Somatic Primary piRNA Biogenesis Driven by \textit{cis}-Acting RNA Elements and \textit{trans}-Acting Yb

**Graphical Abstract**

**Highlights**

- \textit{cis} elements for producing somatic primary piRNAs were identified
- Yb primary binding to \textit{cis} elements in piRNA precursors initiates piRNA production
- Yb secondary binding downstream of \textit{cis} elements does not initiate piRNA production
- Artificial piRNAs from the Yb-\textit{cis}-element system elicit transcriptional silencing

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**In Brief**

piRNAs protect the germline genome from transposons, although the underlying mechanism remains elusive. Ishizu et al. focused on understanding how piRNA sources are selectively determined from all cellular RNAs. They successfully identified the particular RNA elements necessary and sufficient for producing piRNAs and determined Yb protein as the \textit{trans}-acting partner.

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Somatic Primary piRNA Biogenesis Driven by cis-Acting RNA Elements and trans-Acting Yb

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SUMMARY

Primary piRNAs in Drosophila ovarian somatic cells arise from piRNA cluster transcripts and the 3′ UTRs of a subset of mRNAs, including Traffic jam (Tj) mRNA. However, it is unclear how these RNAs are determined as primary piRNA sources. Here, we identify a cis-acting 100-nt fragment in the Tj 3′ UTR that is sufficient for producing artificial piRNAs from unintegrated DNA. These artificial piRNAs were effective in endogenous gene transcriptional silencing. Yb, a core component of primary piRNA biogenesis center Yb bodies, directly bound the Tj-cis element. Disruption of this interaction markedly reduced piRNA production. Thus, Yb is a trans-acting partner of the Tj-cis element. Yb-CLIP revealed that Yb binding correlated with somatic piRNA production but Tj-cis element downstream sequences produced few artificial piRNAs. We thus propose that Yb determines primary piRNA sources through two modes of action: primary binding to cis elements to specify substrates and secondary binding to downstream regions to increase diversity in piRNA populations.

INTRODUCTION

PIWI-interacting RNAs (piRNAs) interact with PIWI proteins to form piRNA-induced silencing complexes (piRISCs), which repress target genes, mostly transposons, either transcriptionally or at the post-transcriptional level by cleaving transcripts in the cytoplasm (Aravin et al., 2007; Brennecke et al., 2007; Ghildiyal and Zamore, 2009; Ishizu et al., 2012; Juliano et al., 2011; Khurana and Theurkauf, 2010; Siomi et al., 2011). Interestingly, not all cells in the gonads use both mechanisms. Follicle cells in Drosophila ovaries use transcriptional silencing but lack piRISC-mediated post-transcriptional silencing, while germ cells possess both transcriptional and post-transcriptional piRISC machineries (Ishizu et al., 2012). In Bombyx ovaries, only post-transcriptional silencing occurs (Kawaoka et al., 2009). This variation largely depends on which PIWI proteins are expressed in a given cell type; transcriptional silencing requires nuclear PIWI proteins while post-transcriptional silencing requires cytoplasmic PIWI proteins (Huang et al., 2013; Malone et al., 2009).

Primary piRNAs are produced from single-stranded long noncoding RNAs transcribed from piRNA clusters in a Dicer-independent manner (Houwing et al., 2007; Vagin et al., 2006). The Drosophila genome contains 142 piRNA clusters (Brennecke et al., 2007), whose expression is regulated differently in different cell types. flamenco (flam), a representative of unidirectional piRNA clusters, is expressed only in follicle cells, whereas the bidirectional cluster 42AB is expressed specifically in nurse cells (Brennecke et al., 2007). The types of transposon fragments inserted in individual piRNA clusters also vary; therefore, piRNA populations differ among cell types. piRNAs in nurse cells are rather complex because primary piRNAs are amplified through the amplification loop, yielding secondary piRNAs (Ishizu et al., 2012). Recent studies showed that secondary piRNAs further produce phased trailer piRNAs (Han et al., 2015; Mohn et al., 2015). Follicle cells do not use this amplification system and thus only contain primary piRNAs.

