Respective Functions of Two Distinct Siwi Complexes Assembled during PIWI-Interacting RNA Biogenesis in *Bombyx* Germ Cells

**Graphical Abstract**

**Highlights**
- Siwi forms distinct complexes with BmSpn-E/BmQin and BmVasa
- The requirements of Siwi/BmSpn-E/BmQin and Siwi/BmVasa complexes differ
- BmSpn-E, unlike BmVasa, is necessary for primary piRNA biogenesis
- Target RNAs remaining on Siwi upon cleavage are released by BmVasa

**In Brief**

PIWI-interacting RNA (piRNA) biogenesis consists of two sequential steps: primary piRNA processing and the ping-pong cycle. The molecular functions of the factors involved remain elusive. Nishida et al. propose a mode for piRNA biogenesis where the BmSpn-E/BmQin dimer binds Siwi and functions in primary piRNA processing, whereas BmVasa, by associating with Siwi, ensures target RNA release upon cleavage to facilitate the ping-pong cycle.

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Respective Functions of Two Distinct Siwi Complexes Assembled during PIWI-Interacting RNA Biogenesis in Bombyx Germ Cells

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INTRODUCTION

In germ cells, PIWI-interacting RNAs (piRNAs) arise primarily from long noncoding RNAs transcribed from piRNA clusters through primary piRNA processing and are amplified through the ping-pong cycle. They are involved in germ cell development, localizing to the posterior pole in oocytes, and are necessary for germ cell silencing. However, the underlying mechanisms remain largely unknown.

The ping-pong cycle depends on reciprocal Slicer-mediated RNA cleavage by two PIWI members (Brennecke et al., 2007; Gunawardane et al., 2007). The current ping-pong model suggests that the process begins with target RNA cleavage by a particular PIWI member loaded with primary piRNAs (the primary piRISC). The Slicer-dependent cleavage on the targets occurs at the position across from nucleotides 10 and 11 of the piRNAs. Upon cleavage, the target RNAs are divided into two pieces: namely, 5' and 3' cleavage products. Of those, the 3' product is loaded onto the other PIWI member in the ping-pong cycle and then trimmed down to the size of piRNAs (24–30 nt) from its 3' end by an unidentified enzyme(s) (Kawaoka et al., 2011), giving rise to the secondary piRISC. Because piRNAs within the secondary piRISC originate from the targets of the primary piRISC, the orientation opposes to that of piRNAs in the primary piRISC. Moreover, piRNAs within the primary and secondary piRISCs overlap through ten nucleotides from their 5' ends. Primary piRNAs show a strong bias for the first U (1U), whereas secondary piRNAs show a 10A bias. The characteristics that piRNAs show in germ cells are referred to as the ping-pong signature (Brennecke et al., 2007; Gunawardane et al., 2007).

The piRNA factors functioning in the ping-pong cycle include Vasa, a germ-specific DEAD box RNA helicase that is highly conserved in animal species and is necessary for germ cell development (Schupbach and Wieschaus, 1986; Lasko and Ashburner, 1988; Tanaka et al., 2000; Ishizu et al., 2012). Drosophila melanogaster Vasa (DmVasa) is localized to the posterior pole in oocytes, where it associates with target mRNAs to locally regulate their translation (Lasko and Ashburner, 1990). Recent studies have shown that DmVasa and mouse Vasa homolog (MVH) are pivotal in piRNA biogenesis in the germline (Lim and Kai, 2007; Malone et al., 2009; Kuramochi-Miyagawa et al., 2010; Nagao et al., 2010). In MVH mutant mouse testes, as in wild-type (WT) testes, MIWI, a mouse PIWI member (Aravin et al., 2007; Pillai and Chuma 2012; Siomi et al., 2011), associates with primary piRNAs (Kuramochi-Miyagawa et al., 2010). In contrast, MIWI2, one of three PIWI members and secondary piRNA binder, is devoid of secondary piRNAs in MVH mutant testes. When not loaded with piRNAs, MIWI2 fails to localize to

SUMMARY

PIWI-interacting RNA (piRNA) biogenesis consists of two sequential steps: primary piRNA processing and the ping-pong cycle that depends on reciprocal Slicer-mediated RNA cleavage by PIWI proteins. However, the molecular functions of the factors involved remain elusive. Here, we show that RNAs cleaved by a Bombyx mori PIWI, Siwi, remain bound to the protein upon cleavage but are released by a DEAD box protein BmVasa. BmVasa copurifies with Siwi but not another PIWI BmAgo3. A lack of BmVasa does not affect primary piRNA processing but abolishes the ping-pong cycle. Siwi also forms a complex with BmSpn-E and BmQin. This complex is physically separable from the Siwi/BmVasa complex. BmSpn-E, unlike BmVasa, is necessary for primary piRNA production. We propose a model for piRNA biogenesis, where the BmSpn-E/BmQin dimer binds Siwi to function in primary piRNA processing, whereas BmVasa, by associating with Siwi, ensures target RNA release upon cleavage to facilitate the ping-pong cycle.
the nucleus, where the MIWI2–piRISC complex would normally otherwise repress transposons by inducing DNA methylation of target genes (Kuramochi-Miyagawa et al., 2008). Thus, transposons are derepressed in MVH mutant testes, resulting in male infertility (Kuramochi-Miyagawa et al., 2010).

