SUPPLEMENTARY INFORMATION

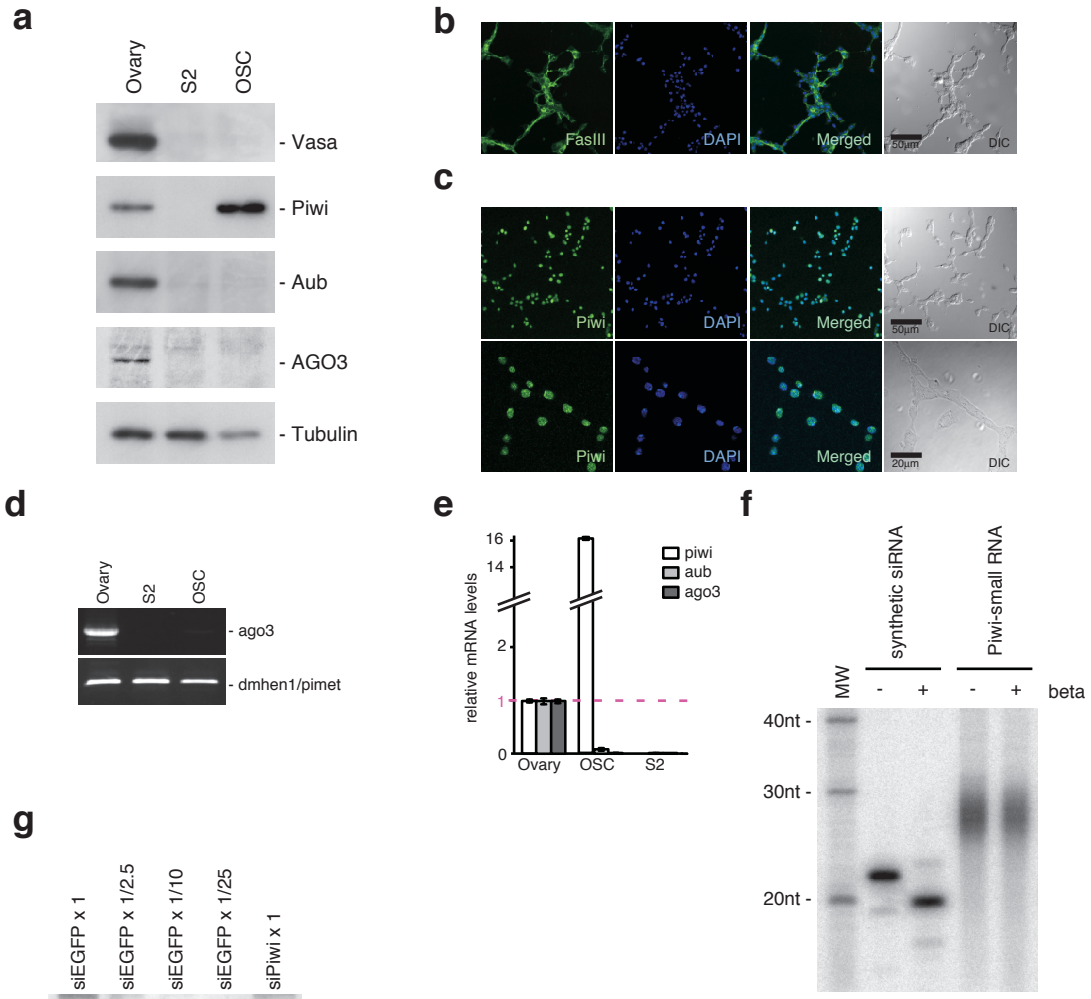

Figure S1. The amplification loop-independent piRNA production in OSC. (a) Western blot analysis was performed with anti-Vasa, -Piwi, -Aub, and -AGO3 antibodies on lysates of S2 cells, fly ovaries, and OSCs. Anti-Tubulin was used as an internal control. (b) OSCs were stained with an anti-FasIII antibody. All cells in culture were FasIII-positive. (c) OSCs were stained with an anti-Piwi antibody. This confirmed that Piwi in OSCs is localized in the nucleus as in ovaries. (d) RT-PCR for *Ago3* and *Dmhen1/Pimet*. Expression of *Ago3* was not detected in OSCs. (e) qRT-PCR confirmed that the level of *ago3* expression is very low in OSCs (100-fold lower compared with that in ovaries and 1,610-fold lower compared with *piwi* mRNA in OSCs). (f) Small RNAs associated with Piwi showed resistance to periodate oxidation/b-elimination treatment, a hallmark for 2'-*O*-methyl modification at the 3' end. (g) Western blotting data (left panel) show that Piwi was successfully depleted from OSCs by siPiwi-mediated RNAi. The sequences of siRNAs (siPiwi and siEGFP) used in these experiments are shown in Supplementary Table 2. siPiwi corresponds to the N-terminal end of Piwi, spanning from glutamic acid58 to glycine64. Even in OSCs, where Piwi was depleted, piRNAs were efficiently loaded onto Piwi (Piwi-DN-DDAA mutant) (right panel). These results support a model in which the primary piRNA processing and the piRNA loading on Piwi appear to occur in the cytoplasm in a Piwi-Slicer-independent manner.

