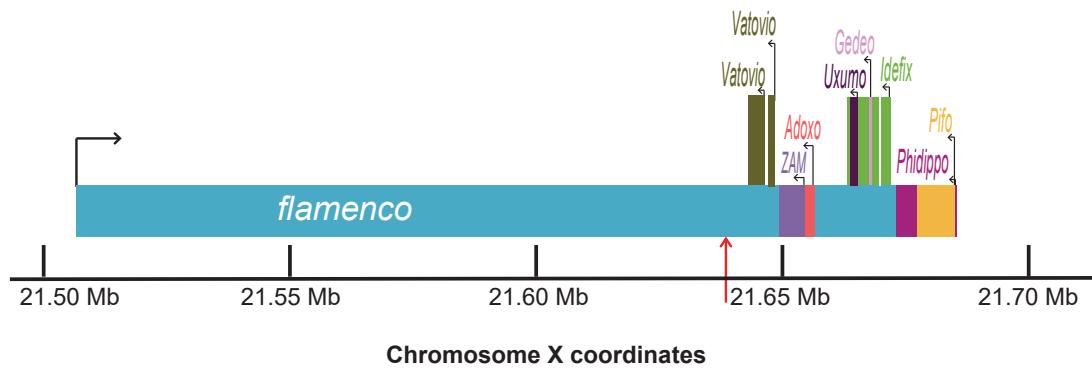


A



B

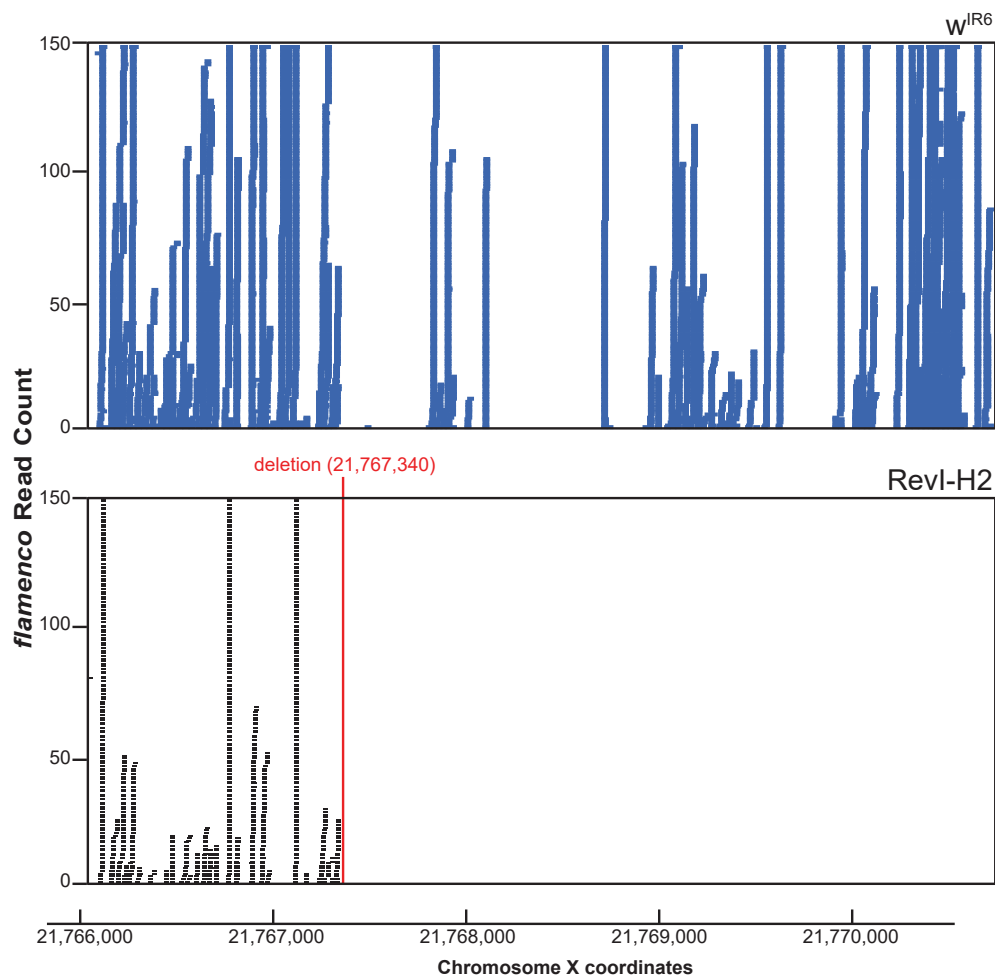


Figure S1. The RevI-H2 line carries a deletion that removes *ZAM* from the *flamenco* piRNA cluster.

a Structure of the *flamenco* piRNA cluster in *D. melanogaster*. TEs located in the region of *flamenco* deleted in RevI-H2 are presented individually above *flamenco* according to [26]. The centromere of the X chromosome is on the right-hand (proximal) side. Sense-strand transcription for *flamenco* and TE orientation are indicated by black arrows. The *flamenco* deletion distal break-point in RevI-H2 [26] is indicated by a red arrow. The chromosome coordinates are according to release 5 of the *D. melanogaster* genome. **b** Genome browser panel showing *flamenco* piRNA levels in *w^{IR6}* and RevI-H2 line. The refined Release 6.03 coordinate for the break-point of the RevI-H2 deletion in *flamenco* reported in [26] is displayed by a red line.

