Gender-specific hierarchy in nuage localization of PIWI-interacting RNA factors in Drosophila

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INTRODUCTION

PIWI-interacting RNAs (piRNAs) are germline-specific small non-coding RNAs that form piRNA-induced silencing complexes (piRISCs) by associating with PIWI proteins, a sub-clade of the Argonaute proteins predominantly expressed in the germline. piRISCs protect the integrity of the germline genome from invasive transposable DNA elements by silencing them. Multiple piRNA biogenesis factors have been identified in Drosophila. The majority of piRNA factors are localized in the nuage, electron-dense non-membranous cytoplasmic structures located in the perinuclear regions of germ cells. Thus, piRNA biogenesis is thought to occur in the nuage in germ cells. Immunofluorescence analyses of ovaries from piRNA factor mutants have revealed a localization hierarchy of piRNA factors in female nuage. However, whether this hierarchy is female-specific or can also be applied in male gonads remains undetermined. Here, we show by immunostaining of both ovaries and testes from piRNA factor mutants that the molecular hierarchy of piRNA factors shows gender-specificity, especially for Krimper (Krimp), a Tudor-domain-containing protein of unknown function(s): Krimp is dispensable for PIWI protein Aubergine (Aub) nuage localization in ovaries but Krimp and Aub require each other for their proper nuage localization in testes. This suggests that the functional requirement of Krimp in piRNA biogenesis may be different in male and female gonads.

Keywords: nuage, piRNA, PIWI, Drosophila, germline
Ago3–piRNA complex, in turn, cleaves antisense transcripts of transposons and the resultant piRNAs are loaded onto Aub. Other factors required for the amplification loop include Vasa, Tudor, Krimp, Spn-E, and Mael (Findley et al., 2003; Lim and Kai, 2007; Malone et al., 2009).

Tud is one of the Tud domain-containing proteins expressed in fly gonads (Boswell and Mahowald, 1985; Thomson and Lasko, 2004; Arkov et al., 2006). The Tud domains in Tud specifically interact with the symmetrical dimethylarginines (sDMAs) in Aub and Ago3 (Kirino et al., 2009; Nishida et al., 2009; Liu et al., 2010a,b; Siomi et al., 2010). The association between Tud and Aub or Ago3 occurs simultaneously and the Tud–Aub–Ago3 complex, in turn, cleaves antisense transcripts of Aub and Ago3 in ovaries, but is placed at the same level with Aub, in fly gonads (Boswell and Mahowald, 1985; Thomson and Lasko, 2004; Arkov et al., 2006). The Tud domains in Tud specifically interact with the symmetrical dimethylarginines (sDMAs) in Aub and Ago3 (Kirino et al., 2009; Nishida et al., 2009; Liu et al., 2010a,b; Siomi et al., 2010). The association between Tud and Aub or Ago3 occurs simultaneously and the Tud–Aub–Ago3 complex contains piRNA intermediate-like RNA molecules but, in the complex, Aub and Ago3 are free from mature piRNAs. Thus, Tud might serve as a platform for the amplification loop (Nishida et al., 2009). Spn–E has a Tud domain (Gillespie and Berg, 1995; Ponting, 1997); however, it does not seem to associate with Aub and Ago3 (Nishida et al., 2009). Krimp also contains a Tud domain (Lim and Kai, 2007). Whether Krimp has the ability to associate with PIWI members through their sDMAs remains undetermined.

Until now, the piRNA biogenesis factors that have been identified in Drosophila are localized in the cytoplasm and are preferentially accumulated in specific cytoplasmic perinuclear structures, the nuage, and Yb bodies in germ and somatic cells, respectively. Hence, both nuage and Yb bodies are considered to be the locations for piRNA biogenesis (Findley et al., 2003; Lim and Kai, 2007; Olivieri et al., 2010; Saito et al., 2010; Qi et al., 2011; for review see Siomi et al., 2011). Zuc is localized to mitochondria (Olivieri et al., 2010; Saito et al., 2010). How Zuc localization to mitochondria is mechanistically involved in piRNA biogenesis remains unknown.

