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Arginine methylation of Aubergine mediates Tudor binding and germ plasm localization

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ABSTRACT

Piwi proteins such as *Drosophila* Aubergine (Aub) and mouse Miwi are essential for germline development and for primordial germ cell (PGC) specification. They bind piRNAs and contain symmetrically dimethylated arginines (sDMAs), catalyzed by dPRMT5. PGC specification in *Drosophila* requires maternal inheritance of cytoplasmic factors, including Aub, dPRMT5, and Tudor (Tud), that are concentrated in the germ plasm at the posterior end of the oocyte. Here we show that Miwi binds to Tdrd6 and Aub binds to Tudor, in an sDMA-dependent manner, demonstrating that binding of sDMA-modified Piwi proteins with Tudor-domain proteins is an evolutionarily conserved interaction in germ cells. We report that in *Drosophila tud[1]* mutants, the piRNA pathway is intact and most transposons are not de-repressed. However, the localization of Aub in the germ plasm is severely reduced. These findings indicate that germ plasm assembly requires sDMA modification of Aub by dPRMT5, which, in turn, is required for binding to Tudor. Our study also suggests that the function of the piRNA pathway in PGC specification may be independent of its role in transposon control.

Keywords: **Argonaute; Aubergine; piRNA; Piwi; Tudor; miRNA**

INTRODUCTION

Ribonucleoprotein complexes composed of Argonaute proteins bound to small RNAs form the essential effector complexes of RNA silencing (Liu et al. 2008). Argonaute proteins contain two characteristic domains termed PAZ and PIWI and are divided into two subclades: Ago and Piwi (Carmell et al. 2002). Ago proteins are typically expressed in most cell types and bind to microRNAs (miRNAs) and short interfering RNAs (siRNAs) (Liu et al. 2008). Piwi family proteins are expressed in the germline and bind to piRNAs that consist of 25–31 nucleotides (nt) (Klattenhoff and Theurkauf 2008; Ghildiyal and Zamore 2009; Kim et al. 2009). The PIWI domain of Ago and Piwi proteins is an RNase H protein domain that may display endonucleolytic activity toward RNAs that are complementary to bound miRNAs or piRNAs (Ghildiyal and Zamore 2009; Kim et al. 2009).

Piwi proteins are essential for germline development and germ cell specification. *Drosophila melanogaster* expresses three Piwi proteins termed Aubergine (Aub) (Harris and Macdonald 2001), Piwi (Cox et al. 1998), and Ago3 (Brennecke et al. 2007; Gunawardane et al. 2007; Li et al. 2009). Mice express three Piwi proteins known as Mili (Kuramochi-Miyagawa et al. 2004), Miwi (Kuramochi-Miyagawa et al. 2001; Deng and Lin 2002), and Miwi2 (Carmell et al. 2007; Girard and Hannon 2008). The sequence diversity of piRNAs is immense, and hundreds of thousands of unique piRNAs have been described in diverse species (Aravin et al. 2003, 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Ruby et al. 2006; Saito et al. 2006; Vagin et al. 2006; Watanabe et al. 2006; Brennecke et al. 2007; Houwing et al. 2007; Kirino et al. 2009). piRNAs originate from piRNA clusters but also from many other genomic areas, including intergenic and genic regions. Many piRNAs are derived from transposable and repetitive elements and also target transposons (Malone and Hannon 2009). However, large classes of piRNAs in different

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species (for example, pachytene piRNAs [Girard et al. 2006] in mice or 21U piRNAs in *Caenorhabditis elegans* [Ruby et al. 2006]) do not appear to be derived from or to target transposons, and their targets and functions remain unknown.

Arginine methylation is an important post-translational modification that is catalyzed by protein methyltransferases (PRMTs) and occurs either as asymmetric arginine dimethylation (aDMA) or symmetric arginine dimethylation (sDMA) (Krause et al. 2007). PRMT5 and its cofactor MEP50/WD45 form the methylosome (Friesen et al. 2001, 2002; Meister et al. 2001) and deposit sDMAs in diverse proteins, such as the Sm proteins, components of small nuclear ribonucleoproteins (snRNPs) (Friesen et al. 2001, 2002; Meister et al. 2001), and histones (Zhao et al. 2009). Methylated arginines, and in particular sDMAs, bind to Tudor domains and regulate protein–protein interactions (Bedford and Richard 2005; Cote and Richard 2005). For example, sDMA modification of Sm proteins promotes their binding to the Tudor domain of the survival of motor neurons (SMN) protein (Selenko et al. 2001), and this interaction facilitates snRNP assembly in mammals (Friesen et al. 2001; Meister et al. 2001; Boisvert et al. 2002; Gonsalvez et al. 2007).

