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Jonathan B. Preall, Benjamin Czech, Paloma M. Guzzardo, et al.

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shutdown is a component of the *Drosophila* piRNA biogenesis machinery

JONATHAN B. PREALL,[1] BENJAMIN CZECH,[1] PALOMA M. GUZZARDO, FELIX MUERDTER, and GREGORY J. HANNON[2]

Howard Hughes Medical Institute, Watson School of Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA

ABSTRACT

In animals, the piRNA pathway preserves the integrity of gametic genomes, guarding them against the activity of mobile genetic elements. This innate immune mechanism relies on distinct genomic loci, termed piRNA clusters, to provide a molecular definition of transposons, enabling their discrimination from genes. piRNA clusters give rise to long, single-stranded precursors, which are processed into primary piRNAs through an unknown mechanism. These can engage in an adaptive amplification loop, the ping-pong cycle, to optimize the content of small RNA populations via the generation of secondary piRNAs. Many proteins have been ascribed functions in either primary biogenesis or the ping-pong cycle, though for the most part the molecular functions of proteins implicated in these pathways remain obscure. Here, we link *shutdown* (*shu*), a gene previously shown to be required for fertility in *Drosophila*, to the piRNA pathway. Analysis of knockdown phenotypes in both the germline and somatic compartments of the ovary demonstrate important roles for *shutdown* in both primary biogenesis and the ping-pong cycle. *shutdown* is a member of the FKBP family of immunophilins. Shu contains domains implicated in peptidyl-prolyl *cis-trans* isomerase activity and in the binding of HSP90-family chaperones, though the relevance of these domains to piRNA biogenesis is unknown.

Keywords: piRNAs; transposon silencing; RNAi; FKBP; germ cells

INTRODUCTION

Eukaryotic genomes are prone to the accumulation of repetitive sequences, including transposable elements, over evolutionary time (McClintock 1953; Kim et al. 1994; Brennecke et al. 2007; Chambeyron et al. 2008; Feschotte 2008). The genomic instability brought about by transposon activity is a double-edged sword. Low levels of transposition can drive evolution in the long term, but loss of control over mobile elements in any individual can threaten reproductive success. Mechanisms for suppressing transposon activation in the germline are therefore both potent and widely conserved (Grimson et al. 2008). In animals, the PIWI-interacting RNA (piRNA) pathway is key to transposon silencing in reproductive tissues (Aravin et al. 2006; Girard et al. 2006; Lau et al. 2006; Vagin et al. 2006; Malone and Hannon 2009; Khurana and Theurkauf 2010; Senti and Brennecke 2010). In *Drosophila*, piRNAs are active both in the germ cell lineage and in a particular somatic lineage that encysts the germ cells and provides growth and maturation signals (Malone et al. 2009).

piRNA clusters sit at the apex of the pathway and, based upon their sequence content, define transposon targets for repression (Brennecke et al. 2007). piRNA clusters give rise to long, single-stranded transcripts (Brennecke et al. 2007) that are thought to be exported to the cytoplasm and processed into primary piRNAs, most likely in specialized cytoplasmic structures (Saito et al. 2010; Handler et al. 2011). A number of proteins have been implicated in primary piRNA biogenesis and their loading into PIWI-family proteins, including Armitage, Zucchini, Vreteno, and the Yb family (Klattenhoff et al. 2007; Pane et al. 2007; Malone et al. 2009; Szakmary et al. 2009; Haase et al. 2010; Olivieri et al. 2010; Saito et al. 2010; Handler et al. 2011; Zamparini et al. 2011). Yet, almost nothing is known about how each of these promotes the production of primary piRNAs.

The soma relies on a single piRNA cluster, *flamenco* (*flam*) (Brennecke et al. 2007). This ~180 kb, centromere-proximal locus on the X chromosome produces a piRNA population that is strongly enriched for species antisense to the *gypsy* family elements. These elements are active in follicle cells and can propagate by infection of germ cells through

[1]These authors contributed equally to this work.

[2]**Corresponding author**
E-mail hannon@cshl.edu

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their capability to form virus-like particles (Pelisson et al. 1994; Chalvet et al. 1999). Somatic piRNAs are produced solely through primary biogenesis (Brennecke et al. 2007; Malone et al. 2009). In the germline, a greater variety of clusters targets a broad spectrum of mobile elements and engages an adaptive cycle, termed ping-pong, through which transposon mRNAs help to shape piRNA populations (Brennecke et al. 2007; Gunawardane et al. 2007). Here, antisense-oriented piRNAs derived from genomic clusters are loaded into Aubergine (Aub) and cleave active transposable element transcripts in an RNAi-like reaction. Unlike classical RNAi, this triggers the production of a new small RNA, derived from the target mRNA and with its 5′ end formed by Aub-mediated cleavage. The new, secondary piRNA is loaded into Ago3, which can then use this sense-oriented species to recognize and cleave cluster-derived transcripts, producing more antisense piRNAs via a similar mechanism.

Mutations in the *Drosophila* piRNA pathway generally result in sterility with stereotypical phenotypes in the male and female germline (Schupbach and Wieschaus 1991; Wilson et al. 1996; Gonzalez-Reyes et al. 1997; Lin and Spradling 1997; Cox et al. 2000; Cook et al. 2004). In part, these are thought to result from DNA double-strand breaks induced by element activity (Chen et al. 2007; Klattenhoff et al. 2007). Such breaks trigger meiotic checkpoint activation mediated by the *Drosophila chk2* ortholog, *loki,* which in turn disrupts dorsal-ventral axis formation during oogenesis. Hence, mutations in secondary piRNA genes such as *aubergine* display fused dorsal appendages and other hallmarks of oocyte ventralization (Theurkauf et al. 2006). Transposon silencing is also critical for the maintenance of germline stem cells (Lin and Spradling 1997; Cox et al. 2000; Houwing et al. 2007). In the male germline, loss of *Su(ste)* piRNAs derepresses the repetitive *Stellate* locus, which disrupts spermiogenesis by causing the overproduction and eventual crystallization of Stellate protein within the testis (Bozzetti et al. 1995; Aravin et al. 2001). Several mutants that are now known to affect the *Drosophila* piRNA pathway—including *aubergine*, *zucchini*, *squash*, *vasa*, and *cutoff*—were first described in a female sterility screen by Schüpbach and Wieschaus over 20 yr ago (Schupbach and Wieschaus 1989, 1991). Of the genes identified in that study that would eventually come to be known as piRNA factors, all but *cutoff* were classified phenotypically as having defects in dorsal appendage formation (Schupbach and Wieschaus 1991).

