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Report

Repression of Retroelements in *Drosophila* Germline via piRNA Pathway by the Tudor Domain Protein Tejas

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Summary

The Piwi-interacting RNAs (piRNAs) have been shown to safeguard the animal germline genome against deleterious retroelements [1–9]. Many factors involved in the production of piRNAs localize to nuage, a unique perinuclear structure in animal germline cells [10], suggesting that nuage may function as a site for processing of germline piRNAs [1, 3–6, 11–14]. Here we report a conserved yet uncharacterized component of the germline piRNA pathway, Tejas (Tej), which localizes to nuage. *tej* is required for the repression of some retroelements and for the production of sufficient germline piRNAs. The localization of Tej to nuage depends on *vasa* (*vas*) [15] and *spindle-E* (*spn-E*) [1, 16, 17] while it regulates the localization of Spn-E, Aubergine (Aub) [3, 4, 14], Argonaute3 (Ago3) [5], Krimper (Krimp) [13], and Maelstrom (Mael) [18] to nuage. Aub, Vas, and Spn-E physically interact with Tej through the N terminus containing the conserved tejas domain, which is necessary and sufficient for its germline function. Aub and Spn-E also bind to the tudor domain at the C terminus. Our data suggest that Tej contributes to the formation of a macromolecular complex at perinuclear region and engages it in the production of germline piRNAs.

Results and Discussions

tejas Encodes a Conserved Tudor Domain Protein and Localizes to Nuage

CG8589 (hereafter referred to as *tejas* (*tej*), meaning "sunshine" in Sanskrit, for its mutant phenotype described below) was initially identified as one of the genes expressed highly in *Drosophila* germline stem cells in a comparative gene expression profile analysis [19]. *tej* is predicted to encode a protein of 559 amino acids that contains a tudor domain at the C terminus (Figure 1A), a conserved motif known to bind the symmetric dimethylated arginine residues [20]. The vertebrate homologs of Tej, such as mouse Tdrd5 and Tdrd7 and zebrafish Tdrd7, have been reported to be expressed in the germline [21–23]. Whereas Tej and Tdrd5 have only one tudor domain, Tdrd7 has three in the C terminus moiety. In addition, we identified a conserved domain in the N terminus of Tej among its homologs (Figures 1A; see also Figure S1A available online).

To analyze the function of *tej* in vivo, we generated a deletion mutant, $tej^{48\text{-}5}$, by excising a nearby *P* element, EY08611 (Figure 1A). $tej^{48\text{-}5}$ lost 1.6 kb of the genomic region encompassing the potential start codon of Tej (Figure 1A, yellow shaded rectangle). Consistent with the annotation of *tej* (Flybase: *CG8589*), northern blot analysis revealed an ~1.7 kb length transcript that was undetectable in $tej^{48\text{-}5}$ and in the transheterozygote $tej^{48\text{-}5}$/*Df(2R)Exel7131* (Figure 1B), suggesting that transcription of *tej* itself is perturbed in $tej^{48\text{-}5}$. Both homozygote and transheterozygote females were viable but sterile (Table S1A; data not shown), indicating the necessity of its function during oogenesis. Either homozygotes or transheterozygotes were used for loss of function in this study.

Immunostaining showed the broad expression pattern of Tej as perinuclear foci in all germline cells except the oocyte (Figure 1C), which is reminiscent of the germline-unique structure, nuage (reviewed in [10]). Costaining with a well-known nuage component, Vasa (Vas) [15] (Figure 1D), and the nuclear envelop marker Lamin (Figure S1B) showed an overlap of virtually all Tej foci with Vas on the cytoplasmic face of the nuclear envelope, confirming that Tej is a nuage component. Similarly, a potential homolog of Tej, Tdrd7, has also been reported to localize to nuage in mouse and zebrafish [22–24]. A transgenic fly harboring HA-Tej placed under upstream activating sequence promoter, when expressed by a germline driver, nanos-Gal4, also exhibited perinuclear nuage foci overlapped with Vas (Figure 1E). The expression of Tej was undetectable in $tej^{48\text{-}5}$ homozygotes and $tej^{48\text{-}5}$/*Df(2R)Exel7131* transheterozygotes (Figure 1D; data not shown), confirming that $tej^{48\text{-}5}$ is a loss-of-function allele.

