, arginine; A, alanine), present in animal PIWI proteins, were suggested by Kirino et al. (2009). The precise positions of sDMAs in mouse and *Drosophila* PIWI proteins were determined later by mass spectrometry (MS). The first R residue identified to have a symmetric dimethyl group was R74 in MILI (Reuter et al. 2009). Interestingly, R74 resides in the sequence FRG (F, phenylalanine), which was not one of the predicted sDMA motifs. More comprehensive mapping of methylated arginines, including monomethylation, in MILI and MIWI was performed later (Chen et al. 2009; Vagin et al. 2009). Vagin et al. (2009) found that R100, R146, R163, and R549 in MILI are dimethylated, whereas R95 in MILI and R49 and R371 in MIWI are monomethylated. R74 in MILI and R14 in MIWI were detected to be both mono- and dimethylated. Chen et al. (2009) used endogenous MILI and MIWI isolated from adult testes and found that R53 in MIWI can be both mono- and dimethylated. R74, R83, R95, and R100 in MILI appear to be both mono- and dimethylated, whereas R45, R146, R156, and R163 are only dimethylated. All dimethylation was most likely symmetrical (sDMAs). There is some discrepancy between the two analyses that might reflect the methylation status from distinct sources. This may suggest that the PIWI methylation can be regulated dynamically through spermatogenesis. The current methylation status of mouse PIWI proteins, as determined by MS, is summarized in Figure 1A. The methylation status of MIWI2 remains undetermined.

In *Drosophila*, endogenous Aub and AGO3 were subjected to liquid chromatography (LC)-MS/MS analysis. R11, R13, and R15 in Aub and R4, R68, and R70 in AGO3 were determined to be sDMAs (Fig. 1B; Nishida et al. 2009). R17 in Aub and R72 in AGO3 were predicted to be dimethylated in silico; however, MS as performed by Nishida et al. (2009) did not reveal these modifications. Monomethylated Rs were not detected in either Aub or AGO3 (MC Siomi, unpubl.). The methylation status of Piwi in ovaries remains undetermined.

Aub, AGO3, and Tud in the pole plasm

The *tud* gene was first discovered in *Drosophila* as a gene required for the assembly of the germ plasm, a specialized cytoplasm containing electron-dense polar granules (Boswell and Mahowald 1985). The *tud* gene encodes an ~280-kDa protein with 11 repeat motifs (Thomson and Lasko 2004, 2005; Arkov et al. 2006). These domains are termed Tud domains (Ponting 1997). Tud domains are commonly found in proteins other than Tud in a wide range of organisms (Ponting 1997; Talbot et al. 1998; Maurer-Stroh et al. 2003).

