Biogenesis pathways of piRNAs loaded onto AGO3 in the
*Drosophila* testis

Akihiro Nagao, Toutai Mituyama, Haidong Huang, et al.

*RNA* 2010 16: 2503-2515 originally published online October 27, 2010
Access the most recent version at doi:10.1261/rna.2270710

**Supplemental Material**
http://rnajournal.cshlp.org/content/suppl/2010/10/18/rna.2270710.DC1.html

**References**
This article cites 61 articles, 26 of which can be accessed free at:
http://rnajournal.cshlp.org/content/16/12/2503.full.html#ref-list-1

**Email alerting service**
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
http://rnajournal.cshlp.org/subscriptions

Copyright © 2010 RNA Society
Biogenesis pathways of piRNAs loaded onto AGO3 in the Drosophila testis

AKIHIRO NAGAO,1,2 TOUTAI MITUYAMA,3 HAIDONG HUANG,4 DAHUA CHEN,4 MIKIKO C. SIOMI,1,5 and HARUHIKO SIOMI1

1Department of Molecular Biology, Keio University School of Medicine, Tokyo 160-8582, Japan
2Institute of Health Biosciences, University of Tokushima, Tokushima 770-8503, Japan
3Computational Biology Research Center (CBRC), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo 135-0064, Japan
4Institute of Zoology, Chinese Academy of Sciences, Beijing, People’s Republic of China
5JST, CREST, Saitama, Japan

ABSTRACT

PIWI-interacting RNAs (piRNAs) silence transposable elements in animal germ cells. In Drosophila ovaries, piRNAs are produced by two distinct pathways: the “ping-pong” amplification cycle that operates in germ cells and a ping-pong-independent pathway termed the primary pathway that mainly operates in somatic cells. AGO3, one of three PIWI proteins in flies, is involved in the ping-pong cycle in ovaries. We characterized AGO3-associated piRNAs in fly testes and found that like in ovaries, AGO3 functions in the ping-pong cycle with Aubergine (Aub) for piRNA production from transposon transcripts. In contrast, most AGO3-associated piRNAs corresponding to Suppressor of Stellate [Su(Ste)] genes are antisense-oriented and bound to Aub. In addition, the vast majority of AGO3-bound piRNAs derived from the AT-chX locus on chromosome X are antisense-oriented and are also found among Aub-associated piRNAs. The presence of very few sense Su(Ste) and AT-chX piRNAs suggests that biogenesis of both Su(Ste) and AT-chX piRNAs by a ping-pong mechanism only is highly unlikely. Nevertheless, the mutual interdependence of AGO3 and Aub for the accumulation of these piRNAs shows that their production relies on both AGO3 and Aub. Analysis of piRNA pathway mutants revealed that although the requirements for piRNA factors for Su(Ste)- and AT-chX-piRNA levels mostly overlap and resemble those for the ping-pong mechanism in the ovaries, Armitage (armi) is not required for the accumulation of AT-chX-1 piRNA. These findings suggest that the impacts of armi mutants on the operation of the piRNA pathway are variable in germ cells of fly testes.

Keywords: AGO3; Aubergine; piRNA; Drosophila; RNA silencing; germline

INTRODUCTION

Recent studies have shown that eukaryotic cells express a large number of different small RNAs, 20- to 30-nucleotide (nt) long, which trigger various forms of sequence-specific gene silencing by guiding the Argonaute complex to target RNAs by base-pairing (Ghildiyal and Zamore 2009; Kim et al. 2009; Siomi and Siomi 2009). This process is referred to as “RNA silencing.” RNA silencing is an evolutionarily conserved nucleic acid–based immunity that restrains the expression of parasitic and pathogenic invaders such as viruses and transposable elements (Girard and Hannon 2008; Siomi and Siomi 2008).

In Drosophila, the endogenous small interfering RNA (endo-siRNA or esiRNA) pathway restrains the expression of transposable elements in somatic cells, whereas the PIWI-interacting RNA (piRNA) pathway represses them in germline cells (Ghildiyal and Zamore 2009; Kim et al. 2009; Siomi and Siomi 2009). esiRNAs are produced by the Dicer2-dependent pathway, indicating that they are derived from double-stranded RNA (dsRNA) precursors (Czech et al. 2008; Ghildiyal et al. 2008; Kawamura et al. 2008; Okamura et al. 2008). Processed esiRNAs are loaded onto AGO2 to form RNA-induced silencing complexes (RISCs) that silence transposable elements by cleaving their transcripts. In contrast, the production of piRNAs is Dicer-independent, indicating that the biogenesis of piRNAs is distinct from that of esiRNAs and does not involve stable dsRNA intermediates (Vagin et al. 2006; Houwing et al. 2008).
2504 RNA, Vol. 16, No. 12

Nagao et al.

2007). In fly gonads, three distinct PIWI proteins of the Argonaute family—AGO3, Aubergine (Aub), and Piwi—are expressed (Williams and Rubin 2002). In fly ovaries, piRNAs associated with Aub and Piwi are derived mainly from the antisense strand of retrotransposons, while AGO3-associated piRNAs arise mainly from the sense strand. Aub- and Piwi-associated piRNAs show a strong preference for uracil (U) at their 5′ ends, while AGO3-associated piRNAs show a preference for adenine (A) at the tenth nucleotide from the 5′ end (Brennecke et al. 2007; Gunawardane et al. 2007). The first 10 nt of Aub-associated piRNAs can be complementary to the first 10 nt of AGO3-associated piRNAs. These PIWI proteins retain the endoribonuclease or Slicer activity that allows them to cleave an RNA substrate across from position 10 of their bound piRNA (Saito et al. 2006; Gunawardane et al. 2007). These observations suggest a Slicer-dependent self-amplifying loop model, called the “ping-pong cycle,” for piRNA biogenesis in which sense piRNAs bound to AGO3 cleave long antisense transcripts and guide the formation of the 5′ end of the antisense piRNA bound to Aub, and vice versa (Brennecke et al. 2007; Gunawardane et al. 2007). Therefore, in this amplification loop, transposons are both a source gene of piRNAs and a target of piRNA-mediated silencing. Signatures of this ping-pong cycle are conserved among metazoans (Aravin et al. 2007; Houwing et al. 2007; Gunawardane et al. 2007; Kawaoaka et al. 2009).