A more-recent study using BmN4 cells, a cultured germ cell line derived from B. mori (silkworm) ovaries, showed that B. mori Vasa (BmVasa), when it lacks the ATP-hydrolyzing activity owing to a single mutation in the DEAD box (E339Q mutant), transiently associates with Siwi and BmAgo3, two PIWI members, and BmQin (also known as BmKumo), a Tudor domain protein, to assemble a piRNA amplifier complex in the ping-pong cycle (Xiol et al., 2014). The requirement of Qin/Kumo in piRNA biogenesis was originally examined in Drosophila (Anand and Kai, 2012; Zhang et al., 2011). The amplifier complex contained three types of RNAs: antisense piRNAs, sense transposon transcripts, and ~12 nt RNAs. The ~12 nt RNAs showed a strong bias for sense strand transcript unwinding and were considered to be Vasa footprints, as the recombinant BmVasa E339Q mutant also bound ~6–10 nt RNAs in E. coli (Xiol et al., 2014). These results supported the idea that the amplifier is the biochemical platform for generating BmAgo3-bound secondary piRNAs from sense transposon precursors. However, the amplifier was detected only when the BmVasa DEAD box (E339Q) mutant was ectopically expressed in BmN4 cells. The underlying mechanism by which the amplifier facilitates secondary piRNA generation also remains unclear.

In this study, by performing in vitro target RNA cleavage assays using immunopurified endogenous piRISCs, we show that target RNAs cleaved by Siwi remain bound to the protein upon cleavage but are released in the presence of BmVasa and ATP. Target RNAs cleaved by Drosophila melanogaster Ago2 (DmAgo2) come off the RISC without the need for additional factors. Under normal conditions, BmVasa copurifies with Siwi, but not with BmAgo3. However, the BmVasa E339Q mutant, which lacks ATP-hydrolyzing and RNA-unwinding activities, forms a complex with Siwi and BmAgo3. The complex contains transposon transcripts in the sense orientation, the sources of BmAgo3-piRNAs, as has been observed (Xiol et al., 2014). However, no ~12 nt RNAs were detected on endogenous BmVasa or the E339Q mutant complex. Immunoprecipitation with an anti-BmSpindle-E (BmSpn-E) antibody showed that BmSpn-E copurifies with Siwi and BmQin. Previous studies showed that Spn-E, a DEXH box/Tudor domain protein (Gillespie and Berg, 1995), is necessary for piRNA biogenesis in Drosophila (Aravin et al., 2004; Vagin et al., 2006; Ishizu et al., 2012). The Siwi/BmSpn-E/BmQin complex excludes the presence of BmVasa and BmAgo3. Depletion of BmVasa does not affect the Siwi-piRNA association, but BmAgo3 is devoid of piRNAs. In contrast, depletion of BmSpn-E caused a severe reduction in primary piRNA accumulation, resulting in Siwi and BmAgo3 not being loaded with piRNAs. Based on these observations, we propose a model for piRNA biogenesis in germ cells, where the BmSpn-E/BmQin dimer binds Siwi to function in primary piRNA processing, whereas BmVasa, despite its association with Siwi, is dispensable for primary piRNA processing but is required for the ping-pong cycle, where it ensures target RNA release from Siwi upon cleavage.

RESULTS AND DISCUSSION

To thoroughly elucidate the mechanism underlying piRNA biogenesis in a germ cell line BmN4, we raised monoclonal antibodies for Siwi and BmAgo3, two silkworm PIWI proteins involved in the pathway (Kawaoka et al., 2009). Western blotting on BmN4 cell lysates prepared before and after depletion of Siwi and BmAgo3 revealed the specificity of the antibodies (Figure 1A). We then used these antibodies to immunoprecipitate endogenous Siwi-piRISC and BmAgo3-piRISC complexes from BmN4 cells. The piRNAs isolated from these complexes showed a slight size difference, apparently only a nucleotide apart, on an acrylamide gel (Figure 1B), showing the high specificity of the immunoprecipitation. Sequencing and bioinformatic analysis of the small RNA pools revealed that both SIWI- and BmAgo3-bound piRNAs are frequently mapped to transposons (Figure S1) and that they show typical ping-pong signatures; namely, piRNAs loaded onto endogenous Siwi (the largest population of which has a length of 28 nt) are predominantly antisense and show a strong 1U bias, whereas piRNAs with endogenous BmAgo3 (the largest population of which has a length of 27 nt) are predominantly sense and show a 10A bias (Figures 1C and S1), as has been previously observed (Xiol et al., 2012).

