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1. Summary

The piRNA-pathway is a small RNA based transposon silencing system that has been found in all animals analyzed so far (Juliano et al., 2011). It acts preferentially in gonads where it protects the reproductive genomes of germ cells from the mutagenic activity of transposons (Klattenhoff et al., 2007; Theurkauf et al., 2006). Indeed, transposons have evolved to be particularly active in germ cells (Shigenobu et al., 2006) due to their mostly vertical transmission strategy and loss of the piRNA pathway leads to sterility due to severe defects in germline genome integrity.

Like the micro-RNA (miRNA) and the short interfering-RNA (siRNA) pathways, the piRNA pathway is based on Argonaute proteins bound to small RNAs, which guide the silencing of complementary RNAs (Aravin and Hannon, 2008; Ghildiyal and Zamore, 2009). Argonaute proteins acting in the piRNA pathway belong to the PIWI clade and their complexed small RNAs are therefore called PIWI interacting RNAs (piRNAs). By being preferentially complementary to transposon sequences, piRNAs guide the selective silencing of these selfish genetic elements.

During my dissertation, I studied the process of piRNA biogenesis, which differs fundamentally from the biogenesis of other small regulatory RNAs. For example, piRNA biogenesis doesn't involve RNase III enzymes such as Dicer (Houwing et al., 2007; Vagin et al., 2006) or double stranded RNA (dsRNA) precursors. In fact no protein acting in the biogenesis of siRNAs or miRNAs is known to participate in piRNA biogenesis.

The major aim of my thesis was to dissect the processes underlying piRNA biogenesis in the *Drosophila* ovary. Towards this end, I combined various genetic approaches with molecular assays such as small RNA sequencing and bioinformatics to ask the following specific questions:

- Which factors are involved in piRNA biogenesis and what are their molecular functions?
- What is the cell biological organization of piRNA biogenesis processes?
- Given that there are multiple PIWI family proteins, are different piRNA biogenesis pathways feeding into distinct PIWI proteins?

The work presented in this PhD thesis shed considerable light on these questions. Using a reverse genetics approach, I could assign essential roles to several proteins in the process of piRNA biogenesis. Genetic and molecular analyses of these allowed me to formulate major conclusions about the cell biological and hierarchical organization of piRNA biogenesis processes in *Drosophila*. Most of the described findings appear to be conserved in vertebrate piRNA pathways highlighting the importance of the *Drosophila* model system for our general understanding of this evolutionarily conserved genome surveillance system. The primary results of my thesis work are represented by two peer-reviewed publications and the broader implications of these findings are discussed in the Discussion chapter.

1.1 Zusammenfassung

Der sogenannte “piRNA-pathway” ist einer der drei zentralen Gen-Repressionssysteme die auf kleinen regulatorischen RNAs beruhen. Er agiert als das Hauptabwehrsystem gegen Transposons und wurde in allen bisher untersuchten Tierarten gefunden (Juliano et al., 2011). Der piRNA-pathway ist vornehmlich in den Gonaden aktiv und schützt die reproduktiven Genome der Keimzellen vor dem mutagenen Einfluss von Transposons (Klattenhoff et al., 2007; Theurkauf et al., 2006). Genau hier nämlich sind Transposons aufgrund ihrer überwiegend vertikalen Übertragungsstrategie besonders aktiv (Shigenobu et al., 2006). Defekte im piRNA-pathway resultieren in starker Anreicherung von Transposons und zu weitreichenden Defekten in der Genomintegrität von Keimzellen was unmittelbar zu Sterilität führt.

Ähnlich wie micro-RNAs (miRNAs) und short interfering RNAs (siRNAs) werden auch piRNAs von Proteinen der Argonaut Familie gebunden. Diese drei Klassen regulatorischer kleiner RNAs verleihen dem jeweiligen Argonaut-Komplex die Sequenz-Spezifität um ganz bestimmte komplementäre RNAs zu erkennen und deren Expression zu verhindern (Aravin and Hannon, 2008; Ghildiyal and Zamore, 2009). Die Argonaut-Proteine, die im piRNA Prozess wirken, gehören zur PIWI Untergruppe und ihre gebundenen kleinen RNAs werden deshalb PIWI interacting RNAs (piRNAs) genannt. Da piRNAs meist komplementär zu Transposon-Sequenzen sind, steuern sie ganz gezielt die Stilllegung dieser egoistischen genetischen Elemente.

Während meiner Dissertation habe ich den Vorgang der piRNA-Biogenese studiert, welcher sich grundsätzlich von der Biogenese der anderen kleinen regulatorischen RNAs unterscheidet. piRNA Biogenese ist beispielsweise unabhängig von RNase III Enzymen wie Dicer (Houwing et al., 2007; Vagin et al., 2006) und auch scheinen doppelsträngige RNA-Vorstufen (dsRNA) keinerlei Rolle zu spielen. In der Tat ist keiner der siRNA- oder miRNA-Biogenese Faktoren in der Biogenese von piRNAs involviert.

Das Hauptziel meiner Arbeit war zentrale Aspekte der piRNA-Biogenese in den Ovarien von *Drosophila* zu beschreiben. Für meine Arbeit habe ich verschiedene genetische Methoden sowie molekularbiologische Ansätze, wie die Sequenzierung von

piRNA Populationen und bioinformatische Analysen kombiniert, um folgende Fragen zu beantworten:

- Welche Faktoren sind an der piRNA-Biogenese beteiligt und was sind deren molekularen Funktionen?
- Wo genau finden die einzelnen Prozesse der piRNA- Biogenese in der Zelle statt?
- Wie unterscheiden sich die jeweiligen piRNA-Biogenese Vorgänge, die mit den drei Typen von PIWI Proteinen verknüpft sind, voneinander?

Die Ergebnisse aus meiner Dissertation tragen erheblich zur Beantwortung dieser Fragen bei. Es wurden mehrere Proteine identifiziert, die für die Herstellung von piRNAs essentiell sind und einigen dieser Faktoren konnten spezifische Rollen zugewiesen werden. Ferner konnten wichtige Schlüsse über die zellbiologische und hierarchische Organisation der piRNA-Biogenese Prozesse gezogen werden. Die gewonnenen Erkenntnisse scheinen auch im piRNA Prozess von Wirbeltieren gültig zu sein -ein klares Argument dafür dass *Drosophila* ein geeigneter Modellorganismus für die Erforschung dieses faszinierenden Genom-Immunsystems ist. Die zentralen Ergebnisse meiner Dissertationsarbeit wurden in zwei Originalartikeln publiziert, deren breitere Implikationen im Diskussionsteil dieser Arbeit näher besprochen werden.

2. Introduction

In this section I will give a comprehensive overview of the current understanding of piRNA biogenesis. Insights based on my thesis have been weaved into this wherever required, but are exhaustively covered in the results and in the discussion chapters.

This section is focused on the literature based on *Drosophila melanogaster*, which is one of the key model organisms for studying the piRNA pathway. Nevertheless, relevant literature from the key vertebrate model organisms mouse and zebrafish is also discussed. In contrast, data from *C. elegans* is not included as its piRNA –or 21U-RNA– pathway has strongly diverged from the other models (readers are referred to (Ashe et al., 2012; Ishizu et al., 2012; Lee et al., 2012; Shirayama et al., 2012) for further reading).

2.1 The stage of the piRNA pathway: the animal gonads

In the majority of the studied animals the expression of the piRNA pathway is restricted to the germline (Juliano et al., 2011). In flies, this is immediately evident from the expression pattern of the different Argonaute proteins (Williams and Rubin, 2002): while the AGO clade Argonaute proteins of the miRNA- and siRNA-pathways are ubiquitously expressed, the Argonaute proteins of the PIWI clade are expressed only in the gonads. The germline needs special protection from the mutagenic activity of selfish genetic elements because transposons are highly transcribed in gonadal cells (Shigenobu et al., 2006) and because the germline genome is the only one being transmitted to next generations.

In *Drosophila*, the female germline arises from germline stem cells, which undergo asymmetric cell division producing another stem cell and a cystoblast (Bastock and St Johnston, 2008). The cystoblast divides four times with incomplete cytokinesis giving rise to a 16-cell cyst (FIG. 1). Early on, one of the cells is determined to be the oocyte while the other 15 become polyploid nurse cells. While the oocyte is transcriptionally inactive, nurse cells are transcriptionally highly active as they nurse the growing oocyte.

Despite being transcriptionally inert, the oocyte genome is vulnerable to TEs (transposable elements) originating from two sources: on the one hand transposons can reach the oocyte from the nurse cells through the ring canals, cytoplasmic bridges that

result from the incomplete cytokinesis; on the other hand some retrotransposons (especially those from the *Gypsy* family) can infect the oocyte from the surrounding somatic cells as they are capable of forming retroviral particles (Lécher et al., 1997). This two-sided threat is probably the reason why the piRNA-pathway in flies is expressed in both, germline and all somatic cells of the ovary (Saito et al., 2006). It is important to note, however, that the germline and the ‘somatic’ piRNA pathways are different. For example, the germline piRNA pathway expresses all three *Drosophila* PIWI proteins (Piwi, Aubergine, Argonaute3) (Brennecke et al., 2007), while the somatic pathway expresses only one (Piwi) (FIG. 2). Indeed, the germline piRNA pathway is much more complex compared to the somatic pathway (TAB. 1).

Also in testes, germline stem cells undergo asymmetric cell divisions (Spradling et al., 2011); the differentiating daughter cells divide four times and then all 16 spermatogonia undergo meiosis producing 64 spermatocytes interconnected by cytoplasmic bridges. The male germline differs from the female as it expresses Aubergine and Argonaute 3 (Nagao et al., 2010), but not Piwi (Saito et al., 2006). Like in the ovary, somatic cells of the testis (the hub cells and the cyst progenitor cells) express a simplified pathway based only on Piwi (Saito et al., 2006). The *Drosophila* male and female piRNA pathways don’t seem to be conceptually very different (Nagao et al., 2010); nevertheless the female has been studied much more than the male, mainly because ovaries are much larger than testes and because many female sterility genes identified in early genetic screens turned out to be piRNA pathway factors (Schüpbach and Wieschaus, 1989, 1991). I therefore focused the Introduction on the female *Drosophila* piRNA pathway.

Also in mice, the piRNA pathway is expressed exclusively or predominantly in the germline (Girard and Hannon, 2007; Lau et al., 2006). In this model organism, studies on the piRNA pathway are nearly exclusively conducted in testes, as the piRNA pathway seems dispensable for female gametogenesis (Aravin et al., 2008). In mouse, a temporal pattern of PIWI protein expression is apparent in testes (Aravin et al., 2008) (TAB. 1): MILI is the first PIWI protein to be expressed in development (already in progenitor germ cells) and it is expressed throughout spermatogenesis till the round spermatid stage; MIWI2 can be detected in spermatogonia only between 15.5 dpc to 3 dpp; MIWI is expressed only from the pachytene stage of meiosis till the round spermatid stage.

The Zebrafish piRNA pathway is expressed and essential both in male and in female gonads (Houwing et al., 2007). Both Zebrafish PIWI proteins (Ziwi and Zili) are expressed throughout male and female gametogenesis (Houwing et al., 2008; Houwing et al., 2007) (TAB. 1).

Taken together, PIWI protein expression patterns indicate that the germline specificity of the piRNA pathway is a conserved feature. In some cases (e.g. *Drosophila* somatic gonadal cells) the germline specificity has been overcome during evolution, likely as a consequence to an acute somatic transposon threat.

Why has such an efficient transposon silencing mechanism not been co-opted by all somatic cells? Maybe other transposon silencing systems are in place in somatic tissues (Czech et al., 2008; Slotkin and Martienssen, 2007) but these are not capable to efficiently protect the germline because they are not efficient enough or because they are suspended during gametogenesis in some organisms (Hackett et al., 2012).

2.2 The genomic origin of piRNAs

Despite the considerable complexity of piRNAs being predominantly derived from repetitive sequences, we have a fairly good understanding of their genomic origins. This is owed primarily to advances in massive parallel sequencing technologies and the availability of assembled genome sequences including large parts of heterochromatin.

Mapping piRNAs to the genome identified three major sources. These are: 1) specific genomic loci that spawn large amounts of piRNAs (termed piRNA clusters), 2) individual transposons, and 3) several protein coding genes with a strong bias for piRNAs being derived from 3'UTR portions.

piRNA clusters give rise to most piRNAs. In flies, they range in size between a few and several hundred Kbp (Brennecke et al., 2007; Grivna et al., 2006) and are often located in peri-centromeric or sub-telomeric heterochromatin. *Drosophila* piRNA clusters have a very high content of transposon sequences. Besides this, no common sequence or structural features have been identified that would allow to distinguish clusters from random heterochromatic loci. It is important to note that the TE sequences in clusters are often transposon remnants or fragments; clusters can therefore be considered as TE graveyards that literally store TE sequence information. In stark contrast to miRNA and

siRNA originating loci whose encoded RNAs fold into predictable secondary dsRNA structures (Aravin and Hannon, 2008; Ghildiyal and Zamore, 2009), no RNA secondary structure features have been detected in piRNA clusters. Thus the diversity of piRNA clusters sparks questions about their genomic regulation, their recognition, and their processing, which cannot be answered based on the paradigm of other small RNA pathways.

According to piRNAs that unequivocally map to piRNA clusters (genome-unique mappers), two major types of clusters can be distinguished: these are uni-strand and dual-strand clusters (Brennecke et al., 2007; Malone et al., 2009).

In uni-strand clusters, all piRNAs map to one DNA strand, meaning that these clusters are transcribed in only one direction (Brennecke et al., 2007; Malone et al., 2009). They are the main source of piRNAs in the ovarian follicle cells of *Drosophila* (Brennecke et al., 2007; Malone et al., 2009). Remarkably, the orientation of TE fragments in uni-strand clusters is heavily biased and almost always antisense to the transcription direction, yielding primarily piRNAs antisense to TEs (Brennecke et al., 2007; Malone et al., 2009). Also the composition of transposon fragments in uni-strand clusters is biased as primarily fragments of co-expressed transposons are enriched. This is particularly evident for the *flamenco* cluster (FIG. 3), the major piRNA cluster of somatic follicle cells and potentially the only cluster that is specifically expressed in these cells and not in germline cells (Malone et al., 2009). The *flamenco* cluster is strongly enriched in sequences from *Gypsy* family TEs (FIG. 3), which are predominantly or exclusively expressed in follicle cells (Pélisson et al., 1997). Based on the analysis of mutants with defective *flamenco* transcription and on RT-PCR experiments, it is believed that uni-strand clusters give rise to single long transcripts from a single tissue-specific and developmentally regulated promoter (Brennecke et al., 2007; Malone et al., 2009; Robert et al., 2001).

In contrast, dual-strand clusters spawn piRNAs, which map uniquely to both genomic strands at similar frequencies and are thus transcribed in both directions (Brennecke et al., 2007; Malone et al., 2009). Following the argumentation above, this alleviates the evolutionary pressure for biased orientation of TE fragments and indeed, no such bias is apparent in dual strand clusters (Brennecke et al., 2007; Malone et al.). All available data suggest that dual strand clusters are specifically expressed in the germline and

consequently, the TE repertoire of these clusters is much broader and targeted at the various classes of germline-specific transposons (Malone et al., 2009).

It is unclear whether uni- and dual-strand clusters are just variations of a common theme or whether they reflect two independent strategies to encode piRNAs in the genome. One study on heterochromatic marks of piRNA clusters suggests a shared biology: according to Rangan et al. (2011), transcription of both uni- and dual-strand clusters depends on heterochromatin formation. Egless, the enzyme that deposits the 3rd methyl group on H3K9me2 localizes to pericentromeric and subtelomeric heterochromatin and loss of it leads to reduced transcript levels of uni- and dual-strand clusters (Rangan et al., 2011). More compelling, however, are findings on two germline-specific factors that genetically are required for piRNA production from dual strand clusters, yet not from single strand clusters. These are Rhino (Klattenhoff et al., 2009), an ovary specific HP1 homolog (Vermaak and Malik, 2009), and Cutoff (Pane et al., 2011), which is related to the yeast transcription termination factor Rai1. Both proteins co-localize in specific foci of germline nuclei and have been suggested to associate with dual-strand, yet not with single strand clusters. Rhino and Cutoff physically interact and their subcellular localization (presumably on dual strand clusters) is mutually interdependent (Pane et al., 2011). Lack of either factor causes loss of dual-strand cluster transcripts and of corresponding piRNAs, but doesn't affect uni-strand clusters (Klattenhoff et al., 2009; Pane et al., 2011). As loss of Cutoff might have different effects on RNA levels of different regions within a dual-strand cluster, it is possible that dual-strand clusters do not produce large transcripts from two flanking promoters (Pane et al., 2011). It has to be noted, however, that these experiments were based on ovary samples, which contain three major cell types, and that the repetitive nature of clusters complicates these experiments considerably. Strong conclusions on this important question are therefore not yet possible.

How Rhino and Cutoff are recruited specifically to dual strand clusters is unknown. As Rhino is an HP1 homolog with a chromo domain (Vermaak and Malik, 2009), it seems plausible that H3K9me3 is involved in aspects of its biology. On the other hand, it is unlikely that H3K9me3 is the only link considering that this mark is clearly not specific for dual-strand clusters. Of note, neither Rhino nor Cutoff are encoded in

vertebrate genomes. Either these genes are particularly fast evolving (Vermaak and Malik, 2009) or the regulation of dual strand clusters is a recent event in insect evolution.

piRNA clusters have been also found in the vertebrate species that have been studied. In Zebrafish, a large portion of testis and ovary piRNAs are repetitive sequences and their origin cannot be clearly traced. Most of the non-repeat piRNAs derive from intergenic regions or piRNA clusters (Houwing et al., 2007). Both repeat and intergenic piRNAs map to the same piRNA clusters and these have a pronounced antisense bias in transposon orientation (Houwing et al., 2007). A special feature of Zebrafish piRNA clusters is that -although they're always being transcribed only from one strand- the transcription direction appears to change several times within a cluster (Houwing et al., 2007).

piRNA clusters were originally identified in mouse. A major advantage of the mouse model is that spermatogenesis occurs in highly synchronous differentiation waves. This allowed a temporal dissection of piRNA populations during early, mid and late spermatogenesis. During embryogenesis, mouse piRNAs are predominantly derived from individual transposon insertions in the genome. Also several piRNA clusters are prominent sources and these resemble fly clusters in many aspects. The mouse genome harbors uni-strand (Aravin et al., 2007) and dual-strand (Aravin et al., 2008) clusters. Uni-strand clusters have a pronounced antisense bias in their transposon orientation (Aravin et al., 2007), while dual-strand clusters are not particularly enriched in transposon sequences (Aravin et al., 2008). Right after birth, piRNA clusters moderately enriched in TE insertions are the major piRNA source. Remarkably, upon onset of the pachytene stage of meiosis, piRNAs are predominantly derived from so-called pachytene clusters, large genomic regions that are not particularly enriched in TE sequences. Cellular targets of these pachytene piRNAs are unclear, but TEs are certainly among them (Reuter et al., 2011).

The transition from transposon to cluster piRNAs might constitute a general piRNA pathway strategy: first transposon RNAs are processed into piRNAs and then these piRNAs trigger secondary piRNAs originating from cluster transcripts (see below for secondary biogenesis). Potential temporal transitions of piRNA populations have not

been probed in *Drosophila*, as isolation of sufficient material from early oogenesis stages is technically challenging.

Although piRNA clusters are the major source of piRNAs in vertebrates and invertebrates, all organisms analyzed so far also produce piRNAs that originate from coding genes. In the *Drosophila* ovary these genic piRNAs represent only ~5% of the total (Brennecke et al., 2007; Robine et al., 2009; Saito et al., 2009), but in mouse pre-pachytene spermatogonia they represent as much as 30% (Aravin et al., 2007). The function of these piRNAs is still unclear. Most genic piRNAs map to the 3'UTR and it is highly unlikely that they regulate their gene of origin, as they are derived from the same strand (Aravin et al., 2007).

2.3 How to specify piRNA sources

Although piRNAs are derived from loci as diverse as coding genes or telomeric heterochromatin, only specific transcripts give rise to piRNAs. Hence, there must be a system that selects piRNA precursor transcripts. In other words, piRNA producing loci must have some features that identify them as such. How piRNA cluster transcripts are recognized and how mRNAs that trigger piRNA production are distinguished from other mRNAs is not known.

One can envision different mechanisms how the cell funnels only selected RNAs into piRNA biogenesis. One possibility, for example, is that mRNAs are processed due to some secondary structure feature or due to specific sequence features. Alternatively, piRNA cluster transcripts might be marked by some factor recruited to their unique chromatin context instead.

Changing the genomic localization of a piRNA cluster is a powerful experimental approach to address the role of chromatin context in the specification of piRNA clusters. A recent study (Muerdter et al., 2012) reported on such piRNA clusters and found that they are able to produce piRNAs with a similar pattern to the endogenous loci even when inserted into euchromatin both in mouse and in fly. This does not rule out a dependence of cluster identity on the chromatin context, as the relevant chromatin features might have been preserved for the transgenic clusters. Of note, piRNAs from artificial euchromatic

clusters in mouse show a non-physiological size profile, hinting at some defects in the handling of these cluster transcripts (Muerdter et al., 2012).

When an intron-harboring sequence is inserted into a piRNA cluster, piRNAs are produced from intronic sequences as well (Muerdter et al., 2012). This indicates that piRNA clusters are potentially immune against splicing, which could be an essential feature to avoid the disruption of cluster transcription by transposon-derived splicing or polyadenylation signals. Genic piRNAs instead always derive from fully spliced transcripts (Muerdter et al., 2012).

2.4 The evolution of piRNA clusters

piRNA clusters cannot be classified as a previously known genetic category and therefore represent a new type of genetic unit. One of their most striking features is their extremely high transposon content (note, however, that this seems to be much less pronounced in mouse). This is in contrast to any other type of gene, where natural selection disfavors transposon insertions. Clearly, the selection of piRNA clusters must follow special criteria.

A popular model for the evolution of piRNA clusters could explain this feature and also the ability of a single piRNA pathway to silence transposons with extremely diverse sequence and integration strategies. This model hypothesizes that the piRNA pathway is taking advantage of the only feature that all transposons share: transposition itself. If an element is mobile, sooner or later it will integrate into a piRNA cluster, thus providing the pathway with the sequence information to target it throughout the genome. The ability to silence an active transposon is clearly an advantageous trait, so that piRNA clusters will accumulate and store transposon sequences over time. This model effectively describes a ‘genomic sequence memory’ that gathers and conserves the sequence information that is necessary to recognize and eventually silence transposons.

This rather fluid model of piRNA cluster composition could also explain other features of piRNA clusters. A transposon insertion into a cluster can be beneficial or not depending on its orientation and on the cluster type. Insertions in uni-strand clusters provide silencing competent information only if they are inserted antisense to the cluster transcription direction. On the other hand, insertions in dual-strand clusters are useful

independently of their orientation. This is the likely basis for the transposon orientation bias in uni-strand clusters that is absent in dual-strand clusters (Brennecke et al., 2007; Malone et al., 2009). Along the same lines, a transposon insertion in a cluster is useful only if the cluster is expressed in the same tissue as the target transposon. This could underlie the different composition of piRNA clusters expressed in different tissues that has been observed in *Drosophila* (Brennecke et al., 2007; Malone et al., 2009). piRNA clusters don't need full length transposon insertions to produce ~23-30 nt long piRNAs that guide transposon silencing. Therefore, there is little selection towards preserving transposon sequences fully or against insertions of new transposons into old ones. Indeed, piRNA clusters are mainly composed of nested and degenerated transposon fragments.

Parts of this adaptative process can be observed over the course of two generations, so that it has been possible to experimentally support this theory of piRNA cluster evolution. It has been shown that flies that inherit novel transposon insertions into piRNA clusters have lower transposition rates for the integrated transposon (Khurana et al., 2011). Furthermore, the probability that a transposon inserts into a piRNA cluster is significantly, albeit only moderately, higher than average (Khurana et al., 2011). In light of the 'genomic memory' theory the evolutionary advantage of such a bias is obvious, but there is no mechanism known that would specify piRNA clusters as transposon sinks.

Despite these theories about the evolution of piRNA clusters, one of the most remarkable features of these loci –namely their predominant localization at the euchromatin/heterochromatin boundary– is difficult to explain. It is possible that this location is the best compromise between guaranteeing low meiotic recombination frequency and still being present in a transcription-friendly environment.

Not surprisingly, piRNA clusters are fast evolving loci due to their key role in the evolutionary 'arms race' between TEs and the genome. The study of the evolution of rodent piRNA clusters (Assis and Kondrashov, 2009) has revealed that piRNA clusters have the highest expansion rate among all genes and that there is virtually no cluster loss. This reflects the extremely high evolutionary pressure caused by transposons. The mechanism of such a fast expansion seems to be ectopic homologous recombination, which is made possible by the repetitive nature of piRNA clusters (Assis and Kondrashov, 2009).

2.5 Primary piRNA biogenesis

A single stranded transcript that has been selected for piRNA biogenesis will be processed into ~23-30 nt small RNAs that are complexed with PIWI family proteins to form an active pi-RISC (piRNA induced silencing complex). This process is termed primary piRNA biogenesis and is distinguished from secondary piRNA biogenesis, which requires an RNA cleavage event that is triggered by a pre-existing pi-RISC, as described in detail below.

piRNA biogenesis differs fundamentally from the biogenesis of other small regulatory RNAs. piRNAs often overlap in a continuous fashion and their ends do not coincide (Brennecke et al., 2007), as if they would be processed via a relatively random process. On the contrary, miRNAs have a highly defined sequence (Ghildiyal and Zamore, 2009) and siRNAs often exhibit a phased character (Kawamura et al., 2008; Okamura et al., 2008). In contrast to miRNA and siRNA precursors, no RNA secondary structure correlates with piRNA production. This is consistent with genetic evidence that rules out the involvement of Dicer enzymes, which require double stranded RNA, in piRNA biogenesis (Houwing et al., 2007; Vagin et al., 2006). To date, several primary biogenesis factors have been identified and some of the primary biogenesis steps have been characterized *in vitro*.

