A novel organelle, the piNG-body, in the nuage of *Drosophila* male germ cells is associated with piRNA-mediated gene silencing

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**ABSTRACT** Proteins of the PIWI subfamily Aub and AGO3 associated with the germline-specific perinuclear granules (nuage) are involved in the silencing of retrotransposons and other selfish repetitive elements in the *Drosophila* genome. PIWI proteins and their 25- to 30-nt PIWI-interacting RNA (piRNAs) are considered as key participants of the piRNA pathway. Using immunostaining, we found a large, nuage-associated organelle in the testes, the piNG-body (piRNA nuage giant body), which was significantly more massive than an ordinary nuage granule. This body contains known ovarian nuage proteins, including Vasa, Aub, AGO3, Tud, Spn-E, Bel, Squ, and Cuff, as well as AGO1, the key component of the microRNA pathway. piNG-bodies emerge at the primary spermatocyte stage of spermatogenesis during the period of active transcription. Aub, Vasa, and Tud are located at the periphery of the piNG-body, whereas AGO3 is found in its core. Mutational analysis revealed that Vasa, Aub, and AGO3 were crucial for both the maintenance of the piNG-body structure and the silencing of selfish *Stellate* repeats. The piNG-body destruction caused by *csul* mutations that abolish specific posttranslational symmetrical arginine methylation of PIWI proteins is accompanied by strong derepression of *Stellate* genes known to be silenced via the piRNA pathway.

**INTRODUCTION**

In many eukaryotic species, germ cells contain specific electron-dense cytoplasmic granules. During *Drosophila* oogenesis, these granules form a perinuclear organelle called nuage, which is believed to be involved in the selection and translational control of mRNAs transported from the nucleus (Findley et al., 2003; Snee and Macdonald, 2004). Recent studies accentuated the participation of nuage in the PIWI-interacting RNA (piRNA) biogenesis and piRNA-dependent silencing of transposons and some other harmful selfish elements (Lim and Kai, 2007; Lim et al., 2009). The piRNA pathway is a genomic defense mechanism conserved in germinial tissues of many eukaryotes (Aravin et al., 2007; Thomson and Lin, 2009). In flies, proteins of the PIWI subfamily Aubergine (Aub) and Argonaute 3 (AGO3), which associate with small, 25- to 30-nt piRNAs and possess an RNA-slicing function, are considered as the main players of the piRNA pathway (Aravin et al., 2001; Vagin et al., 2006; Brennecka et al., 2007; Gunawardane et al., 2007; Li et al., 2009). In the ovaries, Aub and AGO3 proteins realize posttranscriptional silencing via the piRNA ping-pong amplification cycle (Brennecka et al., 2007; Gunawardane et al., 2007). It has been shown that Aub mainly associates with the antisense piRNAs derived from specialized genomic “master” loci, whereas AGO3 preferentially associates with the sense ones (Li et al., 2009; Nishida et al., 2007). Long sense piRNA precursors are believed leave the nucleus to be recognized by the Aub-containing antisense piRNA-induced silencing complex (piRISC), where the secondary sense piRNAs are generated by the transcript cleavage. These newly generated sense piRNAs are subsequently loaded into the AGO3-containing sense piRISC, which presumably recognizes the long antisense transcripts.
producing the antisense piRNAs, which in turn enter the Aub-containing silencing complex, and so on (Brennecke et al., 2007; Gunawardane et al., 2007; Ghildiyal and Zamore, 2009). The amplification cycle appears to take place in the perinuclear RNP-containing nuage granules of nurse cells in Drosophila ovaries (Lim and Kai, 2007; Lim et al., 2009; Li et al., 2009; Malone et al., 2009). Along with Aub and AGO3, several other proteins taking part in silencing of retrotransposons and endogenous Stellate repeats via the piRNA pathway (Maelstrom, Krimper, Spindle E, Squash, Zucchini, Cutoff, Tejas) and also in mRNA degradation (DCP1, Me31B, Pacman) were identified as components of ovarian nuage (Harris and Macdonald, 2001; Findley et al., 2003; Sne€ and Macdonald, 2004). It was shown that the proper perinuclear localization of Aub, Krimper, and Maelstrom in the ovaries depends on Vasa (Lim and Kai, 2007); however, its exact role in nuage remains elusive.