Both AGO3 and Aub are cytoplasmic proteins expressed only in germline cells in the ovaries (Harris and MacDonald 2001; Brennecke et al. 2007; Gunawardane et al. 2007; Nishida et al. 2007). In contrast, Piwi is nuclear and is expressed in both germline cells and somatic support cells such as follicle cells in the ovaries (Cox et al. 2000; Megosh et al. 2006; Saito et al. 2006; Brennecke et al. 2007; Gunawardane et al. 2007; Nishida et al. 2007). Piwi is therefore spatially separated from AGO3 and Aub at the cell-type and subcellular levels in the ovaries. The ping-pong cycle in fly ovaries operates specifically in germ cells and engages mainly AGO3 and Aub (Brennecke et al. 2007; Malone et al. 2009). piRNAs produced by the ping-pong cycle are often referred to as secondary piRNAs. Classification of piRNAs according to their origins has indicated that piRNAs derived from a particular piRNA cluster locus, the flamenco locus, are exclusively loaded onto Piwi and not further amplified (Brennecke et al. 2007; Malone et al. 2009; Saito et al. 2009). These piRNAs are overwhelmingly antisense. The flamenco locus was originally identified as a repressor of transposon expression in somatic follicle cells (Pelisson et al. 1994), where Piwi, but not AGO3 and Aub, is expressed. These results indicate that piRNAs from the flamenco locus are produced by a pathway independent of the ping-pong cycle in ovarian somatic cells. This pathway is called the “primary piRNA pathway” (Brennecke et al. 2007; Malone et al. 2009; Saito et al. 2009; Siomi and Kuramochi-Miyagawa 2009). It is believed that the primary pathway also generates piRNAs that can initiate the ping-pong cycle in the ovarian germ cells (Brennecke et al. 2007; Malone et al. 2009). Although Aub receives some piRNAs via a primary biogenesis pathway operating in the ovarian germ cells, AGO3 contains mostly secondary piRNAs (Brennecke et al. 2007; Li et al. 2009; Malone et al. 2009).

In Drosophila testes, the X-linked Stellate locus is silenced by piRNAs derived from antisense transcripts of the homologous Suppressor of Stellate [Su(Ste)] repeats on chromosome Y (Balakireva et al. 1992; Bozzi et al. 1995; Aravin et al. 2001, 2004; Vagin et al. 2006). Aub is required for accumulation of Su(Ste) piRNAs (Aravin et al. 2004; Vagin et al. 2006). Mutations in Aub result in the formation of Stellate protein crystals in primary spermatocytes, which causes male sterility (Bozzi et al. 1995; Aravin et al. 2001, 2004; Kotelnikov et al. 2009). We previously demonstrated that, among piRNAs associated with Aub in fly testes, those derived from Su(Ste) antisense transcripts were the most abundant (Nishida et al. 2007). The second largest class of piRNAs associated with Aub in the testes is derived from a repetitive region on chromosome X, termed AT-chX. One of these piRNAs, termed AT-chX-1, shows strong complementarity to vasa (vas) miRNA, a germline-specific transcript involved in oocyte differentiation and cyst development (Lasko and Ashburner 1988; Styhler et al. 1998). The AT-chX-1 piRNA down-regulates the protein levels of Vas (Nishida et al. 2007; Li et al. 2009). piRNAs from the two loci, Su(Ste) and AT-chX, are not bound to Piwi (Nishida et al. 2007). Recently, Li et al. (2009) produced Ago3 mutants and demonstrated that Ago3 is required for accumulation of both Su(Ste) and AT-chX piRNAs in fly testes. However, how piRNAs are produced in fly testes remains largely unknown.

We sought to determine whether these abundant piRNAs in fly testes were produced in a ping-pong-dependent manner, as is the case for piRNAs derived from transposons in ovaries. Here, we analyzed piRNAs associated with AGO3 and Aub immunopurified from fly testes. Our data provide support for the ping-pong cycle in which transposon-derived piRNAs are amplified by AGO3 and Aub in fly testes. However, a large number of piRNAs with exactly the same sequences, derived from antisense strands of the two loci, Su(Ste) and AT-chX, were associated with both AGO3 and Aub. Therefore, biogenesis of these piRNAs through a ping-pong mechanism only is highly unlikely. We examined the accumulation of Su(Ste) and AT-chX piRNAs in mutant testes defective for nine piRNA pathway proteins. We found that AGO3, Aub, spindle-E (Spn-E), Krimper (Krimp), Maelstrom (Mael), and VAS are required for the production of both types of piRNAs. However, the production of Su(Ste) piRNAs, but not AT-chX-1 piRNA, depends on the RNA helicase Armitage (Armi). Together, these results suggest that distinct piRNA pathways, with different genetic requirements probably depending on the piRNA loci, operate in germ cells of fly testes.
RESULTS

Expression of AGO3 in fly testes

To biochemically investigate piRNA biogenesis in fly testes, we produced antibodies against AGO3 (Nishida et al. 2009). Western blotting of testis lysates prepared from yellow white wild-type (WT), trans-heterozygous ago3 mutants (ago3^T2/ago3^T3), and aub mutants (aub^N2/aub^C42) using the anti-AGO3 antibody revealed that the amount of AGO3 protein was severely reduced in aub mutant testes (Fig. 1A). The ago3 mRNA levels were also significantly affected in aub mutant testes (Fig. 1A, lower panel). In contrast, levels of AGO3 protein were not affected in aub mutant ovaries, suggesting that Aub is required for stabilizing ago3 mRNA and AGO3 protein in testes. Conversely, levels of Aub protein were not altered in either testes or ovaries of the ago3 mutants (Fig. 1A). Immunofluorescent staining of AGO3 in testes revealed that AGO3 was present in the cytoplasm of germ-line stem cells (GSC), gonialblasts, and spermatogonia, as was the case for Aub (Fig. 1B). However, AGO3 was below the level of detection in primary spermatocytes, where Aub is expressed. No expression of AGO3 or Aub was detected in somatic cells surrounding the gonialblasts and spermatogonia, or in the hub (Fig. 1B), where the strong expression of Piwi was observed (Cox et al. 2000; Saito et al. 2006). Both AGO3 and Aub accumulated in the nuage (Snee and Macdonald 2004; Brennecke et al. 2007), a ring around the cytoplasmic face of the nuclei in germline cells (Eddy 1975). Recent work has suggested the nuage to be a potential site for RISC-mediated transposon silencing and piRNA biogenesis (Brennecke et al. 2007; Lim and Kai 2007; Li et al. 2009; Malone et al. 2009). In aub mutant testes, no nuage staining of AGO3 was observed. Instead, AGO3-positive large dots were occasionally observed in the cytoplasm, which was also the case for aub mutant ovaries (Malone et al. 2009). However, mutations in ago3 did not disrupt the localization of Aub to the nuage in the testes. These results show that Aub is required for AGO3 to be stabilized and to localize to the nuage in the testes. Thus, in the testes, the dependence of these PIWI proteins for their stability and localization to the nuage is different from that observed in the ovaries, where loss of AGO3 does not affect Aub protein levels and localization of AGO3 and...
Aub to the nuage is mutually interdependent (Li et al. 2009; Malone et al. 2009).