$tej^{48\text{-}5}$ Exhibits Defects in the Formation of Karyosome But Not in Polarity Establishment

Many of the nuage component mutants are reported to share some common phenotypes: defects in karyosome morphology and in polarity establishment [12, 13, 18, 25, 26]. We first examined the karyosome phenotype in *tej* mutant by immunostaining with a synaptonemal marker, C(3)G [27]. In the wild-type, C(3)G staining was observed in a single oocyte nucleus from region 3 of germarium onward, and it became extrachromosomal by stage 3, when the oocyte nucleus compacted into karyosome [27] (Figure 1F). In contrast, in *tej* mutant, in about 90% of the ovarioles, C(3)G was observed in two pro-oocytes up to stage 1 of oogenesis and remained chromosomal until later stages, indicating a delay in the oocyte commitment and failure in compaction of the oocyte nucleus into a karyosome (Figure 1F; Table S2). These defects were rescued by HA-Tej when expressed in germline cells by nos-Gal4-Vp16 (Figure 1F; Table S2).

Unlike other nuage mutants, however, *tej* mutant did not show polarity defects [12, 13, 18, 25, 26]. The dorsal marker Gurken was properly localized to the anterior-dorsal region of the oocyte by stage 8 in *tej* mutant, as was that in wild-type (Figure 1G). The posterior marker *oskar* was translated only around stage 9, and its protein was appropriately localized to the posterior (Figure 1G). Furthermore, the embryos from *tej* mutant mothers also exhibited normal dorsal

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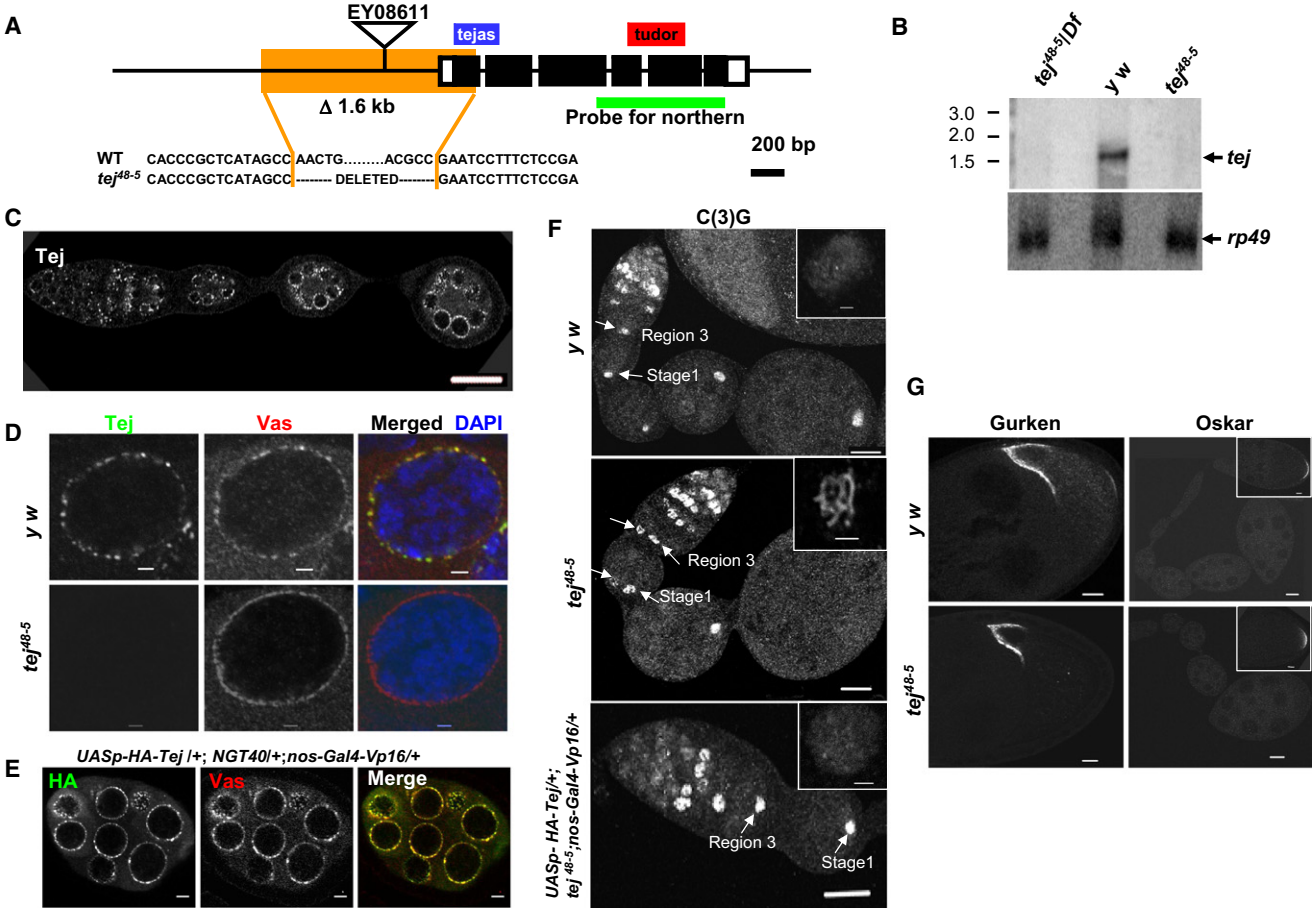

Figure 1. *tej* Encodes a Tudor Domain Protein that Localizes to Perinuclear Nuage

(A) Schematic representation of *tej*. Tej protein harbors a predicted tudor domain (C terminus) and a conserved tejas domain (N terminus) (see also Figure S1A). Orange shaded area represents the deleted 1.6 kb region in tej^{48-5}.