Accumulated evidence has shown that in ovaries, nuage localization of the piRNA factors occurs in a molecular hierarchical fashion. The current understanding is that Vasa, a DEAD-box RNA helicase that is detected specifically in the germ cells, is the first to be located to the nuage (Findley et al., 2003); Vasa localization is followed by Spn–E, Aub, Ago3, and Mael. Whether this hierarchy is female-specific or occurs also in males remains unexamined. To address this question, we performed immunofluorescence analyses using monoclonal antibodies against individual piRNA factors and found that the nuage localization hierarchy was gender-specific; an obvious difference was seen for Krimp. Krimp is placed between Aub and Ago3 in ovaries, but is placed at the same level with Aub, but upstream of Ago3 in testes. These results suggest that the functional requirement of Krimp in piRNA biogenesis may be different between male and female gonads.

**MATERIALS AND METHODS**

**DROSOPHILA STRAINS**

The yellow white (y w) and Oregon-R strains were used as wild-type (wt) strains. The tud allele used was tud1 bw sp/CyO l(2)DT5S131 (Drosophila Genetic Resource Center stock number: 106505). The Aub alleles used were AubH22 cn bw/CyO and AubQC42 cn bw/CyO. AubH22 cn bw/CyO and AubQC42 cn bw/CyO were crossed to yield Aub heterozygous mutant flies; AubQC42/AubH22 (Nishida et al., 2007). The Ago3 alleles used were ago32/TM6B Tb and ago33/TM6B Tb. ago32/TM6B Tb and ago33/TM6B Tb were crossed to yield ago3 heterozygous mutant flies; ago32/ago33 (Li et al., 2009). The krimp allele used was w1118; PBac(WH)krimp86583/CyO (Bloomington Drosophila Stock Center; 18990). w1118; PBac(WH)krimp86583/CyO and w1118; Df(2R)BSC309/CyO (Bloomington Drosophila Stock Center; 23692) were crossed to yield krimp transheterozygote mutant flies; krimp86583/Df. Transheterozygotes krimp86583/Df exhibited male and female sterility and an extent of loss in krimp perinuclear cleaving similar to that exhibited by homozygous krimp86583 (Lim and Kai, 2007, and data not shown). Hence, krimp86583 was used as a loss-of-function allele to characterize krimp phenotypes in this study. All stocks were maintained at 25°C.

**PRODUCTION OF ANTIBODIES AND WESTERN BLOTTING**

An anti-Tud monoclonal antibody was raised specifically against the C-terminus of the protein. A 327 amino acid fragment at the C-terminal end of Tudor (amino acid 2189 to the end) was fused with glutathione S-transferase (GST) and used to immunize mice. An anti-Krimp monoclonal antibody was raised specifically against the N-terminus of the protein. A 200 amino acid fragment at the N-terminal end of Krimp was fused with GST and used to immunize mice. Anti-Tud and anti-Krimp monoclonal antibodies were produced essentially as described previously (Ishizuka et al., 2002) and purified from culture supernant of hybridoma cells under standard procedures using Thiophilic-Superflow resin (BD Biosciences). Western blot analysis was performed as described previously (Miyoshi et al., 2005). Anti-Aub and anti-Ago3 antibodies were used as described previously (Nishida et al., 2007, 2009).

**IMMUNOHISTOCHEMISTRY**

Testes and ovaries were stained with the various antibodies as previously described (Nishida et al., 2007; Nagao et al., 2010). An anti-Aub antibody was directly labeled using the HiLyte Fluor 555 Labeling Kit-NH2 (Dojindo Molecular Technologies). Anti-Ago3 (1:5,000 dilution; Nagao et al., 2010), anti-Tud (1:250 dilution; Saito et al., 2010, anti-Krimp (1:250 dilution) antibodies were used as primary antibodies. Alexa 488-conjugated or Alexa 546-conjugated anti-mouse IgG (Molecular Probes; 1:1,000 dilution) were used as secondary antibodies. DNA was stained with DAPI. All images were collected using a confocal microscope (Zeiss LSM5 EXCITER). All immunohistochemical analyses were performed at least three times and representative images are shown in the figures.

