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Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm

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Abstract

The conserved Piwi family of proteins and piwi-interacting RNAs (piRNAs) play a central role in genomic stability, which is inextricably tied with germ cell formation, by forming ribonucleoproteins (piRNPs) that silence transposable elements (TEs)¹. In *Drosophila melanogaster* and other animals, primordial germ cell (PGC) specification in the developing embryo is driven by maternal mRNAs and proteins that assemble into specialized mRNPs localized in the germ (pole) plasm at the posterior of the oocyte^{2,3}. Maternal piRNPs, especially those loaded on Aubergine (Aub), a Piwi protein, are transmitted to the germ plasm to initiate transposon silencing in the offspring germline^{4,7}. Transport of mRNAs to the oocyte by midoogenesis is an active, microtubule-dependent process⁸; mRNAs necessary for PGC formation are enriched in the germ plasm at late oogenesis via a diffusion and entrapment mechanism, whose molecular identity remains unknown^{8,9}. Aub is a central component of germ granule RNPs, which house mRNAs in the germ plasm^{10,12} and interactions between Aub and Tudor are essential for the formation of germ granules^{13,16}. Here we show that Aub-loaded piRNAs use partial base pairing characteristic of Argonaute RNPs to bind mRNAs randomly, acting as an adhesive trap that captures mRNAs in the germ plasm, in a Tudor-dependent manner. Strikingly, germ plasm mRNAs in Drosophilids are generally longer and more abundant than other mRNAs, suggesting that they provide more target sites for piRNAs to promote their preferential tethering in germ granules. Thus complexes containing Tudor, Aub piRNPs and mRNAs couple piRNA inheritance with germline specification. Our findings reveal an unexpected function for Piwi ribonucleoprotein

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Author Contributions

A.V. and Z.M. conceived, and Z.M. supervised, the study. A.V. and N.V. performed the experiments. P.A. performed bioinformatic analyses with contribution by M.M. and A.V. A.V., P.A., N.V., M.M. and Z.M. interpreted data. A.V. wrote the manuscript, with contribution from all authors.

Author Information

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complexes in mRNA trapping that may be generally relevant to the function of animal germ granules.

We performed stringent immunoprecipitations for Aub after ultraviolet crosslinking (UV CLIP)¹⁷ (**Fig. 1a**) and standard small RNA immunoprecipitations (IP) employing a highly specific antibody that we generated (**Extended Data Fig. 1a**) from wild-type (*yw*) ovaries and from *yw* and Tudor null (*tud*) embryos collected up to 2 h post-laying (0-2 h embryos); this is prior to zygotic transcription and degradation of maternal mRNAs. Crosslinked RNA-Aub complexes yielded strong, specific signals that were absent from non-immune serum (NRS) and no-UV controls (**Fig. 1a**). CLIP and IP libraries contained essentially identical 23-29 nt piRNAs (Fig. 1b, Extended Data Figs. 1b-g, 2a-f, Extended Data Table 1). We verified minimal changes in the piRNA load of Aub in *tud* versus *yw* ovaries (**Extended Data Fig. 2g**)¹³, and found no changes in the piRNA load of 0-2 h embryos compared to ovaries in both genotypes (**Extended Data Fig. 2h, i**). Larger CLIP tags (IgClips, 36 nt) are present in libraries prepared from larger RNP complexes (Fig. 1a-c, Extended Data Fig. 1d, Supplementary Results).

We observe considerable overlap of retrotransposon IgClips with complementary piRNAs (**Extended Data Fig. 3a, Supplementary Table 1**) and strong positive correlation of their abundances (**Extended Data Fig. 3b, c**). Relative distance analysis reveals high occurrence of IgClips with a 10-nucleotide (nt) overlap to complementary piRNAs (**Fig. 1d**, peak at position +9) for all three genotypes. The majority of such IgClips bear an adenine at the tenth position (**Fig. 1e**) and show prominent 5'-5' end coincidence with Ago3 piRNAs (**Fig. 1f**), indicating that they correspond to ping-pong intermediate fragments produced by Aub slicing¹. Furthermore, a second peak at position -15 (**Fig. 1d**), which is 25 nt (the median Aub piRNA length) from position +9, represents 5' ends of fragments of trigger piRNA targets undergoing phased piRNA biogenesis¹⁸. The above results indicate that CLIP captures piRNA biogenesis, complementary retrotransposon targeting and the transient products of Aub slicing activity (**Fig. 1g**).

A significant percentage (~50-66%) of IgClips from all CLIP libraries are mRNA-derived (**Fig. 1c, Extended Data Fig. 1g**). Most Aub-bound mRNAs are not substrates for piRNA processing (**Extended Data Fig. 4a**). Aub IgClip density is relatively higher within 3' UTRs compared to RNA-Seq, and overall IgClip abundance is not correlated with mRNA abundance (**Extended Data Fig. 4b-d**), suggesting specific target mRNA recognition. We cross-indexed Aub-bound mRNAs with the mRNA localization categories (compiled in ref. 19). Strikingly, posterior localization categories are significantly enriched in all three sets of Aub CLIP libraries (embryo: *yw* and *tud*, ovary: *yw*) (**Supplementary Table 2**). Most importantly, we find 15 posterior and germ cell localization categories significantly depleted, and ubiquitous mRNAs enriched in *tud* embryo compared to *yw* embryo CLIP libraries (**Supplementary Table 3**). Posteriorly localized mRNAs appear marginally upregulated compared to other localization categories in *tud* versus *yw* embryo RNA-Seq libraries (two-sided t-test, p=0.01594), ruling out the possibility that the reduced Aub binding is due to reduced posterior mRNA levels in *tud* embryos. Both Aub (**Extended Data Fig. 1a**) and germ plasm mRNAs^{15,20} are uniformly distributed throughout *tud* embryos; therefore the

observed loss of binding specificity towards posterior mRNAs in the absence of Tudor can only be attributed to the disruption of the germ plasm. Thus our experimental approach allows the identification of the mRNAs specifically bound by Aub in the germ plasm, irrespective of the function of Aub in the clearance of maternal mRNAs in the somatic part of the embryo^{21,22}. To identify the primary mRNA targets of Aub within the germ plasm during the formation of germ cells, we calculated the rank product of the normalized IgClip values for mRNAs in the 12 posterior localization categories marked with an asterisk in **Supplementary Table 3**, from three replicate *yw* embryo libraries (p-value <0.05). The list contains 220 genes, many of which appear enriched or selectively protected in germ cells¹⁰, and with established roles in germ cell specification and development such as *cycB*, *nos*, *osk*, *gcl*, *pgc*, *hsp83* (**Supplementary Table 4**). Characterization of Aub RNPs from early embryos provides independent support for the association of germ plasm mRNAs with Aub (**Supplemental Results, Extended Data Fig. 5**). Four separate analyses provide strong evidence that the extent of the observed Aub binding of mRNAs cannot be explained by piRNA targeting of transposon sequences embedded in mRNAs (**Supplemental Results, Extended Data Fig. 6**).

To further investigate the potential of piRNAs to direct Aub to complementary mRNA sequences, we analyzed chimeric IgClips^{23,24} that each contains an intact piRNA, ligated with a sequence fragment (20 nt) that is uniquely aligned on mRNAs (**Fig. 2a, Supplementary Table 5**). To uncover complementarity patterns we implemented unweighted local alignment between the piRNA (in reverse complement orientation) and the mRNA fragment, scoring matches (+1), mismatches (-1) and indels (-2), and reporting the best alignment for every chimeric read. The search was performed within ± 100 bases around the midpoint of the mRNA fragment; this allows the identification of the entire complementary sequence that might be missing from the chimeric fragment, and also provides a reliable estimate of the signal-to-noise ratio. We observed prominent peaks of hundreds of thousands of complementarity events forming around the midpoint and within ± 25 nt, in *yw* and *tud* embryo CLIP libraries (**Fig. 2b-c**). Most events score between 7 and 12; therefore, the complementarity is not extensive. The distribution of the complementarity events in the negative control (random piRNA) is completely flat across the search area and has lower scores (**Extended Data Fig. 7a**), suggesting that the chimeric reads capture genuine sequence-dependent Aub-piRNA:mRNA contacts.

piRNAs in chimeric reads are typical Aub piRNAs (**Extended Data Fig. 7b-e**). piRNA:mRNA complementarities with alignment score ≥ 7 congregate within a 50-nt window (**Fig. 2b-d**), so we focused on events that have such scores and locations. piRNA complementarity towards posterior and non-posterior mRNAs is indistinguishable (**Fig. 2d, Extended data Fig. 7f**), suggesting that the basis of mRNA binding preference by Aub is not sequence specificity. Chimeric reads show substantial overlap (**Fig. 2a**) and the same enrichment in posterior-localized mRNAs with non-chimeric IgClips (**Supplementary Tables 5 and 6**), suggesting that they both capture the same RNA binding events.

Base-paired nucleotides for every piRNA from three replicate CLIP libraries are summarized in a comprehensive plot (**Fig. 3a, Extended Data Fig. 7g**), revealing a bimodal distribution of the complementary regions within the piRNA. Many are found at the 5' end

of the piRNA starting at positions 1 and 2 (reminiscent of miRNA seed-type binding); additional base-paired stretches start at positions 9-17 (**Fig. 3a, b**). This pattern is absent from the negative control (**Fig. 3a**). Net density of base-paired nucleotides reveals a clear preference for piRNAs to utilize nucleotides at positions 2-6 with additional base pairs in positions 16-24 (**Fig. 3c, Extended Data Fig. 7h, i**). This profile is strikingly similar in *yw* and *tud* libraries, and differs slightly from the miRNA hybridization profile²⁴ in the less frequent base-pairing in the 2-6 region, suggesting that piRNAs do not utilize a conserved seed sequence. The periodicity of the graph in **Fig. 3c (Extended Data Fig. 7i)** evokes the helical conformation and base-pairing availability of the small RNA in the context of an Ago-miRNA-target RNA tripartite complex²⁵, suggesting that despite the absence of a conserved seed, the mechanics of piRNA complementary binding are analogous to those of microRNAs. Analysis of the evolutionary conservation of paired, unpaired and flanking nucleotides on the mRNA sequence reveals that the piRNA:mRNA contact sites are not preferentially conserved (**Fig. 3d**).