(B) Northern blot analysis showing an expected ~1.7 kb *tej* transcript, which was undetectable in tej^{48-5} and tej^{48-5}/*Df* ovaries. The green line in (A) represents the region complementary to the probe.

(C) Ovaries immunostained for Tej, showing its perinuclear localization. Scale bars represent 20 μm.

(D) Closer view of a single nurse cell showing colocalization of Tej (green) with Vas (red) at the perinuclear region (nucleus marked with DAPI [blue]) in wild-type, whereas tej^{48-5} completely lacked Tej protein (see also Figure S1B). Scale bars represent 2 μm.

(E) HA-Tej expressed by nanos-Gal4 in germline cells (green) and Vas (red). Scale bars represent 5 μm.

(F) Immunostaining for a synaptonemal marker, C(3)G, showing karyosome defects in tej^{48-5}. In tej^{48-5}, two pro-oocyte nuclei are discernible until stage 1 egg chamber (arrows). The insets show the closer view of stage 3 oocyte nuclei where C(3)G signal remains chromosomal in tej^{48-5}. The expression of HA-Tej in the germline cells of tej^{48-5} restored this defect (bottom). Scale bars represent 20 μm (2 μm in insets).

(G) Ovaries immunostained with anti-Gurken or anti-Oskar. The localization and expression pattern of both proteins appear unaffected in tej^{48-5}. Scale bars represent 5 μm. All of the images are positioned with anterior at the left (see also Table S1).

appendages (Table S1B), suggesting the proper establishment of polarity in *tej* mutant embryos.

tej Is Required for Repressing Retroelements via the Germline Piwi-Interacting RNA Pathway

In the *Drosophila* female germline, two distinct groups of Piwi-interacting RNAs (piRNAs) are involved in repressing the retroelements in different cell types: one in germline cells and the other in the ovarian somatic cells [5, 6, 28]. The production of germline piRNAs depends on two Piwi-subfamily proteins, Aubergine (Aub) and Argonaute 3 (Ago3), both of which localize to nuage, whereas in the soma, piRNAs are generated in an Aub- and Ago3-independent manner [5, 6, 28]. Not only Piwi-subfamily proteins but also many of the nuage components are required for the production of sufficient germline piRNAs: a germline DEAD-box RNA helicase, Vas [15], a *Drosophila* tudor domain protein, Krimper (Krimp) [13], a high-mobility-group box-containing protein, Maelstrom (Mael) [18, 29, 30], predicted nucleases, Squash (Squ) and Zucchini (Zuc) in *Drosophila* [12], and mouse tudor domain proteins Tdrd1 and Tdrd9 [24, 31]. The involvement of several nuage component proteins in the piRNA production suggests that nuage may be a processing site of germline piRNAs [5, 12, 13].

In order to determine whether *tej* also regulates retroelement expression via the piRNA pathway, we examined the levels of representative retroelements by semiquantitative and quantitative reverse transcriptase-polymerase chain reaction with ovarian RNA. In *tej* as well as in *spn-E* mutant ovaries, the retroelements *I-element*, *Het-A*, and *TART* that are known to be repressed by germline piRNAs were highly derepressed (Figure 2A; Figure S2A). The derepression of those retroelements was successfully suppressed by expressing HA-Tej in the germline cells in *tej* mutant background (Figure 2A). On the contrary, in *tej* mutant, no increase was detected in