Subsequently, Munn and Steward (2000) mapped another of these female sterile mutations, *shutdown (shu, CG4735),* to an immunophilin gene of the FK506-binding protein (FKBP) family. Mutations in *shu* disrupt germ cell division, eventually causing the germline stem cells to fail entirely. Two strong alleles caused sterility in both males and females, while a third point mutant allele did not affect male fertility. In mutant females, stem cells that successfully divide generally produce faulty egg chambers that arrest mid-oogenesis. Germline clones for strong alleles of *shu* can produce mature oocytes, though they display typical patterning defects such as fused dorsal appendages. Considered together, these observations implicate *shu* as a component of the *Drosophila* piRNA pathway. This conjecture is supported by the presence of FKBP6, the mammalian protein most similar to Shutdown, in complexes with mammalian Piwi-family proteins, Miwi and Miwi2 (Vagin et al. 2009).

FKBPs play diverse biological roles ranging from facilitating protein folding to modulating transport (Ahearn et al. 2011), receptor signaling (Li et al. 2011), and meiotic recombination (Crackower et al. 2003; Kang et al. 2008). The FKBP domain is annotated as a peptidyl-prolyl *cis-trans* isomerase (PPIase), though there are many instances of well-conserved FKBP domains that lack PPIase activity (Gollan and Bhave 2010). The macrolide immunosuppressants FK506 (tacrolimus) and rapamycin (sirolimus) bind with sub-nanomolar affinity to the FKBP domain and block a key protein–protein interaction surface, but as is the case with PPIase activity, many family members display much reduced affinities for these compounds (DeCenzo et al. 1996; Gollan and Bhave 2010).

FKBP-class immunophilins display a variety of domain architectures. One arrangement, conserved from protozoa to humans, places a tetratricopeptide repeat (TPR) domain downstream from the FKBP domain (Pratt et al. 2004). The TPR domain is a protein–protein interaction module that binds heat shock proteins (HSPs), primarily of the HSP90 family in higher eukaryotes (Pratt 1998; Allan and Ratajczak 2011). Several crystal structures are available that highlight key conserved residues that participate in this interaction (Van Duyne et al. 1993; Ward et al. 2002). Connections between small RNA silencing pathways and HSP activity have been observed in several model systems (Smith et al. 2009). In particular, RNA-induced silencing complex (RISC) loading is facilitated by HSP90 and ATP hydrolysis (Iki et al. 2010; Iwasaki et al. 2010; Miyoshi et al. 2010; Iki et al. 2011).

Here, we report that *shutdown* is a critical element of the *Drosophila* piRNA pathway. Tissue-specific depletion of Shu results in derepression of transposon expression and a near-complete loss of mature piRNAs in both the somatic and germline lineages. Shu is cytoplasmically localized, and its loss disrupts the localization of all three piRNA effectors, Piwi, Aub, and Ago3. We hypothesize that Shu is an essential component of both primary and ping-pong–derived piRNA biogenesis, likely acting at a very early step that is shared between both piRNA systems.

RESULTS AND DISCUSSION

Clues to a role for FKBPs in the piRNA pathway

We previously carried out a proteomic analysis of mammalian PIWI proteins, Miwi and Miwi2 (Vagin et al. 2009). Among the components of these complexes were murine

FKBP6 and multiple HSPs. FKBP5 was also detected in Miwi immunoprecipitates with roughly half the coverage seen for FKBP6. Given the greater convenience of manipulating the piRNA pathway in *Drosophila*, we chose to examine potential roles for FKBP proteins in that model system.

The *Drosophila* genome encodes eight FKBP family members (Fig. 1A,B). Three, CG1847, CG5482, and FKBP59, are annotated to share the domain architecture of FKBP6, with their FKBP domains followed by a TPR. Shutdown is a potential fourth member of this group. Though its TPR