Specification of primordial germ cells (PGCs) in the developing *Drosophila* embryo requires maternal inheritance of cytoplasmic factors that are concentrated in the posterior pole in an area known as the pole or germ plasm (Ephrussi and Lehmann 1992; Jongens et al. 1992; Williamson and Lehmann 1996; Houston and King 2000; Mahowald 2001; Strome and Lehmann 2007; Bastock and St Johnston 2008; Dansereau and Lasko 2008). Pole plasm contains electron-dense granules and related amorphous material that is rich in ribonucleoproteins and mitochondria; it is related to nuage, which surrounds the nurse cell nuclei and contains some of the same components (Dansereau and Lasko 2008; Chuma et al. 2009). Similar electron-dense amorphous material often in close apposition to mitochondria is found in the cytoplasm of germ cells in various species and is known as P granules in *C. elegans*, germinal granules in *Xenopus*, and intermitochondrial cement and chromatoid bodies in mice (Dansereau and Lasko 2008; Chuma et al. 2009). A set of maternally expressed genes (often referred to as posterior group or *grandchildless* genes) are required for PGC specification (Schupbach and Wieschaus 1986), and invariably the protein or RNA products of these genes are concentrated in the pole plasm and are incorporated in PGCs (Ephrussi and Lehmann 1992; Williamson and Lehmann 1996; Houston and King 2000; Mahowald 2001; Strome and Lehmann 2007; Bastock and St Johnston 2008; Dansereau and Lasko 2008). Loss-of-function mutations of *grandchildless* genes lead to offspring that do not form PGCs and are thus sterile (Williamson and Lehmann 1996; Houston and King 2000; Mahowald 2001; Strome and Lehmann 2007; Bastock and St Johnston 2008; Dansereau and Lasko 2008).

Among these genes are *aub*, which encodes the piRNA-binding protein Aub (Harris and Macdonald 2001; Brennecke et al. 2007; Gunawardane et al. 2007); *csul/dart5*, the *Drosophila* homolog of PRMT5 (dPRMT5) (Gonsalvez et al. 2006; Anne et al. 2007); *valois*, the *Drosophila* homolog of MEP50 (dMEP50) (Anne and Mechler 2005; Cavey et al. 2005); and *tudor* (Boswell and Mahowald 1985; Thomson and Lasko 2004, 2005; Arkov et al. 2006). In *valois*-null mutants, dPRMT5 is destabilized, resulting in a loss of sDMA modifications of target proteins, indicating that dMEP50 is required for sDMA production in concert with dPRMT5 (Gonsalvez et al. 2006). Piwi family proteins, including Aub, from diverse species, contain sDMAs, and the sDMAs in *Drosophila* are catalyzed by dPRMT5 (Kirino et al. 2009). These findings explain the genetic relationship between *aub*, *csul*, and *valois* by demonstrating that Csul/dPRTM5 (and presumably its Valois/dMEP50 cofactor) methylate Aub (Kirino et al. 2009).

Tudor is an ∼285-kDa protein that contains 11 Tudor domains (Boswell and Mahowald 1985; Thomson and Lasko 2004, 2005; Arkov et al. 2006). Tudor domains of other proteins have been shown to bind to methylated amino acids and specifically to sDMAs, suggesting that an important function of Tudor domains is to bind to sDMA-containing proteins (Selenko et al. 2001; Bedford and Richard 2005; Cote and Richard 2005). In mice, several Tudor-domain-containing proteins (Tdrd1, Tdrd4, Tdrd5, Tdrd6, and Tdrd7) are expressed in the germline (Smith et al. 2004; Hosokawa et al. 2007). Genetic disruption of *Tdrd1* leads to arrest in spermatogenesis and male sterility (Chuma et al. 2006). In *Tdrd1*-null spermatocytes, there is a strong reduction of the intermitochondrial cement, but the chromatoid body shows a milder disruption of its architecture (Chuma et al. 2006). Tdrd1 contains four Tudor domains and associates with Mili (Reuter et al. 2009; Vagin et al. 2009; Wang et al. 2009). It was recently shown that sDMA modifications in Mili are required for binding to Tdrd1, and in the absence of Tdrd1, there is up-regulation of L1 retrotransposons (Reuter et al. 2009; Vagin et al. 2009). Tdrd6 is the mouse homolog of *Drosophila* Tudor; it contains seven Tudor domains and associates predominantly with Miwi (Vagin et al. 2009; Vasileva et al. 2009), and also with Mili (Vagin et al. 2009; Vasileva et al. 2009). Interestingly, in *Tdrd6*-null mice, the architecture of the chromatoid body is severely disrupted (Vasileva et al. 2009). In contrast to the transposon up-regulation that is seen in *Tdrd1*-null mice, *Tdrd6*-null mice do not show any de-repression of transposons (Vasileva et al. 2009), and the piRNA profile of *Tdrd6*-null spermatocytes is not altered (Vagin et al. 2009).

Here we demonstrate that Aub binds to Tudor and that the sDMAs of Aub are essential for this binding. We also show that Miwi binds to Tdrd6 and sDMAs of Miwi mediate Tudor binding in vitro. Thus, the binding of sDMA-modified Piwi family proteins with Tudor-domain-containing proteins is an evolutionarily conserved interaction in germ cells.