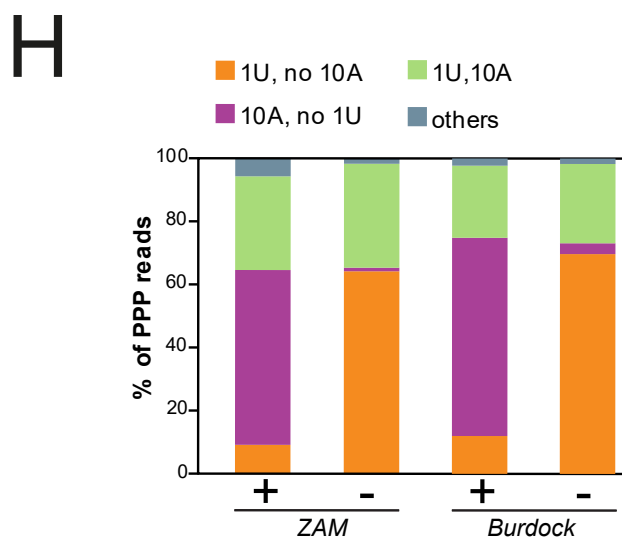
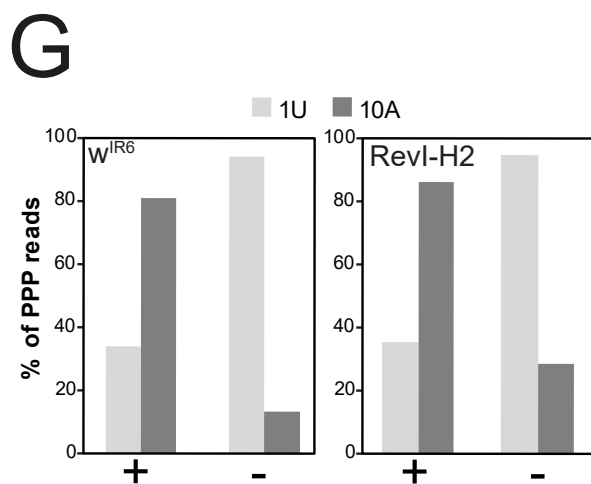
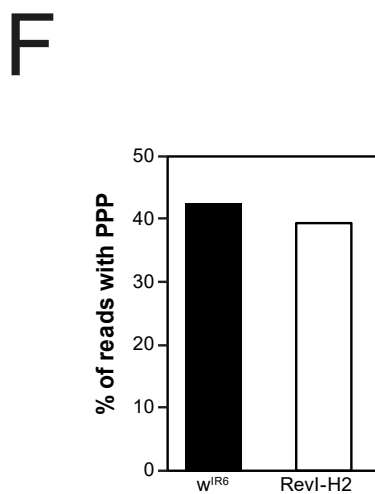
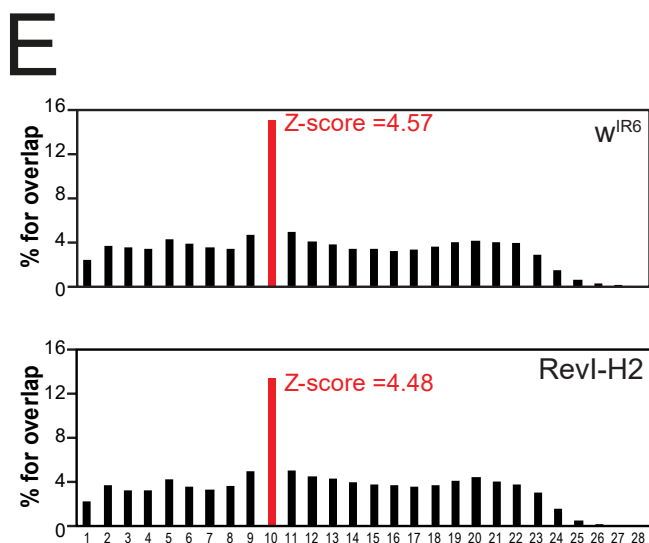
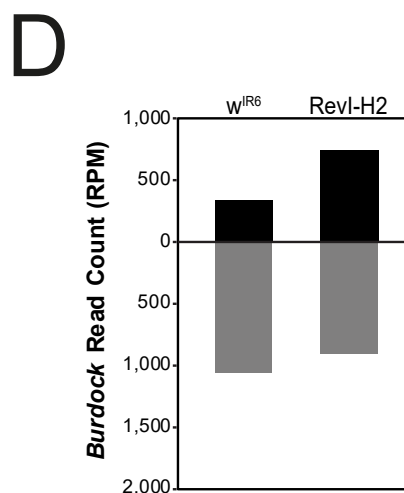
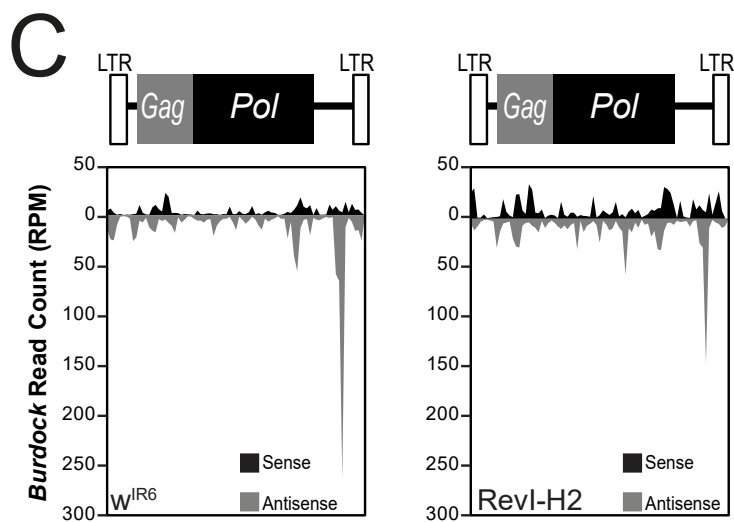
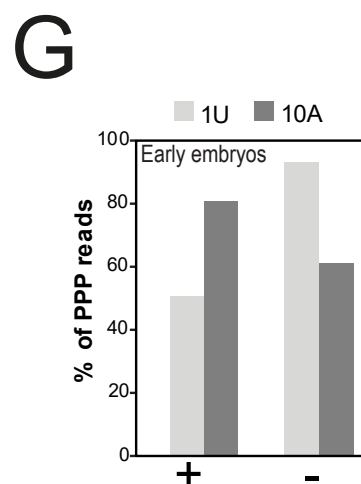
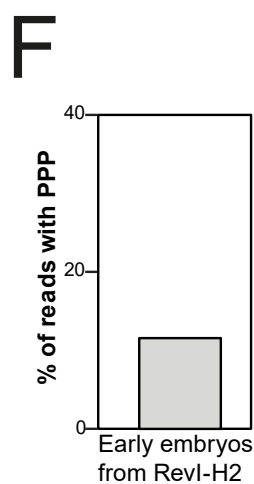
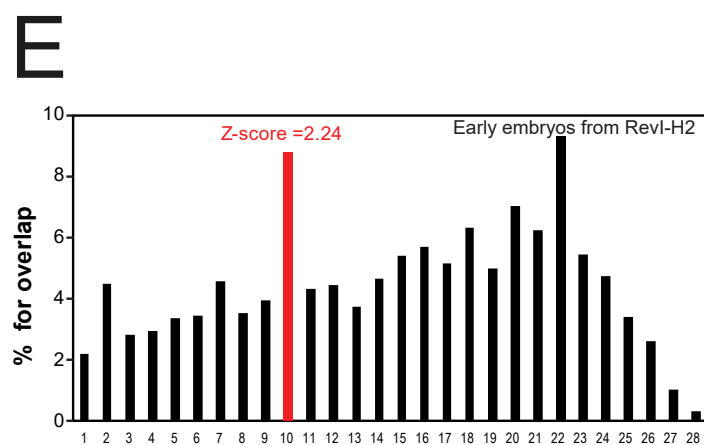
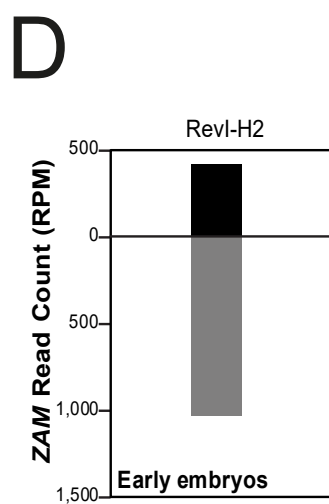
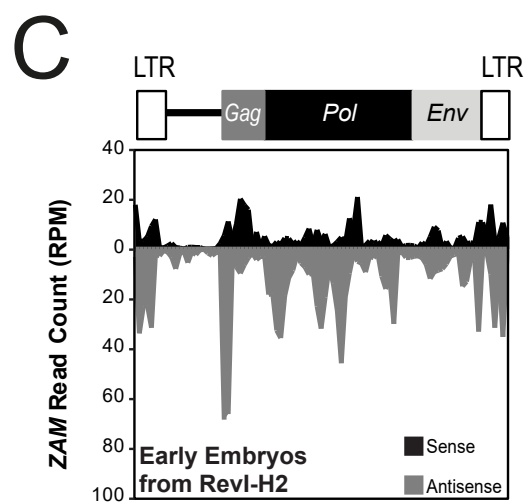
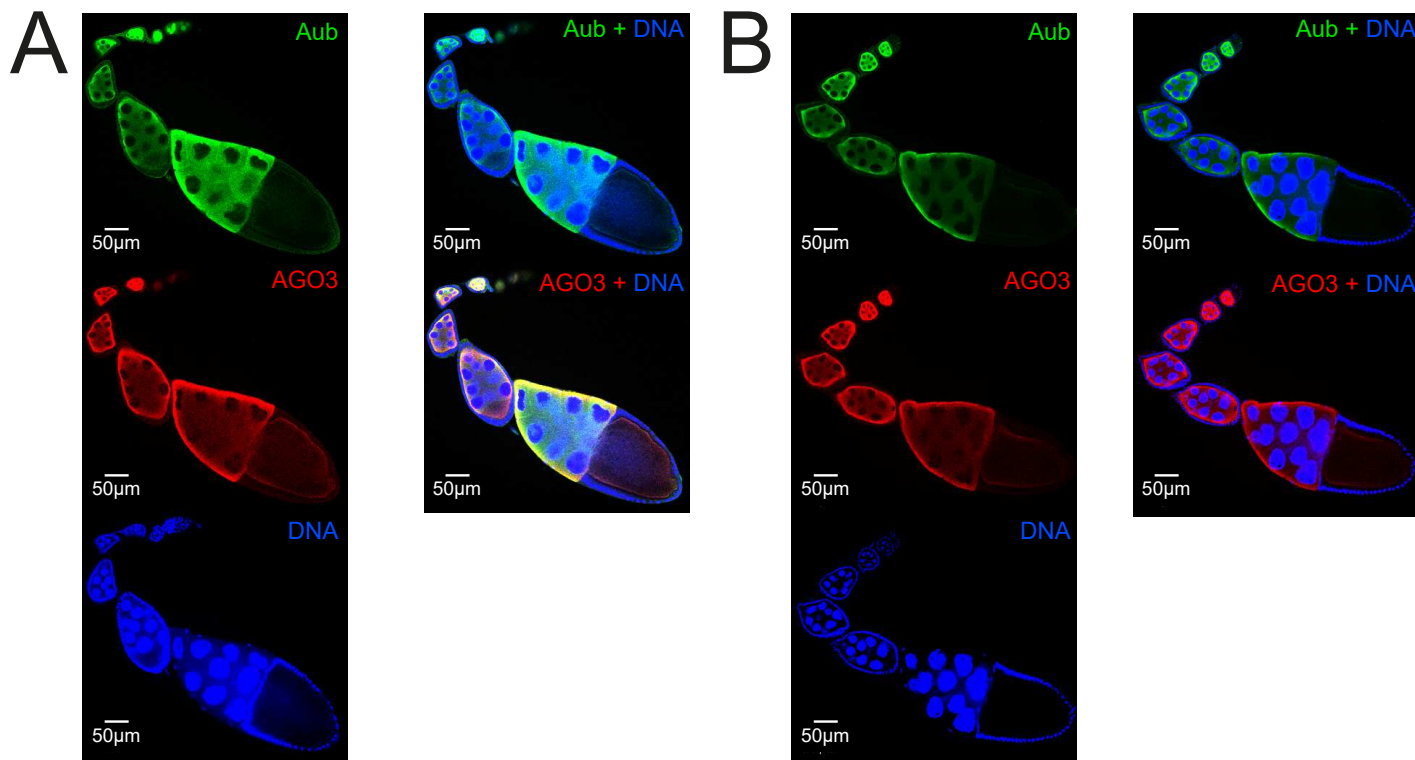
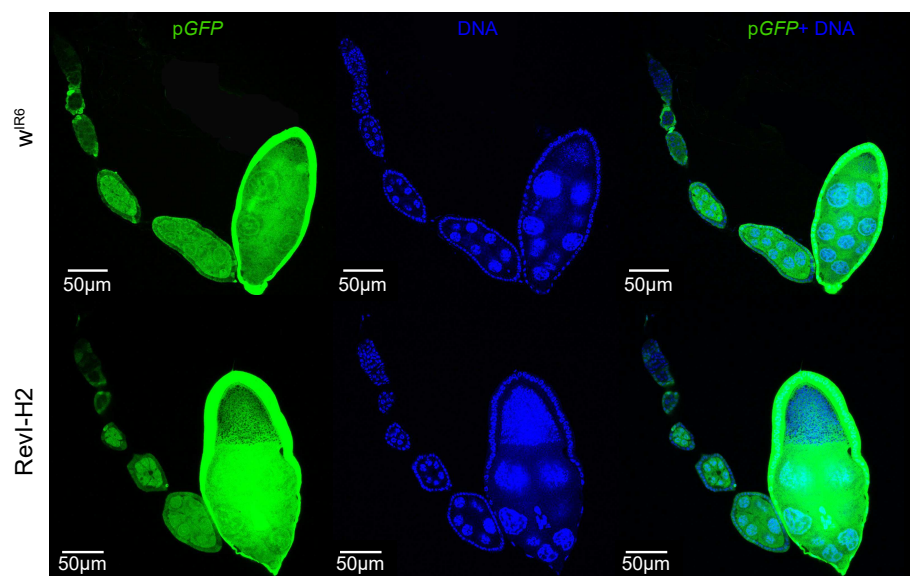