Drosophila primary piRNA biogenesis has been mainly studied in somatic cells of the ovary, whether *in vivo* or in cell culture (OSCs, ovarian somatic cells), because these cells only harbor primary, but not secondary piRNA biogenesis (Malone et al., 2009). During the last five years, several primary biogenesis factors have been described (TAB. 2): Zucchini (Zuc) (Olivieri et al., 2010; Pane et al., 2007; Saito et al., 2009), an RNA-nuclease (Ipsaro et al., 2012; Nishimasu et al., 2012), Armitage (Armi) (Cook et al., 2004; Malone et al., 2009; Tomari et al., 2004), an RNA-helicase, Fs(1)Yb (Olivieri et al., 2010), an RNA helicase, Vreteno (Vret) (Handler et al., 2011), an RNA-binding protein, Sister of Yb (SoYb) (Handler et al., 2011), an RNA-helicase, and Shutdown (Shu) (Munn and Steward, 2000; Olivieri et al., 2012; Schüpbach and Wieschaus, 1991; Xiol et al., 2012), an Hsp90 cochaperone.

Besides Zucchini all of these factors co-localize in a cytoplasmic granule, the so-called Yb-body (Szakmary et al., 2009) (FIG. 4). The Yb-body is in contact with mitochondria (Szakmary et al., 2009) and is typically in close proximity to the nuclear envelope (FIG. 5). Interestingly, Zucchini is a protein of the outer mitochondrial membrane with its nuclease domain facing into the cytoplasm (Saito et al., 2010). Hence, also Zucchini is likely in close proximity to biogenesis factors of the Yb-body (FIG. 5). This makes the Yb-body a very likely center for primary piRNA biogenesis.

In order to understand piRNA biogenesis, it will be essential to also identify and characterize any piRNA intermediate molecules. So far only one such candidate has been described, namely piRNA intermediate-like molecules (piR-ILs), which are 30-70 nucleotides (nt) in size and copurify with the RNA-helicase Armitage (Saito et al., 2010). As the termini of piR-ILs and mature piRNAs do typically not coincide (Saito et al., 2010), piR-ILs either represent partially degraded intermediates or still require subsequent 5' and 3' processing events. This would be certainly true for the 5' end, as piR-ILs do not exhibit the strong 1U bias that characterizes mature primary piRNAs (Saito et al., 2010).

Some steps of piRNA biogenesis have been characterized *in vitro* (Kawaoka et al., 2011). Together with the genetic data summarized above and biochemical data about some biogenesis factors (Kamminga et al., 2010; Nishimasu et al., 2012), a relatively plausible molecular model that accounts for the characteristics of mature piRNAs can be formulated. According to this, primary piRNA biogenesis can be divided into three phases:

- 1) The endonuclease Zucchini cuts a ssRNA precursor and generates a 5' phosphate on one of the cleavage products (Ipsaro et al., 2012; Nishimasu et al., 2012), which would be loaded into a PIWI protein. Thereby, Zucchini would define the 5' end of primary piRNAs. Roughly 80% of Piwi bound primary piRNAs have a Uridine at their 5' end (Brennecke et al., 2007), but Zucchini does not appear to exhibit any sequence specificity (Ipsaro et al., 2012; Nishimasu et al., 2012). The silkworm data might resolve this apparent conundrum as it shows that Siwi, a protein that is loaded with primary piRNAs has a preference for RNAs with a 5' Uridine (Kawaoka et al., 2011), possibly due to the nucleotide selectivity of its MID domain (Ma et al., 2005).

2) A piRNA precursor bound to a PIWI protein is trimmed at its 3' end; the PIWI protein protects the RNA from the trimming activity and thus would determine the final size of the piRNA. The trimming after loading is characteristic of piRNAs and might explain why different PIWIs bind piRNA populations of different sizes (Aravin et al., 2008; Brennecke et al., 2007). *In vitro* experiments show that PIWI proteins can bind 50 bp and longer ssRNAs that are then trimmed to their final size only from their 3' end by a Mg^{2+} -dependent exonuclease (Kawaoka et al., 2011). This process is fundamentally different from the 3' end formation of miRNAs and siRNAs, which is enzymatically determined before Argonaute protein loading (Ghildiyal and Zamore, 2009).

3) All piRNAs undergo a final maturation step that is 2'-O-methylation at their 3' end by the Hen1 enzyme (Ji and Chen, 2012). The methylation takes place only if piRNAs have been trimmed to their final size (Kawaoka et al., 2011), as if trimming and methylation would be somehow coupled. Among animals this modification is piRNA specific with the exception of *Drosophila* siRNAs (Ji and Chen, 2012). The structure of the PAZ domain of the murine Miwi protein in complex with a methylated piRNA mimic has been solved and reveals an enlarged hydrophobic binding pocket to accommodate the bulky methylated 3' end (Simon et al., 2011). It is believed that 3' end methylation protects small RNAs that extensively anneal to their target from degradation (Ameres et al., 2010). Extensive base pairing with a target RNA probably dislocates the 3' end of a small RNA from the PAZ domain of the Argonaute protein exposing it to the action of nucleases. The 3' end methylation protects against nucleases and thus stabilizes small RNAs. The stabilization of piRNAs by this modification has been observed *in vivo*: in the absence of Hen1, piRNAs are being uridylated and degraded by a 3'->5' exonuclease (Kamminga et al., 2010). This suggests that also piRNAs undergo extensive base pairing with their targets. The effects of a lack of piRNA methylation on transposon silencing are only moderate, probably as only stability but not production and function of piRNAs are affected (Kamminga et al., 2010).

All organisms studied so far have more than one PIWI protein, but not all of them bind primary piRNAs (TAB. 1) (Aravin et al., 2008; Brennecke et al., 2007; Grivna et al., 2006; Gunawardane et al., 2007; Houwing et al., 2008; Houwing et al., 2007; Vagin et

al., 2006). This implies that primary biogenesis actively selects the appropriate PIWI proteins as acceptors of primary piRNAs.

Primary piRNAs are bound by Piwi and Aubergine in *Drosophila* (Brennecke et al., 2007; Gunawardane et al., 2007), by MILI and MIWI in mouse (Aravin et al., 2008; Grivna et al., 2006) and by Zivi in Zebrafish (Houwing et al., 2008; Houwing et al., 2007) (TAB. 1).

The study of mouse primary biogenesis has yielded a picture that is overall in agreement with the *Drosophila* data, beside a couple of exceptions (TAB. 2).

MOV10L1, the mouse ortholog of Armitage, is an essential primary biogenesis factor (Frost et al., 2010; Zheng et al., 2010): it associates with MILI and MIWI, the mouse PIWI proteins that bind primary piRNAs; only primary piRNAs can be detected in MOV10L1 pull-downs; and MOV10L1 mutant mice are defective in piRNA production.

MITOPLD is the murine ortholog of Zucchini and it is an outer mitochondrial transmembrane protein (Choi et al., 2006; Nishimasu et al., 2012; Watanabe et al., 2011). Just like lack of Zucchini, lack of MITOPLD causes a strong loss of primary piRNAs but doesn't block secondary biogenesis (Watanabe et al., 2011). An interesting additional phenotype in *MitoPLD* mutant gonads is the clustering of mitochondria (Choi et al., 2006; Huang et al., 2011a; Watanabe et al., 2011); it is, however, not clear whether this is related to piRNA biogenesis.

In addition to the genes that are also known in *Drosophila*, GASZ is a piRNA biogenesis factor that has been described only in mouse so far (Ma et al., 2009). Its phenotype hints at an involvement in primary biogenesis: lack of GASZ causes loss of piRNAs, loss of MILI and MIWI proteins and *Gasz* mutants phenocopy *Mili* mutants.

The main difference between mouse and *Drosophila* primary biogenesis is the roles of TDRD1 (a possible murine ortholog of Vreteno) (Kojima et al., 2009; Reuter et al., 2009) and FKBP6 (murine ortholog of Shutdown) (Xiol et al., 2012). While these factors are necessary for primary as well as for secondary biogenesis in *Drosophila*, in mouse they seem to function exclusively in secondary biogenesis.

The mouse primary piRNA biogenesis factors MOV10L1, TDRD1, and GASZ co-localize with MILI in a sub compartment of nuage called the pi-body (Aravin et al., 2009b). Nuage is an electron-dense speckled structure that rims the nuclei of the germline

of most species (Chuma et al., 2009), including *Drosophila* nurse cells. Nuage is protein and RNA rich and, like the Yb-body, is closely associated with mitochondria (hence also the name “inter-mitochondrial cement”).

In zebrafish TDRD1 is the only described piRNA biogenesis factor. TDRD1 binds both Ziwi and Zili and co-immunoprecipitates their piRNAs (Huang et al., 2011b). Loss of TDRD1 causes a mild reduction of both primary and secondary piRNAs (Huang et al., 2011b) and therefore it is functionally more similar to *Drosophila* Vret rather than mouse TDRD1.

2.6 Secondary piRNA biogenesis and the ping-pong cycle

pi-RISCs are active slicers, as they possess endonucleolytic activity towards complementary RNA molecules. When a mature pi-RISC cleaves a complementary RNA, the cleavage product with a 5' phosphate can be a substrate for the biogenesis of a new, so-called secondary, piRNA. This process is called secondary piRNA biogenesis and serves different functions. First of all, it produces a piRNA with the opposite orientation of the trigger piRNA. In mouse, where primary piRNAs are initially derived from transposon transcripts, this is necessary to yield piRNAs that are antisense to transposons and are thus silencing competent (Aravin et al., 2008). Furthermore, when secondary piRNAs trigger further secondary piRNAs, a positive feed-back loop, which is called the ping-pong cycle, arises and enhances piRNA production (Brennecke et al., 2007; Gunawardane et al., 2007). Last but not least, secondary biogenesis relies on the availability of substrate RNAs, so that actively transcribed transposons are specifically enhancing the production of piRNAs against themselves.

Secondary biogenesis appears to be as conserved as the piRNA pathway itself (Grimson et al., 2008), hinting at the possibility that this might be part of the ancestral biogenesis mechanism.

Just as primary biogenesis, also secondary biogenesis appears to be selective towards specific PIWI proteins (TAB. 1). In *Drosophila*, Aubergine and AGO3, but not Piwi, are relevant contributors to ping-pong (Li et al., 2009b). Both, Aubergine and AGO3 possess active slicer activity (Gunawardane et al., 2007), a prerequisite to trigger secondary piRNAs (TAB. 1). Secondary piRNAs triggered by Aubergine are typically loaded onto

AGO3 (heterotypic ping-pong) but can also end up in Aubergine (homotypic ping-pong) (Li et al., 2009b). In contrast, AGO3 triggered piRNAs are believed to depend strongly on Aubergine. The molecular basis for this specificity is unknown, but it might be fundamental to produce sufficient antisense piRNAs. Indeed, in the absence of AGO3 ping-pong produces an excess of sense piRNAs (Li et al., 2009b). This could imply that specific PIWIs are being defined as trigger and/or acceptor of secondary piRNA biogenesis (TAB. 1). It seems plausible that specific scaffold proteins would be required as adaptors for specific PIWIs. In fact, several secondary biogenesis factors possess multiple Tudor domains (see below and TAB. 2), which have been shown to be able to bind symmetrically dimethylated arginine residues (sDMAs) at the N-terminus of PIWI proteins.

Mouse secondary biogenesis feeds piRNAs specifically into MIWI2 and MILI (Aravin et al., 2008; De Fazio et al., 2011) (TAB. 1). But while MIWI2 is only a secondary piRNA acceptor and its slicer activity is dispensable for piRNA production, MILI both accepts and triggers secondary piRNAs (De Fazio et al., 2011) (TAB. 1). This means that in mouse MILI is autonomously engaged in ping-pong, and MIWI2 instead relies on MILI to get loaded. Also Zebrafish PIWI proteins are involved in ping-pong: Ziwi, which is loaded with primary piRNAs, can trigger the production of secondary piRNAs that are loaded onto Zili that can in turn do the same for Ziwi (Houwing et al., 2008) (TAB. 1).

Secondary biogenesis and ping-pong have been proposed soon after the discovery of piRNAs simply on the basis of piRNA sequence analysis (Brennecke et al., 2007; Gunawardane et al., 2007). According to these models, secondary biogenesis starts with a PIWI protein cleaving a complementary substrate RNA. This means that only PIWI proteins that harbor slicer activity can trigger secondary piRNAs. The cleavage site is precisely determined by the structure of PIWI proteins and it is always opposite to the 10th/11th nucleotide of the guide piRNA. The 5' end that is generated by this cleavage event corresponds to the 5' end of the secondary piRNA, so that the first ten nucleotides of a secondary piRNA are the reverse complement of the first ten nucleotides of the trigger piRNA. This process is what gives rise to the frequent ten nucleotide complementarity within piRNA populations and it is known as 'the ping-pong signature'.

Given that primary piRNAs have a strong tendency to start with a Uridine, secondary piRNAs frequently have an Adenosine at their 10th position.

The existence of secondary piRNAs can be assessed via the ping-pong signature. However, as far as the biochemical identity is concerned, secondary piRNAs are indistinguishable from primary piRNAs. It is in fact rather likely that secondary biogenesis resembles in parts primary biogenesis and that both processes utilize shared factors. Indeed, after slicer mediated 5' end formation (rather than by Zucchini), secondary piRNA intermediates need to be trimmed and 2'-O-methylated at the 3' end, just like primary piRNA precursors.

Secondary piRNAs must be at some point triggered by primary piRNAs. This considerably complicates the genetic dissection of the involved processes. In this regard, the *Drosophila* system is an ideal study object, as here secondary biogenesis can be experimentally uncoupled from primary biogenesis. In the complete absence of primary biogenesis, a subset of piRNAs continues to undergo efficient ping-pong. It is believed that maternally deposited pi-RISCs are triggering this cycle, which then is self-sustained.

Although several factors essential for secondary biogenesis have been identified (TAB. 2), we largely lack mechanistic insight into this process.

In *Drosophila*, secondary biogenesis takes place only in the germline, where Aubergine and AGO3, the main ping-pong players (TAB. 1), are expressed (Brennecke et al., 2007). Aubergine and AGO3 localize to nuage together with all known secondary biogenesis factors (TAB. 2). Specifically, these are Kumo (Anand and Kai, 2012), Vasa (Vagin et al., 2004), Tejas (Patil and Kai, 2010), SpnE (Vagin et al., 2004), Krimper (Lim and Kai, 2007), Papi (Liu et al., 2011), Vret (Handler et al., 2011), SoYb (Handler et al., 2011), BoYb (Handler et al., 2011), and Shutdown (Olivieri et al., 2012). This spurs the hypothesis that nuage, which has long been used as a universal germline marker, is a secondary piRNA biogenesis 'organelle'. Of note, nuage localization of the various biogenesis factors shows a largely linear epistatic dependency (FIG. 4).

Several mouse orthologs of *Drosophila* secondary biogenesis factors have a conserved role in this process, but at the same time significant differences do exist (TAB. 2).

Loss of Mouse Vasa Homolog (MVH) (Kuramochi-Miyagawa et al., 2010), TDRD1 (the potential Vreteno ortholog) (Reuter et al., 2009), FKBP6 (the Shutdown ortholog)

(Xiol et al., 2012), or TDRD5 (the Tejas ortholog) (Yabuta et al., 2011) does not seem to have an impact on MILI-MILI ping-pong, but affect specifically MILI-MIWI2 secondary biogenesis. Loss of any of these factors affects localization and/or loading of MIWI2, but does not impact MILI, MILI-bound piRNAs, or the 1U-10A ping-pong signature. This is radically different from the loss of Vasa, Vreteno, Shutdown or Tejas in *Drosophila*, where Ago3 is delocalized and presumably unloaded, but where also Aubergine bound piRNAs are lost.

It has been suggested that loss of TDRD9 (the Spn-E ortholog) (Shoji et al., 2009) doesn't affect MILI and MIWI2 loading, but it causes an increase in the amount of sense piRNAs. It is difficult to speculate about the role of this factor only from the piRNA profile of the mutant; however, from the available data it appears as if TDRD9 has a modulatory function in secondary biogenesis, maybe in the selection of the proper RNA substrates.

All known mouse secondary biogenesis factors localize to nuage, yet to different sub-compartments (Aravin et al., 2009b). Nuage can be divided into pi-bodies and piP-bodies: they are very often juxtaposed and they are both enriched in MVH, the universal nuage marker. TDRD1, MILI, and TDRD5 localize to the pi-body, while MIWI2 and TDRD9 define the piP-body. In addition, piP-bodies are seemingly enriched in several P-body components such as GW182, DCP1a, DDX6/p54, and XRN1, although the relevance of this for piRNA biogenesis or function is unclear.

Not all murine orthologs of the *Drosophila* secondary biogenesis factors have been characterized yet (TAB. 2). The single mouse ortholog of SoYb and BoYb (TDRD12) has not been studied so far. Kumo's ortholog TDRD4/RNF17 is male sterile (Pan et al., 2005), a common feature of piRNA pathway mutants, but it hasn't been formally linked to the piRNA pathway. The ortholog of Papi (TDRD2/TDRKH) (Chen et al., 2009) instead is known to interact with MIWI, but the lack of a mouse mutant prevents deeper analysis of its function. TDRD2/TDRKH co-localizes with MVH and is most probably an integral protein of the outer mitochondrial membrane like its fly ortholog Papi.

As already mentioned above, TDRD1, the only Zebrafish piRNA biogenesis factor described so far, is important for both primary and secondary biogenesis (Huang et al., 2011b). In agreement with its role in secondary biogenesis, TDRD1 co-localizes with

perinuclear Zili and its loss affects Zili localization. Besides co-immunoprecipitating Ziwi and Zili bound piRNAs, the TDRD1 complex also contains longer RNA species that are likely sliced piRNA targets (Huang et al., 2011b). These TDRD1-associated transcripts (TATs) often show a 10nt overlap with piRNAs of the opposite strand and the same 5' end as piRNAs of the same strand, suggesting that these molecules might be secondary biogenesis intermediates that have not yet been trimmed at the 3' end.

2.7 The function of Tudor domains in piRNA biogenesis

One of the most apparent features among piRNA biogenesis factors is that many harbor so-called Tudor domains (TAB. 2). This observation has led to the hypothesis that Tudor domains might constitute a structural code for piRNA biogenesis processes.

Tudor domains owe their name to the *Drosophila* protein Tudor, which harbors 11 Tudor domains and is involved in the piRNA pathway, although it doesn't seem to have an integral role in piRNA biogenesis (Kirino et al., 2009). Tudor domains bind symmetrical dimethylarginines (sDMAs) that occur within repeats of arginines flanked by glycine or alanine residues (GRG, GRA, and ARG repeats) (Chen et al., 2011).

Interestingly, these repeats are the only apparent shared feature within the N-terminus of almost all PIWI proteins and in fact all PIWI proteins analyzed so far have conserved sDMAs (Kirino et al., 2009; Kirino et al., 2010b; Nishida et al., 2009; Vagin et al., 2009). Also Vasa harbors evolutionarily conserved GRG/GRA/ARG repeats that have been shown to be symmetrically dimethylated (Kirino et al., 2010a).

Symmetrical dimethylation of PIWI proteins and Vasa is catalyzed by a specific methyltransferase (PRMT5 in mouse, Capsuleen in *Drosophila*) (Kirino et al., 2009; Kirino et al., 2010a) and a cofactor (MEP50 in mouse, Valois in *Drosophila*).

Different proteomic studies have revealed several complexes composed of PIWI proteins and various Tudor domain proteins in mouse (Aravin et al., 2009a; Chen et al., 2009). The biochemical analysis of many of these interactions confirmed the direct binding of Tudor domains to the N-terminus of PIWI proteins (Chen et al., 2009; Huang et al., 2011b; Kirino et al., 2010b; Liu et al., 2011; Mathioudakis et al., 2012). Based on co-immunoprecipitations and *in vitro* and structural studies, sDMAs are necessary for the interaction between Tudor domains and their partners. Tudor domains accommodate a

single sDMA residue into an aromatic pocket but also establish additional contacts with neighboring residues, so that the final affinity and therefore also specificity is determined by the peptide context of an sDMA residue (Mathioudakis et al., 2012).

Surprisingly, a complete loss of sDMAs upon Capsuleen mutation does not have a strong impact on either primary or secondary piRNA biogenesis (Kirino et al., 2009); the observed effects on piRNAs appear limited to a modest loss of Aub-bound piRNAs and are much weaker than those observed upon loss of most individual Tudor domain biogenesis factors. This might suggest that Tudor domains are not absolutely necessary for the function of their proteins and instead might increase the efficiency or accuracy of piRNA biogenesis processes. An alternative and more moderate interpretation is that the interaction between Tudor domains and their partners *in vivo* is less dependent on sDMAs than what *in vitro* studies show. This is for example the case for the interaction between Vasa and TDRD1 (Kirino et al., 2010a), which seems independent of sDMAs.

What could be the function of the various Tudor domains during piRNA biology? Tudor domain-sDMA interactions have the potential to build a meshwork because several proteins have multiple Tudor domains and these could bind different PIWI proteins simultaneously. As PIWI proteins harbor several sDMA residues they also could interact with multiple Tudor proteins simultaneously. This meshwork could bring PIWI proteins, Tudor domain proteins and Vasa in close contact, which might be needed for efficient secondary biogenesis or for the recruitment of other relevant factors; indeed most Tudor proteins are involved in secondary piRNA biogenesis. This model is supported by the conserved co-localization of Tudor domain proteins, PIWI proteins, and Vasa to nuage (FIG. 4).

2.8 piRNA loading

The process by which an empty Argonaute protein becomes complexed with an RNA is called 'loading'. It is an active process that requires energy, rather than a diffusion mediated interaction. As already mentioned, it has been shown that PIWI proteins can bind long ssRNAs that still have to be trimmed to the right size and 2'-O-methylated at the 3' end. Only after the piRNA is mature it will establish the full range of interactions with its PIWI protein. The exact steps of PIWI protein loading are still unclear but

different lines of evidence are pointing towards similarities with siRNA and miRNA loading into AGO family proteins, which has been studied in detail in *Drosophila*.

According to the current model, miRNA loading onto Ago1 and siRNA loading onto Ago2 follow the same general principle. Empty Ago2 is bound in a complex of chaperones including Hsp90, Hsc70, Hop, and Droj2 that keep it in an open conformation, so that it can accommodate a small RNA duplex (Iwasaki et al., 2010). The energy for this process is provided by the hydrolysis of ATP by Hsp90 (Iwasaki et al., 2010). Data about miRNA loading in plants is consistent with this model (Iki et al., 2010) and adds another component to this picture (Earley and Poethig, 2011; Iki et al., 2012): CYP40, a cochaperone that is necessary for the maturation of the miRISC (miRNA-mediated silencing complex).

The work of several groups has highlighted an important role for Hsp90 and cochaperones also in the piRNA pathway: the deficiency or the inhibition of Hsp90 causes transposon upregulation and decrease of piRNAs in *Drosophila* (Specchia et al., 2010). Furthermore, Piwi can be found in a complex with Hsp90 and Hop (Gangaraju et al., 2011). Unloaded Ago3 accumulates with Hsp90 and the cochaperone Shutdown (Olivieri et al., 2012), a factor related to CYP40 that is necessary for all piRNA populations. Also in mouse, Hsp90 has been shown to interact with MIWI (Chen et al., 2009) and with FKBP6 (Xiol et al., 2012), a mouse secondary biogenesis factor. All of these data suggest that piRNA, miRNA, and siRNA loading could be relatively similar conserved processes based on the same chaperone machinery.

It has also been shown that in a silkworm ovarian cell culture system Hsp90 is necessary for the removal of secondary biogenesis byproducts from PIWI proteins (Xiol et al., 2012). This might indicate a specific role of Hsp90 in secondary biogenesis or in the mature pi-RISC.

3. Articles

3.1 An in vivo RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in Drosophila

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An *in vivo* RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in *Drosophila*

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In *Drosophila*, PIWI proteins and bound PIWI-interacting RNAs (piRNAs) form the core of a small RNA-mediated defense system against selfish genetic elements. Within germline cells, piRNAs are processed from piRNA clusters and transposons to be loaded into Piwi/Aubergine/AGO3 and a subset of piRNAs undergoes target-dependent amplification. In contrast, gonadal somatic support cells express only Piwi, lack signs of piRNA amplification and exhibit primary piRNA biogenesis from piRNA clusters. Neither piRNA processing/loading nor Piwi-mediated target silencing is understood at the genetic, cellular or molecular level. We developed an *in vivo* RNAi assay for the somatic piRNA pathway and identified the RNA helicase Armitage, the Tudor domain containing RNA helicase Yb and the putative nuclease Zucchini as essential factors for primary piRNA biogenesis. Lack of any of these proteins leads to transposon de-silencing, to a collapse in piRNA levels and to a failure in Piwi-nuclear accumulation. We show that Armitage and Yb interact physically and co-localize in cytoplasmic Yb bodies, which flank P bodies. Loss of Zucchini leads to an accumulation of Piwi and Armitage in Yb bodies, indicating that Yb bodies are sites of primary piRNA biogenesis.

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Introduction

Selfish genetic elements such as transposons populate all eukaryotic genomes. Though in a few cases the host benefits from transposons, they overall have a negative impact on the host's reproductive fitness because of their mutagenic character (Slotkin and Martienssen, 2007).

Genetic studies on *Drosophila melanogaster* illustrate the threat emanating from active transposons. Here, uncontrolled

activity of a single transposable element (e.g. the *I*-element, a retro-element or the *P*-element, a DNA-element) leads to defects in gametogenesis and sterility (Rubin *et al*, 1982; Bucheton *et al*, 1984). Given that the *D. melanogaster* genome harbours over a 100 different transposon families (Bergman *et al*, 2006), many of which are still active, a strong selective pressure to silence transposons must exist.

After the discovery of small RNA-silencing pathways, it has become clear that this regulatory mechanism is at the root of transposon control in animals and that different lineages deploy the basic principle of small RNA pathways in different ways to guarantee specific and efficient silencing of selfish genetic elements (reviewed in Girard and Hannon, 2008; Malone and Hannon, 2009).