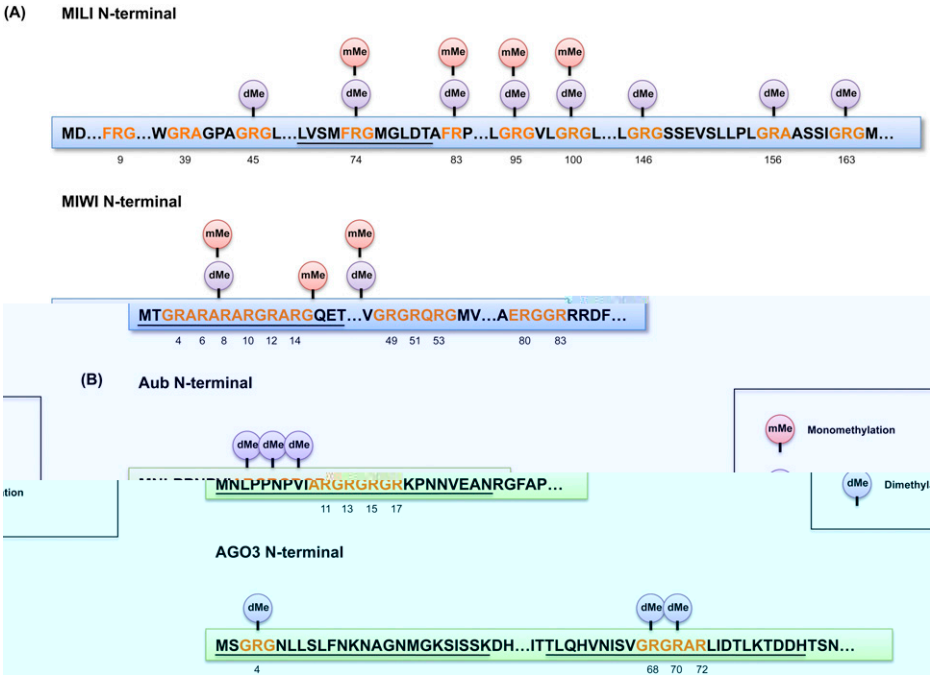

Figure 1. Arginine methylation status of PIWI proteins. Mouse PIWI (*A*; MILI and MIWI) and fly PIWI (*B*; Aub and AGO3) N-terminal sequences are shown with putative sDMA motifs (red). Identified methylation sites (dimethylation [dMe] or monomethylation [mMe]) are shown *above* the relevant arginine, with the residue numbers *below*. Underlined sequences indicate the synthetic peptides that have been used in studies for pull-down assays. The MILI peptide corresponds to L69–T80 and R74 is an sDMA (Reuter et al. 2009). The MIWI peptide corresponds to M1–T18, and R4, R6, R8, R10, R12, and R14 are sDMAs (Kirino et al. 2010). The Aub peptides correspond to M1–N20 (Kirino et al. 2010) and M1–N25 (Nishida et al. 2009), and R11, R13, and R15 are sDMAs. The AGO3 peptides correspond to M1–K25 and T58–H82, and R4 in the M1–K25 peptide and R68 and R70 in the T58–H82 peptide are sDMAs (Nishida et al. 2009).

Based on data in the NCBI HomoloGene database, mice express 26 members of the Tud family (Table 1). Of these, 10 proteins are considered to be members of the Tud domain-containing (TDRD) family (Table 1). TDRD1, which contains four Tud domains along with a MYND (myeloid–nervy–DEAF-1) domain, is necessary for spermatogenesis (Chuma et al. 2006) and interacts with MILI (Table 2; Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009). MIWI also interacts with TDRD1 (Table 2; Chen et al. 2009; Kojima et al. 2009; Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009), although whether TDRD1 shows a tight association with MIWI2 remains controversial (Kojima et al. 2009; Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009). This type of discrepancy can be caused by different groups using different conditions to analyze protein–protein interactions.

In vivo interaction assays with various MILI deletion mutants determined a domain necessary for TDRD1 interaction (Reuter et al. 2009). This domain was in the N-terminal region of MILI and contained dimethylated R74 (Reuter et al. 2009). Treatment with MTA [5′-deoxy-5′-(methylthio)-adenosine], a methyltransferase inhibitor, disrupted MILI-sDMA modification and abolished the association of MILI with TDRD1 (Reuter et al. 2009; Vagin et al. 2009). However, unlike full-length MILI, the N-terminal region was not detected by Y12 (Reuter et al. 2009); this implies that sDMA may not be absolutely necessary for the interaction between MILI and TDRD1. Loss of the MYND domain in TDRD1 does not affect the MILI–TDRD1 interaction (Reuter et al. 2009), indicating that the association occurs through Tud domains.

Tud domain-containing proteins that can associate with PIWI proteins in mouse testes are not limited to TDRD1. Seven Tud domain-containing proteins—TDRD1 to TDRD9, except TDRD3 and TDRD5—were found to be associated with PIWI proteins by various means, both in vivo and in vitro (Table 2; Chen et al. 2009; Kojima et al. 2009; Shoji et al. 2009; Vagin et al. 2009; Vasileva et al. 2009; Wang et al. 2009; Kirino et al. 2010). sDMA dependency of some TDRD proteins to interact with PIWI proteins has been determined. For example, TDRD6—considered the mouse homolog to *Drosophila* Tud, although TDRD6 contains seven Tud domains (Hosokawa et al. 2007), whereas Tud contains 11 Tud domains (Thomson and Lasko 2004, 2005; Arkov et al. 2006)—was able to associate with MILI and MIWI (Chen et al. 2009; Vagin et al. 2009; Vasileva et al. 2009; Kirino et al. 2010), and the interaction most likely occurred in an sDMA-dependent manner (Kirino et al. 2010). TDRD9 interacts specifically with MIWI2 (Table 2; Shoji et al. 2009; Vagin et al. 2009). TDRD1 may also associate with MIWI2 (Vagin et al. 2009; Wang et al. 2009). Whether the TDRD1–MIWI2 and TDRD9–MIWI2 interactions require sDMAs remains unknown.