More than a century ago, the chromatoïd body (CB), a presumed nuage homologue, was found in the cytoplasm of mammalian male germ cells from early spermatocytes to round spermatids (Yokota, 2008). The CB consists of an electron-dense material and seems to move around the nuclei in close contact with the nuclear envelope and pore complexes (Parvinen and Parvinen, 1979; Ventela et al., 2003). Its main function is supposedly related to posttranscriptional mRNA sorting and processing (Kotaja and Sassone-Corsi, 2007). An essential CB component is the mammalian homologue of Drosophila Vasa protein MVH (mouse Vasa homologue). Many other CB constituents, including principal participants of the piRNA silencing pathway, have been found (Yokota, 2008; Kotaja et al., 2006; Vagin et al., 2009).

The presence of nuage in Drosophila melanogaster spermatocytes was supported by live-imaging experiments (Snee and Macdonald, 2004); however, its structure and functions remain poorly explored. It was shown that the repression of repeated Stellate genes in spermatocytes is realized via the piRNA pathway (Aravin et al., 2001; Vagin et al., 2006; Nishida et al., 2007), and several ovarian nuage proteins (Aub, AGO3, Spindle E, Zucchini, Squash, and Tejas) were found to take part in Stellate silencing (Aravin et al., 2001; Vagin et al., 2006; Li et al., 2009; Nishida et al., 2007; Pane et al., 2007; Patil and Kai, 2010; Nagao et al., 2010).

Here we present the results of nuage analysis in the testes of D. melanogaster. We demonstrated size heterogeneity of Vasa-containing cytoplasmic particles and found a new large organelle in the primary spermatocytes, the piNG-body, enriched with Vasa, Aub, and AGO3. We detected several other ovarian nuage proteins as components of the piNG-body and determined their mutual arrangement within the body. It was shown recently that the functions and protein interactions of the PIWI proteins depend on symmetrical arginine methylation (SAM) catalyzed by Csl methyltransferase (Kirino et al., 2009). We found that csul mutations lead to piNG-body disruption accompanied by derepression of testis-specific repeated Stellate genes.

RESULTS

Visualization of the piNG-body and determination of its protein composition

To visualize nuage in the testes of adult flies, we used antibodies against Vasa protein, a well-known component and specific marker of this structure. Immunofluorescence staining and confocal microscopy analysis of the whole-mount testis preparations from wild-type
flies clearly demonstrated Vasa-stained nuage granules of at least two types located near the nucleus in the primary spermatocytes. The conventional nuage granules were scattered on the nuclear surface arranged in discontinuous rings around the nuclei on confocal slices. Among these small, dot-like particles ∼0.62 ± 0.13 μm in diameter (n = 229, where n is the number of the measured particles), we observed significantly larger structures of ∼2.38 ± 0.35 μm (n = 70), mainly one per cell (Figure 1, B and C, and Supplemental Figure S1, A and B). In a spherical approximation, the volume of the larger granules was more than 50 times that of the smaller ones.

It was shown previously that Aub and AGO3 proteins colocalize with Vasa in nuage in the ovaries (Harris and Macdonald, 2001; Sne and Macdonald, 2004; Lim and Kai, 2007; Malone et al., 2009), and their localization is interdependent (Li et al., 2009). Piwi, the third member of the PIWI subfamily of Argonaute proteins, is known to be expressed only in the germinal proliferative center located at the testis apical tip, which contains somatic hub cells and germ stem cells (Cox et al., 2000; Saito et al., 2006; Nishida et al., 2007).

Because Aub and AGO3 are considered as the main participants of the piRNA machinery in male germinal cells (Vazin et al., 2006; Nishida et al., 2007; Nagao et al., 2010), we studied their localization in different types of nuage granules in the testes. Using anti-Aub and anti-AGO3 antibodies, we found significant but incomplete colocalization of the Aub (67.9% ± 5.1) or AGO3 (52.0% ± 6.8) signals with the Vasa signal in the smaller perinuclear particles (Supplemental Figure S3). The Vasa signals colocalized with Aub (68.5% ± 9.2) and AGO3 (54.3% ± 13.1), respectively. In the larger structures, the Aub or AGO3 signal always colocalized with the Vasa signal (Figures 1, B and C, and 2A). Therefore we called them piNG-bodies (piRNA nuage giant bodies).