**RESULTS AND DISCUSSION**

To ensure that we had a constant supply of anti-Tud and anti-Krimp antibodies for this study, we produced Tud and Krimp monoclonal antibodies. We used recombinant Tud and Krimp peptide fragments expressed in and purified from E. coli to immunize mice. Western blotting of total ovary and cultured Schneider 2 (S2) lysates showed that the antibodies reacted with proteins of the expected sizes of Tud and Krimp (Figures 1A,B, respectively). The Tud band in S2 lysate migrated slightly faster than that in ovary lysate (Figure 1A). This may reflect post-translational modification of Tud protein, although clear evidence for this is still required. We also performed western blotting on tud and krimp mutant ovaries (Figures 1A,B). The bands corresponding to Tud...
and Krimp were not present in the mutants, indicating a high specificity of the antibodies for the individual antigens.

We previously examined the subcellular localization of Tud in aub mutant ovaries using an anti-Tud antibody that was a kind gift from A. Nakamura (CDB-RIKEN, Japan; Nishida et al., 2009). Here, we used the newly produced anti-Tud antibody to repeat this immunofluorescence study on both control and aub mutant ovaries. Very little Tud was localized to the nuage when Aub was not expressed (Figure 2A), confirming the previous observation that Aub is necessary for Tud nuage localization (Nishida et al., 2009). Tud localization has not been previously examined in ago3 mutant ovaries. We immunostained for Tud in ago3 mutant ovaries and detected Tud in the nuage (Figure 2A), suggesting that Ago3 is dispensable for Tud nuage localization. Thus, the nuage localization of Aub, but not of Ago3, is necessary for Tud nuage localization. Aub localization to nuage is not affected by loss of ago3 expression (Figure 2A), although Ago3 localization to nuage is affected by loss of aub expression (Figure 2A). Hence, for nuage localization, Ago3 requires Aub but Aub does not require Ago3.

We then examined the subcellular localization of Tud in aub and ago3 mutant testes. The Tud signal was detected in the nuage in ago3 mutant testes (Figure 2B) the same as in control. In aub mutant testes, however, the Tud signal was hardly detected in the nuage; instead, it appeared to be in the nucleus in particular in spermatogonia cells (Figure 2B). These results suggest that Aub controls the Tud nuage localization even in testes. Aub was localized to the nuage in ago3 testes (Figure 2B), confirming that Aub nuage localization does not require Ago3 in both female and male gonads. Ago3 is not localized to nuage in aub mutant testes (Figure 2B). Thus, Ago3 nuage localization requires Aub in both males and females.

We then investigated how the subcellular localization of Aub and Ago3 was affected by loss of tud function. In ovaries, Aub was detected only slightly in the nuage in tud mutant flies (Figure 3A), as has been reported previously (Nishida et al., 2009). In contrast, Ago3 was localized to the nuage, the same as in the control. These results suggest that under conditions where tud function is absent, Ago3 finds an alternative way to be localized to the nuage in a Tud-independent manner. Another possible interpretation is that a small amount of Aub in the nuage is sufficient for Ago3 to be localized to the nuage. Also in testes, Ago3 localization was barely affected by loss of tud function, whereas Aub localization was drastically changed (Figure 3B); Aub was sparsely localized in the nuage and instead was dispersed in the cytosol. These results indicate that the requirement and dispensability of Tud for nuage localization of Aub and Ago3, respectively, are similar in male and females.

We also examined how loss of krimp affects the localization of Aub and Ago3 in the germline. In krimp mutant ovaries, Ago3 lost its ability to localize to the nuage, although Aub was accumulated in the nuage the same as in the control (Figure 4A). Thus, Krimp is necessary for nuage localization of Ago3, but not of Aub. Tud nuage localization was maintained even after krimp expression was lost (Figure 4A). In contrast, in krimp mutant testes, Aub, Ago3, and Tud nuage localization was abolished (Figure 4B). The requirement of Krimp for Aub and Tud nuage localization is different in ovaries and testes.

