

LETTERS

A regulatory circuit for *piwi* by the large *Maf* gene *traffic jam* in *Drosophila*

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PIWI-interacting RNAs (piRNAs) silence retrotransposons in *Drosophila* germ lines by associating with the PIWI proteins Argonaute 3 (AGO3), Aubergine (Aub) and Piwi^{1,2}. piRNAs in *Drosophila* are produced from intergenic repetitive genes and piRNA clusters by two systems: the primary processing pathway and the amplification loop^{1–7}. The amplification loop occurs in a Dicer-independent, PIWI-Slicer-dependent manner^{3,4,8}. However, primary piRNA processing remains elusive. Here we analysed piRNA processing in a *Drosophila* ovarian somatic cell line where Piwi, but not Aub or AGO3, is expressed; thus, only the primary piRNAs exist. In addition to *flamenco*, a Piwi-specific piRNA cluster³, *traffic jam* (*tj*)⁹, a large *Maf* gene, was determined as a new piRNA cluster. piRNAs arising from *tj* correspond to the untranslated regions of *tj* messenger RNA and are sense-oriented. piRNA loading on to Piwi may occur in the cytoplasm. *zucchini*¹⁰, a gene encoding a putative cytoplasmic nuclease, is required for *tj*-derived piRNA production. In *tj* and *piwi* mutant ovaries, somatic cells fail to intermingle with germ cells and Fasciclin III is overexpressed. Loss of *tj* abolishes Piwi expression in gonadal somatic cells. Thus, in gonadal somatic cells, *tj* gives rise simultaneously to two different molecules: the TJ protein, which activates Piwi expression, and piRNAs, which define the Piwi targets for silencing.

Genetic studies have shown that *piwi* and *aub* are essential in germline stem-cell self-renewal and pole-cell formation, respectively^{11,12}. Mutations introduced into *piwi* and *aub* cause de-repression of retrotransposons and a loss of piRNA accumulation in ovaries⁸. A recent study has revealed that strong loss-of-function mutations in *AGO3* also increase expression of selfish genetic elements in germ lines⁵. Thus, the PIWI proteins, with their associated piRNAs, function in retrotransposon silencing. piRNA production in *Drosophila* ovaries occurs in a Dicer-independent manner⁸. A model for piRNA biogenesis—the piRNA amplification loop^{3,4}—was proposed as a result of deep-sequencing and bioinformatic analyses of *Drosophila* piRNAs. In this model, Aub/Piwi and AGO3 reciprocally guide the 5' end formation of piRNAs.

Classification of piRNAs according to their origins indicated that piRNAs derived from a particular piRNA cluster locus—*flamenco* (*flam*)—on the X chromosome are exclusively loaded on to Piwi^{1,3}, indicating that those piRNAs are produced by a pathway independent of the amplification loop^{1,2}. This pathway is called the primary processing pathway^{1,2}. Recently, two independent groups deduced the existence of the primary piRNA processing pathway from extensive bioinformatic analyses of piRNAs in a broad range of piRNA mutants; these studies reconfirmed that primary piRNAs derived from *flam* are most likely loaded directly on to Piwi and not further amplified^{5,6}.

However, a molecular mechanism of the primary processing pathway remains elusive.

We established a stable cell line of ovarian somatic cells (OSCs) from the parental cell line fGS/OSS, comprising germline stem cells and sheets of somatic cells (OSS)¹³. fGS/OSS culture was shown to be Vasa-positive¹³, whereas our OSCs are Vasa-negative (Supplementary Fig. 1a). OSCs express Fasciclin III (FasIII; Supplementary Fig. 1b) and undergo rounds of passage in culture for several months. All these data support the idea that OSCs contain only mitotically active early follicle (somatic) cells.

Piwi is expressed in somatic gonadal cells^{3,14,15}, whereas Aub and AGO3 are not expressed in this cell type^{3,4,16}. Western blot analysis revealed that Piwi, but not Aub and AGO3, was detectable in OSCs (Supplementary Fig. 1a). As in ovaries, Piwi in OSCs was localized in the nucleus (Supplementary Fig. 1c). The absence of *AGO3* expression in OSCs was further confirmed by polymerase chain reaction with reverse transcription (RT-PCR; Supplementary Fig. 1d, e).

Piwi in OSCs was detected in a form bound to small RNAs of 24–30 nucleotides (Fig. 1a). The size distribution of these small RNAs was very similar to that of gonadal Piwi-associating piRNAs (Piwi piRNAs)^{3,4,14}. In addition, they showed resistance to periodate oxidation and β -elimination treatments, which are hallmarks of 2'-O-methyl modification at the 3' end, as in ovarian piRNAs^{8,17,18} (Supplementary Fig. 1f). Thus, the small RNAs associated with Piwi in OSCs could be categorized as piRNAs and, importantly, these piRNAs are produced in an Aub/AGO3-independent manner.