Furthermore, we report that in *Drosophila tud*[1] mutants, neither the levels of Piwi proteins nor of piRNAs are affected in the female germline. However, the localization of Aub in the pole plasm is severely reduced. These findings indicate that pole plasm assembly requires arginine methylation of Aub by dPRMT5, which, in turn, is required for binding to Tudor. The finding that loss of Tudor results in only very mild transposon de-repression suggests that the function of the piRNA pathway in PGC specification may be independent of its function in transposon control.

RESULTS

We have previously shown the presence of symmetrically dimethylated arginines (sDMAs) in the amino termini of Piwi proteins from diverse species, including *Drosophila* Aubergine (Aub) and mouse Miwi, and we hypothesized that sDMAs might mediate interaction with Tudor-domain-containing proteins. To identify proteins that bind specifically to sDMAs of Aub and Miwi, we performed binding experiments using biotinylated peptides derived from the amino termini of Aub and Miwi that contain sDMAs (Fig. 1A). As controls, we used peptides with the same sequence but containing either unmodified arginines or arginines containing asymmetrical dimethyl groups (aDMAs) (Fig. 1A). We immobilized equal amounts of each biotinylated peptide on streptavidin-Sepharose and then incubated the Miwi peptides with mouse testis lysates and the Aub peptides with *Drosophila* ovary lysates. After extensive washes, we analyzed bound proteins with NuPAGE and silver staining. As shown in Figure 1B, Miwi peptide containing unmodified arginines bound numerous proteins. In contrast, very few proteins bound to sDMA or aDMA Miwi peptides (Fig. 1B). Two prominent bands at ~200 kDa were specifically bound to the sDMA-Miwi peptide (Fig. 1B), and they were identified as Tdrd6 by mass spectrometry (see Supplemental Table). The upper band corresponded to full-length Tdrd6, and the lower band corresponded to a naturally found C-terminally truncated form of Tdrd6 (known as ΔC-Tdrd6) (Vasileva et al. 2009). To confirm the results of the mass spectrometry analysis, we performed Western blotting on the eluates of the Miwi peptides, and we detected Tdrd6 and ΔC-Tdrd6 only in the eluates of sDMA-Miwi peptides (Fig. 1B).

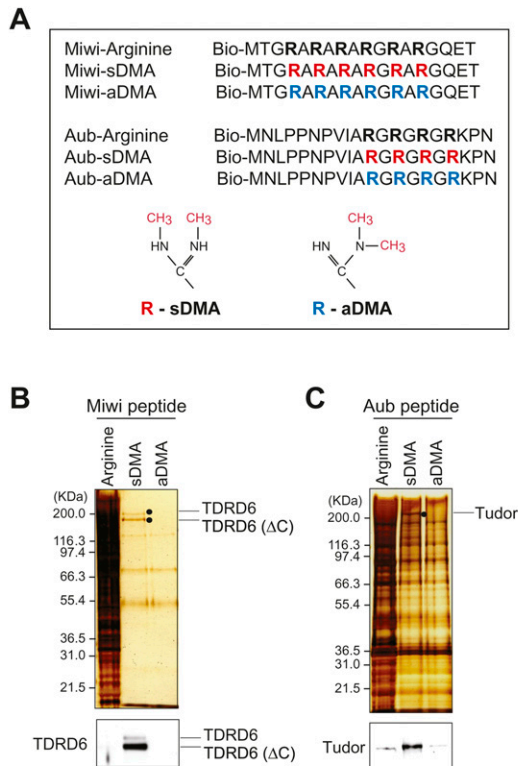

FIGURE 1. *Drosophila* Tudor protein and its mouse homolog, Tdrd6, bind specifically to sDMAs of peptides derived from Aub and Miwi proteins, respectively. (*A*) Biotinylated peptides used for pull-downs. (*B*) Mouse testis or (*C*) *Drosophila* ovary lysates were incubated with indicated peptides; bound proteins were visualized by silver staining and identified by mass spectrometry. Western blots of indicated proteins from pull-downs are shown.

We performed a similar experiment and analysis with Aub peptides. As shown in Figure 1C, a >200-kDa protein band was specifically seen in eluates from sDMA-Aub and was identified by mass spectrometry as *Drosophila* Tudor protein (Supplemental Table). Although the calculated molecular weight of Tudor is ~285 kDa, its mobility is faster in NuPAGE. Western blot analysis of the eluates with anti-Tudor antibody confirmed the presence of Tudor in sDMA-Aub eluates. These findings show that *Drosophila* Tudor protein binds specifically to sDMA-Aub, and Tdrd6, the mouse homolog of Tudor, binds to sDMA-Miwi. It is interesting to note that numerous proteins bind to Aub and Miwi peptides with unmodified arginines, while far fewer proteins bind to aDMA-, or sDMA-modified Aub and Miwi peptides, and only Tudor or Tdrd6 binds specifically to sDMA-Aub and sDMA-Miwi, respectively.