Furthermore, we performed immunofluorescence staining using antibodies against other proteins known to be associated with ovarian nuage: Tudor (Tud; Arkov et al., 2006), Spindle E (Spn-E; Lim and Kai, 2007), Squash (Squ; Pane et al., 2007), Cutoff (Cuff; Chen et al., 2007), and Belle (Bel; Johnstone et al., 2005). We found that Tud, Spn-E, Squ, and Cuff were present in nuage of spermatocytes and enriched in the piNG-bodies (Figure 2, B, D, E, and G). A special pattern of distribution (the cytoplasm, nuage, piNG-bodies, and nucleolus-like structures within the nuclei) was shown for RNA-helicase Bel (Figure 2C).

We also wondered whether the piNG-body was related to microRNA (miRNA) processing similar to mammalian CB, which contains Dicer (Kotaja et al., 2006). The miRNA pathway was shown to be Dicer 1–dependent in Drosophila (Lee et al., 2004); however, we detected no Dicer 1 signal in the piNG-body (Supplemental Figure S4A). Partial nuage-like localization of Dicer 1 was observed in spermatogonia and very early spermatocytes (Supplemental Figure S4C), whereas in mature spermatocytes, the cytoplasm was stained homogeneously (Supplemental Figure S4, A and C). Vasa intronic gene protein (VIG), a RISC component (Caudy et al., 2002; Gracheva et al., 2009), was revealed preferentially in the nuclei but not in nuage and piNG-bodies (Supplemental Figure S4B). However, AGO1, another Argonaute protein and the principal component of the miRNA silencing complexes, was found in both the regular small nuage granules and piNG-bodies (Figure 2F).

Piwi signals colocalized with the Vasa signal (Figure 1, B and C, and 2A).

**FIGURE 2:** Protein components of the piNG-body and conventional nuage granules: AGO3 (A), Tud (B), Bel (C), Squ (D), Cuff (E), AGO1 (F), and Spn-E (G). Testes of yw flies were immunofluorescently stained with anti-Vasa (green) and anti-lamin (violet) antibodies; chromatin was stained with DAPI (blue). Signals from AGO3, Tud, Bel, Squ, Cuff, AGO1, and Spn-E are shown in red. Scale bars, 5 μm.

**piNG-body formation is dynamic and associated with the meiotic prophase**

The piNG-bodies were detected in primary spermatocytes, whereas the smaller, Vasa-stained nuage perinuclear granules could be seen...
After meiosis, no detectable nuage structures were seen in round spermatid cells containing small spherical nuclei (4–6 μm in diameter) with highly compact chromatin (Figure 1, A and D, and Supplemental Figure S2); however, vestigial homogeneous Vasa and Aub signals were still visible in the cytoplasm. Thus the presence of the regular nuage structures and piNG-bodies was strongly restricted to the prolonged primary spermatocyte stage.

Architecture of the piNG-body
To investigate the piNG-body structure, we scanned the immunostained testes with a high-magnification lens (63x, with 4x zoom). The confocal images were examined using three-dimensional reconstruction to reveal the mutual arrangement of the piNG-body components (Figure 3). Vasa and Aub proteins showed complete colocalization and always occupied the external parts of the piNG-body. Their signals reflected the existence of substructures of various shapes, from horseshoe to torus or pyramid, with the internal space free from the signals (Figure 3A). At the same time, AGO3 protein was found inside the body colocalizing with Vasa only at the boundary lines (Figure 3B and Supplemental Figure S5). Thus Aub and AGO3 occupied separate but closely located zones within the piNG-body. Similar to Aub and Vasa, Tud occupied the peripheral shell, leaving the core free from the staining. However, the Tud signal showed only partial overlap with the Vasa signal (Figure 4, A and B). Finally, Spn-E, Squ, Cuff, and AGO1 completely colocalized with the Vasa-stained zones at the periphery of the piNG-body (Supplemental Figure S6).