We examined whether the nuclear localization of Piwi is required for piRNA production and for Piwi loading in OSCs. A mutant of Piwi (Piwi- Δ N) in which its putative nuclear localization signals were deleted, resulting in cytoplasmic localization of the mutant (Fig. 1b), was loaded with piRNAs in a similar manner to wild-type Piwi (Fig. 1c). We also observed that a Slicer-deficient Piwi mutant—Piwi-DDAA, where two aspartic acids (D614 and D685) in the PIWI domain, which are required for Slicer activity, are altered to alanines—was loaded with piRNAs, similarly to wild-type Piwi (Fig. 1c). Depletion of endogenous Piwi from OSCs did not affect piRNA loading on to a double mutant of Piwi (Piwi- Δ N-DDAA) (Supplementary Fig. 1g). Piwi does not seem to homodimerize *in vivo* (data not shown). These results support a model in which the primary piRNA processing and the piRNA loading on Piwi may occur in the cytoplasm in a Piwi-Slicer-independent manner.

Piwi-associating piRNAs in OSCs (OSC piRNAs) are mainly derived from the antisense strand of retrotransposons (Fig. 2a and Supplementary Fig. 2a), similar to the derivation of ovarian Piwi piRNAs^{3,4,14,19}. The size distribution of OSC piRNAs is shown in

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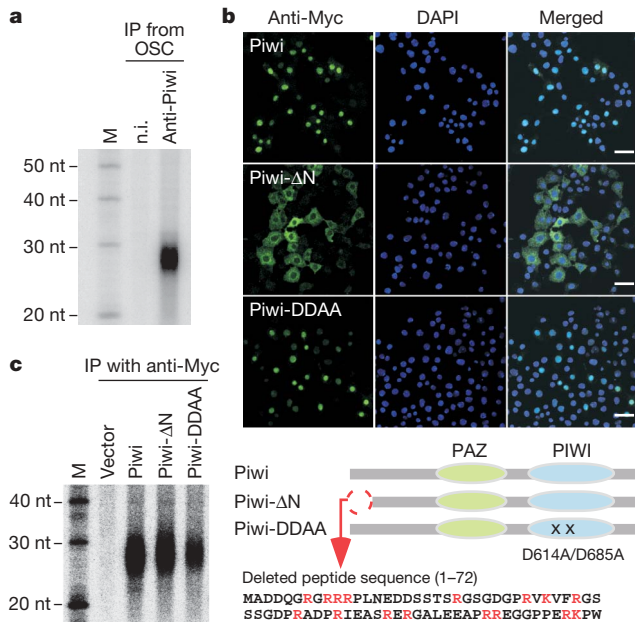


Figure 1 | *Piwi* in OSCs is associated with endogenous small RNAs. **a**, *Piwi*-associating small RNAs in OSCs were visualized by 32 P-labelling. n.i., non-immune IgG was used as a negative control. **b**, Subcellular localization of Myc-tagged wild-type *Piwi*, *Piwi*- Δ N and *Piwi*-DDAA in OSCs. Scale bars, 20 μ m. DAPI, 4', 6-diamidino-2-phenylindole. **c**, Myc-tagged wild-type *Piwi*, *Piwi*- Δ N and *Piwi*-DDAA expressed in OSCs are bound with piRNAs. M, molecular mass marker.

Supplementary Fig. 2b. Examination of their nucleotide bias indicated that OSC piRNAs mostly have a bias for U as the first nucleotide in the sequence (1st-U), but no other prominent bias was observed throughout their entire sequence (Fig. 2b). Exclusion of piRNAs with 1st-U from the piRNA pool did not uncover any obvious bias, including 10th-A (Supplementary Fig. 2c). piRNA pairings through the ten nucleotides from the 5' ends were negligible (Supplementary Fig. 2d). Thus, *Piwi* does not self-amplify piRNAs. All these observations correlate well with the data obtained from a recent deep-sequencing study that was performed using a *Drosophila* ovarian somatic sheet (OSS)⁷.

The origins of OSC piRNAs were examined (Fig. 2c and Supplementary Fig. 3). Unique mapping (see Methods for definition) of OSC piRNAs on the *Drosophila* genome revealed that *flam* is the main source (1,365 perfectly matched and 186 one-base mismatched piRNAs; Supplementary Figs 3 and 4), as it is for ovarian *Piwi* piRNAs³ and OSS piRNAs⁷. In addition to *flam*, we found another locus on chromosome 2L that also produces piRNAs uniquely mapped to the particular region (322 perfectly matched and 29 one-base mismatched piRNAs; Fig. 2c; chromosome 2L, unique). This locus corresponds to the 3' untranslated region (UTR) of a protein-coding, single-exon gene, *traffic jam*⁹ (*tj*, Fig. 2d).