We next performed immunoprecipitations using an antibody against Tdrd6 or non-immune rabbit serum (NRS, negative control) from mouse testis, and we probed the immunoprecipitates with Tdrd6 and Miwi antibodies. The properties of our anti-Miwi antibody are shown in Figure 2A. As shown in Figure 2B, Miwi was present in Tdrd6 immunoprecipitates, consistent with recent reports of Miwi–Tdrd6 interaction (Vagin et al. 2009; Vasileva et al. 2009). We also performed immunoprecipitations using anti-Tudor or NRS from wild-type (wt) and *dPRMT5/csul*-null *Drosophila* ovary lysates, and we probed the immunoprecipitates with anti-Tudor or anti-Aub antibodies. *csul*-null flies cannot produce symmetrical methylation on arginines, and we have previously shown that Aub does not contain sDMAs in ovaries of *csul*-null flies. As shown in Figure 2C, Aub was found in the anti-Tudor immunoprecipitates from wild-type (wt) but not

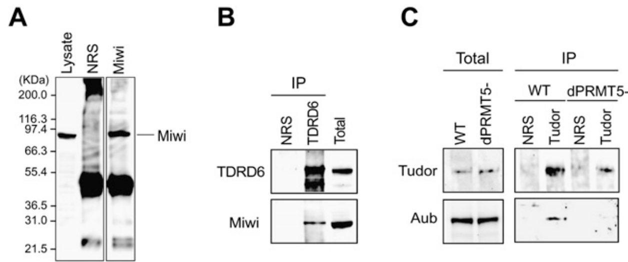

FIGURE 2. Miwi interacts with Tdrd6 in vivo, and sDMAs of Aub are required for Tudor binding in vivo. (*A*) Properties of anti-Miwi antibody. Mouse testis cell lysate and immunoprecipitates (IP) using anti-Miwi or non-immune rabbit serum (NRS, negative control) were probed with anti-Miwi antbody. (*B*) Tdrd6 or NRS immunoprecipitates from mouse testis were probed with indicated antibodies; total lysates prior to immunoprecipitation are shown. (*C*) Tudor or NRS immunoprecipitates from wild-type (wt) and dPRMT5-null ovaries were probed with indicated antibodies.

from *csul*-null ovaries, indicating that sDMA modifications of Aub are essential for Tudor interaction in vivo.

To investigate the Aub–Tudor interaction further, we performed in vitro binding experiments. We have previously shown that in Aub, the four arginines that are substrates for symmetrical dimethylation are found in tandem very close to the N-terminus (Fig. 3A, Aub-WT) and mutation of these arginines into lysines abolishes sDMAs in Aub (Fig. 3A, Aub-4K). We immunopurified Flag-tagged Aub-WT or mutant Aub-4K expressed stably in *Drosophila* S2 cells (which express dPRMT5), and equivalent amounts (Fig. 3B, bottom panel) were used in binding experiments with in vitro translated and radiolabeled Tudor protein. As shown in Figure 3B (upper panel), Tudor bound to Aub-WT but not Aub-4K, indicating that Aub protein interacts with Tudor and that the four arginines that can be modified to sDMAs are required for this interaction.

Previous work has shown that a deletion mutant of Tudor termed Tud-Δ3, expressing Tudor domains 1 and domains 7–11, rescues germ cell formation in a strong loss of function tud^1 mutant background (Fig. 3C). In contrast, deletion mutants of Tudor domains 7–11 (Fig. 3C,Tud-Δ1) and deletion of domains 1–9 (Fig. 3C,Tud-Δ2) were unable to rescue germ cell formation in strong loss-of-function *tud* alleles, indicating that domains 7–11 of Tudor are critical for germ cell formation (Arkov et al. 2006). We tested the ability of these deletion mutants to interact with Aub protein using in vitro binding experiments. As shown in Figure 3D, Tud-Δ3 but not Tud-Δ2 bound specifically to Aub-WT. We observed a strong, nonspecific binding of Tud-Δ1 to the beads (that we did not observe with full-length Tudor) (Fig. 3B), suggesting that the protein produced from the Tud-Δ1 construct might be misfolded and prone to nonspecific binding. Overall, these binding experiments suggest that Tudor domains 7–11, which are required for germ cell formation in vivo, interact with Aub.

Next, we analyzed Piwi proteins and the piRNA pathway in tud^1 mutant ovaries. tud^1 is a strong loss-of-function mutant allele (K1036UAG) that encodes a prematurely truncated form of Tudor that is not detectable in immunoblots (Fig. 4A; Arkov et al. 2006). However, the levels of all three *Drosophila* Piwi proteins (Piwi, Aub, and Ago3) and of the miRNA-binding Ago1 protein were the same between wild-type and tud^1 ovaries. Similar amounts of Aub and Piwi proteins were immunoprecipitated between wild-type and tud^1 ovaries (Fig. 4B), and the amount of bound piRNAs was the same between wild-type and tud^1 ovaries (Fig. 4C). We have previously reported a reduction of Aub and Ago3 protein levels in *csul* ovaries (Kirino et al. 2009). We have found that there is variability in the levels of Aub in *csul* ovaries that may correlate with culture conditions. It is also possible that loss of dPRMT5 may affect an as-yet-unidentified factor(s) that leads to reduction of Aub protein levels.