An earlier study showed that Krimp is localized to the nuage in ovaries (Lim and Kai, 2007). In the present study, we confirmed this localization (Figure 5A) using our newly produced anti-Krimp antibody (Figure 1B). We then examined by immunostaining of ovaries and testes how Krimp nuage localization is affected by loss
FIGURE 2 | Tud subcellular localization in aub and ago3 mutant ovaries and testes. (A) Ovaries: the control (a), and aub (aubHN2/aubQC42) (b), and ago3 (ago3²/ago3³) (c) mutant ovaries were stained with anti-Tud antibody; the control (d), and aub (aubHN2/aubQC42) (e) ago3 (ago3²/ago3³) (f) mutant ovaries were stained with anti-Aub antibody; and the control (g), and aub (aubHN2/aubQC42) (h), and ago3 (ago3²/ago3³) (i) mutant ovaries were stained with anti-Ago3 antibody. Scale bars in all ovary images indicate 10 μm. (B) Testes: the control (a), and aub (aubHN2/aubQC42) (b), and ago3 (ago3²/ago3³) (c) mutant testes were stained with anti-Tud antibody. Enlarged images of the regions that are boxed with white dotted lines in (B) (a–c) are shown in (B) (d–f), respectively. The control (g), and aub (aubHN2/aubQC42) (h), and ago3 (ago3²/ago3³) (i) mutant testes were stained with anti-Aub antibody; and the control (j), and aub (aubHN2/aubQC42) (k), and ago3 (ago3²/ago3³) (l) mutant testes were stained with anti-Ago3 antibody. Scale bars in all testis images indicate 20 μm. All immunohistochemical analyses were performed at least three times and representative images are shown.
of other piRNA factors. We found that loss of ago3 and tud expression in ovaries did not affect Krimp localization (Figure 5A). However, in aub mutant ovaries, Krimp signals were hardly detected in the nuage; instead, Krimp accumulated to unknown cytoplasmic structures (Figure 5A). Thus, Aub, but not Ago3 or Tud, is required for Krimp nuage localization. Why Krimp accumulates in cytoplasmic structures remains unknown. The Krimp signal of immunostaining was lower in aub and tud mutant testes than that in the control, while Krimp expression was unchanged by loss of ago3 expression (Figure 5B). Western blotting confirmed a severe reduction in the expression level of Krimp in aub and

![FIGURE 3](image_url)  
**FIGURE 3** | Loss of tud function affects the subcellular localization of Aub and Ago3 in ovaries and testes similarly. (A) Ovaries: tud heterozygous and homozygous mutant ovaries were immunostained with anti-Tud, anti-Aub, and anti-Ago3 antibodies [(a): tud hetero/anti-Tud; (b): tud homo/anti-Tud; (c): tud hetero/anti-Aub; (d): tud homo/anti-Aub; (e): tud hetero/anti-Ago3; (f): tud homo/anti-Ago3]. Scale bars in all ovary images indicate 10 μm. (B) Testes: tud mutant and control testes were immunostained with anti-Aub and anti-Ago3 antibodies [(a): tud hetero/anti-Aub; (b): enlarged image of the region boxed with a white line in (a); (c): tud homo/anti-Aub; (d): enlarged image of indicated region in (c); (e): tud hetero/anti-Ago3; (f): enlarged image of indicated region in (e); (g): tud homo/anti-Ago3; (h): enlarged image of indicated region in (g)]. Scale bars in all testis images indicate 20 μm. All immunohistochemical analyses were performed at least three times and representative images are shown.

![FIGURE 4](image_url)  
**FIGURE 4** | Loss of krimp function affects the subcellular localization of Aub and Ago3 in ovaries and testes differently. (A) Ovaries: the control and krimp mutant ovaries were immunostained with anti-Aub, anti-Ago3, and anti-Tud antibodies [(a): control/anti-Aub; (b): krimp mutant/anti-Aub; (c): control/anti-Ago3; (d): krimp mutant/anti-Ago3; (e): control/anti-Tud; (f): krimp mutant/anti-Tud; (g): control/anti-Krimp; (h): krimp mutant/anti-Krimp]. Scale bars in all ovary images indicate 10 μm. (B) Testes: the control and krimp mutant testes were immunostained with anti-Aub, anti-Ago3, anti-Tud, and anti-Krimp antibodies [(a): control/anti-Aub; (b): krimp mutant/anti-Aub; (c): control/anti-Ago3; (d): krimp mutant/anti-Ago3; (e): control/anti-Tud; (f): krimp mutant/anti-Tud; (g): control/anti-Krimp; (h): krimp mutant/anti-Krimp]. Scale bars in all testis images indicate 20 μm. All immunohistochemical analyses were performed at least three times and representative images are shown.
tud mutant testes (Figure 5C). Thus, the requirement of Tud in Krimp nuage localization is different between males and females. Why loss of aub and tud expression causes a severe reduction in the Krimp expression requires further investigation. Krimp was hardly detected in the tud mutant testes. However, Ago3 was able to accumulate in the nuage, possibly because Aub was, at least partially, localized to the nuage in tud mutant testes. It could be postulated that Ago3 nuage localization necessitates either Aub or Krimp in the nuage.