Destruction of the piNG-body and derepression of Stellate genes
It was shown that transcripts of the X-linked Stellate tandem repeats are the main target of the piRNA silencing pathway in the testes of D. melanogaster (Nishida et al., 2007; Nagao et al., 2010). In the absence of the Y-linked crystal locus, which encodes the Suppressor of Stellate [Su(Ste)] repeats responsible for the production of antisense piRNAs taking part in the Stellate silencing, high-level Stellate expression occurs in male germinal tissues. Stellate derepression leads to the accumulation of protein crystals and to abnormalities in meiotic chromosome condensation and segregation in spermatocytes, which cause male sterility (Hardy et al., 1984; Livak, 1984). Transcription of Stellate genes starts in primary spermatocytes (Aravin et al., 2004) and, according to our data, temporally coincides with the piNG-body formation (Figure 1B). Loss-of-function aub, ago3, and spn-E mutations lead to the loss of Su(Ste) piRNAs, derepression of Stellate genes, and translation of their transcripts (Aravin et al., 2001, 2004; Vagin et al., 2006; Li et al., 2009). Among the mutations affecting other piNG-body components (Vasa, Bel, Tud, Squ, and Cuff), only vasa and squ

significantly earlier, in germinal stem cells and spermatogonial cells (Figure 1, A and B; see also Supplemental Figure S1A), where the Aub staining could also be observed in the perinuclear area, albeit less clearly. During development, spermatocytes undergo significant growth, which includes a remarkable increase in the nuclear size, up to 25 times in volume. This growth is accompanied by active transcription of constitutive and testis-specific genes, as well as by drastic chromatin reorganization and chromatid pairing events (Cenci et al., 1994; Vazquez et al., 2002). We detected distinct piNG-bodies starting from the S2b stage (as defined by the classification of Cenci et al. [1994]; nuclear diameter of ∼6–10 μm), during the prolonged S3–S4 stages (nuclear diameter of ∼12–15 μm and chromatin separated into three clumps), and up to the S5 stage (nuclear diameter of ∼16–20 μm and completely formed discrete chromosome territories; Figure 1, B and C, and Supplemental Figure S1, A and B). At the end of the S stage (nuclear diameter of ∼15 μm), we still observed the small nuage granules but not the piNG-bodies.
induced strong hyperexpression of Stellate protein (Figure 5, C, E, and F), whereas bel, tud, and cuff did not lead to Stellate derepression (Figure 5, A, B, and D). Often mutations in the vasa locus disturb the expression of both vasa and vig genes (Figure 5E). However, we found that the vasa<sup>W127</sup>/vasa<sup>Y11</sup> allelic combination was characterized by undisturbed expression of VIG (Figure 5E) and clearly demonstrated Stellate derepression. These observations indicate that Vasa is responsible for the piRNA pathway disturbances and Stellate derepression in all mutant alleles.

We wondered whether the mutations leading to Stellate derepression also affected piNG-body formation. In the testes of aub<sup>D1</sup>/vigo<sup>C42</sup> transheterozygous flies, we observed neither the piNG-bodies nor the smaller Aub-stained nuage granules, although Vasa and AGO3 were detectable in nuage (Figure 6, A and F). In the ago<sup>Y11</sup>/ago<sup>Y11</sup> testes, we also observed Aub- and Vasa-stained small nuage granules together with several discrete bright fragments of Aub/Vasa–stained dense particles (~1 μm) randomly distributed on the nuclear surface and in the cytoplasm of spermatocytes (Figure 6B). These particles demonstrated no internal structure typical for the piNG-body and appeared to be its precursors unable to finish the assembly process. Complete piNG-body disassembly was discovered in the vasa testes (vasa<sup>E142</sup>/E21), where Aub relocation from nuage to the cytoplasm was revealed (Figure 6C). Thus strong Stellate derepression and piNG-body disassembly occur in case of aub, ago3, or vasa mutations. However, we clearly detected Vasa- and Aub-stained piNG-bodies in spermatocytes in spn-E and squ mutant testes, spn-E<sup>516</sup> (Figure 6E) and squ<sup>D32</sup>/DF<sup>D28</sup> (Figure 6D), where Stellate derepression was shown (Figure 5C). Thus we suggest the existence of defective piNG-bodies in the case of certain mutations.