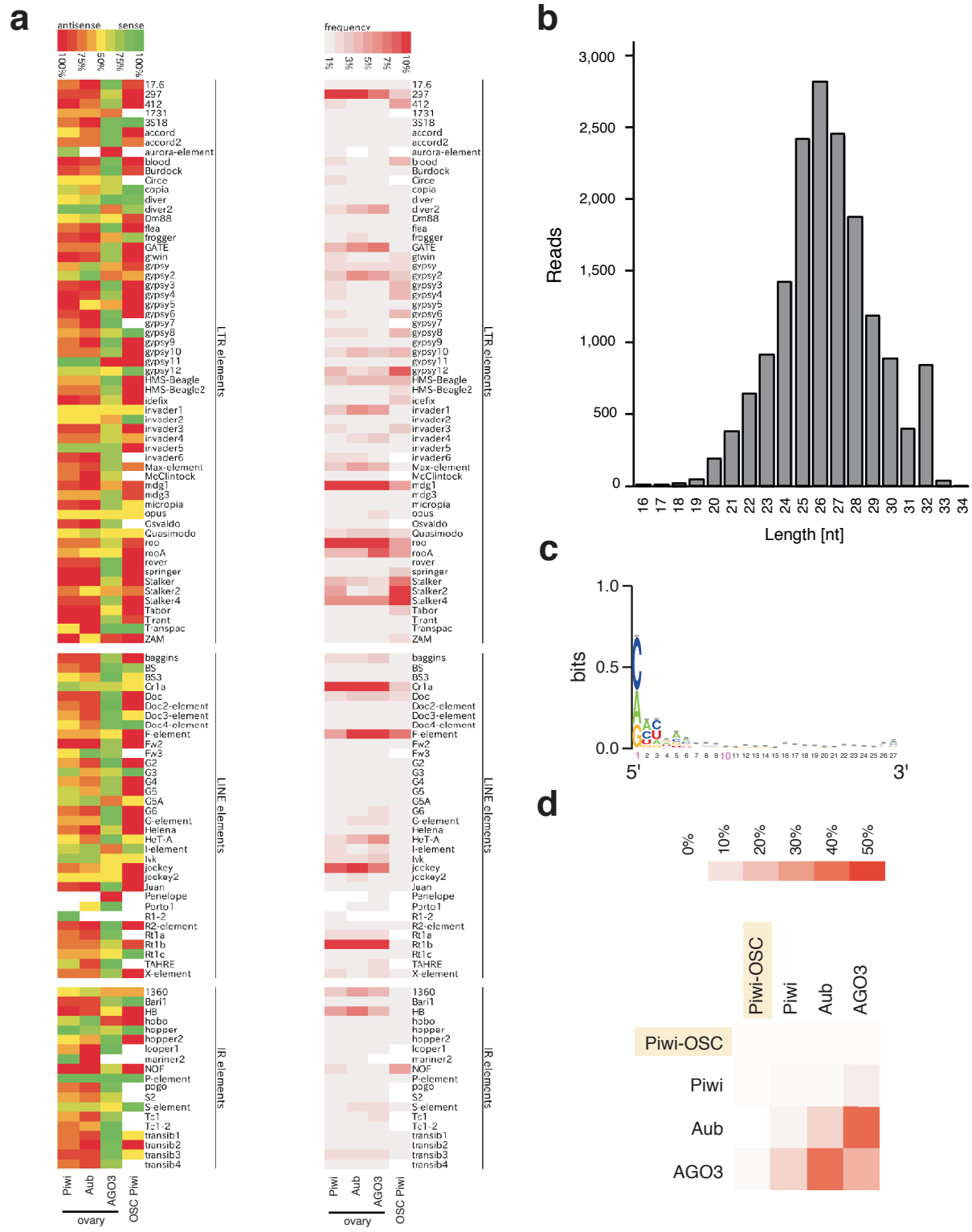

Figure S2. Bioinformatic analysis for piRNAs in OSC. (a) The heat map (left panel) indicates the strand bias of cloned piRNAs and piRNAs[3] with respect to canonical transposon sequences (indicated on the right side). Transposons are grouped into long terminal repeat (LTR), long interspersed nuclear (LINE) and inverted repeat (IR) elements. The color intensities indicate the degree of strand bias (green, sense; red, antisense; yellow, unbiased). The cloning frequencies of individual transposons in all four complexes (this study and ref. 3) are indicated as a heat map (right panel). (b) Distributions of OSC-piRNA base sizes. (c) Exclusion of piRNAs with 1st-U from the piRNA pool did not uncover any other obvious bias, including 10th-A. (d) Profiles of 10-base binding partners representing the ratio of piRNAs in a dataset that form 10-base binding to another piRNA dataset. 153 pairs were found in total 16,511 piRNAs in OSCs.