sDMA-mediated binding of PIWI proteins with Tud family members is evolutionarily conserved. Pull-down assays from *Drosophila* ovary lysates using synthetic Aub

T . 01. *Tud family members in mice*

Tud protein	Motif	Function	References
TDRD1/MTR-1	TUDOR × 4, MYND	Spermatogenesis, piRNA biogenesis	Chuma et al. 2006; Reuter et al. 2009
TDRD2/TDRKH	TUDOR, KH-I	Implicated in spermatogenesis	Chen et al. 2009
TDRD3	TUDOR, DUF1767	Unknown	
TDRD4/RNF17	TUDOR × 5, BBC	Implicated in spermatogenesis	Pan et al. 2005
TDRD5	TUDOR	Implicated in spermatogenesis	Smith et al. 2004
TDRD6	TUDOR × 7	Spermatogenesis, miRNA expression	Hosokawa et al. 2007; Vasileva et al. 2009
TDRD7 /TRAP	TUDOR × 3	Implicated in spermatogenesis	Hosokawa et al. 2007
TDRD8/STK31	TUDOR, polC, PKc-like	Implicated in spermatogenesis	Chen et al. 2009
TDRD9	TUDOR, DEXDc, HELICc, HA2	Spermatogenesis, piRNA biogenesis	Shoji et al. 2009
TDRD12	TUDOR	Unknown	
AKAP1/AKAP121	TUDOR, KH-I	Mitochondrial metabolism	Livigni et al. 2006
ARID4A	TUDOR, RBB1NT, ARID, CHROMO	Implicated in chromatin remodeling	Wu et al. 2008
ARID4B	TUDOR, RBB1NT, ARID, CHROMO	Implicated in chromatin remodeling	Wu et al. 2008
KDM4A/JMJD2A	TUDOR × 2, JmjN, JmjC, PHD × 2	Histone demethylation, self-renewal in embryonic stem cells	Loh et al. 2007
KDM4B/JMJD2B	TUDOR × 2, JmjN, JmjC, PHD × 2	Histone demethylation	Fodor et al. 2006
KDM4C/JMJD2C	TUDOR × 2, JmjN, JmjC, PHD	Histone demethylation, self-renewal in embryonic stem cells	Loh et al. 2007
LBR	LBR-tudor, ICMT	Implicated in morphological granulocyte maturation	Cohen et al. 2008
MTF2	TUDOR, PHD × 2	Unknown	
SETDB1/ESET	TUDOR × 2, MBD, Pre-SET, SET × 2	Histone methylation, maintenance of embryonic stem cell state	Bilodeau et al. 2009
SMN1	TUDOR	snRNP assembly	Gabanella et al. 2005
SMNDC1	TUDOR	SMN-related protein	Talbot et al. 1998
SND1/P100	TUDOR, SNc × 5	Coactivators for signal transducer, implicated in RNA editing and degradation, fly Tudor-SN ortholog	Paukku et al. 2003; Scadden. 2005
PHF19	TUDOR, PHD	Unknown	
PHF20	TUDOR, MBT, PHD, DUF3776	Unknown	
PHF20L1	TUDOR, MBT, PHD, DUF3776	Unknown	
ZGPAT	TUDOR, zf-CCCH, G-patch	Unknown	