Deficiency of several other piNG-body components caused no Stellate derepression (Figure 5, A, B, and D). Analysis of bel<sup>D1</sup>/be<sup>Y</sup> flies revealed severe testes atrophy and complete loss of germ cells prior to the spermatocyte growth stage (when Stellate transcription starts and piNG-bodies emerge in wild type; Supplemental Figure S7A). We investigated the piNG-body and convectional nuage maintenance in case of strong tud<sup>Y</sup> mutation and found no disturbance (Supplemental Figure S7B). Thereby, Tud is not crucial for the piNG-body formation. We also observed piNG-body and nuage preservation in cuff<sup>RM50</sup>/cuff<sup>RM50</sup> transheterozygous testes (Figure S7C).

**Capsuleen affects the piNG-body assembly**

Loss-of-function capsuleen (csul) mutations lead to transposon derepression in the ovaries (Kirino et al., 2009) and male sterility (Gonsalvez et al., 2006). Csul, or Dart5, is an arginine methyltransferase responsible for SAM of Piwi, Aub, AGO3, and Vasa (Kirino et al., 2009, 2010) that has been shown to be essential for piRNA pathway functioning in the ovaries (Kirino et al., 2009). We analyzed Stellate silencing and piNG-body formation in the testes of csul<sup>RM50</sup>/Df(2R)Jp7 and csul<sup>RM50</sup>/Df 23692 transheterozygous males and found strong Stellate derepression (Figure 7A) along with disappearance of the piNG-body in spermatocytes (Figure 7C). Vasa-containing small nuage granules were clearly detectable in the csul mutant testes, whereas the Aub signals were randomly distributed in the cytoplasm of spermatocytes and did not colocalize with the Vasa-stained perinuclear granules, although miRNA and endoribonuclease–prepared short interfering RNA (esiRNA) levels remained unaffected (Figure 7B). Thus disassembly of the piNG-body was concomitant with the loss of SAM modification of Aub and AGO3 and with the piRNA pathway disruption.

**DISCUSSION**

Here we studied the localization and compartmentalization of the piRNA pathway components in nuage of Drosophila male germinal tissues, the structure, and protein composition of which have been poorly investigated to date. Using the immunofluorescence approach, we identified two types of granules comprising nuage. Along with the conventional small nuage particles evenly scattered on the nuclear surface in primary spermatocytes, we found a novel large granular organelle, one per cell. This organelle contained Vasa, Aub, AGO3, Tud, Spn-E, Bel, Squ, Cuff, and AGO1 proteins (Figures 1 and 2). Because most of the identified components are known to be participants of the piRNA pathway, we called the organelle the piNG-body. piNG-bodies appeared in spermatocytes at the S2b stage and were recognizable until the S5 stage (Figure 1, B and C, and Supplemental Figure S1, A and B). Drosophila spermatocytes are characterized by active transcription, which drops dramatically after meiotic divisions (White-Cooper, 2010). We detected neither piNG-bodies nor other nuage granules in round spermatids (Figure 1D and Supplemental Figure S2) and thus supposed that piNG-body formation was strongly associated with the period of active transcription in primary spermatocytes, where defects in the piRNA system caused derepression of Stellate genes and transposons (Aravin et al., 2004; Nagao et al., 2010).

The mammalian CB is another large granular organelle involved in RNA processing in male germinal tissues. Although it appears in
late pachytene spermatocytes and exists through the round spermatid stage, its formation, like that of to the piNG-body, coincides with a well-ordered disposition of its organelle, the piNG-body, due to a functional specialization and compartmentalization of the piRNA machinery.

We observed that aub, ago3, and vasa mutations affecting Stellate silencing also impaired piNG-body formation and caused its disruption (Figure 6, A–C and F). In contrast, the deficiency of Squ, a putative nuclease believed to be responsible for piRNA biogenesis (Pane et al., 2007), led to Stellate hyperexpression (Figure 5C) but caused no visible disturbances in the Vasa- and Aub-stained piNG-bodies (Figure 6D).