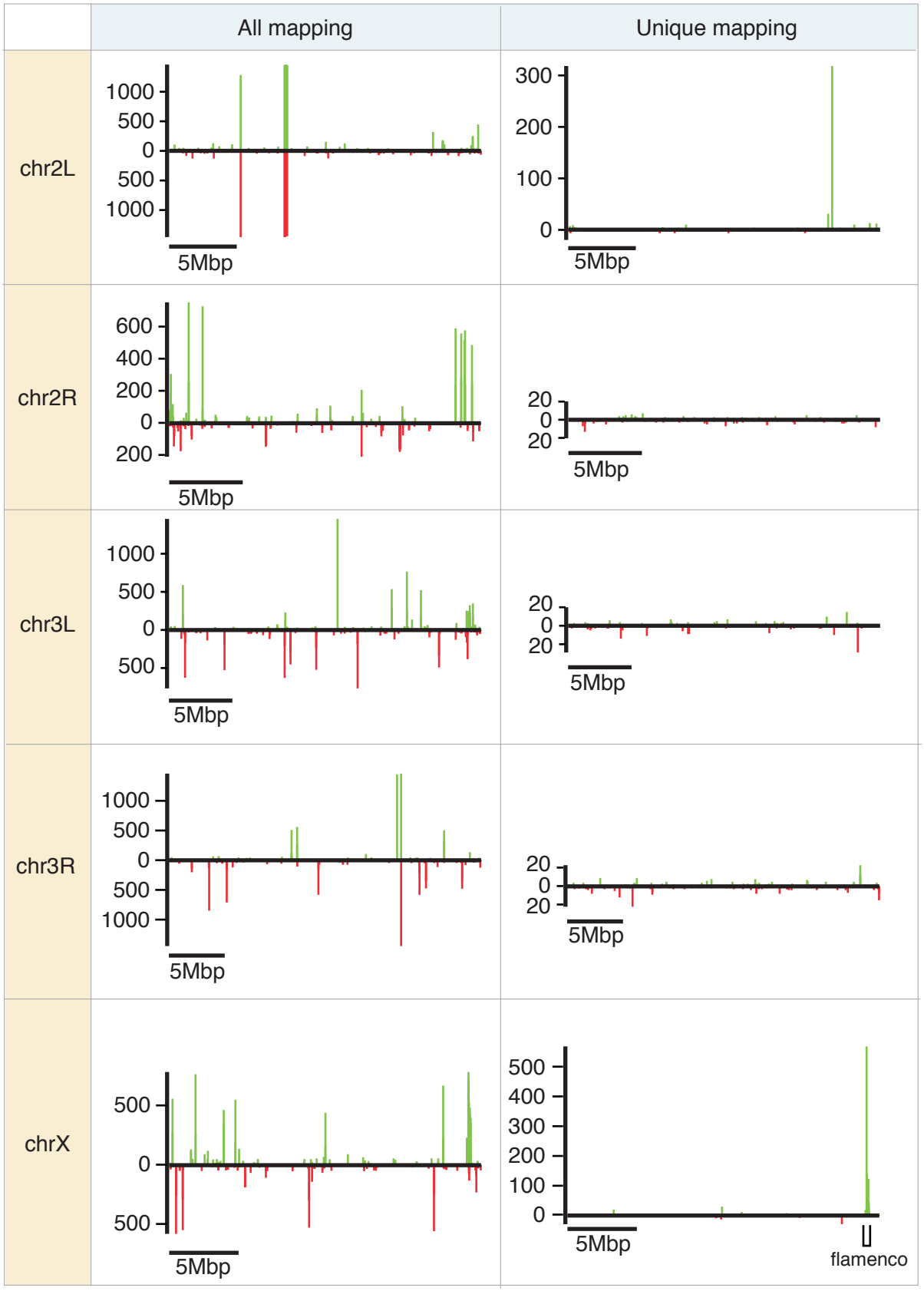

Figure S3. Frequency maps of OSC-piRNAs. Frequencies of mapped piRNAs are depicted as bar graphs aligned on chromosomes. The green bars represent the forward strand while the red bars are the reverse strand. Frequency maps for piRNAs that have a single copy in the genome are presented on the right column. Apparent spikes are found in a neighboring region of the centromere of chromosomes 2L and X.

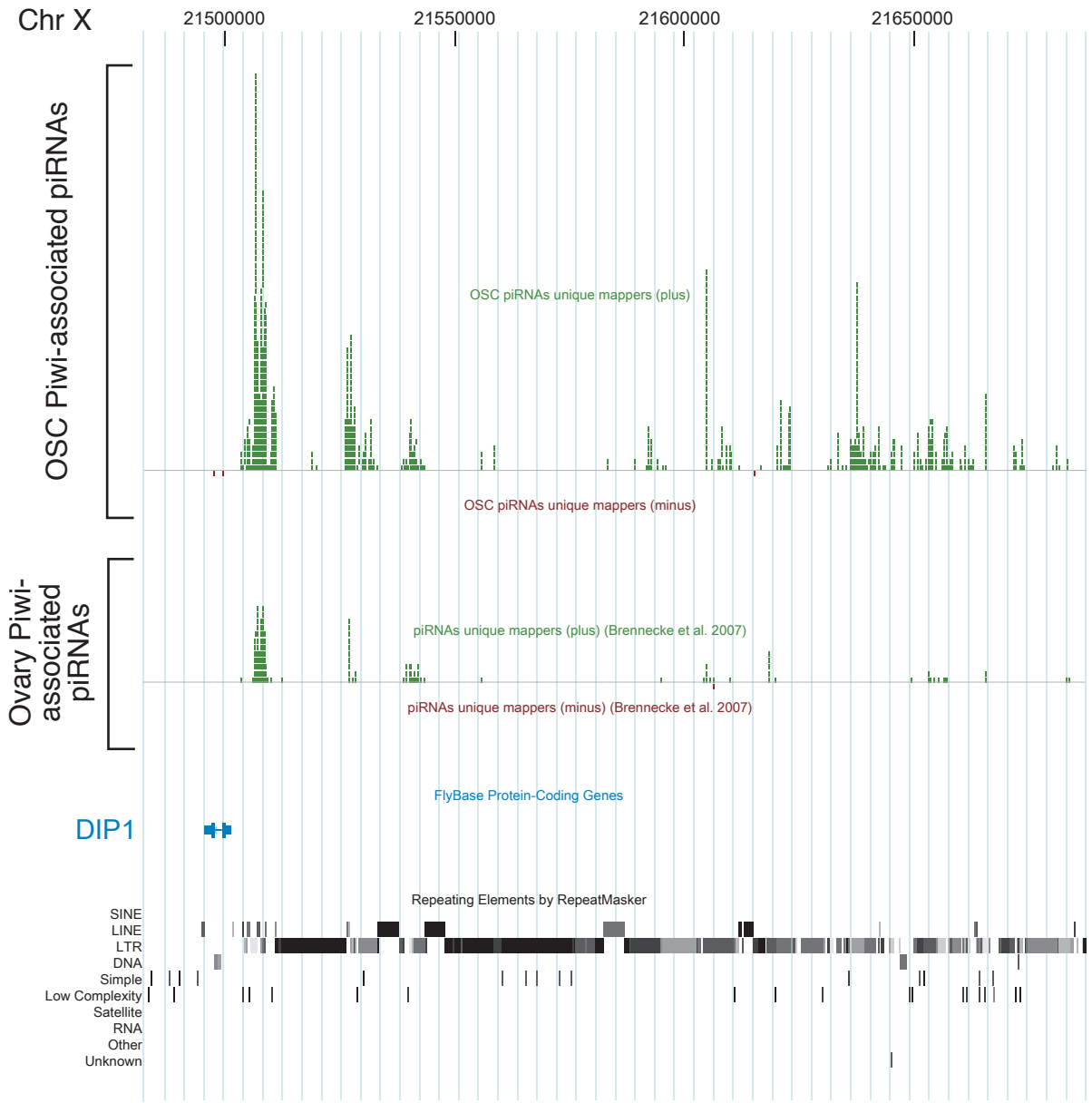

Figure S4. piRNAs (unique mappers) derived from *flam* are summarized. The top panel (OSC Piwi-associated piRNAs) shows *flam*-piRNAs found in our study. The middle panel (ovary Piwi-associated piRNAs) shows *flam*-piRNAs found in the previous study by Brennecke et al.[3]. The bottom shows repetitive elements found in the region. The location of *DIP1*, a protein-coding gene located in *flam*, is also shown.