The hierarchy between Siwi and BmAgo3 in the piRNA amplification process, to determine the primary and secondary piRISCs, has not been examined. It is notable that the piRNA strand bias that individual PIWI proteins show does not help us to understand their hierarchy. For example, mouse MILI, which is higher in the hierarchy for the piRNA amplification cycle than MIWI2 (i.e., MILI loaded with primary piRNAs [MILI-piRISC] initiates the amplification cycle in which MIWI2 is loaded with secondary piRNAs produced by MILI-piRISC in the pathway), shows a strong bias for sense piRNAs (Aravin et al., 2008; De Fazio et al., 2011), whereas a MILI homolog in Drosophila, Aubergine, shows a strong bias for antisense piRNAs (Brennecke et al., 2007; Gunawardane et al., 2007). Ago3, a second PIWI member in Drosophila, is loaded solely with secondary piRNAs, the products of the ping-pong cycle, as is MIWI2 in mouse testes (Brennecke et al., 2007; Gunawardane et al., 2007). However, piRNAs loaded onto Ago3 are predominantly sense, whereas those loaded onto MIWI2 are biased toward antisense. Likewise, we cannot exclude the possibility that primary piRNAs in silkworm show a 10A bias. Thus, we set out to determine the hierarchy between Siwi and BmAgo3 in the ping-pong cycle by expressing FLAG-Siwi and FLAG-BmAgo3 in BmAgo3- and Siwi-depleted BmN4 cells, respectively (Figure S2A). RNAs isolated from the complexes were visualized by 32P-labeling at the 5′ end (Figure 1D). FLAG-Siwi expressed in BmN4 cells upon effective depletion of BmAgo3 was loaded with piRNAs, although the signal was slightly lower (~80%) than that in control cells (siLuc). These piRNAs loaded onto FLAG-Siwi in BmAgo3-depleted and control cells were sequenced and analyzed bioinformatically (Figure S2B). In sharp contrast, piRNA loading onto FLAG-BmAgo3 was severely impaired by Siwi loss (~10%; Figure 1D). These results reveal that Siwi is higher in the hierarchy for the ping-pong cycle than BmAgo3; i.e., primary piRNAs originating from piRNA clusters, whose expression is independent of BmAgo3-Slicer (target RNA cleavage) activity, are loaded onto
Siwi, whereas secondary piRNAs, the products of Siwi-piRISC cleavage in the ping-pong cycle, are loaded onto BmAgo3 (Figure 1E).

To understand the molecular function of BmVasa in the ping-pong cycle, we generated an anti-BmVasa monoclonal antibody (Figure 2A) and used it for immunoprecipitation. The protein components were visualized by silver staining (Figure 2B). The existence of BmVasa (~75 kDa) in the complex was confirmed by western blotting (Figure 2B). Besides BmVasa, an ~100 kDa protein band (the expected size of Siwi) appeared on the silver-stained gel. Western blotting confirmed it to be Siwi (Figure 2B). Notably, BmAgo3 was not detected in the complex (Figure 2B). Under more-stringent conditions for immunoprecipitation (i.e., high salt), which presumably disrupt most protein-protein interactions, Siwi disappeared from the complex (Figure S3A), excluding cross-reactivity of anti-BmVasa antibody to Siwi. RNase A treatment of lysate prior to immunoprecipitation did not affect the interaction between BmVasa and Siwi (Figure 2C). Thus, the Siwi-BmVasa interaction is RNA independent. It is noted that this finding agrees with the previous observation that MVH interacts with MILI through the N-terminal region, which presumably has no RNA-binding property (Kuramochi-Miyagawa et al., 2004).

To examine whether BmVasa would preferably bind piRNA-free Siwi or Siwi-piRISC, Siwi was immunoprecipitated individually using anti-Siwi and anti-BmAgo3 antibodies. Preferable binding of BmVasa to piRNA-free Siwi would suggest an involvement of BmVasa in primary piRNA processing. An alternative idea may be that BmVasa plays a role in Siwi loading with secondary piRNAs produced by BmAgo3-piRISC in the ping-pong cycle. After equalizing the amount of Siwi in both immunoprecipitates by adequate dilution, RNAs were isolated from the fractions and 32P labeled at the 5’ end. Siwi in the BmVasa complex was loaded with piRNAs to a similar extent to that in the anti-Siwi immunoprecipitates (Figure 2D). This suggests that BmVasa binds Siwi-piRISC. FLAG-BmAgo3 expressed in BmVasa-depleted BmN4 cells was scarcely associated with piRNAs, whereas piRNA loading onto FLAG-Siwi was hardly affected (Figures 2E and S3B). Moreover, deep sequencing of piRNAs loaded onto FLAG-Siwi upon deletions of BmVasa and BmAgo3 revealed a similar sequence variety of Siwi-bound piRNAs in BmVasa- and BmAgo3-depleted BmN4 cells (Figures S2C–S2E). A unilateral collaboration of BmVasa with Siwi-piRISC in the ping-pong cycle is suggested (Figure 2F).