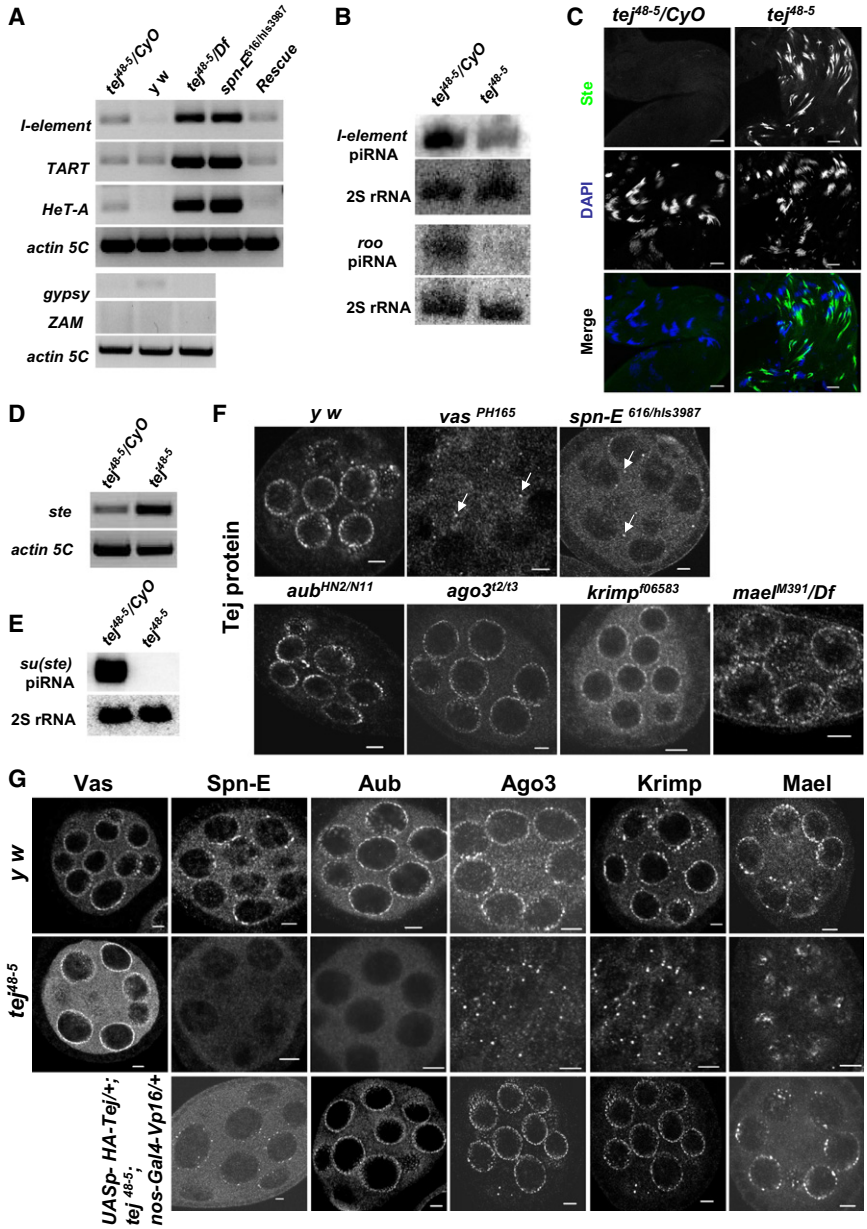

Figure 2. *tej* Is Required for Repression of Retroelements via the Piwi-Interacting RNA Pathway, and It Genetically Interacts with Other Components of the Pathway

(A) Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for expression of retroelements in the wild-type and the *tej* mutant ovarian RNA. *I-element*, *TART*, and *HetA* were derepressed in $tej^{48\text{-}5}$*/Df*, whereas *gypsy* and *ZAM* were not. The expression of HA-Tej in the germline cells of $tej^{48\text{-}5}$ successfully suppressed the derepression of retroelements ("rescue") (see also Figure S2A).

(B) Polyacrylamide gel electrophoresis (PAGE) northern analysis showing the reduction of *roo* and *I-element* Piwi-interacting RNAs (piRNAs) in the *tej* mutant ovary.

(C and D) Immunostaining for Ste (green) with DAPI (blue) in the primary spermatocytes (C), and semiquantitative RT-PCR showing the upregulation of *ste* in the *tej* mutant testis (D). Scale bars represent 20 μm.

(E) PAGE northern analysis showing a great reduction in the *su(ste)* piRNA level in $tej^{48\text{-}5}$.

(F) Tej localization in various nuage component mutants. Tej mislocalizes as tiny foci in the cytoplasm of *vas* and *spn-E* mutants (arrows) but remains unaffected as perinuclear foci in *aub*, *ago3*, *krimp*, and *mael* mutants. Scale bars represent 5 μm.

(G) *tej* mutant ovaries immunostained for the other nuage component proteins. Vas localization is unaffected, whereas others are mislocalized: Spn-E and Aub become dispersed into the cytoplasm, Krimp and Ago3 mislocalize as foci in the cytoplasm, and Mael is observed in the nucleus as a blob-like structure. All of the mislocalized proteins were brought back to nuage by expressing HA-Tej in the *tej* mutant germline cells (bottom). Scale bars represent 5 μm (see also Figures S2B–S2D).

the levels of *gypsy* and *ZAM* transcripts that are repressed by somatic piRNAs (Figure 2A) [5, 6, 28]. We further examined the levels of representative germline piRNAs, *I-element*, and *roo* piRNAs by polyacrylamide gel electrophoresis northern blotting. Both piRNAs were significantly reduced in *tej* mutant ovary compared to those of wild-type (Figure 2B), suggesting that *tej* contributes to the production of germline piRNAs, but not to that of somatic piRNAs, to repress retroelements.

