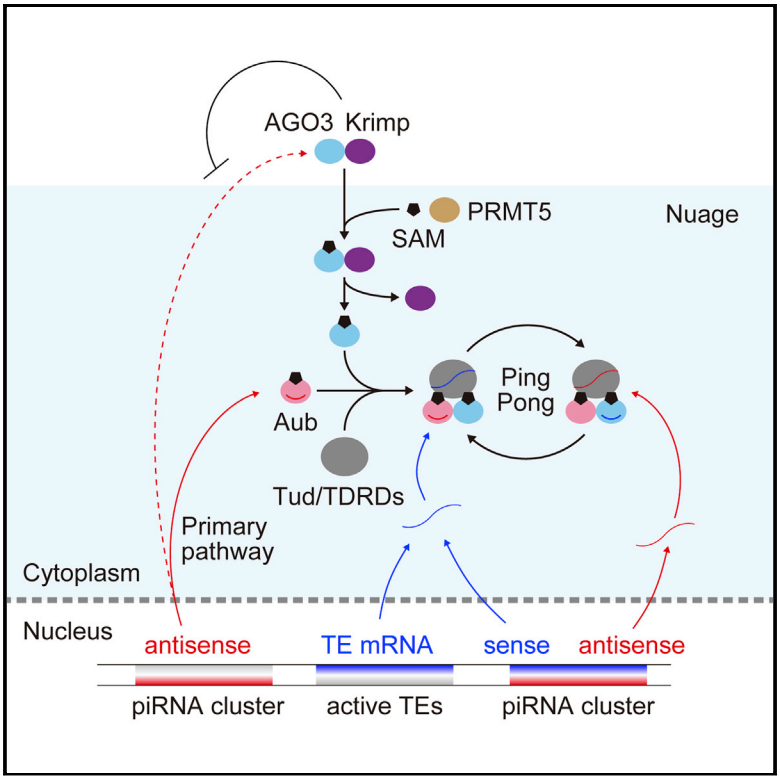
Article

Molecular Cell

Krimper Enforces an Antisense Bias on piRNA Pools by Binding AGO3 in the *Drosophila* Germline

Graphical Abstract



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In Brief

Sato et al. show that Krimper, a Tudor-domain protein, interacts with unmethylated AGO3 to promote AGO3 methylation, localization to nuage, and Aub-AGO3 piRNA amplification. Krimper also blocks primary piRNA loading onto AGO3, enforcing an antisense bias on the piRNA pool.

Highlights

- Krimp binds unmethylated AGO3 to ensure sDMA modification and heterotypic ping-pong
- Loss of Aub allows primary piRNA loading onto AGO3
- Loss of Krimp in OSC cells allows AGO3 primary piRNA loading
- Krimp blocks access of AGO3 to primary piRNAs enforcing an antisense bias on piRNAs

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Krimper Enforces an Antisense Bias on piRNA Pools by Binding AGO3 in the *Drosophila* Germline

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SUMMARY

Piwi-interacting RNAs (piRNAs) suppress transposon activity in animal germ cells. In the *Drosophila* ovary, primary Aubergine (Aub)-bound antisense piRNAs initiate the ping-pong cycle to produce secondary AGO3-bound sense piRNAs. This increases the number of secondary Aub-bound antisense piRNAs that can act to destroy transposon mRNAs. Here we show that Krimper (Krimp), a Tudor-domain protein, directly interacts with piRNA-free AGO3 to promote symmetrical dimethylarginine (sDMA) modification, ensuring sense piRNA-loading onto sDMA-modified AGO3. In *aub* mutant ovaries, AGO3 associates with ping-pong signature piRNAs, suggesting AGO3′s compatibility with primary piRNA loading. Krimp sequesters ectopically expressed AGO3 within Krimp bodies in cultured ovarian somatic cells (OSCs), in which only the primary piRNA pathway operates. Upon *krimp*-RNAi in OSCs, AGO3 loads with piRNAs, further showing the capacity of AGO3 for primary piRNA loading. We propose that Krimp enforces an antisense bias on piRNA pools by binding AGO3 and blocking its access to primary piRNAs.

INTRODUCTION

In most animals, the PIWI clade of Argonaute family proteins is required for fertility and silencing of germline transposable elements (TEs) (Ghildiyal and Zamore, 2009; Ishizu et al., 2012; Juliano et al., 2011; Kim et al., 2009; Siomi et al., 2011). PIWI proteins are loaded with Piwi-interacting RNAs (piRNAs) of 25 to 35 nt in length to form piRNA-induced silencing complexes (piRISCs). In these, piRNAs guide the repressive activities of PIWI proteins toward TEs by means of base pairing to silence TEs and thereby maintain genome integrity in the gonad (Siomi et al., 2011).

Two main pathways exist to generate piRNAs in the *Drosophila* ovary: the primary processing pathway and the ping-pong amplification cycle (Brennecke et al., 2007; Gunawardane et al., 2007; Ishizu et al., 2012; Malone et al., 2009; Siomi et al., 2011). In the primary processing pathway, which operates in both germline cells and surrounding somatic cells, the majority of piRNAs are produced from long single-stranded RNAs, which are transcribed from TE-rich genomic regions called piRNA clusters. The majority of primary piRNAs are antisense with respect to TE coding strands. These piRNAs guide TE silencing in *trans* (Malone et al., 2009; Saito et al., 2009; Yamanaka et al., 2014). Studies in cultured ovarian somatic cells (OSCs) revealed that the somatic primary pathway is a linear pathway in which piRNA precursors are cleaved into intermediates by one or more nucleases, including Zucchini (Zuc). These intermediates are loaded exclusively onto Piwi in the cytoplasmic Yb bodies, and subsequently, their 3′ ends are defined, probably by trimming, and methylated, yielding mature piRISCs, which are then transported into the nucleus to exert TE silencing (Haase et al., 2010; Ipsaro et al., 2012; Ishizu et al., 2012; Olivieri et al., 2010; Saito et al., 2010; Qi et al., 2011; Nishimasu et al., 2012). The primary processing pathway in germline cells produces piRNAs that are loaded onto both Piwi and Aub but not AGO3 (Brennecke et al., 2007; Li et al., 2009; Malone et al., 2009; Siomi et al., 2011). Although primary piRNAs in both germline and surrounding somatic cells show a strong antisense bias (with respect to TE coding strands) and exhibit a preference for a 5′ uridine (1U) residue (Saito et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007; Malone et al., 2009; Siomi et al., 2011), the primary piRNA pathways in the two cell types do not seem to be identical, because the requirement of piRNA factors differs between the two. How primary piRNAs in germline cells are produced and how these sequence and strand biases are enforced remains to be elucidated.

The ping-pong cycle operates specifically in germline cells and engages AGO3 and Aub, both of which accumulate in the nuage, perinuclear structures found at the cytoplasmic face of the nuclear envelope in animal germline cells (Brennecke et al., 2007; Gunawardane et al., 2007; Lim and Kai, 2007; Nishida et al., 2007). Multiple components of the piRNA pathway are enriched in the nuage, causing this structure to be implicated in piRNA production and TE silencing (Lim and Kai, 2007). Piwi does not participate in this pathway, probably because of its

sequestration into the nucleus upon piRNA loading (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2010). The pathway depends on the endoribonuclease or Slicer activities of AGO3 and Aub, which cleave antisense and sense transcripts of both piRNA clusters and active TEs to produce two classes of piRNAs: piRNAs associated with Aub, which are derived mainly from the antisense strands of TEs and show a strong preference for U at their 5′ ends, and piRNAs associated with AGO3, which arise mainly from the sense strands and show a preference for adenine (A) at nucleotide 10 (10A) but no 5′ nucleotide preference (Brennecke et al., 2007; Gunawardane et al., 2007; Siomi et al., 2011). The two classes of piRNAs overlap by precisely 10 nt at their 5′ ends, which is evidence of piRNA amplification by reciprocal cycles of Slicer-mediated cleavage (Brennecke et al., 2007; Gunawardane et al., 2007; Siomi et al., 2011). These features of piRNAs (1U/10A partners with a 10 nt, 5′ overlap) are often referred to as the ping-pong signature (Malone et al., 2009). Both AGO3 and Aub act catalytically, thereby mediating not only repeated rounds of piRNA production but also post-transcriptional silencing of TEs by consuming TE transcripts (Brennecke et al., 2007; Gunawardane et al., 2007). The ping-pong pathway is believed to be primed by both maternally deposited Aub-bound piRNAs and zygotic primary piRNAs that are loaded onto Aub (Brennecke et al., 2007, 2008; Gunawardane et al., 2007; Siomi et al., 2011).