The cellular localization of BmVasa along with Siwi and BmAgo3 in BmN4 cells was examined. Although Siwi, but not
Figure 2. BmVasa Binds Siwi, but Not BmAgo3, in the Ping-Pong Cycle
(A) Western blotting shows the specificity of anti-BmVasa monoclonal antibody raised in this study.
(B) BmVasa copurifies with Siwi, but not BmAgo3, from BmN4 cells. Left: silver staining of the protein components in the anti-BmVasa immunoprecipitated complex. Right: western blotting shows that BmVasa binds Siwi, but not BmAgo3. h.c., heavy chains of the antibodies.
(C) RNase A treatment of the lysate prior to immunoprecipitation barely affected the interaction between BmVasa and Siwi.
(D) BmVasa associates with Siwi, regardless of its association with piRNAs. Top and middle panels show western blotting results. Bottom panel shows piRNAs labeled with $^{32}$P.
(E) A lack of BmVasa causes BmAgo3 to be devoid of piRNAs. piRNA loading onto Siwi was not affected by BmVasa loss. Upper and lower panels show western blotting results and piRNAs labeled with $^{32}$P, respectively.
(F) A model for BmVasa function with Siwi in the ping-pong cycle.
(G) BmVasa strongly colocalizes with Siwi at the nuage in BmN4 cells.
BmAgo3, localizes diffusely in the cytosol, localization of both PIWI proteins at the nuage (Eddy, 1975; Mahowald, 1971), electron-dense, nonmembranous perinuclear structures considered to be the center for piRNA biogenesis, was clearly observed to be the center for piRNA biogenesis, was clearly observed (Figure 2G). The Siwi-positive and BmAgo3-positive nuage signals only partially overlapped (Figures 2G and S3C). BmVasa was strongly colocalized with Siwi at the nuage, whereas its colocalization with BmAgo3 was partial (Figures 2G and S3C). A unilateral collaboration of BmVasa with Siwi-piRISC is further supported.

The current model of the ping-pong cycle involves Siwi- and BmAgo3-Slicer activity, although the endonuclease activity of neither protein has been examined. Thus, in vitro target RNA cleavage assays were performed using Siwi-piRISC immunopurified from BmN4 cells using the anti-Siwi antibody under stringent conditions. The purity of the complex was examined by silver staining and western blotting (Figures 3A and S3D). The RNA target (a piRNA-4 target) used in this assay contains a sequence fully complementary to piRNA-4 (Figure S3E), one of the piRNAs associated with Siwi in BmN4 cells. Northern blotting confirmed the existence of piRNA-4 in Siwi-piRISC, but not in BmAgo3-piRISC (Figure S3B). As expected, the piRNA-4 target was cleaved by Siwi-piRISC, but BmAgo3-piRISC failed to do so (Figure 3C). The cleavage required Mg^{2+} ions (Figure S3F), as has been shown with DmAgo2-Slicer activity (Schwarz et al., 2004; Miyoshi et al., 2005). A mutant piRNA-4 target, in which 3 nt at the center were mutated (Figure S3G), could not be cleaved by Siwi-piRISC (Figure 3D). Thus, piRNA-4 triggers piRNA-4 target cleavage when it is loaded onto Siwi.

Fractionation of the supernatant from the beads with Siwi-piRISC attached, upon cleavage, revealed that the vast majority of both 5' and 3' products remained bound to Siwi-piRISC (Figure 3E; data not shown). We examined if this was also the case with DmAgo2, a ubiquitously expressed Argonaute protein in Drosophila (Miyoshi et al., 2005). A 21 nt small interfering RNA (siRNA) duplex, whose guide strand is identical to that of piRNA-4 in sequence over 21 nt from the 5' end, was incubated with Schneider 2 cell lysate to form the DmAgo2-siRISC. The resultant siRISC was immunopurified using anti-DmAgo2 antibody (Miyoshi et al., 2005). The 5' product cleaved by DmAgo2 was detected mostly in the supernatant (Figure 3F). It should be noted that, after cleavage, the state of the ten-base overlap between the small RNA (either piRNA or siRNA) and the 3' cleaved product ought to be identical in both cases (Figure S3H). The 3' cleaved product of BmAgo3-piRISC (Figure S3I) was also retained on the effector complex (Figure 3G). These results indicate a uniqueness of piRISC; i.e., piRISC, unlike siRISC, requires cofactor(s) to release cleaved RNAs after slicing event. Without this step, the piRISC fails to be free from target RNAs, and therefore, the piRNA amplification cycle cannot proceed properly, leaving BmAgo3 devoid of secondary piRNAs.