The biogenesis of somatic primary piRNAs has been studied using ovaries and an ovarian somatic cell (OSC) line (Olivieri et al., 2010; Saito et al., 2009). A current model suggests that upon transcription flam-piRNA precursors are localized to perinuclear Flam bodies (Murota et al., 2014) and processed at adjacent Yb bodies (Olivieri et al., 2010; Saito et al., 2010). Yb bodies contain many piRNA factors besides Yb (Haase et al., 2010; Olivieri et al., 2012; Qi et al., 2011; Saito et al., 2009; Saito et al., 2010; Zamparini et al., 2011). Zucchini (Zuc), an endonuclease required for processing piRNA intermediates into mature piRNAs, is localized on the surface of mitochondria (Choi et al., 2006; Han et al., 2015; Ipsaro et al., 2012; Mohn et al., 2015; Nishimatsu et al., 2012; Olivieri et al., 2012). Yb bodies tend to be observed in inter-mitochondrial regions (Murota et al., 2014; Nishimatsu et al., 2012; Szakmary et al., 2009). This arrangement of organelles appears crucial for accelerating piRNA processing because it centralizes all the necessary factors in the cytoplasm. Upon maturation, piRNAs associate with Piwi, a Drosophila PIWI protein, to form piRISCs, which are then translocated to the nucleus to implement nuclear transposon silencing through chromatin modifications on target transposon loci with support from co-factors such as GTSF1/Asterix and Maelstrom (Döbert et al., 2013; Ishizu et al., 2012).
The 24-nt arbitrary sequence inserted in EGFP-tj MT is shown in red. WT and EGFP-tj MT in OSCs were subjected to northern blotting. Upper right: total RNAs and piRNAs in the Piwi immunoprecipitated from OSCs expressing EGFP-tj WT and EGFP-tj MT. Lower right: the amounts of Piwi-piRISC fraction immunopurified from OSCs expressing EGFP-tj WT and EGFP-tj MT were examined by western blotting using an anti-Piwi antibody. Some protein-coding genes such as *Traffic jam* (*Tj*) also act as primary piRNA sources (Robine et al., 2009; Saito et al., 2009), and genic piRNA sources express proteins in OSCs and follicle cells. The Tj protein, encoded by Tj, is a large Maf transcriptional factor necessary for controlling gonad morphogenesis (Li et al., 2003). Loss of Tj function abolishes Piwi expression in follicle cells. However, Piwi expression in nurse cells is not influenced by Tj loss. Thus, the dependence of Piwi expression on Tj differs between follicle cells and germ cells (Saito et al., 2009).

Only a limited number of transcripts serve as somatic primary piRNA precursors. However, the mechanism underlying the recognition and selection of these transcripts as piRNA precursors is poorly understood. To better understand the mechanism, we used the Tj 3’ UTR as a representative of somatic primary piRNA sources to identify a cis element and its trans-acting partner necessary for producing primary piRNAs in OSCs.

**RESULTS**

**Production of Artificial Primary piRNAs from a Random Sequence Inserted into the Tj 3’ UTR**

First, we constructed a plasmid, EGFP-tj WT, consisting of the EGFP coding sequence (CDS; 717 nt) and the Tj 3’ UTR (1,467 nt) (Figure 1A). We previously showed that piRNAs are not produced uniformly along the Tj 3’ UTR; rather, it contains two hotspots that generate substantial amounts of Tj-piRNAs (Saito et al., 2009). In the current study, the first hotspot (24 nt) in EGFP-tj WT was replaced with a 24-nt arbitrary sequence, yielding a mutant construct, EGFP-tj MT (Figure 1A). The 24-nt sequence did not match any region in the *Drosophila* genome (data not shown).

OSCs were transfected individually with EGFP-tj WT and EGFP-tj MT constructs. After transfection, northern blotting was performed with two specific probes, WT and MT, designed to detect natural Tj-piRNAs originating from the first hotspot in the Tj 3’ UTR and artificial piRNAs produced from the 24-nt random sequence embedded in EGFP-tj MT, respectively. The MT probe detected small RNAs of 23–29 nt in length among total RNAs isolated from OSCs after transfection with EGFP-tj MT (Figure 1B). Interestingly, the signals were highly enriched in the Piwi-piRISC fraction immunopurified from OSCs, demonstrating that the artificial piRNAs produced from the 24-nt random sequence in EGFP-tj MT were loaded onto Piwi (Figure 1B). These artificial piRNAs (MT-piRNAs) were undetected in OSCs transfected with EGFP-tj WT, confirming MT probe specificity (Figure 1B). The WT probe detected Tj-piRNAs in both cells (Figure 1B). However, the signal intensity appeared slightly higher in OSCs transfected with EGFP-tj WT, likely because of exogenous expression of Tj-piRNAs from the construct.

**Identification of a cis-Acting Element in the Tj 3’ UTR**

To narrow down the region that acts as a cis element in the expression of MT-piRNAs from EGFP-tj MT, three deletion mutants, MT-1 to MT-3, were produced (Figure 2A). A control

Figure 1. Artificial piRNA Production from an Inserted Fragment into the 3’ UTR of Tj

(A) Top: schematic drawings of EGFP-tj WT and EGFP-tj MT constructs consisting of EGFP CDS (green) and the Tj-3’ UTR (orange). The “hotspot” element (24 nt) in the Tj 3’ UTR of EGFP-tj WT was substituted with a 24-nt arbitrary sequence (shown by a red box) in EGFP-tj MT. Center: schematic drawing of Tj mRNA. Tj-piRNAs (green bars) (Saito et al., 2009) are mapped. Tj 3’ UTR (415–490) is shown by a black bar. Bottom: alignments of Tj-piRNAs originating from the first hotspot in the Tj 3’ UTR (415–490). The hotspot sequence is shown in orange. The 24-nt arbitrary sequence inserted in EGFP-tj MT is shown in red. (B) Left: scheme of the experiments for detecting piRNAs derived from EGFP-tj WT and EGFP-tj MT in OSCs. Upper right: total RNAs and piRNAs in the Piwi complex immunopurified from OSCs were subjected to northern blotting analysis. WT and MT probes detect natural Tj-piRNAs originating from the first hotspot in the Tj 3’ UTR and artificial piRNAs produced from the 24-nt random sequence embedded in EGFP-tj MT, respectively. Artificial MT-piRNAs associated with Piwi in OSCs expressing EGFP-tj MT. Lower right: the amounts of Piwi immunoprecipitated from OSCs expressing EGFP-tj WT and EGFP-tj MT were examined by western blotting using an anti-Piwi antibody.