We also tested the levels of several transposon transcripts, whose expression is sensitive to mutations that disrupt piRNA-mediated transposon silencing (Vagin et al. 2006; Li et al. 2009) in *csul* (dPTMT5-null) and tud^1 ovaries. As shown in Figure 4D, transcript levels of *Diver*, *HeT-A*, *Accord2*, and *Blood* were clearly up-regulated in *csul* ovaries. However, in tud^1 ovaries, only the *Blood* retrotransposon was up-regulated. Next, we analyzed by confocal microscopy the localization of Aub in wild-type and tud^1 ovaries. As shown in Figure 4E, the levels and localization of Aub were the same between wild-type and tud^1 early egg chambers. However, there was a marked reduction of Aub that is localized in the pole plasm in tud^1 ovaries (Fig. 4F). Collectively, these findings indicate that in the absence of Tudor, neither the levels of Piwi proteins nor piRNAs are affected in the female germline, and silencing of most transposons is intact. However, the localization of Aub in the pole plasm is severely affected.

DISCUSSION

We have recently shown that dPRMT5 (Csul/dart5) catalyzes sDMA modifications of Aub (Kirino et al. 2009). We predict that a similar requirement for dMEP50/Valois in Aub methylation is required since dPRMT5 stability and function require dMEP50. In this study, we identify that an important and evolutionarily conserved function of sDMA modifications of Piwi family proteins is to direct their binding to Tudor-containing proteins. One such interaction is between sDMA-modified Aub and Tudor in *Drosophila* oocytes. Collectively these findings provide an explanation for the relationship between the protein products of four posterior-group genes that has been previously elusive: dPRMT5 and dMEP50 produce sDMAs in Aub, which, in turn, are required for binding to Tudor (Fig. 5).

A general role of Tudor-domain-containing proteins and Piwi family proteins in germline development is now

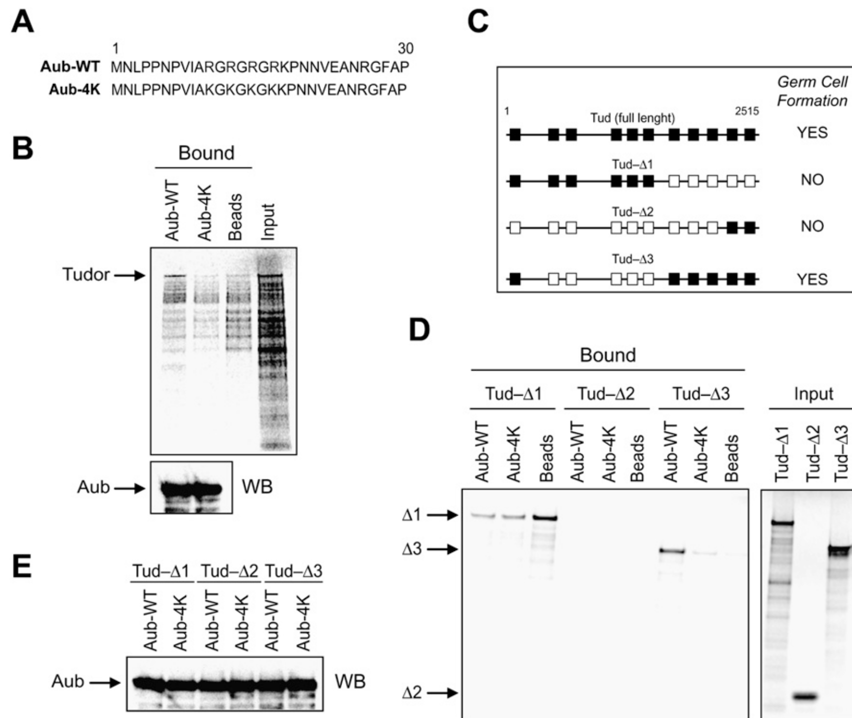

FIGURE 3. sDMAs of Aub are required for binding to Tudor in vitro. (*A*) Sequences of Aub-WT and mutant Aub-4K, showing the four arginines that are substrates for methylation and that were substituted for lysines. (*B*) In vitro translated and radiolabeled full-length Tudor was incubated with immunopurified Flag-tagged wild-type Aub (wt) or mutant Aub (Aub-4K, not containing sDMAs) produced is S2 cells, or with Flag-beads only. Bound proteins were analyzed by NuPAGE and visualized by autoradiography. Input shows 10% of in vitro translated Tudor. Amounts of Aub-wt and Aub-4K were analyzed by Western blot using Flag antibody. (*C*) Schematic of domain architecture and deletion mutants of Tudor; (boxes) individual Tudor domains; (white boxes) deleted domains. Ability to support germ cell formation is indicated (Arkov et al. 2006). (*D*) In vitro translated and radiolabeled deletion mutants of Tudor were incubated with immunopurified Aub-wt or Aub-4K, or with Flag-beads only. Bound proteins were analyzed by NuPAGE and visualized by autoradiography. Input shows 10% of in vitro translated Tudor deletion mutants. (*E*) Amounts of Aub-wt and Aub-4K of each binding reaction were analyzed by Western blot using Flag antibody.