TJ is a soma-specific large Maf factor necessary for controlling gonad morphogenesis in *Drosophila*⁹. TJ is the only *Drosophila* orthologue of the transcriptional factors c-Maf and MafB/Kleisler in vertebrates⁹. In *tj* mutant gonads, somatic cells fail to intermingle and properly develop germ cells. This eventually causes an early blockage in germ-cell differentiation and no follicle cells are detected in adult ovaries of *tj* mutants⁹. All of the OSC piRNAs derived from *tj* were sense-oriented (Fig. 2d), indicating that the *tj* transcript may serve not only as the template for TJ synthesis but also as the precursor of the piRNAs. The *tj* transcript in OSCs was not dedicated to piRNA production, because the TJ protein was strongly expressed in OSCs (Supplementary Fig. 5a). The existence of piRNAs derived from the 3' UTR of *tj* was further confirmed by northern blot analysis (Supplementary Fig. 5b). *tj*-derived piRNAs also appeared in ovarian total

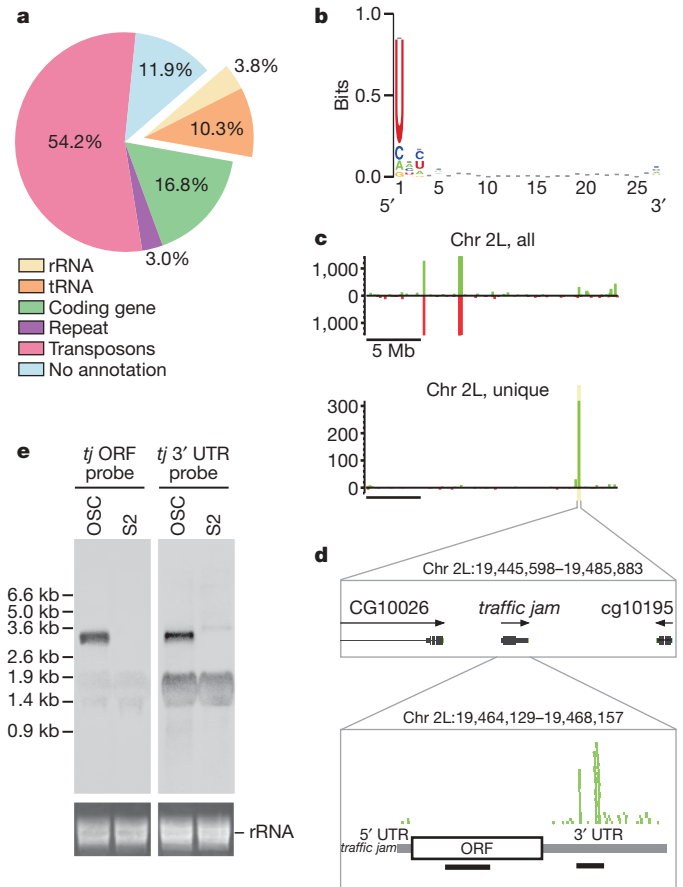


Figure 2 | *Piwi*-associated piRNAs in OSCs. **a**, The contents of *Piwi*-associated small RNAs in OSCs perfectly match the *Drosophila* genome sequence. **b**, Examination of nucleotide bias indicates that *Piwi* piRNAs have mostly uracil at the 5' ends (1st-U). **c**, Mapping data on chromosome 2L. Unique mapping data (bottom; shown as 'unique') indicate that *tj* is the source of OSC piRNAs. y axis shows the read number. **d**, The locus containing *tj* (shown in **c**) is enlarged. Green bars (bottom) indicate piRNAs corresponding to *tj*. Two thick black lines show probes (ORF and 3' UTR) in **e**. **e**, Northern blot analysis. Both ORF and 3' UTR probes visualized a single band of the same length corresponding to the *tj* transcript in OSCs.

small RNA libraries produced by ref. 20 (Supplementary Fig. 6) and ref. 6 (see later).

We assessed whether the transcriptional unit of *tj* is first divided into two parts, each with an individual function—one for TJ synthesis and the other for piRNA production—or if one full-length *tj* transcriptional unit contains both functions. Northern blot analysis, using two probes corresponding to either the open reading frame (ORF) or the 3' UTR of *tj*, visualized a single discrete band of the same length (Fig. 2e), indicating that the latter scenario might be the case.

piRNAs derived from 3' UTRs (and from slightly extended regions) of protein-coding genes other than *tj* were also found (Supplementary Table 1). For example, the 3' UTRs of *brat*²¹ and *Klp10A*²² also produce piRNAs (Supplementary Fig. 7). Interestingly, they are all derived from the sense strand; thus the parental genes are apparently not the targets for gene silencing by *Piwi*. The parental genes are also not repetitive. By contrast, piRNAs derived from retrotransposons or *flam* in OSCs are mainly antisense-oriented and are thought to arise from much longer, repetitive precursors¹.