BmAgo3 is a DEAD box protein that tightly interacts with Siwi-piRISC in BmN4 cells (Figure 2B). We hypothesized that BmAgo3 might act as the factor releasing cleaved RNAs from Siwi-piRISC. To test this hypothesis, FLAG-BmAgo3 was purified from BmN4 cells after its ectopic expression by transfection (Figure S4A) and incubated with Siwi-piRISC bound to cleavage products. FLAG-BmAgo3, but not FLAG-EGFP employed as a negative control, released significant amounts of the 3' cleaved product from the effector complex (Figure 4A). In the absence of

Figure 3. RNAs Cleaved by Siwi-piRISC Remain Associated with the Complex upon Cleavage

(A) A silver-stained gel showing Siwi immunopurified from BmN4 cells under stringent conditions. (B) Northern blotting confirms the specific loading of piRNA-4 onto Siwi. (C) Siwi-piRISC, but not BmAgo3-piRISC, cleaves the piRNA-4 target in vitro target RNA cleavage assays. Control: nonimmune IgG. (D) Siwi-piRISC cleaves the piRNA-4 wild-type target (wt), but not the mutant target (mutant). Control: nonimmune IgG. (E) The cleaved piRNA-4 target remains bound with Siwi-piRISC upon cleavage. Control: nonimmune IgG. cf., centrifuge. (F) Cleaved piRNA-4 target dissociates from DmAgo2-siRISC upon cleavage. (G) Cleaved target remains bound with BmAgo3-piRISC upon cleavage.
Figure 4. The RNA-Unwinding Activity of BmVasa Plays a Role in Releasing the Cleaved Targets from Siwi-piRISC upon Cleavage
(A) FLAG-BmVasa releases the cleaved targets from Siwi-piRISC in the presence of ATP.
(B) D338A and E339Q mutations in BmVasa impair the RNA-releasing activity of BmVasa.
(C) The BmVasa E339Q mutant forms a complex with not only Siwi but also BmAgo3. l.c., light chains of the antibodies.

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ATP, the activity was negligible (Figure 4A). The 5' cleavage product was also released from the piRISC upon incubation with BmVasa (data not shown). It seems that uncleaved, full-length target RNAs were also released from piRISC after incubation with FLAG-BmVasa in the presence of ATP (Figure 4A). The mutant piRNA-4 target that was not cleaved by Siwi-Slicer (Figure 3D), and therefore remained bound with the complex, was also released upon incubation with BmVasa (Figure S4B), suggesting that BmVasa liberates Siwi-piRISC from the complex containing uncleavable target RNAs.

The crystal structure of DmVasa bound to an ATP analog and a single-stranded RNA revealed that Glu400 (the second amino acid in the DEAD motif), His575, and Gly552 interact with a water molecule necessary for ATP hydrolysis (Sengoku et al., 2006). Eukaryotic translation initiation factor 4A (eIF4A), another DEAD box protein, facilitates translation by relaxing the 5' end secondary structure of mRNA substrates (Gingras et al., 1999). A Glu183 mutation to glutamine in eIF4A, which is equivalent to Glu400 in DmVasa, significantly reduced the ATP hydrolysis and RNA-unwinding activities of eIF4A (Pause and Sonenberg, 1992). Therefore, we altered Glu339 in BmVasa (corresponding to Glu400 in DmVasa) to glutamine and examined how this mutation affects the BmVasa activity. Incubation of FLAG-BmVasa E339Q (Figure S4A) with Siwi-piRISC failed to release the 3' product from the effector complex (Figure 4B). We also mutated Asp338 in BmVasa to alanine (the first amino acid in the DEAD motif). Asp338 in BmVasa corresponds to Asp399 in DmVasa, which is necessary for ATP binding. This mutant behaved similarly to the E339Q mutant (Figures 4B and S4A). These results indicate that the ATP-binding/hydrolysis and RNA-unwinding activities of BmVasa are required to displace the cleavage products from Siwi-piRISC. It is notable that BmVasa WT, but not its mutants, also displaced uncleavable target RNAs from Siwi-piRISC (Figure 4B).

To further characterize the BmVasa mutants, communoprecipitation experiments were performed. The BmVasa E339Q complex contained much more Siwi than did the BmVasa WT complex (Figures 4C and S4C). An ~105 kDa protein was present in the E339Q complex (Figure S4C) and was confirmed to be BmAgo3 by western blotting (Figure 4C), consistent with earlier observations (Xiol et al., 2014). Northern blotting showed that BmAgo3 within the E339Q complex was loaded with much less piRNAs compared with BmAgo3 immunoprecipitated using anti-BmAgo3 antibody from the crude lysate (Figure 4D), suggesting that BmAgo3 bound to the BmVasa mutant complex is not yet in the piRISC form. Sense, but not antisense, piRNA intermediates were detected in the E339Q mutant complex (Figures 4E and S4D). The sense piRNA intermediates were observed also in the BmVasa WT complex, although the signal strengths were significantly lower and the lengths of the RNAs may be longer, suggesting aberrant accumulation of the molecules in the mutant complex. The amplifier complex composed of BmVasa E339Q mutant, Siwi, and BmAgo3 contained RNAs of ~12 nt (Vasa footprints; Xiol et al., 2014). However, in our study, neither the BmVasa E339Q mutant complex nor the endogenous BmVasa complex contained ~12 nt RNAs (Figure 4F). This discrepancy may have been caused by differences in the tags and antibodies used for immunoprecipitation.