Similarly, *tej* mutant testis also exhibited derepression of *stellate* (*ste*), which is known to be silenced by the germline piRNAs derived from the *suppressor of ste* (*su(ste)*) locus [1, 5, 32]. We detected high expression of Ste protein-forming crystals and significant upregulation of *ste* transcript in *tej* mutant testis (Figures 2C and 2D). As expected, the *su(ste)* piRNAs were greatly reduced in *tej* mutant as compared to the control (Figure 2E). Hence, our data suggest that *tej* is required for the production of sufficient piRNAs in both the female and male germline.

tej Is Required for the Localization of Piwi-Interacting RNA Component Proteins to Nuage

Drosophila nuage components are previously shown to mutually depend on each other for the localization of their encoded proteins to nuage [12, 13, 18]. Among those examined, *vas* was placed at the most upstream position, followed by *spn-E*, *aub*, *krimp*, and *mael*, with respect to their localization to nuage. We examined such genetic interactions between *tej* and other piRNA pathway genes (Figures 2F and 2G). In *vas* and *spn-E* mutant ovaries, Tej mislocalized as tiny foci into the cytoplasm, whereas in *aub*, *ago3*, *krimp*, and *mael* mutants, its perinuclear localization remained unaffected (Figure 2F). This is similar to what was observed in mouse: the nuage localization of tudor domain proteins Tdrd1, Tdrd6, and Tdrd7 depends on mouse *vas* homolog, *mvh* [23], suggesting that such genetic hierarchy is conserved to some extent across the species. Reciprocally, in *tej* mutant ovaries, localization of Vas remains unaffected, whereas other examined components are mislocalized from perinuclear nuage (Figure 2G).

Spn-E, a conserved piRNA pathway component [1, 31], also appeared as perinuclear foci colocalized with Aub in