The nuage localization of piRNA factors, Aub, Tud, Krimp, and Ago3, in ovaries and testes from wt and the piRNA factor mutant flies was summarized (Figure 6A) and their molecular hierarchy was compared (Figure 6B). Their expression patterns in wt ovary and testis were also summarized (Figure 6C). An obvious difference in the hierarchy is the location of Krimp between Aub and Ago3 in ovaries but at the same level as Aub and upstream of Ago3 in testes. Krimp expression in testis is limited to developmentally early stage cells, such as germline stem cells, gonialblasts, and spermatogonia (Figure 6C) and this may be the cause of the gender difference observed in the location of Krimp in the molecular hierarchy.

We reported previously that, in testes, Ago3 functions in the amplification loop with Aub for producing transposon-derived piRNAs (Nagao et al., 2010). However, most Ago3-associated testis piRNAs corresponding to Suppressor of Stellate [Su(Ste)] genes that function in silencing the Stellate gene, are antisense-oriented and are also detected in piRNAs associated with Aub (Nagao et al., 2010). In addition, the vast majority of Ago3-associated piRNAs derived from the AT-chX locus on chromosome X, some of which may function in silencing vasa, are also antisense-oriented and are found among Aub-associated piRNAs (Nagao et al., 2010). The piRNAs that show characteristics similar to those of AT-chX and Su(Ste) piRNAs have so far not been found in ovaries (Nagao et al., 2010). Our current finding, that Krimp is located in a different position in the nuage localization hierarchy in ovaries and testes, may further support the idea that some mechanisms of piRNA biogenesis differ in ovaries and testes. Our interpretation is that Krimp may be one of the important factors for producing AT-chX and Su(Ste) primary piRNAs and may enabling their loading into not only Aub, but also Ago3, in testes. In ovaries, Krimp is placed between Aub and Ago3 in the hierarchy and this may be why primary piRNAs are specifically loaded into Aub, but
FIGURE 6 | Molecular hierarchy of the piRNA factors in their localization to nuage in ovaries and testes. (A) The nuage localization of piRNA factors, Aub, Tud, Krimp, and Ago3 in wild-type (wt) and their mutant ovaries and testes: n.d.: none detected. Since none of the piRNA factors in this figure were detected in the nucleus of wt ovaries and testes, they were considered to be cytoplasmic proteins. In tud mutant ovaries and testes, Aub is only weakly accumulated in the nuage; thus, the nuage is indicated with a thinner line. The Krimp signals are hardly detected inaub and tud mutant testes. (B) The nuage localization hierarchy: Aub is placed upstream of Ago3 in both ovaries and testes. However, Krimp shows gender-specificity; in ovaries Krimp lies between Aub and Ago3, in testes Krimp is placed at the same level as Aub in testes. Tud is required for Aub localization in both male and female nuage. However, Ago3 can be localized to the nuage without tud function in both males and females. It is likely that Ago3 nuage localization occurs in both Aub-dependent and Tud-independent manners. (C) The expression patterns of Aub, Tud, Krimp, and Ago3 in ovaries and testes. GSCs, germline stem cells; CBs, cystoblasts; NCs, nurse cells; Oo, oocyte; GBs, gonioblasts; SG, spermatogonia.

not into Ago3. Krimp has a Tud domain and, thus, it is reasonable to postulate that Krimp associates with Aub and/or Ago3 through their sDMA modifications in both ovaries and testes. However, the mode of their associations could be different in the two tissues: the extent of sDMA modification of Aub and Ago3 may be different and/or the sDMA requirements in the association between Krimp and Aub/Ago3 may be different. These possible differences could be the cause of the gender difference for Krimp in the piRNA factor hierarchy. We are currently investigating the biochemical function of Krimp in piRNA biogenesis and its relationship with Aub and Ago3, as well as the modification status of Aub and Ago3 in both ovaries and testes, which
will help further our understanding of piRNA biogenesis in general and of the gender differences in the molecular mechanisms in particular.

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