Although transposon control is important for all cells, their silencing is of pivotal importance in the germline, the only cell lineage that passes its genetic information onto the next generation. Indeed, multi-cellular animals possess a unique small RNA pathway targeted towards silencing selfish genetic elements in their gonads. At the centre of this pathway is a subclass of Argonaute proteins, the so-called PIWI proteins, complexed with 23–30 nt long PIWI-interacting RNAs (piRNAs). Mutations in PIWI proteins result in strong de-repression of transposable elements and lead to widespread defects in gametogenesis and sterility (reviewed in Klattenhoff and Theurkauf, 2008; Malone and Hannon, 2009).

The piRNA pathway is best understood in *Drosophila*, where insight from three research areas has accumulated. First, studies on transposon biology have uncovered important concepts of the host control system and have also identified a number of essential genetic loci involved (Prud'homme *et al*, 1995; Aravin *et al*, 2001; Jensen *et al*, 2002; Desset *et al*, 2003; Ronsseray *et al*, 2003; Sarot *et al*, 2004; Pelisson *et al*, 2007). Second, sterility and egg patterning screens have uncovered more than a dozen factors that turned out to be piRNA pathway members (Schupbach and Wieschaus, 1991; Clegg *et al*, 1997; Lin and Spradling, 1997; Cook *et al*, 2004; Chen *et al*, 2007; Pane *et al*, 2007). And finally, research centred on small RNA pathways and bioinformatics analysis of piRNA populations has allowed combining the diverse genetic findings into a coherent model of transposon control in *Drosophila* (Aravin *et al*, 2003; Vagin *et al*, 2006; Brennecke *et al*, 2007; Gunawardane *et al*, 2007). According to this, PIWI proteins are loaded with piRNAs in a Dicer and presumably double-stranded RNA (dsRNA)-independent manner (Vagin *et al*, 2006). Most piRNAs originate from transposon- and other repeat regions in the genome (Aravin *et al*, 2003; Saito *et al*, 2006; Vagin *et al*, 2006; Brennecke *et al*, 2007; Gunawardane *et al*, 2007). A hallmark of the piRNA pathway is that discrete genomic loci (piRNA clusters) that harbour a diverse collection of transposon and repeat fragments are major sources of piRNAs (Brennecke

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et al, 2007). Another unique feature of the piRNA pathway is the involvement of PIWI proteins in a target-dependent amplification loop (Brennecke *et al*, 2007; Gunawardane *et al*, 2007). In this so-called ping-pong cycle, the PIWI proteins Aubergine and AGO3 cleave reciprocally sense (from active elements) and antisense (from piRNA clusters) transcripts, respectively. It is postulated that each cleavage event triggers the production of a novel piRNA from the cleaved RNA. Thus, the ping-pong cycle leads to a preferential amplification of silencing competent piRNAs. Gonad-specific activity of the piRNA pathway, repeat enriched piRNA clusters and signatures of the ping-pong cycle are all conserved features in vertebrates (reviewed in Malone and Hannon, 2009).

Major open questions are how the cell distinguishes transcripts from piRNA clusters, transposons and endogenous genes and how piRNA biogenesis and loading into PIWI proteins is controlled. Equally unclear is how PIWI-piRNA complexes silence the array of selfish genetic elements in the genome. Various studies indicate that post-transcriptional control through target slicing and degradation and transcriptional control through guiding DNA and/or chromatin modifications both have an active function (Carmell *et al*, 2007; Klenov *et al*, 2007; Kuramochi-Miyagawa *et al*, 2008; Lim *et al*, 2009).

In *Drosophila*, germline cells and somatic support cells of male and female gonads possess a functional piRNA pathway. However, in somatic support cells, only one of the three PIWI family proteins (Piwi) is expressed and a simplified pathway (the somatic piRNA pathway) lacking the ping-pong cycle is active (Sarot *et al*, 2004; Pelisson *et al*, 2007; Lau *et al*, 2009; Li *et al*, 2009; Malone *et al*, 2009; Saito *et al*, 2009). Deep sequencing of small RNA populations indicates that single-stranded RNAs from a subset of piRNA clusters and to a lower extent also from over a 1000 genes are processed into piRNAs (Lau *et al*, 2009; Malone *et al*, 2009; Robine *et al*, 2009). The most prominent piRNA cluster in somatic support cells is the genetically identified *flamenco* or *COM* locus (Prud'homme *et al*, 1995; Desset *et al*, 2003). It contains an exceptionally high density of transposon fragments, nearly all of which belong to the *gypsy* family of retro-elements. As nearly all fragments are oriented antisense to the transcription direction, piRNA processing from *flamenco* yields almost exclusively antisense piRNAs (Pelisson *et al*, 2007; Brennecke *et al*, 2007; Malone *et al*, 2009). Many *gypsy* family transposons expressed in somatic support cells encode functional *gag*, *pol* and *env* genes. In the absence of efficient silencing, they form viral particles that invade the neighbouring oocyte potentially through cellular transport vesicles (Pelisson *et al*, 1994; Chalvet *et al*, 1999; Leblanc *et al*, 2000; Brasset *et al*, 2006).

The somatic cells of the *Drosophila* gonad are the only described cell type with an active piRNA pathway that lacks the ping-pong cycle. These cells are, therefore, ideally suited for genetic and biochemical approaches towards elucidating the core concepts of the piRNA pathway. Here, we describe a genetic assay that allows for testing any gene of interest for its involvement in the somatic piRNA pathway. We use it to identify the three evolutionarily conserved proteins Armitage (Armi), Zucchini (Zuc) and Yb (King *et al*, 2001; Cook *et al*, 2004; Pane *et al*, 2007) as essential components of primary piRNA biogenesis in *Drosophila* and link the Yb body, a

previously identified cellular structure (Szakmary *et al*, 2009) to piRNA biogenesis.

Results

An in vivo assay for the identification of novel piRNA pathway components

To facilitate the identification of novel somatic piRNA pathway members, we constructed a tester fly line that could be combined with the Vienna *Drosophila* RNAi collection (VDRC) (Dietzl *et al*, 2007). The VDRC library consists of Gal4-inducible UAST lines allowing expression of dsRNA hairpins against >90% of the *Drosophila* genes. The tester line combines a GAL4 driver specific for somatic support cells with a sensor capable of monitoring the integrity of the somatic piRNA pathway.

We identified the previously reported *traffic jam* GAL4 line (*tj*-GAL4) as an optimal driver for RNAi in somatic support cells (Tanentzapf *et al*, 2007). GAL4 expression mirrored the restricted expression of the host gene *tj* to gonadal somatic support cells when crossed to UAST-GFP (Figure 1A) (Li *et al*, 2003). GFP signal was only detected in somatic support cells of the ovary including somatic stem cells in the germarium. To determine the tissue specificity of *tj*-GAL4, we crossed the line to UAST-Luciferase and measured reporter activity in whole females, dissected ovaries and carcasses (flies with manually removed ovaries). The *tj*-GAL4 driver induced Luciferase signal ~50-fold and on average, 94% of this signal originated from ovaries (Figure 1B).

Crosses to a broad spectrum of RNAi lines targeting essential genes resulted in missing or rudimentary ovaries with no impact on viability (Supplementary Table S1). This is significant, as it has been reported that a number of strong follicle cell GAL4 drivers were incapable of inducing efficient RNAi (Zhu and Stein, 2004). Importantly, *tj*-GAL4-driven RNAi against *piwi*, the only undisputed member of the somatic piRNA pathway, resulted in ovaries that phenocopied *piwi* mutant ovaries (Figure 1C) (Cox *et al*, 2000). A known target of the somatic piRNA pathway, the retroviral *gypsy* transposon (Sarot *et al*, 2004), was de-repressed in these ovaries with a strong accumulation of the *gypsy* Env protein at the follicle cell cortex (Figure 1D). No staining by the *gypsy* Env antibody was detectable in wild-type ovaries and staining was restricted to patches of cells in which *piwi* RNAi was induced clonally (Supplementary Figure S1). Quantitative RT-PCR analysis further indicated that besides *gypsy*, the retro-elements *ZAM* and *Tabor*, two additional targets of the somatic piRNA pathway (Desset *et al*, 2003; Li *et al*, 2009; Malone *et al*, 2009), are strongly de-silenced in *piwi* RNAi crosses compared with *aubergine* RNAi crosses (*aub* shows no detectable expression in follicle cells) (Figure 1E). Notably, RNA levels of the telomeric retro-elements *HeT-A* and *TART*, which are sensitive to defects in the germline piRNA pathway (Vagin *et al*, 2006; Klattenhoff *et al*, 2009), were unaffected. These results are in line with the abundance of piRNAs against *gypsy*, *ZAM* and *Tabor* and the low level of piRNAs against *HeT-A* and *TART* in cultured ovarian somatic cells (OSCs), which are derived from somatic support cells (Supplementary Figure S2) (Niki *et al*, 2006; Lau *et al*, 2009).

We combined the *tj*-GAL4 driver with a *gypsy*-lacZ reporter that in flies carrying a restrictive allele of the *flamenco* cluster is fully silenced by the somatic piRNA pathway (Sarot *et al*,

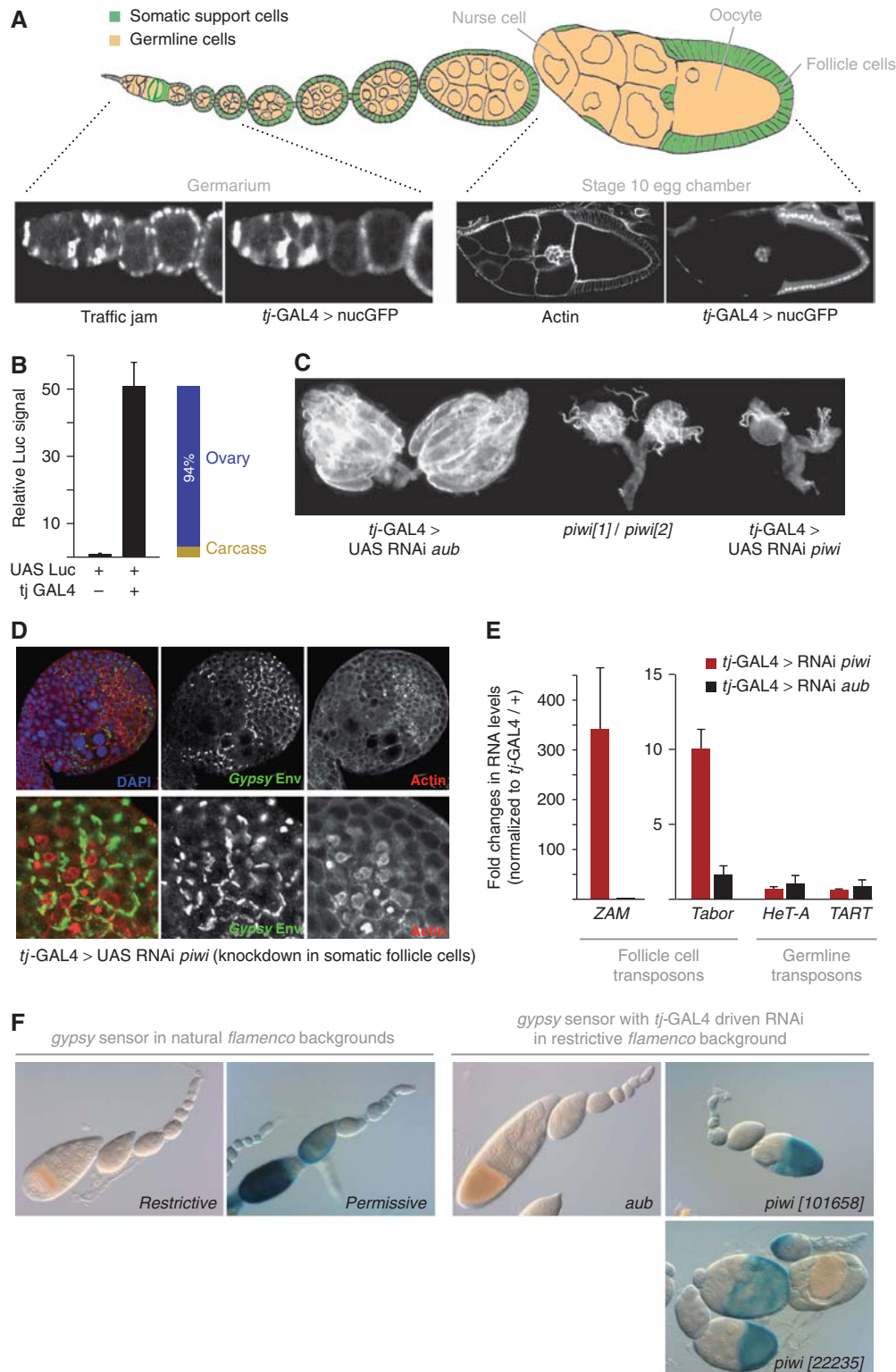
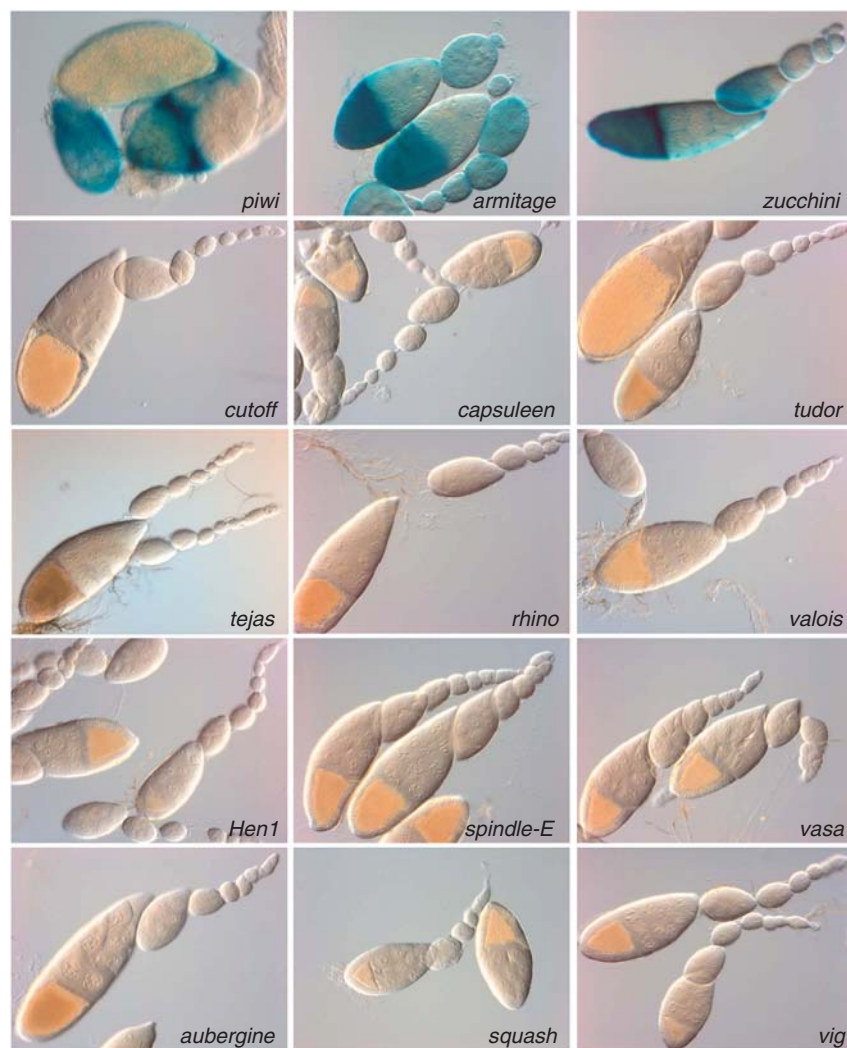


Figure 1 An *in vivo* RNAi-based assay for the somatic piRNA pathway. **(A)** Cartoon depicting the two major cell types in the ovary with somatic cells in green and germline cells in beige. The *tj*-GAL4 driver is expressed at all developmental stages in somatic support cells (labelled with Tj) as judged by a cross to UAS-GFP. **(B)** Relative Luciferase reporter activity is shown from flies of the indicated genotypes (left) and from ovaries and carcasses of *tj*-GAL4 > UAS-Luc flies. **(C)** Morphology of ovaries from flies expressing dsRNA against *piwi* and *aub* through *tj*-GAL4 compared with ovaries from *piwi* mutants. **(D)** Immuno-staining of the retroviral *gypsy* Env protein (green) in ovaries with *tj*-GAL4 induced Piwi knockdown. The lower panels show a zoom onto the follicular epithelium. Actin (red) marks the cell cortex. **(E)** Shown are changes in steady-state RNA levels ($n = 3$) of the endogenous retro-elements ZAM and *Tabor* upon Piwi (red) or Aubergine (black) knockdown in comparison with *tj*-GAL4 / + flies. The germline-controlled *HeT-A* and *TART* elements serve as controls. **(F)** Shown is the *gypsy*-lacZ reporter in restrictive and permissive *flamenco* backgrounds (left) and in a restrictive background upon RNAi against *aub* and *piwi* (two independent hairpins). β -Gal staining (blue) is strongest in the columnar follicle epithelium of stages 9–10 egg chambers.



gypsy sensor with *tj*-GAL4 driven RNAi against indicated gene (restrictive *flam.* background)

Figure 2 Comprehensive analysis of the involvement of piRNA pathway members in the somatic pathway. Shown are β -Gal stainings as readout for *gypsy* silencing for genetically identified piRNA pathway genes in which a VDR line was available using *gypsy*-lacZ and *tj*-GAL4 in a restrictive *flamenco* background.

2004). Lack of the somatic piRNA pathway or homozygosis for a permissive *flamenco* allele strongly de-silences β -Gal expression (Figure 1F) (Sarot *et al*, 2004). When the *tj*-GAL4; *gypsy*-lacZ line was crossed to an *aub*-RNAi line, no β -Gal signal was detectable. In contrast, crosses to two independent *piwi* RNAi lines resulted in strong β -Gal activity (Figure 1F). Thus, given the near genome-wide collection of RNAi lines at the VDR, almost any gene can be tested for its involvement in the somatic piRNA pathway with a single genetic cross.

Armitage and Zucchini are essential components of the somatic piRNA pathway

Over a dozen genes have been implicated in the *Drosophila* piRNA pathway (Boswell and Mahowald, 1985; Gillespie and Berg, 1995; Cox *et al*, 1998; Harris and Macdonald, 2001; Cook *et al*, 2004; Vagin *et al*, 2004; Anne *et al*, 2007; Chen *et al*, 2007; Horwich *et al*, 2007; Lim and Kai, 2007; Pane *et al*, 2007; Saito *et al*, 2007; Klattenhoff *et al*, 2009; Li *et al*, 2009; Malone *et al*, 2009; Patil and Kai, 2010). To test their involvement in the somatic piRNA pathway, we

crossed the *tj*-GAL4; *gypsy*-lacZ tester strain to RNAi lines for all candidates if a VDR line was available. Most candidates are seemingly not essential components of the somatic piRNA pathway (Figure 2). Consistent with this, loss of *spindle-E*, *squash*, *aubergine*, *rhino* or *vasa* does not affect *flamenco*-derived piRNA levels, indicating their specific involvement in the germline-specific ping-pong amplification cycle (Klattenhoff *et al*, 2009; Malone *et al*, 2009). In addition, loss of the piRNA methyl-transferase Hen1 has been reported to impact transposon silencing only very mildly (Horwich *et al*, 2007). It is of interest to note that lack of symmetrically methylated Arginines (mediated by knocking down the arginine methyl-transferase *capsuleen* or its cofactor *valois*) did not lead to detectable sensor activation. Thus, this post-translational modification of PIWI proteins (Kirino *et al*, 2009; Nishida *et al*, 2009; Vagin *et al*, 2009) has no or only a modulatory function in the somatic piRNA pathway, although we cannot exclude the involvement of other arginine methyl transferases.

In contrast, the *gypsy* sensor was strongly de-silenced in the cross to the *zucchini* (*zuc*) RNAi line (Figure 2). This

is in agreement with published findings that loss of *Zuc* affects *flamenco*-derived piRNA levels as well as those derived from the *tj*-3'UTR, a gene expressed in somatic support cells only (Malone *et al*, 2009; Robine *et al*, 2009; Saito *et al*, 2009).

To our surprise, knockdown of *armitage* (*armi*) led to a robust de-silencing of the sensor (Figure 2). This contradicts earlier findings that *armi* mutations specifically affect the germline piRNA pathway (Malone *et al*, 2009): in *armi*[1]/*armi*[72.1] mutants, Piwi-bound piRNAs and nuclear localization of Piwi were only lost in germline cells. Furthermore, *flamenco*-derived piRNA levels were unchanged in those mutants. In support of the previous genetic results, the *gypsy-lacZ* sensor was not de-silenced in follicle cells of *armi*[1]/*armi*[72.1] ovaries (Figure 3A). In these ovaries, we observed weak β -Gal activity in nurse cells, indicating a slight activity of the *gypsy* promoter and an *Armi*-dependent silencing mechanism in ovarian germline cells.

The *armi*[1] and *armi*[72.1] alleles are not null alleles as noted previously (Cook *et al*, 2004). Nevertheless, they cause a severe reduction of *Armi* protein in germline cells. No difference in immuno-staining, however, has been detected in somatic follicle cells of these mutants, which has been interpreted as antibody cross-reactivity (Cook *et al*, 2004). The following observations, however, indicate that *Armi* is expressed in follicle cells and that it is an essential component of the somatic piRNA pathway. We first verified our knockdown result with an independent RNAi line (Figure 3A). Second, knockdown of *Armi* led to a strong de-silencing of the *ZAM* and *Tabor* retro-elements with no impact on *HeT-A* and *TART* (Figure 3B). Similar results were obtained from flies expressing the *zuc* RNAi construct. We next verified robust *Armi* expression in somatic support cells by three independent means: first, a transgenic fly line expressing *Armi* as a C-terminal GFP fusion under its own promoter showed robust GFP signal in germline and somatic cells of the ovary, similar to the published immuno-stainings (Figure 3D) (Cook *et al*, 2004). Second, the follicle cell staining of *Armi* was specifically lost in clones of cells expressing the *armi* RNAi construct (Figure 3E). Third, western analysis showed that ovary lysate contains two *Armi* isoforms that appear as a duplet and that both isoforms are also present in a lysate from OSCs (cultured somatic cells) (Figure 3C). To test for the specificity of the western signal, we generated a genomic deletion that removes the entire *armi* locus including two downstream genes (*armi*[Δ 1]). Homozygous *armi*[Δ 1] flies are viable, contain malformed ovaries and do not lay eggs. Extracts from these ovaries lack both *Armi* isoforms (Figure 3C).

This indicates that the two published alleles *armi*[1] and *armi*[72.1] are not null alleles at the protein level. Indeed, both *Armi* isoforms were detected by western analysis in *armi*[1]/*armi*[72.1] flies albeit at ratios that resemble that found in OSCs (Figure 3C). Furthermore, *Armi* protein was detectable in follicle cells only of *armi*[1]/*armi*[Δ 1] and *armi*[72.1]/*armi*[Δ 1] mutant ovaries (Figure 3D). We, therefore, suspected that the *armi* locus contains two promoters and, therefore, gives rise to two distinct mRNAs encoding the two observed protein isoforms. Indeed, two mRNA isoforms have been observed by northern analysis with only the larger one being severely affected by the published alleles (Cook *et al*, 2004). We performed 5'RACE analysis on OSC RNA and

identified two distinct mRNA 5' ends with one corresponding to the Flybase annotated transcription start site (TSS) (Figure 3F). The second TSS is a few nucleotides downstream of the annotated ATG start codon and, therefore, is predicted to generate an ~ 4 kDa smaller isoform. In this model, the insertion site of the P-element in *armi*[1] and the small deletion in the *armi*[72.1] allele, which was derived from *armi*[1] by imprecise excision are expected to affect only the longer isoform. Although the 5'RACE analysis indicates that both promoters are active in follicle cells, a western analysis from lysate obtained from 0 to 0.5 h old embryos identified only the larger isoform, indicating that the downstream promoter is not active in germline cells (Figure 3C). Our results, therefore, offer a consistent explanation for the discrepancy of the published literature with our result that *Armi* is an essential component of the somatic piRNA pathway.

Yb co-localizes with Armitage in cytoplasmic foci and is essential for the somatic piRNA pathway

Both GFP tagged and endogenous *Armi* localized to the cytoplasm of follicle cells and OSCs with a strong enrichment in 1–3 peri-nuclear foci (Figure 4A and B). Within the ovary, *Armi* foci were much more prominent in the germarium and younger egg chambers and became progressively smaller from stage 5–6 onwards (not shown). At this stage, follicle cells switch from a mitotic cycle to an endo-replication cycle in a Notch-signalling-dependent manner (Deng *et al*, 2001). Interestingly, disruption of the Notch-signalling pathway by RNAi against Notch led to a strong increase in *Armi* levels in older (>stage 6), but not in younger egg chambers (Figure 4C; not shown), suggesting that Notch signalling dampens *Armi* expression in endo-replicating cells.

The distinct localization of *Armi* in follicle cells was reminiscent of processing bodies (P bodies), cytoplasmic foci involved in RNA degradation and storage (Eulalio *et al*, 2007a). Co-labelling of *Armi* and the P-body marker DCP1 indicated that *Armi* foci are in most cases directly adjacent to P bodies (Figure 4D). There appear to be many more P bodies than *Armi* foci, but typically the most prominent P bodies flank *Armi* foci. In germline cells, we observed that areas with *Armi* staining typically exhibit also strong DCP1 labelling (Supplementary Figure S3A). The significance of this correlation remains to be determined, but we note that knockdown of two critical P-body components (*Lsm1* and *Me31b*) with *tj*-GAL4 did not lead to defects in *gypsy* sensor silencing. Moreover, RNAi flip-out clones for *Lsm1* did not affect number or integrity of the *Armi* foci (not shown).