(MYND) Myeloid, Nervy, and DEAF-1 Zinc finger; (KH-I) K homology RNA-binding domain type I; (DUF1767) domain of unknown function; (SMN) Survival motor neuron protein; (BBC) B-box C-terminal domain; (polC) DNA polymerase III PolC; (PKc-like) protein kinases catalytic domain; (DEXDc) DEAD-like helicases superfamily; (HELICc) helicase superfamily C-terminal domain; (HA2) helicase-associated domain; (RBB1NT) N terminus to the ARID/BRIGHT domain in DNA-binding proteins of the Retinoblastoma-binding protein 1 family; (ARID) ARID/BRIGHT DNA binding domain; (CHROMO) chromatin organization modifier domain; (Jmj) jumonji domain; (PHD) plant homeodomain finger; (LBR-tudor) Lamin-B receptor of TUDOR domain; (ICMT) isoprenylcysteine carboxyl methyltransferase family; (MBD) methyl-CpG-binding domains; (SET) Su–Enhancer–Trithorax domain; (SNc) staphylococcal nuclease homologs; (MBT) malignant brain tumor repeat; (DUF3776) domain of unknown function; (zf-CCCH) Zinc finger C-x8-C-x5-C-x3-H type; (G-patch) G-patch domain.

peptides, with and without sDMAs, show that a protein of ~280 kDa associates only with the Aub-sDMA peptide (Kirino et al. 2009; Nishida et al. 2009). This protein was identified as Tudor by MS, and the association was confirmed by Western blot analysis using anti-Tud antibodies. Tud members in *Drosophila* are listed in Table 3. Of these, CG14303 is the closest homolog of mouse TDRD1; however, CG14303 does not show an sDMA-dependent association with Aub peptides (Nishida et al. 2009). Also, Spn-E, the *Drosophila* homolog of mouse TDRD9 (Shoji et al. 2009), shows no detectable association with the peptides (Nishida et al. 2009). Thus, association of Aub with Tud is rather specific. Similar assays were performed for AGO3 using peptides that correspond to two different sDMA-modified regions of the protein (Nishida et al. 2009). The sDMA peptide corresponding to the N-terminal end of AGO3 (Fig. 1B) showed no association with Tud, but the second sDMA

T . O2. *Interaction between PIWI and TDRD proteins in mice*

PIWI protein	TDRD protein	References
Adult testis		
MILI	TDRD1/MTR-1	Chen et al. 2009; Kojima et al. 2009; Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD6	Vagin et al. 2009
MIWI	TDRD1/MTR-1	Chen et al. 2009; Kojima et al. 2009; Vagin et al. 2009
	TDRD2/TDRKH	Chen et al. 2009; Vagin et al. 2009
	TDRD4/RNF17	Vagin et al. 2009
	TDRD6	Chen et al. 2009; Vagin et al. 2009; Vasileva et al. 2009; Kirino et al. 2010
	TDRD7/TRAP	Chen et al. 2009
	TDRD8/STK31	Chen et al. 2009
Transgenic mouse		
Adult testis MIWI (sDMA)	TDRD1/MTR-1	Vagin et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD4/RNF17	Vagin et al. 2009
	TDRD6	Vagin et al. 2009
	TDRD7/TRAP	Vagin et al. 2009
	TDRD9	Vagin et al. 2009
Embryonic testis MILI	TDRD1/MTR-1	Vagin et al. 2009
Embryonic testis MIWI2	TDRD1/MTR-1	Vagin et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD9	Vagin et al. 2009
HEK293T/HEK293		
MILI (sDMA [Reuter et al. 2009; Vagin et al. 2009])	TDRD1/MTR-1	Kojima et al. 2009; Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009; Wang et al. 2009
	TDRD9	Vagin et al. 2009
MIWI (sDMA [Chen et al. 2009; Vagin et al. 2009])	TDRD1/MTR-1	Kojima et al. 2009; Wang et al. 2009; Vagin et al. 2009
	TDRD2/TDRKH	Chen et al. 2009; Vagin et al. 2009
	TDRD9	Vagin et al. 2009
MIWI2	TDRD1/MTR-1	Kojima et al. 2009; Wang et al. 2009
	TDRD9	Shoji et al. 2009
Rabbit reticulocyte lysate system		
MILI	TDRD6	Vasileva et al. 2009
MIWI	TDRD6	Vasileva et al. 2009

Summary of protein–protein interactions observed between PIWI and TDRD proteins in mice.

peptide, spanning amino acids 58–82 of AGO3, interacted strongly with Tud. These data indicate clearly that sDMA modifications alone are not sufficient to promote the PIWI–Tud association, and that the association occurs in a peptide sequence-specific manner. None of the AGO3 peptides associate with Spn-E (Nishida et al. 2009). AGO3 is considered the *Drosophila* homolog of MIWI2, in the sense that both proteins interact specifically with secondary piRNAs produced by the amplification loop, which is operated by Aub and AGO3 in *Drosophila* and by MILI and MIWI2 in mice (Aravin et al. 2008). Here, a species difference is obvious with regard to the PIWI–Tud association.