Figure S2. In Revl-H2 ovaries, piRNAs derived from *Burdock*, the prototypic germinal TE, present similar features as those derived from *ZAM*.

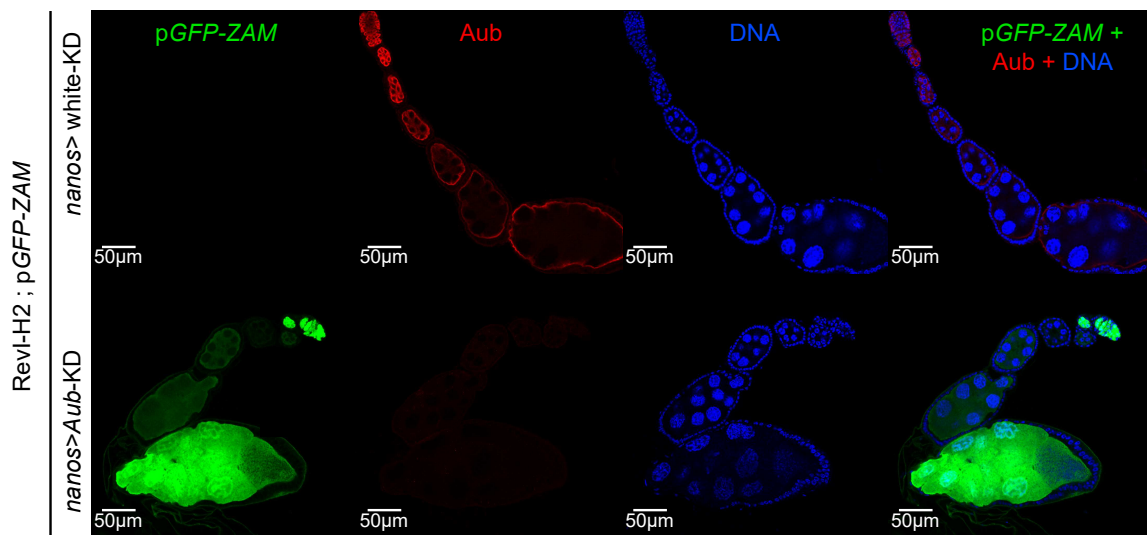
a-b Logo of nucleotide bias for the first ten positions of sense (*a*) and antisense (*b*) *ZAM*-derived piRNAs with ping-pong partner (PPP) produced in Revl-H2 ovaries. The nucleotide height represents its relative frequency at that position. **c** Density profile of *Burdock*-derived piRNAs along the 6.4kb *Burdock* sequence in *w^{IR6}* (left) and Revl-H2 (right) ovaries (all mapping piRNAs allowing up to 3 mismatches). Sense and antisense reads are in black and grey, respectively. The organization of *Burdock* is displayed above the profiles. **d** The total amount of *Burdock*-derived piRNAs produced in *w^{IR6}* and Revl-H2 ovaries was quantified from the profiles in *c*. **e** Histogram showing the percentage of 5'-overlaps between sense and antisense *Burdock*-derived piRNAs (23-29nt) in *w^{IR6}* (top) and Revl-H2 (bottom) ovaries. The peak in red defines the proportion of 10nt-overlapping pairs and the Z-score is indicated. **f** Bar diagram indicating the percentage of *Burdock*-derived piRNAs with ping-pong partner (PPP) in the *w^{IR6}* and Revl-H2 lines. **g** Analysis of the nucleotide bias for sense (+) and antisense (-) *Burdock*-derived piRNAs with PPP in *w^{IR6}* (left) and Revl-H2 (right). The percentages of PPPs with a 1U and 10A are displayed. **h** Analysis of nucleotide bias for sense (+) and antisense (-) *ZAM*- and *Burdock*-derived piRNAs with PPPs in Revl-H2 ovaries.