Analysis of piRNAs associated with AGO3 and Aub in fly testes by sequencing

We previously performed a small-scale sequencing study to identify small RNAs associated with Aub, immunoprecipitated from testis lysate with an anti-Aub antibody (Nishida et al. 2007). To gain further insight into piRNA biogenesis in testes, we immunopurified AGO3 and Aub with specific antibodies from fly testes and performed a large-scale sequencing to comprehensively examine their associated small RNAs. Both immunopurified AGO3 and Aub in testes (Fig. 2A) were associated with small RNAs 23–28 nt long (Fig. 2B).

To characterize the piRNAs associated with AGO3 and Aub, we generated 70,323 and 108,439 sequencing reads, respectively, for AGO3- and Aub-associated piRNAs. The list of piRNAs obtained is shown in Table 1 and Supplemental Table 1. piRNAs were mapped to the *Drosophila* genome and annotated (Fig. 2C). Fifty-four percent of the AGO3-associated piRNAs corresponded to transposons. In sharp contrast, only 7% of the Aub-associated piRNAs corresponded to transposons. The Aub-associated piRNAs corresponding to transposons mainly arose from antisense transcripts and showed a strong preference for U at their 5’ ends, while the AGO3-associated transposon piRNAs were mainly derived from sense transcripts and showed a strong preference for A at the tenth nucleotide from the 5’ end (Fig. 3A,B). Among transposon-derived piRNAs in Aub, ~17% have ping-pong partner piRNAs, while ~27% of transposon-derived piRNAs in AGO3 have ping-pong partner piRNAs (Supplemental Fig. 1). Thus, piRNAs corresponding to transposons in the testes show signatures of the ping-pong amplification cycle. These results suggest that piRNAs of transposon origin are produced by the amplification loop in testes as in ovaries. Curiously, we found that the expression levels of transposons in the testes were only slightly affected by the loss of *aub* or *ago3* functions (Supplemental Fig. 2). This is in agreement with findings that the testis expression of several retrotransposons was not significantly affected by *aub* mutations (Aravin et al. 2001), and these findings together raise the possibility that there exist unknown AGO3/Aub-independent mechanism(s) controlling transposon silencing in germline cells in the testes.

Although most transposon-derived piRNA species identified in the ovaries were sequenced only once (Brennecke}

**FIGURE 2.** Analyses of small RNAs associated with AGO3 and Aub in testes. (A) Immunoprecipitation was performed from wild-type fly testes using anti-AGO3 and anti-Aub antibodies. (B) RNA molecules extracted from the immunoprecipitated complexes were visualized by 32P-ATP labeling on denaturing acrylamide gel. Small RNAs, 23–28 nt long, were observed associated with AGO3 and Aub in the testes. (C) Profiles of small RNAs associated with AGO3 and Aub in fly testes. The most abundant class of piRNAs associated with Aub was those derived from the *Suppressor of Stellate* [Su(Ste)] antisense transcripts. The second most abundant class of piRNAs associated with Aub was made up of those derived from an intergenic repetitive region on the X-chromosome, termed AT-chX. On the other hand, the majority of piRNAs associated with AGO3 were derived from transposons and other repetitive DNA elements (repeats) found in the genome. Su(Ste) piRNAs (~5%) as well as AT-chX piRNAs (~10%) were also present in the AGO3 small RNA library.
et al. 2007; Li et al. 2009; Malone et al. 2009), we noted that piRNAs derived from some transposons including Helena, Opus, and Mgd3 elements in the testes, were sequenced multiple times (Table 1; Supplemental Table 1). For example, a piRNA derived from a sense strand of Helena was sequenced 4187 times in the AGO3 library and 314 times in the Aub library. These piRNAs with multiple reads tended to be derived from the sense strands of transposons (Fig. 3A). We found no antisense piRNAs that paired with these multiple-read sense piRNAs with signatures of an amplification cycle, namely, 1U/10A partners with a 10-nt, 5′-overlap.