Amplification of the silencing signal in the ping-pong cycle requires a system that acts to enforce an antisense bias on piRNA pools to efficiently silence TEs by cleaving TE sense transcripts or mRNAs. Indeed, sense piRNAs are less abundant and disproportionately bound to AGO3, which enforces the characteristic antisense bias of Aub-bound piRNAs. This increases the number of piRNAs that can act to destroy TE mRNAs (Gunawardane et al., 2007; Huang et al., 2014; Li et al., 2009). There are at least two possible scenarios for how piRNA pools in AGO3 complexes display a sense bias. First, AGO3 is actively prevented from being loaded with primary piRNAs. Second, the primary pathway is inherently incompatible with AGO3 loading. A recent study suggests that the latter may be the case (Olivieri et al., 2012); however, several findings argue against this scenario. For example, ectopic expression of AGO3 in OSCs increases the expression of the *gypsy* transposon, which is normally silenced by the primary piRNA processing pathway in OSCs (Li et al., 2009). This has been interpreted to mean that the primary pathway is compatible with AGO3 loading, meaning that AGO3 can compete with Piwi for piRNAs, but unlike Piwi, it cannot act directly to silence TEs. Furthermore, a large number of piRNAs with exactly the same sequences, derived from antisense strands of two loci, *Su(Ste)* and *AT-chX*, are associated with both AGO3 and Aub in the fly testis (Nagao et al., 2010). This also implies that AGO3 can be loaded with the same primary and secondary piRNAs that are loaded onto Aub. These results suggest that AGO3 is actively prevented from being loaded with primary piRNAs in the fly ovary. Thus, it is tempting to speculate that a factor(s) exists that interacts with AGO3 to block loading of primary piRNAs onto AGO3.

Besides AGO3 and Aub, the ping-pong cycle in the fly ovary requires the functions of many other factors, including the RNA helicases Spindle-E (Spn-E) and Vasa and the Tudor domain proteins Tudor (Tud), Tejas (Tej), Qin/Kumo, and Krimper (Krimp), as well as Maelstrom (Mael), a component of the microtubule-organizing center (Lim and Kai, 2007; Malone et al., 2009; Nishida et al., 2009; Patil and Kai, 2010; Anand and Kai, 2012; Sato et al., 2011; Zhang et al., 2011, 2014). All of these factors accumulate in the nuage (Findley et al., 2003; Lim and Kai, 2007; Malone et al., 2009; Nishida et al., 2009; Patil and Kai, 2010; Anand and Kai, 2012; Zhang et al., 2011; Sato et al., 2011). Among them, proteins that contain multiple Tudor domains are known to possess the potential to interact simultaneously with several proteins through symmetrical dimethylarginines (sDMAs) and to function as a scaffolding platform for the assembly of multi-protein complexes (Chen et al., 2011; Siomi et al., 2011). This might be particularly important for the ping-pong mechanism, which requires close association between two PIWI proteins (Nishida et al., 2009). Here, we show that Krimp, a Tudor-domain protein, specifically interacts with AGO3. AGO3 in Krimp-associated complexes is mainly in a non-sDMA form and free from piRNAs. In *krimp* mutant ovaries, AGO3 is not sDMA modified. Loss of Krimp has pronounced impacts on piRNA levels and ping-pong signatures; a striking depletion of AGO3-associated piRNAs manifests, Aub-Aub homotypic ping-pong prevails, and there is a relative increase in Aub-bound sense piRNAs. In *aub* mutant ovaries, piRNAs with ping-pong signatures are still loaded onto AGO3. This suggests that primary piRNAs can be loaded onto AGO3, although their AGO3 loading is normally blocked in the presence of Aub. In OSCs, where only the primary pathway operates and neither AGO3 nor Aub are expressed (Saito et al., 2009), Krimp accumulates at cytoplasmic granules termed Krimp bodies, and transfected AGO3 is sequestered into these bodies (Olivieri et al., 2012). However, interestingly, upon *krimp* RNAi in OSCs, AGO3 becomes dispersed in the cytoplasm and loaded with primary piRNAs. Together, these findings suggest a model in which Krimp prevents AGO3 from becoming loaded with primary piRNAs, thereby enforcing an antisense bias on piRNA pools in the ping-pong cycle.

RESULTS

Krimp Binds to Unmethylated AGO3

In *krimp* mutant ovaries, AGO3 loses its ability to localize to the nuage (Nagao et al., 2011), while Aub remains accumulated in the nuage as in the control (Lim and Kai 2007; Nagao et al., 2011). These findings prompted us to examine if Krimp has a specific physical interaction with AGO3 in the ovary. We immunopurified Krimp-associated complexes from ovary lysates and determined if AGO3 was present in the complexes. As shown in Figure 1A, Krimp co-purified with AGO3, but not with other PIWI proteins, suggesting a specific interaction of Krimp with AGO3.

Krimp is a Tudor-domain protein. PIWI proteins contain sDMAs, which are incorporated by the dPRMT5/Capsuleen/Dart5 enzyme and which can be recognized by Tudor-domain proteins (Kirino et al., 2009; Nishida et al., 2009). Thus, we wanted to determine if the association of Krimp with AGO3 is sDMA modification dependent. To this end, we produced a monoclonal antibody that specifically recognizes sDMA-containing AGO3 (sDMA-AGO3) (Figures S1A and S1B). Using the sDMA-specific antibody, we compared the amounts of sDMA-AGO3 in

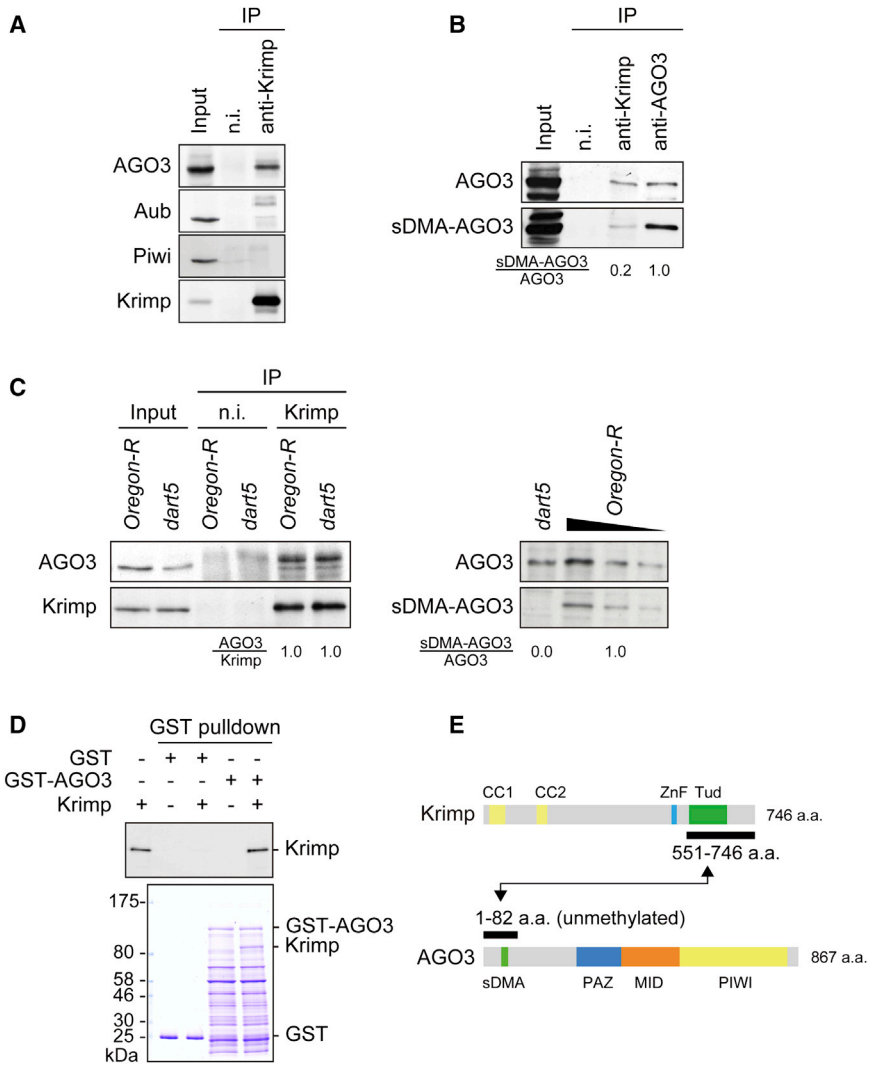

Figure 1. Krimp Specifically Associates with AGO3 in a sDMA-Independent Manner

(A) Krimp-IP complexes from *Oregon-R* ovary lysate were immunoreacted using specific antibodies against Piwi subfamily proteins. "n.i." indicates non-immune IgG used as a negative control.