The subcellular localization of *Armi* resembles that of the *Yb* protein. *Yb* is encoded by the *fs(1)Yb* gene and is a putative RNA helicase that contains a predicted Tudor domain (King and Lin, 1999; Szakmary *et al*, 2009). *Yb* is expressed in somatic support cells of the male and female gonad and accumulates in cytoplasmic foci that have been termed *Yb* bodies (Szakmary *et al*, 2009). *Yb* mutants are female sterile and exhibit defects in the maintenance of germline and somatic stem cells (King and Lin, 1999; King *et al*, 2001). Immuno-fluorescence experiments indicated that *Armi* and *Yb* proteins co-localize and accumulate in *Yb* bodies of follicle cells (Figure 4E) and OSCs (Supplementary Figure S3B). In addition, a specific interaction between the two proteins was observed in co-IP experiments from OSC lysate using FLAG-tagged *Yb* (Figure 4F). To test for a

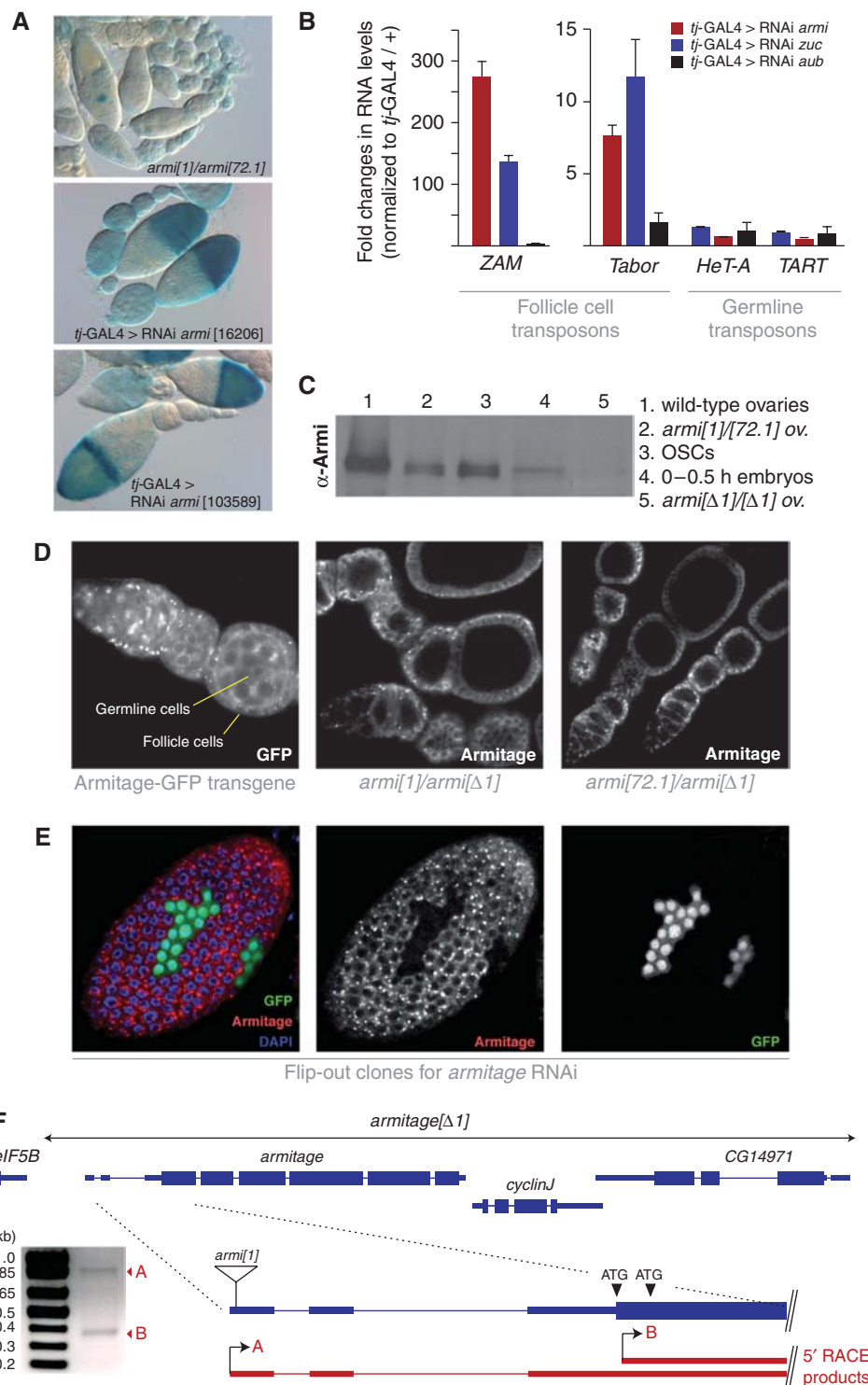


Figure 3 Armitage is an essential player in the somatic piRNA pathway. (A) *gypsy-lacZ* reporter activity in *armi*[1]/*armi*[72.1] ovaries compared with ovaries in which *armi* was knocked down using *tj*-GAL4-mediated RNAi. (B) Shown are fold changes in steady-state RNA levels ($n = 3$) for the ZAM, Tabor, HeT-A and TART retro-elements in ovaries with *tj*-GAL4-driven RNAi against *armi*, *zuc* and *aub* (control). (C) Western blot analysis showing two Armi isoforms expressed in ovaries and OSCs, one isoform in early embryos, persistent expression of Armi in published mutant alleles and the absence of Armi in the *armi*[Δ1] allele. (D) Shown are Armi stainings of an ovariole expressing Armi-GFP under its endogenous promoter (left) and of ovarioles trans-heterozygous for the published alleles *armi*[1] and *armi*[72.1] over the *armi*[Δ1] allele. (E) Armi staining (red) of the follicular epithelium in which *armi*-RNAi has been clonally activated. GFP signal (green) labels cells expressing the *armi* dsRNA hairpin. (F) Cartoon of the *armi* genomic locus showing the extent of the *armi*[Δ1] deletion, the two identified 5' RACE products (red), their respective transcription start sites and the insertion site of the P-element in the *armi*[1] allele. To the left, an Agarose gel with the two sequenced RACE products is shown.

functional involvement of Yb in the somatic piRNA pathway, we crossed two independent *fs(1)Yb* RNAi lines to our tester strain and observed strong de-repression of the *gypsy* reporter

(Figure 4G). Depletion of Yb also led to a de-silencing of the ZAM and Tabor retro-transposons, but not the germline-controlled HeT-A and TART elements (Figure 4H). These

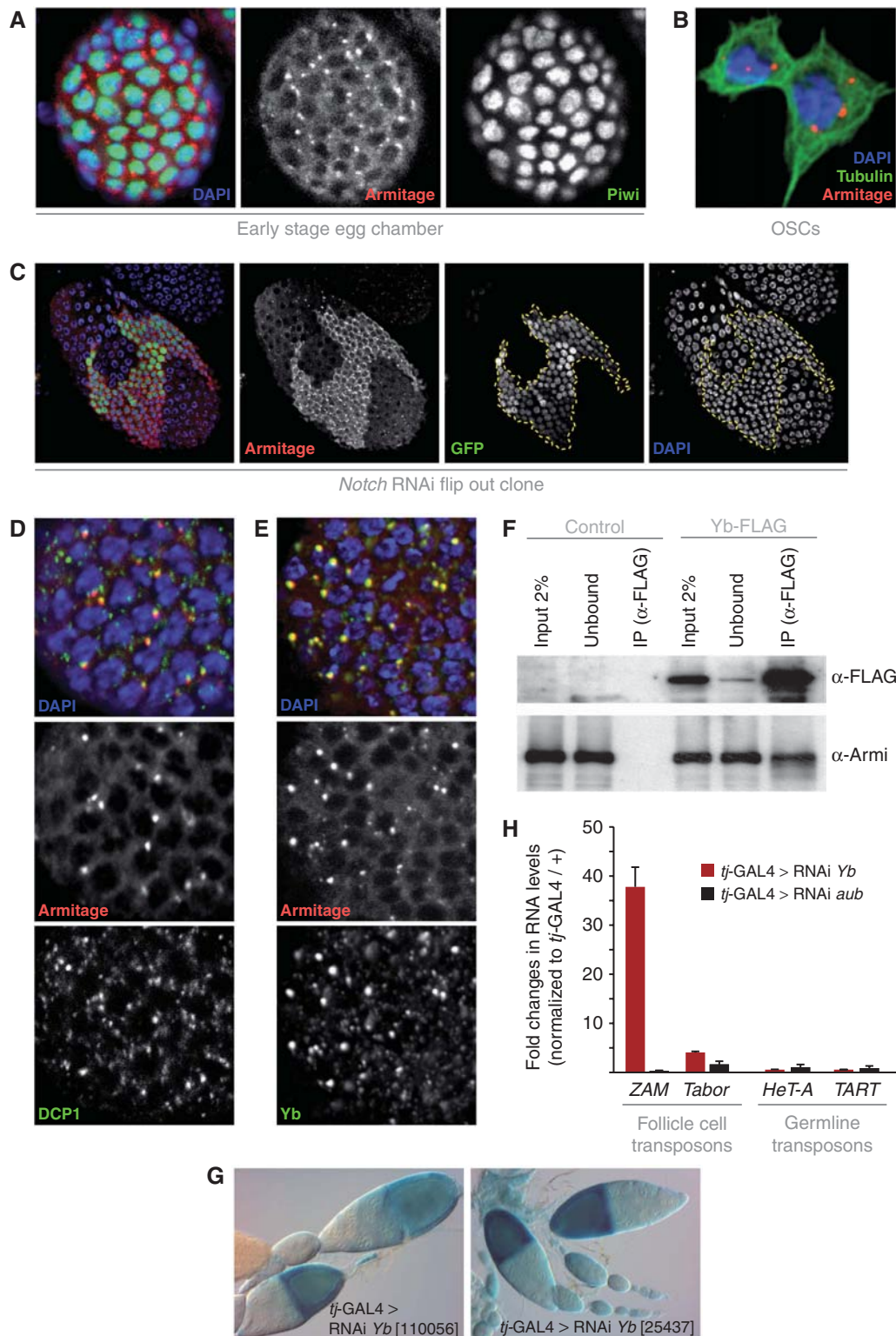


Figure 4 Armi localizes to Yb bodies and Yb is an essential pathway component. **(A)** Shown are immuno-fluorescence stainings for Armi (red) and Piwi (green) in somatic follicle cells. DNA was labelled with DAPI (blue). **(B)** Immuno-fluorescence staining for Armi (red), Tubulin (green) and DNA (blue) in OSCs. **(C)** Immuno-fluorescence analysis of Armi (red) in the follicular epithelium of an egg chamber containing a Notch dsRNA-expressing clone (clone marked with GFP (green) and clone borders indicated by the dashed line). DAPI staining (blue) confirms that Notch knockdown impairs endo-replication. **(D)** Co-labelling of Armi (red) and DCP1-GFP (green) in somatic follicle cells. **(E)** Co-labelling of Armi (red) and Yb (green) in somatic follicle cells. **(F)** Co-immuno-precipitation of endogenous Armi with FLAG-tagged Yb. Shown are western blots against FLAG to detect Yb (top) and against endogenous Armi (bottom). Control cells did not express FLAG-Yb. **(G)** Shown are β-Gal stainings of ovarioles expressing the gypsy reporter and two different dsRNA hairpins against *fs(1)Yb* with *tj-GAL4*. **(H)** Shown are fold changes in steady-state RNA levels ($n = 3$) of *ZAM*, *Tabor*, *HeT-A* and *TART* retro-elements in ovaries in which *fs(1)yb* and *aub* (control) were knocked down through *tj-GAL4*-mediated RNAi. Values are normalized to a *tj-GAL4* / + control.

results identified Yb as an essential component of the somatic piRNA pathway and link the previously described Yb bodies to piRNA biology.

Loss of Armitage, Zucchini and Yb impairs nuclear localization of Piwi

To gain insight into the defects exerted onto the somatic piRNA pathway upon loss of Armi, Zuc and Yb, we performed a systematic analysis of Armi, Piwi and Yb localization in cells mutant for each factor (a lack of reagents prevented localization studies for Zuc).

RNAi-mediated knockdown of Yb in follicle cell clones led to a loss of nuclear Piwi accumulation (Figure 5A). Furthermore, no Armi foci were detectable when Yb was depleted by RNAi. Instead, Armi was uniformly distributed throughout the cytoplasm (Figure 5A). Identical results were obtained in FRT-mediated mitotic clones using the *Yb[72]* loss of function allele (Supplementary Figure S4).

RNAi-mediated knockdown of Armi also caused a loss of Piwi from the nucleus (Figure 5B). In addition, Yb bodies were reduced in size, but did not disappear entirely (Figure 5C). We, therefore, analysed Yb localization in *armi* null mutants and observed a loss of detectable Yb bodies in nearly all cells (Supplementary Figure S5). Thus, Armitage and Yb are each required for their reciprocal localization to Yb bodies and potentially for Yb-body formation *per se*.

The most interesting effects were seen upon Zuc knockdown. Here, Piwi was not only lost from the nucleus, but instead accumulated in peri-nuclear foci (Figure 5D). Co-labelling experiments showed that these correspond to Armi bodies (Figure 5D, bottom). In addition, the size of Armi foci increased several fold in Zuc mutant cells (Figure 5D). We also observed elevated intensity of Yb foci in these cells, but the effects were much weaker than those observed for Armi (not shown). Identical results were obtained in FRT-mediated mitotic clones using the *zuc[HM27]* loss of function allele (Supplementary Figure S6). The co-localization of Piwi and Armi in *zuc* mutant cells suggests a rapid transit of Piwi through Armi/Yb bodies in wild-type cells, as we never observed accumulation of endogenous Piwi protein in cytoplasmic foci. However, OSCs chemically transfected with GFP-tagged Piwi often exhibit co-localization of Piwi with Armi foci, potentially as a consequence of Piwi over-expression (Supplementary Figure S7).

To test whether Armi is required for Piwi's accumulation in Yb bodies, we simultaneously knocked down Armi and Zuc in follicle cell clones. In these cells, Piwi did not accumulate in cytoplasmic foci (Supplementary Figure S8). We note, however, that Yb-body size is severely reduced upon Armi knockdown, thus complicating this interpretation. No defects on Armi localization were seen in cells expressing an RNAi construct against Piwi or in cells homozygous for the *piwi[1]* loss of function allele (Figures 5E and 8D). This indicates that Armi does not depend on Piwi for its localization to Yb bodies and that Yb-body formation occurs in the absence of Piwi.

Armitage and Piwi interact physically in OSCs and ovaries

In an attempt to identify Piwi-interacting proteins, we immuno-precipitated endogenous Piwi from OSC lysate using polyclonal antibodies directed against the Piwi N-terminus. Silver staining identified two protein bands at

~140 kDa that were specific for the Piwi-IP compared with a control IP (Figure 6A). Mass spectrometric analysis identified Armi as the major protein in both bands (32 and 27% sequence coverage). This result is at odds with Armi being a cytoplasmic protein, whereas Piwi is enriched in the nucleus under wild-type conditions. However, our observation that Piwi localizes transiently with Armi to Yb bodies offers an explanation for the observed interaction. Western blot analysis indicated that this interaction is also robust in IPs from ovary lysate (Figure 6B). The major interacting Armi isoform in ovaries is the germline-specific larger isoform, whereas the smaller isoform predominates in OSCs. To test the specificity of the Armi Piwi interaction, we immuno-precipitated transfected FLAG-tagged Piwi from OSC lysate. Again, endogenous Armi protein was only detected in the FLAG-Piwi-IP, but not in the FLAG-IP from non-transfected cells (not shown). Finally, endogenous Piwi was co-immuno-precipitated with Armi using flies expressing C-terminally GFP-tagged Armi under its own promoter (not shown). In this experiment, we also noted that Armi forms oligomeric complexes *in vivo*, as endogenous Armi protein was efficiently co-immuno-precipitated with GFP-tagged Armi (Figure 6C).

An immuno-precipitation of Piwi in the presence of RNase A and T1 indicates that the interaction between Armi and Piwi depends at least in part on RNA (Figure 6D). Although we cannot exclude incomplete RNA digestion, we suggest that the interaction between Armi and Piwi is direct, but enhanced by RNA. Such a direct interaction is supported by a co-immuno-precipitation of mouse PIWI proteins and the mouse Armi orthologue MOV10L1 in 293 cells that contain no active piRNA pathway (Zheng *et al*, 2010).

Armitage, Zucchini and Yb are required for primary piRNA biogenesis

Loss of Armi in germline cells leads to a failure of nuclear Piwi accumulation accompanied by a loss of germline-specific Piwi-bound piRNAs (Malone *et al*, 2009). A correlation between nuclear localization and intact small RNA biogenesis has also been observed for mouse MIWI2 and *Tetrahymena* Twi1 (Aravin *et al*, 2008; Noto *et al*, 2010; Zheng *et al*, 2010). We sequenced Piwi-bound piRNAs from ovaries in which we depleted Piwi, Armi, Zuc or Yb in all ovarian somatic support cells by RNAi using the *tj-GAL4* driver. RNAi against *aub* was used as a control. An immuno-fluorescence analysis showed the efficiency of the *tj-GAL4*-driven knockdown in follicle cells as they lacked Piwi-nuclear accumulation (Figure 7A). We prepared ovary lysates and immuno-precipitated Piwi-piRNA complexes. From these, small RNAs were isolated and used for the construction of cDNA libraries, which were sequenced on the Illumina G2 platform. After removal of small RNAs mapping to abundant cellular non-coding transcripts (rRNAs, tRNAs, snoRNAs), the composition of all libraries was highly similar with ~84% mapping to annotated repeats (Supplementary Table S2). As Piwi function within germline cells was unaffected, we used the germline-specific piRNA cluster at position 42AB to normalize the individual libraries. Three other peri-centromeric piRNA clusters (*cluster 20A*, *cluster 38C*, *cluster 80E*) with predominant or exclusive piRNA processing in the germline (Lau *et al*, 2009; Malone *et al*, 2009) showed similar piRNA levels across all libraries (Figure 7B). In contrast, piRNAs

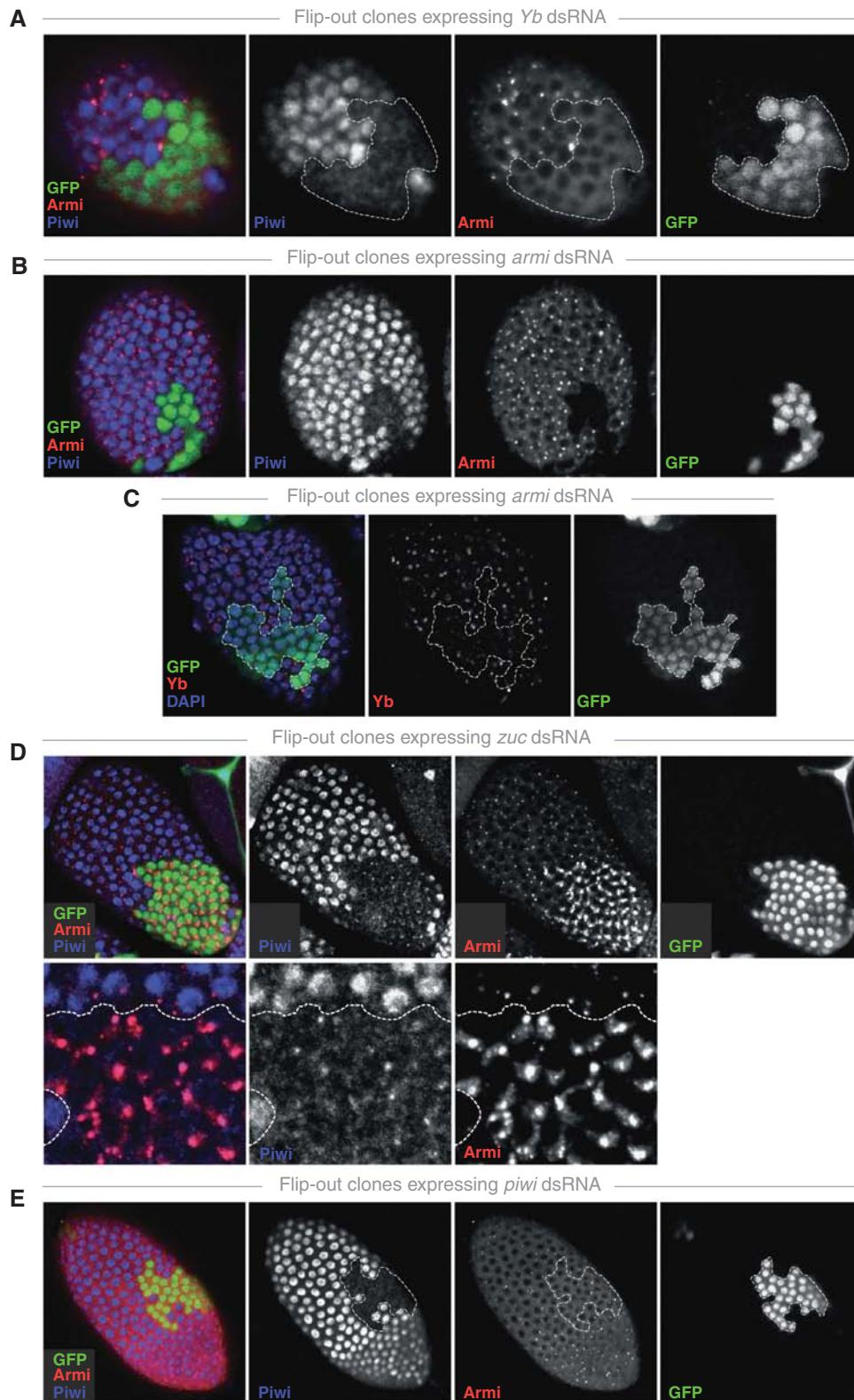


Figure 5 Comprehensive analysis of Armi, Piwi and Yb localization upon their reciprocal knockdown. (A–E) Shown are surface views of the follicular epithelium of egg chambers containing clones expressing dsRNA hairpins against the indicated genes stained for Piwi (blue), Armi (red) and Yb (red). Clones are marked by the presence of GFP (green) and clone borders are indicated (dashed line). In all rows, the merged RGB image is shown alongside with the individual channels in black and white. In (D), the lower row shows higher magnification views.

uniquely mapping to the *flamenco* locus, a piRNA cluster that is exclusively or predominantly processed into piRNAs in somatic support cells, were 6- to 10-fold reduced in *piwi*, *armi*, *zuc* and *fs(1)Yb* knockdown ovaries. The *tj*-3'UTR is another source for

abundant piRNAs in somatic support cells (Robine *et al*, 2009; Saito *et al*, 2009). Levels of *tj*-derived piRNAs also decreased 6- to 10-fold in all libraries in comparison with the control (Figure 7B). Figure 7C depicts the normalized piRNA profiles

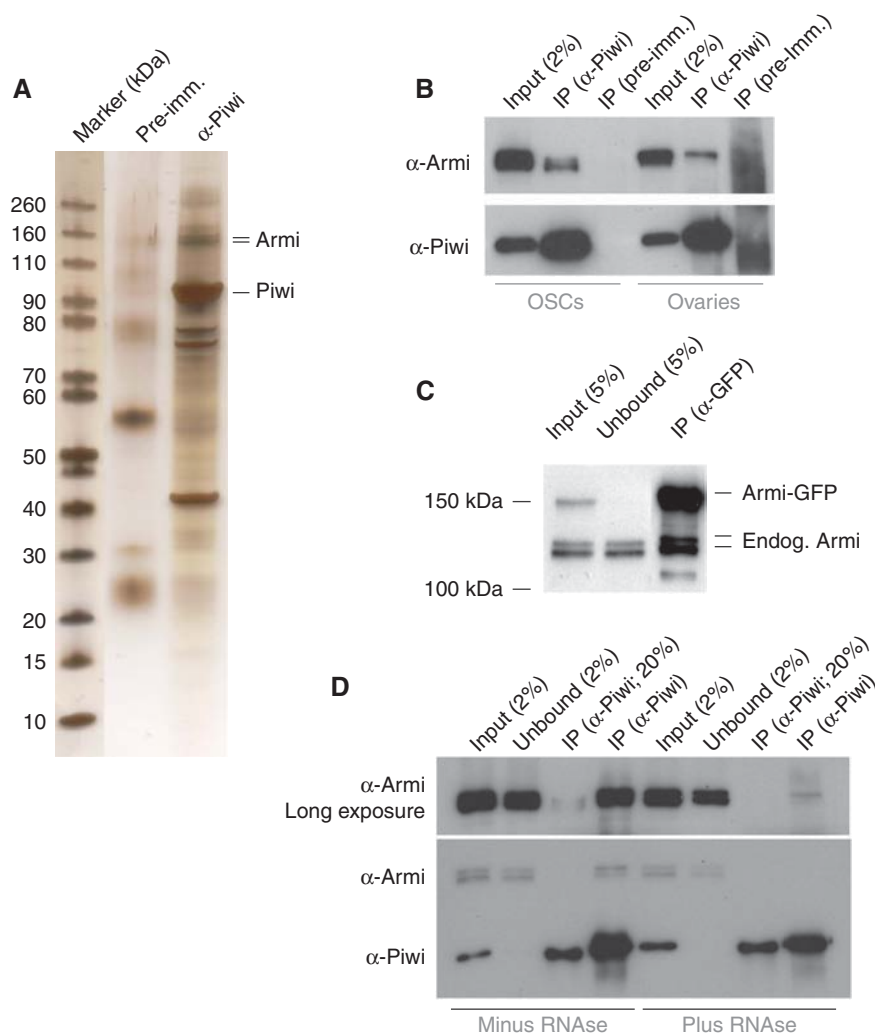


Figure 6 Piwi and Armitage interact physically. **(A)** SDS-PAGE of a Piwi immuno-precipitation (IP) alongside a control IP from OSC lysate stained with silver. A molecular weight marker is shown to the left and Piwi and Armi bands are indicated. **(B)** Western blot showing co-IP of endogenous Armi with Piwi from OSCs and ovaries. Pre-immune serum was used for the control IP. **(C)** Western blot showing co-IP of endogenous Armi with GFP-tagged Armi from ovary lysate. **(D)** Western blot showing co-IP of endogenous Armi with Piwi in the presence or absence of RNase.

(sense and antisense) originating from *cluster 42AB*, the *flamenco* cluster and the *tj* locus.