The piNG-body volume is about 50 times that of a conventional nuage granule, and we succeeded in revealing its internal structure. We suggest that the observed relative positions of Aub and AGO3 proteins within the piNG-body (Figure 3 and Supplemental Figure S5) are essential for correct functioning of the piRNA processing. Aub, which plays a role complementary to AGO3 in silencing of transposons and other repetitive selfish elements, is located at the periphery of the piNG-body (Figure 3A), whereas AGO3 is found in the core (Figure 3B and Supplemental Figure S5). We suppose that the observed piNG-body architecture reflects the existence of separate functions of the sense and antisense piRISCs, keeping both parts in close contact necessary for a rapid exchange of piRNA intermediates. Testis transposon piRNAs show clear signatures of the ping-pong cycle (Nagao et al., 2010). Although this mechanism appears to be nonprevalent in Stellate silencing, both Aub and AGO3 are strongly required for the accumulation of Su(Ste) piRNAs in the testes (Vagin et al., 2006; Li et al., 2009; Nagao et al., 2010). The concentration and compartmentalization of macromolecular complexes are believed to be essential for efficient kinetics of RNA sorting and processing (Kurland et al., 2006).

Recent papers described two types of the nuage granules in mouse fetal gonocytes (Aravin et al., 2009; Shoji et al., 2009). The first type, pi-bodies, contain MILI and TDRD1 proteins, whereas the second type, piP-bodies, harbor MIWI2, TDRD9, and Maelstrom, as well as known P-body proteins (DCP1, XRN1, and GW182). PIWI family proteins MILI and MIWI2 play complementary roles in transposon silencing in mice embryos, being involved in the piRNA amplification cycle (Aravin et al., 2008). The pi- and piP-bodies are closely located in the cytoplasm of gonocytes but exist as separate particles preserving functional specialization and compartmentalization of the piRNA pathway modules. We demonstrated that a similar separation of piRISC complexes in flies could be achieved within a single organelle, the piNG-body, due to a well-ordered disposition of its components.

We observed that aub, ago3, and vasa mutations affecting Stellate silencing also impaired piNG-body formation and caused its disruption (Figure 6, A–C and F). In contrast, the deficiency of Squ, a putative nuclease believed to be responsible for piRNA biogenesis (Pane et al., 2007), led to Stellate hyperexpression (Figure 5C) but caused no visible disturbances in the Vasa- and Aub-stained piNG-bodies (Figure 6D). spn-E mutation also did not lead to visible loss of the Vasa- and Aub-stained piNG-bodies.
and disruption of SAM modification caused Stellate derepression (Figure 7A). Thus the absence of Stellate hyperexpression in the tud testes was unexpected. Here we found that in the piNG-body, the Tud-stained zones significantly overlapped with Vasa and Aub at the periphery rather than with AGO3 in the core (Figure 4). However, we showed that tud\(^{−}\) mutation did not lead to Stellate derepression (Figure 5B) and piNG-body disappearance (Figure 5B). In Drosophila, 25 Tudor domain–containing proteins have been described (Siomi et al., 2010), and some of them, such as Krimper (Lim and Kai, 2007) and Tejas (Patil and Kai, 2010), were shown to be involved in the piRNA pathway in the ovaries and testes. It is possible that yet- unidentified proteins carrying Tudor domains also interact with the PIWI proteins in the piNG-body and their functions can be interchangeable.

We demonstrated that SAM provided by Csul arginine methyltransferase was essential for the assembly of the piNG-body but not of the conventional small nuage granules. Strong Stellate derepression associated with the loss of Su(Ste) piRNAs was observed in the csul testes (Figure 7, A and B) lacking the piNG-body (Figure 7C). Because SAM is required for at least Aub and AGO3 functionality (Kirino et al., 2009, 2010b; Nishida et al., 2009), our observations highlight the functional involvement of the piNG-body in the piRNA-mediated silencing in the testes. Similar to ovarian nuage (Anne et al., 2007), testis nuage localization of Vasa appears to be SAM independent (Figure 7C).