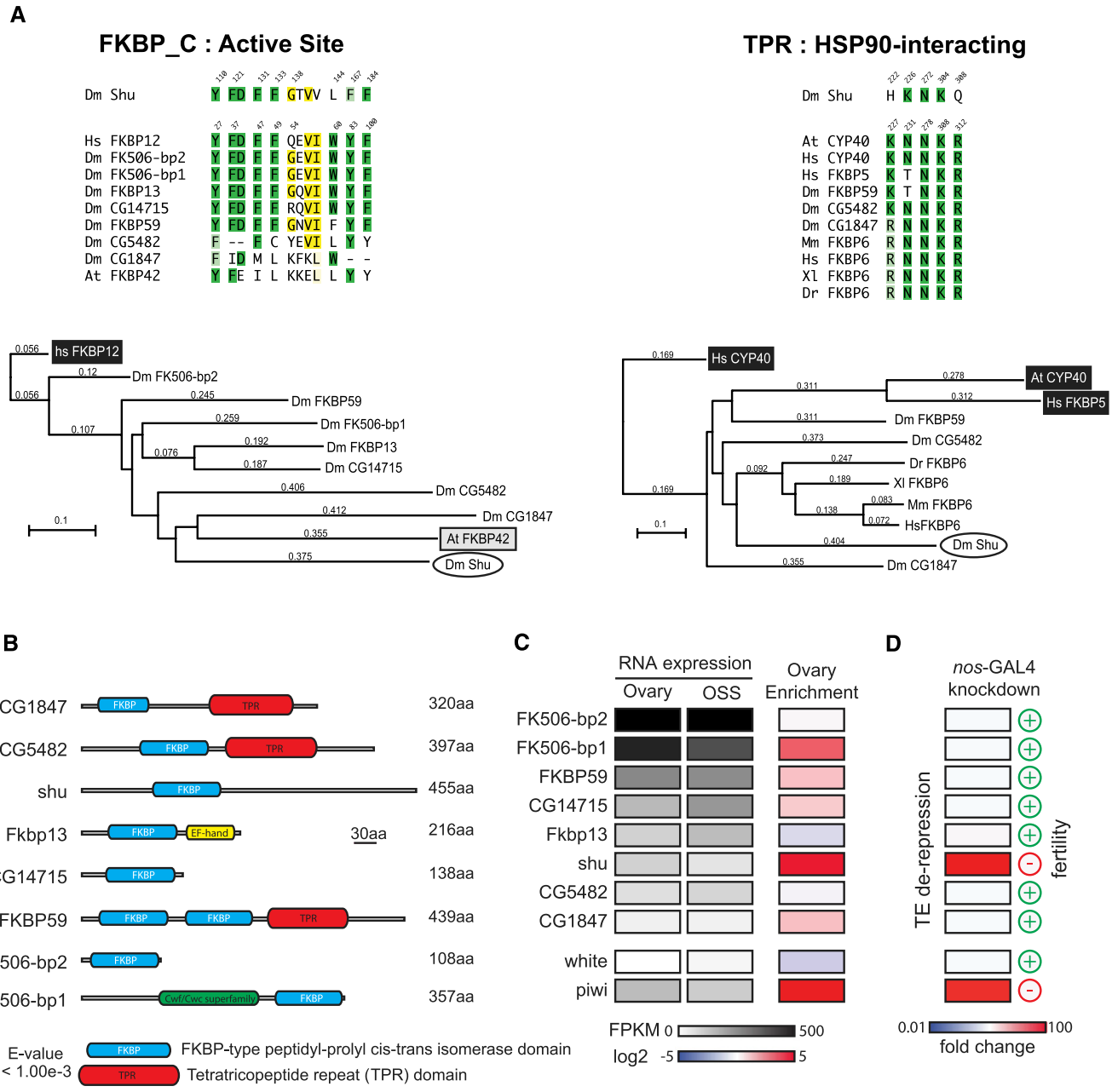

FIGURE 1. Shutdown is the only FKBP-family protein required for transposon silencing. (*A*) *Above* are shown the critical residues for the FKBP family peptidyl-prolyl *cis-trans* isomerase active site and the HSP90-interacting region in the TPR, as indicated, comparing the site in *Drosophila* Shutdown with those present in other family members. Residues in green indicate highly conserved residues with a known impact on PPIase activity, while those in yellow indicate a more poorly conserved region that has also been implicated. *Below* are evolutionary trees comparing each domain with family members present in other species. (*B*) The domain structures of the eight *Drosophila* FKBP family members are shown schematically. (*C*) Relative expression levels of *Drosophila* FKBP family members are shown for ovary and OSS RNAseq data sets. Relative enrichment in ovary versus other tissues is also shown. (*D*) Shown are relative *HetA* expression levels detected in ovaries from *Drosophila* engineered to express dsRNAs corresponding to each family member in the germline lineage. To the *right* is indicated whether dsRNA-expressing females are fertile (+) or sterile (−).

domain has substantially diverged in comparison to its paralogs (TPR_2, Pfam e-value = 0.0026), secondary structure predictions using the Phyre2 algorithm (www.sbg.bio.ic.ac.uk/phyre2) annotate the putative TPR as such with high confidence. Among *Drosophila* FKBPs, Shutdown is most closely related to FKBP6 ($E = 2 \times 10^{-36}$) overall, whereas FKBP59 is a potential FKBP5 ortholog ($E = 2 \times 10^{-46}$).

An examination of RNAseq both from the *Drosophila* ovarian somatic sheet (OSS) cell line and from a published ovarian data set revealed that several FKBPs are expressed in female reproductive tissues (Fig. 1C; Gan et al. 2010). A broader set of published microarray data (Chintapalli et al. 2007) suggested that the expression pattern of *shu* is much more biased to the ovary than is expression of other family members (Fig. 1C), a bias shared by many piRNA pathway components.

The FKBP_C domain is broadly conserved across evolution, though its PPIase activity is not (Kamphausen et al. 2002). Phylogenetic comparison of *Drosophila* FKBP_C domains to known active (*Homo sapiens* FKBP12) and inactive (*Arabidopsis thaliana* FKBP42) PPIase domains suggested that *shu* is more similar to inactive variants (Fig. 1A, bottom). Shutdown does retain more of the active site residues shown to be essential for PPIase activity in human FKBP12 (Fig. 1A, left) than does *AtFKBP42*. A conserved tryptophan residue (W60 in *Hs*Fkbp12) has been replaced by a leucine in Shutdown. Introduction of this change into Fkbp12 reduces PPIase activity by approximately eightfold and rapamycin and FK506 binding affinity by 10- and 75-fold, respectively. It is therefore likely that Shutdown does not represent an optimally active PPIase and may instead utilize the domain as a protein interaction interface, as do other FKBP family members (Gollan and Bhave 2010).

The Shu TPR domain is less well conserved and, in fact, shows little similarity to other TPRs known to bind HSP90 (Fig. 1A, right). In particular, nonconservative amino acid changes at two key residues suggested that the affinity of this domain for the C-terminal MEEVD of HSP90 is likely to be dramatically reduced compared with other family members (Ratajczak and Carrello 1996; Ward et al. 2002). Still, a *shu* allele (shu^{PB70}) bearing a point mutation at a nonconserved residue in the putative TPR is sufficient to cause female sterility, indicating that this region is essential for some aspects of Shu function.

Shutdown is implicated in transposon silencing

Recent work has suggested that Dcr-2 is a limiting factor that prevents conventional dsRNA triggers from inducing potent RNAi in *Drosophila* germ cells, but that this restriction could be overcome by enforced Dcr-2 expression (Handler et al. 2011; Wang and Elgin 2011). We took advantage of this observation by bringing UAS-driven dsRNA constructs from the Vienna *Drosophila* RNAi Center (VDRC) into a background containing a germline-specific GAL4-driver ({GAL4-nos.NGT}40; aka *nos*-GAL4) and a UAS-*Dcr-2* transgene. Among dsRNAs targeting all fly FKBP proteins, only those corresponding to *shu* had significant impacts on expression levels of the *HetA* transposon (Fig. 1D). Moreover, only dsRNAs targeting Shu caused female sterility, a property typical of piRNA mutants (Fig. 1D).

To validate *shu* as a novel piRNA pathway component, we compared the impact of its depletion to knockdowns of known piRNA pathway genes, *armi* and *piwi*. Germline silencing of each gene resulted in a similar level of derepression for 17 transposons, measured by quantitative PCR (qPCR) (Fig. 2A). The tissue specificity of our knockdown strategy was supported by the fact that germline-specific, telomeric transposons *TAHRE*, *HetA*, and *TART* were the most heavily derepressed (greater than 150-fold, $P < 0.01$), whereas RNA levels for primarily somatic elements, such as *ZAM,* remained unchanged (about 1.2- to 1.5-fold).