We further analysed piRNA levels mapping to transposons, which have been classified as germline dominant (*HeT-A*, *F-element*, *TART*, *GATE*) or soma dominant (*gypsy5*, *ZAM*, *Tabor*, *412*, *Idefix*) (Li *et al*, 2009; Malone *et al*, 2009). Using the same normalization from before (*cluster 42AB*), piRNA levels for the germline-dominant elements were essentially unchanged in all libraries (Supplementary Figure S9A). In contrast, piRNAs mapping to the soma-dominant elements were strongly reduced in number and those remaining had a higher proportion of sense-derived piRNAs, which in wild-type ovaries make up <5% of all *ZAM*- and *Tabor*-specific piRNAs (Supplementary Figure S9A,B). We speculate that the de-silencing of the *ZAM* and *Tabor* elements leads to disproportionate piRNA processing directly from their active sense transcripts.

Our combined results show that primary piRNA biogenesis or loading into Piwi is dependent on Armi, Yb and Zuc in ovarian somatic support cells.

Similarities between somatic and germline Piwi pathways

It has been suggested that Piwi biology in soma and germline of the *Drosophila* ovary differs (Malone *et al*, 2009). This was based, however, on *armi* alleles, which based on our data are germline specific. It has also been reported that *zuc* null mutants exhibit no defects in Piwi-nuclear accumulation (Malone *et al*, 2009). This prompted us to re-investigate the similarities of the somatic and the germline Piwi pathways using null mutants for *armi*, *piwi*, *fs(1)Yb* and *zuc*.

Other than previously reported, *zuc* mutant ovaries exhibited a severe loss of Piwi from the nucleus in somatic and germline cells of the ovary (Figure 8A and B). While Piwi protein accumulated in Yb bodies in follicle cells, it appeared to concentrate in clouds at the nuclear periphery of germline cells (asterisks in Figure 8B). These clouds were also prominently stained for Armi (Figure 8B, right). To test whether this could represent disintegrating nuage, we stained *zuc* mutant ovarioles for Aub and AGO3, two proteins localizing to nuage. No obvious defects in nuage pattern were detect-

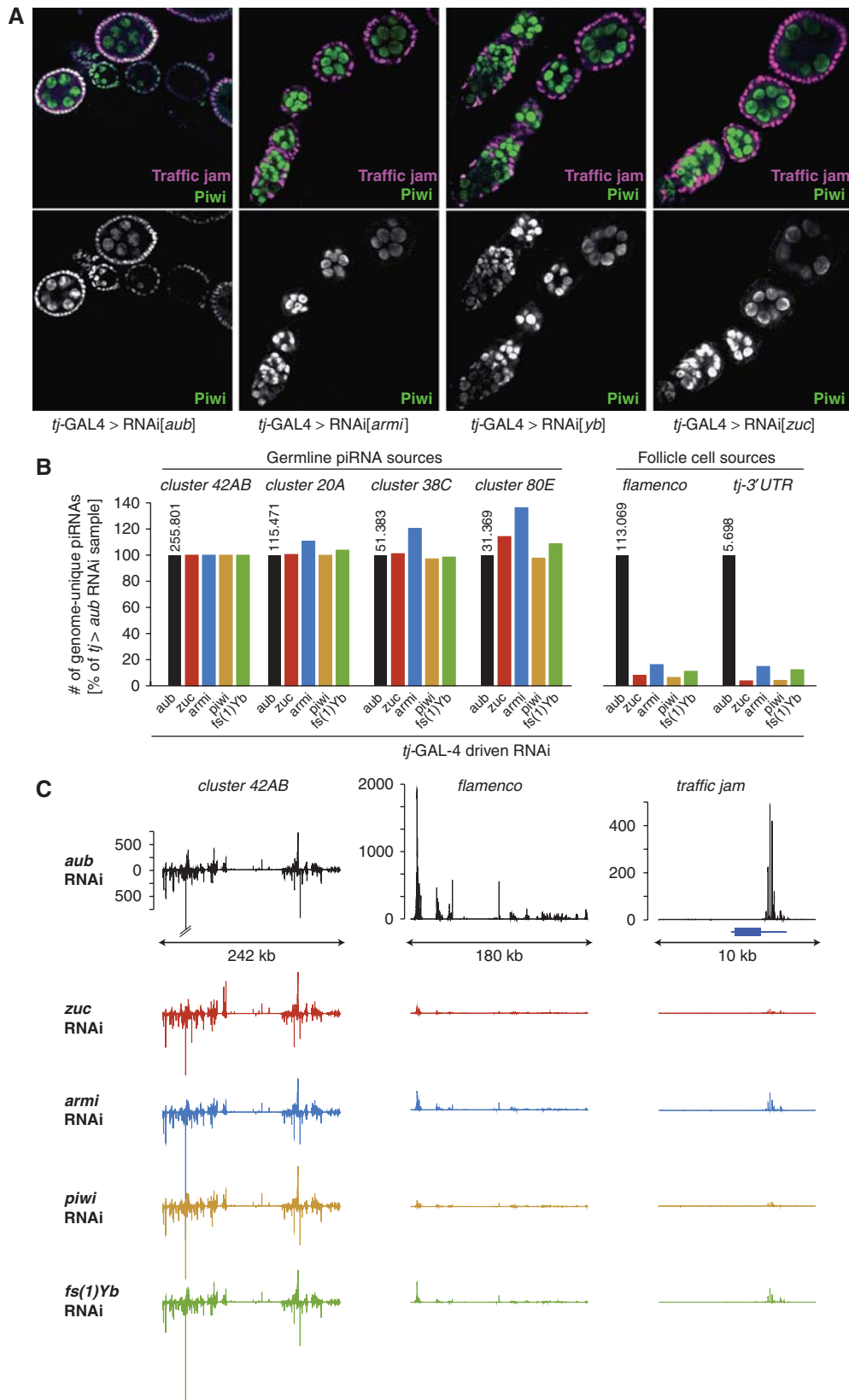


Figure 7 piRNA biogenesis defects in somatic cells lacking Armi, Piwi, Zuc and Yb. (A) Shown are ovarioles stained for Piwi (green) and Traffic jam (magenta) from flies expressing the indicated dsRNAs with *tj-GAL4*. Note the lack of nuclear Piwi in follicle cells that are marked by Tj (*aub* knockdown serves as negative control). (B) Shown are relative levels of genome-unique Piwi-piRNAs mapping to the indicated piRNA clusters in ovaries of the indicated genotypes. Libraries were normalized through the genome-unique mappers to 42AB, a germline-specific piRNA cluster. The *tj-GAL4 > aub*-RNAi sample serves as control. The respective raw read numbers from the *tj > aub*-RNAi library are indicated above the black bars. (C) Detailed piRNA profiles (only genome-unique piRNAs are shown) across the piRNA clusters 42AB and *flamenco* as well as the major genic piRNA precursor *tj*. The colour code is as in (B) and the annotated *tj* gene is indicated in blue. The y axis indicates the number of sequences per 200 nt window normalized to 1 million sequenced reads and graphs within a vertical row are shown at the same scale.

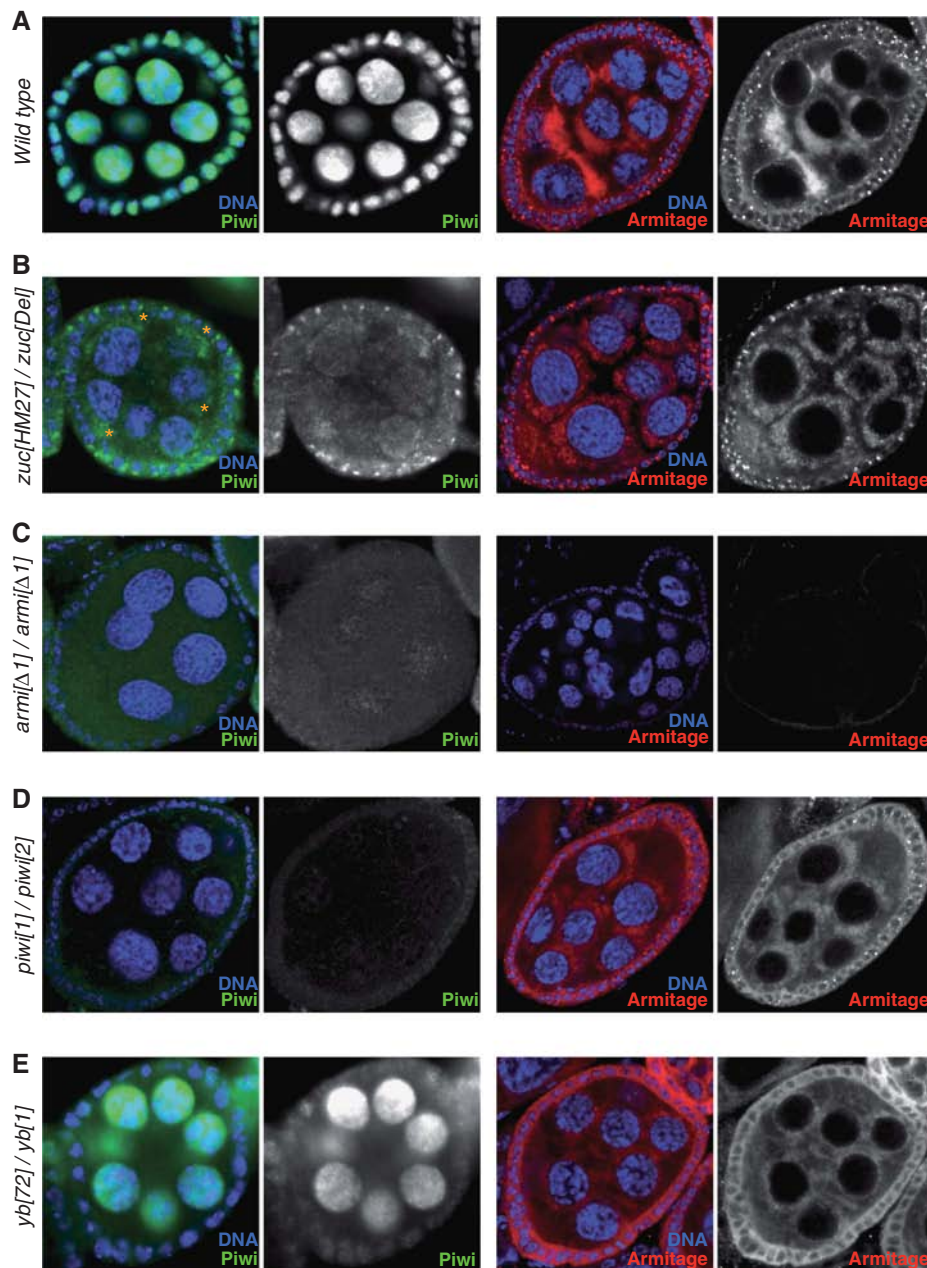


Figure 8 Similarities of Piwi biology in soma and germline of the ovary. (A–E) Shown are optical sections through mid stage egg chambers of indicated genotypes (left) stained for DNA (blue), Piwi (green) and Armi (red). Peri-nuclear accumulation of Piwi in germline cells of *zuc* mutant ovaries is marked by asterisks in (B).

able based on Aub localization, but we observed frequent accumulation of AGO3 in cytoplasmic foci (Supplementary Figure S10). Loss of *Zuc* thus leads to a significant co-localization of Piwi and Armi in soma (Yb bodies) and germline (peri-nuclear clouds, distinct from nuage). As expected, loss of Armi de-localized Piwi from the nucleus in germline and soma (Figure 8C). No changes in Armi localization either to Yb bodies in somatic cells or peri-nuclear clouds in germline cells were seen in ovaries lacking Piwi (Figure 8D). These results suggest that Piwi biology is similar in somatic and germline cells of the ovary.

The only protein that acts differentially in the two cell types is Yb. Ovaries mutant for Yb lost nuclear Piwi accumulation in somatic but not in germline cells (Figure 8E).

Similarly, although somatic Armi localization to Yb bodies was lost, the pattern of Armi in germline cells was wild type (Figure 8E). This is consistent with Yb being specifically expressed in somatic cells of the ovary only (Szakmary *et al*, 2009), and raises a question to as whether a redundant protein might serve Yb's function in germline cells (see Discussion).

Our immuno-fluorescence analysis indicated that Piwi levels were reduced in cells mutant for *armi*, *zuc* or *fs(1)Yb*. To test this, we performed western blot analysis using ovary extracts obtained from null mutants for each respective gene (Supplementary Figure S11). This indicated indeed strongly reduced Piwi levels in *armi* and *zuc* mutants, whereas *fs(1)Yb* mutants were less affected, consistent with

it being only important for Piwi biology in the somatic support cells. No changes in Armis levels were observed in *piwi*, *zuc* or *fs(1)Yb* mutants (not shown). We conclude that failure to load Piwi with piRNAs leads to Piwi destabilization and failure in nuclear accumulation.

Discussion

In this study, we present genetic, cell biological and molecular data that shed light on primary piRNA biogenesis in *Drosophila*.

We describe a robust *in vivo* assay that allows the identification of genes with a critical involvement in the somatic piRNA pathway. It takes advantage of the recently constructed genome-wide RNAi library at the VDRC and, therefore, will allow the conduction of a genome-wide RNAi screen. The somatic support cells of the *Drosophila* ovary are the only described cells containing a piRNA pathway without ping-pong cycle. Elucidation of this pathway will, therefore, not only uncover the basic concepts behind the piRNA biogenesis and silencing machineries. It will also simplify the genetic and functional characterization of identified candidates, aided by the availability of the OSC culture system (Niki *et al*, 2006). Similar to any screen system, our assay has drawbacks such as availability of an RNAi line, potential off-target effects or inefficient knockdown. However, an RNAi screen has the major advantage that knockdown of genes essential for other vital functions in the cell might still allow the development of ovaries suitable for analysis, whereas EMS generated null alleles would often prevent this.

We used this assay to assign essential functions to the piRNA pathway proteins Armis and Zuc within the somatic pathway and identified Yb as a novel piRNA pathway gene. Detailed cell biological analyses in conjunction with protein interaction studies and piRNA sequencing efforts place all three factors upstream of the active Piwi-piRNA complex, either in piRNA biogenesis or in piRNA loading into Piwi. All three proteins are required for piRNA accumulation and for the nuclear accumulation of Piwi. The cytoplasmic localization of Armis, Yb and Zuc suggests that piRNA biogenesis/loading occurs in the cytoplasm. This is supported by the observation that N-terminally truncated Piwi that cannot translocate into the nucleus is still loaded with piRNAs (Saito *et al*, 2009). In addition, piRNAs derived from cellular mRNAs typically map to the 3'UTR (Robine *et al*, 2009), indicating that ribosomal association with those mRNAs did precede their funnelling into piRNA biogenesis.

Our data indicate that unloaded Piwi does not translocate into the nucleus. This is highly reminiscent to the situation in mouse and *Tetrahymena*. The mouse PIWI family protein MIWI2 as well as the *Tetrahymena* Twi1 protein accumulate in the cytoplasm under conditions that do not allow piRNA biogenesis or loading into these factors (Aravin *et al*, 2008; Noto *et al*, 2010; Zheng *et al*, 2010). Furthermore, unloaded Piwi appears to be unstable *in vivo*. This effect is most apparent in ovaries lacking Armis and Zuc, two factors that are essential for Piwi-piRNA biogenesis/loading in soma and germline.

Our results further indicate that aspects of piRNA biogenesis and/or piRNA loading take place in discrete peri-nuclear foci that have been previously termed Yb bodies (Szakmary

et al, 2009). The two RNA helicases Armis and Yb localize both to Yb bodies and interact physically. Both proteins are required for their reciprocal localization to cytoplasmic foci and potentially for Yb-body formation *per se*. A strong argument for the importance of Yb bodies in the piRNA pathway stems from the observation that *fs(1)Yb[1]* mutant ovaries also show Piwi loss from the nucleus (not shown). In this allele, a conserved Arginine residue in the Tudor domain is mutated. This still allows Yb protein expression, but blocks Yb-body formation (Szakmary *et al*, 2009). Ultra-structurally, Yb bodies were described as electron dense cytoplasmic spheres that are directly adjacent to an RNA containing electron dense structure (Szakmary *et al*, 2009). Our localization studies with DCP1 indicate that these previously identified neighbouring structures are likely a subset of cellular P bodies. The functional significance of this is unclear, as we do not detect any defects in gypsy silencing upon knockdown of two central P-body components LSM1 and Me31b, which have been reported to lead to a dispersal of P bodies in *Drosophila* S2 cells (Eulalio *et al*, 2007b). Nevertheless, the physical neighbourhood of these two bodies is intriguing and is reminiscent of the neighbouring and/or coinciding localization of a set of piRNA pathway proteins and P-body components in mouse germ cells and *Drosophila* nurse cells (Aravin *et al*, 2009; Lim *et al*, 2009).

Of the three identified proteins, Yb is the only factor that seems specific for the Piwi pathway in somatic support cells. This is consistent with genetic analyses showing that germline-specific mutants for *fs(1)Yb* are fertile (King *et al*, 2001). The function of Yb in the somatic support cells is required for maintenance of somatic and indirectly also for germline stem cells (King *et al*, 2001). In this, *fs(1)Yb* mutants strongly resemble *piwi* mutants and indicate once again that the Piwi-dependent piRNA pathway in the somatic support cells is essential for germline stem cell niche maintenance. The specificity of Yb for the somatic cells raises the question, whether there is a related protein with equivalent functions in germline cells. Protein BLAST analysis indeed identified two proteins (CG31755 and CG11133) with similarity to Yb over most of their sequence (Szakmary *et al*, 2009). Similar to Yb, CG31755 and CG11133 also contain a recognizable Tudor domain downstream of their helicase domain. Both proteins are selectively expressed in ovaries and testes (FlyAtlas). These observations indicate that CG31755 and CG11133 could serve Yb's function within the germline. We note that knockdown of these proteins individually does not impact the somatic piRNA pathway (not shown). CG31755 and CG11133 share considerable similarity to mouse TDRD12, a gene with testis and oocyte-specific expression.

The function of Armis as an essential factor in primary piRNA biogenesis is conserved in mouse (Frost *et al*, 2010; Zheng *et al*, 2010). In the absence of the germline-specific orthologue MOV10L1, spermatogenesis is blocked at a similar stage as in MILI mutants and MILI and MIWI2, which is thought to function downstream of MILI in the mouse ping-pong cycle are lacking bound piRNAs. In *Drosophila*, Armis is *per se* not required for the ping-pong cycle (Malone *et al*, 2009), indicating that the function of this RNA helicase is restricted to primary piRNA processing or Piwi loading. Two Armis isoforms are expressed in ovaries with germline cells expressing only the larger one. Although this might suggest functional specialization, we note that the two isoforms show

an identical distribution in a glycerol gradient, interact both with Piwi and show identical subcellular localization in follicle cells based on GFP fusions (data not shown).

Similar to *Armi*, *Zuc* is well conserved in vertebrates. The mouse orthologue *PLD6* is expressed specifically in testes (<http://www.biogps.gnf.org>) consistent with a function in the piRNA pathway. *Zuc* contains a single phospho-lipase D domain, which is predicted to confer endo-nucleolytic activity (Zhao *et al*, 1997). The three important residues for catalysis are conserved in *Zuc* and a point mutation in the active site (*zuc[SG]*; Pane *et al*, 2007) causes a similar delocalization of Piwi from the nucleus as the null mutant (not shown). Our data indicate that *Zuc* function (potentially endo-nucleolytic generation of piRNA 5' or 3' ends) is required to release Piwi and *Armi* from Yb bodies. We further show that *Zuc* is essential for Piwi-piRNA biogenesis. This casts doubts on the rather weak effects of *zuc* mutations on *flamenco* piRNA biogenesis reported previously (Malone *et al*, 2009). Genotyping of *trans*-heterozygous *zuc* mutants (*zuc[HM27]/zuc[Def]*) from stocks balanced over *CyO* is complicated by a dominant wing phenotype of the *zuc[Def]* allele interfering with confident selection against the *CyO* balancer. We suspect that this led to a mix of homozygous and heterozygous ovaries analysed in that study. It is unclear, however, how the defects in Piwi localization went unnoticed in such a situation.

The phenotype of *armi* and *zuc* mutant ovaries indicates that Piwi biology is similar in germline and somatic support cells. We note that the analysis of piRNAs bound by Piwi and AGO3 also showed a participation of Piwi in the germline-specific ping-pong cycle (Brennecke *et al*, 2007; Li *et al*, 2009). However, in *armi* germline mutants, the ping-pong cycle is still active, yet Piwi is not loaded effectively (Malone *et al*, 2009). This suggests that primary piRNA biogenesis through *Zuc/Armi* is the major pathway feeding into Piwi in germline cells.

In summary, we identified the RNA helicases *Armi* and *Yb* and the predicted nuclease *Zuc* as essential components of primary piRNA biogenesis. We further link peri-nuclear Yb bodies to piRNA biogenesis and show that Piwi biology in germline and soma follows a common logic. The described genetic assay system and the availability of the cultured OSC line will be important resources to work towards a mechanistic understanding of piRNA biogenesis and the silencing mechanism, two of the most mysterious open questions in the small RNA field.

Materials and methods

Drosophila stocks

All experiments were performed at 25°C. The used fly strains were *tj-GAL4* (DGRC stock 104055); *flam[restrictive]*; ; *gypsy-lacZ* (Sarot *et al*, 2004); *attP2-UAST-Luciferase* (Markstein *et al*, 2008); RNAi lines were from the Vienna *Drosophila* RNAi Centre: *armi* (16205GD; 103589KK), *zuc* (48764GD; 110430KK), *piwi* (22235GD; 101658KK), *fs(1)Yb* (25435GD; 110056KK), *tejas* (103264KK); *Notch* (100002KK); no RNAi lines are available at the VDRC for *krimp* and *AGO3*. YFP-Dcp1 (Lin *et al*, 2006); *armi[1]/TM3* and *armi[72.1]/TM3* (Cook *et al*, 2004); *zuc[HM27]/CyO* and *Df(2L)PRL/CyO* (Pane *et al*, 2007); *piwi[1]/CyO* and *piwi[2]/CyO* (Lin and Spradling, 1997); *fs(1)Yb[72]/FM6* and *fs(1)Yb[1]/FM6* (Swan *et al*, 2001).

armi[Δ1] is an FRT-mediated deletion based on DGRC stocks e001160 and d07385; it deletes the entire *armi*, *CycJ* and *CG14971* genes;

Armi-GFP flies carry a genomic rescue construct with an EGFP cassette inserted at the C-terminus by bacterial recombineering; *act-GAL4* flip-out clones were generated using *y,w,hsflp¹²²*; *act<CD2>GAL4,UAS-GFP*; newly enclosed females were heat shocked 1 h at 37°C and dissected 4–5 days later; FRT-based mitotic clones were based on *hsflp¹²²,FRT19A,ubi-GFP* and *hsflp¹²²,FRT40A* armadillo-lacZ; clones were induced by heat-shocking freshly enclosed females on two consecutive days for 1 h at 37°C; flies were dissected 5 days later.

Luciferase assay

Three female flies, carcasses or pairs of ovaries of the genotype [*tj-GAL4/+*; *attP2-UAST-Luciferase/+*] and three females of the genotype [*attP2-UAST-Luciferase*] were homogenized in 100 μl of Glo Lysis Buffer (Promega). A total of 20 μl lysate were mixed 1:1 with Steady-Glo Assay Reagent (Promega). Luminescence was measured with a Synergy Plate Reader after 10 min with a sensitivity of 160 and an integration time of 1 s (results were the average of three experiments).

β-Gal stainings

Ovaries from 2- to 3-day-old flies were dissected in PBS, kept on ice (maximum 30 min), fixed in 0.5% glutaraldehyde/PBS at room temperature (RT; 20 min) and rinsed with PBS. The chromogen reaction was run in staining solution (10 mM PBS, 1 mM MgCl₂, 150 mM NaCl, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 0.1% Triton, 0.1% X-Gal) for 2 h at RT.

Immuno-cytochemistry

Ovaries were dissected in PBS on ice, fixed with 4% paraformaldehyde/PBS with 0.2% Triton X-100 at RT for 20 min and rinsed three times with PBT (PBS with 0.2% Triton X-100). After blocking in BBT (PBT with 0.1% BSA) for 2 h at RT, the ovaries were incubated with primary antibodies in BBT overnight at 4°C. After three PBT washes, secondary antibodies (1:500; Molecular Probes) were incubated 3 h at RT.

OSCs were plated on Concanavalin-A-coated coverslips, fixed with 4% paraformaldehyde in PBS (RT, 20 min), permeabilized with 0.1% Triton X-100 in PBS (10 min) and blocked with 1% BSA in PBS.

Primary antibodies used were α-Tj (1:500, rat; Li *et al*, 2003), α-Piwi (1:1000, rabbit), α-Piwi (1:1000, mouse; Saito *et al*, 2006), α-Armi (1:1000, rabbit; Cook *et al*, 2004), α-β-Gal (1:50, mouse 40-1a from Developmental Studies Hybridoma Bank), α-Yb (1:1000, rabbit; Szakmary *et al*, 2009), α-gypsy-Env (1:1000, rabbit).

5' RACE

Total RNA from OSCs was used for the 5' RACE determination of *armi* mRNAs (Invitrogen). Primers used were TCCCAGGAATGCT CAAGG (reverse transcription) and CACATCCGACGTGTAGATCCA AG (PCR).

Cell culture

OSCs were cultured as described (Niki *et al*, 2006). OSCs were transfected with the Cell Line Nucleofector kit V (Amaya Biosystems), selecting the programme T-029 (K Saito, personal communication).

Piwi-IP MS analysis

OSCs were harvested by trypsinization and lysed in two volumes of lysis buffer (10 mM Hepes pH7, 150 mM NaCl, 5 mM MgCl₂ 10% glycerol, 1% Triton X-100, complete protease inhibitors (Roche), 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF), followed by manual disruption using a Wheaton dounce homogenizer and a 10 min centrifugation step at 4 degrees. The pellet was re-extracted twice and supernatants were pooled. A total of 3 mg of total protein were incubated (2 h, 4°C) with peptide purified Piwi antibodies (cross-linked to Dynabeads ProteinG (Invitrogen) using a standard dimethyl pimelimidate cross-linking protocol). Pre-immune serum from the same rabbit was used as control. Beads were washed five times with low detergent wash buffer (10 mM Hepes pH7, 150 mM NaCl, 5 mM MgCl₂ 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF), five times with wash buffer (Hepes pH7, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA) and once

with 150 mM NaCl. The beads were boiled with SDS sample buffer and analysed by one-dimensional gel electrophoresis and silver staining.