2012; Muehrdter et al., 2013; Ohtani et al., 2013; Olivieri et al., 2010; Saito et al., 2010)

*flam* is the major source of primary piRNAs in OSCs and follicle cells in the ovaries (Brennecke et al., 2007; Malone et al., 2009; Saito et al., 2009). *flam* is largely occupied by transposon remnants, whose orientation predominantly opposes that of the parental transposons; thus, most primary piRNAs arising from the piRNA cluster act as antisense oligos to repress parental transposons (Brennecke et al., 2007; Malone et al., 2009; Saito et al., 2009). Only a limited number of transcripts serve as somatic primary piRNA precursors. However, the mechanism underlying the recognition and selection of these transcripts as piRNA precursors is poorly understood. To better understand the mechanism, we used the Tj 3’ UTR as a representative of somatic primary piRNA sources to identify a cis element and its trans-acting partner necessary for producing primary piRNAs in OSCs.
Figure 2. Identification of a cis-Regulatory Element in the Tj 3' UTR

(A) Schematic drawings of EGFP-tj MT and its mutant constructs. MT-4 contains the Actin42A 3' UTR (360 nt) (light blue) instead of the Tj 3' UTR. The 24-nt artificial sequence (red) exists also in MT-4.

(B) WT-piRNA and MT-piRNA production was monitored by northern blotting using WT and MT probes as in Figure 1B.

(C) Schematic drawings of MT-2 and its mutant constructs. The 100-nt random sequence inserted in MT-2-1 and MT-2-2 is shown (purple). A stable stem-loop structure was inserted into the 5' UTR of MT-2 to inhibit EGFP translation.

(D) WT-piRNA and MT-piRNA production was monitored by northern blotting using WT and MT probes as in Figure 2B.

(E) Western blotting shows the expression levels of EGFP protein in OSCs after transfection. β-tubulin was detected as a loading control.

Figure 3. Deletion of the Tj-cis Element from the Drosophila Genome using the CRISPR/Cas9 System

To examine whether deletion of the Tj-cis element from the Drosophila genome would cause defective Tj-piRNA biogenesis, we used the CRISPR/Cas9 system (Hsu et al., 2014). We constructed three plasmids expressing short-guide RNAs (sgRNAs) that target specific sites in the Tj locus: targets 1–3 (Figure 3A). sgRNAs were expressed in OSCs for targeting either target 1 + target 2 or target 1 + target 3. We then performed PCR to examine if genomic deletion occurred as expected. DNA fragments of the expected sizes, 599 nt (target 1 + target 2) and 530 nt (target 1 + target 3), were detected only when sgRNAs had been expressed (Figure S2A). Single PCR bands were produced after cloning of each cell line (Figure 3A). Sequencing of the PCR fragments determined the purity of the clones and the deletion sites (Figure S2B), confirming the generation of two deletion mutant cell lines: OSC-Δtj-cis 1 and OSC-Δtj-cis 2.

We conducted northern blotting using a WT probe (Figure 1B) to detect endogenous Tj-piRNAs in both wild-type and mutant OSC lines. This revealed that the piRNAs arising from the first Tj 3' UTR hotspot (Figure 1A) in normal OSCs were barely detected in OSC-Δtj-cis 1 and OSC-Δtj-cis 2 cells (Figure 3B). The production of Idefix-piRNAs arising from other genomic loci was barely affected by Tj-cis element deletion (Figure 3B). Deep sequencing and genome mapping of piRNAs co-immunoprecipitated with Piwi showed that the numbers of Tj-piRNAs, particularly from the 200-nt region neighboring the Tj-cis element, were severely decreased in OSC-Δtj-cis 1 and
piRNAs corresponding to the deletion sites were no longer detected, confirming deletion of the genomic elements. piRNAs mapped to the Tj3’UTR showed little difference between OSC-Dtj-cis 1 and OSC-Dtj-cis 2, agreeing with the observation that MT-3 failed to produce MT-piRNAs (Figure 2B). Dm and Brat 3’UTRs are known as genic piRNA sources (Saito et al., 2009) and the levels of Dm-piRNAs and Brat-piRNAs originating from them were unaffected in both mutant cell lines (Figure 3D). These results suggest that the Tj-cis element identified in this study acts as a cis-regulatory element for producing endogenous Tj-piRNAs from the neighboring region in OSCs.