Shu RNAi also recapitulated the ventralized egg phenotype of shu^{PB70} germline clones, as evidenced by a high incidence of fused or abnormal dorsal appendages (Fig. 2B; Munn and Steward 2000). Surprisingly, the ventralization phenotype was not penetrant in *armi* and *piwi* knockdowns eggs, despite the eggs being nonviable (Fig. 2C). For *armi*, prior studies of mutants produce a clear expectation of ventralization upon potent knockdown (Klattenhoff et al. 2007; Orsi et al. 2010). For *piwi*, the prediction is less clear. Germline *piwi* clones were reported not to show this distinctive phenotype; however, RNAi-mediated *piwi* knockdown did produce eggs with a spindle morphology (Cox et al. 2000; Wang and Elgin 2011). In addition to causing sterility, *shu* depletion also reduced the number of nonviable eggs laid, suggesting that there may be additional requirements for *shu* function outside of piRNA-mediated transposon silencing.

Shu is required for Piwi, Aub, and Ago3 localization

In wild-type tissues, Piwi is localized to the nucleus of germline and somatic cells (Cox et al. 2000; Saito et al. 2006; Brennecke et al. 2007). Aub and Ago3 are expressed exclusively in the germline and are enriched in a perinuclear organelle called nuage (Lim and Kai 2007; Li et al. 2009). Proper localization depends upon normal piRNA production and loading into PIWI family proteins, and disruption of this pattern is an indicator of impaired biogenesis (Malone et al. 2009).

Depletion of *shu* using the *nos*-GAL4 driver resulted in redistribution of Piwi from nurse cell nuclei to the syncytial cytoplasm of the developing egg chamber, while neighboring somatic follicle cells retain proper nuclear Piwi localization (Fig. 2D). Similarly, the ping-pong factors Ago3 and Aub were redistributed from nuage to cytoplasmic foci, while the localization of the core nuage component Vasa was not altered (Fig. 2D). Driving the *shu* dsRNA using

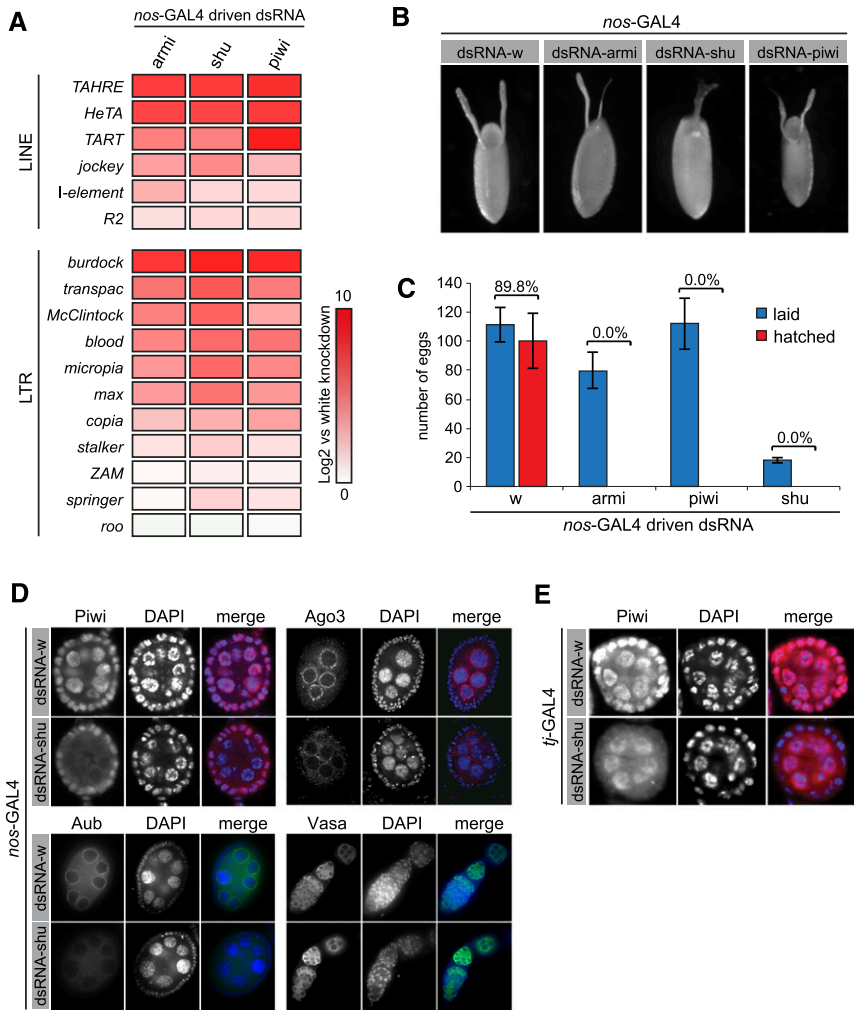

FIGURE 2. Phenotypes of *Drosophila* with germline-specific *shu* knockdown. (*A*) Depletion of *shu* in the germline results in derepression of multiple, unrelated transposons from the LINE and LTR families. Derepression, relative to *white* RNAi, is displayed as log2 fold change in heat map form. Analysis of flies with germline knockdown of Armi and Piwi, two known piRNA components, is displayed for comparison. (*B*) Germline-knockdown of *shu* causes patterning defects as indicated by the presence of fused dorsal appendages. (*C*) Depletion of *shu* causes female sterility. *shu* RNAi females lay fewer eggs compared with controls or animals depleted of other piRNA pathway factors. Hatching rates for all knockdown animals are zero, indicating complete sterility. (*D*) Depletion of *shu* in the germline using *nos*-GAL4 results in Piwi delocalization from nuclei and in Aub and Ago3 delocalization from nuage. Vasa localization is not changed. Depletion of *white* is shown as control. (*E*) *Tj*-GAL4–driven knockdown of *shu* in somatic follicle cells also causes Piwi delocalization. RNAi against *white* is shown as control.

GAL4 expressed from the soma-specific *traffic jam* promoter (*tj*-GAL4) caused delocalization of Piwi from the nuclei of follicle cells, while germline Piwi remained unaffected (Fig. 2E).