Here we detected a novel spermatocyte-specific nuage-associated organelle, the piNG-body, significantly larger than the conventional small nuage granules. We found that the piNG-body contained the PIWI subfamily proteins Aub and AGO3 and demonstrated that the body disassembly was accompanied by the derepression of Stellate repeats. We also showed SAM modifications of Aub and AGO3 to be essential for both piNG-body assembly and piRNA pathway functions. We suggest that the internal structure of the piNG-body provides effective compartmentalization and functioning of the piRNA pathway components, as well as cross-talk between the piRNA and miRNA pathways, since AGO1 was shown to be a component of the piNG-body.

**FIGURE 6:** Mutational analysis of the piNG-body. In all experiments, the testes were stained with anti-Vasa (green) and anti-Aub (red) antibodies, except for F, in which they were stained with anti-AGO3 (red) antibodies. Scale bars, 5 μm. (A, F) The aub\(^{−/}\)/aub\(^{+/}\) tests, small AGO3- and Vasa-stained nuage granules are visible, but no piNG-body formation is observed. (B) The ago3 mutation effect on the piNG-body formation. No normal-sized piNG-bodies are visible; however, up to several granules are visible, but no piNG-body formation is observed. (C) No nuage is found in the testes of aub\(^{−/}\)/aub\(^{+/}\) flies (the same results were obtained with aub\(^{+/}\)/aub\(^{−/}\) flies). (D) piNG-bodies and conventional nuage granules are distinctly seen in the squ\(^{+/}\)/squ\(^{−/}\) flies. Strong immunostaining is observed in the piNG-body in the piRNA-mediated silencing zone (Figure 6E). Although the process of piNG-body formation is not understood mechanistically, we can assume a hierarchic order of protein recruitment: Vasa and Aub can be considered as basic architectural components, the incorporation of which is independent of Squ and Spn-E. Several piNG-body components (Vasa, Aub, and AGO3) are responsible for both general piNG-body assembly and Stellate silencing, whereas others (Squ and Spn-E) may determine the correct internal structure of the body, and their deficiency may result in the assembly of a defective piNG-body lacking some essential functions.

It is known that SAM modification is responsible for Aub and AGO3 recognition by Tud (Kirino et al., 2010b; Nishida et al., 2009), and disruption of SAM modification caused Stellate derepression (Figure 7A). Thus the absence of Stellate hyperexpression in the tud testes was unexpected. Here we found that in the piNG-body, the Tud-stained zones significantly overlapped with Vasa and Aub at the periphery rather than with AGO3 in the core (Figure 4). However, we showed that tud\(^{−}\) mutation did not lead to Stellate derepression (Figure 5B) and piNG-body disappearance (Figure 5B). In Drosophila, 25 Tudor domain–containing proteins have been described (Siomi et al., 2010), and some of them, such as Krimper (Lim and Kai, 2007) and Tejas (Patil and Kai, 2010), were shown to be involved in the piRNA pathway in the ovaries and testes. It is possible that yet- unidentified proteins carrying Tudor domains also interact with the PIWI proteins in the piNG-body and their functions can be interchangeable.

**MATERIALS AND METHODS**

**Fly stocks**

Germinant tissues of adult D. melanogaster males raised at 23°C were used. The Df(1)w\(^{67C23}\);P{pCaSpeR-6Ste-lacZ} line (designated as cry) carrying a deletion of the crystal locus on the Y chromosome...
derivation and immunostaining. The cuffRNAl/cuffRNAl1 and cuffRNAl/cuffRNAl transheterozygous allelic combinations (opposite-direction crosses; the first indicated parent is the mother) and the tud homozygous allele also were used for Western blot analysis of Stellate derepression and immunostaining. The be1F08/nos30/be1F08 and be1F08/be1F08nos30 transheterozygous allelic combinations, as well as be1F08−1 and be1F08nos30 homozygous alleles, were used for the same purpose. The csulRNAl/Df(2R)Usp7 and csulRNAl/Df 23692 flies were used for Western and Northern blot analyses and immunostaining.