Spn-E is a DEAD box/Tudor domain protein necessary for accumulating piRNAs in Drosophila ovaries (Aravin et al., 2004; Vagin et al., 2006; Malone et al., 2009; Ishizu et al., 2012). Depletion of BmSpn-E in BmN4 cells caused a severe reduction in the accumulation of sense (secondary) piRNAs associated with BmAgo3 (Figure 5A). Depletion of Siwi and BmAgo3 produced similar results (Figure 5A). Thus, the requirement of BmSpn-E in the ping-pong cycle is conserved in silkworm.

To understand the function of BmSpn-E in the ping-pong cycle, we raised an anti-BmSpn-E antibody (Figure S5A) and performed immunoprecipitation experiments. Mass spectrometric analysis revealed that BmSpn-E forms a complex with Siwi and BmQin (Figure 5B). Western blotting on the complex components confirmed the existence of Siwi and BmQin in the complex (Figures 5B and S5B). Because Siwi was detected in the complex, we assumed the presence of BmVasa in the complex. However, this was not the case. Western blotting detected no BmVasa (Figure 5B). It seems that Siwi assembles two distinct complexes: one with BmVasa and one with the BmSpn-E/BmQin dimer. This idea was further supported by the observation that the Siwi/BmVasa complex (Figure 2B) contains neither BmSpn-E nor BmQin (Figure 5C). Immunofluorescence using monoclonal antibodies specific to BmVasa, BmSpn-E, and BmQin showed that the BmVasa-positive nuage is overlapped with the BmSpn-E/BmQin-positive nuage, although the signals for BmSpn-E and BmQin overlapped considerably (Figures 5D and S5C). These results also suggest that the Siwi/BmSpn-E/BmQin complex is separable from the Siwi/BmVasa complex.

Our finding that Siwi forms two distinct complexes in BmN4 cells prompted us to examine if BmSpn-E also plays a different role in primary piRNA processing. Northern blotting experiments revealed that a lack of BmSpn-E, but not a loss of BmVasa, causes a severe decrease in Siwi-bound piRNA accumulation (Figure 5E). We then examined the amounts of piRNAs loaded onto Siwi and BmAgo3 under conditions where BmSpn-E was depleted (Figure S5D). Not only BmAgo3-piRNAs but also Siwi-bound piRNAs were hardly detected by 32P labeling under these conditions (Figure 5F). These results suggest that the requirements for BmVasa and BmSpn-E in piRNA biogenesis in germ cells differ: whereas BmVasa is required specifically for production of BmAgo3-secondary piRNAs, BmSpn-E is necessary for primary piRNA biogenesis. Thus, the ping-pong cycle, which largely depends on the existence of primary piRNAs, is also abolished by loss of BmSpn-E. Siwi residing in the BmSpn-E complex was devoid of piRNAs (Figure 5G), supporting the idea that BmSpn-E binds Siwi prior to its loading with primary piRNAs. This is consistent with the original idea that BmSpn-E functions in primary piRNA biogenesis in germ cells.
Figure 5. Siwi Forms Two Distinct Complexes in piRNA Biogenesis in Germ Cells

(A) Depletion of BmSpn-E (siBmSpn-E) causes a severe reduction in sense (secondary) piRNA accumulation in BmN4 cells. Depletion of Siwi and BmAgo3 produced a similar result. U6 small nuclear RNA (snRNA): a loading control.

(B) Left: silver staining of the protein components in the anti-BmSpn-E immunoprecipitated complex. Right: western blotting shows that Siwi forms a complex with BmSpn-E and BmQin. BmVasa was not detected in the complex.

(C) Western blotting shows that the Siwi/BmVasa complex (Figure 2B) involves neither BmSpn-E nor BmQin.

(D) Immunofluorescence shows that BmSpn-E and BmQin only partially colocalize with BmVasa. The signals for BmSpn-E and BmQin largely overlap, as expected.

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A recent study showed that the DExH box helicase domain of Spn-E, which most likely is required for the ATP-binding/hydrolysis and RNA-unwinding activities of Spn-E (Lüking et al., 1998), is necessary for transposon silencing in Drosophila germline (Ott et al., 2014). Thus, we asked if BmSpn-E’s DExH box is required for Siwi-bound piRNA production in BmN4 cells. Loss of BmSpn-E drastically decreased the amount of Siwi-bound piRNAs (Figures 5E and 5F). Ectopic expression of myc-tagged BmSpn-E WT, but not its DExH-box mutants, D250A and E251Q, increased the levels of Siwi-bound piRNAs in BmN4 cells. However, two BmSpn-E DExH box WT in BmN4 cells rescued this defect in the ping-pong cycle (Figure 6).