Despite its effects on the localization of PIWI-family proteins, we found that the bulk of Shutdown was not associated with domains characteristic of those piRNA pathway components. We generated N- and C-terminal GFP fusions of Shu expressed under the control of the ubiquitous *Actin5c* promoter. We examined the localization of Shutdown fusion proteins by transfection of OSS cells. Control constructs showed the expected localization with GFP-Piwi accumulating in nuclei and with GFP-Armi showing strong perinuclear localization consistent with its association with Yb-bodies. Zucchini features sequence homology with phospholipase D and was reported to localize to the outer membrane of mitochondria. In our studies, it displayed considerable overlap with the mitochondrial stain MitoTracker CMXRos (Supplemental Fig. S2). While cytoplasmic foci of GFP-tagged Shu were visible using both N- and C-terminal constructs, they did not overlap with the previously characterized localization patterns of other piRNA pathway proteins. Considered together, these data indicate that Shu is neither enriched in known structures associated with silencing nor required for assembly of a core nuage component.

Shu is essential for accumulation of both primary and secondary piRNAs

Strong derepression of germline and somatic transposons and the loss of characteristic localization patterns for Piwi-family proteins suggested that *shu* might function as a core piRNA biogenesis component, similar to *armi*. To address this possibility, we cloned and sequenced small RNAs from ovaries in which we drove the expression of *white* (*w*), *shu*, and *piwi* dsRNAs in the germline (*nos*-GAL4) or soma (*tj*-GAL4), as described above. Germline small RNAs libraries were normalized using the number of unique reads mapping to the *flam* locus, which is unaffected by germline-specific knockdowns. Germline-specific *shu* knockdown dramatically reduced the observed piRNA population compared with the *white* knockdown control. Small RNA reads with the characteristic piRNA size (23–29 nucleotides [nt]) mapping to the germline-specific, dual-strand *42AB* cluster were reduced 11.4-fold overall (8.2× on plus strand, 14.4× on minus strand). In contrast, *piwi* knockdown produced only a 2.8-fold overall reduction (2.8× on plus strand, 2.7× on minus strand) (Fig. 3A).

The incomplete loss of piRNAs in the *piwi* knockdown likely reflects the fact that piRNAs from *42AB* are normally loaded into each of the three *Drosophila* PIWI proteins