(B) Krimp- and AGO3-IP complexes from *Oregon-R* ovary lysate were immunoreacted using antibodies against AGO3 and sDMA-modified AGO3 (sDMA-AGO3). The ratio of sDMA-AGO3/AGO3 signal intensity was calculated.

(C) Krimp-IP complexes from ovary lysates of *Oregon-R* and *dart5* mutant were immunoreacted using antibodies against Krimp and AGO3 (left panel). Ovary lysates of *Oregon-R* and *dart5* mutant were immunoreacted using antibodies against AGO3 and sDMA-AGO3 (right panel). The ratio of sDMA-AGO3/AGO3 signal intensity was calculated.

(D) GST pull-down assay using recombinant GST, GST-AGO3, and Krimp.

(E) Summary of the functional domains required for the Krimp-AGO3 interaction (for more detail, see Figures S1E–S1I). See also Figure S1 and S2.

immunoprecipitates obtained with an anti-Krimp antibody and an anti-AGO3 antibody that recognizes both sDMA and unmethylated AGO3. The results showed that AGO3 in Krimp complexes was mostly devoid of sDMA modification (Figure 1B). We further found that Krimp interacted with AGO3 in *dart5* mutant ovaries (Figure 1C), where AGO3 was not sDMA-modified (Figure 1C). To confirm these results, we performed glutathione S-transferase (GST) pull-down assays using GST-AGO3 and Krimp (non-tagged) proteins expressed in and purified from *E. coli*. GST-AGO3 purified from *E. coli* was not sDMA-modified (Figure S1A). Krimp expressed in *E. coli* specifically associated with GST-AGO3, but not with GST itself (Figure 1D). Together, these results show that Krimp is capable of interacting with unmethylated AGO3 and also show that this interaction is direct, not requiring other factors.

To corroborate these findings, we performed GST pull-down experiments with synthetic oligo-peptides with/without sDMA modification (AGO3-2 and AGO3-2-sDMA) (Nishida et al., 2009). Only an unmodified (AGO3-2) but not a sDMA-modified (AGO3-2-sDMA) oligo-peptide bound to GST-Krimp (Figure S1C), showing that the Krimp interaction is sensitive to the methylation status of AGO3. We also performed peptide pull-down experiments using ovary lysates and found that Tud interacts only with AGO3-2-sDMA (Figure S1D), confirming our previous findings that Tud interacts specifically with sDMA-modified AGO3 (Nishida et al., 2009). Krimp in ovary lysates interacts with both AGO3-2 and AGO3-2-sDMA (Figure S1D). Together these results suggest that Krimp initially forms a complex with unmethylated AGO3, in which sDMA modification of AGO3 occurs. sDMA modified AGO3 then dissociates from Krimp to interact with Tud.

We undertook domain mapping of both AGO3 and Krimp using GST fusion proteins and a series of in-vitro-translated deletion products to identify the regions responsible for their interaction. Krimp is predicted to contain N-terminal coiled-coil domains, a zinc finger, and a canonical Tudor domain (Figures 1E and S1E). We found that the C-terminal region containing the canonical Tudor domain of Krimp is required for the interaction with non-sDMA-modified AGO3 (Figure S1F). A series of AGO3 deletion products revealed that an N-terminal portion containing 82 aa is required for the interaction with Krimp (Figures S1G–S1I). These results demonstrate that AGO3 directly interacts with the C-terminal Tudor domain-containing region of Krimp through its N-terminal region, which contains sDMA-modifiable arginine residues (Figure 1E) (Nishida et al., 2009).

Krimp-Associated AGO3 Is Devoid of piRNAs

Recent findings indicate that Krimp has no significant impact on primary piRNA biogenesis but is required for the nuage

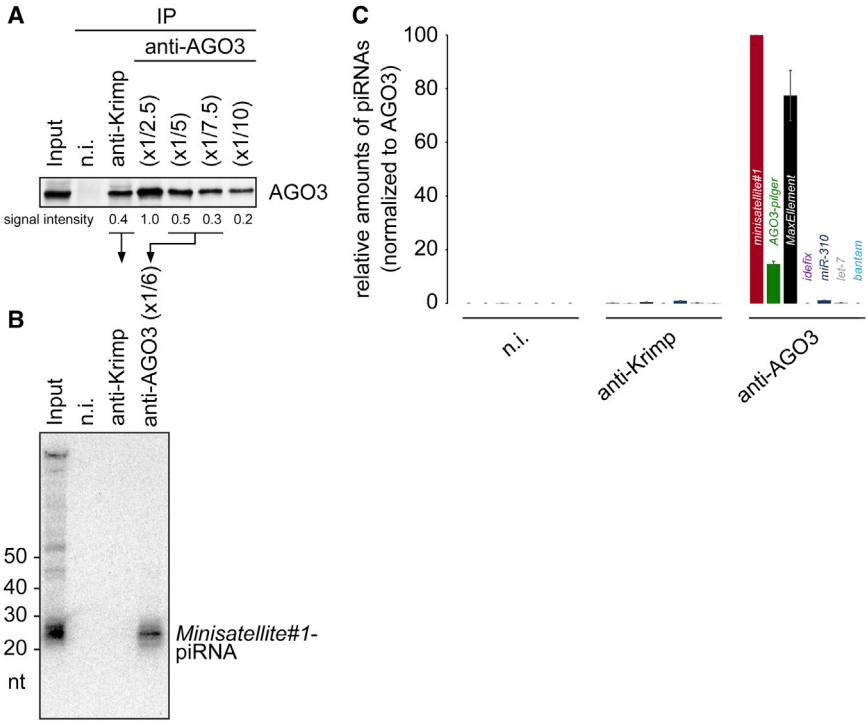

Figure 2. AGO3 Coimmunoprecipitated with Krimp Is Not Loaded with piRNAs

(A) Krimp- and AGO3-IP complexes from *Oregon-R* ovary lysate were immunoreacted using an antibody against AGO3. The amount of Krimp-associated AGO3 was approximately equal to that of AGO3 immunoprecipitated with anti-AGO3 after a 1:6 dilution. "n.i." indicates non-immune IgG used as a negative control.

(B and C) Quantification of piRNAs in the anti-Krimp and anti-AGO3 IP complexes by northern blot analysis (B) and real-time PCR (C). *Minisatellite#1*, *AGO3-pilger*, and *MaxEllement* are AGO3-bound piRNAs (Gunawardane et al., 2007). *idefix* piRNA is a Piwi-bound somatic piRNA (Saito et al., 2009). *miR-310*, *let-7*, and *bantam* are miRNAs. N = 3; error bars indicate SEM.

localization of AGO3, suggesting a specific role in the ping-pong cycle (Malone et al., 2009; Zhang et al., 2011). Our findings that Krimp preferentially interacts with unmethylated AGO3 suggest that Krimp may well regulate sDMA modification of AGO3 and, subsequently, the ping-pong cycle.