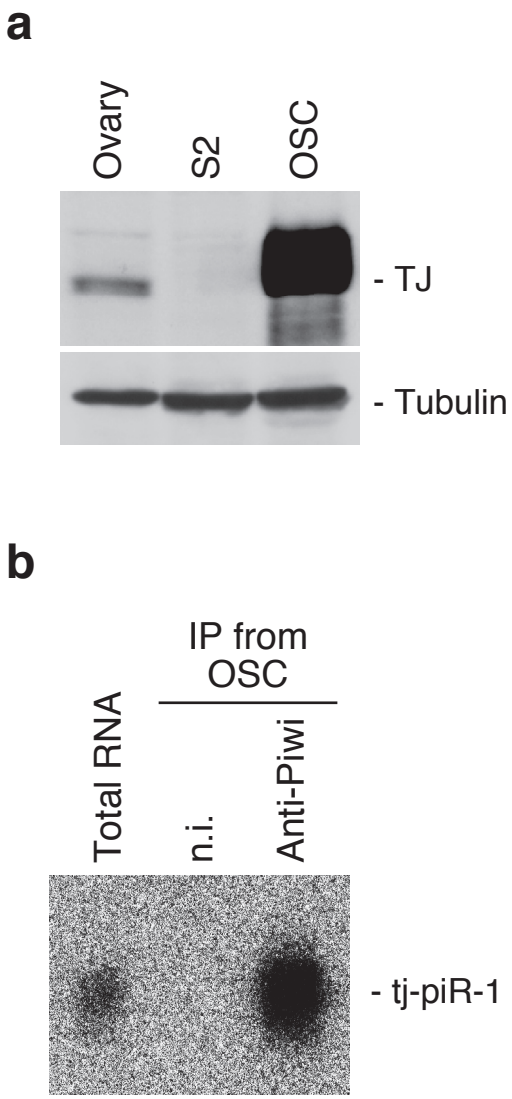

Figure S5. The *tj* gene gives rise to TJ protein and *tj*-piRNAs in OSC. (a) Western blotting analysis shows that TJ is strongly expressed in OSCs. Anti-Tubulin was used as an internal control. (b) Northern blotting analysis shows that one piRNA originating from *tj* mRNA, tj-piR-1, indeed exists in small RNAs associating with Piwi in OSCs. n.i.: non-immune IgG was used as a negative control. Total RNA: 5 mg of total RNA of OSCs was used.

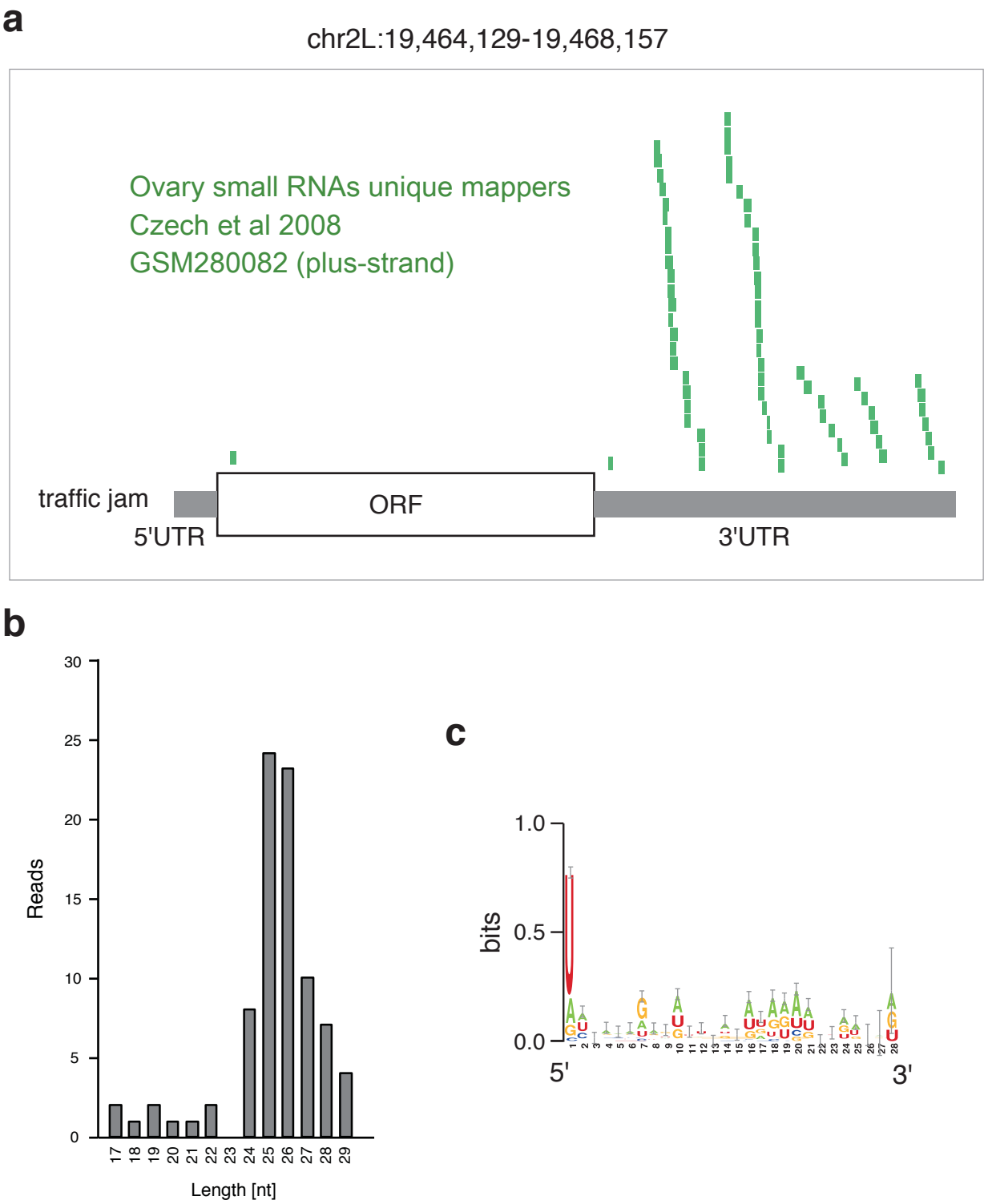

Figure S6. *tj*-derived piRNAs found in the ovarian total small RNA library by Czech *et al*[20]. (a) piRNAs originating from *tj* mRNA 3'UTR (green bars; all sense-oriented) were also found in the ovarian small RNA library produced by Czech *et al.*[20]. (b) The size distribution of *tj*-piRNAs shown in (a). (c) Examination of nucleotide bias indicated that *tj*-piRNAs shown in (a) have mostly uracil at the 5' ends (1st-U).