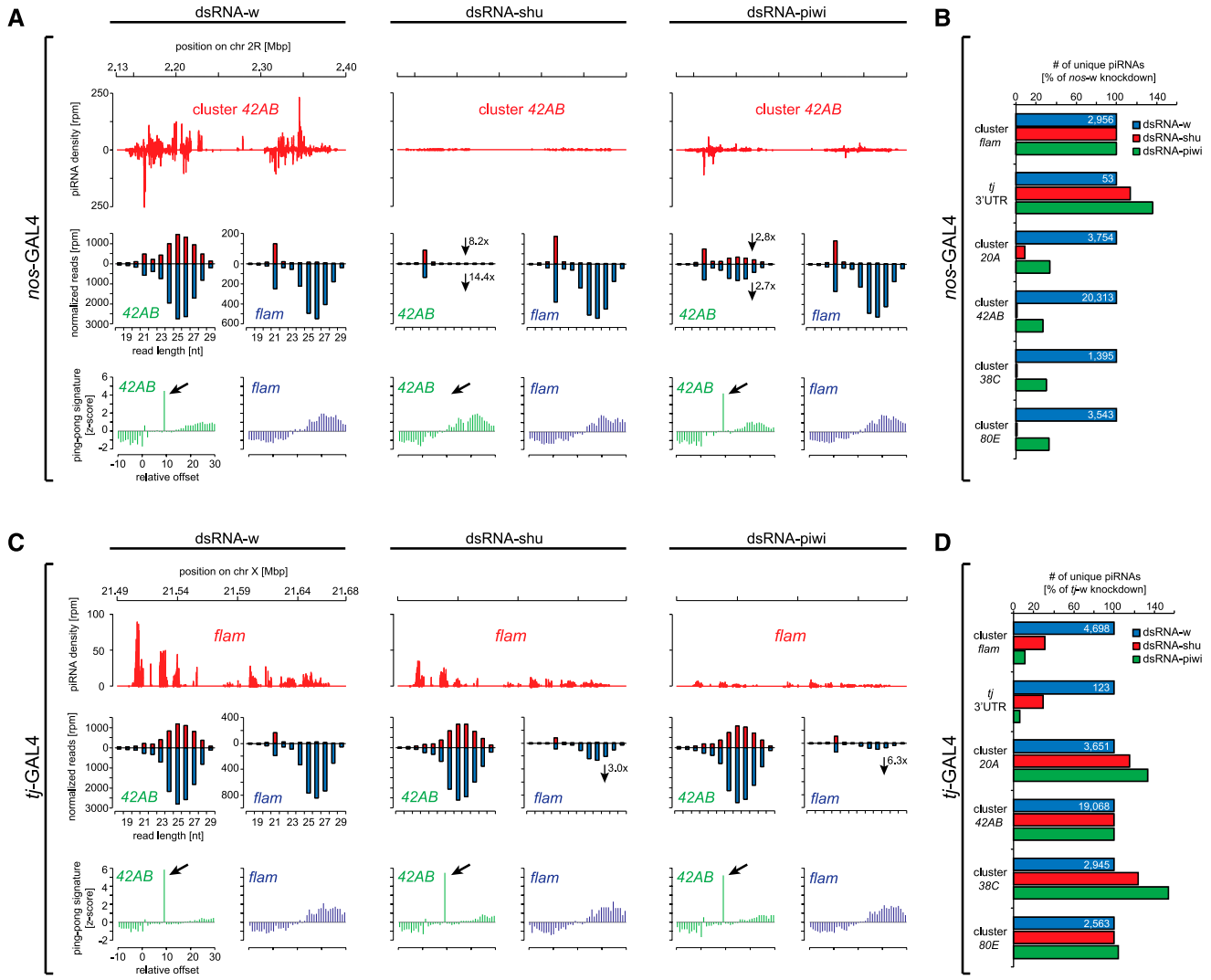

FIGURE 3. Knockdown of *shu* causes loss of cluster-derived piRNAs in both somatic and germline tissues. (*A*) At the *top* is shown a histogram of small RNAs mapping to the germline-specific *42AB* cluster in flies expressing the indicated dsRNAs specifically in germ cells. In the *middle*, the size distribution of RNAs derived from each strand of the *42AB* and *flamenco* clusters is shown as a histogram. At the *bottom* are histograms reflecting the relative enrichment of RNAs overlapping by the indicated number of nucleotides, plotted by *Z*-score, for the *42AB* and *flamenco* clusters in the indicated knockdown animals. The peak at position 9 (arrow) is indicative of a ping-pong interaction. (*B*) A histogram shows relative piRNA levels for a series of germline and somatic clusters. Total reads were normalized across libraries to piRNAs mapping to *flamenco*, which is unaffected in germline-specific knockdowns. For each cluster, changes in mapping piRNAs are shown with reference to the *white* control, which is set to 100%. *C* and *D* are similar to *A* and *B* except that dsRNA expression is driven by a follicle cell–specific *tj*-GAL4 driver. In *C*, at the *top*, reads are shown mapping to the soma-specific *flamenco* cluster. In *D*, reads are normalized across libraries to those derived from *42AB*, whose activity is not affected in the soma-specific knockdown.

(Brennecke et al. 2007), with loading of Aub and Ago3 occurring independently of Piwi function. Small RNAs mapping to this cluster in the *shu*-depleted germline also showed a clear reduction in ping-pong signatures, defined as the frequency of reads with a paired opposite strand read overlapping by 10 nt (Fig. 3A). In contrast, *piwi*, which does not participate significantly in ping-pong amplification, had no effect on ping-ping signatures upon knockdown. The effects of *shu* knockdown appear to be specific to the piRNA pathway. Reads corresponding to miRNAs were not reduced in *shu* knockdown animals (Supplemental Fig. S3). Though enforced Dicer-2 expression generally increased the endo-siRNA fraction, we did not note any further effect of *shu* knockdown, even on endo-siRNAs mapping to piRNA clusters (e.g., *42AB*) (Fig. 3A).

We also analyzed effects of *shu* or *piwi* depletion on other piRNA clusters. We compared reads that could be uniquely mapped to each annotated cluster to the *white* knockdown controls. Reads were set to 100% in the *white* library (normalized read number for the *white* knockdown library is show as a blue bar). piRNAs derived from the 3′ UTR of *traffic jam*, a genic locus that produces piRNAs only in

follicle cells, showed no impact of *shu* and *piwi* knockdowns (Fig. 3B), as expected. In contrast, all germline clusters analyzed showed a dramatic reduction of piRNA levels upon expression of *nos*-GAL4–driven *shu* dsRNAs (<10% remaining as compared to *white* RNAi). Depletion of Piwi had similar effects, although the reduction was less profound (~30% of *white* levels, as seen for *42AB*), probably due to intact Ago3 and Aub loading.

Primary and secondary piRNA biogenesis mechanisms in the germline exhibit some degree of interdependence. For example, disruption of ping-pong in *ago3* mutants or upon Aub knockdown feeds back and reduces the number of primary piRNAs loading into Piwi through unknown mechanisms (Li et al. 2009; Wang and Elgin 2011). Follicle cells, which are of somatic origin, express no detectable Aub or Ago3 and do not use ping-pong amplification. Thus, we directly tested the involvement of *shu* in primary piRNA production by sequencing small RNAs from *tj*-GAL4–driven dsRNA in ovaries. PiRNA-sized small RNAs were normalized using the number of unique reads mapping to the germline-specific *42AB* locus, which is unaffected by *tj*-GAL4–mediated knockdowns.

The sole somatic, unidirectional *flamenco* cluster produces abundant piRNAs that load only the Piwi protein. Thus, as expected, depletion of *piwi* caused a significant reduction in piRNAs derived from this locus (5.2-fold) (Fig. 3C,D). Follicular knockdown of *shu* also produced a marked reduction in *flam* piRNAs (2.9-fold) (Fig. 3C,D). As expected, piRNAs uniquely mapped to *flam* showed no ping-pong signature in any of the somatic knockdowns. Reads corresponding to germline clusters remained unchanged in piRNA abundance, with no shift in size profiles or ping-pong signatures, indicating that, as expected, the pathway remains fully functional in germ cells of animals that have lost *shu* expression only in the soma. As in germ cell–specific knockdowns, miRNA abundance was unaffected (Supplemental Fig. S3).

Mapping small RNAs from our germline-specific knockdown animals to a set of known *Drosophila* transposon consensus sequence further supported a general requirement for *shu* in piRNA accumulation. We retained in our analysis only the 75 transposons with the highest abundance of corresponding piRNAs. Previous reports have demonstrated substantial expression biases for many transposons, with some showing preferential expression in the somatic lineage and others being found predominantly in germline lineages (Malone et al. 2009). For the set of germline-enriched transposons, *nos*-GAL4–driven dsRNA-*shu* substantially affected all known elements, reducing overall piRNA levels (Fig. 4A). In general, sense and antisense piRNAs were depleted to roughly similar extents, suggesting that loading of all three PIWI clade proteins is affected by loss of *shu*. In contrast, only a subset of transposons showed depletion of piRNAs in the *nos*-GAL4–driven *piwi* knockdowns. Elements with a known somatic expression bias, including *ZAM*, *tabor*, *gypsy*, and others (indicated by red dots), show little or no reduction in piRNA levels upon germline knockdown of either *shu* or *piwi* (Fig. 4A). Transposable elements with strong germline signatures (green asterisks), like the LINE element *Rt1b* or the LTR transposon *roo* (pao family), not only showed a severe reduction of their corresponding piRNA levels but also demonstrated a dramatic loss of ping-pong signatures (Fig. 4B). In contrast, soma-specific elements retain their piRNA levels and generally lack ping-pong signatures. As an example of such an element, piRNA levels for the LTR element *ZAM* (gypsy family) are shown (Fig. 4B, bottom).

Summary

A combination of biochemical and genetic approaches are beginning to link a substantial number of proteins to functions in the piRNA pathway. Some act exclusively in primary piRNA biogenesis and affect small RNAs in both the germline and somatic compartments of the *Drosophila* ovary (Malone et al. 2009; Haase et al. 2010; Olivieri et al. 2010; Saito et al. 2010; Handler et al. 2011; Zamparini et al. 2011). Others function exclusively in the germline, and these tend to selectively affect the ping-pong cycle that hones piRNA populations in response to the expression of transposon mRNAs or factors implicated in germline cluster transcription (Klattenhoff et al. 2009; Li et al. 2009; Patil and Kai 2010; Pane et al. 2011; Zhang et al. 2011; Anand and Kai 2012). Here, we followed clues initially provided by proteomic analysis of Piwi-family protein complexes in mice to link *shutdown*, a gene previously shown to be required for fertility in *Drosophila* females (Schupbach and Wieschaus 1991; Munn and Steward 2000), to the piRNA pathway.