Mutations in *zucchini* (*zuc*), a gene encoding a putative nuclease, cause female sterility¹⁰ and a reduction of *roo*- and *flam*-derived piRNAs in ovaries^{6,10}. However, whether or not *zuc* is expressed in gonadal somatic cells and, if it is expressed, where *Zuc* accumulates was unknown. We observed that OSCs express *zuc* (data not shown)

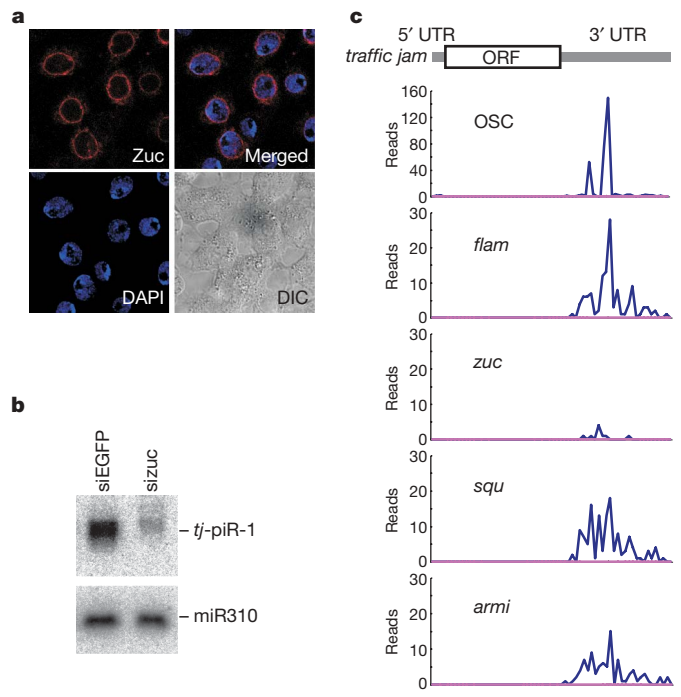


Figure 3 | Involvement of *zuc* in the *tj*-piRNA production pathway. **a**, Myc-tagged Zuc protein was overexpressed in OSCs and its subcellular localization was determined using an anti-Myc antibody (red). Zuc accumulates in the perinuclear region in the cytoplasm of OSCs. DIC, differential interference contrast. **b**, Accumulation of *tj*-derived piRNAs, but not of miRNAs, requires *zuc* in OSCs. **c**, The read numbers of *tj*-derived piRNAs in various piRNA mutants. These data, obtained from the data set of ref. 6, strongly support the idea that *zuc* is required for *tj*-piRNA production.

and that Zuc is predominantly localized in the cytoplasm of OSCs, particularly in the perinuclear region (Fig. 3a). We then sought to determine whether *zuc* is necessary for *tj* piRNA production in OSCs. Depletion of *zuc* by RNA interference (RNAi) significantly reduced the expression level of *tj* piRNAs, but not of microRNAs (Fig. 3b), suggesting that *zuc* is involved in the *tj* piRNA production pathway. This was further supported by analysis of the data set of ref. 6, which indicated that the read number of *tj* piRNAs in *zuc* mutants was much lower compared with those in other mutants (Fig. 3c).

It was previously reported that *tj* mutant somatic cells show a failure to intermingle with germ cells in third instar larval ovaries⁹. Notably, we noticed that the *piwi* mutants²³ *piwi*² and *piwi*³ showed a similar phenotype: somatic cells in the ovaries of third instar larvae of *piwi* mutants adhere to each other and exclude Vasa-positive germ cells (Fig. 4a and Supplementary Fig. 8a). *aub* mutants did not phenocopy the *piwi* mutants (Supplementary Fig. 8b). *piwi* mutants express the *tj* transcript (data not shown) and the TJ protein (Fig. 4a and Supplementary Fig. 8a) at approximately wild-type levels. Thus it is unlikely that *tj* piRNAs target the parental *tj* gene.

We next examined the expression of Piwi in larval ovaries of *tj* mutants. Vasa and TJ are known to be expressed in germline stem cells (and in their developing cells) and somatic cells, respectively, whereas Piwi is known to be expressed in both cell types^{3,11,14}. In wild-type larval ovaries, Vasa- and TJ-positive cells are mutually exclusive, but Piwi is expressed in both cell populations (Fig. 4b and Supplementary Fig. 9a). By contrast, in *tj* mutants, Piwi expression was restricted to Vasa-positive cells (Fig. 4b and Supplementary Fig. 9a). These results indicate that TJ is the activator of *piwi* expression in gonadal somatic cells.

Expression of Piwi in *tj* mutant testes was also examined. As in ovaries, *tj* mutations in testes caused a lack of Piwi expression in gonadal somatic cells, including hub, cyst progenitor cells and early cyst cells. In contrast, Piwi signals were clearly seen in these cell types of wild-type testes (Supplementary Fig. 9b). Interestingly, without functional TJ, germline stem cells and their developing cells in testes, which normally show a faint signal for Piwi, highly express Piwi (Supplementary Fig. 9b). It seems that TJ in testes negatively controls