We used the local alignment approach by which we analyzed the chimeric CLIP tags, to identify potential piRNA target sites in the *D. melanogaster* transcriptome. In 206,400,271 total sites, the vast majority (99.6%) are of scores 7-11 (**Fig. 4a**). Importantly, the densities of putative piRNA target sites on mRNA regions are essentially identical for mRNAs with or without posterior localization, and very similar to that of the chimeric mRNA fragments (higher densities in the UTRs compared to CDS; **Fig. 4b, c, Extended Data Fig. 8**).

mRNAs in the 12 posterior localization categories are significantly longer than non-posterior localized mRNAs (**Fig. 4d**)²⁶ and so contain a higher number of piRNA target sites (**Fig. 4e**); nevertheless, transcript length normalization eliminates this difference (**Fig. 4f, g**). This holds true when the scores of the predicted sites are accounted for (**Fig. 4g**), and also when the scores are weighted for the preference of piRNA nucleotides 2-6 and 16-24 to base-pair (not shown). Posterior mRNAs are also more abundant than non-posterior; when factored in, this increases the difference of the target site abundance per transcript for the two localization categories (**Fig. 4h**). Posterior and non-posterior mRNAs are equally targeted (per kb) by each piRNA even when piRNA copy number is accounted for (**Extended Data Fig. 9a**). Notably, the size differential (and not the absolute length) of posterior and non-posterior mRNAs is conserved among *Drosophilids*: the intra-species size differential always favors posterior mRNAs, although non-posterior mRNAs from one species might be longer than the posterior mRNAs of another (**Fig. 4i**). Therefore, although piRNAs randomly base pair with non-conserved mRNA sequences, this mechanism is biased towards a specific class of mRNAs for germ plasm anchoring. Additionally, from the two categories of posterior localized mRNAs, Localized and Protected¹⁰, Localized mRNAs have longer 3' UTRs than Protected, further supporting the notion that mRNA length positively affects germ plasm enrichment (**Extended Data Fig. 9b, c**).

The concept of mRNA entrapment at the germ plasm during ooplasmic streaming is well established^{8,9,27}, but the mechanism at the molecular level has been so far elusive. We propose that germ plasm localized Tud-Aub-piRNA complexes play the role of a nondiscriminatory adhesive trap that can form numerous, non-conserved piRNA:mRNA contacts to capture mRNAs and form germ plasm mRNPs (**Figure 4j, Supplementary**

Discussion). This mechanism likely shows preference for posterior mRNAs because they are significantly longer and more abundant²⁶. We believe that the above mechanism acts in addition to specific protein-protein, protein-RNA and RNA-RNA interactions that are necessary for mRNA transfer and anchoring to the posterior, and for translational control^{10,12,28,30}. The multivalence of Aub-Tudor interactions likely contributes to the formation of multimeric germ granule complexes. We propose that germ cell specification and function by maternal mRNAs, and piRNA inheritance converge in Aub. Coupling germ cell specification with piRNA inheritance could be a strategy that increases reproductive fitness by ensuring the propagation of robust transposon silencing mechanisms to germ cells across generations and across the population.

METHODS

Wet-lab methods

Drosophila strains – Tissue collection—The following strains and heteroallelic combinations were used: *y¹w¹¹¹⁸* as the wild-type stock (*yw*), *aub^{HN2/QC42}* (*aub*), *tud^{1/Df(2R)PurP133}* (*tud*), for *aub* and *tud* mutant (loss of function) fly stocks, respectively^{31,32,33,15}. All stocks were grown at 25 °C with 70% relative humidity on a 12 h light-dark cycle. 2-4 d female flies were crossed to *yw* males for 2 d in standard cornmeal food supplied with yeast paste before ovary dissection. Embryos harvested at well-defined time-windows were dechorionated in 50% commercial bleach for 2 min, washed extensively in water and collected in PBS or HBSS or fixation solution, depending on downstream applications.

Antibodies—Antibody against Aubergine (Aub-83) was produced by immunizing rabbits with Aub peptide (HKSEGDPRGGSVRGRC, where terminal cysteine was used to couple to KLH; Genscript) and selected with peptide-affinity purification of sera. Other antibodies that were used in this study: mouse monoclonal anti-PABP (6E2 clone)³⁴, E7 mouse monoclonal anti- β -tubulin (Developmental Studies Hybridoma Bank) and anti-Tudor mouse monoclonal (gift from M. Siomi).

Immunofluorescence—Fixation and immunohistochemistry of dissected ovaries and embryos was performed according to standard protocols. Primary antibodies against Aub and Tud were used at 1 ng/ μ L final concentration. Secondary antibodies conjugated to Alexa 488 and 594 (Life technologies) were used at 1:1000 dilution. Ovary and embryo samples were imaged on Leica TCS SPE confocal microscope.

Aub HITS-CLIP—CLIP was performed as previously described for Mili, Miwi and MOV10L1^{17,35,36}. The protocol is described in detail in³⁶ and uses stringent buffer conditions to ensure high specificity. 40 mg of *Drosophila* embryos (0-2 h) or ~80 ovaries from 4-6 d females were collected in ice-cold HBSS and UV-irradiated (3 \times) at 254 nm (400 mJ/cm²). The tissues were pelleted, washed with PBS and the final tissue pellet was flash-frozen in liquid nitrogen and kept at -80°C. UV light-treated tissues were lysed in 350 μ L 1 \times PMPG [1 \times PBS (no Mg²⁺ and no Ca²⁺), 2% Empigen] with protease inhibitors and

RNasin (2 U/ μ L) and no exogenous ribonucleases; lysates were treated with DNase I (Promega) for 5 min at 37 °C, and then were centrifuged at 100,000 \times g for 30 min at 4 °C.

For each IP, approximately 10 μ L of our anti-Aub antibody was bound on 150 μ L (slurry) of protein A Dynabeads in Ab binding buffer (0.1 M Na-phosphate pH 8 and 0.1% NP-40) at RT for 2 h; Ab-bound beads were washed 3 \times with 1 \times PMPG. Antibody beads were incubated with lysates (supernatant of 100,000 \times g) for 3 h at 4 °C. Low- and high-salt washes of immunoprecipitation beads were performed with 1 \times and 5 \times PMPG (5 \times PBS, 2% Empigen). RNA linkers (RL3 and RL5), as well as 3' adaptor labeling and ligation to CIP (calf intestinal phosphatase)-treated RNA CLIP tags were performed as previously described³⁶.

Immunoprecipitation beads were eluted at 70 °C for 12 min using 30 μ L of 2 \times Novex reducing loading buffer. Samples were analyzed by NuPAGE (4%-12% gradient precast gels, run with MOPS buffer). Cross-linked RNA-protein complexes were transferred onto nitrocellulose (Invitrogen LC2001), and the membrane was exposed to film for 1–2 h. Membrane fragments containing the main radioactive signal and fragments up to ~15 kDa higher were excised (**Fig. 1a**). RNA extraction, 5' linker ligation, Reverse-transcriptase (RT)-PCR and second PCR step were performed with the DNA primers (DP3 and DP5, DSFP3 and DSFP5) as described previously³⁶. cDNA from two PCR steps was resolved on and extracted from 3% Metaphor 1xTAE gels. Size profiles of cDNA libraries prepared from the main radioactive signal and higher MW were similar (**Fig. 1a**). DNA was extracted with QIAquick Gel Extraction kit and submitted for deep sequencing. The cDNA libraries were sequenced with Hi-Seq Illumina at 100 cycles.

Solid-support directional (SSD) RNA-Seq—SSD RNA-Seq was performed as previously described¹⁷, using total RNA (depleted of ribosomal RNA with Ribozero - EpiCentre-) isolated from 0-2 h embryos of appropriate genotypes.

Nycodenz density gradient ultracentrifugation and subsequent analyses—Nycodenz density gradient separation of RNPs was performed as previously described¹⁷ with modifications. A 20%-60% (top to bottom) Nycodenz gradient (4.8 mL) in 1 \times KMH150 (150 mM KCl, 2 mM MgCl₂, 20 mM HEPES pH 7.4, 0.5% NP-40, 0.1 U/ μ L rRNasin, and protease inhibitors) was prepared as a step gradient by overlaying 5 equal parts of Nycodenz solutions and was let to diffuse overnight at 4 °C. 0.2 mL of post nuclear *yw* embryo lysate in 1xKMH was laid over the gradient and centrifuged at 150,000 \times g for 20 h. We used embryos of stages 4-6, to avoid earlier stages were mRNAs at the soma form distinct mRNPs than the ones formed in the pole plasm – PGCs. The gradient was collected in 12 equal fractions. Samples from each fraction were used for protein determination by Bradford and RNA extraction with Trizol LS. Right before RNA extraction, 500 ng of *in vitro* transcript of Renilla Luciferase mRNA was spiked in each fraction for normalization purposes in subsequent steps.

qRT-PCR—Equal volume of RNA extracted from each fraction was reverse transcribed by Superscript III (Invitrogen 18080-051) in the presence of random hexamers. Equal volume of the cDNA was mixed with primers (*gcl*, *osk*, *hsp83*, *dhd*, *cycB*: Qiagen QuantiTect Assay;

Renilla Luciferase (rLuc), F: 5'-CGCTGAAAGTGTAGTAGATGTG and R: 5'-TCCACGAAGAAGTTATTCTCCA) and Power SYBR Green reaction mix (Applied Biosystems 4367659). The reactions were run on a StepOnePlus™ System (Applied Biosystems) using the default program.

Immunoprecipitation and detection of piRNAs, and preparation of cDNA libraries—Aub immunoprecipitation, 5' end labeling of piRNAs and cDNA library preparation were carried out as previously described^{37,38}.

Bioinformatic analyses

Code availability—We used CLIPSeqTools³⁹, a bioinformatics suite that we created for analysis of CLIP-Seq datasets (accessible at: <https://github.com/mnsmar/clipseqtools> and <http://mourelatos.med.upenn.edu/clipseqtools/tutorial/>) and a Perl programming framework that we developed (M.M., P.A. and Z.M., manuscript in preparation; preprint available at: <http://biorxiv.org/content/early/2015/11/03/019265>). The latter framework is named GenOO and has been specifically developed for analysis of High Throughput Sequencing data. The source code for GenOO has been deposited in GitHub and can be accessed at <https://github.com/genoo/>.