Indicated bands were excised from the gel, processed by a standard in-gel tryptic digest protocol and further analysed by Tandem Mass Spectrometry. Data analysis was with Mascot 2.2.04.

Co-immuno-precipitation

act>3xFLAG-Yb and act>3xFLAG-Piwi constructs were based on the *Drosophila* Gateway collection (<http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html>) and were transfected in OSCs. Non-transfected OSCs were used as negative control. Per sample, a 10 cm plate of OSCs was harvested 4 days after transfection and lysed in 200 µl lysis buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P40). Lysates were cleared for 10 min at 16 000 g at 4°C and the supernatant incubated for 4 h at 4°C with 7 µg of α-FLAG antibody (SIGMA M2) cross-linked to 30 µl of Invitrogen ProteinG Dynabeads. Beads were washed six times for 10 min with washing buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5 mM EDTA) and eluted by boiling in 2 × western blot sample buffer.

Small RNA cloning and analysis

Ovaries were dissected in PBS (on ice). Lysate from ~100 µl ovaries was obtained by extracting them three times in lysis buffer (10 mM Hepes pH7, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1% Triton X-100, complete protease inhibitors (Roche), 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF). Piwi was immuno-precipitated using polyclonal rabbit antiserum and small RNAs were extracted with phenol chloroform. Small RNA libraries were generated as previously described (Brennecke *et al*, 2007) and sequenced using the Illumina G2 platform. Only sequences matching the *Drosophila* genome (release 5; excluding Uextra) 100% were retained. Libraries were normalized through their unique mappers to the 42AB piRNA cluster to allow for cross-comparisons.

Transposon QPCR analysis

First strand cDNA was obtained by using random primers on Trizol extracted total ovarian RNA from 2- to 3-day-old flies. Quantitative PCR was performed using BioRad IQ SYBR Green Super Mix. Steady-state RNA levels were calculated from the threshold cycle for amplification using the 2^{-ΔΔC_T} method (Livak and Schmittgen, 2001). Fold enrichments are in comparison with a *tj*-GAL4/+ control sample. Throughout rp49 was used for the normalization

and comparable results were obtained by using actin5C for normalization. Average levels and standard deviations were calculated from three biological replicates according to Livak and Schmittgen (2001). Control experiments with template dilution indicated that the efficiencies of target amplification and control genes (rp49 and actin5C) were comparable.

Primers for QPCR analysis were

rp49_for: CCGCTTCAAGGGACAGTATCTG
rp49_rev: ATCTCGCGCAGTAAACGC
actin5C_for: AAGTTGCTGCTCTGGTGTCTG
actin5C_rev: GCCACACGCAGCTCATTGTAG
ZAM_for: CTACGAAATGGCAAGATTAATTCCACTTCC
ZAM_rev: CCGTTTCCTTTATGTCGCAGTAGCT
Tabor_for: ACGTTGTTTCACGACATTAGCCG
Tabor_rev: GGGTTGTTTCGGATCTGACG
HeT-A_for: CGCGCGGAACCCATCTTCAGA
HeT-A_rev: CGCCGCAGTCGTTTGGTGAT
TART_for: TTTTCCGGATCCAAGTGAAC
TART_rev: TCTGTCGTCGGAAGTTGTT

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Anne J, Ollo R, Ephrussi A, Mechler BM (2007) Arginine methyltransferase Capsuleen is essential for methylation of spliceosomal Sm proteins and germ cell formation in *Drosophila*. *Development* **134**: 137–146
- Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T (2003) The small RNA profile during *Drosophila melanogaster* development. *Dev Cell* **5**: 337–350
- Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Gvozdev VA (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Curr Biol* **11**: 1017–1027
- Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ (2008) A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. *Mol Cell* **31**: 785–799
- Aravin AA, van der Heijden GW, Castaneda J, Vagin VV, Hannon GJ, Bortvin A (2009) Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet* **5**: e1000764
- Bergman CM, Quesneville H, Anxolabehere D, Ashburner M (2006) Recurrent insertion and duplication generate networks of transposable element sequences in the *Drosophila melanogaster* genome. *Genome Biol* **7**: R112
- Boswell RE, Mahowald AP (1985) tudor, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. *Cell* **43**: 97–104
- Braslet E, Taddei AR, Arnaud F, Faye B, Fausto AM, Mazzini M, Giorgi F, Vaury C (2006) Viral particles of the endogenous retrovirus ZAM from *Drosophila melanogaster* use a pre-existing endosome/exosome pathway for transfer to the oocyte. *Retrovirology* **3**: 25
- Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* **128**: 1089–1103
- Bucheton A, Paro R, Sang HM, Pelisson A, Finnegan DJ (1984) The molecular basis of I-R hybrid dysgenesis in *Drosophila melanogaster*: identification, cloning, and properties of the I factor. *Cell* **38**: 153–163
- Carmell MA, Girard A, van de Kant H, de Rooij D, Bestor T, Hannon GJ (2007) Miwi2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev Cell* **12**: 503–514
- Chalvet F, Teyssset L, Terzian C, Prud'homme N, Santamaria P, Bucheton A, Pelisson A (1999) Proviral amplification of the Gypsy endogenous retrovirus of *Drosophila melanogaster* involves env-independent invasion of the female germline. *EMBO J* **18**: 2659–2669
- Chen Y, Pane A, Schupbach T (2007) Cutoff and aubergine mutations result in retrotransposon upregulation and checkpoint activation in *Drosophila*. *Curr Biol* **17**: 637–642
- Clegg NJ, Frost DM, Larkin MK, Subrahmanyam L, Bryant Z, Ruohola-Baker H (1997) maelstrom is required for an early step in the establishment of *Drosophila* oocyte polarity: posterior localization of grk mRNA. *Development* **124**: 4661–4671
- Cook HA, Koppetsch BS, Wu J, Theurkauf WE (2004) The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* **116**: 817–829

- Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev* **12**: 3715–3727
- Cox DN, Chao A, Lin H (2000) piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**: 503–514
- Deng WM, Althausen C, Ruohola-Baker H (2001) Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells. *Development* **128**: 4737–4746
- Desset S, Meignin C, Dastugue B, Vaury C (2003) COM, a heterochromatic locus governing the control of independent endogenous retroviruses from *Drosophila melanogaster*. *Genetics* **164**: 501–509
- Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblaue S, Couto A, Marra V, Keleman K, Dickson BJ (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* **448**: 151–156
- Eulalio A, Behm-Ansmant I, Izaurralde E (2007a) P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol* **8**: 9–22
- Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E (2007b) P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol* **27**: 3970–3981
- Frost RJ, Hamra FK, Richardson JA, Qi X, Bassel-Duby R, Olson EN (2010) MOV10L1 is necessary for protection of spermatocytes against retrotransposons by Piwi-interacting RNAs. *Proc Natl Acad Sci USA* **107**: 11847–11852
- Gillespie DE, Berg CA (1995) Homeless is required for RNA localization in *Drosophila* oogenesis and encodes a new member of the DE-H family of RNA-dependent ATPases. *Genes Dev* **9**: 2495–2508
- Girard A, Hannon GJ (2008) Conserved themes in small-RNA-mediated transposon control. *Trends Cell Biol* **18**: 136–148
- Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi MC (2007) A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. *Science* **315**: 1587–1590
- Harris AN, Macdonald PM (2001) Aubergine encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. *Development* **128**: 2823–2832
- Horwich MD, Li C, Matrangola C, Vagin V, Farley G, Wang P, Zamore PD (2007) The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr Biol* **17**: 1265–1272
- Jensen S, Gassama MP, Dramard X, Heidmann T (2002) Regulation of I-transposon activity in *Drosophila*: evidence for cosuppression of nonhomologous transgenes and possible role of ancestral I-related pericentromeric elements. *Genetics* **162**: 1197–1209
- King FJ, Lin H (1999) Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during *Drosophila* oogenesis. *Development* **126**: 1833–1844
- King FJ, Szakmary A, Cox DN, Lin H (2001) Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the *Drosophila* ovary. *Mol Cell* **7**: 497–508
- Kirino Y, Kim N, de Planell-Saguer M, Khandros E, Chiorean S, Klein PS, Rigoutsos I, Jongens TA, Mourelatos Z (2009) Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. *Nat Cell Biol* **11**: 652–658
- Klattenhoff C, Theurkauf W (2008) Biogenesis and germline functions of piRNAs. *Development* **135**: 3–9
- Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, Zhang F, Schultz N, Koppetsch BS, Nowosielska A, Seitz H, Zamore PD, Weng Z, Theurkauf WE (2009) The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. *Cell* **138**: 1137–1149
- Klenov MS, Lavrov SA, Stolyarenko AD, Ryazansky SS, Aravin AA, Tuschl T, Gvozdev VA (2007) Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the *Drosophila melanogaster* germline. *Nucleic Acids Res* **35**: 5430–5438
- Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri TW, Hata K, Li E, Matsuda Y, Kimura T, Okabe M, Sakaki Y, Sasaki H, Nakano T (2008) DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* **22**: 908–917
- Lau NC, Robine N, Martin R, Chung WJ, Niki Y, Berezikov E, Lai EC (2009) Abundant primary piRNAs, endo-siRNAs, and microRNAs in a *Drosophila* ovary cell line. *Genome Res* **19**: 1776–1785
- Leblanc P, Desset S, Giorgi F, Taddei AR, Fausto AM, Mazzini M, Dastugue B, Vaury C (2000) Life cycle of an endogenous retrovirus, ZAM, in *Drosophila melanogaster*. *J Virol* **74**: 10658–10669
- Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM, Kittler EL, Zapp ML, Klattenhoff C, Schulz N, Theurkauf WE, Weng Z, Zamore PD (2009) Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* **137**: 509–521
- Li MA, Alls JD, Avancini RM, Koo K, Godt D (2003) The large Maf factor Traffic Jam controls gonad morphogenesis in *Drosophila*. *Nat Cell Biol* **5**: 994–1000
- Lim AK, Kai T (2007) Unique germ-line organelle, nuage, functions to repress selfish genetic elements in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **104**: 6714–6719
- Lim AK, Tao L, Kai T (2009) piRNAs mediate posttranscriptional retroelement silencing and localization to pi-bodies in the *Drosophila* germline. *J Cell Biol* **186**: 333–342
- Lin H, Spradling AC (1997) A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124**: 2463–2476
- Lin MD, Fan SJ, Hsu WS, Chou TB (2006) *Drosophila* decapping protein 1, dDcp1, is a component of the oskar mRNP complex and directs its posterior localization in the oocyte. *Dev Cell* **10**: 601–613
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods* **25**: 402–408
- Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, Hannon GJ (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. *Cell* **137**: 522–535
- Malone CD, Hannon GJ (2009) Small RNAs as guardians of the genome. *Cell* **136**: 656–668
- Markstein M, Pitsouli C, Villalta C, Celniker SE, Perrimon N (2008) Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nat Genet* **40**: 476–483
- Niki Y, Yamaguchi T, Mahowald AP (2006) Establishment of stable cell lines of *Drosophila* germ-line stem cells. *Proc Natl Acad Sci USA* **103**: 16325–16330
- Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, Inagaki S, Huang H, Chen D, Kodama T, Siomi H, Siomi MC (2009) Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in *Drosophila* germlines. *EMBO J* **28**: 3820–3831
- Noto T, Kurth HM, Kataoka K, Aronica L, DeSouza LV, Siu KW, Pearlman RE, Gorovsky MA, Mochizuki K (2010) The Tetrahymena argonaute-binding protein Giw1p directs a mature argonaute-siRNA complex to the nucleus. *Cell* **140**: 692–703
- Pane A, Wehr K, Schupbach T (2007) zucchini and squash encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. *Dev Cell* **12**: 851–862
- Patil VS, Kai T (2010) Repression of retroelements in *Drosophila* germline via piRNA pathway by the Tudor Domain Protein Tejas. *Curr Biol* **20**: 724–730
- Pelisson A, Sarot E, Payen-Groschene G, Bucheton A (2007) A novel rasiRNA-mediated silencing pathway downregulates sense gypsy transcripts in the somatic cells of the *drosophila* ovary. *J Virol* **81**: 1951–1960
- Pelisson A, Song SU, Prud'homme N, Smith PA, Bucheton A, Corces VG (1994) Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila* flamenco gene. *EMBO J* **13**: 4401–4411
- Prud'homme N, Gans M, Masson M, Terzian C, Bucheton A (1995) Flamenco, a gene controlling the gypsy retrovirus of *Drosophila melanogaster*. *Genetics* **139**: 697–711
- Robine N, Lau NC, Balla S, Jin Z, Okamura K, Kuramochi-Miyagawa S, Blower MD, Lai EC (2009) A broadly conserved pathway generates 3'UTR-directed primary piRNAs. *Curr Biol* **19**: 2066–2076
- Ronsseay S, Josse T, Boivin A, Anxolabehere D (2003) Telomeric transgenes and trans-silencing in *Drosophila*. *Genetica* **117**: 327–335
- Rubin GM, Kidwell MG, Bingham PM (1982) The molecular basis of P-M hybrid dysgenesis: the nature of induced mutations. *Cell* **29**: 987–994

- Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, Kotani H, Asai K, Siomi H, Siomi MC (2009) A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. *Nature* **461**: 1296–1299
- Saito K, Nishida KM, Mori T, Kawamura Y, Miyoshi K, Nagami T, Siomi H, Siomi MC (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes Dev* **20**: 2214–2222
- Saito K, Sakaguchi Y, Suzuki T, Siomi H, Siomi MC (2007) Pimet, the *Drosophila* homolog of HEN1, mediates 2'-O-methylation of Piwi-interacting RNAs at their 3' ends. *Genes Dev* **21**: 1603–1608
- Sarot E, Payen-Groschene G, Bucheton A, Pelisson A (2004) Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* flamenco gene. *Genetics* **166**: 1313–1321
- Schupbach T, Wieschaus E (1991) Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**: 1119–1136
- Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* **8**: 272–285
- Swan A, Hijal S, Hilfiker A, Suter B (2001) Identification of new X-chromosomal genes required for *Drosophila* oogenesis and novel roles for fs(1)Yb, brainiac and dunce. *Genome Res* **11**: 67–77
- Szakmary A, Reedy M, Qi H, Lin H (2009) The Yb protein defines a novel organelle and regulates male germline stem cell self-renewal in *Drosophila melanogaster*. *J Cell Biol* **185**: 613–627
- Tanentzapf G, Devenport D, Godt D, Brown NH (2007) Integrin-dependent anchoring of a stem-cell niche. *Nat Cell Biol* **9**: 1413–1418
- Vagin VV, Klenov MS, Kalmykova AI, Stolyarenko AD, Kotelnikov RN, Gvozdev VA (2004) The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of *Drosophila melanogaster*. *RNA Biol* **1**: 54–58
- Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**: 320–324
- Vagin VV, Wohlschlegel J, Qu J, Jonsson Z, Huang X, Chuma S, Girard A, Sachidanandam R, Hannon GJ, Aravin AA (2009) Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. *Genes Dev* **23**: 1749–1762
- Zhao Y, Stuckey JA, Lohse DL, Dixon JE (1997) Expression, characterization, and crystallization of a member of the novel phospholipase D family of phosphodiesterases. *Protein Sci* **6**: 2655–2658
- Zheng K, Xiol J, Reuter M, Eckardt S, Leu NA, McLaughlin KJ, Stark A, Sachidanandam R, Pillai RS, Wang PJ (2010) Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. *Proc Natl Acad Sci USA* **107**: 11841–11846
- Zhu X, Stein D (2004) RNAi-mediated inhibition of gene function in the follicle cell layer of the *Drosophila* ovary. *Genesis* **40**: 101–108

3.2 Supplemental information

An *in vivo* RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in *Drosophila*

Daniel Olivieri, Martina M. Sykora, Ravi Sachidanandam, Karl Mechtler and Julius Brennecke

Table S1. Phenotypic Summary of *tj*-GAL4 driven RNAi against a collection of house-keeping genes

CG #	Gene Name	VDRC ID	Library	Phenotype
CG13410	mitochondrial ribosomal protein L35	13443	GD	viable; very small ovaries
CG13410	mitochondrial ribosomal protein L35	103388	KK	viable; very small ovaries
CG1913	α -Tubulin at 84B	33427	GD	viable; no ovaries
CG1913	α -Tubulin at 84B	107109	KK	pharate lethal
CG5353	Threonyl-tRNA synthetase	7752	GD	viable; no ovaries
CG5353	Threonyl-tRNA synthetase	107265	KK	viable; very small ovaries
CG5363	<i>cdc2</i>	41838	GD	viable; no ovaries
CG5363	<i>cdc2</i>	106130	KK	viable; no ovaries
CG6253	Ribosomal protein L14	44629	GD	viable; no ovaries
CG6253	Ribosomal protein L14	102011	KK	viable; no ovaries
CG6349	DNA polymerase α 180kD	11227	GD	viable; no ovaries
CG6349	DNA polymerase α 180kD	103699	KK	viable; no ovaries
CG9075	Eukaryotic initiation factor 4a	42202	GD	viable; no ovaries
CG9075	Eukaryotic initiation factor 4a	100310	KK	viable; no ovaries

Table S2. Content Analysis of small RNA libraries obtained from Piwi-IPs.

A. Statistics for reads

	tj > <i>aub</i> RNAi	tj > <i>piwi</i> RNAi	tj > <i>armi</i> RNAi	tj > <i>zuc</i> RNAi	tj > <i>Yb</i> RNAi
Total reads	9.547.451	7.785.649	5.409.376	9.000.057	13.466.053
% of reads matching the genome 100%	7.471.255	5.499.917	3.998.721	5.005.875	10.933.088
# of reads mapping to rRNA, tRNA, snoRNA	522.429	779.521	210.185	645.291	899.389
# of analyzed reads	6.948.826	4.720.396	3.788.536	4.360.584	10.033.699
repeats	83.0 %	84.4 %	85.1 %	84.2 %	83.3 %
no annotation	9.6 %	9.0 %	9.2 %	9.1 %	9.0 %
exons	5.0 %	4.4 %	3.7 %	4.0 %	5.8 %
introns	1.7 %	1.5 %	1.6 %	1.3 %	1.4 %
Pre-miRNAs	0.6 %	0.6 %	0.3 %	1.2 %	0.4 %

B. Statistics for species

	tj > <i>aub</i> RNAi	tj > <i>piwi</i> RNAi	tj > <i>armi</i> RNAi	tj > <i>zuc</i> RNAi	tj > <i>Yb</i> RNAi
Total species	2.433.473	1.988.666	1.796.123	1.566.130	3.193.174
% of species matching the genome 100%	1.336.025	1.001.251	961.431	647.626	1.907.157
# of species mapping to rRNA, tRNA, snoRNA	28.943	28.163	23.412	18.752	44.430
# of analyzed species	1.307.082	973.088	938.019	628.874	1.862.727
repeats	72.8 %	75.8 %	76.9 %	77.7 %	70.1 %
no annotation	11.2 %	10.5 %	10.9 %	10.0 %	11.2 %
exons	11.4 %	10.4 %	8.6 %	9.4 %	15.2 %
introns	4.6 %	3.3 %	3.6 %	2.8 %	3.5 %
Pre-miRNAs	0.0 %	0.1 %	0.0 %	0.1 %	0.0 %

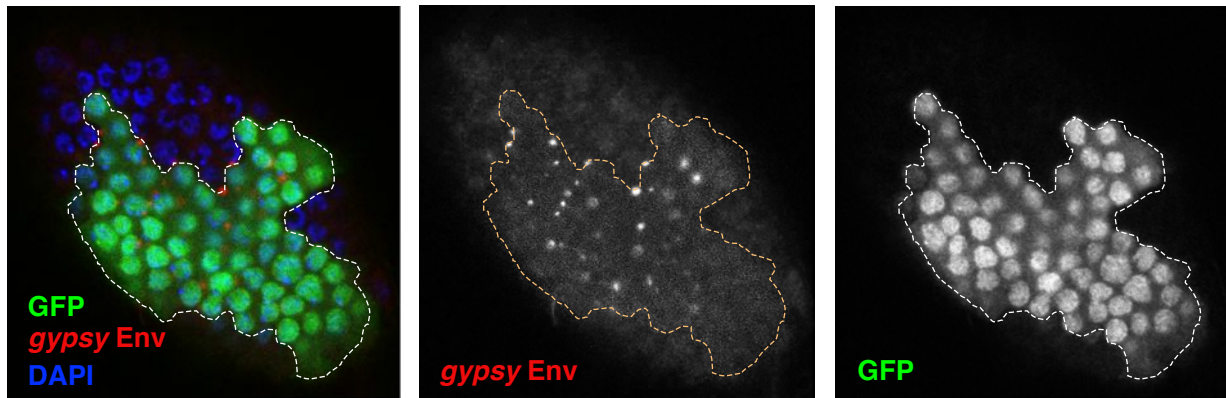


Figure S1. Specific de-silencing of *gypsy* in clones expressing *piwi*-dsRNA.

Shown is a surface view on the follicular epithelium of an egg chamber stained with *gypsy*-Env (red), GFP (green) and DAPI (blue). Cells expressing the dsRNA hairpin against *piwi* are marked with GFP.

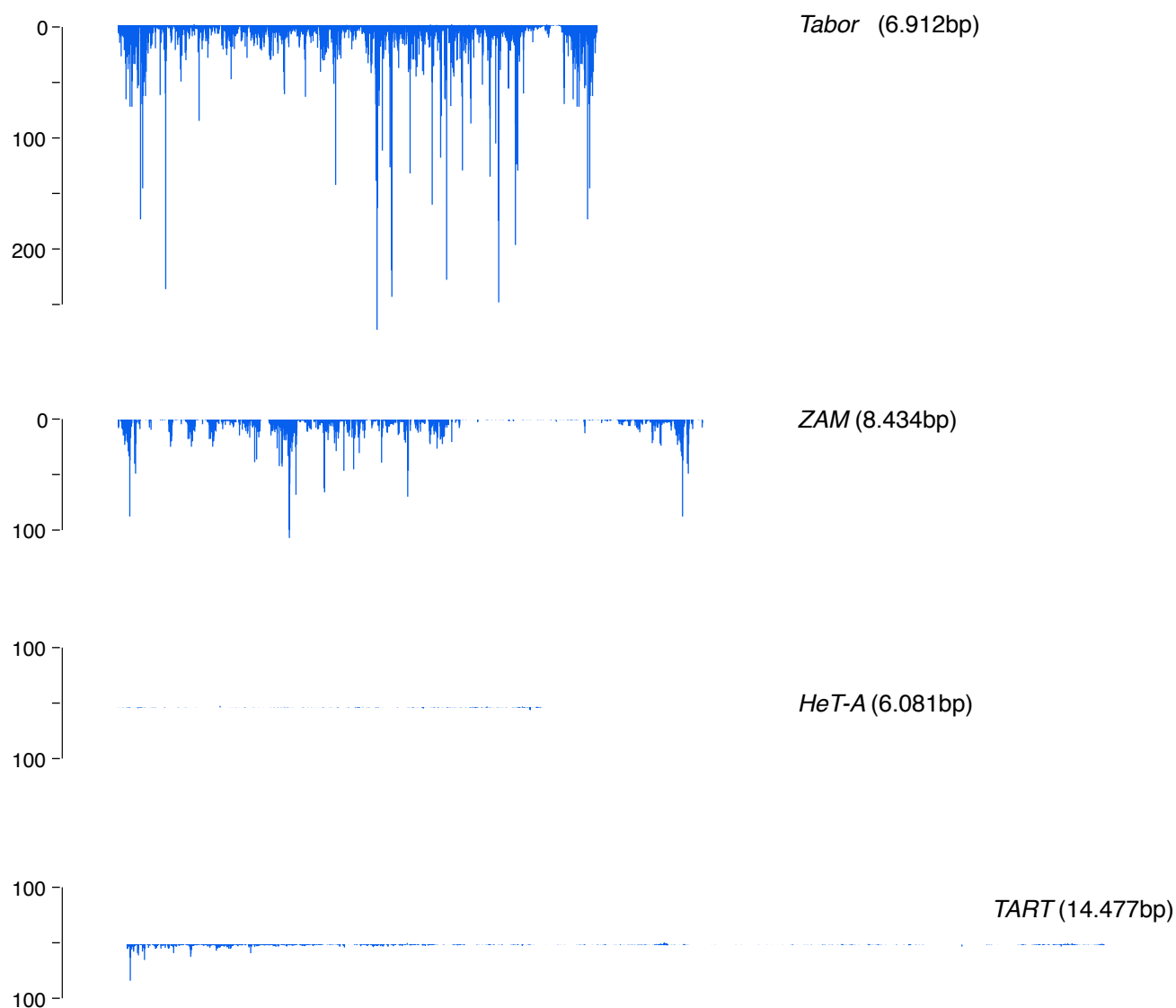
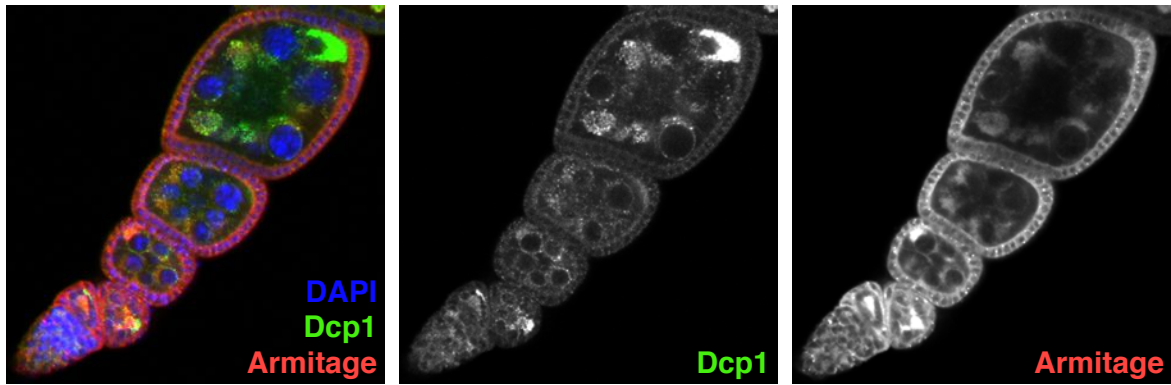


Figure S2. piRNA profiles mapping to *Tabor*, *ZAM*, *HeT-A* and *TART* elements in OSC cells.