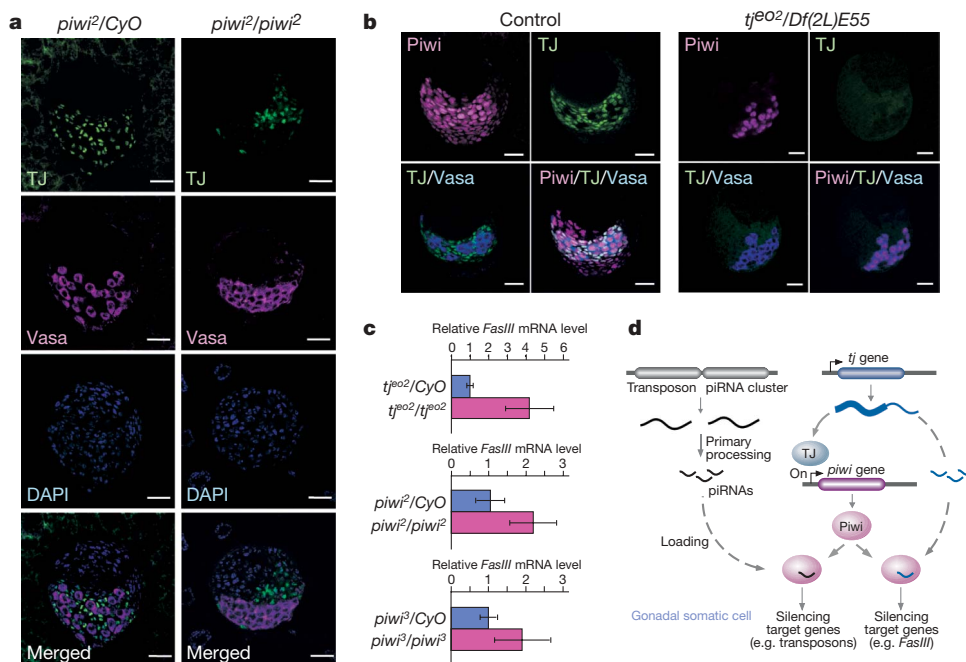


Figure 4 | Phenotypes of *tj* and *piwi* mutant ovaries and testes. **a**, In control (*piwi*²/*CyO*) ovaries, TJ-positive cells and primordial germ cells (PGCs) form a mixed cell population. In *piwi*²/*piwi*² larval ovaries, TJ-positive cells do not intermingle with PGCs, but instead form coherent clusters. TJ and Vasa are shown in green and magenta, respectively. Scale bars, 20 μ m. **b**, In *tj* mutant (*tj*^{eo2}/*Df(2L)E55*) larval ovaries, Piwi (magenta)

is expressed only in Vasa-positive (blue) cells. TJ is shown in green. Scale bars, 20 μ m. **c**, Quantitative RT-PCR shows that the expression level of *FasIII* is upregulated by loss of *piwi* or *tj* expression. **d**, Two functions of *tj* in the regulation of the function of *piwi* in gonadal somatic cells. The first function of *tj* is to activate *piwi* expression. The second is to supply piRNAs for Piwi. Genes targeted by the complex might include *FasIII*.

piwi expression and indirectly controls expression in germline stem cells and their developing cells.

This study has uncovered two functions of *tj* in the regulation of *piwi*'s functions. In gonadal somatic cells, TJ supposedly controls transcription of various genes. Our study indicates that *piwi* is highly likely to be one of the genes under strong TJ control because loss of *tj* in gonadal somatic cells abolished Piwi expression. Further support for the hypothesis that TJ controls *piwi* expression was provided by DNA sequences near the putative transcriptional start site of the *piwi* gene, which show a weak but significant similarity to the Maf binding consensus sequence²⁴, and which were bound with TJ in OSCs (Supplementary Fig. 10a, b). Thus, the first function of *tj* is to activate the expression of Piwi in gonadal somatic cells. The second function is to supply piRNAs for Piwi. Without the supplement of *tj* piRNAs, Piwi would lose the activity to target genes that should be silenced by Piwi and the *tj*-piRNA complex. A likely target of such silencing is *FasIII* because *FasIII*, a cell adhesion molecule concentrated at the hub cell junction, is ectopically overexpressed in other somatic cells in *tj* larval testes⁹. Indeed, the *FasIII* expression level was higher in *piwi* mutant testes than in control testes (Fig. 4c). Some of the *tj* piRNAs identified in this study showed strong complementarity to the *FasIII* primary transcript (Supplementary Fig. 10c). Although all the *tj*-derived piRNAs are sense-oriented and thus unlikely to target the *tj* mRNA, given the nuclear localization of Piwi, it is conceivable that the Piwi-piRNA complex could associate with the *tj* gene.

These findings suggest a novel regulatory circuit where *tj* mRNA simultaneously produces two types of functional molecules (Fig. 4d): TJ protein, which activates expression of Piwi, and piRNAs, which are loaded on to Piwi to silence specific target genes, such as *FasIII* and other, as yet undiscovered, genes.