Statistics—In statistical analyses, we ensured that the assumptions of each statistical test are met and that the statistical test used is appropriate for the analysis. In all analyses the statistical tests and methods used are clearly stated in relevant sections.

Data—*Drosophila* (assembly dm3) transcript, exon and repeat genomic locations were downloaded from the UCSC genome browser (downloaded 22 March 2011 from <http://genome.ucsc.edu>). Repeat consensus sequences were downloaded from Flybase (<http://flybase.org/> - transposon_sequence_set v9.42). Localization categories for *Drosophila* genes were taken from Lécyer et al., 2007¹⁹. The localization annotation matrix was downloaded from (<http://fly-fish.cabr.utoronto.ca> annotation_matrix.csv). Transposon categories were as in Malone et al., 2009³¹.

Preprocessing—The 3' end ligated adaptor (GTGTCACTCACTTCCAGCGGTCGTATGCCGTCTTCTGCTTG) was removed from the sequences using the cutadapt software and a 0.25 acceptable error rate for the alignment of the adaptor on the read. To eliminate reads in which the adaptor was ligated more than one time, adaptor removal was performed 3 times.

Alignment—Reads for all samples were aligned against the dm3 *Drosophila melanogaster* genome assembly using the aligner *bwa v0.6.2-r126*, with the default settings⁴⁰. Reads were also aligned against the Repeat consensus sequences using the same aligner.

Genomic distribution—All mapped reads were divided in the following genomic categories: repeat, antisense repeat, non-coding RNA, coding RNA. The remaining reads were considered as intergenic reads.

Correlation of replicates—Gene expression was defined as the number of reads that map on each gene and the values were normalized by the upper quartile normalization method⁴¹. The log₂ gene expression levels of replicates are compared using the Pearson Correlation function in R.

Coincidence with IP—Reads mapping in the same position (same 5' end mapping) were considered as coinciding. When comparing CLIP with IP libraries, the percentage of piRNA-size CLIP reads that had a coinciding start with any standard IP read were counted as positive.

Significant Localization—For each localization category, the quartile-normalized IgCLIP binding level (“mRNA expression level” in each CLIP library) is compared via two sided t-test between genes that belong to the category vs genes that do not belong to it. To compare two samples, we measure the difference in binding (per gene) between the two conditions ($\log_2(\text{gene.expr.cond1} / \text{gene.expr.cond2})$) and then perform a t-test of differences in genes belonging to the category vs genes not belonging in the category.

Early embryo posterior localization categories—The following twelve mRNA localization categories¹⁹ were found significantly depleted in *tud* embryo Aub CLIP libraries compared to *yw* embryo libraries, and were used in analyses were “posterior localized mRNAs” are mentioned: “1:41:RNA islands”, “1:42:Pole buds”, “1:40:Pole plasm”, “3:265:Perinuclear around pole cell nuclei”, “4:370:Germ cell localization”, “4:403:Germ cell enrichment”, “3:348:Pole cell enrichment”, “2:141:Pole cell localization”, “2:153:Perinuclear around pole cell nuclei”, “2:142:Pole cell enrichment”, “3:347:Pole cell localization”, “1:59:Perinuclear around pole cell nuclei” (<http://fly-fish.cabr.utoronto.ca/>). The remaining mRNAs are mentioned as non-posterior localized mRNAs. The following three posterior localization categories were also depleted in *tud* embryo Aub CLIP libraries compared to *yw*: “1:39:Posterior localization”, “2:124:Posterior localization”, “3:352:Posterior localization”. Almost all of the mRNAs contained in the above twelve categories are also contained in these three, but these three categories also contain some mRNAs that do not actually localize in the pole plasm or the germ cells (i.e. with apical localization), therefore mRNAs belonging in any of these three localization categories but not in any of the above mentioned twelve posterior categories were not considered for the generation of the Supplementary Table 4. Many mRNAs do not have a designated localization pattern, and they are mentioned as “undetermined localization”. It is worth mentioning that this category contains at least a few mRNAs with clear posterior – pole plasm localization. Through manual searches of the Berkeley Drosophila Genome Project chromogenic ISH database (<http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl>) we noticed that many Aub bound mRNAs, whose localization is not annotated in the Fly-FISH database, are indeed localized in the germ plasm/cells (such as CG4735/shu, CG7070/PyK, CG4903/MESR4, CG5452/dnk, CG9429/Calr), therefore our analysis is most likely underestimating the true number of Aub bound mRNAs that are important for germline specification and function. Because of this, mRNAs with “undetermined localization” were never mixed with “non-posterior localized” mRNAs in our analyses.

Highly Bound Genes—To identify highly bound genes, we used the rank product method⁴². Specifically, genes are sorted by expression per sample, and for each gene the product of their ranks is calculated. The probability of this rank product produced by chance is calculated by permutations of all non-zero value genes.

Transcript expression calculation—We calculated the expression for protein-coding transcripts by counting the number of RNA-Seq reads that map within the exons of each transcript. The counts were normalized using RPKM (reads per million divided by the length in kb of the exonic region of the mRNA) and upper quartile normalization, effectively dividing each count by the upper quartile of all counts⁴¹. The transcript with the highest RPKM score was used (“best transcript”) unless otherwise noted.

Transcript Aub binding calculation—We calculated the expression for protein-coding transcripts by counting the number of CLIP reads that map within the exons of each transcript in the sense orientation. The counts were normalized using RPM (reads per million) and upper quartile normalization, effectively dividing each count by the upper quartile of all counts⁴¹.

RNA-Seq correlation vs CLIP—Upper quartile normalized RPKM for RNA-Seq was compared to similarly normalized CLIP binding levels defined as average number of reads per transcript in CLIP replicates. Correlation was calculated using the Pearson Correlation function in R.

Chimeric CLIP tags—Identification of hybrid reads:

- 1) Identified IgCLIP size reads (read length >35) that did not align to the genome.
- 2) Made a set of substrings from both ends of reads from (1) of piRNA size (L=[23,29]).
- 3) Identified the substring from (2) to full-length piRNAs (L=[23,29]) from corresponding Low samples (table1)
- 4) The longest aligning piRNAs are retained and coupled with the remainder of the read as piRNA-IgCLIP couples.
- 5) The piRNA aligning fragment is cut from the read. Very small remainder reads (L=[<,20]) are discarded.
- 6) The remainders are aligned to the genome (using bwa default settings).
- 7) Remainders aligned in one single position that is on a known mRNA are retained.

Alignment of piRNAs to regions

- 1) Regions of 200nt length were cut around the midpoint of the genomic alignment region from step 7 of previous routine.

Specifically, if ($d=200$ the length of the final region we want and L is the length of the read), a genomic region flanking the read on each side of length $d/2$ was excised from the chromosome sequence. If the alignment was located in the minus strand the sequence was reversed and complemented at this point. This total region has length $d+L$. We discard an equal number of nucleotides from each side to reach a final length of L (specifically we substring starting from $int(L/2)$ and for d nucleotides. NB: *int* will always round down). At this point we have a region of length 200nt centered around the alignment region of the fragment.

- 2) We use a slightly modified Smith-Waterman⁴³ alignment method [weights: match=+1, mismatch=-1, gap=-2] to align piRNAs on the 200-nt long regions from (1).

Differences of our alignment versus Smith-Waterman:

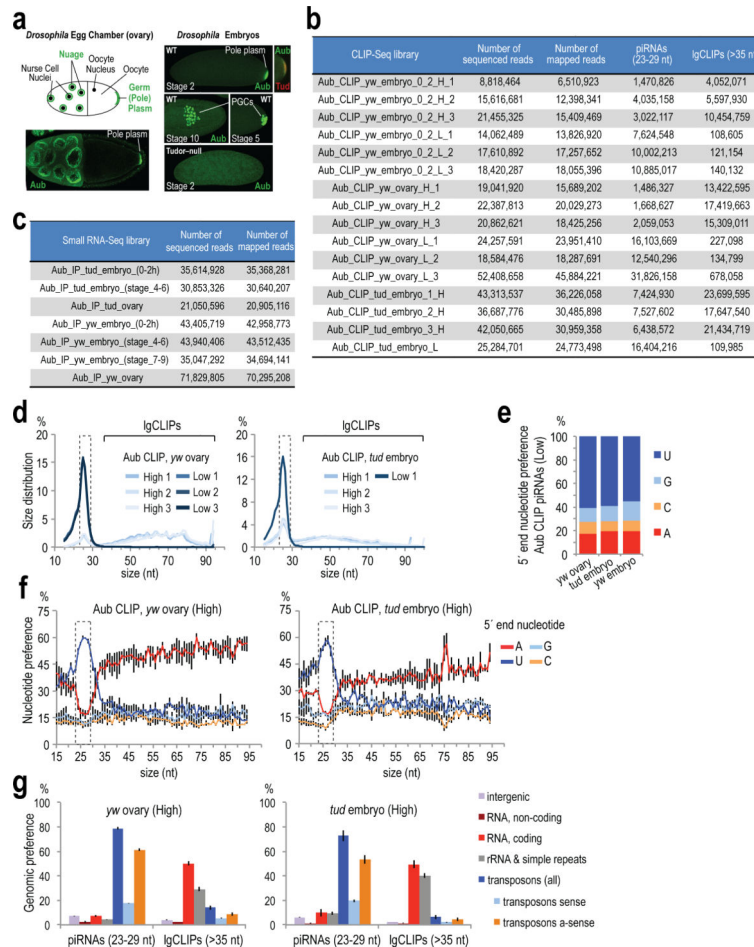
- a) No penalties are given to non-matching nucleotides on the edges of the alignment.
 - b) If there are multiple optimal alignment scores, one is picked randomly.
 - c) Alignments in which part of one sequence is outside the boundaries of the other sequence are not considered.
- 3) The midpoint of the alignment (if k nucleotides matched that is the $int(k/2)$ nucleotide) is used for graphs of alignment positioning on regions.

mRNA target prediction for the top 2000 expressed piRNAs—We grouped piRNA sequences into families based on the first 23nt of each piRNA. Using the alignment algorithm described above we aligned one piRNA (the most abundant) for each of the top 2000 families to the longest annotated transcript for each protein-coding gene. These 2000 piRNA families represent ~37% of piRNA reads from Low *yw* CLIP libraries. To factor in transcript abundance, we multiplied the RNA-Seq (*yw* embryo 0-2 h) RPKM value for each mRNA with the number of predicted piRNA target sites found within the mRNA. This provides a “targeting potential” of every mRNA species, corrected for its abundance.