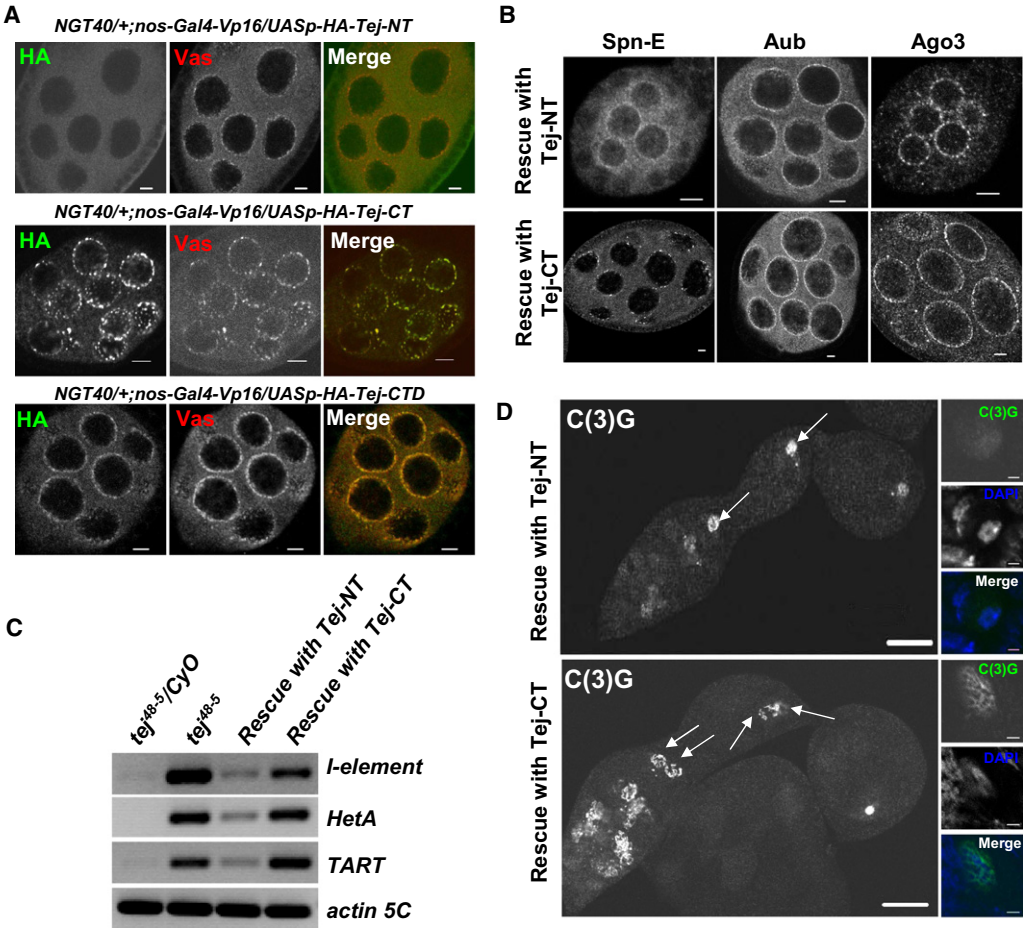

Figure 3. Conserved Tejas Domain Is the Functional Domain, and Tudor Domain Is Important for Formation of Perinuclear Foci

(A) Immunostained ovaries expressing HA-tagged Tej-NT, Tej-CT, and Tej-CTD (see Figure 4B for the schematic) with anti-HA (green) and anti-Vas (red). Tej-CT and Tej-CTD form perinuclear foci overlapping with Vas, whereas Tej-NT is uniformly observed in the cytoplasm. Scale bars represent 5 μm.

(B–D) HA-Tej-NT and HA-Tej-CT carrying transgenes were expressed in the *tej* mutant germline (rescue construct).

(B) The localization of Spn-E, Aub, and Ago3 was brought back to the perinuclear region by Tej variants. Whereas HA-Tej-NT rescued it in both earlier and later stages (stage 3–5 egg chambers are shown), HA-Tej-CT rescued it only in later stages (stage 6–8). Scale bars represent 5 μm.

(C) The derepression of retroelements in tej^{48-5} was rescued to a greater extent by Tej-NT expressed in germline cells, but not by Tej-CT.

(D) C(3)G staining showing the rescue of the defect in the oocyte decision and karyosome morphology (right) by Tej-NT, but not by Tej-CT. Scale bars represent 20 μm and 2 μm, for ovarioles and oocyte nucleus (right panels), respectively (see also Figure S3 and Table S2).

wild-type ovaries, whereas it was undetectable in *spn-E* mutant ovaries (see Figure S2B for immunostaining; see Figure S2D for western), indicating that Spn-E is also a component of nuage. This is consistent with that of mouse TDRD9, a homolog of Spn-E, although nuclear fraction of Spn-E in fly germline cells was not seen, unlike in mouse germline cells [24, 31]. Perinuclear Spn-E is lost and disperses into the cytoplasm of *vas* and *tej* mutants, whereas it remains unaffected in *aub* mutant (Figure 2G; Figure S2C). All of the mislocalized nuage components in *tej* mutant were brought back to perinuclear by the expression of HA-Tej in germline (Figure 2G, bottom). Taken together, these results suggest that *tej* and *spn-E* are downstream of *vas* and upstream of *aub*, *ago3*, *krimp*, and *mael*, whereas they appear to function at the same level for their localizations to the nuage. This hierarchical relation may be an indication of their order of function in the nuage. However, we cannot exclude the possibility that the mislocalization of Aub and Spn-E in *tej* mutant ovaries could be due to the slight reduction of the expression (Figure S2D).

Conserved Tejas Domain, But Not Tudor Domain, Is Essential for the Function of *tej* In Vivo

Other than a tudor domain at the C terminus, Tej contains another conserved domain at the N terminus, termed the "tejas domain" (Figure 1A; Figure S1A). To examine the function of each domain in vivo, we generated transgenic flies harboring variants of Tej fused to HA and expressed them in the germline with nanos-Gal4 (Figure 3). Tej-NT is devoid of tudor domain onward, Tej-CT is devoid of tejas domain alone, and lastly Tej-CTD contains tudor domain onwards (see Figure 4B for schematic). When expressed in the germline, Tej-CT and Tej-CTD formed perinuclear foci colocalized with Vas, whereas Tej-NT was observed uniformly in the cytoplasm (Figure 3A), suggesting that the tudor domain is necessary and sufficient to form perinuclear nuage foci. Consistent with our results, tudor domains of mouse Tdrd1 and Tdrd6 were reported to be essential for their localization to nuage [23], suggesting that the tudor domains may play important roles in forming the perinuclear foci.