METHODS SUMMARY

The OSC line was developed from fGS/OSS¹³. Piwi was immunopurified from OSCs using an anti-Piwi antibody¹⁴. Cloning of small RNAs associated with Piwi in OSCs was carried out as described²⁵. Genome mapping and annotation was performed as described in the Methods. Western blotting⁴, RT-PCR, periodization/ β -elimination^{4,17}, northern blotting¹⁴ and immunostaining²⁶ were performed as described.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.S., S.I., Y.K. and H.K. conducted biochemical experiments. T.M., Y.O., E.S. and K.A. performed bioinformatics. K.S., T.M., H.S. and M.C.S. designed experiments, interpreted data and prepared the manuscript.

Author Information Small RNA sequences have been deposited at the GEO database under accession number GSE15137. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.S. (awa403@sc.itc.keio.ac.jp) or M.C.S. (siomim@sc.itc.keio.ac.jp).

METHODS

OSC culture. Culture medium was prepared essentially as previously described¹³. Cross and Sang's M3 (BF) medium was prepared from Shields and Sang M3 Insect Medium supplemented with 0.6 mg ml⁻¹ glutathione, 10% FBS, 10 mU ml⁻¹ insulin and 10% fly extract. We collected adult Oregon R flies and measured their total weight. Flies were treated with 70% ethanol for 5 min, and then washed several times with PBS. Flies were transferred to a mortar and 10 ml of M3 medium supplemented with 10% FBS (M3/FBS) was added. Flies were homogenized using a pestle on ice, after which M3/FBS was added to the homogenate, giving a final concentration of 0.2 g (fly weight) per ml. This homogenate was centrifuged at 6,000g for 10 min. The supernatant was divided into 500 µl aliquots and then heat treated at 60 °C for 5 min. This preparation was spun at 17,000g for 10 min. The supernatant was centrifuged at least twice more and the clear supernatant then collected. The supernatant (fly extract) was stored at -30 °C before use. Fly extract was sterilized by filtration and added to the culture medium to a final concentration of 10%. For passaging, OSCs were washed in PBS and then exposed to a solution of trypsin-EDTA for 1 min at 37 °C. Culture medium was then added to neutralize the trypsin. The cells were washed by aspiration in the medium and aliquots were pipetted into a 6-well plate (Falcon) or a 90-mm cell culturing dish (FPI). These were then incubated at 26 °C (the detailed protocol is available on request).

Drosophila strains. *yellow white* (*y w*) and Oregon R were used as wild-type strains. The *piwi* alleles used were *piwi*² and *piwi*³ (Bloomington stock number 12225) (gift from H. Lin). *Df(2L)BSC145* (Bloomington stock number 9505) is a deficiency line that covers the *piwi* locus. The *tj* alleles used were *tj*⁹⁰² and *tj*⁴⁷³⁵ (gift from D. Golt, S. Kobayashi and H. Sano). *Df(2L)E55* (DGRC stock number 106822) is a deficiency line that covers the *tj* locus. The *aub* alleles used were *aub*^{HN2} *cn bw/CyO* and *aub*^{QC42} *cn bw/CyO*. *aub*^{HN2} *cn bw/CyO* and *aub*^{QC42} *cn bw/CyO* were crossed to yield *aub* heterozygous flies, *aub*^{QC42}/*aub*^{HN2}. To recognize *piwi*^{-/-}, *tj*^{-/-} and *aub*^{QC42}/*aub*^{HN2} larvae, *w*^{*}; *L*² *Pin*¹/*CyO*, *P{GALA-Kr.C}DC3 P{UAS-GFP.S65T}DC7* (Bloomington stock number 5194) were used as balancer chromosomes. All stocks were maintained at 25 °C.

Immunoprecipitation and cloning of small RNAs associated with Piwi in OSCs. Piwi was immunopurified from OSCs using a specific antibody¹⁴. 1 × 10⁸ OSCs were homogenized in a hypotonic buffer (30 mM HEPES, pH 7.3, 2 mM magnesium acetate, 5 mM dithiothreitol (DTT) and 1 mg ml⁻¹ Pefablock SC) to prepare OSC lysate. Piwi and AGO1 were immunopurified using anti-AGO1 (ref. 27) and anti-Piwi (P3G11) antibodies¹⁴ immobilized on GammaBind beads (GE Healthcare). Just before immunoprecipitation, potassium acetate and NP-40 were added to the lysates to 150 mM and 0.1%, respectively. Reaction mixtures were rocked at 4 °C for 2 h and beads were washed five times with washing buffer (30 mM HEPES, pH 7.3, 150 mM potassium acetate, 2 mM magnesium acetate, 5 mM DTT, 0.1% NP-40 and 1 mg ml⁻¹ Pefablock SC). After immunoprecipitation, total RNAs were isolated from the immunoprecipitates with phenol-chloroform and precipitated with ethanol. RNAs were dephosphorylated with CIP (NEB) and labelled for visualization with ³²P-γ-ATP using T4 polynucleotide kinase (Takara). Cloning of small RNAs associated with Piwi in OSCs was carried out as previously described²⁵. Deep-sequencing was performed on a GS FLX system (Roche).