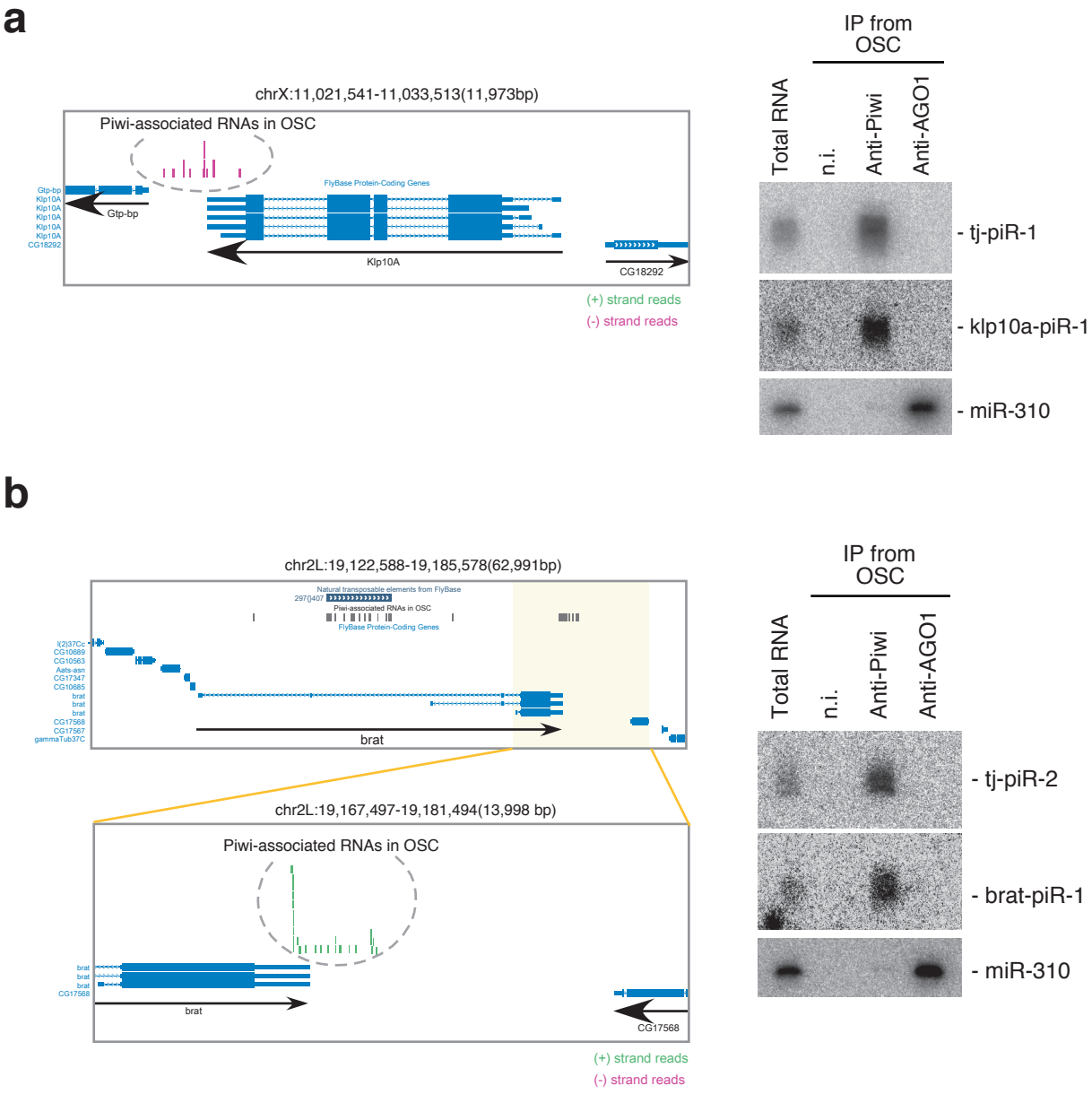

Figure S7. *klp10a*- and *brat*-derived piRNAs in OSC. (a) Some OSC-piRNAs were found to be derived from the 3' UTR of the *klp10a* located on the X chromosome. Note that some others originated from the flanking region of *klp10a* at its 3' end. These might arise from the *klp10a* mRNA precursor or mature mRNA that contains a longer 3' UTR than those registered in the FlyBase as FlyBase Protein-coding genes. Northern blot analysis (right panel) shows that klp10a-piR-1 indeed exists in small RNAs associating with Piwi in OSCs, but was not loaded onto AGO1 in OSCs. n.i.: non-immune IgG was used as a negative control. Total RNA: 5 mg of total RNA of OSCs was used. (b) OSC-piRNAs derived from the 3' UTR of *brat*, located on chromosome 2L were also observed. Note that some others originated from the flanking region of *brat* at its 3' end. These might arise from the mRNA precursor or mature mRNA that contains a longer 3' UTR than those registered in the FlyBase. Northern blotting analysis (right panel) shows that brat-piR-1 exists in small RNAs associating with Piwi in OSCs, but was not loaded onto AGO1 in OSCs. n.i.: non-immune IgG was used as a negative control. Total RNA: 5 mg of total RNA of OSCs was used.