S , , O T PI I , , PI I , RNA, O ,

Many Tud family members interact with PIWI proteins. Thus, it can be speculated that this might be the most prominent pathway for Tud biology. Many groups have looked into how Tud members affect the association between PIWI and piRNAs. Immunoprecipitation from adult mouse testes using an anti-MILI antibody copurified MILI with piRNAs (Kirino et al. 2009; Reuter et al. 2009). In addition, immunoprecipitation using Y12 copurified MILI with piRNAs (Kirino et al. 2009; Reuter et al. 2009). Y12 (antibody) and TDRD1 (protein) recognize the same sDMA region at the N terminus of MILI; this implies that a fraction of MILI that is not associated with TDRD1 is recognized by Y12. This suggests that, even without association with TDRD1, Mili is able to maintain binding with piRNAs. MILI has additional sDMAs (Fig. 1; Chen et al. 2009; Vagin et al. 2009); however, the MILI mutant (termed m1), in which R9, R39, R45, and R74 were mutated to lysines (Ks), showed no association with TDRD1 or TDRD9 (Reuter et al. 2009). Thus, TDRD1 is not likely to be required for MILI to maintain the association with piRNAs once the small RNAs have

T . O3. *Tud family members in* Drosophila

Tud protein	Motif	Function	Closest homolog[a]	References
Tudor	TUDOR × 11	Germline development, piRNA biogenesis	TDRD6	Schupbach and Wieschaus 1986; Nishida et al. 2009
Spindle E	TUDOR, DEXDc × 2, HA2, cas3-core	piRNA biogenesis, histon methylation	TDRD9	Pal-Bhadra et al. 2004; Lim and Kai 2007
Tudor-SN	TUDOR, SNc × 4	RNAi pathway, implicated in RNA editing and degradation	TDRD8	Caudy et al. 2003; Scadden 2005
CG7082	TUDOR, KH-I × 2	Unknown	TDRD2	
CG8589	TUDOR	Unknown	TDRD5	
CG8920	TUDOR × 3	Unknown	TDRD7	
CG13472	TUDOR, DUF1767, UBA	Unknown	TDRD3	
CG14303	TUDOR × 5	Unknown	TDRD1	
Female sterile (1) Yb	TUDOR, SrmB	Male germline stem cell maintenance		Szakmary et al. 2009
Krimper	TUDOR	piRNA biogenesis		Lim and Kai 2007
MBD-R2	TUDOR, THAP, MBD, PHD	Unknown		
Ovarian tumor	TUDOR, OTU	RNA localization nurse cell chromosome dispersion		Goodrich et al. 2004
Polycomblike	TUDOR, PHD × 2	Chromatin modification		Lonie et al. 1994
Survival motor neuron	TUDOR	snRNP assembly		Miguel-Aliaga et al. 2000
Yu	TUDOR, KH-I	Formation of long-term memory, protein kinase A binding		Lu et al. 2007
CG4771	TUDOR × 2	Unknown		
CG9684	TUDOR × 2, MYND	Unknown		
CG9925	TUDOR × 3, MYND	Unknown		
CG15042	TUDOR	Unknown		
CG15930	TUDOR	Unknown		
CG17454	TUDOR	Unknown		
CG30390	DUF1325	Unknown		
CG31755	TUDOR, SrmB, DEXDc	Unknown		

aBased on FlyBase BLAST search.
(DEXDc) DEAD-like helicases superfamily; (HA2) helicase-associated domain; (cas3-core) CRISPR-associated helicase Cas3; (SNc) staphylococcal nuclease homologs; (KH-I) K homology RNA-binding domain type I; (DUF1767) domain of unknown function; (UBA) ubiquitin-associated domain; (SrmB) superfamily II DNA and RNA helicases; (THAP) thanatos-associated protein domain; (MBD) methyl-CpG-binding domains; (PHD) plant homeodomain finger; (OTU) ovarian tumor-like cysteine protease; (MYND) myeloid, Nervy, and DEAF-1 Zinc finger; (DUF1325) SGF29 tudor-like domain. Determined from data in the NCBI HomoloGene and FlyBase database.

been loaded onto MILI. Whether Y12 copurifies TDRD proteins with MILI remains undetermined.