We first addressed whether AGO3 in Krimp complexes is loaded with piRNAs. We compared the amounts of piRNAs in anti-Krimp and anti-AGO3 immunoprecipitates. To equalize AGO3 quantities, the anti-AGO3 immunoprecipitates were sequentially diluted before western blot analysis. The results showed that a 1:6 dilution equalized the amount of AGO3 in the anti-AGO3 and anti-Krimp immunoprecipitates (Figure 2A). We then performed northern blot analysis to examine the amount of *minisatellite#1* piRNA in the complexes. *Minisatellite#1* piRNA, a sense piRNA known to be specifically loaded onto AGO3 (Nishida et al., 2009), was detected in the AGO3 complexes (which had been diluted 1:6 before analysis) but not in the Krimp complexes (Figure 2B). Additionally, piRNA detection by qRT-PCR revealed that other piRNAs that are loaded onto AGO3 are not present in anti-Krimp immunoprecipitates (Figure 2C). These results show that most AGO3, in a form associated with Krimp, is not loaded with piRNAs. Thus, Krimp interacts with AGO3 before AGO3 enters the ping-pong cycle, which likely occurs in the nuage.

Krimp Is Required for sDMA Modification of AGO3 and Secondary piRNA Loading

AGO3 in Krimp complexes is mostly not sDMA modified and free from piRNAs. We thus asked if AGO3 is sDMA modified in *krimp* mutants. We found that the majority of AGO3 is not sDMA modified (Figures 3A and S2A), but that both Aub and Piwi remain sDMA modified in *krimp* ovaries (Figures S2B and S2C), which suggests that Dart5 is functional in *krimp* mutants. As a consequence, sDMA-dependent interaction between AGO3 and Tud is impaired in *krimp* mutant ovaries, where Aub still interacts with Tud (Figures S2D and S2E). These results suggest that Krimp is required for sDMA modification of AGO3. We found no significant association of Krimper with Dart5 and Valois, which are components of arginine-methylation complexes (methylosomes), (Anne and Mechler, 2005) (Figures S2F and S2G), suggesting that Krimp-AGO3 complexes only transiently interact with methylosomes. Because AGO3 lost its ability to localize to the nuage in *krimp* mutant ovaries (Malone et al., 2009; Nagao et al., 2011; Zhang et al., 2011), Krimp is likely to promote sDMA modification of AGO3 by localizing AGO3 in nuage where methylosome components are also accumulated (Anne and Mechler, 2005).

The requirement of Krimp in the sDMA modification of AGO3 prompted us to ask how piRNA loading onto individual PIWI proteins is affected in *krimp* mutant ovaries. We immunopurified AGO3, Aub, and Piwi from control (*krimp* heterozygous, *krimp*/*CyO*) and *krimp* mutant (trans-heterozygous, *krimp*/*Df*) ovaries (Figure S3) and examined the piRNAs that are associated with equivalent amounts of each protein (Figure 3B). Although the amounts of piRNAs associated with Piwi were only slightly reduced in *krimp* mutant ovaries, Aub- and AGO3-bound piRNAs were markedly reduced. In particular, almost no AGO3-associated piRNAs were detected in *krimp* mutant ovaries. Thus, our data show that Krimp has a huge impact on the abundance of germline piRNAs bound to Aub/AGO3, especially their binding to AGO3. Our results also explain the previous findings showing that loss of *krimp* causes a substantial reduction in overall piRNA populations and also results in a slight shift in size toward longer piRNAs, characteristic of Piwi-bound piRNAs (Malone et al., 2009).

Loss of Krimp Permits Aub-Aub Homotypic Ping-Pong

We then performed deep sequencing of AGO3/Aub/Piwi-bound piRNAs in control and *krimp* mutant ovaries and compared them

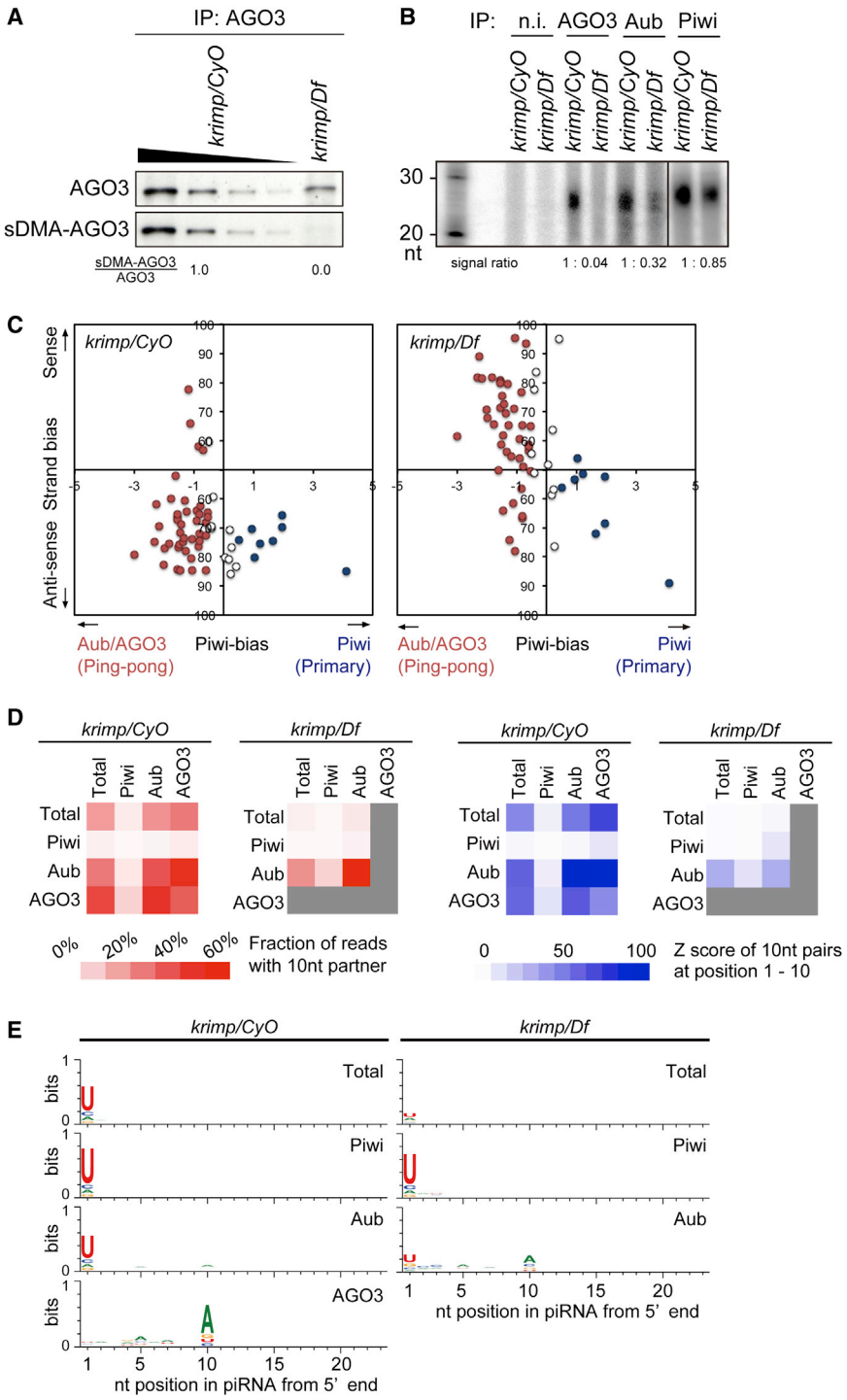

Figure 3. *krimp* Is Required for sDMA Modification and the Aub-AGO3 Heterotypic Ping-Pong Amplification Loop

(A) The level of AGO3 sDMA modification in the *krimp* mutant. AGO3-IP complexes from ovary lysates of control (*krimp/CyO*) and *krimp* mutant (*krimp/Df*) were immunoreacted using antibodies against AGO3 and sDMA-AGO3. The ratio of sDMA-AGO3/AGO3 signal intensity was calculated. (B) IPs were performed from ovary lysates of *krimp/CyO* and *krimp/Df* using antibodies against each Piwi subfamily protein. Equal amounts of RNA molecules were isolated from complexes shown in (Figure S3) and then ^{32}P labeled.