Analysis of transposon expression patterns and small RNA libraries in *shu* knockdown cells and animals suggests a role either in piRNA biogenesis or in piRNA stabilization, perhaps by fostering loading of piRNAs into PIWI-family proteins. Shutdown is a member of the FKBP family and its constituent domains have been ascribed PPIase activity and the ability to interact with the HSP90 family of chaperone proteins. Either of these activities could underlie the role of Shutdown in the piRNA pathway. In particular, studies of the Argonaute clade have implicated HSP family chaperones as critical cofactors for small RNA loading (Iki et al. 2010; Iwasaki et al. 2010; Miyoshi et al. 2010; Iki et al. 2011). However, evolutionary comparisons indicate that both the PPI and HSP90-binding domains harbor variations that reduce activity when introduced into other well-studied FKBP family members. Thus, understanding the true role of Shutdown in both primary biogenesis and the ping-pong cycle will await further genetic analysis and the development of biochemical systems that recapitulate aspects of the piRNA pathway in vitro.

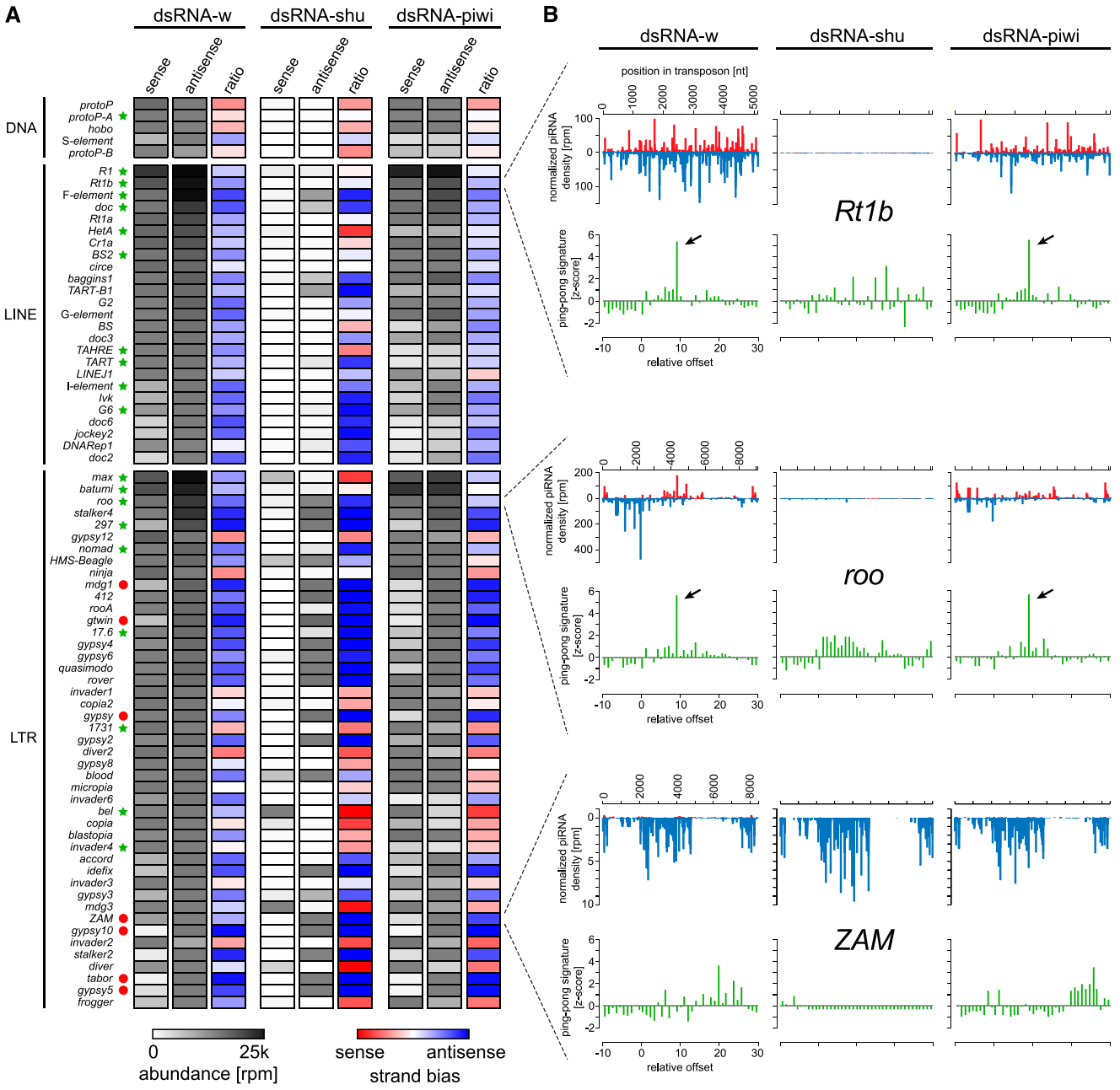

FIGURE 4. Loss of transposon control in *shu* knockdowns is a consequence of piRNA loss. (*A*) The heat map displays changes in piRNA abundance for each germline knockdown (as indicated) for the 75 elements most heavily targeted in our strain. Sense and antisense, with respect to the transposon coding strand, are quantified separately (gray heat maps), and their ratio is also indicated (red-blue heat map). (*B*) For three transposons, piRNAs are plotted along the length of the consensus sequence (*upper*) and a histogram of overlap between sense and antisense species (*lower*) is presented to indicate the degree of ping-pong (arrow highlights peak at position 9). Data are presented for *shu* and *piwi* knockdown and for a control (*white*). Two transposons with strong expression in the germline, *Rt1b* and *roo* (*top* and *middle*), are shown in comparison to a somatically biased element, *ZAM*. Since knockdown is germline specific, *ZAM* piRNAs are unaffected.