OSC-Dtj-cis 2 cells (Figure 3C). piRNAs corresponding to the deletion sites were no longer detected, confirming deletion of the genomic elements. piRNAs mapped to the Tj 3’UTR showed little difference between OSC-Dtj-cis 1 and OSC-Dtj-cis 2, agreeing with the observation that MT-3 failed to produce MT-piRNAs (Figure 2B). Dm and Brat 3’UTRs are known as genic piRNA sources (Saito et al., 2009) and the levels of Dm-piRNAs and Brat-piRNAs originating from them were unaffected in both mutant cell lines (Figure 3D). These results suggest that the Tj-cis element identified in this study acts as a cis-regulatory element for producing endogenous Tj-piRNAs from the neighboring region in OSCs.

The Tj-cis Element Is Sufficient for Producing piRNAs from Downstream Regions
To determine whether the Tj-cis element is sufficient for producing piRNAs from unintegrated DNA, we constructed plasmid EGFP-tj-cis, consisting of the EGFP CDS, the Tj-cis element, and four tandem repeats of a 25-nt fragment from which artificial piRNAs were designed to be exogenously expressed (Figure 4A). We confirmed that the sequence was not found in the Drosophila genome (data not shown). Another mutant, MT-5, was also constructed, in which the Tj-cis element and tandem repeat positions in EGFP-tj-cis were swapped (Figure 4A). Northern blotting showed that in sharp contrast to EGFP-tj-cis, MT-5 expressed few piRNAs from the tandem repeats (Figure 4B). The expression levels of the two constructs were similar (Figure S3). These results verify that the Tj-cis element is sufficient to express artificial piRNAs from downstream, but not upstream, arbitrary elements.

We then deep-sequenced primary piRNAs associated with Piwi in OSCs expressing EGFP-tj-cis and mapped them on the construct. This revealed that piRNAs can be generated throughout the 3’UTR, not only from the random sequence consisting of four identical repeats, but also from the Tj-cis element and poly linker regions (Figure 4C). Both endogenous and exogenous piRNAs mapped to the Tj-cis element, and only a few sequence reads corresponding to the EGFP CDS were detected. These results corroborate that the Tj-cis element expressed piRNAs from downstream, but hardly at all from upstream elements. It also agrees with the observation that a limited number of piRNAs mapped upstream of the Tj-cis element, including the Tj CDS and Tj 5’UTR (Robine et al., 2009). Characteristics of the artificial piRNAs derived from the EGFP-tj-cis construct are summarized (Figures 4D–4F).
Primary piRNA Production Largely Depends on Yb Binding to the cis element

We previously performed HITS crosslinking immunoprecipitation (CLIP) experiments in OSCs using an anti-Yb antibody (Murota et al., 2014). Here, to expand the read numbers, we constructed two new Yb-CLIP libraries (Figure S4A), which showed a high correlation for tag sequences (Figure S4B). Bioinformatic analysis revealed that Yb bound strongly and persistently with the Tj 3’ UTR, but not the Tj CDS or 5’ UTR (Figure 5A). The Tj-cis element resided in one of the strong, if not the strongest, Yb association sites in the 3’ UTR (Figure 5A). Comparison of Yb-CLIP mapping data with piRNA mapping data obtained in this study (Figure 3C; wild-type) showed that they greatly overlap (Figure S4C), suggesting the direct involvement of Yb in determining substrates in somatic primary piRNA biogenesis.

Unlike MT-2, MT-3 failed to produce artificial MT-piRNAs in OSCs (Figures 2A and 2B). However, Yb-binding marks were observed on regions shared with MT-2 and MT-3 (Figures 2A and 5A). Tj-R1, a 121-nt sequence adjacent to the Tj-cis element, was within the shared regions, so we next investigated whether Tj-R1 could also produce artificial piRNAs. The Tj-cis element in EGFP-tj-cis (Figure 4A) was replaced with Tj-R1 to yield construct MT-6 (Figure 5B). Northern blotting revealed that limited artificial piRNAs were produced from MT-6 in sharp contrast to EGFP-Tj-cis (Figures 5B and S4D). RNA immunoprecipitation (RIP) experiments followed by qRT-PCR showed that MT-6 transcripts only weakly bound Yb (Figure 5C). This suggested that despite the close proximity of Tj-R1 to the Tj-cis element and Yb-binding marks determined by CLIP, it was ineffective in driving piRNA production. We also constructed MT-7, in which Tj-R1 was replaced with Tj-R2, encompassing another 100-nt sequence following Tj-R1. MT-7 transcripts showed little Yb-binding capacity and produced only a small amount of artificial piRNAs (Figures 5B, 5C, and S4D). Yb-RNA binding determined by CLIP may therefore reflect primary binding to cis elements that provokes piRNA production, and secondary binding that does not provoke piRNA production but determines the domains to
be processed in piRNA production. HITS-CLIP experiments using OSC-ΔTj-cis1 (Figure 3A) revealed that when the Tj-cis element was deleted from the Drosophila genome, Yb only slightly bound the Tj-R1 and Tj-R2 regions within the genome (Figure 5D). These results agree with the observation that MT-6 and MT-7 only weakly bound Yb in OSCs. The primers used for qPCR are indicated by arrows in (B). Bars represent means ± SD of three independent experiments (*p < 0.05).