We then evaluated the targeting potential of each piRNA-mRNA pair using three different scoring schemes. For the first we sum the alignment score of all putative piRNA binding sites on the mRNA. For the second we calculated a weighted alignment score for each putative piRNA binding site and then we sum all scores similar to the previous scheme. The weighted score for each binding site is calculated based on the following formula $\sum x_i * A_i$ where x_i is 1 or 0 based on whether the nucleotide at position i of the piRNA is bound or not and A_i is the weight for nucleotide i . For the third, we multiplied the total number of predicted complementary sites per piRNA, with the piRNA copy number.

Study of the lengths of *D. melanogaster* orthologous mRNAs in other *Drosophila* species—Transcript sequences (fasta file) for each species were downloaded from Flybase (<ftp://ftp.flybase.net/genomes/> on Sep. 1st 2015, current version used for each genome). For each gene (identified as the “parent” tag in the fasta file header), the longest transcript length was identified. For the analysis of the expressed mRNAs (Fig. 4d), we utilized our *yw* embryo RNA-Seq data to identify the longest transcript with the highest length normalized abundance. Ortholog gene tables were downloaded from Flybase (gene_orthologs_fb_2015_03.tsv.gz) and were used to identify ortholog genes across species. For each species, all genes that mapped to localized and unlocalized *Drosophila melanogaster* genes were used in the comparison and were assigned to the corresponding group as their *D. melanogaster* ortholog. Boxplots were created using the lattice package in R (bwplot) and omitting outliers, p-values were calculated using the Wilcoxon exact rank test (wilcox.test in R) one-sided with the hypothesis that localized genes are longer than nonlocalized.

Extended Data



Extended Data Figure 1. Endogenous Aub localization in genotypes used, sequenced and mapped reads of CLIP-Seq and RNA IP libraries used in this study, and general characteristics of *yw* ovary and *tud* embryo (0-2 h) CLIP-Seq libraries

a. Immunofluorescence of ovary and early embryo of indicated genotypes using antibodies against Aubergine (Aub-83; green) and Tudor (red), and schematic representation of the egg chamber. Aub is localized in the nuage and germ (pole) plasm of WT ovaries, in the germ plasm of early WT embryos (stage 2) and within PGCs as they form in the posterior pole (stage 5) and as they migrate during gastrulation (stage 10). Tud colocalizes with Aub in the germ plasm of early embryos but it is not detected after PGC formation. In Tudor mutant early embryos, Aub is not concentrated in the posterior but it is diffusely present throughout the embryo; PGCs are never specified resulting in agametic adults (see also **Extended Data Fig. 9**).

b. Sequenced and mapped reads of CLIP-Seq libraries prepared in this study.

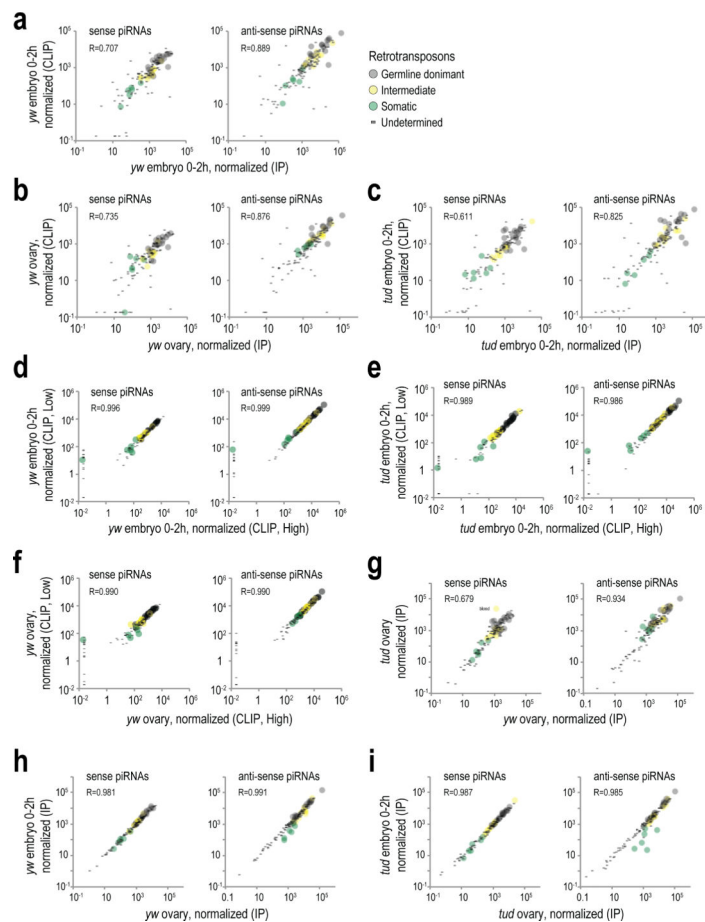
c. Sequenced and mapped reads of RNA IP deep sequencing libraries prepared in this study.

d. Size distribution for the three Low and three High *yw* ovary and *tud* embryo (0-2 h) Aub CLIP-Seq libraries. The size range of piRNAs (23-29 nt) is indicated with a dashed box.

e. Average 5' end nucleotide composition for piRNAs (23-29 nt) from three Low *yw* ovary, *tud* embryo (0-2 h) and *yw* embryo (0-2h) Aub CLIP-Seq libraries.

f. Average 5' end nucleotide composition of CLIP tags from three High *yw* ovary and *tud* embryo (0-2 h) Aub CLIP-Seq libraries. piRNAs (23-29 nt) are indicated with a dashed box. Error bars represent one standard deviation (\pm S.D.; n=3).

g. Genomic distribution of CLIP tags for three High *yw* ovary and *tud* embryo (0-2 h) Aub CLIP-Seq libraries. Error bars: \pm S.D.; n=3. Overlap of piRNAs from CLIP and IP libraries.



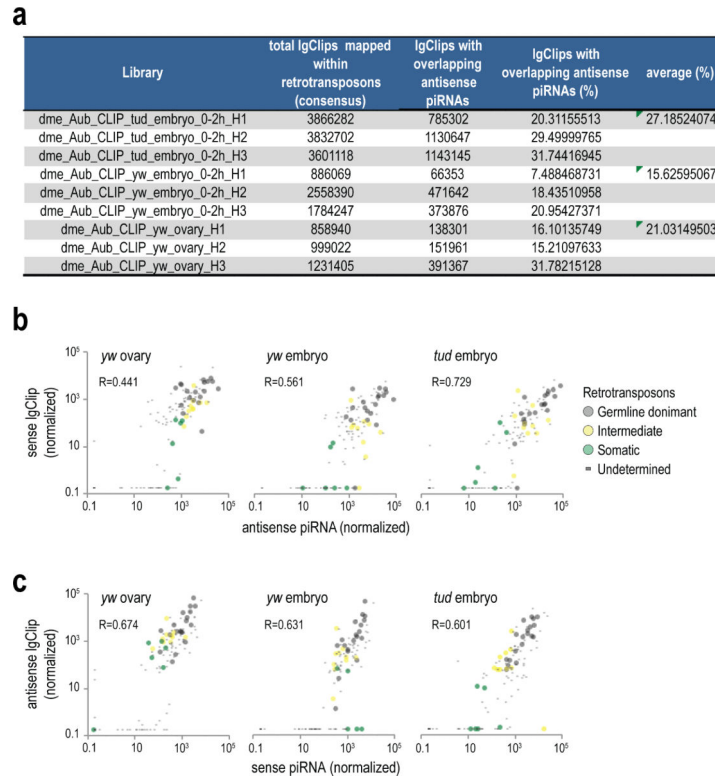
Extended Data Figure 2. Pairwise comparisons of transposon piRNA populations from various libraries

a, b, c. Scatterplot comparison of normalized abundance of piRNAs mapped on consensus retrotransposon sequences (sense and antisense), from *yw* embryo (0-2 h) standard Aub IP and Aub CLIP libraries (**a**); from *yw* ovary libraries (**b**); and from *tud* embryo 0-2 h libraries (**c**). Pearson correlation is shown for all elements in every plot. Retrotransposon categories are set as in Malone et al., 2009³¹.

d, e, f. Scatterplot comparison of normalized abundance of transposon-derived piRNAs in Aub CLIP libraries prepared from higher MW (**Fig. 1a**, marked with a light blue line) with the piRNAs found in the libraries prepared from the main radioactive signal (**Fig. 1a**, marked with a dark blue line) from *yw* embryo 0-2 h (**d**); from *yw* ovary Aub CLIP “High” and “Low” libraries (**e**); and from *tud* embryo 0-2 h Aub CLIP “High” and “Low” libraries (**f**). These comparisons indicate that the piRNA loads in Low and High CLIP libraries are essentially identical.

g. Scatterplot comparison of normalized abundance of transposon derived piRNAs for *yw* ovary and *tud* ovary Aub IP libraries, to evaluate changes of piRNA load in the absence of Tudor. While antisense derived piRNAs are largely unchanged, a few sense-derived piRNAs are changed (blood retrotransposon is indicated).

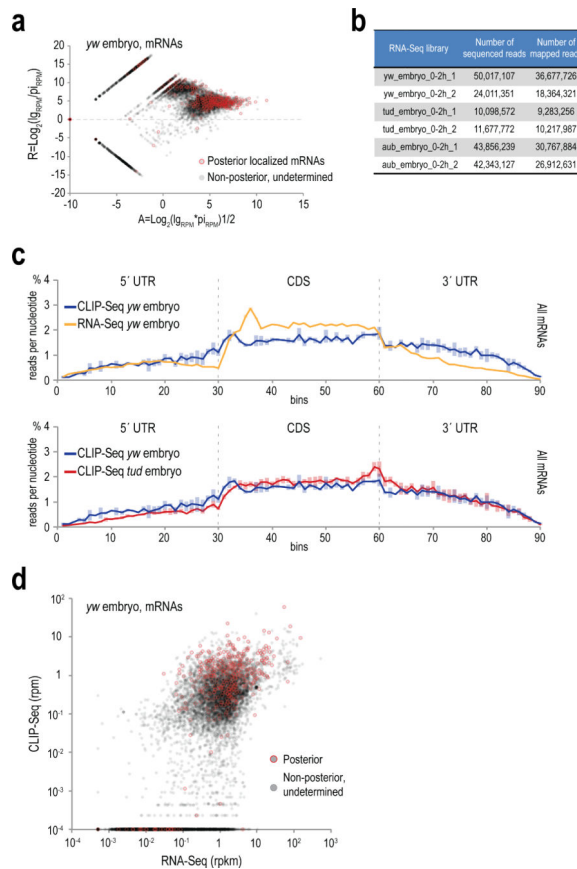
h, i. Scatterplot comparison of normalized abundance of transposon derived piRNAs for *yw* ovary and *yw* embryo 0-2 h Aub IP libraries (**h**); and for *tud* ovary and *tud* embryo 0-2 h libraries (**i**).