(C) Strand-bias was compared with Piwi-bias for each piRNA associated with Aub in *krimp/CyO* and *krimp/Df*. The plots are colored according to their Piwi-bias; piRNAs bound to Piwi indicating primary piRNAs (higher Piwi-bias; blue dots), piRNAs bound to Aub/Ago3 indicating ping-pong piRNAs (lower Piwi-bias; red dots), and piRNAs with intermediate Piwi-bias (white dots).

(D) Heatmaps of the ping-pong signature (left panel) and ping-pong z score (right panel) indicating the degree to which complementary 5′ 10-mers are found in pairwise library comparisons, with a key to the intensity of the signal shown below. Ping-pong signals were frequently observed between Aub and AGO3 in *krimp/CyO*, whereas Aub-Aub homotypic ping-pong was observed to prevail in *krimp/Df*.

(E) Sequence logos of Piwi-, Aub-, and AGO3-bound total piRNAs in *krimp/CyO* and *krimp/Df*. See also Figures S3 and S4.

with piRNAs in total small RNAs (Figures 3C–3E and S4; Table S1). Total small RNA libraries were normalized to endo-siRNA reads, and AGO3/Aub/Piwi-IP libraries were normalized to identical numbers of reads. We first confirmed that loss of *krimp* results in both a substantial reduction in overall piRNA populations and a shift in size toward Piwi-bound piRNAs (Malone et al., 2009) (Figure S4A). For the piRNAs mapped to TEs, loss of *krimp* affected the populations bound to Aub/AGO3 rather than those bound to Piwi (Figures S4B–S4D).

We then quantified the ping-pong signature (10 nt overlap at their 5′ ends) of *krimp* heterozygous and mutant piRNA reads mapped to TEs by calculating the likelihood of piRNAs to have a complementary ping-pong partner with the indicated overlapping base pairs (Figure S4E). A strong ping-pong signature among piRNAs, which correlates with germline silencing, was observed in piRNAs from *krimp* heterozygous flies. Interestingly, piRNAs isolated from *krimp* mutant ovaries also showed a weak but evident ping-pong signature. From the data showing that AGO3 piRNAs are almost completely ablated in *krimp* mutant ovaries (Figure 3B), we hypothesized that this may be caused by the ability of Aub piRNAs to engage in piRNA amplification by homotypic Aub-Aub cycling. The disruption of Aub/AGO3 ping-pong amplification was also observed by focusing on strand bias of piRNAs bound to Aub. The antisense strand bias of Aub, which was clearly seen in *krimp* heterozygous files, could no longer be detected in mutant flies (Figure S4F), and Aub

started to load sense piRNAs rather than antisense piRNAs. This was particularly observed for piRNAs originating from ping-pong amplification rather than primary biogenesis, based on the result that piRNAs from transposons with a low Piwi-bias were significantly biased toward the sense strand only within *krimp* mutants compared with those in heterozygous flies ($p < 0.001$) (Figure 3C). In contrast to Aub-bound, ping-pong participating piRNAs having a bias for the sense strand, the strand bias of Piwi-bound primary piRNAs remained unchanged (Figure S4F). To test our hypothesis that the Aub-mediated ping-pong amplification operates in *krimp* mutant ovaries, we analyzed the complementary relationships between the first ten0 nucleotides of total small RNAs and AGO3/Aub/Piwi-bound piRNAs (Figure 3D). In *krimp* heterozygous libraries, we observed that >52% (z score > 106.5) of small RNAs in the Aub library have complementary partners in the AGO3 library. In contrast, Aub-AGO3 heterotypic ping-pong was not present in krimp mutant ovaries, but significantly, the self-complementarity among Aub-bound piRNAs was observed in *krimp* mutant ovaries (>57%, z score = 30.9; Figure 3D). The identification of a nucleotide bias for piRNAs in *krimp* mutants also supports the possibility of Aub-Aub self-ping-pong amplification. Primary piRNAs, which are known to bind to both Aub and Piwi (Brennecke et al., 2008), show a strong antisense bias and typically begin with U (1U). Sense piRNAs typically bear an A at position 10 (10A) and bind AGO3 (Figure 3E). Although this 1U-bias is seen in the *krimp* mutant Aub library, the degree was much lower than that in heterozygous flies. Moreover, a 10A-bias was also observed, indicating that an Aub-Aub homotypic ping-pong amplification loop was active in *krimp* mutants (Figure 3E). This shows that Aub-Aub homotypic ping-pong dominates in *krimp* mutants, resulting in an increased piRNA sense fraction (Figure 3C and Figure S4F), thereby leading to derepression of TEs (Lim and Kai, 2007). Together, our data indicate that Krimp is required for the biogenesis of AGO3-bound piRNAs and piRNAs generated by the Aub-AGO3 ping-pong amplification loop, while its effects on primary biogenesis and Aub-Aub homotypic ping-pong are limited.

Primary piRNAs Are Loaded onto AGO3 in the Absence of Aub

Loss of *aub* impairs the production of germline piRNAs, and the remaining piRNAs display no ping-pong cycle (Malone et al., 2009). This suggests that only primary piRNAs are produced, which are then loaded onto Piwi and/or AGO3 in *aub* mutant ovaries. To test if remaining primary piRNAs in *aub* mutant ovaries are loaded onto AGO3, we immunopurified AGO3 complexes from *aub* mutant ovaries. This revealed that small RNAs were still loaded onto AGO3 in *aub* mutant ovaries (Figure 4A), although the amounts of associated small RNAs were reduced. Sequencing of these AGO3-associated small RNAs further revealed that characteristic features of AGO3-associated piRNAs are largely lost; they showed a decrease of 10A with a concomitant increase of 1U (Figure 4B). Additionally, piRNAs derived from both germline-specific dual-stranded piRNA cluster, *42AB*, and uni-stranded primary piRNA cluster, *Cluster 2*, were loaded onto AGO3 in *aub* mutant ovaries (Figure 4C). The degree of ping-pong amplification of AGO3-bound piRNAs mapped to the *42AB* cluster was reduced, compared with control (Figure 4D). These findings show that primary piRNAs derived from piRNA clusters are loaded onto AGO3 in *aub* mutant ovaries and are not significantly further amplified by AGO3-AGO3 homotypic ping-pong. Furthermore, these findings suggest that primary piRNAs can be loaded onto AGO3, but that this loading is actively blocked in the presence of Aub. In *aub* mutant ovaries, the nuage localization of both AGO3 and Krimp is lost; instead, Krimp accumulates in large cytoplasmic foci termed Krimp bodies, where AGO3 is also accumulated (Lim and Kai, 2007; Nagao et al., 2011; Olivieri et al., 2012). Thus, the reduced loading of piRNAs onto AGO3 is probably caused by the sequestration of AGO3 to Krimp bodies.

Recapitulation of AGO3-Primary piRNA Loading in OSCs

Krimp accumulates at Krimp body-like cytoplasmic foci in OSCs (Figures 5A and 5B) (Olivieri et al., 2012) where neither Aub nor AGO3 are expressed and, therefore, only the primary piRNA biogenesis pathway operates (Saito et al., 2009). These foci in OSCs are probably equivalent to the Krimp bodies in *aub* mutant ovaries. Interestingly, although ectopically expressed Aub is distributed almost evenly in the cytoplasm and does not change Krimp body formation in OSCs (Olivieri et al., 2012), ectopically expressed AGO3 colocalizes with Krimp bodies in OSCs (Figures 5A and 5B) (Olivieri et al., 2012), suggesting a physical interaction of Krimp with AGO3. Ectopically expressed Aub in the heterologous system was efficiently loaded with primary piRNAs, while ectopically expressed AGO3 was sequestered into Krimp bodies and did not appear to associate with piRNAs (Figures 5A–5C) (Olivieri et al., 2012). We surmised that AGO3 might also be loaded with primary piRNAs if Krimp was depleted, thereby releasing AGO3 from Krimp bodies in OSCs. Ectopically expressed Aub was found to co-purify with small RNAs in both normal and *krimp*-depleted OSCs (Figures 5C–5G). We thus wanted to test whether AGO3 exists in a complex with primary piRNAs in Krimp-depleted OSCs, where production of primary piRNAs is not affected (Figure S5A). We first examined the cellular localization of myc-tagged AGO3 after depletion of Krimp by RNAi. As shown in Figure 5A, AGO3 was distributed throughout the cytoplasm in *krimp* RNAi knock-down OSCs. We then immunopurified myc-AGO3 complexes from Krimp-depleted OSCs using an anti-AGO3 antibody and examined whether small RNAs were present in purified complexes. AGO3 in Krimp-depleted OSCs was found to co-purify with small RNAs (Figure 5C).