Processing sequence tags. The adaptor sequences attached to the 5' and 3' ends of every sequence produced from the Roche/454 FLX system were removed. Ideally, the raw sequence belonged to one of the following canonical patterns: 5'-ATCGTCTCGGGATGAAA(N...)/TTTCATCCCGAGACGAT-3' and 3'-ATTGACCCGAGTTACAG(N...)/TTTCATCCCGAGACGAT-5', where (N...) represents an arbitrary nucleotide sequence for subsequent analyses. The adapters can have deletions at their 5'/3' ends. We used the NCBI bl2seq program (-p BLASTN -g F -W 10 -e 10 -D 1) to detect fragments of the adaptor sequences in the raw sequence and extracted 21,211 sequences belonging to the canonical/near-canonical patterns. Sequences containing ambiguous Ns were discarded.

Genome mapping and annotation. We followed a previously described procedure to map and annotate sequences²⁵. 21,089 sequences were mapped to the *Drosophila melanogaster* (dm3) genome. The annotation results are shown as a pie chart in Fig. 2a. Supplementary Fig. 2a compares the characteristics of small RNAs associated with ovarian Piwi, Aub, AGO3³ and OSC Piwi in terms of strand bias (left) and cloning frequency (right). The strand bias is computed by comparing the mapping strand of a piRNA with the strand of a coinciding transposon (112 popular classes are used) of the natural transposable element (FlyBase²⁸) track of the UCSC GenomeBrowser for Functional RNA²⁹. To distinguish a piRNA solely mapped on to a single genomic locus from piRNAs mapped on to multiple genomic loci, we use a term 'unique mapping'.

Sequence logo. We used the Web Logo program³⁰ to produce Fig. 2b and Supplementary Fig. 2c. The sequences, ranging from 25 to 27 bases, are aligned to the 5' end with appended '1' to fill the gaps for sequences shorter than 27 bases.

Frequency map. Frequency maps for OSC piRNAs were generated (Fig. 2c and Supplementary Fig. 3) representing the number of piRNAs that are 100% identical to the genomic sequence in a 5-kb sliding window. piRNAs in the forward strand are rendered in green whereas piRNAs in the reverse strand are rendered in red.

Surveying ten-base binding partners. We performed a sequence similarity search using the NCBI BLASTN program among small RNAs associated with ovarian Piwi, Aub, AGO3³ and OSC Piwi to detect potential binding partners that bind each other with 10-base full complementarity. We used the sequences that were 100% identical to the genomic sequence.

Northern blot, western blot and β-elimination analyses. Northern blot analysis was carried out essentially as previously described¹⁴. Total RNAs of OSCs and S2 cells were isolated using ISOGEN (Wako). Total RNAs from the immunoprecipitates were isolated with phenol-chloroform and precipitated with ethanol. DNA fragments for detecting *tj* transcripts (accession number AY325814) were cloned into pBS SK+. The ORF probe corresponded to nucleotides 801–1300 and the 3' UTR probe corresponded to nucleotides 2143–2402. Sequences of the primers were as follows: *tj*-ORF-forward, 5'-CATGACATGATGTGGCTGAC-3'; *tj*-ORF-reverse, 5'-GATCTGGTCGAGCTGGCG-3'; *tj*-utr-forward, 5'-TTTCAAGAGAAGTGCATTCCC-3'; *tj*-utr-reverse, 5'-TATCTCATCTATCTCAATCTCTGTC-3'. PCR products were used as templates. DNA probes were synthesized using a random primed labelling kit (Takara) in the presence of ³²P-dCTP. For small RNA northern blot analyses, probes used for miR-310, *tj*-piR-1, *tj*-piR-2, *kfp10a*-piR-1 and *brat*-piR-1 were as follows: miR-310, 5'-AAAGCCGGGAAGTGTGCAATA-3'; *tj*-piR-1, 5'-GGTAATGGGAATGCACCTTCTCTTCAA-3'; *tj*-piR-2, 5'-TCTCATCTATCTCAATCTCTGTCGA-3'; *kfp10a*-piR-1, 5'-GATGTCAGTTGGTTTGGTGTGA-3'; *brat*-piR-1, 5'-TTGTGTCGCGGTTTCGGTTTGGGT-3'. The DNA oligonucleotides were labelled with T4 polynucleotide kinase in the presence of ³²P-γ-ATP. Western blot analysis was performed as described previously⁴. Ten micrograms of protein from each sample was loaded on gels. Culture supernatants of anti-Piwi hybridoma cells (P3G11)¹⁴, a mouse monoclonal antibody for Aub¹⁶, culture supernatants of anti-AGO3 hybridoma cells⁴, a rabbit polyclonal antibody to Vasa (1:1,000 dilution), a guinea-pig antibody to TJ (1:1,000 dilution)⁹ and anti-tubulin (from DSHB) (1:5,000 dilution) were used. Periodate oxidation/β-elimination treatment was performed as previously described¹⁷.