Extended Data Figure 3. Retrotransposon targeting by complementary piRNAs identified by Aub CLIP

a Overlap of IgClips with complementary piRNAs from CLIP libraries, mapping on retrotransposons.

b, c. Scatterplot of normalized abundance of antisense piRNAs and sense IgClips (**b**) and for sense piRNAs and antisense IgClips (**c**) mapped on retrotransposons for the indicated Aub CLIP libraries. Pearson correlation is shown for all elements in every plot. Retrotransposon categories are set as in Malone et al., 2009³¹.



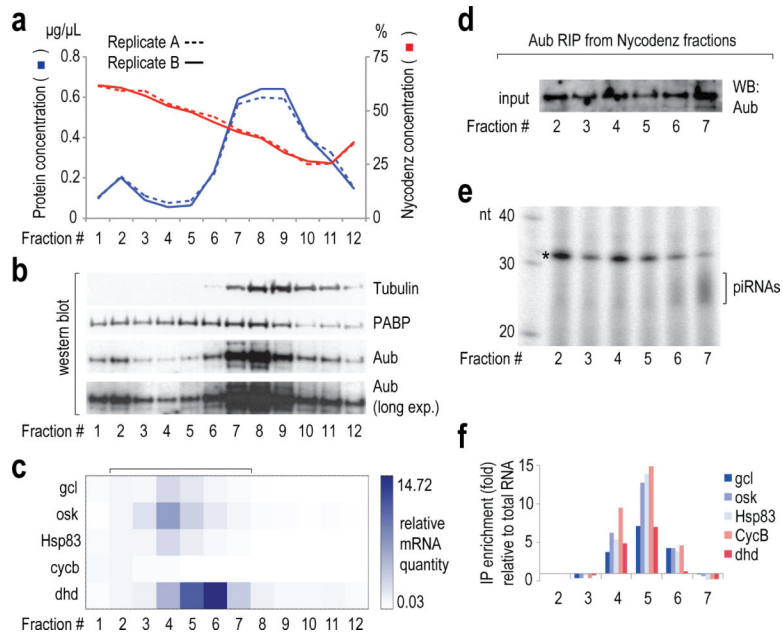
Extended Data Figure 4. CLIP identifies extensive mRNA binding by Aub

a. Ratio Average (RA) plot of normalized (RPM) Aub CLIP tag (pi , piRNA ; lg , lgCLIP) abundance (A value) versus lgClips over piRNA abundance (R value), for all mRNAs. Outlined circles (red) correspond to genes that belong in the 12 posterior localization categories depleted in *tud* versus *yw* Aub CLIP libraries. Zero values are substituted with a small (smallest than the minimum) value so that log calculations are possible. This graph strongly suggests that mRNA binding by Aub as captured by CLIP is not for piRNA biogenesis purposes.

b. Sequenced and mapped reads of RNA-Seq libraries prepared in this study.

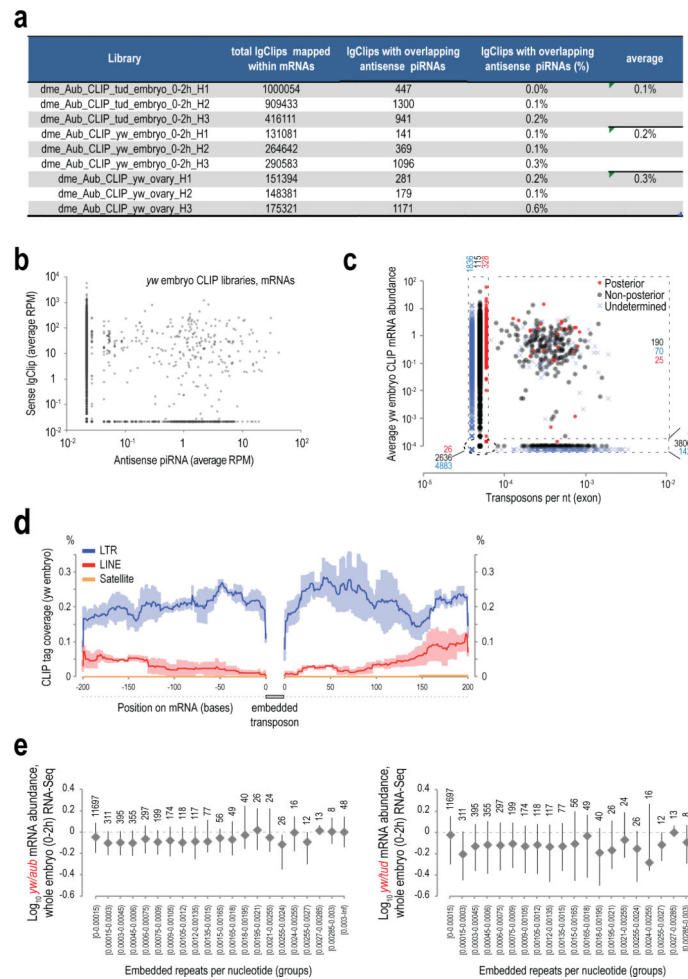
c. Density of Aub CLIP-Seq tags (*yw* embryo, and lower panel: *tud* embryo) and RNA-Seq reads (upper panel: *yw* embryo) within the untranslated regions and the coding sequence of the meta-mRNA. Each mRNA region is divided in 30 bins and the number of the chimeric mRNA fragments (genomic coordinate of the mRNA fragment midpoint) mapped within each bin is counted. Error bars indicate one S.D. ($n=3$) for CLIP-Seq; min and max values for the two RNA-Seq replicate libraries.

d. Scatterplot of average normalized mRNA abundance for *yw* embryo RNA-Seq (rpkm) and Aub CLIP-Seq (rpm). Aub highly bound mRNAs with posterior localizations (**Supplementary Table 4**) are marked with a red circle. Zero values are substituted with a small (smallest than the minimum) value so that log calculations are possible. CLIP-Seq identifies mRNAs that span the whole expression range of RNA-Seq libraries, indicating that Aub CLIP does not capture transcripts simply based on abundance.



Extended Data Figure 5. Partial purification of Aub RNPs from early embryo supports piRNA independent binding of germ plasm mRNAs by Aub

- a.** Fractionation of isopycnic Nycodenz density gradients of post-nuclear *yw* embryo lysate. Protein and Nycodenz concentration for every fraction is plotted.
- b.** Western blot detection of indicated proteins in gradient fractions. A short and a long exposure (long exp.) for Aub is shown. Uncropped gels for panels b, d and e can be found in Supplementary Figure 1.
- c.** Heat map of levels of indicated germ plasm mRNA determined by qRT-PCR, normalized to spiked luciferase RNA, and with fraction 2 as a reference.
- d.** Western blot detection of Aub in indicated diluted Nycodenz fractions used for Aub RNA IP.
- e.** Electrophoretic analysis on denaturing polyacrylamide gels of ³²P-labeled small RNAs immunoprecipitated with Aub from indicated gradient fractions. A bracket denotes piRNAs, detected primarily in fractions 6 and 7 (asterisk: 2S rRNA).
- f.** Bar plot showing -fold enrichment (over fraction-extracted total RNA) of indicated germ plasm mRNAs in Aub IPs from gradient fractions, measured by qRT-PCR. Luciferase mRNA was used as a spike.



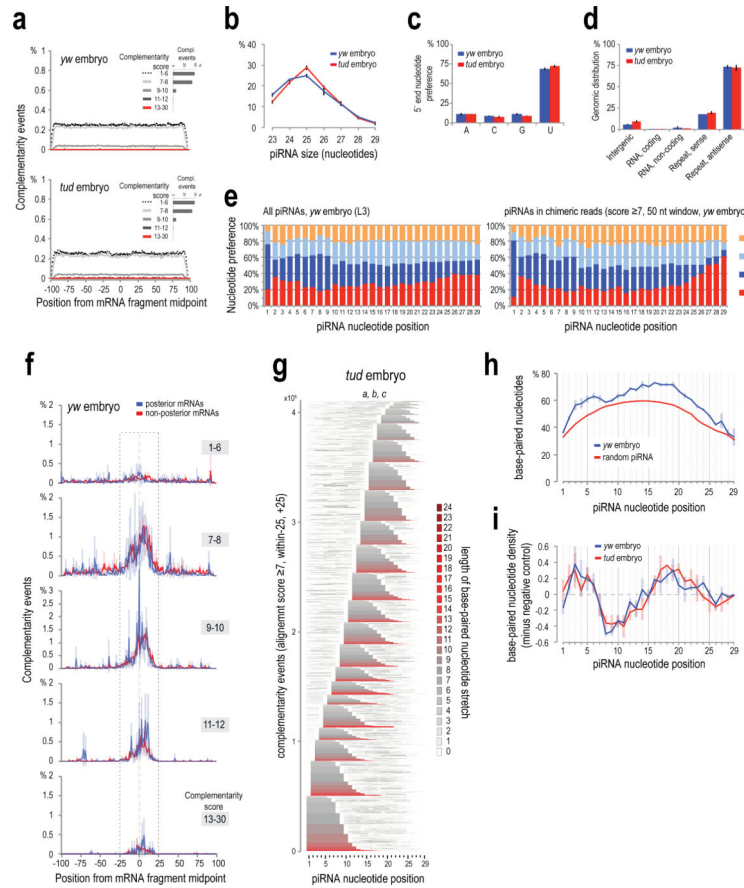
Extended Data Figure 6. Analysis of Aub CLIP tags mapping to mRNAs with regard to the presence of mRNA embedded transposons