becoming apparent. Mouse Tdrd1 binds to Mili (Reuter et al. 2009; Wang et al. 2009), and this interaction is dependent on sDMA modifications of Mili (Reuter et al. 2009; Vagin et al. 2009). Although the levels of Mili protein and bound piRNAs are not changed in *Tdrd1*-null spermatocytes (Reuter et al. 2009; Wang et al. 2009), the identity of the Mili-bound piRNAs is altered with over-representation of genic piRNAs at the expense of transposon-derived piRNAs (Reuter et al. 2009). This shift correlates with L1 transposon de-repression (Reuter et al. 2009; Vagin et al. 2009) and DNA demethylation (Reuter et al. 2009), which is similar to the phenotype observed in *mili*-null spermatocytes (Aravin et al. 2007). Furthermore, in the absence of Tdrd1 or Mili, Miwi2, which is normally a nuclear protein, delocalizes to the cytoplasm (Reuter et al. 2009; Vagin et al. 2009), and in the absence of Mili, the cytoplasmic Miwi2 is devoid of piRNAs (Aravin et al. 2007). Miwi2 also associates with Tdrd1 (Vagin et al. 2009). These findings indicate that the function of Tdrd1 is closely related to Mili and Miwi2, and Tdrd1 has an important role in specifying the piRNA content of Mili (Reuter et al. 2009) and the operation of the ping-pong cycle of piRNA amplification (Vagin et al. 2009).

Mouse Tdrd6 interacts with Miwi and Mili (Vagin et al. 2009; Vasileva et al. 2009), and we show in this study that Tdrd6 binds to sDMA-modified peptide of Miwi. In contrast to the *Tdrd1*-null mice, transposons are not de-repressed in *Tdrd6*-null spermatocytes (Vasileva et al. 2009), and the piRNA profile of *Tdrd6*-null spermatocytes is not altered (Vagin et al. 2009). This is consistent with our finding that most transposons are not up-regulated in *tud[1]* *Drosophila* ovaries, with the exception of the *Blood* transposon. This may indicate that Tudor is required directly or indirectly for the biogenesis or function of a subset of piRNAs that may target *Blood*. Future deep sequencing studies of *tud[1]* piRNAs will be required to address whether *Blood* de-repression correlates with loss of cognate piRNAs. However, we note that the relationship between transposon de-repression and loss of piRNAs is complex and not well understood. For example, despite widespread changes in the content and levels of piRNAs in *Aub*-null and *Ago3*-null ovaries, there is only partial overlap between the altered piRNAs and the de-repressed transposons (Li et al. 2009; Malone et al. 2009). It is also possible that piRNAs and piRNA-associated proteins target and regulate the expression of mRNAs whose protein products are important for the specification of germ cells. For example, the *Drosophila* AT-chX-1 and AT-chX-2 piRNAs are antisense to Vasa mRNA and down-regulate the expression of Vasa protein, which is essential for germ cell specification (Nishida et al. 2007; Li et al. 2009). An important goal of future studies will be to identify RNAs that are bound by piRNPs in the germ plasm.

It appears that specific members of the Piwi family of proteins function together with Tudor-domain-containing proteins. In that regard, Ago3 and Piwi will likely interact with specific Tudor-domain-containing proteins. It is interesting to note that Spindle E contains a Tudor domain along with an RNA helicase domain and is an important factor for piRNA biogenesis in *Drosophila* (Savitsky et al. 2006; Vagin et al. 2006; Malone et al. 2009). The mouse homolog of Spindle E is Tdrd9 and shows weak association