(D) Yb-CLIP mapping data show that Yb binding of the Tj-R1 and Tj-R2 regions in the Drosophila genome is low in OSC-ΔTj-cis1 (Figure 3A). Signals are displayed in RPM.

UV crosslinking has been shown to occur preferentially at U-rich stretches (Sugimoto et al., 2012), it does not exclude the possibility that Yb shows a U bias in its RNA binding. CIMSs were found in the Tj-cis element, Tj-R1, and Tj-R2 (Figure S4F), suggesting that it would be difficult to distinguish primary and secondary Yb-RNA binding via bioinformatic approaches. We sought to computationally identify any shared structural motifs in the Yb-CLIP sequence tags but found no obvious motifs (data not shown).

cis-Regulatory Elements in flam and Other Genic piRNA Sources

Genome-wide analysis of Yb-CLIP tags revealed that Yb binds various but particular regions across flam transcripts, and that the regions greatly overlap with flam-piRNA mapping regions (Figure 6A). Neither piRNAs nor Yb-CLIP tags were mapped on the neighboring gene, Dip1 (Figure 6A). This further verifies a pivotal role for Yb in piRNA biogenesis substrate determination.

flam exons 1 and 2 are shared with flam isoforms in ovarian somatic sheet cells (equivalent to OSCs in this study) and fly ovaries (Goriaux et al., 2014). Both exons are also good targets for Yb binding (Figure 6A), so we examined if they act as cis-regulatory elements for piRNA production. For this, the Tj-cis element in the EGFP-Tj-cis construct was replaced with either flam exon 1 or exon 2, yielding EGFP-flam-e1 and EGFP-flam-e2 (Figure 6B). Both exons produced artificial piRNAs from their downstream region (Figures 6C and S5A). We then fragmented exon 1 into R1 and R2 and repeated the assays. In accordance with Yb-CLIP results, R1 was predicted to bind Yb only weakly but it produced few artificial piRNAs. By contrast, R2, predicted to bind Yb relatively strongly, produced piRNAs similarly to...
full-length exon 1 (Figures 6D, 6E, and S5B). Yb-RNA binding therefore plays a crucial role in driving piRNA production.

We further extended our experiments to the genic piRNA gene CG9257 (Saito et al., 2009). Yb-CLIP showed that Yb bound almost the entire 3' UTR of the transcripts (Figure 6F), and regions R1 and R2 in the CG9257 3' UTR acted as cis-regulatory elements similar to the Tj-cis element (Figures 6F and 6G). However, a deletion mutant of R2, R3, produced few piRNAs, despite its moderate binding with Yb. R3 may therefore represent a secondary binding site of Yb, as well as Tj-R1 and Tj-R2.

**Exogenously Expressed piRNAs Are Sufficiently Abundant to Elicit the Silencing of Endogenous Genes**

Next, we assessed whether artificial piRNAs expressed under the control of the Tj-cis element were able to repress endogenous genes in OSCs. We chose Krimper (Krimp) as an endogenous gene target because its knockdown previously had no effect on piRNA biogenesis or function (Olivieri et al., 2012). We constructed four plasmids (Krimp-5'0, Krimp-CDS-1, Krimp-CDS-2, and Krimp-3'0) expressing piRNAs targeting the 5' UTR, CDS-1 (1–50), CDS-2 (591–640), and the 3' UTR of Krimp mRNA, respectively (Figures 7A and 7B). All plasmids were based on the EGFP-tj-cis construct by inserting three identical copies of Krimp targeting sequences (50 nt each; except for the sequence targeting the 5' UTR, which was 72 nt) in front of the tandem repeats. The insertion was made in an antisense orientation; thus, piRNAs arising from the insertions should act as antisense to Krimp mRNAs. Northern blotting confirmed the expression of Krimp-piRNAs (Figure 7C).

To determine the efficiency of Krimp-piRNAs in Krimp silencing, OSCs were transfected with Krimp-5', Krimp-CDS-1, Krimp-CDS-2, and Krimp-3', together with another plasmid containing a blasticidin-resistance gene. On day 3 after transfection, blasticidin selection was applied to remove untransfected cells (Figure S6A). Western blotting with an anti-Krimp antibody (Nagao et al., 2011) showed that Krimp was significantly repressed in OSCs when Krimp-piRNAs targeting the Krimp CDS (1–50) and 3' UTR were expressed (Figure S6B). Conversely, Krimp-piRNAs targeting the 5' UTR and CDS (591–640) repressed Krimp to a lesser extent (Figure S6B).
Considering that blasticidin treatment might not fully eliminate untransfected cells, leading to an apparent weakness in the silencing effect, we performed immunofluorescence using an anti-Krimp antibody. This determined the silencing effect only in cells in which Krimp-piRNAs had been generated, because cells considered EGFP-positive showed high EGFP mRNA expression, suggesting a high level of Krimp-piRNA expression.