### Table 1. Top 50 small RNAs in AGO3 complexes in testes

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Read (AGO3)</th>
<th>Read (Aub)</th>
<th>mismatch</th>
<th>Annotation (category)</th>
<th>Annotation (detail)</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGUUUCAUGCUGUAGACGGCUCGCGG</td>
<td>2642</td>
<td>13,615</td>
<td>0</td>
<td>AT-chX</td>
<td>AT-chX-B</td>
<td>Antisense</td>
</tr>
<tr>
<td>AAAACCUAGAGAUAGGUGAGGCGG</td>
<td>2229</td>
<td>119</td>
<td>0</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>UGGCUUUCGAGAGAUAGGUGAGGCAA</td>
<td>2069</td>
<td>17</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>ULCUGAGCUGUAGAGAAGAAAGGGCG</td>
<td>1670</td>
<td>43,207</td>
<td>0</td>
<td>SU(Ste)</td>
<td>SU(Ste)</td>
<td>Antisense</td>
</tr>
<tr>
<td>UGUGUCGAGAUGAGACGGCACGCGG</td>
<td>875</td>
<td>3013</td>
<td>0</td>
<td>AT-chX</td>
<td>AT-chX-B</td>
<td>Antisense</td>
</tr>
<tr>
<td>AUGGCUGUUAGUAGGGUGUGGCGG</td>
<td>857</td>
<td>3</td>
<td>0</td>
<td>ncRNA</td>
<td>rRNA</td>
<td>Sense</td>
</tr>
<tr>
<td>UGUGUCGAGAUGAGACGGCACGCGG</td>
<td>729</td>
<td>4</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>UGUGUCGAGAUGAGACGGCACGCGG</td>
<td>693</td>
<td>91</td>
<td>0</td>
<td>AT-chX</td>
<td>AT-chX-B</td>
<td>Antisense</td>
</tr>
<tr>
<td>UGUGUCGAGAUGAGACGGCACGCGG</td>
<td>693</td>
<td>8</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>UGAGCAGAUAACGCGAUAGCGGACK</td>
<td>606</td>
<td>0</td>
<td>0</td>
<td>No annotation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGCUUCCUCAAGCCAGCGCUCUACC</td>
<td>479</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>ACGGAAUAGAUCAGGCGCCCCAAC</td>
<td>441</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>opus</td>
<td>Sense</td>
</tr>
<tr>
<td>UGCAGAGAUCAGAAGCGGCGGCA</td>
<td>374</td>
<td>2</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>AAAACCUAGAGAUGAGGUGAGGCAA</td>
<td>366</td>
<td>24</td>
<td>0</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>AAAACCUAGAGAUGAGGUGAGGCAA</td>
<td>357</td>
<td>24</td>
<td>0</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>UGUGUCGAGAUGAGACGGCACGCGG</td>
<td>308</td>
<td>134</td>
<td>0</td>
<td>AT-chX</td>
<td>AT-chX-B</td>
<td>Antisense</td>
</tr>
<tr>
<td>CCCGAACGAGACGAGAUCAGGAC</td>
<td>283</td>
<td>16</td>
<td>0</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>ACGGAAUAGAUCAGGCGCCCCAAC</td>
<td>274</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>F-element</td>
<td>Sense</td>
</tr>
<tr>
<td>AACUGAUAAGCAGGAAAAGCAGAA</td>
<td>255</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>invader4</td>
<td>Sense</td>
</tr>
<tr>
<td>UGCAGAUAAGAUCAGGCGCCCCAAC</td>
<td>241</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>opus</td>
<td>Sense</td>
</tr>
<tr>
<td>AAAACCUAGAGAUGAGAUCAGGAC</td>
<td>241</td>
<td>17</td>
<td>1</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>UUCAAGGUGUAACCCAGAGAUCUAC</td>
<td>238</td>
<td>80</td>
<td>0</td>
<td>Repeat</td>
<td>trf</td>
<td></td>
</tr>
<tr>
<td>UACUGUAGUUCCCGCCGAUUAGGAGUAC</td>
<td>234</td>
<td>0</td>
<td>0</td>
<td>ncRNA</td>
<td>rRNA</td>
<td>Sense</td>
</tr>
<tr>
<td>GAUCAUAGAUCAGAGAGUUAGGCU</td>
<td>216</td>
<td>42</td>
<td>0</td>
<td>ncRNA</td>
<td>rRNA</td>
<td>Sense</td>
</tr>
<tr>
<td>ACAUCAUAAAGCAGGCAUCUCAGU</td>
<td>188</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>GATE</td>
<td>Sense</td>
</tr>
<tr>
<td>ACAGAAAUAACGAGCCGUGGCGAAGA</td>
<td>181</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>mdg3</td>
<td>Sense</td>
</tr>
<tr>
<td>UGGUUCCAGAAGAUGAGGCUCGGCGGCC</td>
<td>175</td>
<td>0</td>
<td>0</td>
<td>AT-chX</td>
<td>AT-chX-B</td>
<td>Antisense</td>
</tr>
<tr>
<td>AACUGAUAAGAUCAGGCGCCCCAAC</td>
<td>165</td>
<td>41</td>
<td>0</td>
<td>Transposon</td>
<td>invader4</td>
<td>Sense</td>
</tr>
<tr>
<td>GCCGAACGAGAUCGAGCGAAGC</td>
<td>164</td>
<td>41</td>
<td>0</td>
<td>Transposon</td>
<td>invader1</td>
<td>Sense</td>
</tr>
<tr>
<td>CAACCCAGAUAGUACUCUAGGAGGAC</td>
<td>158</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>Doc</td>
<td>Sense</td>
</tr>
<tr>
<td>UGGCAAAGACCCGCGGUCGUGUUUGGUGGCU</td>
<td>152</td>
<td>2</td>
<td>0</td>
<td>ncRNA</td>
<td>SsrRNA</td>
<td>Sense</td>
</tr>
<tr>
<td>UGcaGAGAAGAGAGAGGCGGAGU</td>
<td>151</td>
<td>6212</td>
<td>0</td>
<td>Su(Ste)</td>
<td>Su(Ste)</td>
<td>Antisense</td>
</tr>
<tr>
<td>ACCGAAUAACGAGGAGGAC</td>
<td>147</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>mdg3</td>
<td>Sense</td>
</tr>
<tr>
<td>CAGGAUAGUAGAUAUCAGGAGGGCA</td>
<td>147</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>Doc</td>
<td>Sense</td>
</tr>
<tr>
<td>AAAACCGAAGAGAUGAUCAGGCGGCCA</td>
<td>143</td>
<td>41</td>
<td>0</td>
<td>Transposon</td>
<td>Helena</td>
<td>Sense</td>
</tr>
<tr>
<td>CCGCAGUAGAUCAGGAGGCCGAGGACC</td>
<td>139</td>
<td>0</td>
<td>0</td>
<td>Su(Ste)</td>
<td>Su(Ste)</td>
<td>Antisense</td>
</tr>
<tr>
<td>AACUGGAGAUAACGAGAUCAGGAGA</td>
<td>131</td>
<td>35</td>
<td>0</td>
<td>ncRNA</td>
<td>rRNA</td>
<td>Antisense</td>
</tr>
<tr>
<td>UACAGCAGAAGAGAUAAGGAGAAGGAGAAGGCA</td>
<td>124</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>frogger</td>
<td>Sense</td>
</tr>
<tr>
<td>CAACCGAGCAUCACAAAGCCCAGGCAA</td>
<td>123</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>G2</td>
<td>Sense</td>
</tr>
<tr>
<td>UUCAAGGUGUAAACCCAGAGAUCUGGUGU</td>
<td>121</td>
<td>19</td>
<td>0</td>
<td>Repeat</td>
<td>trf</td>
<td></td>
</tr>
<tr>
<td>UUGCAAGAACGAGCAAGGAGAC</td>
<td>113</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>micropia</td>
<td>Sense</td>
</tr>
<tr>
<td>GCCCAAGUAACUAGAUCAGAGCGGCGG</td>
<td>111</td>
<td>3</td>
<td>0</td>
<td>Transposon</td>
<td>opus</td>
<td>Sense</td>
</tr>
<tr>
<td>GUGGCUGAUUGAGGGCGGCGGCUGAGUUA</td>
<td>109</td>
<td>0</td>
<td>0</td>
<td>No annotation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGCUUCAUCAAGCCAGGAGGUGGCGG</td>
<td>104</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>R1-element</td>
<td>Antisense</td>
</tr>
<tr>
<td>ACCCUUGAUAACCGGUGACUCUUCU</td>
<td>103</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>R1b</td>
<td>Sense</td>
</tr>
<tr>
<td>UGAGAUAAGCUGAAGGAGGAAAGA</td>
<td>102</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>G2</td>
<td>Sense</td>
</tr>
<tr>
<td>UCGGACCGAACCACCUUCUGUUGUGU</td>
<td>102</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>baggins</td>
<td>Sense</td>
</tr>
<tr>
<td>AACCGAUAAGAAGGAGAAAGGAGA</td>
<td>101</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>invader4</td>
<td>Sense</td>
</tr>
<tr>
<td>ACCUUGAUAACGCGGUGACUCUAC</td>
<td>98</td>
<td>0</td>
<td>0</td>
<td>Transposon</td>
<td>R1b</td>
<td>Sense</td>
</tr>
<tr>
<td>UCAGCCGGAAACCCUUCUGUGUGUGU</td>
<td>96</td>
<td>1</td>
<td>0</td>
<td>Transposon</td>
<td>baggins</td>
<td>Sense</td>
</tr>
</tbody>
</table>