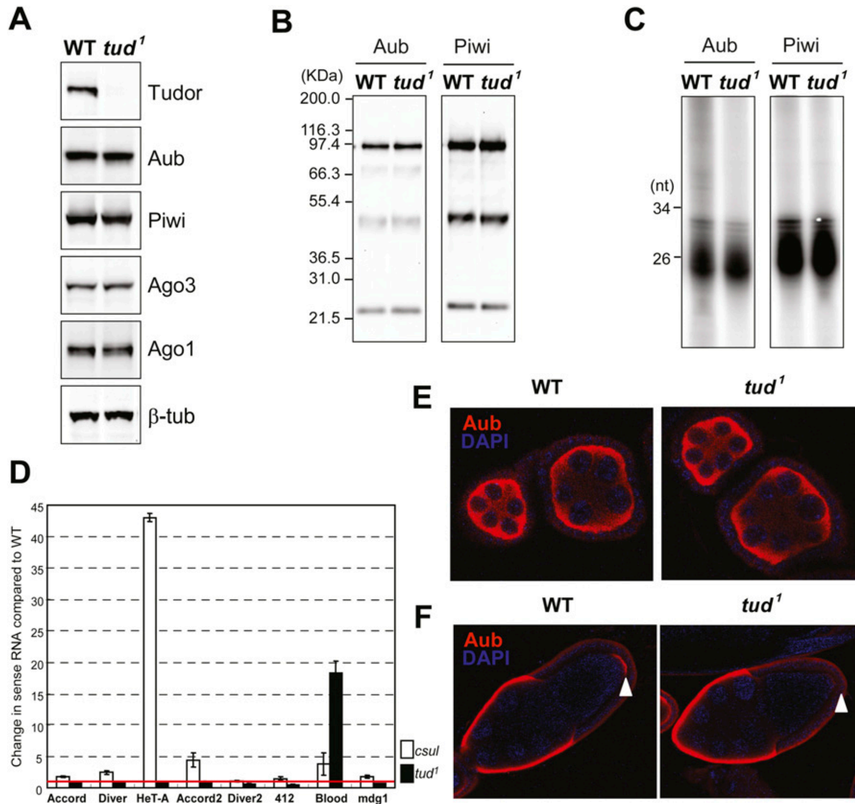

FIGURE 4. Tudor is essential for localization of Aub to the pole plasm but does not affect the levels of Piwi proteins or piRNAs or of most transposon transcripts. (*A*) Western blots of *Drosophila* ovary lysates from wild-type (WT) or *tudor* (*tud*^{1}) with indicated antibodies. (*B*) Aub or Piwi immunoprecipitates from ovary lysates were probed with Aub and Piwi antibodies, and (*C*) bound piRNAs were 5′-end-radiolabeled and analyzed by UREA-PAGE. (*D*) Levels of indicated transposon transcripts in ovaries of wild-type, *tud*^{1}, and *csul/dPRMT5*-null were examined by qRT-PCR. (Red line) Transcript levels from wild-type ovaries were set as 1, and fold-changes are indicated; averages of three independent experiments with SD values are shown. Localization of Aub in wt and *tud*^{1} ovaries in (*E*) early or (*F*) late egg chambers; (arrow) pole (germ) plasm.

with Miwi and Miwi2 (Vagin et al. 2009). Krimper, which is also required for piRNA biogenesis in *Drosophila*, contains a Tudor domain (Lim and Kai 2007). Interactions between these proteins and Piwi family proteins will be interesting to elucidate. At the same time, the occurrence of multiple Tudor domains in Tudor and other Tdrd's suggests that they may form landing pads for additional DMA-containing or other proteins. It is likely that Tudor has multiple binding partners. The functions of Tudor with regard to germ cell specification and posterior patterning appear to involve different regions of the protein (Arkov et al. 2006), and ME31B, eIF4A, Aub, and TER94 have been identified as components of Tudor complexes (Thomson et al. 2008), although direct binding to Tudor has not been demonstrated for any of these proteins other than Aub. Further analysis of Tudor-binding proteins will be important to future studies of germ cell specification and will shed additional light on its function.

MATERIALS AND METHODS

Analysis of the proteins interacting with peptides containing arginine methylations

Miwi and Aub peptides (Fig. 1A) were synthesized by Millipore. Each peptide was immobilized on Streptavidin Sepharose High Performance (GE Healthcare) and incubated in the lysate produced from mouse testis (Pel-Freez Biologicals) (Miwi peptide) or *Drosophila* ovary lysate (Aub peptide) for 1.5 h at 4°C in a lysis buffer (20 mM Tris-HCL at pH 7.5, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.5% NP-40, and 0.1% Triton X-100 in the case of Miwi peptides and complete EDTA-free protease inhibitors [Roche]; and the same buffer but containing 150 mM NaCl in the case of Aub peptides). After extensive washings, bound proteins were resolved by NuPAGE and visualized by silver staining.

Western blots and immunoprecipitations

Western blots and immunoprecipitations were performed as described (Kirino et al. 2009). Anti-Mili (17.8) (Kirino et al. 2009), anti-Flag M2 (Sigma), and anti-β-tubulin (E7; Developmental Studies Hybridoma Bank) were used in this study. Antibodies against the *Drosophila* Ago1, Aub, Piwi, and Ago3 were gifts from M.C. Siomi and H. Siomi (Keio University) (Miyoshi et al. 2005; Saito et al. 2006; Gunawardane et al. 2007). *Drosophila* Tudor antibody was described by Thomson et al. (2008). Anti-Tdrd6 antibodies were gifts from R. Jessberger (Mount Sinai School of Medicine) (Vasileva et al. 2009), and S. Chuma (Kyoto University) (Chuma et al. 2006). Anti-Miwi antibody was prepared by immunizing rabbits with a synthetic peptide coupled to KLH (C-ERGGRRRDFHD; Genscript); sera were affinity-purified over a column containing immobilized peptide.