Despite the cytoplasmic localization of Tej-NT, however, its expression in germline rescued the phenotypes of *tej* mutant

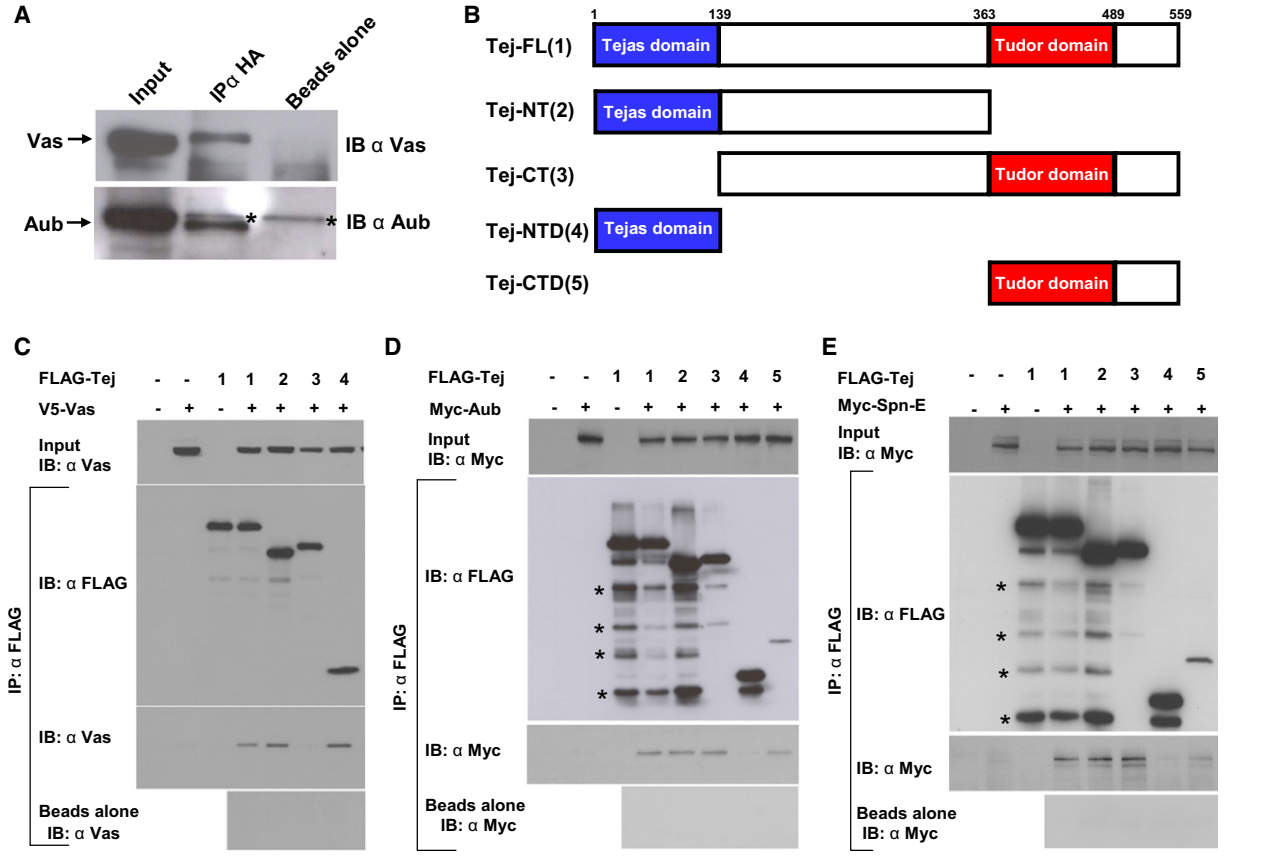

Figure 4. Tej Physically Interacts with Vas, Spn-E, and Aub through Distinct Domains

(A) Immunoprecipitation (IP) experiments with the ovary extract expressing full-length HA-Tej. Vas and Aub were coimmunoprecipitated (arrows) with HA-Tej in vivo.

(B) The schematics of Tej variants tagged to FLAG that are transfected into S2 cells.

(C–E) IP experiments with S2 cell extract cotransfected with variants of Tej and one of V5-Vas, Myc-Aub, or Myc-Spn-E. The numbers at the top of each blot denote the constructs of Tej listed in (B).

(C) Vas was specifically pulled down with FL, NT, and NTD of Tej.

(D and E) Aub and Spn-E were pulled down with FL, NT, CT, and CTD, but not with NTD. Asterisks denote nonspecific bands (see also Figure S4).

(sterility [Table S1A], derepression of retroelements [Figure 3C], and the delay in oocyte decision, as well as failure in karyosome compaction of *tej* mutant [Figure 3D; Table S2]) to a similar extent as full-length Tej (Tej-FL). On the contrary, Tej-CT rescued the sterility to a much lower level (Table S1A) and could not restore other defects (Figures 3C and 3D; Table S2). The mislocalized Piwi-interacting RNA-induced silencing complex (piRISC) components, Aub and Ago3, and RNA helicase, Spn-E, in *tej* mutant ovaries were brought back to perinuclear by Tej-FL and Tej-NT at all stages, but only at the later stages by Tej-CT (Figure 2G; Figure 3B). These results suggest that cytoplasmic Tej-NT sufficiently functions in germline cells, possibly by mediating a formation of macromolecular complex engaging in piRNA production. However, the expression of *tej* from the transgenes appears to be much higher than that of the endogenous *tej* (Figure S3). Hence, we cannot exclude the possibility that Tej-NT may also have reduced activity, but it was masked by the high expression.