Immunofluorescence staining and confocal microscopy

Testes of 0- to 2-d-old adult male flies were dissected in phosphate-buffered saline (PBS) at 4°C, washed with PBT (1x PBS, 0.1% Tween 20), and fixed in 3.7% formaldehyde and PBT for 30 min at room temperature. All of the following procedures were carried out as described previously (Egorova et al., 2009). Staining was detected by laser scanning confocal microscopy using a Carl Zeiss LSM 510 META machine (Carl Zeiss, Jena, Germany) in multichannel mode with 63x oil objective (numerical aperture 1.4); frame size 1024 × 1024 pixels; scan speed 7, with four repetitions. All images, except for magnified ones (4–5x digital zoom) recorded with the z-resolution of 0.5 μm, were taken with the z-resolution of 1 μm. The obtained pictures were imported into Imaris 5.0.1 (Bitplane, Zurich, Switzerland) for subsequent processing. Colocalization was calculated using Coloc software (Imaris 5.0.1, Advanced Coloc method.

Antibodies

The following antibodies were used for immunofluorescence staining: a mix of murine monoclonal anti-lamin Dm0 ADL67.10 and ADL84 antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), 1:500; rabbit polyclonal anti-lamin antibodies (Osouda et al., 2005), 1:500; rabbit polyclonal anti-Aub antibodies (Brennecke et al., 2007), 1:200; murine monoclonal anti-Aub 4D10 antibody (Nishida et al., 2007), 1:100; rabbit polyclonal anti-AGO3 antibodies (Brennecke et al., 2007), 1:300; rat monoclonal anti-Vasa antibody (Developmental Studies Hybridoma Bank), 1:300; rabbit polyclonal anti-Bel antibodies (Johnstone et al., 2005), 1:250; rabbit polyclonal anti-Tud antibodies (Arkov et al., 2006), 1:300; rabbit polyclonal anti–VIG CSH1801 antibodies (Gracheva et al., 2009), 1:500; rabbit polyclonal anti–Dicer 1 ab4735 antibodies (Abcam, Cambridge, MA) 1:300; murine monoclonal anti-Squ antibody (Developmental Studies Hybridoma Bank), 1:50;...
murine monoclonal anti-Cuff antibody (Developmental Studies Hybri-
 domic Bank), 1:50; murine monoclonal anti-AGO1 1B8 antibody (Miyoshi et al., 2005), 1:100; murine monoclonal anti-β-actin ab8224 antibody (Abcam), 1:800. Alkaline phosphatase–conjugated anti-
mouse antibodies (Sigma-Aldrich, St. Louis, MO) were used as a secondary reagent at a dilution of 1:1,000. DAPI (4',6-diamidino-2-phenylindole) was used for chromo-
tin staining.

For Western blot analysis, the following antibodies were used: murine polyclonal anti-Stellate antibodies described in Egorova et al. (2009), 1:3000; murine monoclonal anti-β-actin ab8224 antibody (Abcam), 1:800. Alkaline phosphatase–conjugated anti-
mouse antibodies (Sigma-Aldrich, St. Louis, MO) were used as a secondary reagent at a dilution of 1:20,000. Samples were resolved by SDS–PAGE and blotted onto Immobilon-P PVDF membrane (Sigma-Aldrich). Blots were developed using the Immun-Star AP de-
tection system (Bio-Rad Laboratories, Hercules, CA) in accordance with the recommendations of the manufacturer.

Northern blot analysis

A total of 5 μg of RNA isolated from testes by TRIzol reagent (Invitro-
gen) was resolved by 20% denaturing PAGE. After that, the RNA was transferred onto the Hybond N+ membrane (Amersham-
Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ) and cross-linked by UV radiation (Stratagene, Santa Clara, CA). The subsequent prehybridization and hybridization were performed in Church buffer at 37°C for 1 h and overnight, respectively. For hybridiza-
tion, 10 pmol of DNA oligonucleotides radiolabeled with [32P]- 
P[TPAT] by T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) was used. The following probes were used for the detection of Su(Ste) piRNAs, 5′-GGGCTGTCTTACGACGATGA-3′; dme-mir-8 miRNA, 5′-GAACATCTTACCTCGACAGATTA-3′; endo-
siRNA-1, 5′-GGAGGCAAATGTTGGAGTCAA-3′; and 2S rRNA, 5′-TACAACCTCCTACATGAGTTCCAGCA-3′. After hybridiza-
tion, the blots were washed twice with 2x 150 mM sodium chloride, 15 mM sodium citrate, and 0.05% SDS at 37°C and analyzed by the
STORM PhosphorImager system (Amersham-Pharmacia).

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