In this study, we showed for that BmVasa, a germ-specific DEAD box RNA helicase, uses RNA-unwinding activity to release target RNAs from Siwi-piRISC, upon cleavage, in the ping-pong cycle in germ cells. The BmVasa complex immunopurified from BmN4 cells contained Siwi, but not BmAgo3 (Figure 2B). The cleavage products of BmAgo3-piRISC are also retained on the complex upon cleavage (Figure 3G), suggesting that a protein factor like BmVasa, but not BmVasa itself, is required to dissociate these products from the complex. Indeed, we showed by performing in vitro assays that BmVasa failed to displace the cleavage products from BmAgo3-piRISC (Figure S5G). We are currently aiming to identify BmAgo3-associating proteins. This will hopefully help us understand the counterpart of BmVasa functioning specifically for BmAgo3-piRISC in the ping-pong pathway.

**EXPERIMENTAL PROCEDURES**

**Production of Monoclonal Antibodies**

Monoclonal antibodies for BmVasa, Siwi, BmAgo3, BmSpn-E, and BmQin were produced essentially as previously described (Saito et al., 2006). Mice were immunized with glutathione S-transferase (GST)-tagged BmVasa (full-length), the N-terminal region of Siwi (amino acids 2–100) tagged with GST, the N-terminal region of BmAgo3 (amino acids 2–100) tagged with GST, the N-terminal region of BmAgo3 (amino acids 2–100) tagged with GST, the N-terminal region of BmAgo3 (amino acids 2–100) tagged with GST, the N-terminal region of BmAgo3 (amino acids 2–100) tagged with GST, the N-terminal region of BmAgo3 (amino acids 2–100) tagged with GST.

**Small RNA Isolation from the Immunoprecipitated Materials**

Immunoprecipitation was performed essentially as described previously (Saito et al., 2005). BmN4 lysates prepared in binding buffer (30 mM HEPES [pH 7.4], 150 mM potassium acetate, 5 mM magnesium acetate, 5 mM dithiothreitol, 0.1% Tergitol-type NP-40, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 0.5% aprotonin) were incubated with antibodies bound to Dynabeads Protein G (Invitrogen). The beads were then washed three times with binding buffer containing 500 mM sodium chloride and twice with binding buffer with no supplement. Total RNAs were eluted from the beads by phenol-chloroform treatment and precipitated with ethanol. RNAs were dephosphorylated with CIP (New}

(E) Northern blotting shows that BmSpn-E is necessary for Siwi-bound piRNA accumulation in BmN4 cells. U6 snRNA: a loading control.

(F) A lack of BmSpn-E drastically affected both Siwi-piRNA and BmAgo3-piRNA associations.

(G) Siwi in the BmSpn-E complex is devoid of piRNAs. The top two panels show western blotting results. The bottom panel shows piRNAs labeled with ³²P.

(H) Ectopic expression of BmSpn-E WT, but not its DExH-box mutants, D250A and E251Q, increased the levels of Siwi-bound piRNAs in BmN4 cells lacking endogenous BmSpn-E. The top two panels show western blotting results. The bottom four panels show northern blotting results.

Figure 6. A New Model for piRNA Biogenesis in Germ Cells Consisting of Two Sequential Steps: Primary piRNA Processing and the Ping-Pong Cycle

The BmSpn-E/BmQin dimer binds Siwi to function in primary piRNA processing, whereas BmVasa, via associating with Siwi, ensures target RNA release from the protein upon cleavage to facilitate the ping-pong cycle. A BmAgo3-like protein (shown as X) may dissociate from the BmAgo3-piRISC complex in the process. BmAgo3 remains associated with the Siwi-BmVasa E339Q mutant complex.
England Biolabs) and radiolabeled with \(^{32}\)P-\(\gamma\)-ATP using T4 PNK (NEB). RNA cloning was carried out as previously described (Hirano et al., 2014).

**Small RNA Sequencing and Data Analysis**

Sequencing of the small RNA libraries generated from the Siwi and BmAgo3 immunoprecipitates was performed using MiSeq (Illumina), resulting in a total of 2,048,705 Siwi small RNA reads and 2,916,872 BmAgo3 small RNA reads. Adaptor sequences were removed from obtained reads and mapped to the \(B. \text{ mori}\) genomic sequence (Mita et al., 2004) with Bowtie2 (Langmead and Salzberg, 2012), allowing zero mismatches. Using genome-mapped reads, length distribution and nucleotide bias were calculated. Weblogo3 was used to produce sequence logos (Crooks et al., 2004). Sequences were aligned to the 5\(^\prime\) end to search for nucleotide bias.