Deep sequencing of the AGO3-associated small RNAs in OSC cells revealed that they show characteristics of somatic primary piRNAs (Figures 5D–5G and S5B): they possess a strong 1U-bias (with no 10A bias) and correspond to somatic piRNA clusters, including *flamenco* and *traffic jam*, but not to germline clusters such as *42AB*. The profile of AGO3-bound piRNAs in OSCs is almost identical to that of Piwi-bound piRNAs. This clearly indicates that AGO3 was efficiently loaded with primary piRNAs in OSCs when Krimp was depleted. Thus, AGO3 is compatible with primary piRNA loading in OSCs, but Krimp blocks the loading.

It was previously shown that ectopic expression of UAS-AGO3 driven by *actin5c*-Gal4 in the ovarian soma increases the expression of the *gypsy* transposon, which is otherwise

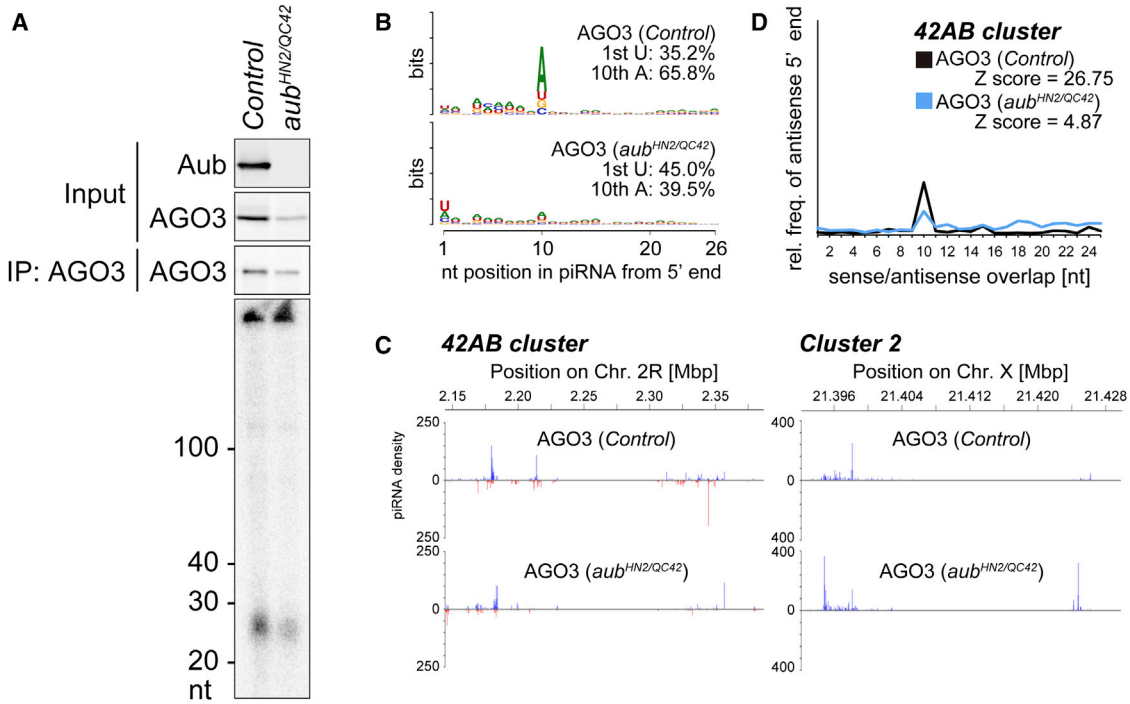

Figure 4. piRNAs Are Loaded onto AGO3 in *aub* Mutant Ovaries

(A) AGO3-interacting piRNAs are barely detectable in *aub* mutant ovaries. IPs were performed from ovary lysates of control and *aub* mutant (*aub* $^{HN2/QC42}$) using antibodies against AGO3.

(B) Sequence logos of AGO3-bound piRNAs in the control and *aub* mutant.

(C) Uniquely mapping piRNAs are plotted over the germline-specific dual-stranded piRNA cluster *42AB* and the uni-stranded piRNA cluster *Cluster 2* (sense and antisense piRNAs are indicated with peaks pointing upward and downward, respectively). Libraries were normalized to allow for a direct comparison of piRNA densities between all libraries (see Experimental Procedures).

(D) Depiction of the ping-pong signature, defined as the value at nucleotide position 10. Graphs indicate the relative frequency with which a complementary piRNA exists with a 5′ end (y axis) at the indicated distance (x axis) for the uniquely mapped piRNAs plotted over the cluster *42AB*.

silenced by the primary piRNA processing pathway in OSCs (Li et al., 2009). This finding suggests that AGO3 can compete with Piwi for primary piRNAs but that, unlike Piwi, it cannot act to efficiently silence TEs, probably caused by failure of nuclear localization or the inefficiency of its Slicer activity. In agreement with the observation by Li et al. (2009), we found that ectopic expression of Aub in OSCs resulted in derepression of the *mdg1* transposon regardless of Krimp expression, while AGO3 in the heterologous system caused derepression of the *mdg1* transposon only when Krimp was depleted (Figure 5H). These results show that both Aub and AGO3, when they are loaded with piRNAs, increase the expression of TEs in OSCs. This is probably because they can compete with Piwi for primary piRNAs, but they cannot silence TEs. We previously demonstrated that the nuclear localization but not the Slicer activity of Piwi is required for TE silencing in OSCs (Saito et al., 2010), indicating that TE silencing via the somatic primary piRNA pathway occurs only in the nucleus via a Slicer-independent mechanism. Because both Aub and AGO3 remain in the cytoplasm in transfected OSCs, they cannot silence TEs in OSCs. Taken together, these results further support the idea that AGO3 is competent to be loaded with primary piRNAs in the absence of Krimp.

DISCUSSION

Our data suggest that Krimp regulates sDMA modification of AGO3 through direct binding to the N-terminal portion of the protein, thereby controlling interactions of other Tudor-domain proteins, such as Tud (Figure 6A).

The Role of Krimp in the Ping-Pong Cycle

A recent study showed that a lack of Qin, another Tudor domain protein, disrupts the interaction between AGO3 and Aub and triggers Aub-Aub homotypic ping-pong in fly ovaries, resulting in an increase in the abundance of sense piRNAs while the overall abundance of piRNAs is preserved (Zhang et al., 2011, 2014). Therefore, Qin seems to act to suppress Aub-Aub homotypic ping-pong by promoting the Aub-AGO3 interaction, which leads to an increase in heterotypic Aub-AGO3 ping-pong (Figure 6B). A considerable amount of Aub-Aub homotypic ping-pong occurs even in controls, so Aub-Aub homotypic ping-pong is probably a default pathway for piRNA biogenesis; however, this pathway increases the proportion of sense piRNAs in the piRNA pool and so does not efficiently repress TEs. Therefore, there should be a system(s) that promotes heterotypic Aub-AGO3 ping-pong and that also increases the proportion of antisense piRNAs. Although