H



I



J

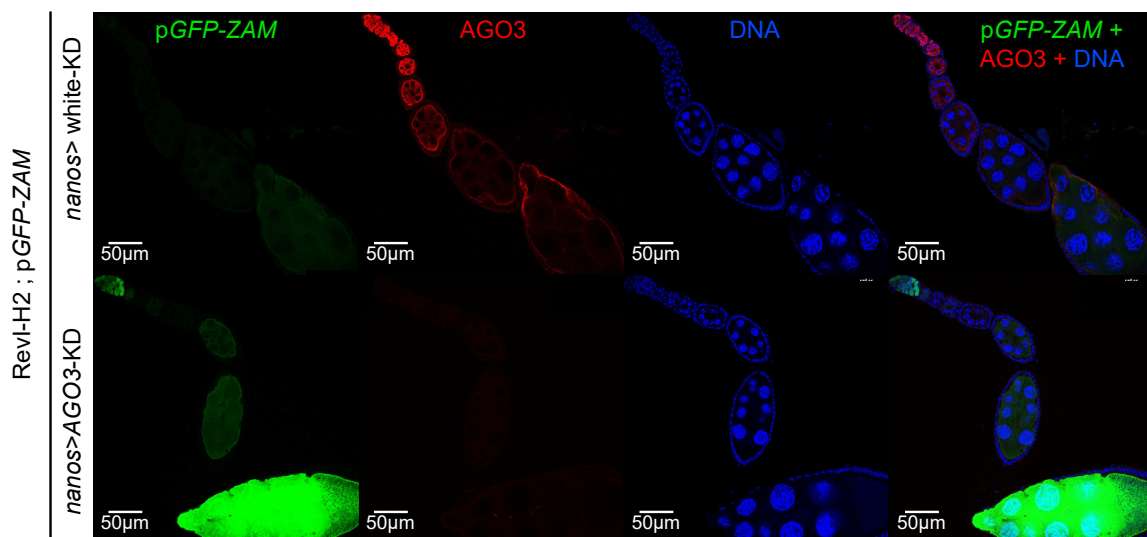
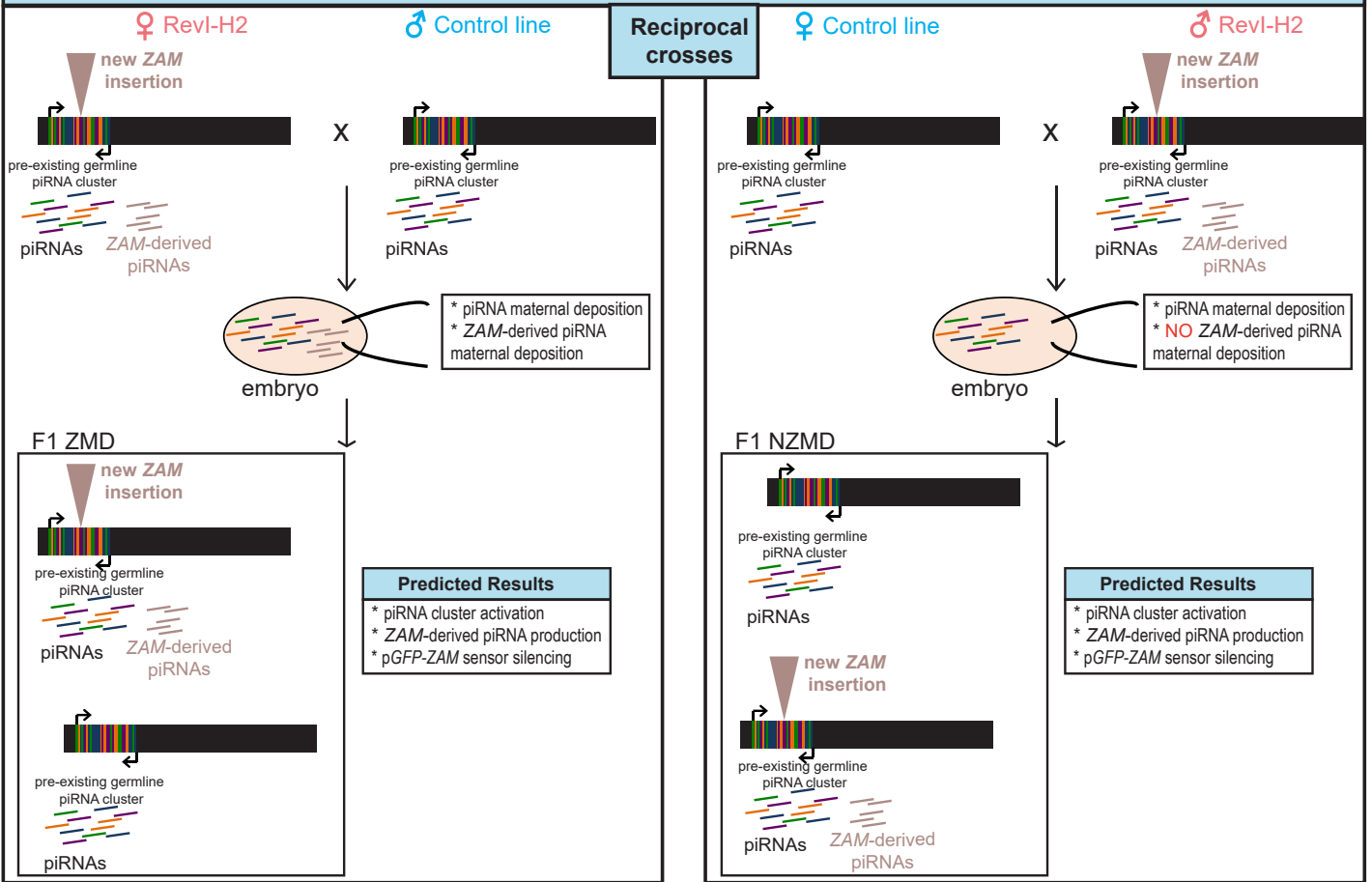


Figure S3. ZAM-derived piRNAs are *de novo* produced by the germline of Revl-H2 ovaries.