As expected, Krimp bodies (Nagao et al., 2011; Olivieri et al., 2012), cytoplasmic granules in which Krimp strongly accumulates, disappeared in EGFP-positive cells regardless of the constructs used for transfection (Figures 7D and S6C). This indicated that Krimp silencing occurred regardless of where Krimp-piRNAs targeted Krimp mRNA.

Krimp-piRNAs exogenously expressed from the constructs were loaded onto Piwi to form piRISCs (Figure 7C). To examine if Krimp silencing occurred Piwi-dependently, the efficiency of Krimp silencing was assessed in Piwi-depleted OSCs. When Piwi was present in OSCs, Krimp expression was strongly down-regulated by Krimp-piRNAs (Figure S6D). However, when Piwi was depleted, Krimp expression was restored (Figure S6D).

Thus, Krimp silencing mediated by exogenous piRNAs appears to be Piwi dependent.

To assess whether Krimp silencing mediated by exogenous Krimp-piRNAs is transcriptional, we performed chromatin-immunoprecipitation (ChIP). The association of RNA pol II with Krimp was significantly reduced by Krimp-piRNAs targeting the Krimp CDS (1–50) and 3’ UTR (Figure S6E). Under these conditions, the accumulation of H3K9me3 at Krimp increased (Figure S6E). The expression of Krimp-piRNAs in the sense orientation only slightly affected RNA pol lI and H3K9me3 binding to Krimp (Figure S6F). These results suggest that exogenously expressed piRNAs trigger transcriptional silencing in OSCs.

We also set out to determine the silencing effect of artificial piRNAs against Tj. Primary piRNAs targeting Tj mRNAs (Figure 7E) were expressed in OSCs. Two plasmids were produced based on the EGFP-tj-cis construct, as in Figure 7A, by inserting three identical Tj targeting sequences (Tj-5’ and Tj-CDS; 50 nt each) in front of the tandem repeats. Northern blotting confirmed the expression of corresponding piRNAs (Figure 7F). Immunofluorescence using anti-Tj antibodies showed that EGFP-positive cells expressing artificial Tj-5’-piRNAs and Tj-CDS-piRNAs lacked Tj signals in the nucleus (Figure 7G) (note that these artificial piRNAs were “antisense” to Tj mRNA while endogenous
Tj-piRNAs are "sense" to Tj mRNA). Thus, artificial piRNAs produced under control of the Tj-cis element appear capable of silencing endogenous genes.

**DISCUSSION**

Yb bodies and Flam bodies in OSCs are considered to be the centers for primary piRNA maturation/piRISC formation and piRNA intermediate storage, respectively, and exist in close proximity (Murota et al., 2014; Olivieri et al., 2010; Saito et al., 2010). The formation of both bodies depends on the Yb protein, particularly its RNA-binding activity (Murota et al., 2014). In the absence of this, piRNA processing fails, resulting in piRNA loss, although piRNA intermediates and processing factors are present in the cytosol. Thus, Yb binding to piRNA sources centralizes all necessary ingredients for piRNA biogenesis, which is crucial for primary piRNA production (Murota et al., 2014). In this study, we continued the study on Yb and discovered that the direct association of Yb with a specific ~100-nt element (i.e., cis element) within the piRNA precursors provokes somatic primary piRNA biogenesis from downstream regions. Insertion of the Yb-binding element within RNA molecules that do not otherwise serve as piRNA precursors converts the RNA transcripts into piRNA sources. Artificial primary piRNAs were mapped only downstream, but not upstream, of regions of the Yb-binding element. Previous studies demonstrated that natural genomic piRNAs mostly arise from 3' UTRs rather than mRNA CDS or 5' UTRs (Robine et al., 2009; Saito et al., 2009). The present study also showed that few Tj-piRNAs mapped to the Tj CDS (Figure 3C), and that few Yb-CLIP tags were also found in the Tj CDS (Figure 5A). Thus, Yb determines not only substrate specificity but also processing directionality in the somatic primary piRNA biogenesis pathway. This may occur through the Yb-controlled recruitment of other piRNA factors, such as another putative RNA helicase Armi and endonuclease Zuc, only to downstream sequences. We are currently investigating this possibility.

Yb-CLIP tags greatly overlap with primary piRNA-producing loci in the genome. This strongly supports the idea that Yb is the central player in determining substrates in the piRNA pathway. An unexpected but intriguing observation in our study is that Tj-R1 and Tj-R2 in the Tj 3' UTR show strong Yb-binding marks, as does the Tj-cis element, but provoked very little artificial piRNA production in contrast to the Tj-cis element (Figure 5B). Yb-CLIP experiments showed that Yb binding to Tj-R1 and Tj-R2 within the Drosophila genome largely depends on Yb binding to its upstream Tj-cis element (Figure 5D). We therefore propose a model in which Yb determines primary piRNA sources by two sequential modes of action: primary binding to cis elements that represents selection of piRNA precursors among cellular RNAs, then secondary binding to downstream regions, representing the defining domains to be processed by precursors (Figure S7). This complexity in determining piRNA precursors could ensure the high diversity in piRNA populations, which is a unique feature of piRNAs (Aravin et al., 2007; Brennecke et al., 2007; Lau et al., 2009).