Note that a large number of piRNAs with exactly the same sequences are present in both AGO3- and Aub-associated complexes.
Su(Ste) piRNAs

The most abundant class of Aub-associated piRNAs identified comprised those derived from Su(Ste) antisense transcripts (70.4%) (Figs. 2C, 4; Supplemental Table 2). Of all the Su(Ste) piRNAs associated with Aub, ~65% comprised only one piRNA, termed Su(Ste)-4 piRNA (Nishida et al. 2007). If up to two mismatches were permitted, ~87% of

FIGURE 3. Bioinformatics analysis of piRNAs in testes. (A) The heat map (left panel) indicates the strand bias of cloned piRNAs with respect to canonical transposon sequences. Transposons are grouped into long terminal repeat (LTR), long interspersed nuclear (LINE), and inverted repeat (IR) elements. The color intensities indicate the degree of strand bias: (green) sense; (red) antisense; (yellow) unbiased. Aub-associated piRNAs mainly arose from antisense transcripts of transposons, and AGO3-associated piRNAs were mainly derived from sense transcripts. The cloning frequencies of individual transposons are also indicated as a heat map (right panel). (B) The basic composition of piRNAs from transposons indicates that Aub-associated piRNAs show a strong preference for U at their 5’ ends. On the other hand, AGO3-associated piRNAs contain predominantly A at the tenth nucleotide from the 5’ end. piRNAs corresponding to the Su(Ste) and AT-chX loci were not included in this analysis.
the Aub-associated Su(Ste) piRNAs (58,055 out of 66,734 reads) mapped to the Su(Ste)-4 piRNA (Supplemental Table 2). The over-representation of the Su(Ste)-4 piRNA in the Aub library is consistent with our previous results obtained by analyzing a small Aub library that was independently produced and sequenced by a different method (Nishida et al. 2007). We also confirmed by Northern blot analysis that Su(Ste)-4 is a highly abundant piRNA in the Aub library. The over-representation of Su(Ste)-4 piRNA sequences loaded onto both AGO3 and Aub, with the read numbers, is also listed below. The vast majority of Su(Ste) piRNAs loaded onto both AGO3 and Aub comprised those derived from Su(Ste) antisense transcripts, including the most abundant Su(Ste)-4 piRNA. Half of Su(Ste) piRNA sequences loaded onto AGO3 alone (AGO3 Uniq.) comprised those derived from Su(Ste) sense transcripts and only a portion of AT-chX piRNAs associated with AGO3 (AGO3 Uniq.) were sense-oriented. In contrast, Su(Ste) piRNA sequences loaded onto Aub alone (Aub Uniq.) were all antisense. AT-chX piRNA sequences loaded onto both AGO3 and Aub (Common) comprised almost exclusively those derived from AT-chX antisense transcripts, including the most abundant AT-chX-1 piRNAs.

AT-chX piRNAs

A second large class of piRNAs associated with Aub in the testes was made up of those derived from a short repeated region, termed AT-chX, on chromosome X (Nishida et al. 2007). One of these piRNAs, termed AT-chX-1 (Nishida et al. 2007), showed strong complementarity to vas mRNA and suppresses the expression of VAS protein (Nishida et al. 2007). We analyzed AGO3- and Aub-associated AT-chX piRNAs and found that 10% and 20% of AGO3- and Aub-associated piRNAs, respectively, were derived from the AT-chX piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A). Taken together, these results suggest that the production of most of the Su(Ste) piRNAs only through a ping-pong pathway is unlikely.

Among the AGO3-associated Su(Ste) piRNAs, ~15% (584 out of 3214 reads) were sense-oriented (Fig. 4). To examine whether Su(Ste) piRNAs are produced in a ping-pong-dependent manner, we analyzed all the Su(Ste) piRNAs bound to AGO3 and Aub to determine whether the first 10 nt of Su(Ste) piRNAs in the sense orientation were complementary to the first 10 nt of Su(Ste) piRNAs in the antisense orientation (Fig. 4). We found that only four Aub- and 11 AGO3-associated sense Su(Ste) piRNAs have 78 Aub- and 38 AGO3-associated antisense Su(Ste) piRNA pairs showing signatures of an amplification cycle with 1U/10A partners with a 10-nt, 5'-overlap (Supplemental Table 3). Although this suggests that the ping-pong cycle may still operate for the production of Su(Ste) piRNAs, albeit rarely, we were unable to find sense Su(Ste) piRNA pairs that paired with Su(Ste)-4 piRNA or its variants. We also failed to detect by Northern blotting sense piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A). This suggests that the ping-pong cycle may still operate for the production of Su(Ste) piRNAs, albeit rarely, we were unable to find sense Su(Ste) piRNA pairs that paired with Su(Ste)-4 piRNA or its variants. We also failed to detect by Northern blotting sense piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A). This suggests that the ping-pong cycle may still operate for the production of Su(Ste) piRNAs, albeit rarely, we were unable to find sense Su(Ste) piRNA pairs that paired with Su(Ste)-4 piRNA or its variants. We also failed to detect by Northern blotting sense piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A). This suggests that the ping-pong cycle may still operate for the production of Su(Ste) piRNAs, albeit rarely, we were unable to find sense Su(Ste) piRNA pairs that paired with Su(Ste)-4 piRNA or its variants. We also failed to detect by Northern blotting sense piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A). This suggests that the ping-pong cycle may still operate for the production of Su(Ste) piRNAs, albeit rarely, we were unable to find sense Su(Ste) piRNA pairs that paired with Su(Ste)-4 piRNA or its variants. We also failed to detect by Northern blotting sense piRNAs that pair with Su(Ste)-4 piRNA (Supplemental Fig. 5A).
AT-chX locus (Fig. 2C). These piRNAs were overwhelmingly antisense: Only 32 out of 5,240 reads for AGO3-associated AT-chX piRNAs, and only one (one out of 19,227 reads) for Aub-associated AT-chX piRNAs were sense-oriented (Fig. 4; Supplemental Fig. 4). Only one pair (AT-chX-71 and AT-chX-71 sense) with ping-pong signatures was found among all these AT-chX piRNAs (Supplemental Fig. 4). Ninety-three percent of AGO3-associated AT-chX piRNA species were also found among the Aub-associated AT-chX piRNAs (Fig. 4). If two mismatches were permitted, ~91% of all Aub-associated AT-chX piRNAs, and ~93% of all AGO3-associated AT-chX piRNAs, were mapped to AT-chX-1 and its variants (Supplemental Table 4). However, we failed to find sense piRNAs that pair with AT-chX-1 piRNA among Aub- and AGO3-associated AT-chX piRNAs. We confirmed by Northern blot analysis that the AT-chX-1 piRNA is a very abundant piRNA in the testis (Supplemental Fig. 5A). We also confirmed by Northern blotting the expression of AT-chX-71 piRNA that pairs with AT-chX-71 sense piRNA. However, we failed to detect AT-chX-71 sense piRNA and sense piRNAs that pair with AT-chX-1 piRNA (Supplemental Fig. 5A). Together, these results suggest that the production of piRNAs from the AT-chX locus, particularly for the AT-chX-1 piRNA, through a ping-pong mechanism only is highly unlikely.