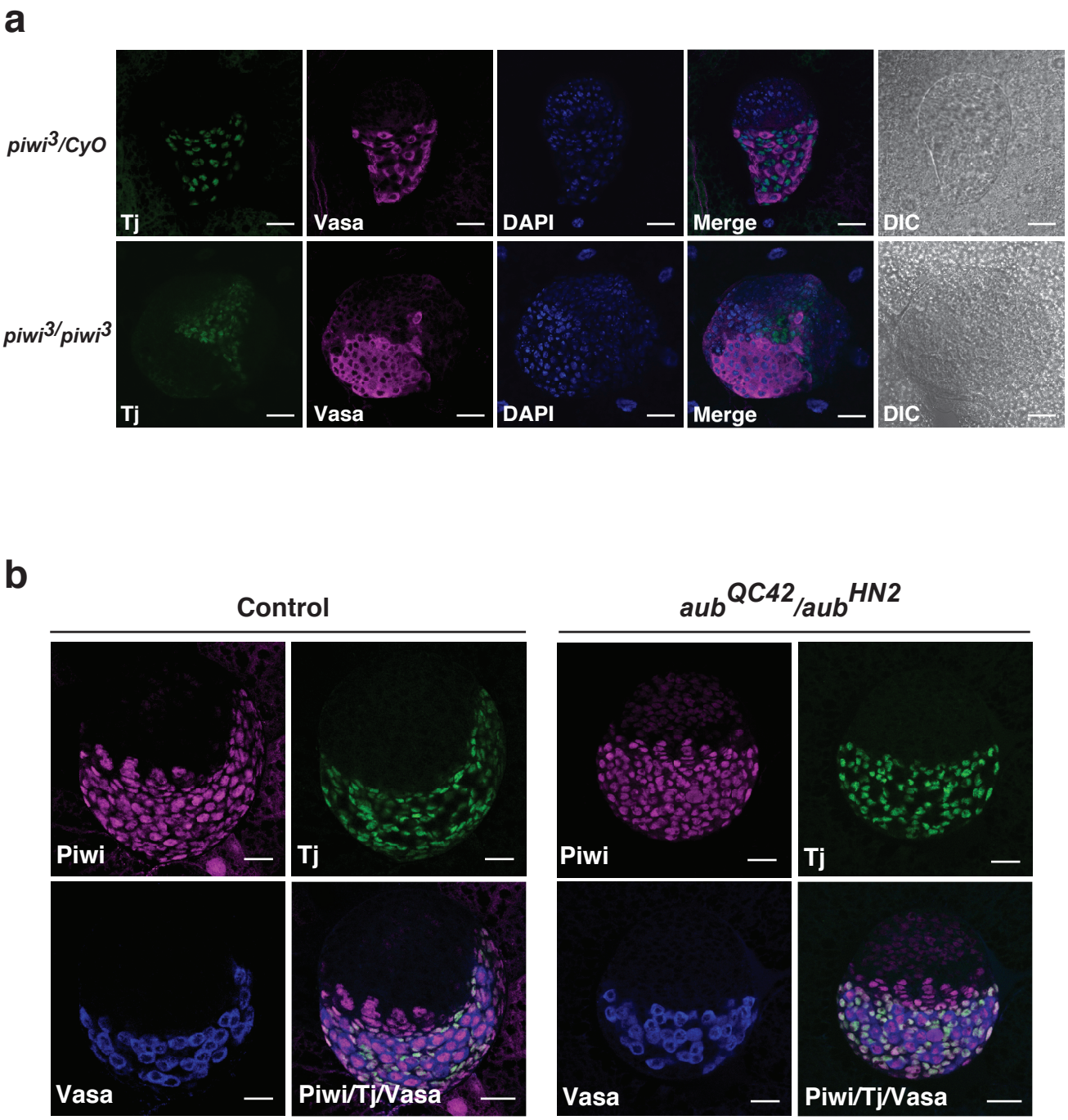

Figure S8. Immunofluorescence analyses of *piwi* and *aub* mutant larval ovaries. (a) In *piwi* mutant larval ovaries, TJ-positive cells fail to intermingle with PGCs. Interstitial cells and GSCs found in late third instar larval ovaries express TJ (green) and Vasa (magenta), respectively. In control (*piwi*^{3}/*CyO*), TJ-positive cells and PGCs form a mixed cell population. By contrast in *piwi* homo mutant (*piwi*^{3}/*piwi*^{3}) ovaries, TJ-positive cells do not intermingle with PGCs, but instead form coherent clusters. Scale bars; 20 μm. (b) *aub* mutants (*aub*^{QC42}/*aub*^{HN2}) did not show the failure of intermingling phenotype. Scale bars; 20 μm.