MATERIALS AND METHODS

Fly stocks and handling

Drosophila UAS-dsRNA strains were obtained from the VDRC. *nos*-GAL4 and *tj*-GAL4 driver lines were obtained from Bloomington and Kyoto, respectively (see Supplemental Table S1). For knockdown experiments, five males from dsRNA stocks were crossed with five virgin females expressing the desired GAL4 driver. Fertility of the FKBP-family F1 knockdown females was estimated by counting the number of eggs laid and crawling larvae 7 d post transfer to fresh media. Quantitative fertility measurements (shown in Fig. 1D) were obtained by transferring 3-d-old male and female F1 offspring (10 each) to grape-agar plates for

4 h and counting the eggs laid. Hatching frequencies were ascertained after 24 h (measurements were carried out in triplicate). For qPCR, small RNA libraries, and immunofluorescence experiments, ovaries were dissected from 2- to 3-d-old females fed with fresh yeast paste.

Expression of tagged transgenes in OSS

Full-length coding sequences of Shu, Piwi, Armi-RB, and Zuc were amplified from *Drosophila* ovary cDNA, cloned into pENTR/D-TOPO, and recombined into N- or C-terminal GFP destination vectors of the *Drosophila* Gateway collection (Terence Murphy, Carnegie Institute of Washington, Baltimore, MD). *Shu* was cloned into pAGW and pAWG, Zuc into pUWG, and Piwi and Armi into pUGW. Cells were transfected using Xfect reagent (Clontech) and costained with DAPI and MitoTracker Red CMXRos (Invitrogen).

RNA isolation, reverse transcription, qPCR

Ovaries were dissected into cold 1× PBS. Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. One microgram RNA was treated with DNase I Amplification Grade (Invitrogen) according to the manufacturer's instructions. Complementary DNA was prepared by reverse transcription using oligo$(dT)_{20}$ primer and SuperScript III Reverse Transcriptase (Invitrogen). qPCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems) and primers listed in Supplemental Table S2 on a Chromo4 Real-Time PCR Detector (BioRad). Transcripts were quantitated by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001), and normalized to transcript levels of *rp49*. Fold changes are expressed relative to control dsRNA-white knockdown RNA. Significance was calculated using a one-tailed heteroscedastic Student *t*-test of *rp49*-subtracted transposon c(t) values. All experiments were carried out in triplicates, with the average results shown.

Immunofluorescence

Ovaries were fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature. Blocking and permeabilization were carried out simultaneously in wash buffer (50 mM Tris at pH 6.8, 150 mM NaCl, 0.5% NP-40) supplemented with bovine serum albumin (5 mg/mL). All primary antibodies were diluted 1:1000 and incubated overnight at 4°C in wash buffer plus 1 mg/mL BSA. Anti-Ago3 and Anti-Piwi were generated in our laboratory (Brennecke et al. 2007); monoclonal mouse anti-Aub was provided by Mikiko Siomi (Nishida et al. 2007); and rabbit anti-Vasa (d-260) was purchased from Santa Cruz. Secondary AlexaFluor-488 and -568 antibodies were purchased from Invitrogen and used at 1:1000. Images were acquired on a Perkin Elmer UltraVIEW spinning disk confocal microscope.

RNAseq data analysis

For transcriptome libraries, 1 μg of total RNA from OSS cells transfected with GFP control dsRNA was used as input for the Illumina mRNA-Seq sample prep kit (catalog no. RS-930-1001). Libraries were made following the instructions by the manufacturer and sequenced on the Illumina GAII platform. RNAseq data were deposited to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession no. GSE38090. Publically available ovarian RNAseq data (GEO accession no. GSM424751) (Gan et al. 2010) were reanalyzed for this study. Raw sequence reads were iteratively mapped to the *Drosophila* genome (version dm3) using Bowtie (Langmead et al. 2009) with a tolerance of up to two mismatches. Remaining reads were also mapped to RefGene-annotated exon junctions with TopHat (Trapnell et al. 2009). Transcripts were quantitated using Cufflinks (Trapnell et al. 2010) and expressed as fragments per kilobase per million reads (fkpm) for relative comparison of FKBP family mRNA expression in the ovary.

Small RNA libraries and bioinformatic analysis

Small RNAs were cloned as described (Brennecke et al. 2007). For this study, the following small RNA libraries from total RNAs were prepared:

19–28 nt from *tj*-GAL4–driven dsRNA against white,
19–28 nt from *tj*-GAL4–driven dsRNA against shu,
19–28 nt from *tj*-GAL4–driven dsRNA against piwi,
19–28 nt from *nos*-GAL4–driven dsRNA against white,
19–28 nt from *nos*-GAL4–driven dsRNA against shu, and
19–28 nt from *nos*-GAL4–driven dsRNA against piwi.

Libraries were sequenced in-house using the Illumina GAII sequencing platform. Small RNA sequences were deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession no. GSE38089. The analysis of small RNA libraries was performed similarly as described (Czech et al. 2008). In brief, Illumina reads were stripped of the 3′ linker and collapsed, and the resulting small RNA sequences were matched to the *Drosophila* release 5 genome (version dm3) without mismatches. Only reads that met these conditions were subjected to further analyses. For annotations we used a combination of UCSC (repeats/transposons; noncoding RNAs), miRBase (microRNAs), and FlyBase (protein coding genes; noncoding RNAs) tracks, as well as custom tracks (for synthetic markers, endo-siRNAs from structured loci, and miR and miR* strands) with different priorities. For comparison of small RNA counts between samples, libraries of dsRNA-white samples were set to 1 million reads. Next, all libraries were normalized based on unique piRNA-size mappers to the *flamenco* (for *nos*-GAL4 knockdowns) or *42AB* (for *tj*-GAL4 knockdowns) piRNA clusters. Heat maps were created by plotting the abundance of sequences (all piRNAs to a given transposable element or individual miRNA strands) as well as their strand bias within the indicated libraries.

Ping-pong analysis

For each piRNA, the relative frequency (*Z*-score) of an existing "neighbor" piRNA on the opposite strand within a certain window (10-nt upstream of and 30-nt downstream from each 5′ end of a piRNA) was calculated. In the case of germline and somatic piRNA clusters, this analysis was based on genomic mapping coordinates. For transposons, the 5′ coordinate of each mapping event to the respective transposon consensus sequence was used. Calculated frequencies were based on total cloning count. A spike at position 9 indicates more than average partners with a 10-nt overlap and is a signature of ping-pong amplified piRNAs.

DATA DEPOSITION

RNAseq data and small RNA sequences were deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession no. GSE38098.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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