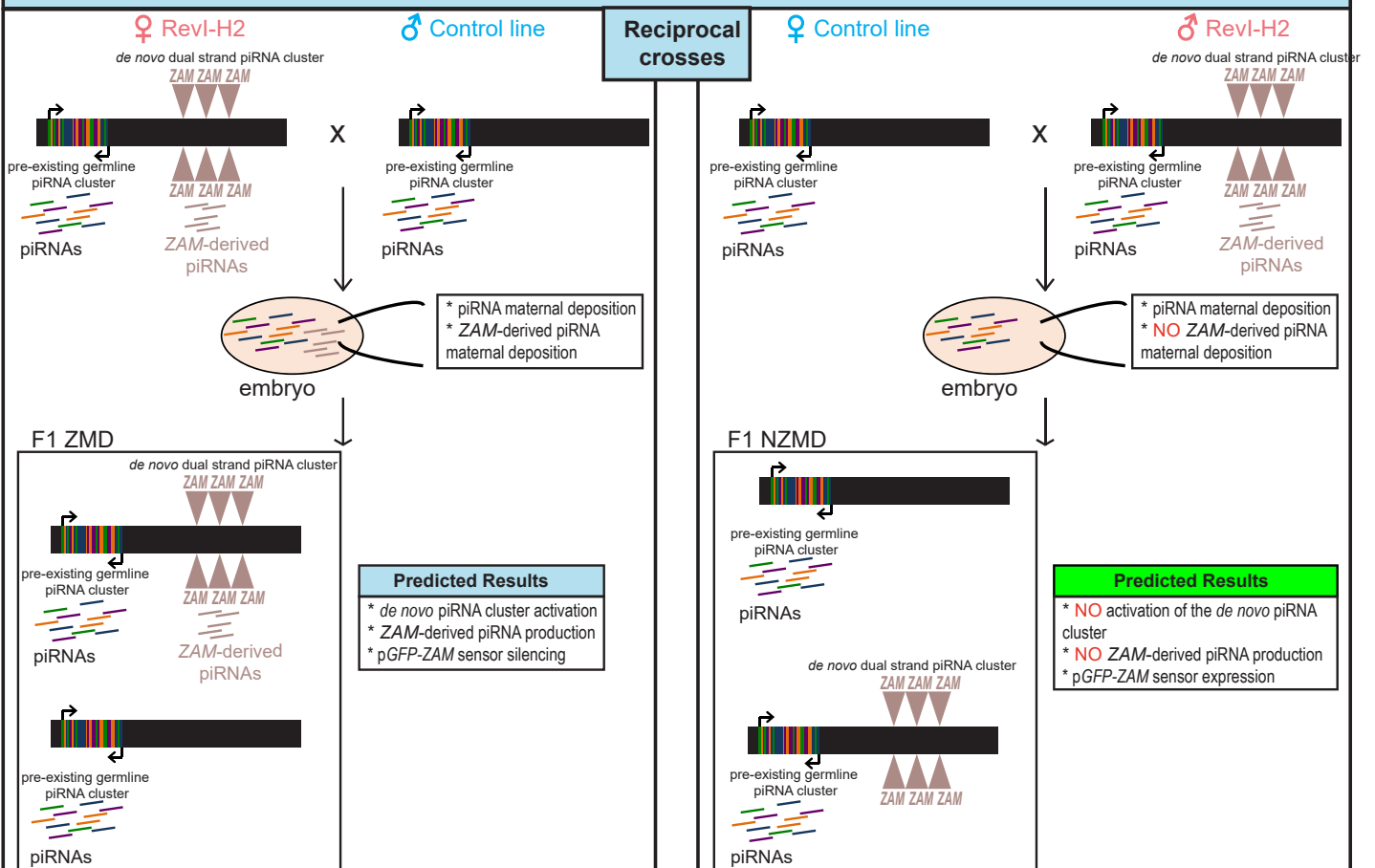
a-b Confocal images of ovarioles from *w^{IR6}* (a) and Revl-H2 (b) ovaries after labeling with anti-Aub (green, top) and -Ago3 (red, middle) antibodies and DNA (blue, bottom) staining. Merged images of the Aub or Ago3 signal and DNA staining are displayed on the right panels. **c** Density profile of ZAM-derived piRNAs along the ZAM sequence produced in early embryos from Revl-H2 (allowing up to 3 mismatches). Sense and antisense reads are represented in black and grey, respectively. ZAM organization is displayed above the profile. **d** The total amount of ZAM-derived piRNAs produced in early embryos from Revl-H2 was quantified from the profile in Fig. S3C. Sense and antisense reads are represented in black and grey, respectively. **e** Histogram showing the percentage of 5'-overlaps between sense and antisense ZAM-derived piRNAs (23-29nt) in early embryos from Revl-H2. The peak in red defines the proportion of 10nt-overlapping pairs and the Z-score is indicated. **f** Bar diagram indicating the percentage of ZAM-derived piRNAs with ping-pong partner (PPP) in early Revl-H2 embryos. **g** Analysis of the nucleotide bias for sense (+) and antisense (-) ZAM-derived piRNAs with PPPs in early Revl-H2 embryos. The percentages of PPPs with a 1U and those with a 10A are displayed. **h** Confocal images of ovarioles after GFP (green, left panels) and DNA (blue, middle panels) staining. Ovarioles were from the progeny of a cross between *w^{IR6}* or Revl-H2 females and males carrying the pGFP-ZAM sensor transgene – obtained after excision of the ZAM sequence upon recombination between the flanking FRTs – driven by *actin*-Gal4. Right panels, merged images of GFP and DNA labeling. **i-j** Confocal images of ovarioles after GFP (green), Aub (red, i) or AGO3 (red, j) and DNA (blue) staining. Ovarioles were from the progeny of a cross between Revl-H2 females carrying the *nanos*-Gal4 driver and males carrying the pGFP sensor transgene with either of the RNAi *Aub*- or AGO3-KD (Knock-Down). The white-KD was used as a control. Right panels, merged images of GFP, Aub (i) or AGO3 (j) and DNA labeling.

A

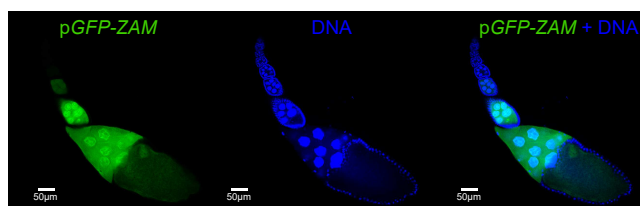
Hypothesis n°1: ZAM new insertion occurred in a pre-existing germline piRNA cluster



Hypothesis n°2: ZAM new insertions formed a *de novo* germline piRNA cluster

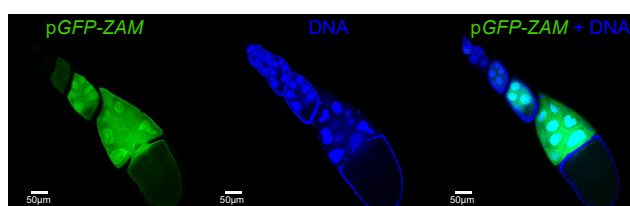


B



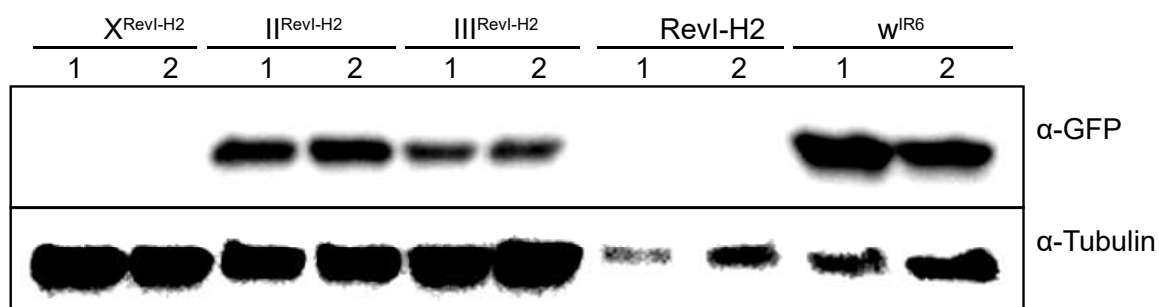
F1 progeny (cross female w^{IR6} with male pGFP-ZAM)

C

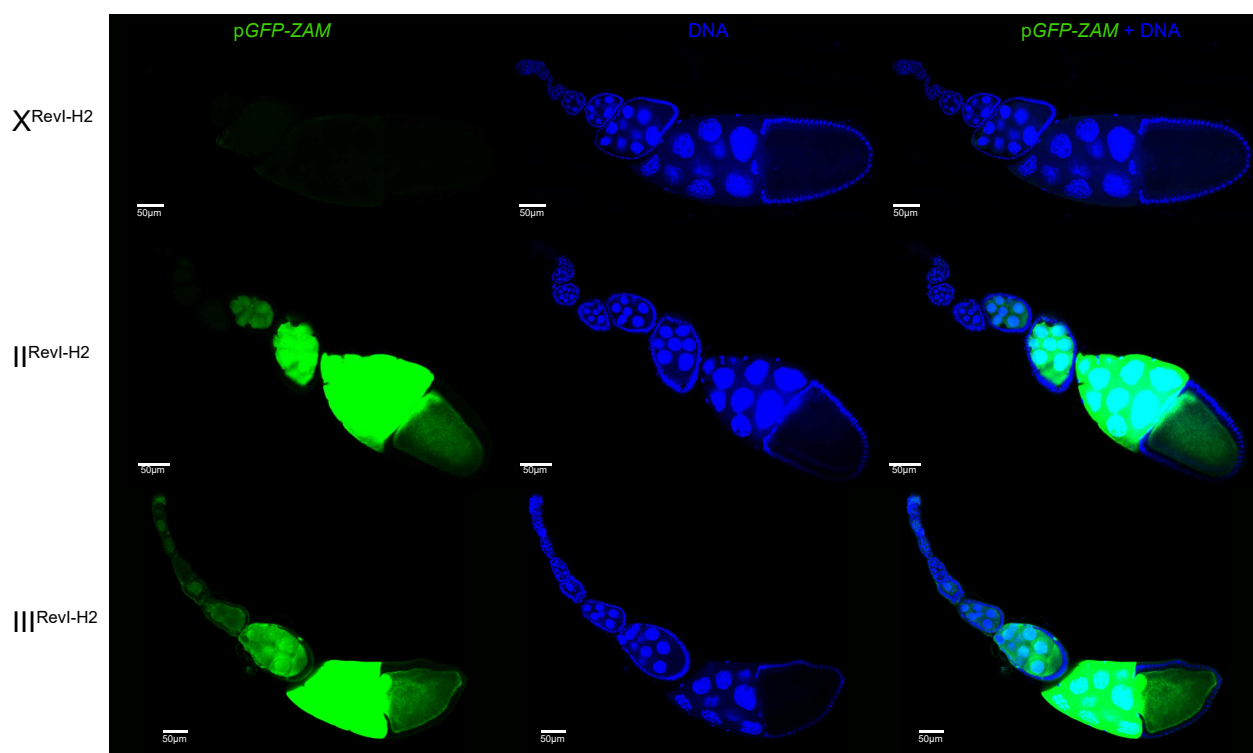


F1 progeny (cross male w^{IR6} with female pGFP-ZAM)