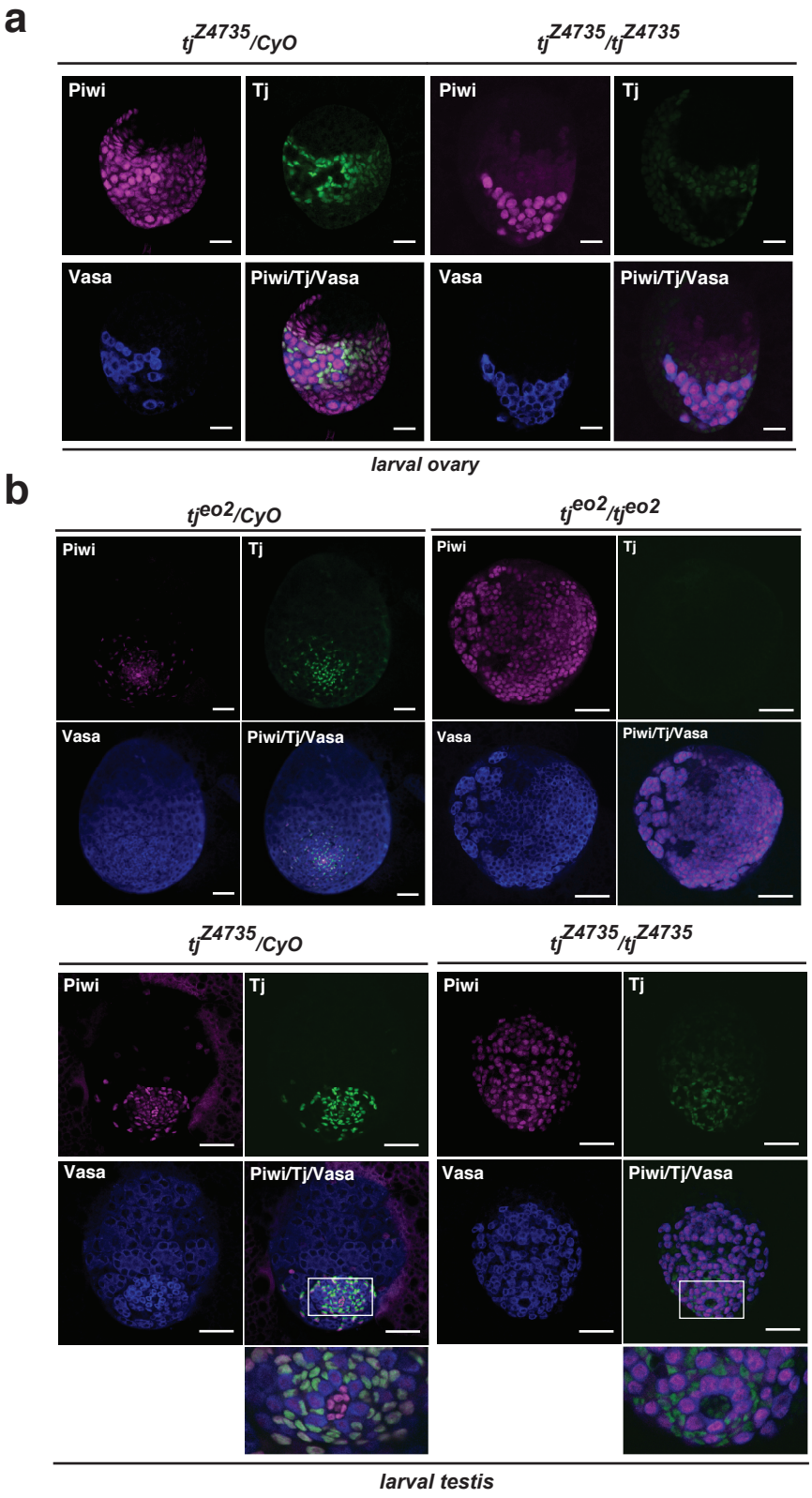

Figure S9. Immunofluorescence analyses of *tj* mutant larval ovaries and testes. (a) In the control (tj^{Z4735}/CyO) Piwi (magenta) is expressed in both Tj- (green) and Vasa (blue)-positive cells. In the *tj* mutant (tj^{Z4735}/tj^{Z4735}), somas do not intermingle with PGCs. Piwi is not expressed in mutant somas. (b) In control (tj^{eo2}/CyO and tj^{Z4735}/CyO) testes, Piwi is strongly detected in somas. In tj^{eo2}/tj^{eo2} and tj^{Z4735}/tj^{Z4735} testes, expression of Piwi is no longer detected in somas but is strongly detected in Vasa-positive GSCs and developing cells. Piwi, TJ and Vasa are shown in magenta, green and blue, respectively. Scale bars; 50 mm. In the *tj* mutant (tj^{Z4735}/tj^{Z4735}) testes, Piwi is not expressed in somatic cells but is highly detected in GSCs and their developing cells. The bottom panels show magnification of the boxed areas in the middle panels. Scale bars; 50 μm.