The RNA-binding activity of Yb is required for primary piRNA production in OSC. Yb mutants carrying a point mutation within the DEAD box showed little RNA binding activity (Murota et al., 2014). When these Yb mutants were expressed individually in OSC lacking endogenous Yb, piRNA precursors were not accumulated in Flam bodies, and few piRNAs were produced. As a consequence, transposons were de-silenced. Therefore, there is little doubt that the RNA-binding activity of Yb through the DEAD-box is indispensable for primary piRNA production. HITS-CLIP experiments clarified direct interaction of Yb with piRNA sources, including Tj mRNA. Insertion of a particular Yb-bound RNA element within Tj mRNA, i.e., the Tj-cis element, upstream of any given RNA molecule enables the arbitrary sequences to produce artificial piRNAs. Deletion of the Tj-cis element from the Drosophila genome significantly abolished piRNA production from its downstream region spanning at least ~200 nt. These observations strongly support our model (Figure S7), in which Yb is the trans-acting factor that recognizes and binds cis elements within piRNA precursors to provoke primary piRNA biogenesis in ovarian somatic cells. However, it does not exclude the possibility that Yb collaboratively achieves this task with unknown factors. Moreover, we are not certain if Yb is the uppermost factor in the cytoplasmic phase of the biogenesis pathway upon nuclear transport of piRNA precursors. We are currently engaged in addressing these challenging questions in the laboratory.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**

OSCs were cultured as described previously (Saito et al., 2009; Niki et al., 2006). OSC transfection was carried out using Xfect Transfection Reagent (Clontech) as described previously (Murota et al., 2014). Blasticidin (Life Technologies) was added to the media at 50 μg/ml upon co-transfection using a plasmid carrying a blasticidin-resistant gene. RNAi was performed as described previously (Murota et al., 2014).

**Plasmid Construction**

To generate the EGFP-tj WT construct, the full-length Tj 3' UTR was first PCR-amplified with the primers tj-3'UTR F/R (Table S1, all oligonucleotides were purchased from Invitrogen) from OSC cDNA samples using KOD plus DNA polymerase (Toyobo) and then cloned between XhoI and BamHI sites of pEGFP-N1 (Clontech). The polylinker region between EGFP and Tj 3' UTR was removed by inverse PCR using the primers delta-mcs F/R. The EGFP-3'UTR insert was subcloned into the Nhel and BamHI sites of pAcM (Saito et al., 2009). The Cas9 plasmid, psp70-Cas9, and the sgRNA plasmid, pU6-BbsI-chiRNA, were purchased from Addgene. Target sequences were synthesized as oligomers and inserted into the BbsI site of pU6-BbsI-chiRNA. Detailed methods for constructing other plasmids are described in the Supplemental Information.

**Derivation of the Tj-cis Element Deletion Mutant OSCs**

OSCs were transfected with 4 μg of Cas9 plasmid, 4 μg of sgRNA plasmids, and 400 ng of the blasticidin resistance gene plasmid as described above. Cells were incubated for 48 hr post-transfection prior to blasticidin selection. Three days after addition of blasticidin, 5.0 × 10^5 cells were passaged in 6-cm dishes and allowed to grow in blasticidin containing medium. After 6–7 days of culture, colonies were picked and passaged in single wells of 24-well plates and allowed to grow to confluence in blasticidin free medium. Genomic DNA was extracted using QuickExtract DNA Extraction Solution (Epicenter) following the manufacturer's protocol. The genomic region flanking the CRISPR target site was PCR amplified and analyzed by electrophoresis on 1% agarose gels.

**Immunoprecipitation**

Immunoprecipitation from OSCs was performed as previously described (Saito et al., 2009). Anti-Piwi antibody (Saito et al., 2006) was immobilized on
Dynabeads protein G (Invitrogen). Total RNAs were isolated from the immunoprecipitates with phenol-chloroform and precipitated with ethanol.

**Northern Blotting**

Total RNA was isolated from OSCs using RNAzol RT (Molecular Research Center). Any contaminating DNA was digested by TURBO DNase (Ambion). For small RNA detection, northern blotting analysis was carried out using 5 μg of total RNA as described previously (Saito et al., 2006). The DNA oligonucleotides used are summarized in Table S1. mRNAs were purified from 20 μg of total RNA using Oligotex-dT30 (Takara) according to the manufacturer’s instructions, and northern blotting analysis was carried out as described previously (Murota et al., 2014). DNA probes were synthesized using a Random Primer DNA Labeling Kit Ver. 2 (Takara) in the presence of 32P-dCTP. Templates of random-primed probes were generated by PCR. For the EGFP probe, the EGFP ORF was amplified using the primers EGFP F/R with EGFP-C1 vector as template. For the GAPDH probe, the GAPDH ORF was amplified using the primers GAPDH F/R with OSC cDNA as template.

**Western Blotting**

Western blotting was performed as described previously (Saito et al., 2006). Anti-Piwi (Saito et al., 2006) (1:1,000 dilution), anti-EGFP (MBL) (1:1,000 dilution), anti-Yb (Murota et al., 2014) (1:1,000 dilution), and anti-tubulin (DSHB) (1:2,000 dilution) antibodies were used.