D



E



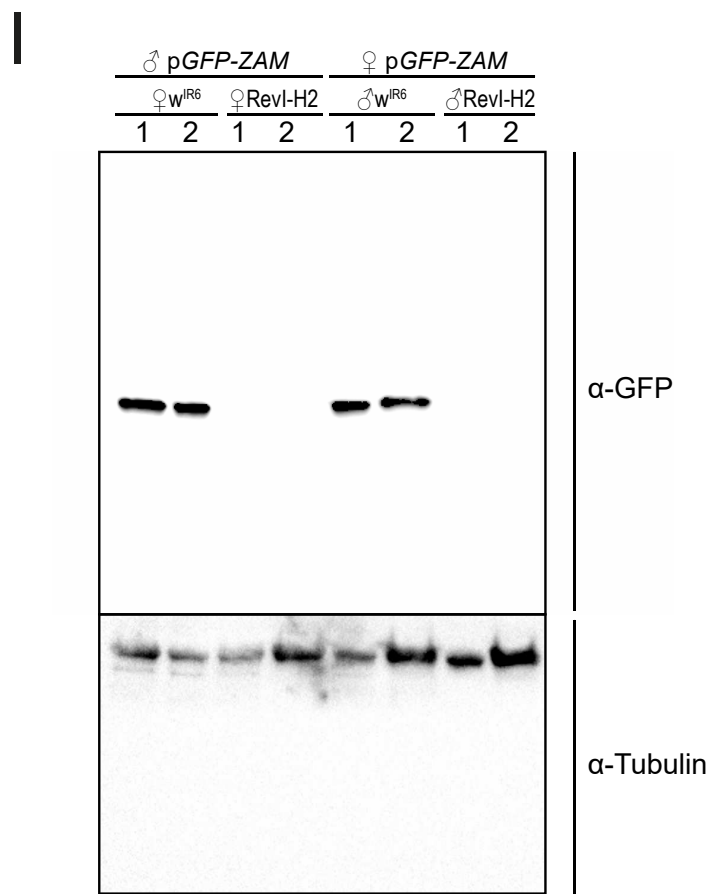
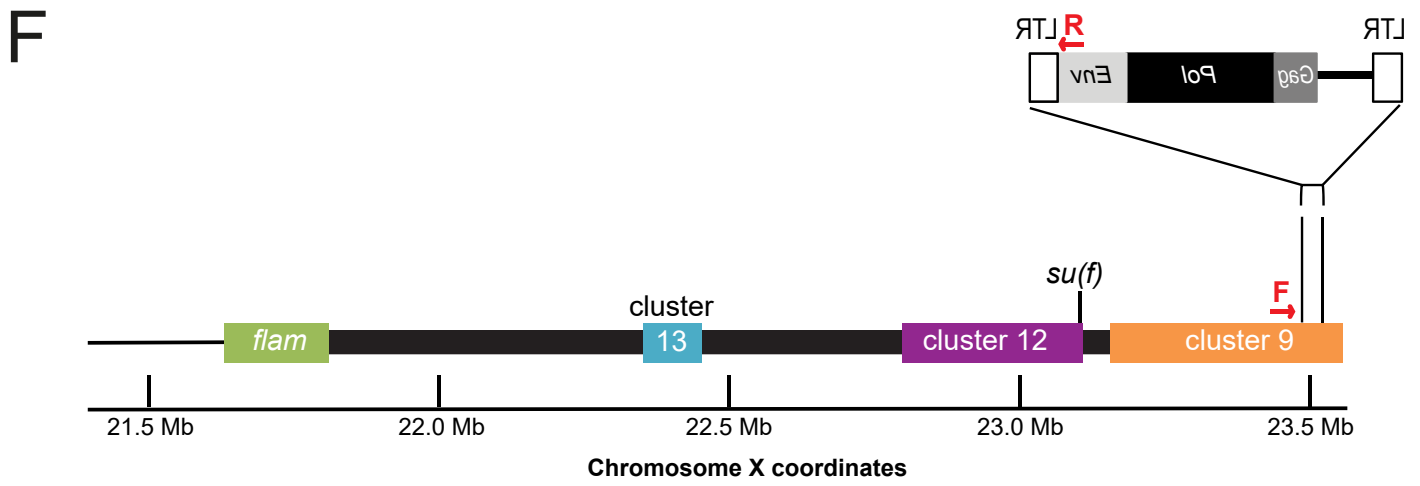


Figure S4. ZAM-derived piRNAs originate from a germline piRNA cluster localized on the X chromosome.

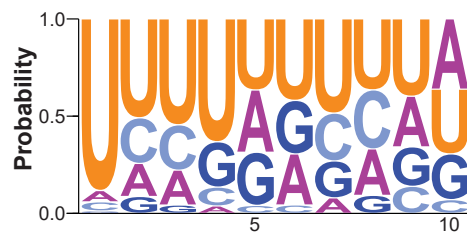
a Schema representing crosses performed to generate the ZMD and NZMD F1 progeny analyzed in Figure 4. The schema details both hypothesis for the new ZAM insertion in the RevI-H2 line. Only the X chromosome is displayed on the schema. The control line is the pGFP-ZAM line in which expression is driven in germline cells by a nanos-Gal4 driver. **b-c** Confocal images of ovarioles after staining for GFP (green, left panels) and DNA (blue, middle panels). Merged images of the GFP and DNA signals are on the right. Ovarioles were from the progeny of a cross between w^{IR6} females and control males (**b**) and from the reciprocal cross between w^{IR6} males and control females (**c**). In both crosses, the pGFP-ZAM line in which expression is driven in germline cells by a nanos-Gal4 driver was the control line. **d** Western blot analysis of proteins extracted from ovaries of progenies of crosses between X^{Rev} , II^{Rev} or III^{Rev} females with control males. The control line was the same as in **b** and **c**. Proteins were from two biological replicates (1&2) prepared from 5 pairs of ovaries and α -tubulin was the loading control. **e** Confocal images of ovarioles after staining for GFP (green, left panels) and DNA (blue, middle panels). Merged images of the GFP and DNA signals are on the right. Ovarioles were from the progeny of a cross between X^{Rev} , II^{Rev} or III^{Rev} females with control males. The control line was the same as in **b** and **c**. **f** Structure of the region where the new ZAM insertion was identified in RevI-H2. The insertion is located in the dual-strand piRNA cluster 9 (according to ranking of piRNA clusters identified in *D. melanogaster* by [30]). ZAM is in genomic minus strand orientation. The chromosome coordinates are according to release 6 of the *D. melanogaster* genome. The su(f) genetic marker is indicated. The pericentromeric part of the X chromosome is displayed by a black box, euchromatin as a black line, *flam* and the other piRNA clusters by colored boxes. Primers (F and R) used to confirm the presence of the ZAM insertion by PCR (Fig. S4H) are depicted by red arrows. The ZAM insertion was localized in region X: 23,474,449..23,513,109 by genome sequencing of RevI-H2 (3 identical possible insertion sites). **g** The sequenced junctions between ZAM and the genomic flanks. The ZAM sequence is displayed in black capital letter and the genomic flanking sequence matching to cluster 9 in orange lower case letters. Target site duplications (TSD) are boxed. **h** PCR analysis of the new ZAM insertion identified in RevI-H2. Used primers are displayed in Fig. S4F. Only RevI-H2 presents an amplicon showing that the w^{IR6} , Iso1A and w^{1118} lines are devoid of this insertion and confirming the presence of this ZAM insertion located in cluster 9 in RevI-H2. **i** Western blotting of proteins extracted from ovaries of progenies of crosses between w^{IR6} or RevI-H2 and the same control line as in Fig. 4a. The lines used for the crosses are indicated above. Proteins were from two biological replicates (1&2) prepared from 5 pairs of ovaries; α -tubulin was used as loading control. Images displayed in this panel are the unprocessed versions of those displayed in Fig. 4c.