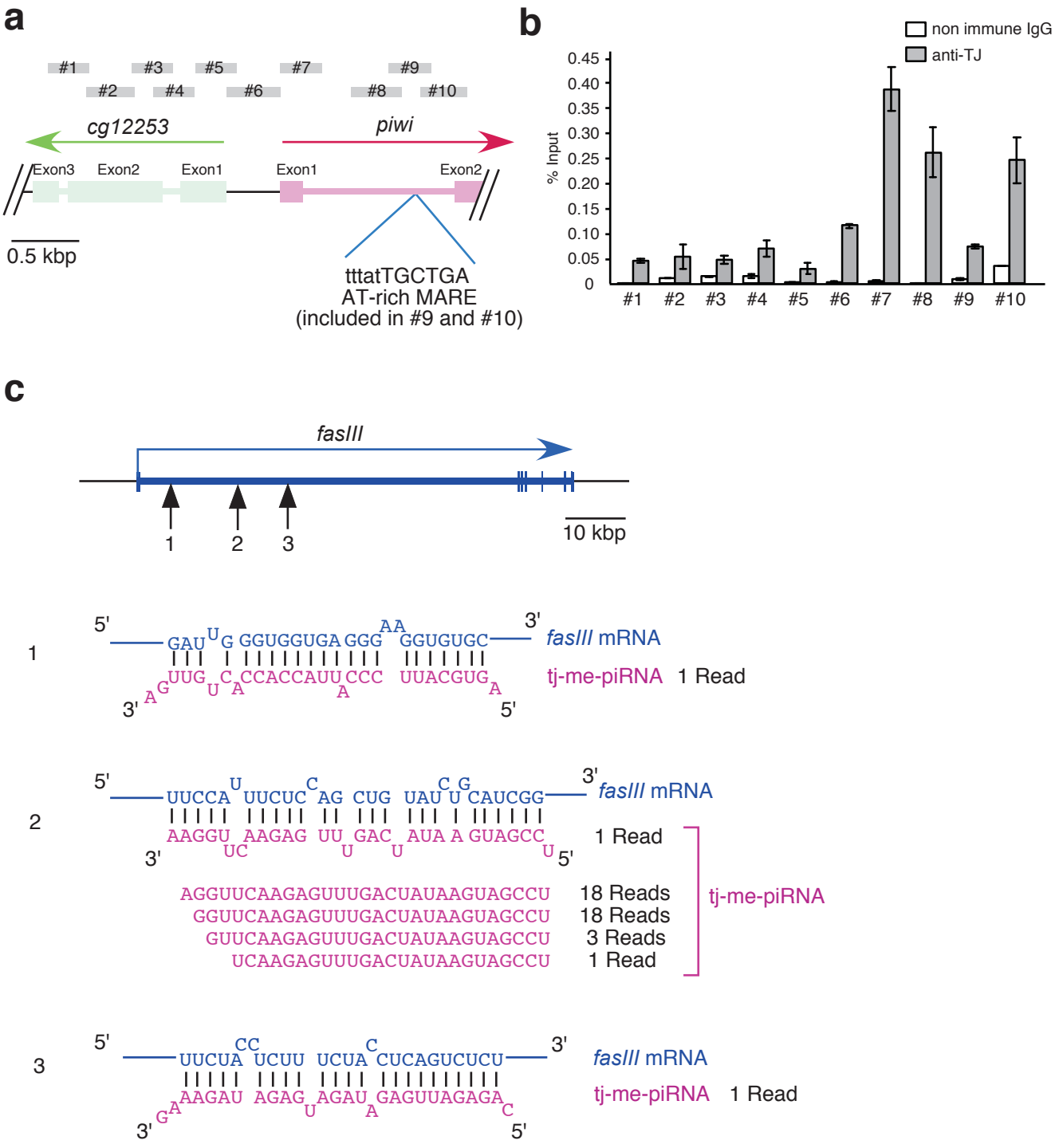

Figure S10. Targeting prediction. (a) ChIP analysis using anti-TJ antibody. Short DNA sequences [summarized in (a); #1 to #10] near the putative transcriptional start site of the *piwi* gene (corresponding to the 5' end of Exon1) were examined for association with TJ in OSCs. AT-rich half MARE (Maf-recognition element), which is conserved in many genes regulated by Maf factors in various species[28], is found in both #9 and #10 DNA fragments. Note that AT-rich half MARE found in *D. melanogaster piwi* gene (tttatTGCTGA) is conserved in a variety of *Drosophila* species, such as *D. pseudoobscura* (aatatTGCTGA) and *D. mojavensis* (aaattTGCTGA). (b) TJ directly associates with *piwi* gene. non-immune IgG was used as a negative control. (c) Some of *tj*-me-piRNAs identified in this study show strong complementarities to the *fasIII* precursor transcript.

Reads	Number of gene	Genes List	Total reads
322	1	traffic jam	322
31	1	CG32000	31
29	1	jim	29
24	1	brat	24
22	3	CG41099, Art4, Nipped-A	66
20	1	dm	20
16	1	Tif-IA	16
14	1	Chd64	14
13	3	trol, CG41580, klp10A	39
12	3	krz, plexA, CG32654	36
10	5	fng, Atpalpha, CG9257, Sema-1a, CG10289	50
1-9	1206		2123
		Total	2770

Table S1. Cloning profiles of piRNAs derived from coding genes. A number of coding genes give rise to piRNAs from their 3'UTRs. This table shows that 1,227 coding genes are sources of at least one copy of a piRNA. A small set of coding genes produce more than 10 copies of piRNAs from their 3'UTRs. Among them, *traffic jam* stands out with an excessive number of piRNAs produced.

Supplementary Table 2 Sequences of primers used for qRT-PCR, construction of Piwi mutants and ChIP analysis and RNAi. All sequences are shown in 5' to 3' direction.

Experiment	Gene	Primer forward	Primer reverse
Mutagenesis Primer	Piwi D614A	GACTGATGACAATTGGCTTTGCC	CTCGTGTGCTCTTCGCAATGGCAAA
		ATTGCGAAGAGCACACGAG	GCCAATTGTCATCAGTC
	Piwi D685A	GCCATCTCGAATCGTATTTTATCG	GAGAGCCGGAGCTCACACCGGCTC
		AGCCGGTGTGAGCTCCGGCTCTC	GATAAAATACGATTCGAGATGGC
qRT-PCR	RP49	CCGCTTCAAGGGACAGTATCTG	ATCTCGCCGCAGTAAACGC
	Piwi	CAAGGCCGGATAATTGGACA	CCATCGCTCGGAGTGGTAAG
	Aubergine	GGGATGACCAGCAAGAAAGG	AAGACCGTCGCAGTCGTGTA
	AGO3	CTGCATTTGTGGCCTCCATA	GGGGAGTTTGCCATTCCTTT
	FasIII	AACCCAACACAGCGCTCCTC	ATCCGGGTGTCTTGCTCCAC
	Traffic Jam	ACCAGTGGCACATGGACGAA	CGCTCCCGAAGATGTGTTCA
	Zucchini	CCCCATTACCACGAACTTGA	CCAAGAGCCGTCCAGTTTAC
ChIP-qPCR	Piwi #1	TGTCATCTCCGGATTTTGGT	ACCTACGCATCGGCTAGAAA
	Piwi #2	TTTCTAGCCGATGCGTAGGT	GTGAGGTTTCAACCGGAAAA
	Piwi #3	TTTTCCGGTTGAAACCTCAC	AACCGTGTGAACTGGCTTTC
	Piwi #4	ACGCGCCTAGTTATTTTTGC	ACGGGATGACTTGGAAATTG
	Piwi #5	AATTCTGCGAAGGTTTTCCT	CAATCGTTGCAAGAGCCTTA
	Piwi #6	GCAGGGTGGCTGTTTTGTAT	GTGACCAAATGGCCAGATTT
	Piwi #7	ACTGAGTCCAAAGCGTCGTT	GGTGTCGAATTCCGAAGAAA
	Piwi #8	ACCATCACCTGACAGGAAGG	CTAACGGTCAAGCTACGAAAA
	Piwi #9	TGCAGGCAAAGCATACTACAA	CGTGCGAGATTCAGCAATAA
	Piwi #10	CATCGCGATTGTTTTCAATG	TCCTCGAGAGCTCTTCTCTCTC

Experiment	siRNA name	siRNA sense	siRNA antisense
RNAi	EGFP-si	*GGCAAGCUGACCCUGAAGU*TT	*ACUUCAGGGUCAGCUUGCC*TT
	Piwi-si	*GCUCCCAGGCGUGAAGGUG*TT	*CACCUUCACGCCUGGGAGC*TT
	Zucchini –si	*GCAUUGCCGUCAGCACUGU*TT	*ACAGUGCUGACGGCAAUGC*TT