**Plasmid Construction**

Expression vectors for FLAG-Siwi, FLAG-BmAgo3, FLAG-BmVasa, and myc-BmVasa were generated by Gateway Technology (Invitrogen) using the pIB-3 \(\times\) FLAG and pIB-myc vectors. The expression vector for myc-BmSpn-E was generated with an In-Fusion HD Cloning kit (Takara). The expression vectors for FLAG-BmAgo3 mutants, myc-BmVasa mutants, and myc-BmSpn-E siRNA-resistant and myc-BmSpn-E mutants were generated by inserting the corresponding coding regions. The sequences of DNA oligos used are shown in Table S1.

**RNAi and Transgene Expression in BmN4 Cells**

BmN4 cells (4 \(\times\) 10\(^5\) cells) were transfected with 500 pmol of siRNA duplex in 10 \(\mu\)l of FuGENE HD transfection reagent (Promega). After transfection, cells were incubated at 27\(^\circ\)C for 72 hr. siRNA transfection was performed again to ensure the RNAi effect. The sequences of the siRNAs used are summarized in Table S1. Luc siRNA (Saito et al., 2009) was used as a control. To exogously express proteins in BmN4 cells, the cells (6 \(\times\) 10\(^5\) cells) were transfected with 2 \(\mu\)g of plasmid in 5 \(\mu\)l of FuGENE HD transfection reagent. After transfection, cells were incubated at 27\(^\circ\)C for 48 hr.

**Immunoprecipitation and Western Blotting**

Siwi and BmAgo3 were immunopurified from BmN4 cells using anti-Siwi and anti-BmAgo3 antibodies, respectively. FLAG-Siwi and FLAG-BmAgo3 were immunopurified from BmN4 cells using anti-FLAG M2 antibody (Sigma). After immunoprecipitation, the beads were washed extensively with binding buffer containing 500 mM sodium chloride and then with binding buffer, myc-BmVasa (both WT and mutant) complexes were immunopurified from BmN4 using anti-myc antibody (Sigma-Aldrich). BmVasa and BmSpn-E were immunopurified from BmN4 cells using anti-BmVasa and anti-BmSpn-E antibodies. After immunoprecipitation, the beads were washed extensively with binding buffer. Western blotting was performed as described previously (Miyoshi et al., 2005). To examine the RNA dependency of Siwi-BmVasa interaction, BmN4 lysates were treated with 10 \(\mu\)g/ml RNaseA at 4\(^\circ\)C for 30 min prior to immunoprecipitation.

**Immunofluorescence**

Immunofluorescence was carried out essentially as described previously (Nishida et al., 2013). Cells were immunostained with anti-Siwi, anti-BmAgo3, anti-BmVasa, anti-BmSpn-E, and anti-BmQin antibodies. Alexa 488/Alexa Fluor 555 goat anti-mouse immunoglobulin G 1 (IgG1)/IgG2a antibodies (Invitrogen) were used as secondary antibodies. Images were taken at the same exposure.

**Northern Blot Analysis**

Northern blot analysis was carried out essentially as described previously (Saito et al., 2010). The sequences of the DNA oligos used are shown in Table S1. Templates for producing probes to detect BmRT3, Bmmar6, L1Bm, R2Bm, Pao, and yamato transcripts were amplified by PCR using primers containing T7 and T3 promoter sequences. BmRT3, Bmmar6, and L1Bm RNA probes were transcribed using a Megascript T3 kit (Life Technologies). R2Bm, Pao, and yamato RNA probes were transcribed with a Megascript T7 kit (Life Technologies).

In Vitro Target RNA Cleavage Assay

Cleavage assays were performed as described previously (Miyoshi et al., 2005). To make the piRNA target template, piRNA target-F and piRNA target-R were annealed and inserted into pBlueScript vector treated with KpnI and EcoRI. The piRNA target template was amplified by PCR using T7 and T3 promoter sequences. The piRNA target was transcribed using a Mega T3 kit and then radiolabeled with \(^{32}\)P-\(\gamma\)-ATP using T4 PNK and was ligated to piRNA-4 target-WT-5′ or piRNA-4 target-mut-5′. RISC formation assays were performed as previously described (Nishida et al., 2007).

Cleaved RNA-Releasing Assays

The beads and supernatant were fractionated after target RNA cleavage assays. The beads were mixed with FLAG-BmVasa WT, FLAG-BmVasa mutants, or FLAG-EGFP (Figure S4A) in the presence of 4 mM ATP, 10 mM creatine phosphate, and 30 \(\mu\)g/ml creatine kinase and then incubated for 2 hr at 27\(^\circ\)C.

**ACCESSION NUMBERS**

Deep sequencing data have been deposited in the NCBI Gene Expression Omnibus and are available under accession number GSE58221.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.013.

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