A

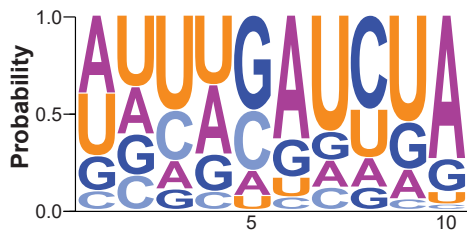
sense *Adoxo*-derived reads with PPP in w^{IR6}



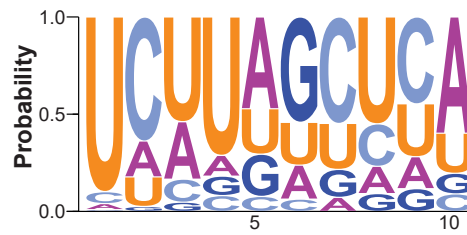
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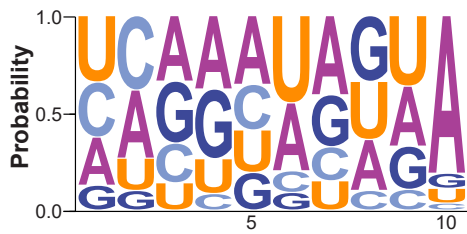
sense *Gedeo*-derived reads with PPP in w^{IR6}



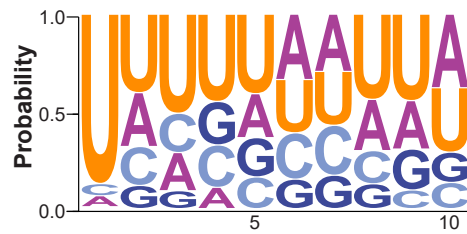
antisense *Gedeo*-derived reads with PPP in w^{IR6}



sense *Idefix*-derived reads with PPP in w^{IR6}



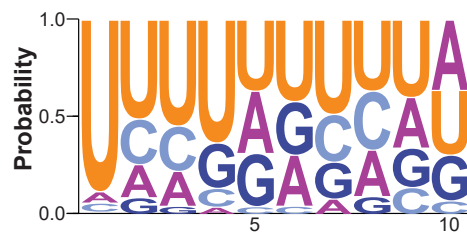
antisense *Idefix*-derived reads with PPP in w^{IR6}