**Mutational analysis to define the genetic requirements for Su(Ste) and AT-chX piRNA production in testes**

It is known that both AGO3 and Aub are required to produce or stabilize Su(Ste) piRNAs (Vagin et al. 2006; Li et al. 2009), suggesting mutual interdependence of the PIWI proteins for the biogenesis of Su(Ste) piRNAs. It is also known that AGO3 is required to produce or stabilize AT-chX piRNAs (Li et al. 2009). We used Northern hybridization to examine Su(Ste)-4 and AT-chX-1 piRNA production in ago3 and aub mutant testes (Fig. 5A). Neither Su(Ste)-4 nor AT-chX-1 piRNAs were detected in aub mutant testes. A marked reduction in both piRNA species was observed in ago3 mutant testes. However, production of these piRNAs was not affected in piwi mutant testes, consistent with the fact that Piwi in the testes is expressed mostly in somatic cells, where AGO3 and Aub are not expressed (Nishida et al. 2007). These results suggest that both Su(Ste)-4 and AT-chX-1 piRNAs are produced in germ cells by a mechanism requiring both AGO3 and Aub, even though these piRNAs have few or no signatures of the ping-pong cycle.

Previous genetic studies have shown that both ago3 and aub mutations cause the formation of Stellate crystals.
in the testes (Fig. 5B; Bozzetti et al. 1995; Aravin et al. 2004; Vagin et al. 2006; Li et al. 2009). We noted that, as reported (Li et al. 2009), Stellate protein crystals form in primary spermatocytes in ago3 testes but not as abundantly as in aub testes. We examined the accumulation of Stellate mRNA in these mutant testes and found that the level of Stellate mRNA was only slightly increased in ago3 testes, compared with that in aub testes (Fig. 5B). This may be accounted for by the low expression level of AGO3 protein in testes. The amount of AGO3 in testes was approximately 10 times lower than that of Aub in the testes (Supplemental Fig. 6). In addition, residual Su(Ste) piRNAs in ago3 mutant testes may still be loaded onto Aub to form an RISC that silences Stellate mRNA, which may account for the weaker phenotype observed in ago3 mutant testes (Fig. 5A,B).

Mutations in a large number of genes affect piRNA production in Drosophila (Vagin et al. 2006; Chen et al. 2007; Lim and Kai 2007; Nishida et al. 2007; Pane et al. 2007; Klattenhoff and Theurkauf 2008; Li et al. 2009; Malone et al. 2009). To understand their involvement in the production of Su(Ste)-4 and AT-chX-1 piRNAs, we examined the molecular phenotypes of a series of eight mutants, including putative helicases, nucleases, and Tudor-domain proteins. We used Northern hybridization to examine Su(Ste)-4 and AT-chX-1 piRNA production in these mutant testes. As shown in Figure 5C, both Su(Ste)-4 and AT-chX-1 piRNAs were strongly reduced in spn-E and krimp mutant testes. spn-E encodes a member of the DExH family of adenosine triphosphatases (ATPases) with a Tudor domain (Gillespie and Berg 1995), and mutations in this gene are known to impair Stellate silencing by eliminating Su(Ste) piRNAs (Aravin et al. 2004; Vagin et al. 2006). krimp encodes a Tudor-domain protein (Barbosa et al. 2007; Lim and Kai 2007). Both genes are critical for silencing of transposons and the accumulation of piRNAs in the Drosophila germ line (Vagin et al. 2006; Lim and Kai 2007). Normal accumulation of both Su(Ste)-4 and AT-chX-1 piRNAs also requires mael and vas. mael was identified as a genetic loss-of-function mutant, whose germ-line cells exhibited incorrect posterior localization of several transcripts (Clegg et al. 1997; Lim and Kai 2007). Mael has an MHG box and a domain homologous to DnaQ-H 3′-to-5′ exonucleases (Zhang et al. 2008). vas is a germline-specific gene that encodes a DEAD-box RNA helicase involved in oogenesis (Lasko and Ashburner 1988; Styhler et al. 1998).

zucchini (zuc) and squash (squ) were identified in a screen for female sterile mutations and cause dorso–ventral patterning defects (Schupbach and Wieschaus 1991; Pane et al. 2007). Both encode proteins with homology with nucleases. Mutations in squ slightly reduced the accumulation of Su(Ste)-4 piRNA and AT-chX-1 piRNA. However, the accumulation of neither piRNA was significantly affected in zuc mutant testes.