- a.** Overlap of IgClips with complementary piRNAs from CLIP libraries, mapping on mRNAs.
- b.** Scatterplot of *yw* embryo Aub IgClips mapped in the sense orientation on mRNAs with piRNAs mapped in the antisense orientation. Zero values are substituted with a small (smallest than the minimum) value so that log calculations are possible. Contrary to retrotransposons (**Extended Data Fig. 3**), there is no correlation, suggesting that extensive piRNA complementarity cannot explain the widespread mRNA binding shown by mRNA IgClips.
- c.** Scatterplot of *yw* embryo Aub IgClips mapped in the sense orientation on mRNAs with per base (nt) mRNA embedded retrotransposons (LINE, LTR, Satellite). Posterior, non-posterior and undetermined localizations are marked as indicated. The graph is separated in four quadrants: clockwise from lower left corner: 0 embedded repeats, 0 CLIP tags; 0 embedded repeats, >0 CLIP tags; >0 embedded repeats, >0 CLIP tags, >0 embedded repeats, 0 CLIP tags. The number of genes in the four quadrants is indicated. Zero values are substituted with a small (smallest than the minimum) value (different small value for every localization category was used for clarity) so that log calculations are possible. This graph

suggests that there is no correlation between the number of CLIP tags and the number of embedded repeats within the mRNAs.

d. Aub IgClips density surrounding (± 200 bases) mRNA embedded retrotransposons (LINE, LTR, Satellite as indicated). This analysis shows that there is no increase in the IgClip density in the areas flanking embedded repeats, suggesting that repeat sequences are not used as target areas for mRNA binding by Aub. Error bars \pm S.D.; $n=3$.

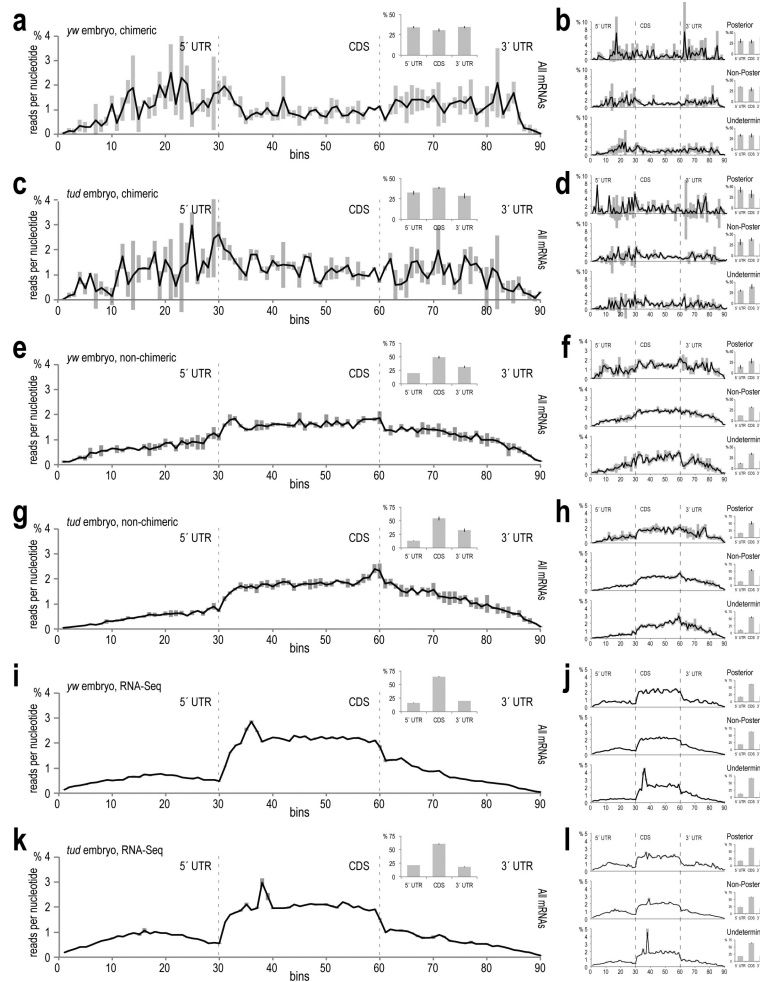
e. Analysis of mRNA expression level in relation to the number of embedded repeats. The number of embedded repeats per nucleotide of exon was plotted with the ratio (log10) of mRNA expression in *yw* embryo (0-2 h) versus *aub^{QC42HN2}* embryo (0-2 h) (left graph) and *yw* embryo (0-2 h) versus *tud* embryo (0-2 h) (right graph). The mRNAs are divided into groups based on the number of embedded repeats. A number above each data point denotes the number of mRNAs in each group. The graphs suggest that there is no proportional or consistent abundance change, decrease or increase, with the number of embedded repeats.



Extended Data Figure 7. Characteristics of piRNA base-pairing with complementary target sites identified from analysis of chimeric CLIP tags

a. piRNA:mRNA complementarity events for a random piRNA (negative control, average of three *yw* (upper panel) and *tud* embryo (lower panel) 0-2 h samples), within ± 100 bases from the midpoint of the mRNA part of the chimeric read. Complementarity events are plotted per alignment score group as indicated, for clarity. Inset (per sample): barplot of average complementarity events per score group, error bars \pm S.D.; $n=3$.

- b.** Size distribution of the piRNAs identified within chimeric CLIP tags, for *yw* and *tud* embryo CLIP libraries. Error bars, \pm S.D.; n=3. Only the piRNAs implicated in the complementarity events occurring within ± 25 nts from the midpoint of the mRNA fragment and with score ≥ 7 are analyzed in this graph, and the graphs in panels (c, d, e, g, h, i).
- c.** 5' end nucleotide preference for the piRNAs identified within chimeric CLIP tags, for *yw* and *tud* embryo Aub CLIP libraries. Error bars, \pm S.D.; n=3.
- d.** Genomic distribution for the piRNAs identified within chimeric CLIP tags, for *yw* and *tud* embryo Aub CLIP libraries. Error bars, \pm S.D.; n=3.
- e.** Per position nucleotide preference for all piRNAs in Aub *yw* embryo 0-2 h CLIP library L3 (left), and for the piRNAs identified within chimeric CLIP tags, for *yw* and *tud* embryo Aub CLIP libraries.
- f.** Complementarity events between piRNAs and mRNA fragments of chimeric reads, for posterior and non-posterior localized mRNAs (*yw* embryo). The plots are separated per score group. Error bars: \pm S.D.; n=3.
- g.** Heatmaps showing base paired nucleotides of piRNAs for all complementarity events identified within chimeric CLIP tags (events occurring within ± 25 nts from mRNA fragment midpoint, score ≥ 7) for *tud* embryo. Color is according to the length of the consecutive stretch of base paired nucleotides that runs over every position (color code shown on the right). Stacked piRNAs are aligned at their 5' ends and sorted (bottom to top) following these rules: *a*) starting position of the longest stretch of consecutive base paired nucleotides, relative to the piRNA end; *b*) length of longest base-paired stretch; *c*) total number of base-paired nucleotides.
- h.** Base-pairing frequency along the piRNA length for *yw* embryo libraries (blue) and their negative control (red). Error bars: \pm S.D.; n=3
- i.** Net base-pairing frequency along the piRNA length (red) and net density of base paired nucleotides (gray) in mRNAs from chimeric CLIP tags from *tud* embryo libraries. Error bars: \pm S.D.; n=3.



Extended Data Figure 8. Non-chimeric Aub CLIP tag (IgClip), chimeric piRNA-mRNA fragment and RNA-Seq read density along the untranslated and coding sequences of mRNAs

a. Average density of chimeric mRNA fragments (Aub CLIP, *yw* embryo 0-2 h) along the three parts of the meta-mRNA. Each mRNA region is divided in 30 bins and the number of the chimeric mRNA fragments (genomic coordinate of the mRNA fragment midpoint) mapped within each bin is counted. Error bars, \pm S.D.; $n=3$. Inset: bar plot showing cumulative density in each mRNA region.

b. Average density of the chimeric mRNA fragments on mRNA regions; mRNAs are separated in three localization groups, posterior localized (12 categories, **Supplementary Table 3**), non-posterior, and undetermined localization as indicated. Error bars, \pm S.D.; $n=3$. Inset: bar plot showing cumulative density in each mRNA region.

c. Same as **(a)** for chimeric mRNA fragments from Aub CLIP libraries, *tud* embryo 0-2 h.

d. Same as **(b)** for chimeric mRNA fragments from Aub CLIP libraries, *tud* embryo 0-2 h.

e. Same as **(a)** for non-chimeric IgClips from Aub CLIP libraries, *yw* embryo 0-2 h.

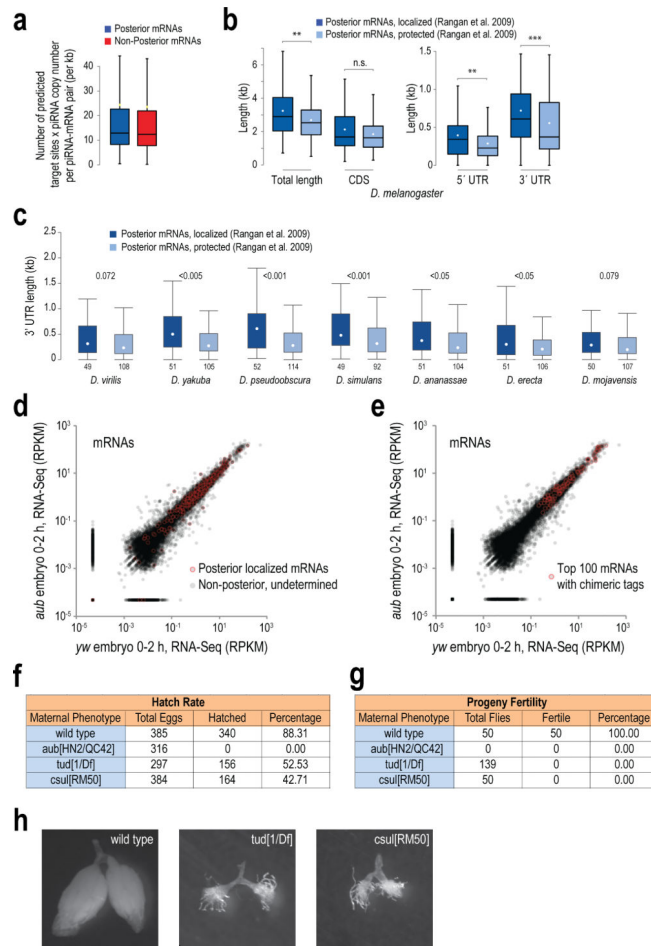
f. Same as **(b)** for non-chimeric IgClips from Aub CLIP libraries, *yw* embryo 0-2 h.

g. Same as **(a)** for non-chimeric IgClips from Aub CLIP libraries, *tud* embryo 0-2 h.

h. Same as **(b)** for non-chimeric IgClips from Aub CLIP libraries, *tud* embryo 0-2 h.

i. Same as **(a)** for RNA-Seq reads, *yw* embryo 0-2 h.