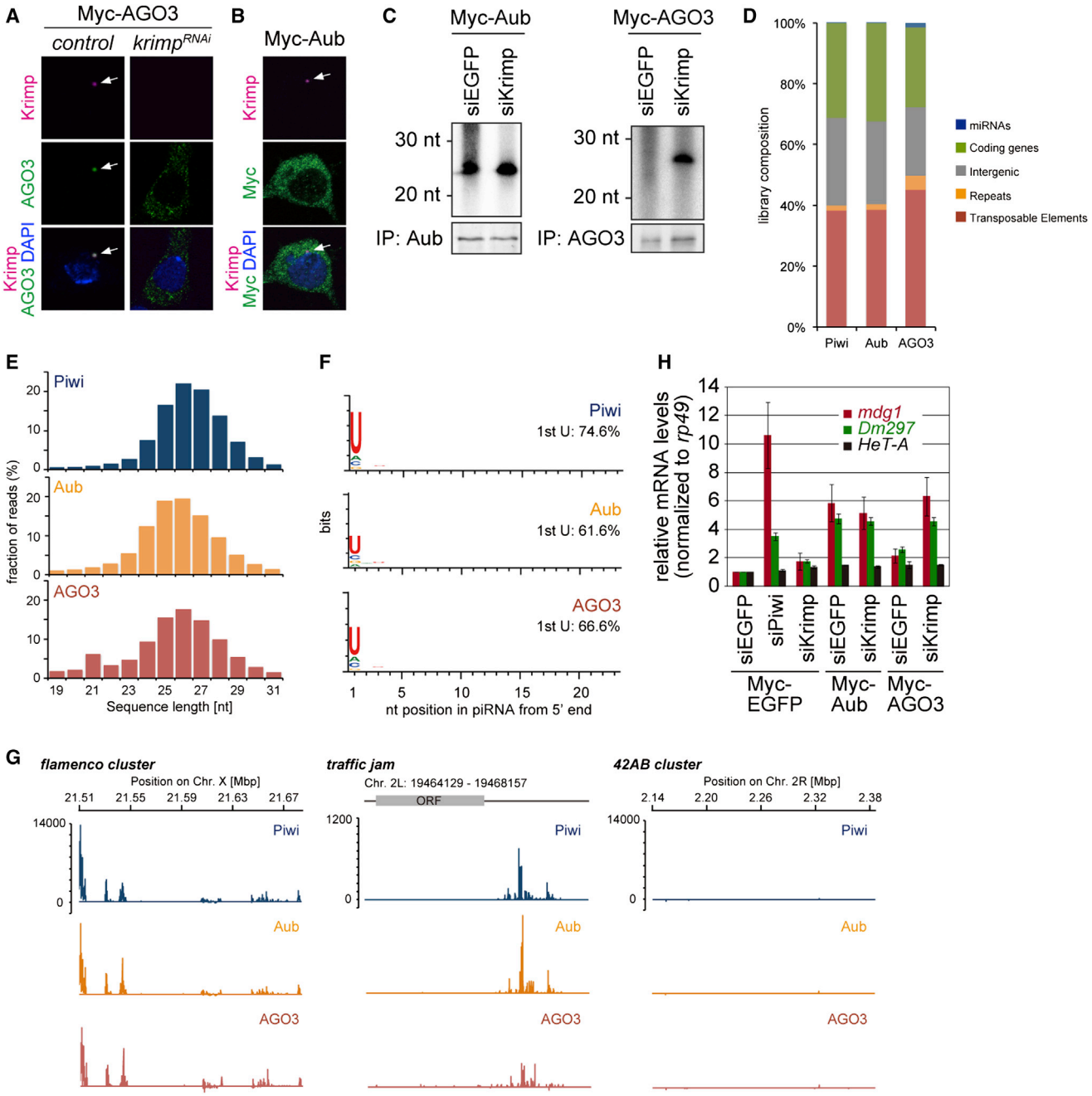

Figure 5. AGO3 Is Compatible with the Primary piRNAs in Krimp-Depleted Somatic Cells

(A) Cellular localization of AGO3 in Krimp-depleted OSCs. Ectopically expressed AGO3 was detected in Krimp bodies (indicated by an arrow). The distribution of AGO3 became diffuse following the depletion of Krimp by RNAi.

(B) Cellular localization of Aub in OSCs. Krimp body was indicated by an arrow.

(C) Aub- and AGO3-IP complexes from OSC lysates overexpressing Myc-Aub or Myc-AGO3 with or without depletion of *krimp* were then immunoreacted using Aub or AGO3 antibody. RNA molecules were isolated from complexes and then ^{32}P labeled.

(D) Chart summarizing the annotation of Piwi-, Aub-, and AGO3-bound piRNA populations.

(E) siRNA-normalized small RNA size profiles are shown for Piwi-, Aub-, and AGO3-bound piRNAs.

(F) Sequence logos of Piwi-, Aub-, and AGO3-bound piRNAs.

(G) Uniquely mapping piRNAs are plotted over the somatic primary piRNA cluster *flamenco*, the genic piRNA locus *traffic jam*, and the germline-specific dual-stranded piRNA cluster *42AB* (sense and antisense piRNAs are indicated with peaks pointing upward and downward, respectively).

(H) Expression levels of several transposons in OSCs overexpressing Aub or AGO3. *mdg1* and *Dm297*, but not *HeT-A*, transposons are silenced by a Piwi-dependent piRNA pathway in OSCs. *rp49* was used as an internal control. See also Figure S5. N = 3; error bars indicate SEM.

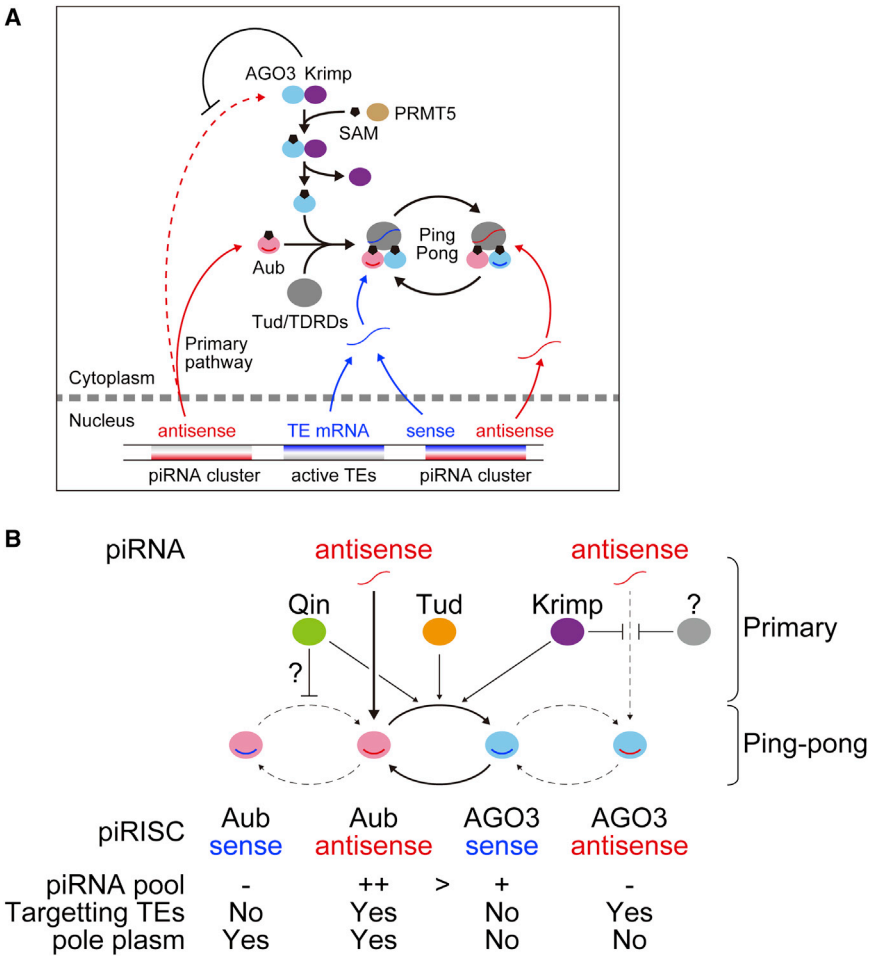

Figure 6. Krimp Enforces an Antisense Bias on piRNA Pools by Assembling AGO3 in the Ping-Pong Cycle

(A) During oogenesis, in nurse cells, Krimp associates with nascent, piRNA-, and sDMA-free AGO3 and translocates it to the nuage, the location of dPRMT5-mediated sDMA modification. Upon sDMA modification, Krimp might then dissociate from sDMA-modified AGO3 prior to its entry into the ping-pong cycle. Additionally, from observations in the OSC system, Krimp keeps AGO3 away from the primary piRNA pathway in germline cells. TDRDs, Tudor domain-containing proteins; SAM, S-adenosylmethionine.