Small RNA data from Robine et al. 2009 was used to map OSC derived 23-32nt small RNAs to the indicated retro-elements. Peaks pointing down indicate antisense piRNAs, those pointing up correspond to sense piRNAs. All four plots are shown at the same scale. The profiles have a single nt resolution along the x-axis and the y-axis indicates reads per 1 million sequenced RNAs (excluding miRNA, rRNA, tRNA and snoRNA species).

A



B

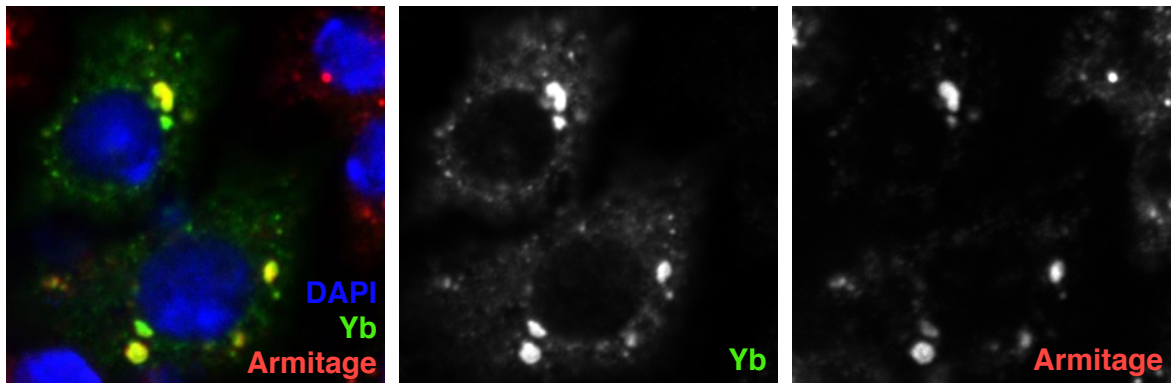


Figure S3. Armitage, Yb and DCP1 localization in OSC cells and ovaries.

(A) Shown is an ovariole expressing YFP-DCP1 (green) stained for Armitage (red) and DNA (blue).
 (B) Shown are OSC cells transiently transfected with GFP tagged Yb (green) and stained for Armitage (red) and DNA (blue).

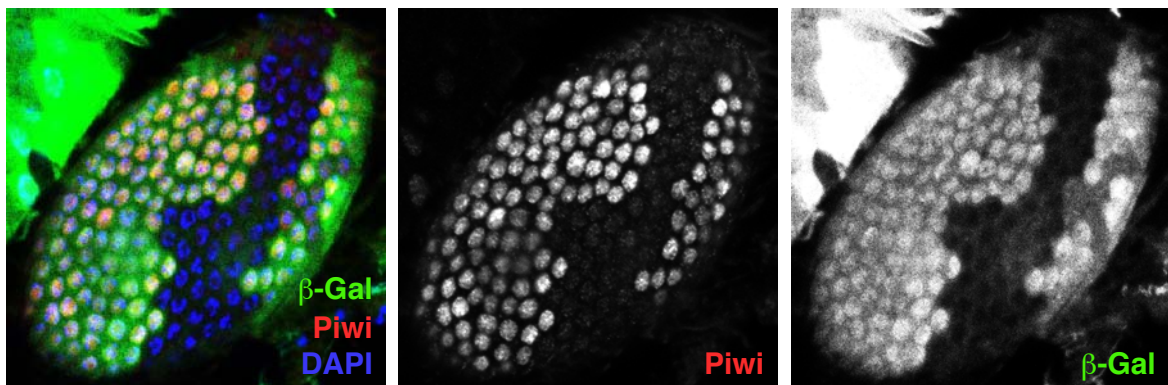


Figure S4. Follicle cells lacking Yb lose nuclear Piwi localization.

Shown is a surface view onto an egg chamber containing a mitotic clone for the *fs(1)Yb[72]* null allele stained for Piwi (red), β-Gal (green) and DNA (blue). The clone is marked by the absence of β-Gal (green).

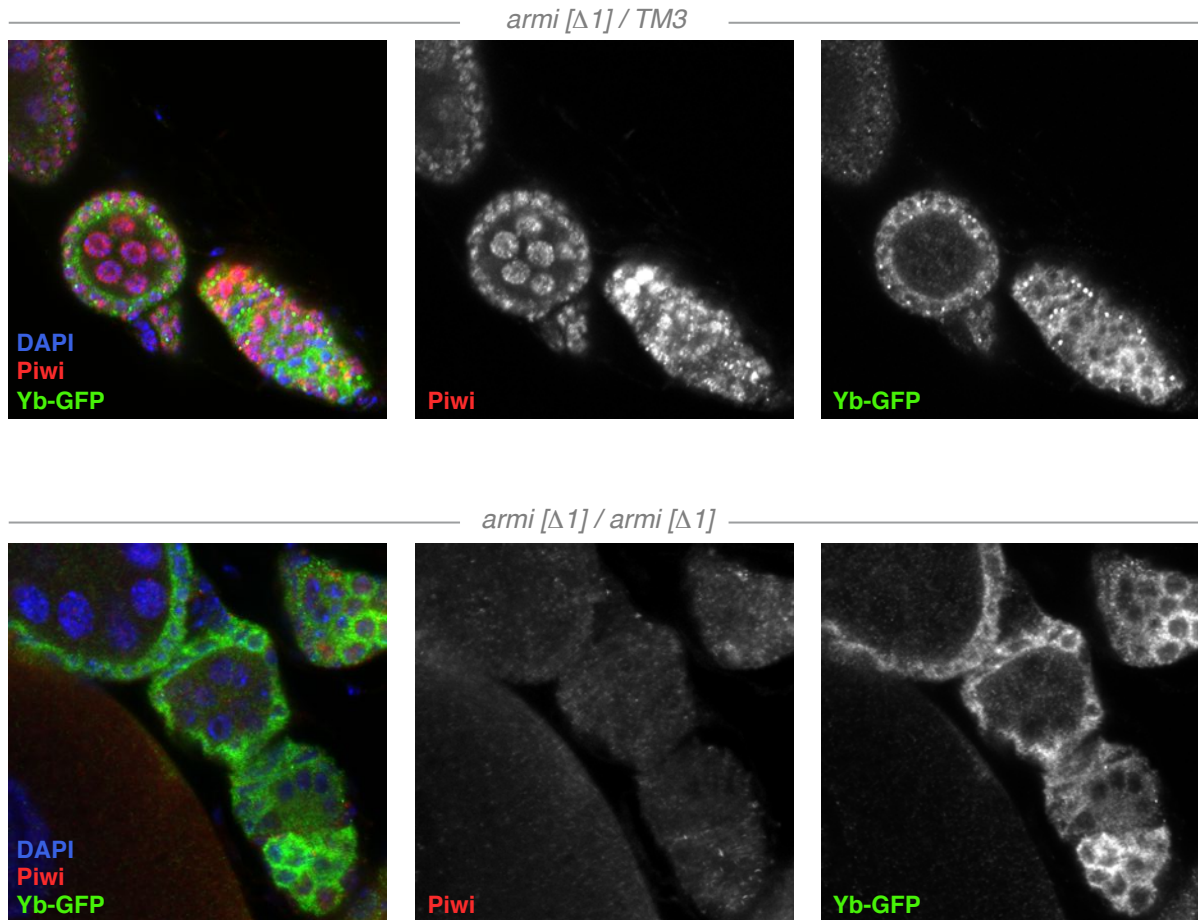
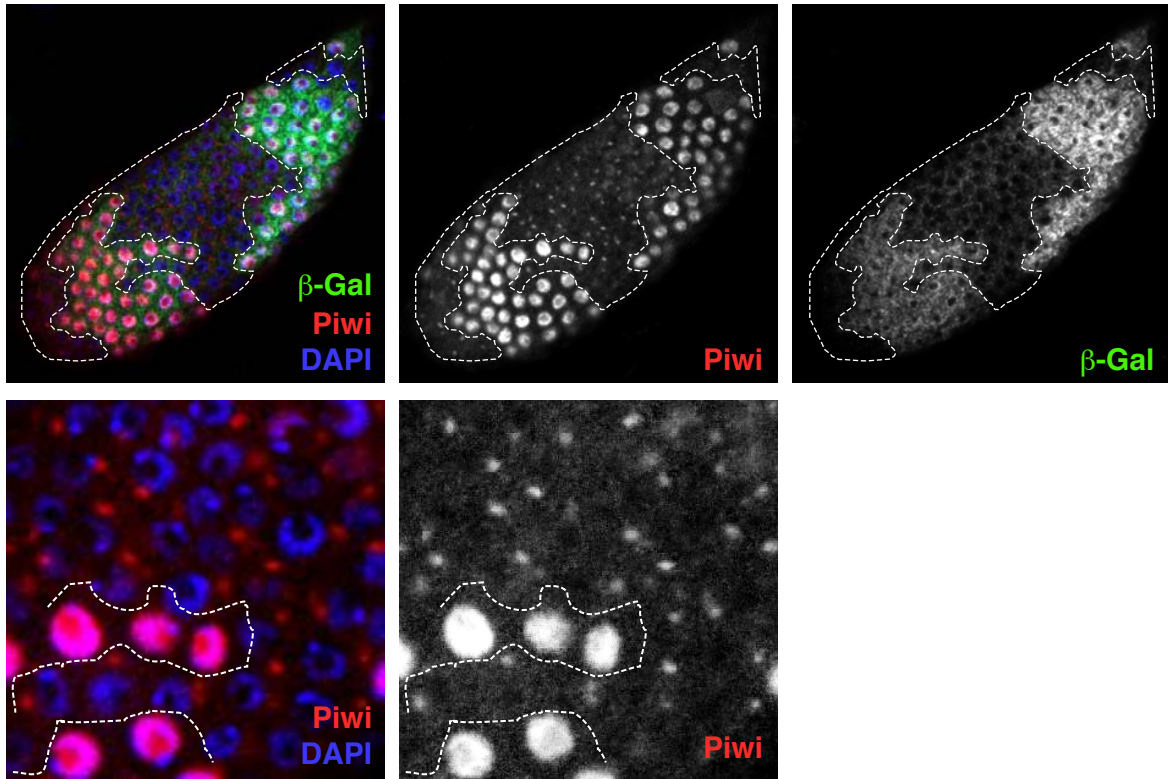


Figure S5. Armitage is required for Yb localization to cytoplasmic foci.

Shown are confocal sections through ovarioles heterozygous (top) or homozygous (bottom) for the *armi*[$\Delta 1$] deletion carrying a Yb-GFP (green) transgene and stained for Piwi (red) and DNA (blue).

A

mitotic FRT clones for *zuc*[*HM27*]



B

mitotic FRT clones for *zuc*[*HM27*]

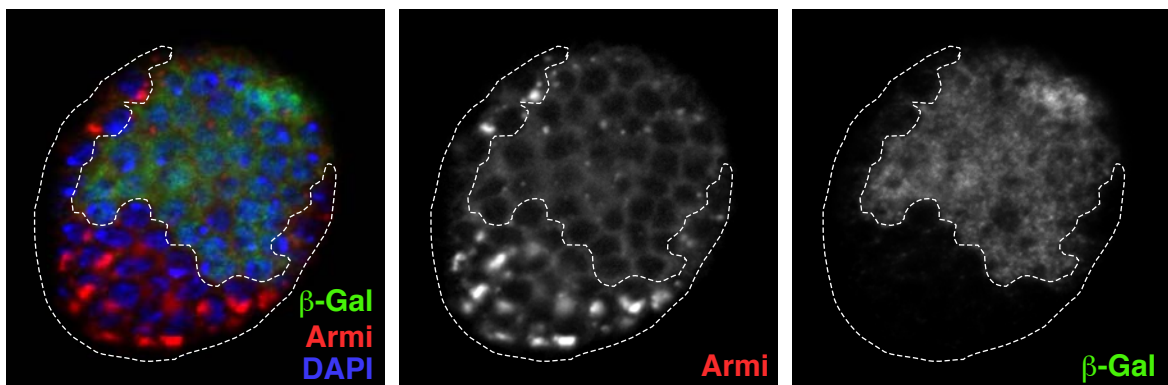


Figure S6. Follicle cells lacking *Zuc* exhibit *Piwi* localization to Yb-bodies.

(A) Shown is a surface view onto an egg chamber containing mitotic clones for the *zuc*[*HM27*] null allele stained for *Piwi* (red), β -Gal (green) and DNA (blue). The clones are marked by the absence of β -Gal (green) and is encircled by a dashed line. The lower panels show a magnified view. (B) Shown is a surface view onto an egg chamber containing mitotic clones for the *zuc*[*HM27*] null allele stained for *Armi* (red), β -Gal (green) and DNA (blue). The clone is marked by the absence of β -Gal (green) and encircled by a dashed line.

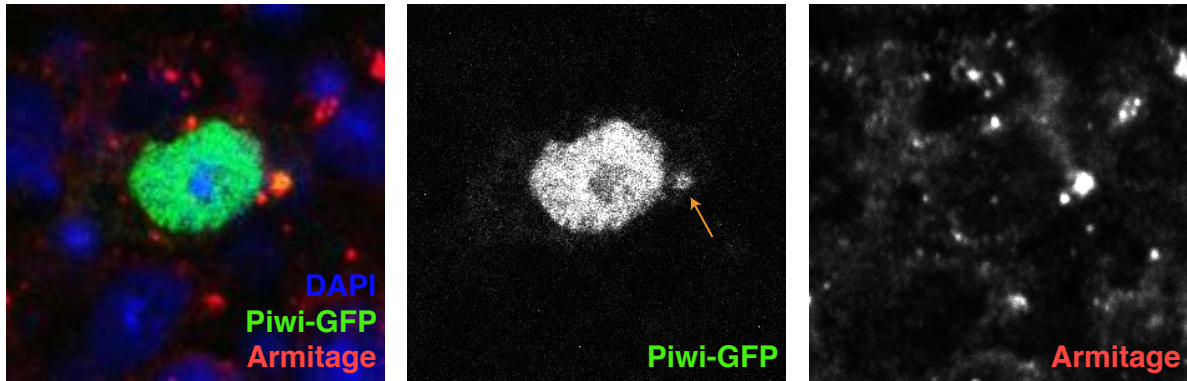


Figure S7. OSC cells transiently expressing GFP-Piwi show Piwi localization to Armi-foci.

Shown are OSC cells chemically transfected with GFP-Piwi (green) stained for Armitage (red) and DNA (DAPI, blue). The arrow points to the Piwi staining co-localizing with Armi.

flip out clones expressing *zuc* and *armi* dsRNA

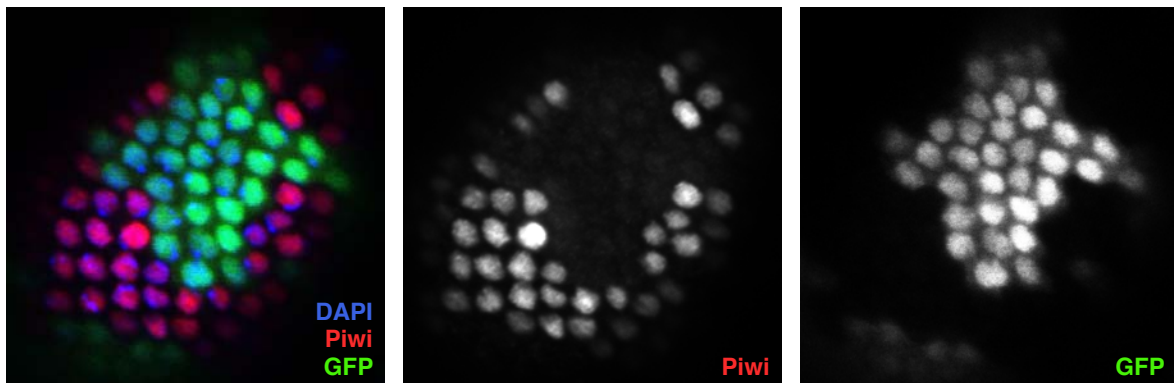


Figure S8. Armitage is required for Piwi localization to Yb-bodies

Shown is a surface view onto the follicular epithelium of an egg chamber containing clones expressing dsRNA hairpins against *zuc* and *armi* stained for Piwi (red) and DNA (DAPI; blue). The clone is marked by the presence of GFP (green).

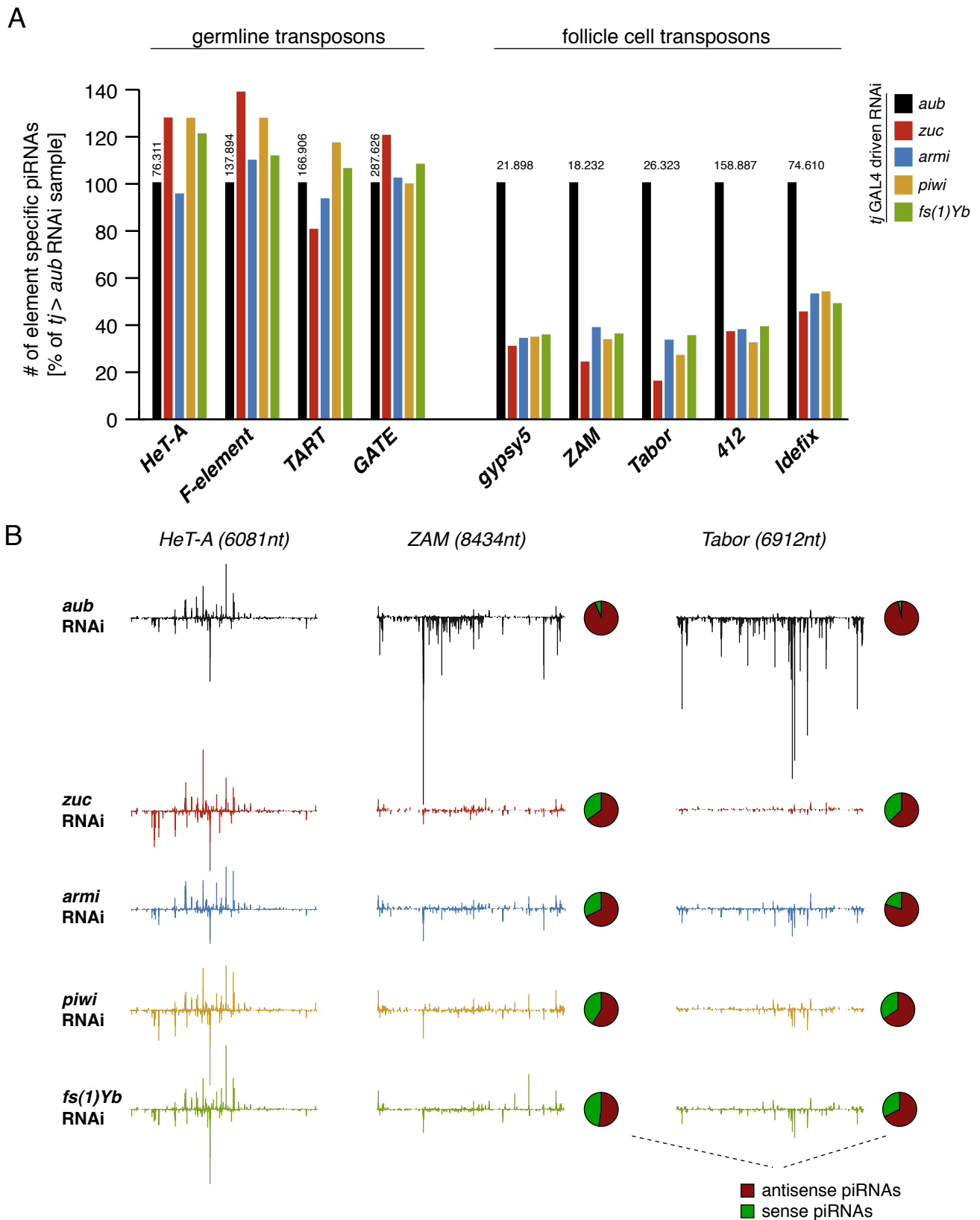


Figure S9. piRNA biogenesis defects in the absence of Zuc, Armii and Yb.

(A) Shown are relative levels of Piwi-piRNAs from ovaries of the indicated genotypes mapping to the indicated transposons. Elements silenced preferentially in the germline are to the left whereas elements with specific silencing in somatic support cells are to the right (based on Malone et al. 2009). Libraries were normalized via the genome-unique mappers to 42AB, a germline specific piRNA cluster (Fig. 5). The *tj-GAL4>aub-RNAi* sample acts as control and the raw numbers of this sample are indicated above the black bars. (B) Detailed piRNA profiles across the transposons *HetA*, *ZAM* and *Tabor*. The color code is as in (A). Cake diagrams indicate the levels of sense (green) and antisense (red) piRNAs mapping to the indicated transposons.

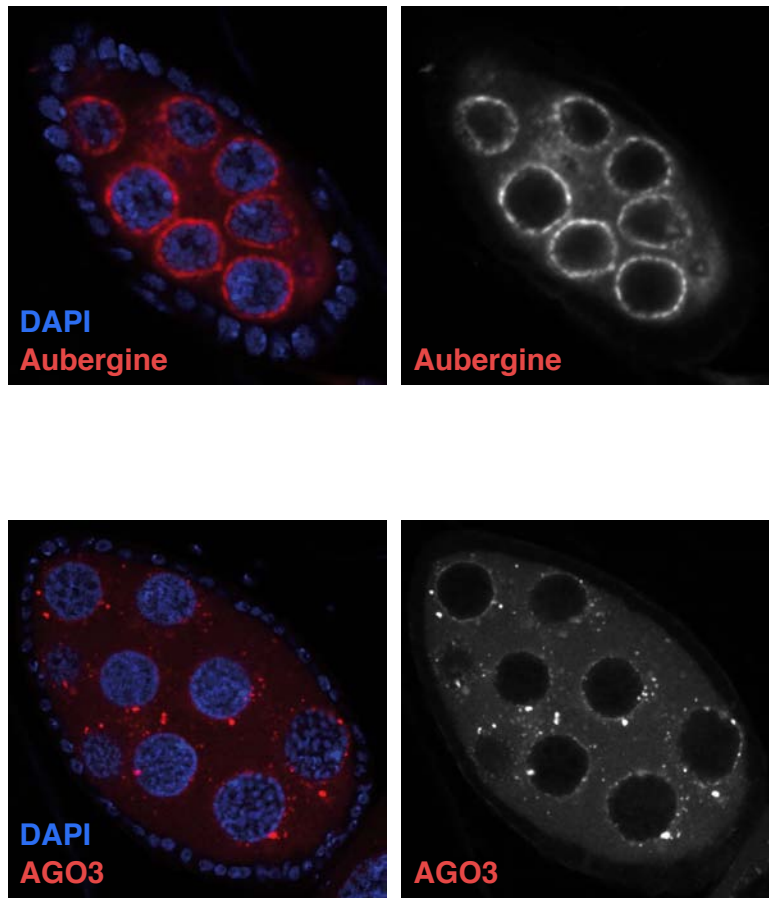


Figure S10. Aubergine and AGO3 localization in *zuc* mutant egg chambers.

Shown are confocal sections through *zuc*[*HM27*]/*zuc*[*Def*] egg chambers stained for Aubergine (top) and AGO3 (bottom). DNA was visualized with DAPI (blue).

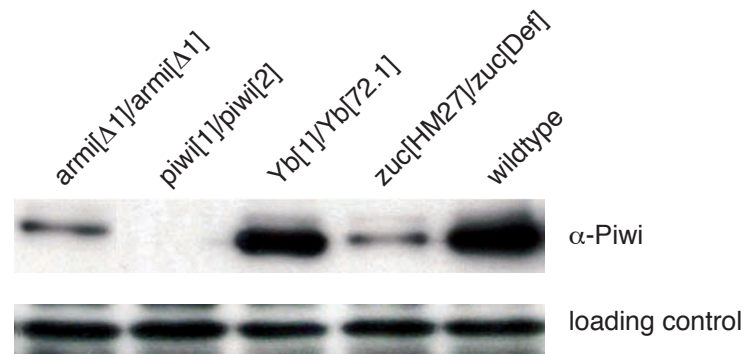


Figure S11. Piwi levels are reduced in primary piRNA biogenesis mutants.

Shown is a western blot against Piwi from ovarian extracts of the indicated mutants. The lower western blot indicates consistent loading.

3.3 The Cochaperone Shutdown Defines a Group of Biogenesis Factors Essential for All piRNA Populations in Drosophila

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Contributions: Daniel Olivieri and Julius Brennecke conceived the experiments, Daniel Olivieri conducted the experiments, Ravi Sachidanandam and Sailakshmi Subramanian provided the small RNA database, Kirsten-André Senti provided genetic tools, Julius Brennecke and Ravi Sachidanandam performed the computational analysis, and Julius Brennecke, Kirsten-André Senti, and Daniel Olivieri wrote the paper.

The Cochaperone Shutdown Defines a Group of Biogenesis Factors Essential for All piRNA Populations in *Drosophila*

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SUMMARY

In animal gonads, PIWI proteins and their bound 23–30 nt piRNAs guard genome integrity by the sequence specific silencing of transposons. Two branches of piRNA biogenesis, namely primary processing and ping-pong amplification, have been proposed. Despite an overall conceptual understanding of piRNA biogenesis, identity and/or function of the involved players are largely unknown. Here, we demonstrate an essential role for the female sterility gene *shutdown* in piRNA biology. Shutdown, an evolutionarily conserved cochaperone collaborates with Hsp90 during piRNA biogenesis, potentially at the loading step of RNAs into PIWI proteins. We demonstrate that Shutdown is essential for both primary and secondary piRNA populations in *Drosophila*. An extension of our study to previously described piRNA pathway members revealed three distinct groups of biogenesis factors. Together with data on how PIWI proteins are wired into primary and secondary processing, we propose a unified model for piRNA biogenesis.