RT-PCR analyses. Total RNAs of ovaries, S2 cells and OSCs were isolated using ISOGEN according to the manufacturer's instructions. Total RNAs were treated with DNase to eliminate DNA contamination. Five-hundred nanograms of total RNA was annealed with an oligo-dT primer. Reverse transcription was performed using Accuscript High Fidelity Reverse Transcriptase (Stratagene) according to the manufacturer's instructions. The resultant cDNAs were amplified using KOD-plus DNA polymerase and primers for each gene. Sequences of the oligonucleotide primers used are: *Dmhen1/Pimet*, 5'-ATGTTTTCGCACAAGTTTATTGCGGG (forward), 5'-GCCCAAACACCAGTCTTGAAC (reverse); *AGO3*, 5'-GCGAGACGAAGTACGGTCAGATAAC (forward), 5'-CAATCAAATAAGCCAATTTGTGAGCG (reverse). For quantitative RT-PCR, total RNA (0.1 µg) was used to reverse transcribe target sequences using a Transcriptor First strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. The resulting cDNAs were amplified with a LightCycler 480 Real-Time PCR Instrument II (Roche) using the LightCycler 480 SYBR Green I Master (Roche). The primers used are shown in Supplementary Table 2.

Myc-tagged protein expression and RNAi in OSCs. A short DNA fragment encoding a Myc-tag was inserted into the KpnI site of pAc5.1/V5-HisA (Invitrogen) to produce pAcM. Full-length *piwi* and *zuc* cDNA were inserted into pAcM to yield Myc-Piwi wild type and Myc-Zuc, respectively. To produce Myc-Piwi-ΔN, a partial fragment of the *piwi* gene (amino acids 73–843) was amplified and inserted into pAcM. Mutagenesis to yield Myc-Piwi-DDAA and Myc-Piwi-ΔN-DDAA was performed with a QuikChange PCR kit using Pfu Turbo (Stratagene). Primers used are shown in Supplementary Table 2. Trypsinized OSCs (3 × 10⁶ cells) were suspended in 100 µl of Solution V of the Cell Line Nucleofector Kit V (Amaxa Biosystems) and mixed with 5 µg of expression plasmid or 200 pmol of siRNA duplex. Transfection was conducted in an electroporation cuvette using the Nucleofector instrument (Amaxa Biosystems). The transfected cells were transferred to fresh OSC medium and incubated at 26 °C. In the case of RNAi, cells were transfected again after 48 h (the detailed protocol is available on request). The siRNA duplexes used are shown in Supplementary Table 2.

Immunostaining. OSCs were fixed with 4% formaldehyde in PBS for 15 min and treated with 0.1% Triton X-100 in PBS for 15 min. After blocking with 2% BSA in PBS, they were incubated with the primary antibody for 1 h. Immunostaining of larval ovaries was performed essentially as previously described²⁶. Briefly, third larval or adult ovaries were dissected manually in PBS. The samples were fixed in 4% paraformaldehyde in PBS for 20 min and treated with methanol and detergent.

The samples were washed three times (20 min each) in PBTx (PBS containing 0.1% Triton X-100) and blocked in PBTxb (PBTx containing 5% bovine serum (Sigma)) for 1.5 h. Subsequently, samples were incubated with primary antibodies in PBTxb for 16 h at 4 °C. After washing three times (20 min each) in PBTx, samples were incubated with secondary antibodies in PBTxb for 16 h at 4 °C and rinsed three times (20 min each) in PBTx. Culture supernatants of anti-Piwi hybridoma cells (P4D2, 1:1 dilution)¹⁴, a mouse monoclonal antibody to Myc-tag (1:1,000 dilution, Sigma), a guinea-pig antibody to TJ (1:2,000 dilution)⁹, a rabbit polyclonal antibody to Vasa (1:1,000 dilution) and a mouse antibody to FasIII (1:50 dilution) were used for primary antibodies. Alexa-488-conjugated anti-mouse IgG (Molecular Probes), Cy3-conjugated anti-mouse IgG (Sigma), Alexa-546-conjugated anti-rabbit IgG (Molecular Probes), Alexa-633-conjugated anti-rabbit IgG (Molecular Probes), Alexa-546-conjugated anti-guinea-pig IgG (Molecular Probes) and Alexa-488-conjugated anti-guinea-pig IgG (Molecular Probes) were used as the secondary antibodies. DNA was stained with DAPI. All images were collected using a confocal microscope (Zeiss LSM5 EXCITER).

ChIP assay. ChIP assays were performed using the EZ ChIP Chromatin Immunoprecipitation Kit (Upstate). Briefly, 2×10^7 cells were crosslinked with

1% formaldehyde for 10 min at room temperature and sheared to produce crosslinked DNA (~200–1,000 bp in length). The DNA–protein complexes were immunoprecipitated overnight with either non-immune IgG antibody or anti-TJ antibody. After reversing crosslinking at 65 °C for 12–16 h, DNA was recovered. Short DNA fragments corresponding to *piwi* gene were amplified by a LightCycler 480 Real-Time PCR Instrument II using SYBR Premix Ex Taq (Takara). The primers used are listed in Supplementary Table 2.

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