Loss of TDRD1 function does not change the ability of MILI to associate with piRNAs in embryonic testes (Reuter et al. 2009), although the total amounts of piRNAs are severely decreased in *Tdrd1* mutants (Vagin et al. 2009). Deep sequencing analysis revealed that loss of TDRD1 function leads to overrepresentation of small RNAs derived from protein-coding transcripts (from both exons and introns) and of 5S ribosomal RNAs (rRNAs) associated with MILI (Reuter et al. 2009). Representation of transposon-derived small RNAs is not altered by *tdrd1* mutations (Reuter et al. 2009). The molecular mechanisms that create this discrepancy observed between wild-type and *tdrd1* mutant testes remain unclear. The overall piRNA profiles in spermatocytes, in contrast, do not seem to be changed by loss of TDRD6 function (Vagin et al. 2009). TDRD6 may also be required for regulation of miRNAs (Vagin et al. 2009; Vasileva et al. 2009); however, the effect may be indirect, because changes in developmental timing in *tdrd6* mutant mice may result in altered miRNA profiles, as has been reported by Vagin et al. (2009).

Loss of Tud function in *Drosophila* ovaries causes Aub to be associated with a greater abundance of piRNAs compared with Aub in wild-type ovaries (Nishida et al. 2009). Deep sequencing for piRNAs associated with Aub in both wild-type and *tud* ovaries revealed that overrepresentation of any kind of Aub-associated small RNAs did not occur in *tud*; however, the population of transposon-derived piRNAs was altered significantly by loss of Tud function. An obvious change in the strand bias of transposon-derived piRNAs was not seen in the mutants. How this alteration is caused by Tud loss remains unclear; however, these results suggest that Tud is required for the quality control of transposon-derived piRNAs. Obvious derepression of transposons in *tud* ovaries has not been reported. This might be because, although the population was altered, Aub is still associated with

transposon-derived piRNAs, even in the absence of Tud function, and thus the Aub–piRNA association state is not "perfect" but is somewhat "manageable" to silence transposons, at least in ovaries. However, offspring of *tud* mutants are devoid of germ cells. One explanation for this might be that the phenomenon is simply independent of the transposon derepression issue.

Depletion of *tdrd9* in mice testes causes a massive increase of LINE1-derived piRNAs, while other piRNAs, such as the ones derived from IAP and Sine B1 transposons, show a decrease in *Tdrd9* mutants (Shoji et al. 2009); hence, the population of transposon-derived piRNAs is changed by TDRD9. In this sense, Tud in *Drosophila* and TDRD9 in mice are functionally similar to each other, although their similarity at the peptide sequence level is not particularly high.

F O PI I O O T

PIWI proteins in *Drosophila* germlines were originally reported to be destabilized by loss of sDMA modification (Kirino et al. 2009). However, this interpretation was revised later (Nishida et al. 2009; Kirino et al. 2010). There now seems to be a consensus that the stability of PIWI proteins is not changed by the loss of sDMA modification. In *tud* mutants as well as in *prmt5* mutants, the total amounts of Aub and AGO3 were not reduced, indicating that Tud association with the PIWI proteins, regardless of the presence of sDMAs, is not required for their stabilization in vivo. However, the localization of PIWI proteins in ovaries was greatly affected by Tud depletion. In *tud* mutants, Aub is not detected at the nuage, although the protein is detected at the posterior pole of oocytes (Nishida et al. 2009). Kirino et al. (2010) reported that Aub localization to the posterior pole is markedly reduced in *tud* mutants. Thus, it remains controversial whether the posterior pole localization of Aub is dependent on its association with Tud.