**Small RNA Cloning and Sequencing**

For piRNA cloning, coimmunoprecipitated piRNA was extracted from Piwi immunoprecipitates using phenol and chloroform, and gel-purified piRNAs were cloned using the NEBNext Small RNA Library Sample Prep Set (NEB). piRNA libraries were analyzed on a HiSeq2000 (illumina).

**Small RNA Sequencing Data Analysis**

Sequencing of small RNA libraries generated from Piwi immunoprecipitates were performed using HiSeq2000. Adaptor sequences were removed from obtained reads and mapped to the Release 5 assembly of the *Drosophila* genome using Bowtie (Langmead et al., 2009), allowing zero mismatch and extracting genome-mapped reads. Given two biological replicate data sets of HITS-CLIP, their reproducibility was assessed as follows. For each CIMS, in one data set (query) and in the other (target), we collected reads that were mapped to the CIMS position and to the same strand using BEDTools (version 2.18.1) and an in-house Perl script. The boundaries of each read cluster were defined as the left-most and right-most positions of the collected reads. For each cluster in query, the cluster in target that overlapped with it by at least 1 bp and whose CIMS position was closest to the query CIMS position was identified. Then, Pearson and Spearman’s rank correlation coefficients between their read numbers were calculated. If multiple clusters in target satisfied the above criterion (i.e., with the same CIMS distances), the averaged read number was used.

**RIP and qPCR**

OSCs (4–5 × 10^7) were homogenized in lysis buffer (20 mM HEPES-KOH [pH 7.3], 150 mM sodium chloride, 1 mM DTT, 1 mM EDTA, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin, 0.5% NP40), and cell debris was removed by centrifugation. Total RNAs were isolated using ISOGEN-LS reagent (Nippongene). Anti-Yb antibody (Murota et al., 2014) (2 μg) was immobilized on Dynabeads protein G. Lysates were incubated with beads for 2 hr at 4°C before washing three times with IP wash buffer (20 mM HEPES-KOH [pH 7.3], 300 mM sodium chloride, 1 mM DTT, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin, 0.05% NP40) and three times with high-salt wash buffer (20 mM HEPES-KOH [pH 7.3], 500 mM sodium chloride, 1 mM DTT, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin, and 0.05% NP40). Total RNAs were isolated from the immunoprecipitates with phenol-chloroform and were precipitated with ethanol. For qPCR analysis following RNA immunoprecipitation, a fixed volume of RNA isolated from input and immunoprecipitates was used for reverse transcription. Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (Toyobo). qPCRs were performed using the specific qPCR primers mcs F/R (Table S1) in the StepOnePlus Real-Time PCR System (Life Technologies). THUNDERBIRD SYBR qPCR Mix (Toyobo) was used as described in the instruction manual. The specific enrichment was analyzed based on a percent input calculation.

**Immunofluorescence**

Immunofluorescence of OSCs was performed using anti-EGFP IgG2b (MBL) (1:500 dilution), anti-Krimp IgG1 (Nagao et al., 2011) (1:250 dilution), and anti-TJ IgG1 (1:250 dilution) antibodies as described previously (Murota et al., 2014). Alexa-Fluor-488-conjugated anti-mouse IgG2b (Molecular Probes) and Alexa-Fluor-594-conjugated anti-mouse IgG1 (Molecular Probes) antibodies were used as secondary antibodies (1:1,000 dilution). Anti-TJ monoclonal antibody was produced by immunizing mice with GST-TJ protein as previously described (Saito et al., 2009).

**ChiP and qPCR**

OSCs (1–2 × 10^8) were crosslinked with 1% formaldehyde for 5 min at room temperature, and crosslinking was then quenched with 125 mM glycine for 5 min at room temperature. Fixed cells were washed twice with PBS, harvested by scraping, lysed with swelling buffer (25 mM HEPES-KOH [pH 7.3], 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.1% NP40, 1 mM DTT, 1 × Halt Protease Inhibitor Cocktail [Thermo Scientific]), and pelleted. Nuclear pellets were extracted by sonication buffer (50 mM HEPES-KOH [pH 7.3], 140 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 × Halt Protease Inhibitor Cocktail), sonicated in a Covaris S220 focused ultrasonicator, and diluted. After removing input, the DNA-protein complexes were incubated with 2 μg of nonimmune IgG antibody, anti-Pol II antibody 4H8 (CST), or anti-H3K9me3 antibody (Active motif) at 4°C overnight. DNA-protein complexes were precipitated using Dynabeads Protein G for 1 hr. The beads were consequently washed with low-salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 2 mM EDTA, 0.05% NP40) and 0.1% sodium deoxycholate, 0.1% SDS, 1 × Halt Protease Inhibitor Cocktail, twice with 70% ethanol, and dried. DNA-protein complexes were solubilized by 5% SDS, and 10 μl of each sample was loaded for PCR analysis using Krimp primers.
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SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at
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