- j.** Same as **(b)** for RNA-Seq reads, *yw* embryo 0-2 h.
k. Same as **(a)** for RNA-Seq reads, *tud* embryo 0-2 h.
l. Same as **(b)** for RNA-Seq reads, *tud* embryo 0-2 h.



Extended Data Figure 9. Lengths of posterior localized mRNAs in *Drosophila* species; characteristics of embryos used in our studies

- a.** Box-and-whisker plot of the number of predicted piRNA target sites (per kb of mRNA sequence) for every mRNA-piRNA pair, multiplied by the piRNA copy number. Posterior and Non posterior mRNAs are as indicated. Median, black line. This graph indicates that the “targeting potential” (number of predicted complementary sites multiplied by the piRNA copy number) of every piRNA against each mRNA is the same for the two localization categories, suggesting that the piRNA copy number is not a contributing factor for the observed preference of posterior localized mRNAs for piRNA adhesion.
- b.** Box-and-whisker plot of the lengths of *D. melanogaster* mRNAs (and their 5' UTR, CDS and 3' UTR parts) that are found in the Enriched and Protected categories, as defined by the Lehmann lab¹⁰. Median, black line; mean, white dot; n.s.: p value >0.05; **: p value <0.01; ***: p value <0.001, one-sided Wilcoxon rank sum test.
- c.** Box-and-whisker plot of the lengths of the 3' UTRs of mRNAs from the indicated *Drosophila* species that are orthologous to the *D. melanogaster* mRNAs found in the

Enriched and Protected categories, as defined by the Lehmann lab¹⁰. Incomplete annotation did not allow us to perform this analysis for all the species shown on **Fig. 4i**. Mean, white dot; the p values of the statistical test (one-sided Wilcoxon test) of whether the lengths of the Localized versus Protected mRNAs are different, are displayed for each species.

d, e. RNA-Seq scatterplots from 0-2 h wild-type (*yw*) and 0-2 h Aub null (*aub*) embryos. Shown in red are posterior localized mRNAs (**d**) or the top 100 mRNAs identified from Aub CLIP piRNA-mRNA chimeric reads (**e**). There is no change in mRNA levels between wild-type and *aub* mutant 0-2 h embryos.

f, g. Hatch rates (**f**) and fertility of progeny (**g**) of embryos from indicated genotypes. Note that, unlike Tud and Csul, the absence of Aub (*aub[HN2/QC42]*) leads to complete embryo lethality.

h. Gross ovary appearance of wild-type (*yw*), tudor mutant (*tud[1/Df]*) and csul mutant (*csul[RM50]*) adult flies. Note complete absence of germline ovarian tissue in adult flies lacking Tudor or Csul; embryos from these flies develop into agametic adults because PGCs are never specified.

Extended Data Table 1

Overlap of piRNAs from CLIP and IP libraries Comparisons of piRNA sequences found in CLIP and IP libraries from same tissues.

Library 1	Library 2	unique piRNA sequences in library 1	unique piRNA sequences in library 2	common	percent1	percent2	average percent 2
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_H1	6913438	348812	150654	2.179147336	43.19060124	42.22806
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_H2	6913438	838891	333876	4.829377222	39.79968792	
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_H3	6913438	694458	284532	4.115636822	40.97180823	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_H1	9938639	560082	286627	2.883966306	51.17589924	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_H3	9938639	293375	156976	1.579451673	53.50694504	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_H2	9938639	332484	176012	1.770986953	52.93848727	
Aub_IP_tud_embryo_0-2h	Aub_CLIP_tud_embryo_0-2h_L1	5147948	1257672	458182	8.900284152	36.43096133	
Aub_IP_tud_embryo_0-2h	Aub_CLIP_tud_embryo_0-2h_H2	5147948	1104187	460392	8.943213879	41.69511143	
Aub_IP_tud_embryo_0-2h	Aub_CLIP_tud_embryo_0-2h_H3	5147948	2567880	948630	18.42734231	36.94214683	
Aub_IP_tud_embryo_0-2h	Aub_CLIP_tud_embryo_0-2h_H1	5147948	1040626	379030	7.362739484	36.4232683	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_L1	9938639	1850192	874693	8.800933407	47.27579624	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_L2	9938639	2407082	1108175	11.15016855	46.03810755	
Aub_IP_yw_ovary	Aub_CLIP_yw_ovary_L3	9938639	3082922	1367516	13.75959022	44.35778784	
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_L1	6913438	2012094	722743	10.45417634	35.91994211	
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_L2	6913438	2161685	769241	11.12675054	35.58524947	
Aub_IP_yw_embryo_0-2h	Aub_CLIP_yw_embryo_0-2h_L3	6913438	2701578	902250	13.0506703	33.39714789	

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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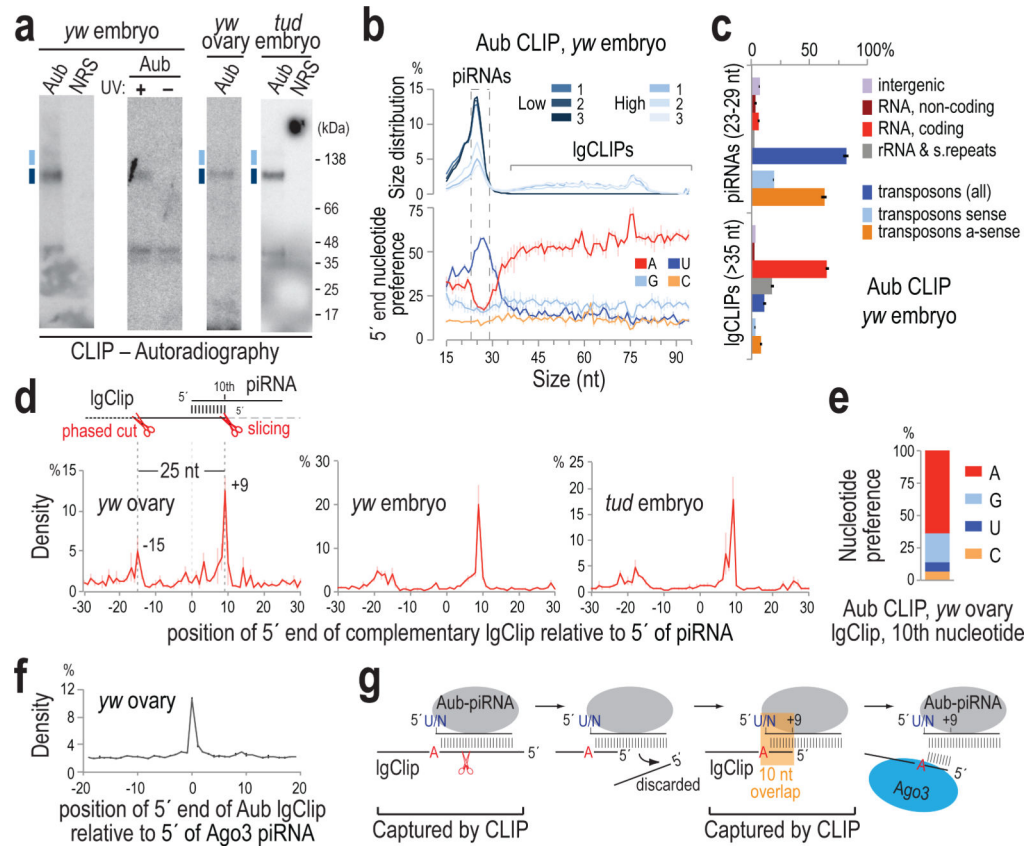


Figure 1. Transcriptome-wide identification of RNAs bound by Aubergine and *in vivo* retrotransposon targeting and slicing captured by CLIP

a. Aub CLIPs; separate libraries were prepared from RNA extracted from indicated positions; uncropped gels can be found in Supplementary Figure 1.

b. Size distribution and 5' end nucleotide composition per size of CLIP tag. Error bars represent one standard deviation (\pm S.D.; $n=3$; same applies to c, e, g).

c. Genomic distribution of CLIP tags for three High *yw* embryo (0-2 h) Aub CLIPs.

d. Position of 5' ends of retrotransposon IgClips relative to 5' ends of complementary piRNAs (0, x-axis).

e. Nucleotide composition at +9 of retrotransposon-derived IgClips with 10-nt overlap to complementary piRNAs.

f. *yw* ovary Aub IgClip 5' end positions relative to the 5' ends of Ago3-loaded piRNAs (0, x-axis).

g. Schematic of processing fragments captured by Aub CLIP.