(B) Piwi subfamily proteins potentially associate with both sense and antisense piRNAs. Antisense piRNAs but not sense piRNAs can act to repress the activation of transposons and can be loaded onto mainly Piwi and Aub in germ cells, because Piwi and Aub but not AGO3 are transmitted to the next generation to maintain germline development in *Drosophila*. The piRNA production shown by dotted lines is hardly observed in the normal state.

Aub-Aub homotypic ping-pong also prevails in *krimp* mutant ovaries, the overall abundance of piRNAs in germline cells is dramatically decreased. This profile of piRNAs in *krimp* mutant ovaries is very similar to that in *ago3* mutant ovaries in which the ping-pong cycle collapses (Huang et al., 2014; Li et al., 2009). The increase in Aub-Aub homotypic ping-pong observed in *krimp* mutant ovaries may largely reflect the loss of AGO3-Aub heterotypic ping-pong, rather than a direct effect on promoting Aub-Aub homotypic ping-pong. Therefore, Krimp plays a very different role to Qin in the ping-pong cycle.

The ping-pong cycle is mediated by the Slicer-dependent mutual cleavage of sense and antisense transcripts of a TE, which should result in equal amounts of sense and antisense piRNAs. Thus, an important question is this: what makes sense piRNAs less abundant and disproportionately bound to AGO3 in the ping-pong cycle, enforcing the characteristic antisense bias of Aub-bound piRNAs? In other words, why is Aub-Aub homotypic ping-pong not sufficient to produce the characteristic antisense bias of Aub-bound piRNAs? In *krimp* mutant ovaries, AGO3 is no longer sDMA modified and is free from piRNAs. Therefore, Krimp not only masks the N-terminal portion of AGO3 where sDMA-modifiable arginine residues reside, but also mediates the sDMA modification that leads to interactions of AGO3 with other Tudor domain proteins, including Tud, and to its nuage localization (Figure 6A). In this way, Krimp promotes the ping-pong cycle. Because sDMA modification of Aub is not affected in *krimp* mutant ovaries, complex formation between Aub loaded with primary piRNAs and Tudor domain proteins may occur before sDMA-modified AGO3 joins the complexes to ensure Aub-AGO3 heterotypic ping-pong. Qin may act in this step to suppress Aub-Aub homotypic ping-pong to further promote Aub-AGO3 heterotypic ping-pong.

AGO3 is clearly compatible with the primary piRNA pathway in OSCs when Krimp is depleted. Therefore, Krimp may not only promote the Aub-AGO3 heterotypic ping-pong cycle but also actively prevent AGO3 from becoming loaded with primary piRNAs in germline cells (Figure 6B). In contrast, in *aub* mutant ovaries where AGO3 no longer accumulates at nuage but is sequestered into Krimp bodies (Olivieri et al., 2012), AGO3 is still loaded with reduced levels of piRNAs that have characteristics of primary piRNAs (Figure 4). These results suggest that complete blockage of primary piRNA loading onto AGO3 in ovarian germline cells requires an additional factor(s). An alternative possibility may be that AGO3 does not selectively accept primary piRNAs when *aub* is mutated but is perhaps loaded in a rather non-specific manner. Thus, together, these findings suggest that the characteristic antisense bias of Aub-bound piRNAs is created by the sum of piRNAs produced in the ping-pong cycle and the continual flow of primary piRNAs onto Aub. Aub is much more abundant than AGO3 in fly ovaries (Brennecke et al., 2007; Gunawardane et al., 2007), further suggesting that the continuous flow of primary piRNAs to Aub should contribute to the antisense bias of Aub-bound piRNAs. Alternatively, it is also conceivable that AGO3-sense piRNA complexes may be

catalytically more active than Aub-antisense piRNA complexes, potentially resulting in the antisense bias of Aub-bound piRNAs. However, in OSCs, AGO3 is loaded with primary piRNAs when Krimp is depleted while AGO3 in germline cells is not loaded with piRNAs. This suggests that in addition to Krimp, a second/backup system may also operate to further prevent AGO3 from associating with primary piRNAs in germline cells.

In *aub* mutant ovaries, Krimp is no longer accumulated at the nuage but forms Krimp bodies (Lim and Kai, 2007; Nagao et al., 2011), suggesting that the nuage localization of Krimp is Aub dependent. However, Krimp does not directly interact with Aub, and Krimp in OSCs forms Krimp bodies when Aub is ectopically expressed. Therefore, there must be a germline-specific factor(s) that links Aub and Krimp (Figure 6B). The N-terminal coiled-coil domains of Krimp are required for Krimp body formation (data not shown). Thus, a factor(s) may exist to mask the coiled-coil domains to prevent Krimp from forming the aggregates in germline cells. The function and/or stability of this putative factor could be Aub dependent in germline cells. The next key challenge will be to identify such a factor that regulates Krimp, thereby contributing to the operation of the ping-pong cycle.

Both AGO3 and Aub have the potential to load sense and antisense piRNAs, and the loading of these two proteins likely relies on the functions of interacting proteins, such as Krimp. We therefore hypothesized that AGO3 and Aub are functionally very similar and that their interacting partners play essential roles in determining the behaviors of the two PIWI proteins, such that they effectively participate in ping-pong amplification. If both Aub and AGO3 are compatible with the primary piRNA pathway, why then are primary piRNAs loaded only onto Aub and Piwi? Perhaps this is a system to ensure a heterotypic ping-pong between Aub and AGO3 and some other as yet unknown mechanism to amplify antisense piRNAs. During *Drosophila* germline development, Piwi and Aub, but not AGO3, are directly deposited from mother to offspring through germline transmission (Brennecke et al., 2007). Antisense piRNAs that can act to repress the activation of TEs and initiate the ping-pong cycle are loaded onto Piwi and Aub (Figure 6B) (Le Thomas et al., 2014). The maternal loading of antisense piRNAs, together with the functions of Tudor domain proteins such as Krimp and Qin, could establish an antisense bias in the ping-pong amplification loop.

There is no apparent homolog of Krimp in mammals. This may be because the ping-pong amplification mechanism in mammals is different from that in *Drosophila*. For example, the mouse PIWI protein, MILI, loads sense rather than antisense primary piRNAs. However, heterotypic ping-pong with ping-pong partner MIWI2 may not operate because, once loaded with secondary piRNAs, MIWI2 is imported into the nucleus to direct specific DNA methylation of transposon loci (Aravin et al., 2007, 2008; De Fazio et al., 2011). It is likely that a Krimp-like protein exists in mammals to enforce a piRNA strand bias.

EXPERIMENTAL PROCEDURES

Drosophila Strains

Fly stocks and details are given in the Supplemental Information.

Production of an Anti-sDMA-AGO3 Monoclonal Antibody

An anti-sDMA-AGO3 monoclonal antibody was raised specifically against a synthetic peptide corresponding to the N terminus of the protein. Details are given in the Supplemental Information.

Immunoprecipitation, Pull-Down Assay, and Western Blot Analysis

Immunoprecipitation (IP) and western blot analysis were performed as described previously (Nishida et al., 2009). Details of these and pull-down assays are given in the Supplemental Information.

Northern Blot Analysis and Total RNA Labeling

Northern blot analysis and total RNA labeling were performed as by Nishida et al., (2009) and Saito et al., (2010). Details are given in the Supplemental Information.

RNAi, Plasmid Transfection, Immunohistochemistry, and qRT-PCR Analysis

OSC culture, RNAi, plasmid transfection, immunohistochemistry in OSCs, and qRT-PCR analysis were performed as in Saito et al. (2006, 2009, 2010) and Sato et al. (2011). Details are given in the Supplemental Information.

Cloning and Analysis of Small RNA Libraries

Cloning and analysis of small RNA libraries were performed as previously described (Saito et al., 2009; Malone et al., 2009) with modifications. Details are given in the Supplemental Information.

ACCESSION NUMBERS

The Gene Expression Omnibus (GEO) accession number for small RNA sequencing data reported in this paper is GEO: GSE69293.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.06.024.

AUTHOR CONTRIBUTIONS

K.S., Y.W.I., M.C.S., and H.S. conceived of the project and designed the experiments. K.S. and Y.W.I. performed all the experiments with the help of A.S., Y.T., P.C., and H.I. Y.W.I. performed the majority of computational analyses. K.S., Y.W.I., M.C.S., and H.S. analyzed the data and wrote the paper.

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