Tej Physically Interacts with Vas, Spn-E, and Aub at Distinct Domains

Genetic analysis of nuage components revealed that *tej* functions downstream of *vas* and upstream of *aub* for its localization to nuage (Figures 2F and 2G). Next we investigated their physical interaction in ovaries by coimmunoprecipitation (co-IP) with ovary extracts expressing HA-Tej in the germline cells. Vas and Aub were successfully pulled down with HA-Tej, suggesting a direct or indirect interaction with Vas and Aub in vivo. To identify the interacting domain of Tej, we generated five different variants of FLAG-tagged Tej and cotransfected individually with V5-Vas, Myc-Aub, or Myc-Spn-E into *Drosophila* S2 cells for IP experiments (see Figure 4B for schematic diagram of the variants). Vas, Aub, and Spn-E were successfully pulled down with Tej-FL (1) in the absence of other germline factors (Figures 4C–4E), suggesting their potential direct interaction. Nevertheless, we could not detect interaction between Tej and Spn-E in ovaries, possibly because of technical problems. Vas was further pulled down with Tej-NT (2) and Tej-NTD (4), but not with Tej-CT (3), indicating that tejas domain is sufficient and necessary for interaction with Vas (Figure 4C). On the other hand, Aub and Spn-E were pulled down with Tej- NT (2), Tej-CT (3), and Tej-CTD (5), but not with Tej-NTD (4) (Figures 4D and 4E), indicating that they interact with Tej both at the center region and the C terminus harboring tudor domain.

Recently it has been reported that Piwi-subfamily proteins are symmetrically dimethylated at the arginine (sDMA) residues residing in the N terminus [24, 33, 34]. Although this

modification is required for their interaction with some of the tudor domain proteins, such as mouse TDRD1 and TDRD9 and *Drosophila* tudor [24, 33–36], it is not necessary for the interaction of *Drosophila* dTdrd1 with Aub [35]. We tested whether the interaction of Tej and Aub is sDMA dependent. The four arginine residues at the N terminus were changed to lysine to abolish their sDMA [34] (Figure S4A), and the mutated Aub was subjected to an IP experiment. We observed that both wild-type and the mutant Aub were equally pulled down with Tej-FL (1), Tej-NT (2), and Tej-CTD (5) (Figure S4B), suggesting that the interaction between Tej and Aub is sDMA independent.

Tej-NT is sufficient to interact with Vas, Spn-E, and Aub in vitro, and its expression in germline cells can rescue the *tej* mutant phenotype. On the other hand, Tej-CT interacts only with Aub and Spn-E and not with Vas, and its expression fails to rescue most of the *tej* mutant phenotypes. These results suggest that not only the interaction of Tej with Spn-E and Aub but also that with Vas is crucial for the germline function in vivo. Tej may engage Aub to process retroelement transcripts that have been unwound by the potential RNA helicases, Vas and Spn-E, through the interaction with them [3–6].

Conclusions

We report that a new member of the germline piRNA pathway, *tej*, is required for the production of sufficient germline piRNAs to repress retroelements in *Drosophila*. *tej* encodes a tudor domain protein localized to nuage, a potential processing site of germline piRNAs. *tej* regulates the localization of other piRNA components, including the piRISC components Aub and Ago3, to nuage. Further, Tej physically interacts with Vas, Spn-E, and Aub, and the interaction with Aub is independent of sDMA. Together with previous observations [12, 37], our genetic and physical interaction studies suggest that piRNA components may form a macromolecular complex at the perinuclear region by being engaged in processing retroelement RNA into piRNAs in the ping-pong cycle. The hierarchical interaction of genes coding for components of the nuage, with regard to localization of the proteins they encode, may indicate that these proteins act sequentially in the ping-pong cycle. Vas may first become loaded onto nuage. Then Tej may act together with Vas and Spn-E to unwind retroelement transcripts and engage Aub in processing them into germline piRNAs. Unlike others, *tej* is an unusual piRNA component mutant that does not show polarity defects. This could be due to piRNA-independent functions of other nuage components in the polarity formation, which does not involve *tej*. Alternatively, the establishment of polarity may depend on one or more specific piRNAs that require other piRNA or nuage components, but not *tej* function, to be generated. Profiling of piRNA in *tej* mutant will provide some insights to address this issue.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at doi:10.1016/j.cub.2010.02.046.

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