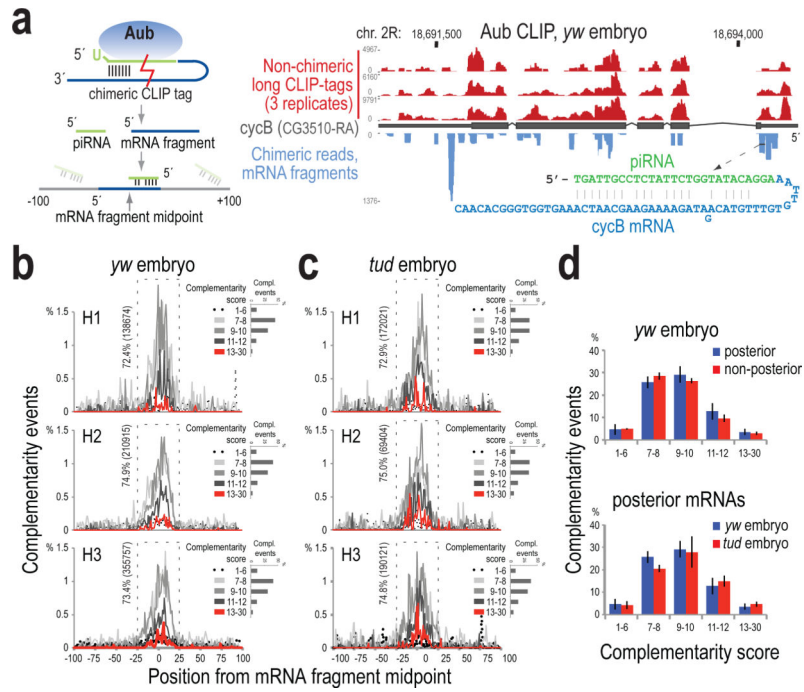


Figure 2. Complementarity analysis between the piRNA and mRNA parts of chimeric CLIP tags
a. Strategy for chimeric CLIP tag analysis, and genome browser illustrating AUB IgClips on *cycB*; sequence and base pairing of a chimeric CLIP tag is shown.

b, c. piRNA:mRNA complementarity events (percent) within ± 100 bases from the midpoint of the mRNA part of the chimeric read, plotted per alignment score for *yw* (**b**) and *tud* embryo (**c**) AUB CLIPs (biological triplicates). Percentage and number of total events occurring within ± 25 bases (dashed rectangle) are shown. Inset, per sample: barplot of number of complementarity events per score group.

d. Barplots of piRNA:mRNA complementarity events occurring within the ± 25 bases window of the midpoint of the search area and with score ≥ 7 , for indicated mRNA localization categories and AUB CLIP libraries. Error bars: \pm S.D.; $n=3$.

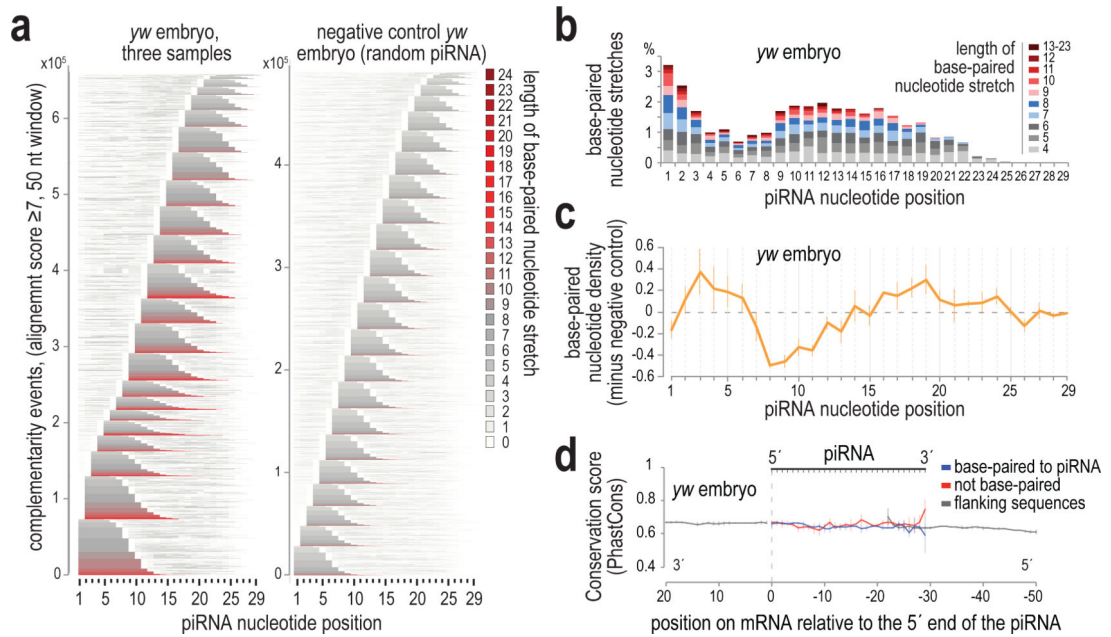


Figure 3. Characteristics of piRNA base-pairing identified by chimeric CLIP tag analysis

a. Heat maps showing base-paired nucleotides within the piRNA sequence, for all complementarity events (score ≥ 7) within ± 25 bases window, for *yw* embryo and negative control. Stacked piRNAs are sorted (bottom to top) by: starting position and length of the longest stretch and total number of base-paired nucleotides. Every nucleotide position is colored according to the length of the stretch of consecutively base-paired nucleotides that runs through that position.

b. Percent of stretches of consecutive base-paired residues per starting position within the piRNA sequence.

c. Base-paired nucleotide density per position minus negative control (random piRNA). Error bars: \pm S.D.; $n=3$.

d. Average mRNA conservation score on and around piRNA:mRNA contact sites. Error bars: \pm S.D.; $n=3$.

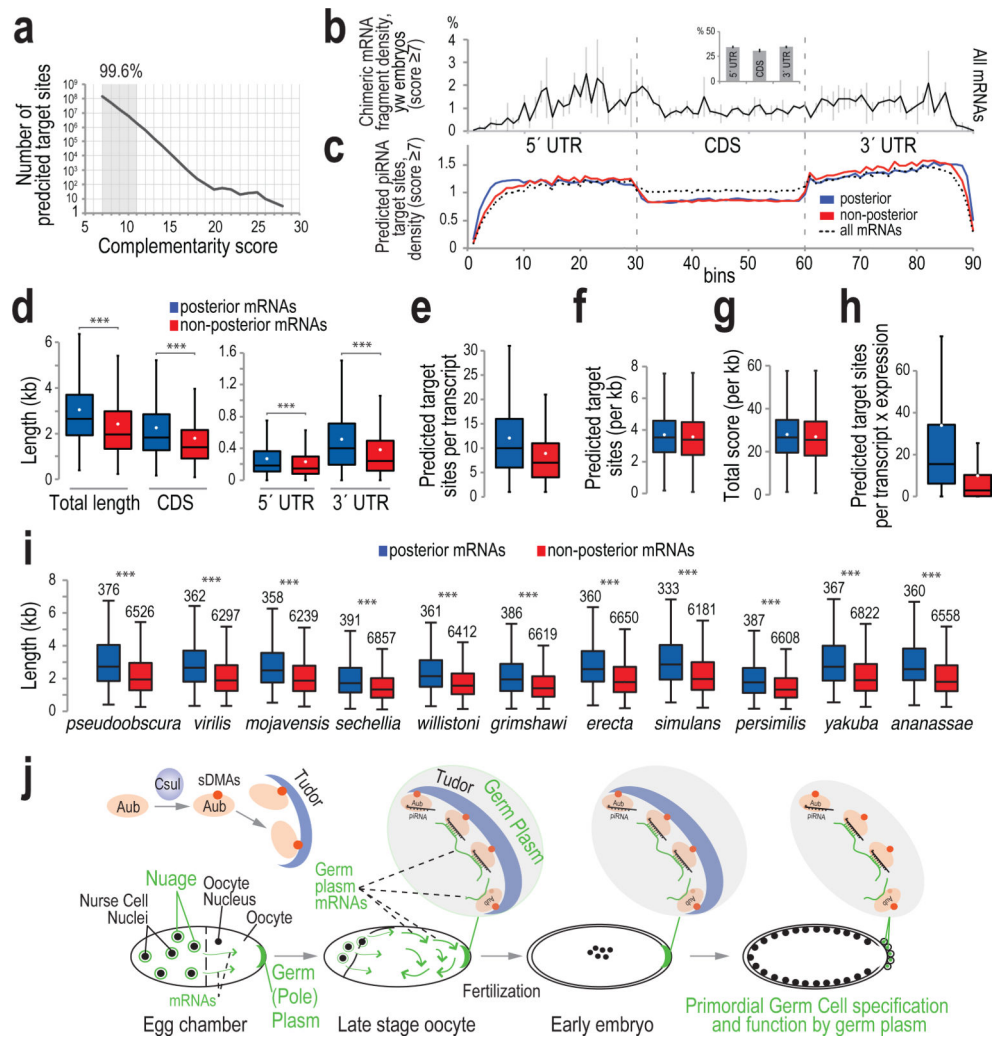


Figure 4. Transcriptome-wide prediction of piRNA target sites and length differential of posterior-localized mRNAs

a. Number of predicted piRNA complementary sites on mRNAs, per score.

b, c. Average binned density of: chimeric mRNA fragments (Aub CLIP, yw embryo 0-2 h) along the meta-mRNA (inset: bar plot showing cumulative density in each mRNA region); error bars, \pm S.D.; $n=3$ (**b**); predicted piRNA complementary sites within all (14058), posterior (380), and non-posterior (6747) localized mRNAs (**c**).

d – i. Box-and-whisker plots of: lengths of mRNAs expressed in yw embryos (0-2 h); median, black line; mean, white dot, ***: p value < 0.005 , one-sided t-test (**d**); number of predicted piRNA complementary sites per mRNA (**e**); length-normalized number of predicted piRNA complementary sites (**f**); length-normalized total score of predicted piRNA complementary sites (**g**); number of predicted piRNA complementary sites per mRNA multiplied with the abundance of each mRNA -RPKM- (**h**); lengths of orthologous mRNAs in other *Drosophila* species, ***: p value $< 10^{-16}$, one-sided Wilcoxon exact rank test (**i**).

j. Aubergine couples piRNA inheritance with germ cell specification in *Drosophila*. Aub, carrying arginines that are symmetrically dimethylated by Csil, interacts with Tudor, and both are localized in the germ plasm during mid-stage oogenesis. Ooplasmic streaming at

later stages promotes diffusion of mRNPs, facilitating random contacts of mRNAs with the germ plasm. AubpiRNAs form an adhesive trap that captures mRNAs forming numerous low complementarity contacts. mRNAs with posterior functions are longer and more abundant than the rest, form more piRNA mediated contacts with the germ plasm, thus their entrapment is enhanced. Tudor-AubpiRNA-mRNA complexes along with other RNA binding proteins form germ granules that contain both piRNAs and mRNAs that induce PGC specification. Aub and its RNA cargo is incorporated in PGCs providing the maternal mRNAs that are necessary for PGC function and the maternal piRNAs that will propagate an RNA immune response against transposons.