sense *Vatovio*-derived reads with PPP in w^{IR6}

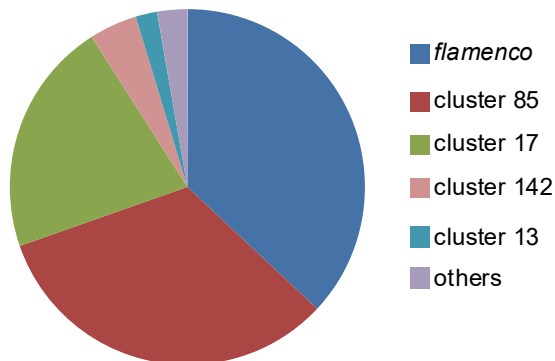


antisense *Vatovio*-derived reads with PPP in w^{IR6}



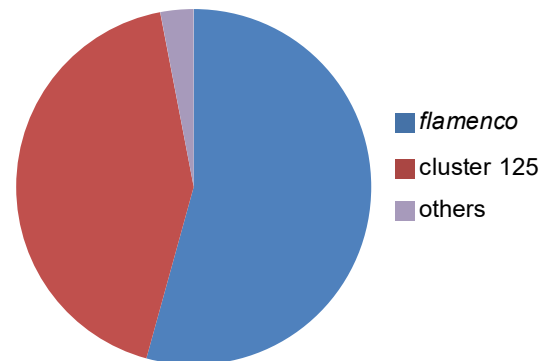
B

Phidippo



C

Pifo



D

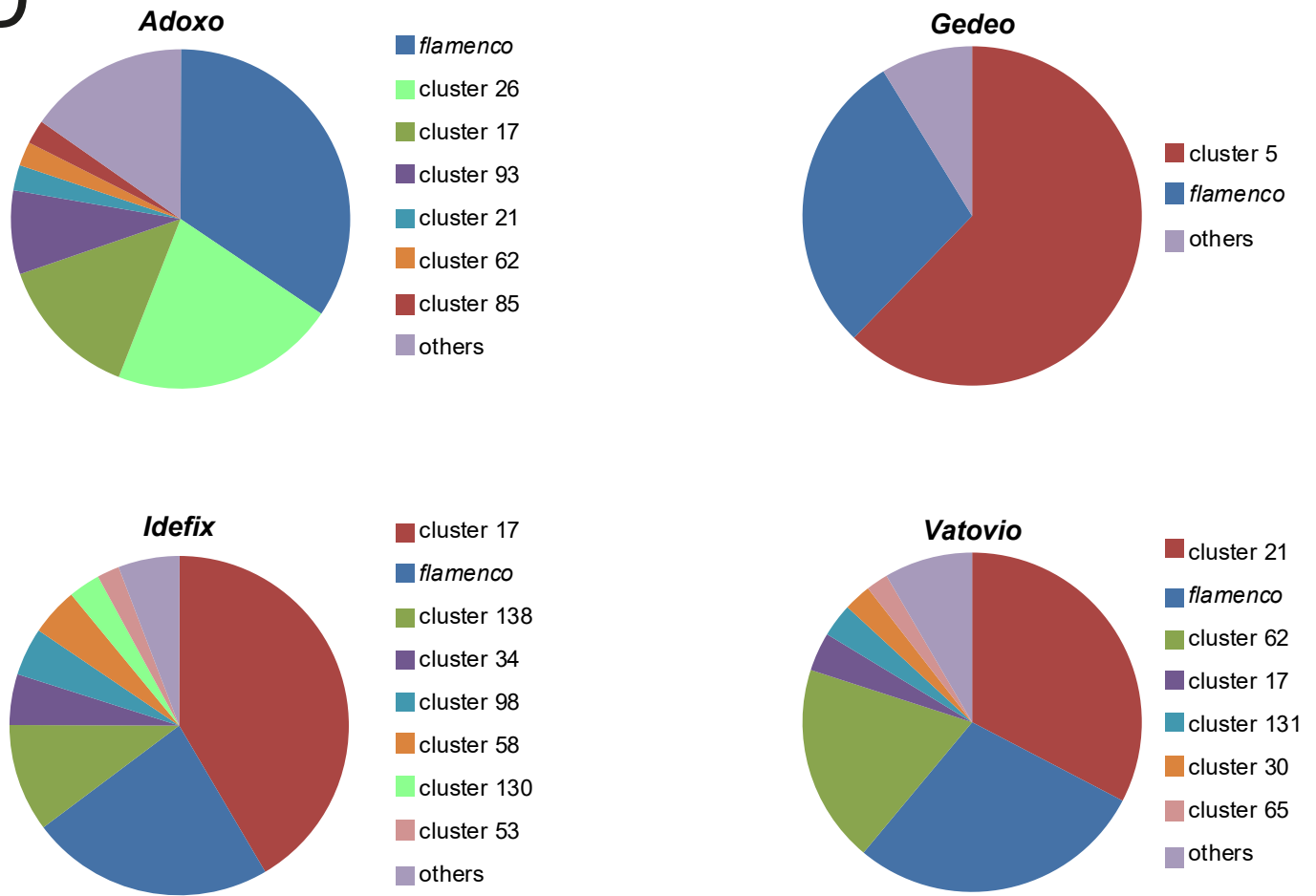
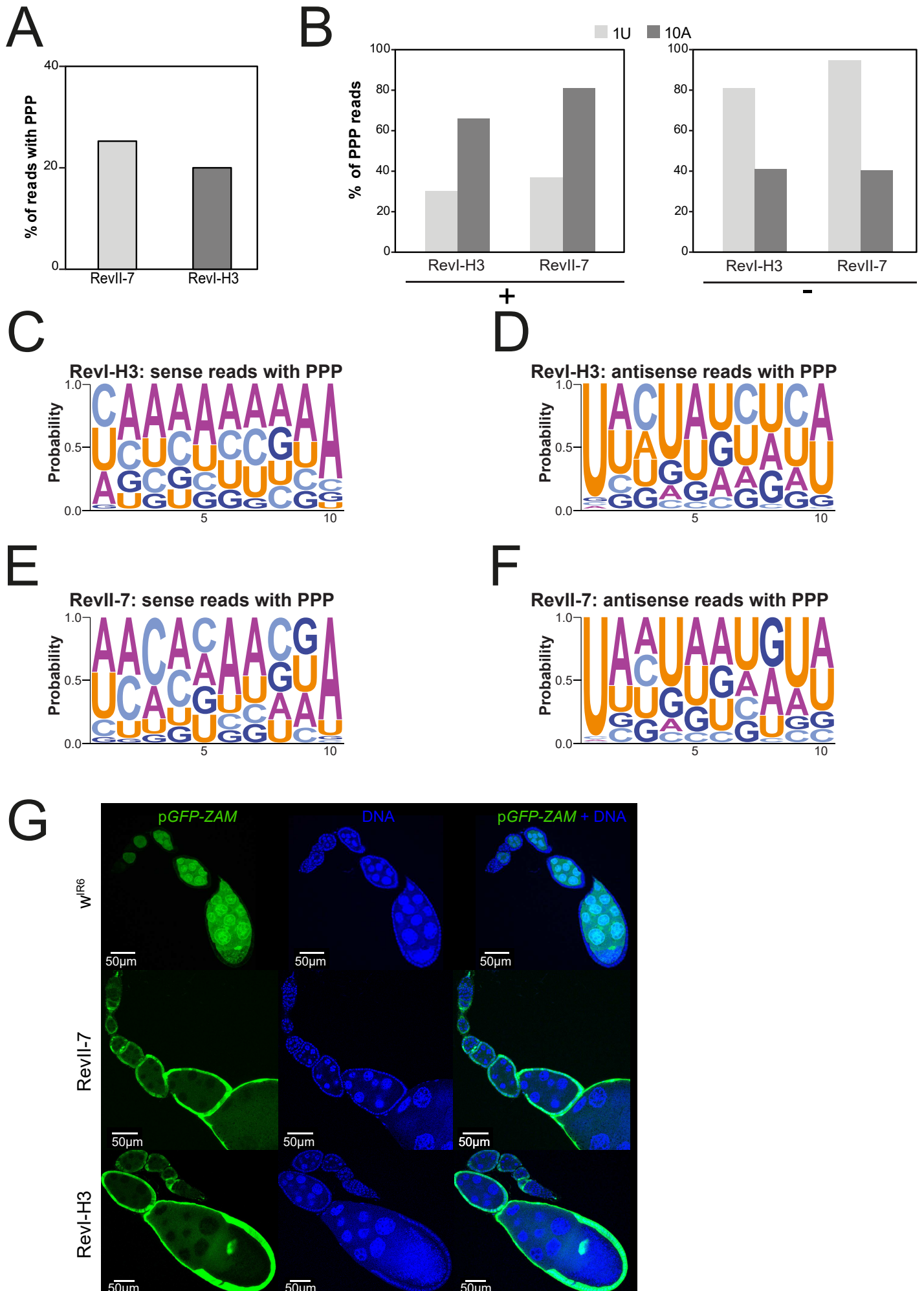


Figure S5. *Phidippo*- and *Pifo*-derived piRNAs are mainly produced by the *flamenco* cluster.

a Logo of nucleotide bias for the first ten positions of *Adoxo*-, *Gedeo*-, *Idefix*- and *Vatovio*-derived piRNAs with ping-pong partner (PPP) produced in w^{IR6} ovaries. The nucleotide height represents its relative frequency at that position. **b-c** Pie charts showing the proportion of *Phidippo*- (*a*) and *Pifo*-derived piRNAs (*b*) mapped (allowing up to 3 mismatches) to the 142 piRNA clusters (allowing no mismatch, piRNA clusters as in [9]) in the w^{IR6} line. **d** Pie charts showing the proportion of *Adoxo*-, *Gedeo*-, *Idefix*- and *Vatovio*-derived piRNAs mapped (allowing up to 3 mismatches) to the 142 piRNA clusters (allowing no mismatch, piRNA clusters as in [9]) in the w^{IR6} line.



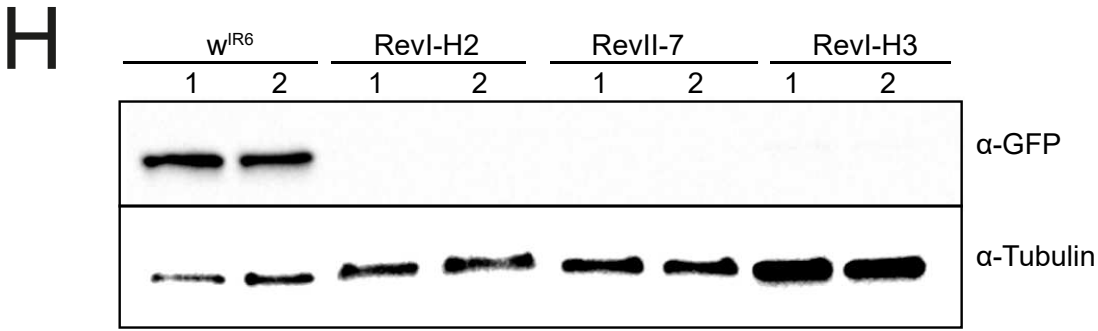


Figure S6. ZAM-derived piRNAs produced in the different Rev lines display similar features.

a Percentage of ZAM-derived piRNAs with ping-pong partner (PPP) in the RevII-7 and RevI-H3 lines. **b** Analysis of the nucleotide bias for sense (+) and antisense (-) ZAM-derived piRNAs with PPPs in the RevII-7 and RevI-H3 lines. The percentages of PPPs with a 1U and a 10A are shown. Both lines had a 10A bias for sense piRNAs and a 1U bias for antisense piRNAs. **c-f** Logo of nucleotide bias for the first ten positions of sense (**c**, **e**) and antisense (**d**, **f**) ZAM-derived piRNAs with ping-pong partner (PPP) produced in RevI-H3 (**c-d**) and RevII-7 (**e-f**) ovaries. The nucleotide height represents its relative frequency at that position. **g** Confocal images of ovarioles after staining for GFP (green, left panels) and DNA (blue, middle panels). Merged images of the GFP and DNA signals are on the right. Ovarioles were from the progeny of crosses between *w^{IR6}* (top), RevII-7 (middle) or RevI-H3 (bottom) females with males that harbor the pGFP-ZAM sensor transgene driven by the *actin*-Gal4 driver. **h** Western blot analysis of proteins extracted from ovaries of progenies of crosses between *w^{IR6}*, RevI-H2, RevII-7 or RevI-H3 females with a control male. The pGFP-ZAM line in which ZAM expression is driven in germline cells by a *nanos*-Gal4 driver was the control line. Proteins were from two biological replicates (1&2) prepared from 5 pairs of ovaries and α-tubulin was the loading control.