In vitro binding experiments

Drosophila full-length Tudor and mini-*tud* constructs Δ1, Δ2, and Δ3 carrying an HA epitope at the N terminus cloned into

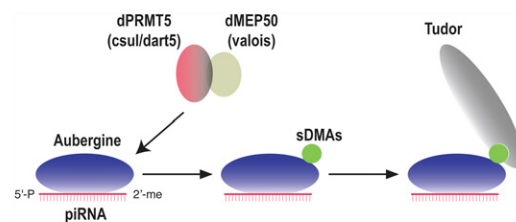

FIGURE 5. Model depicting interactions of indicated proteins in pole (germ) plasm formation.

pP{CaSpeR-2} (gifts from A. Arkov, Murray State University) (Arkov et al. 2006) were used as templates for the amplification of the four coding sequences (CDS) by PCR with *Pfu* Turbo polymerase (Stratagene), following the manufacturer's instructions. PCR primers were:

Forward: 5′-CTCGAGACCATGTACCCGTACGATGTGC-3′; and Reverse: 5′-TCACTGACATTCCTGAAGCT-3′.

After the addition of 3′ adenosine overhangs by incubation with *Taq* DNA polymerase and dATP, the CDS were cloned into pCR-XL-TOPO vector (Invitrogen). Recombinant plasmids were selected for same-strand orientation of CDS with vector T7 RNA polymerase promoter by restriction analysis and were verified by DNA Sequencing. One microgram of recombinant plasmid from each construct, linearized ~100 bases downstream from the stop codon, was used for in vitro translation using TNT reticulocyte lysate system in the presence of 40 μCi of ^{35}S-methionine in a 50 μL reaction for 2 h at 30°C. Recombinant wild-type and mutant Flag-Aub were produced in S2 cells and immunoprecipitated using M2 anti-Flag agarose as previously described (Kirino et al. 2009). Fifteen microliters of either wild-type or mutant Aub-bound beads were incubated for 1 h with 15 μL of reticulocyte reaction containing ^{35}S-labeled Tudor in RSB-150 buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5% glycerol, 2.5 mM $MgCl_2$, 0.05% NP-40) at 4°C and washed three times with the same buffer. Proteins bound on the beads were analyzed on NuPAGE 3%–8% Tris-Acetate gels.

Drosophila *stocks*

Tudor mutant flies (yw; tud^1/CyO) were a gift from A. Arkov (Murray State University) (Arkov et al. 2006).

RNA isolation and labeling, and quantitative RT-PCR analysis

RNA isolation from *Drosophila* ovaries or immunoprecipitates, 5′-end labeling of piRNAs, and quantification of the transposon transcript by quantitative RT-PCR were performed as previously described (Kirino et al. 2009). The following primer pairs as described by Li et al. (2009) were used for quantitative RT-PCR:

Accord: forward (5′-ACAATCCACCAACAGCAACA-3′) and reverse (5′-AAAAGCCAAAATGTCGGTTG-3′);
Diver: forward (5′-GGCACCACATAGACACATCG-3′) and reverse (5′-GTGGTTTGCATAGCCAGGAT-3′);
HeT-A: forward (5′-CGCGCGGAACCCATCTTCAGA-3′) and reverse (5′-CGCCGCAGTCGTTTGGTGAGT-3′);
Accord2: forward (5′-TTGCTTTCGGACTTCGTCTT-3′) and reverse (5′-TTCCACAACGAAAACAACCA-3′);
Diver2: forward (5′-CTTCAGCCAGCAAGGAAAAC-3′) and reverse (5′-CTGGCAGTCGGGTGTAATTT-3′);
412: forward (5′-CACCGGTTTGGTCGAAAG-3′) and reverse (5′-GGACATGCCTGGTATTTTGG-3′);
Blood: forward (5′-TGCCACAGTACCTGATTTCG-3′) and reverse (5′-GATTCGCCTTTTACGTTTGC-3′);
mdg1: forward (5′-AACAGAAACGCCAGCAACAGC-3′) and reverse (5′-CGTTCCCATGTCCGTTGTGAT-3′); and
Rp49 (used as a control): forward (5′-CCGCTTCAAGGGACAGT ATCTG-3′) and reverse (5′-ATCTCGCCGCAGTAAACGC-3′).

Immunofluorescence and confocal microscopy

Drosophila ovaries were dissected from adult flies and immunostained under standard procedures using anti-Aub (Gunawardane et al. 2007) as the primary antibody and Alexa 594-conjugated anti-mouse IgG (Molecular Probe) as the secondary antibody. All images (Fig. 4) were acquired with a Zeiss LSM 510META NLO confocal microscope with the following specifications, settings, and magnification: for early-stage egg chambers (Fig. 4E), Wavelength: Red = 543 nm (100% transmission), Blue (DAPI) = 740 nm (2.5% transmission), Objectives: Plan-Apochromat 63X/1.4, Scan Zoom: 1.0, Stack size: X = 142.86 μm, Y = 142.86 μm, Pinhole: 118 μm for Red, 1000 μm for DAPI, and for later stage (Fig. 4F), Wavelength: Red = 543 nm (100% transmission), Blue (DAPI) = 740 nm (1.5% transmission), Objectives: Plan-Apochromat 20X/0.8, Scan Zoom: 1.0, Stack size: X = 450.00 μm, Y = 450.00 μm, Pinhole: 120 μm for Red, 832 μm for DAPI.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

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