Su(Ste)-4 piRNA was almost absent from armitage (armi) mutant testes, consistent with previous results (Vagin et al. 2006). Mutations in armi disrupt translational repression and localization of oskar mRNA and block RNA interference (RNAi) in Drosophila oocytes (Cook et al. 2004; Tomari et al. 2004). armi encodes a homolog of Arabidopsis SDE-3, an RNA helicase, which plays a role in post-transcriptional gene silencing triggered by transgenes and some viruses (Dalmay et al. 2001). The mammalian Armi homolog Mov10 also plays a role in siRNA-directed RNAi in cultured human cells (Meister et al. 2005). However, loss of Armi function had little impacts on AT-chX-1 piRNA levels.

We probed other piRNAs [Su(Ste)-6 and Su(Ste)-pair4] from the Su(Ste) locus on the Northern blots and found that the genetic requirements for their accumulation are almost identical to those for the Su(Ste)-4 piRNA (Supplemental Fig. 5B,C). We also probed another piRNA (AT-chX-71) from the AT-chX locus on the Northern blots and found that, in contrast with AT-chX-1 piRNA, the accumulation of this piRNA appears dependent on armi, although modest, as compared with piRNAs from the Su(Ste) locus (Supplemental Fig. 5B,C).

Taken together, these results indicate that although production of both Su(Ste)-4 and AT-chX-1 piRNAs requires several common genes, including ago3, aub, spn-E, krimp, and vas, which is similar to the requirements for the ping-pong mechanism in the ovaries (Malone et al. 2009), the impacts of armi mutants on the operation of the piRNA pathway are variable in germ cells of fly testes.

DISCUSSION

The present study demonstrated that piRNAs in the germ cells of fly testes are produced not only by a ping-pong cycle but probably also by other mechanisms whose genetic requirements vary depending on the piRNA loci. A surprisingly large portion of Aub-associated piRNAs in testes comprised only two piRNA species: Su(Ste)-4 and AT-chX-1. These piRNAs also comprise a large proportion of AGO3-associated piRNAs in testes. These piRNAs are not bound to Piwi that is expressed only in somatic cells in testes (Nishida et al. 2007). Therefore, these very abundant piRNAs are produced only in germ cells of the testes. In germ cells of the ovaries, AGO3 and Aub mostly engage in the ping-pong cycle to amplify piRNAs derived from transposons (Li et al. 2009; Malone et al. 2009). Although transposon-derived piRNAs that associate with AGO3 and Aub in testes show signatures of the ping-pong cycle, Su(Ste) and AT-chX piRNAs show very few signatures of the ping-pong cycle. The ping-pong cycle requires initiators to amplify piRNAs. piRNA populations maternaly inherited by germline transmission serve as important initiators of the ping-pong cycle to amplify piRNAs in the ovaries (Blumenstiel and Hartl 2005; Brennecke et al. 2008). However, few or no Su(Ste) piRNAs or AT-chX piRNAs are produced in the ovaries (Nishida et al. 2007). They are
therefore not maternally passed on to the offspring to serve as inputs to the ping-pong cycle. Thus, these piRNAs cannot rely on maternally deposited piRNA populations to initiate the production in testes. In other words, the *Drosophila* testis must have evolved these sets of piRNA pathway genes to produce *Su(Ste)* and *AT- chX* piRNAs. In ovaries, the primary piRNAs, whose production is mostly dependent on Piwi, have been proposed to be important initiators of the ping-pong cycle (Brennecke et al. 2007; Malone et al. 2009). Although Piwi is coexpressed with AGO3 and Aub in germ cells of the ovaries, its expression in testes is largely restricted to somatic cells where AGO3 and Aub are not expressed. Indeed, production of *Su(Ste)* and *AT- chX* piRNAs is Piwi-independent. Together, these findings suggest that production of *Su(Ste)* and *AT- chX* piRNAs through a ping-pong mechanism only is highly unlikely. However, it is still formally possible that, as suggested in Li et al. (2009), very few sense piRNAs might be sufficient to guide the processing of a large number of antisense piRNAs in a ping-pong manner.

How are these very abundant piRNAs produced in germ cells of the testes? Genetic analysis has revealed that in fly ovaries, *ago3*, *aub*, *krimp*, *spn-E*, and *vas* are required for the normal production of the ping-pong-dependent piRNAs derived from transposons in germ cells (Li et al. 2009; Malone et al. 2009). However, only *piwi* and *zuc* mutations specifically decrease levels of piRNAs produced by the primary pathway in ovarian somatic cells (Malone et al. 2009; Saito et al. 2009). *armi* plays an important role in the ping-pong mechanism (Malone et al. 2009). We demonstrated that in testes, *ago3*, *aub*, *krimp*, *spn-E*, and *vas* are required for the production of both *Su(Ste)* and *AT- chX* piRNAs in germ cells. This resembles the genetic requirements of the ping-pong cycle in the ovaries (Malone et al. 2009). However, these piRNAs in testes do not require *piwi* for their production. In addition, the *AT- chX* accumulation does not require *armi*. These results therefore suggest that the *Su(Ste)* and *AT- chX* piRNA pathways rely on different sets of genes.

The production of both *Su(Ste)* and *AT- chX* piRNAs is independent on both AGO3 and Aub. How does such interdependence between the two proteins occur? Mutations in *aub* severely impair the expression of AGO3 protein in testes. Thus, neither AGO3 nor Aub is sufficiently abundant for the loading of piRNAs in *aub* mutant testes. It is conceivable that, without the PIWI proteins, piRNAs are degraded. Supporting this is the observation that increased levels of Argonaute proteins in mammalian cells correlate with increased levels of mature miRNAs (Diederichs and Haber 2007). This effect depends on direct binding of the Argonaute proteins to the miRNA, suggesting that Argonaute proteins are limiting and serve to stabilize miRNAs. In sharp contrast, in *ago3* mutant testes, the levels of Aub are not altered, but piRNA accumulation is markedly reduced. As *Su(Ste)* and *AT- chX* piRNAs are mainly bound to Aub, it is difficult to explain how Aub could rely on AGO3 to stably produce these piRNAs in the testes. Because the accumulation of piRNA precursor- or intermediate-like molecules is not observed in *ago3* and *aub* mutant testes (Fig. 5A), depletion of AGO3 and Aub may not substantially affect the processing of piRNA precursors. A plausible, although unsatisfying, model is that both proteins are required to form protein complexes that promote effective loading of piRNAs onto both AGO3 and Aub. Spn-E and Krimp are essential for the production of *Su(Ste)* and *AT- chX* piRNAs, and both proteins contain Tudor domains that recognize and bind to symmetric dimethyl-arginine residues (sDMAs) on proteins (Côté and Richard 2005; Bedford and Clarke 2009). Recently it has been shown that PIWI proteins in fly ovaries contain sDMAs (Kirino et al. 2009; Nishida et al. 2009; Siomi et al. 2010) and that Tudor-domain-containing proteins such as Tudor interact with PIWI proteins specifically through their sDNA modifications (Nishida et al. 2009). In this context, it is tempting to speculate that AGO3 and Aub in fly testes may also contain sDMAs, which are required for the formation of functional complexes with Tudor-domain-containing proteins including spn-E and Krimp in the piRNA pathway. This model also implies that complexes containing AGO3 with sDMAs and complexes containing Aub with sDMAs are independently required for the piRNA pathway.