The protein levels of MILI and its cytoplasmic localization in mouse testes were barely affected by the loss of TDRD1 expression. However, MIWI2 localization was largely affected. In wild-type testes, MIWI2 is found predominantly in the nucleus and at the nuage. Without TDRD1, MIWI2 becomes mislocalized, being evenly distributed in the cytoplasm (Reuter et al. 2009; Shoji et al. 2009; Vagin et al. 2009). The expression level of MIWI2 was not changed by the loss of TDRD1. Similar mislocalization of MIWI2 was observed previously in MILI mutants, where piRNAs were found not to be loaded onto MIWI2 (Aravin et al. 2008). This was because piRNAs associated with MIWI2 require MILI–Slicer for their production. This suggested that, without piRNA loading, MIWI2 is not imported into the nucleus. A possible scenario might then be that, in *Tdrd1* mutants, the amplification loop for producing MIWI2-associated piRNAs would be somehow unavailable or not functional, as in *mili* mutants, and, consequently, this defect freed MIWI2 from piRNA association, and this absence of piRNAs caused MIWI2 to be localized in the cytoplasm. Indeed, it was found that the levels of piRNAs in the antisense orientation are lowered selectively by TDRD1 loss (Vagin et al. 2009). Antisense piRNAs are thought to be loaded predominantly onto MIWI2 through the amplification loop pathway in mice. Therefore, the decreased amount of piRNAs associated with MIWI2 might indeed be the main cause for MIWI2 mislocalization. Loss of TDRD9 does not cause the mislocalization of MILI, MIWI2, or TDRD1 (Shoji et al. 2009); this is likely to be because piRNAs are loaded onto MIWI2, even in this mutant. It seems that the requirements of TDRD1 and TDRD9 in the piRNA production system differ from each other. In *tdrd6* mutants, MIWI was found to be dispersed in the cytoplasm (Vasileva et al. 2009), although the cause of this observation remains unclear.

Earlier studies have revealed that the loss of MILI and MIWI2 causes the expression levels of both LINE1 and IAP transposons to be much higher compared with those in wild-type testes (Aravin et al. 2007a; Kuramochi-Miyagawa et al. 2008). Loss of MILI and MIWI2 impairs DNA methylation of transposons (Aravin et al. 2007b, 2008; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008). Interestingly, loss of both TDRD1 and TDRD9 derepresses LINE1 (Reuter et al. 2009; Shoji et al. 2009; Vagin et al. 2009); however, silencing of IAP remains as in wild type. The DNA methylation state of LINE1, but not of IAP, was also found to be decreased in *tdrd1* and *tdrd9* mutants (Reuter et al. 2009; Shoji et al. 2009). It is thus obvious that both TDRD1 and TDRD9 have important roles, at least in LINE1 silencing in mouse testes.

The current body of evidence shows clearly that sDMA modifications are made on PIWI proteins by PRMT5. Also, PIWI proteins associate, via their sDMA modifications, with Tud family members. These mechanisms are highly evolutionarily conserved and are indispensable for gametogenesis. In fact, genes encoding protein factors required for this sequential molecular flow are classified mostly as grandchild-less genes. This simply indicates that the system may be germline-specific. Tud family members have impacts on piRNA production and on piRNA association with PIWI proteins; however, no impact on ubiquitously expressed miRNAs or endo-siRNAs has so far been reported (except for the possible involvement of TDRD6 in miRNA regulation). Indeed, none of the ubiquitous AGO proteins that function in RNA silencing by associating with miRNAs and endo-siRNAs have so far been shown to be sDMA-modified, in contrast to PIWI proteins. Thus, regulation by PRMT5-directed sDMA modification and sDMA-specific association with Tud might be a specialized event for the PIWI–piRNA pathway. Why such an elaborative system exists specifically for the piRNA pathway remains unknown. It can be speculated that germlines need to selectively use PIWI proteins containing sDMA and PIWI without sDMAs at certain stages during gametogenesis. To clarify the possibility, it would be interesting to study a dynamic event for PIWI-sDMA modification in germlines.

How and with what factors are piRNA production and piRNA association with PIWI proteins regulated? Until recently, this question was unanswered; however, we

now know that particular members of the Royal Family of Tudor—TDRD proteins in mice and Tudor in *Drosophila*—have roles in piRNA pathways. It is tempting to postulate that Tud members act as the sovereigns of the whole piRNA process. Further investigation should help clarify if this interesting scenario reflects reality, and resolve other related questions regarding the Royal Family of Tudor and the piRNA pathways occurring in germlines.

A _ O O ·

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