Our findings suggest that multiple pathways for piRNA biogenesis may exist in fly testes. More comprehensive bioinformatics, biochemical and genetic characterization of AGO3, Aub, and Piwi and their associated piRNAs in the *Drosophila* testis, should shed light on the molecular pathways of piRNA production.

**MATERIALS AND METHODS**

**Drosophila strains**

The yellow white (yw) strain was used as the wild type. The strains bearing *aub* mutations, *aub<sup>hnc</sup> cn bw/CyO and *aub<sup>5c42</sup> cn bw/CyO, and *ago3* mutations, *ago3<sup>2</sup> /TM6B Tb and *ago3<sup>57</sup> /TM6B Tb (Li et al. 2009), were provided by P. Zamore (University of Massachusetts, USA). The P[6Ste;Ste-lacZ]; Ycry Bs strains were provided by A. Aravin (Cold Spring Harbor Laboratory, NY, USA). Other mutant alleles and allelic combinations used in this work were *armi<sup>1</sup> /TM3 Sb Ser and *armi<sup>2-1</sup> /TM3 Sb P[hs-hid], PBac[WH]krimp<sup>00683</sup>/CyO, piew<sup>1</sup>/CyO (a kind gift from H. Lin, Yale Stem Cell Center), *mae<sup>Akr1</sup>/TM3 Sb (a kind gift from T. Kai, Temasek Lifesciences Laboratory, Singapore) and Df(3L)BSC554/ *TM6C Sb, spn-E<sup>10037</sup> e/TM3, Sb, P[w<sup>1</sup> hs-hid], squ<sup>3p2</sup> cn bw/CyO and Df(2L)Edi109/SM6a, tud<sup>1</sup> bw sp/CyO I(2)DTSS13<sup>1</sup>, vasa<sup>PH105</sup>/CyO (a kind gift from S. Kobayashi, NIBB, Okazaki), and Df(2L)A267 b cn bw/CyO Adh, zuc<sup>988</sup> cn bw/CyO and Df(2L)Exel6031.

**Western blotting**

The anti-Aub monoclonal antibody (Nishida et al. 2007) was used at 1:1000 dilution, and the anti-AGO3 monoclonal antibody
miRNA Cloning Kit (BioDynamics Laboratory). The sequences of Aub and Piwi in the testes were included in the DynaExpress mixtures were rocked for 1 h at 4°C. 1500 testes were used per immunoprecipitation. The reaction was performed at 4°C to enhance detection of small RNA molecules (Pall and Hamilton 2008). After cross-linking, hybridization was performed at 42°C in 0.2 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA with end-labeled antisense oligodeoxynucleotide, and washed at 42°C in 2× saline sodium citrate and 0.1% SDS. The probes used for detecting Si(Sce)-4 piRNAs, AT-chX-1 piRNAs, and U6 snRNAs are listed in Supplemental Table 5.

**Northern blotting**

Total RNA from fly testes was isolated using ISOGEN (Nippon Gene). Northern blotting was performed as reported previously (Saito et al. 2006) with minor modifications. Five micrograms of total RNA from each sample was separated on 12% acrylamide-denaturing gels and transferred onto Hybond-N membrane (Amersham Pharmacia) in distilled water and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride cross-linking reactions for 2 h at 60°C to enhance detection of small RNA molecules (Pall and Hamilton 2008). After cross-linking, hybridization was performed at 42°C in 0.2 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA with end-labeled antisense oligodeoxynucleotide, and washed at 42°C in 2× saline sodium citrate and 0.1% SDS. The probes used for detecting Su(Ste)-4 piRNAs, AT-chX-1 piRNAs, and U6 snRNAs are listed in Supplemental Table 5.

**RT-PCR**

Quantitative RT-PCR (qRT-PCR) was performed as reported previously (Saito et al. 2009). One microgram of total RNA from each sample was used to reverse-transcribe target sequences using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. The resulting cDNAs were amplified with a LightCycler 480 SYBR Green I Master (Roche). The primers used are shown in Supplemental Table 5.

**SUPPLEMENTAL MATERIAL**

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

**ACKNOWLEDGMENTS**

We thank P. Zamore, A. Aravin, H. Lin, S. Kobayashi, and T. Kai for fly stocks and antibodies. We also thank S. Inagaki and...
H. Ishizu for technical advice, and Y. Iyoda for collecting fly testes. We thank members of the Siomi laboratory for discussion and comments on the manuscript. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) grants to H.S. M.C.S. is supported by CREST (Core Research for Evolutional Science and Technology) from JST Japan (Science and Technology Agency) and the New Energy and Industrial Technology Development Organization (NEDO).

Received May 17, 2010; accepted September 16, 2010.

REFERENCES


Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, Hannon GJ. 2009. Specialized piRNA pathways...
piRNAs bound to AGO3 in fly testes

Megosh HB, Cox DN, Campbell C, Lin H. 2006. The role of PIWI and the
Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Lührmann R, 
Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, 
Yoshizawa A, Komori T, Asai K. 2008. The Functional RNA Database 3.0. databases to support mining and annotation of func-
Nishida KM, Saito K, Mori T, Kawamura Y, Nagami-Okada T, 
Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, 
Saito K, Nishida KM, Mori T, Kawamura Y, Miyoshi K, Nagami T, 
Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, 
Siomi MC, Kura-mochi-Miyagawa S. 2009. RNA silencing in germi-
Snee MJ, Macdonald PM. 2004. Live imaging of nuage and polar granules: evidence against a precursor-product relationship and a novel role for Oskar in stabilization of polar granule compo-
Tomari Y, Du T, Haley B, Schwarz DS, Bennett R, Cook HA, 
Zhang D, Xiong H, Shan J, Xia X, Trudeau VL. 2008. Functional insight into Maelstrom in the germline piRNA pathway: a unique domain homologous to the DnaQ-H 3’–5’ exonuclease, its lineage-