INTRODUCTION

PIWI interacting RNAs (piRNAs) are a class of animal small RNAs. They are bound by PIWI family proteins and guide the sequence specific silencing of selfish genetic elements such as transposable elements (TEs). Defects in the piRNA pathway lead to TE derepression, genomic instability and ultimately sterility (Malone and Hannon, 2009; Siomi et al., 2011).

In *Drosophila*, most piRNAs are generated from two sources; on the one hand, these are piRNA cluster transcripts that originate from discrete genomic loci and serve as reservoirs of TE sequences; on the other hand, these are RNAs derived from active TEs that engage—together with cluster transcripts—in a piRNA amplification loop called the ping-pong cycle (Brennecke et al., 2007; Gunawardane et al., 2007; Senti and Brennecke, 2010).

Two modes of piRNA biogenesis exist: (1) during primary piRNA biogenesis, a single stranded RNA is processed into

pre-piRNAs, which are loaded onto PIWI proteins and are subsequently 3' trimmed and methylated, yielding mature piRNA induced silencing complexes (piRISCs) (Kawaoka et al., 2011; Senti and Brennecke, 2010). (2) piRISCs with active slicer activity can trigger secondary piRNA biogenesis, where a new piRNA is formed out of the sliced target RNA. In the presence of corresponding sense and antisense precursor RNAs, secondary piRNA biogenesis acts as the ping-pong amplification loop. The two piRNAs engaged in ping-pong have opposite orientation and exhibit a characteristic ten nucleotide 5' overlap (ping-pong signature; Brennecke et al., 2007; Gunawardane et al., 2007).

Primary and secondary piRNA biogenesis co-occur in germline cells, complicating the genetic and mechanistic dissection of these processes. However, somatic cells of the gonad also harbor a piRNA pathway and this is based exclusively on primary piRNA biogenesis (Lau et al., 2009; Li et al., 2009; Malone et al., 2009; Saito et al., 2009). The *Drosophila* ovary is therefore ideally suited to identify and characterize factors required for either primary or secondary piRNA biogenesis or both.

Somatic support cells of the *Drosophila* ovary express Piwi as the only PIWI family protein. Primary piRNA biogenesis is thought to take place in peri-nuclear Yb-bodies, where the RNA helicases Armitage (Armi) and Yb as well as the TUDOR domain protein Vreteno (Vret) accumulate. In addition to these three factors, the putative mitochondria-localized nuclease Zucchini (Zuc) and the RNA helicase Sister of Yb (SoYb) are essential for piRNA biogenesis in the soma (Haase et al., 2010; Handler et al., 2011; Olivieri et al., 2010; Qi et al., 2011; Saito et al., 2010; Zamparini et al., 2011). Formation of mature Piwi-RISC triggers its nuclear import, while failure in piRNA biogenesis results in destabilization of presumably unloaded Piwi (Haase et al., 2010; Olivieri et al., 2010; Qi et al., 2011; Saito et al., 2010). Mature Piwi-RISC triggers TE silencing by an unknown mechanism that requires Piwi's nuclear localization but not its slicer activity (Saito et al., 2010; Klenov et al., 2011).

With the exception of Yb, the above mentioned biogenesis factors are also essential in germline cells for the formation of Piwi-RISC. Germline cells, however, express two additional PIWI proteins, Aubergine (Aub) and Argonaute 3 (AGO3), which localize to the cytoplasm and are enriched in peri-nuclear nuage. Aub and AGO3 are the main players in the ping-pong cycle. Several factors with essential or modulatory roles in the ping-pong cycle have been identified. These are the RNA helicases Spindle-E and Vasa and the TUDOR domain proteins Krimper,

Tejas (Tej), Qin and Tudor (Anand and Kai, 2011; Lim and Kai, 2007; Malone et al., 2009; Nishida et al., 2009; Patil and Kai, 2010; Zhang et al., 2011).

The analysis of piRNA populations from wild-type and piRNA pathway mutant ovaries indicated that Piwi is mainly a recipient of primary piRNAs, while Aub and AGO3 are predominantly or exclusively recipients of secondary piRNA biogenesis (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009; Malone et al., 2009). Given this, three major questions arise: (1) Are primary and secondary piRNA biogenesis processes genetically and mechanistically separate or do common factors act in both processes? (2) In which processing step do identified piRNA biogenesis factors act? (3) How are the three PIWI family proteins wired into piRNA biogenesis? In other words, are certain PIWI proteins only receiving primary or only secondary piRNAs?

Here, we show that the female sterility gene *shutdown* (Munn and Steward, 2000; Schüpbach and Wieschaus, 1991) encodes a piRNA biogenesis factor. We show that Shu is required for all piRNA populations in ovaries and that it acts downstream of known piRNA biogenesis factors. A comparison of Shu to several other pathway factors led to the definition of three major groups of piRNA biogenesis factors. In combination with data on how PIWI proteins are wired into piRNA biogenesis, we propose a model that accounts for the distinct association of piRNA subpopulations with specific PIWI proteins in *Drosophila*.

RESULTS

The Cochaperone Shutdown Is an Essential piRNA Pathway Factor

In *Drosophila*, mutations in piRNA pathway factors result in sterility. In many cases, however, oogenesis proceeds and mutant females are laying eggs. These exhibit defects in egg asymmetry and fail to develop. Loss of egg asymmetry is triggered by sustained activation of the Chk2 DNA damage checkpoint that is presumably activated by uncontrolled TE activity (Klattenhoff et al., 2007). Based on the characteristic mutant egg morphology, several piRNA pathway genes were identified before piRNAs were described (e.g., Schüpbach and Wieschaus, 1991). We searched the literature for female sterility mutants with egg polarity defects yet no reported link to piRNA biology. One of the candidates was *shutdown* (*shu*), a gene encoding a cochaperone of the immunophilin class that harbors a peptidyl-prolyl-*cis/trans*-isomerase domain (PPIase) and a TPR domain (Munn and Steward, 2000). Notably, the orthologous mouse FKBP6 gene is essential for male fertility (Crackower et al., 2003) and was identified in PIWI immune precipitates (Vagin et al., 2009).

Unlike piRNA pathway mutants that only affect the germline branch and that allow overall oogenesis to occur, *shu* mutant ovaries are rudimentary and resemble mutants that affect also the somatic piRNA pathway (not shown). We compared TE RNA levels in *shu* mutant ovaries to those of heterozygous controls and observed derepression of TEs known to be active either in germline or in somatic support cells (Figure 1B). Sterility, ovary morphology and TE repression were rescued

by a genomic construct harboring the *shu* locus with an additional N-terminal GFP cassette (Figures 1A and 1B). To support a role for Shu in ovarian somatic and germline cells we used tissue specific RNAi based on the *traffic jam* GAL4 driver (soma) or the *nanos* GAL4 driver (germline) (Handler et al., 2011; Ni et al., 2011; Olivieri et al., 2010). Similar to germline knockdowns (GLKD) of the piRNA biogenesis factor Armi, GLKD of Shu resulted in derepression of the germline controlled TE *HeT-A*, while soma knockdowns resulted in derepression of the soma controlled element ZAM. The *blood* element was derepressed in both knockdowns, in agreement with this TE being targeted by soma and germline piRNAs (Malone et al., 2009).

We next analyzed localization and levels of PIWI proteins in cells lacking Shu using mosaic clones. *shu* mutant cells exhibited a near loss of Piwi (Figures 1E and 1F). Similarly, levels and nuage accumulation of Aub were severely affected (Figure 1G). AGO3 levels were moderately decreased and residual AGO3 accumulated in cytoplasmic foci rather than in nuage (Figure 1H). Western blot analysis from *shu*-GLKD ovaries confirmed the strong reductions in Piwi and Aub levels (Figure S1). The only other known piRNA pathway factor with similar requirements for all PIWI proteins is the TUDOR domain containing protein Vret (Handler et al., 2011; Zamparini et al., 2011).

Shutdown Is Required for the Biogenesis of All piRNA Populations

Reductions in PIWI levels such as in the case of *shu* mutants have been linked to defects in piRNA biogenesis. We sequenced 18–30 nt RNAs from ovaries of *shu* mutants and heterozygous controls. Both libraries were normalized to one million miRNA reads. In support of this normalization, levels of endogenous hairpin derived siRNAs were similar in both libraries (Figure 2A). Remarkably, while repeat derived siRNAs (21 nt) were slightly elevated in *shu* mutants, repeat derived piRNAs were nearly ablated (~30-fold reduction; Figures 2A and 2B). An analysis of the 93 Repbase TEs with more than 1000 sequenced antisense piRNAs in wild-type ovaries revealed severe reductions of piRNA levels for all TEs (Figure 2C). We noted a stronger impact on germline dominant elements (~150-fold reduction) compared to intermediate (~23-fold) and soma dominant elements (~15-fold; Figures 2C and 2D). We attribute this to the distorted morphology of *shu* mutant ovaries, which likely contain a relatively greater proportion of somatic cells than wild-type ovaries. A similar trend was observed for piRNAs mapping uniquely to piRNA clusters; the soma dominant cluster *flamenco* was strongly, but less affected than germline dominant clusters such as *cluster 42AB* (Figure 2E). Also gene-derived piRNAs required Shu as piRNAs mapping to the 3'UTR of *traffic jam*, a prominent source of piRNAs in *Drosophila*, were nearly lost (not shown; Robine et al., 2009; Saito et al., 2009).

Shutdown Acts Downstream of Known piRNA Biogenesis Factors

Current models postulate that PIWI proteins are loaded with longer pre-piRNA molecules. Subsequently, an unknown 'trimmer' activity generates the 3' end (Kawaoka et al., 2011).

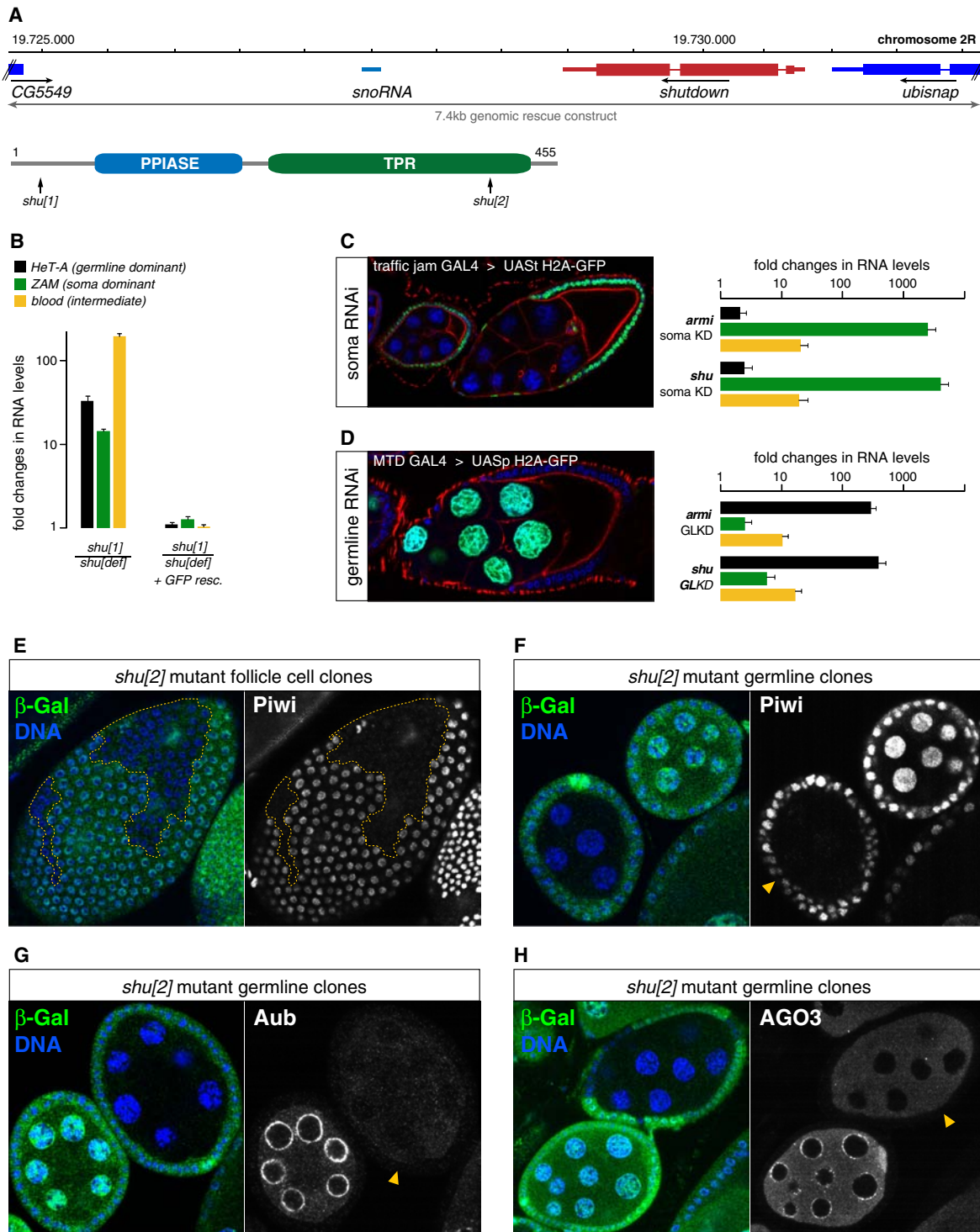


Figure 1. Shutdown Is an Essential piRNA Pathway Factor

(A) Shown is the genomic *shu* locus, the extent of the rescue construct and the domain composition of the 455 amino acid Shu protein (arrows indicate the two nonsense mutations in *shu*[1] and *shu*[2]).

(B) Fold changes in steady state RNA levels of indicated TEs in *shu* mutant and GFP-*shu*-rescued ovaries (normalized to *shu* heterozygotes; n = 3; error bars indicate StDev.).

(C and D) Immunofluorescence images show soma (C) and germline (D) specificity of tj-Gal4 and MTD-Gal4 (crossed to UAS H2A-GFP (green); DNA (blue); actin (red)). Bar diagrams show fold changes in steady state RNA levels of indicated TEs upon soma (C) or germline (D) specific knockdown of *armi* or *shu* (n = 3; StDev.).

This predicts a hierarchy of factors that (1) direct precursor RNAs to the biogenesis machinery, that (2) generate pre-piRNA molecules, that (3) load pre-piRNAs into PIWI proteins and that (4) catalyze piRNA 3' end maturation.

Thus, piRNA biogenesis is a dynamic process and underlying protein-protein and protein-RNA interactions must be transient, challenging the molecular dissection of the biogenesis pathway. A hierarchy of piRNA biogenesis factors can, however, be delineated based on their localization to Yb-bodies in somatic cells or to nuage in germline cells, the proposed sites of piRNA biogenesis (Handler et al., 2011; Lim and Kai, 2007; Olivieri et al., 2010; Saito et al., 2010).

Based on the GFP-Shu rescue construct and antibody stainings, Shu localized to the cytoplasm of ovarian germline and somatic cells and was enriched in nuage (Figure 3A). In follicle cells, Shu levels were considerably lower. We therefore turned to cultured ovarian somatic cells (OSCs; Niki et al., 2006; Saito et al., 2009). Here, GFP-Shu as well as endogenous Shu was cytoplasmic and typically enriched in Yb-bodies (Figure 3B). The primary piRNA biogenesis factors Yb, Armi and Vret colocalize in Yb-bodies (Figure 3C) and a genetic hierarchy exists (Yb → Armi → Vret; Handler et al., 2011; Olivieri et al., 2010; Saito et al., 2010). Comparable to observations in mouse (Watanabe et al., 2011), loss of the essential biogenesis factor Zuc led to clustering of mitochondria (Figure S2). Under those conditions, Yb, Armi and Vret decorated most mitochondria and defined a “giant” Yb-body that also accumulated Shu and—presumably unloaded—Piwi (Figure 3C; Olivieri et al., 2010; Saito et al., 2010). We conclude that Shu is a bona fide Yb-body factor.

We tested Shu's requirement for Yb-body formation. In clones of *shu* mutant follicle cells, Yb and Armi accumulated in discrete foci, indistinguishable from surrounding control cells, arguing that Yb-body formation does not require Shu (Figure 3D). Based on knockdown experiments in OSCs, Yb-body localization of Vret (though dependent on Armi and Yb), was only mildly affected by loss of Shu (Figure 3E). On the other hand, Shu localization to Yb-bodies required all known biogenesis factors (Figure 3E). Notably, knockdown of Piwi also caused delocalization of Shu from Yb-bodies. This suggests the possibility that unloaded Piwi recruits Shu. Indeed, low levels of Piwi colocalize with Shu in Yb-bodies and Piwi copurified with Shu and Armi in IP-experiments (Figure S2).

Taken together, Shu localizes to sites of piRNA biogenesis, potentially interacts with unloaded PIWI proteins and acts downstream of known biogenesis factors.

Shutdown's TPR Domain Is Essential for Its Function and Shutdown and Hsp83 Colocalize with Unloaded AGO3

Heat shock protein 90 (Hsp90) is required for loading siRNA duplexes into Argonaute proteins (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). Recently, plant Cyclophilin 40

(Cyp40) has been shown to be an important Hsp90 cochaperone for siRNA loading into AGO1 (Iki et al., 2011). Cyp40 shares the same domain organization with Shu and the C-terminal TPR domain was shown to be essential for Hsp90 interaction and Cyp40 function. We therefore tested whether Shu cooperates with Hsp83 (*Drosophila* Hsp90) during piRNA biogenesis.

TPR domains interact with the C-terminal MEEVD peptide of Hsp90 (Scheufler et al., 2000). The key amino acid residues for an Hsp90 interaction are present in the Shu TPR domain (Figure 4A). We mutated Lys 304 into Ala (K304A) in the GFP tagged rescue construct and tested whether *shu*[K304A] could rescue *shu* loss of function alleles. The wild-type *shu*-GFP-rescue construct fully restored fertility and PIWI protein localization. In contrast, *shu*[K304A] flies were sterile, lacked Aub/AGO3 nuage localization and showed elevated TE levels (Figures 4B and 4C). Essentially identical results were obtained upon mutating Gln 308 into Glu (Figure S3). In both cases Piwi localization and thus presumably primary piRNA biogenesis was not severely affected although ZAM, a soma specific TE was moderately derepressed. It is possible that the introduced TPR mutations do not result in a complete loss of Shu's function and secondary piRNA biogenesis is more sensitive toward loss of Shu activity.

To support a role of Hsp83 in piRNA biogenesis, we determined its localization in wild-type and piRNA biogenesis defective ovaries using a genomic GFP-tagged *Hsp83* construct. In wild-type nurse cells, Hsp83 localized uniformly to the cytoplasm and weakly to the nucleus (Figure 4D). Hsp83 was enriched in a peri-nuclear rim, potentially reflecting nuage accumulation. Strikingly, upon GLKD of *aub*, *vas*, *vret* or *shu*, Hsp83 colocalized with AGO3 in cytoplasmic foci (Figure 4D). We isolated equivalent amounts of AGO3 from *shu*-GLKD and control ovaries and found no association of AGO3 with piRNAs upon loss of Shu (Figure S3). This suggests that unloaded AGO3 that localizes to cytoplasmic foci interacts with Hsp83.

In support of its proposed interaction with Hsp83, Shu also localized to the cytoplasmic AGO3 foci in every knockdown that disrupted Aub/AGO3 nuage localization (shown for *aub*-GLKD in Figure 4E). In all cases, AGO3-Shu foci were also positive for the piRNA biogenesis factor Krimp. Thus, loss of secondary piRNA biogenesis results in the accumulation of the three nuage factors AGO3, Krimp and Shu in large cytoplasmic foci, which also accumulate Hsp83. These foci depended on Krimper (see below), but also formed in the absence of Shu (Figures 4D and 4E). This indicated that the cochaperone Shu is not required for the recruitment of Hsp83 to AGO3 foci, analogous to the Cyp40/AGO1/Hsp90 biology in plants (Iki et al., 2011).

Taken together, our genetic and cell biological data point toward an important role for the Hsp83 machinery in piRNA biogenesis. Indeed, Piwi has been found in a complex with Hsp83 in embryo lysate (Gangaraju et al., 2011) and Hsp83 has been demonstrated to affect phenotypic variation at least in part via the piRNA pathway (Specchia et al., 2010).

(E) Confocal section of a follicle epithelium with a mitotic *shu*[2] clone (marked by absence of β-Gal (green)) stained for DNA (blue) and Piwi (monochrome). The dashed yellow line marks the clone boundary.

(F–H) Confocal sections of two egg chambers with one being mutant for *shu*[2] in the germline (absence of β-Gal (green); yellow arrowhead in monochrome panels) stained for DNA (blue), Piwi (F), Aub (G) or AGO3 (H).

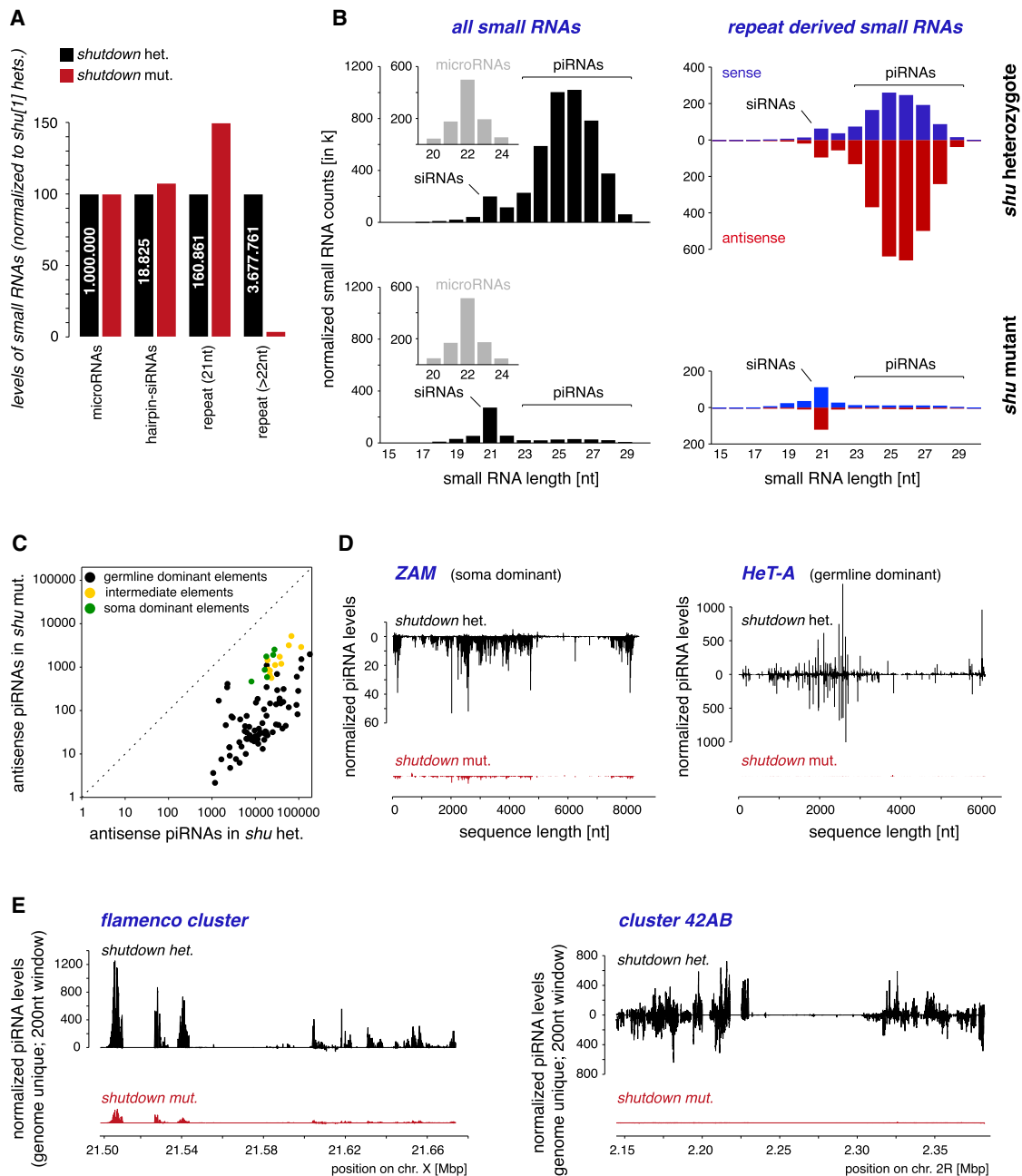


Figure 2. Shutdown Is Required for the Biogenesis of All Ovarian piRNA Populations

(A) Shown are normalized levels of indicated ovarian small RNAs from *shu[1]* heterozygous and *shu[1]/shu[def]* flies.

(B) To the left, length profiles of normalized small RNAs split into miRNAs (small panels) and remaining RNAs (siRNAs and piRNAs) are shown. To the right, length profiles of repeat derived small RNAs (red antisense; blue sense) are shown; top panels show *shu* heterozygotes, bottom panels *shu* mutants.

(C) Scatter plot (log scale) showing levels of antisense piRNAs mapping to soma dominant (green), intermediate (yellow) or germline dominant (black) TEs in *shu* het. or *shu* mut. libraries.

(D) normalized piRNA profiles (sense up; antisense down) from *shu* het. (black) or *shu* mut. (red) libraries mapping to indicated TEs.

(E) normalized profiles of genome unique piRNAs (sense up; antisense down) from *shu* het. (black) or *shu* mut. (red) libraries mapping to indicated piRNA clusters (200nt walking window).

A Controlled System to Compare the Impact of Biogenesis Factors on piRNA Populations

The analysis of small RNAs from *shu* mutant ovaries indicated that Shu is required for all piRNA biogenesis branches. However, this conclusion and also comparisons to other pathway factors were hampered by the distorted morphology of *shu* mutant ovaries. We therefore turned to robust germline knockdown systems (Ni et al., 2011; Handler et al., 2011) and analyzed the resulting impacts on piRNAs. This offered two advantages: First, overall ovarian morphology was unaffected as somatic cells that maintain the stem cell niche were unperturbed (full mutants of *piwi*, *shu*, *armi*, *zuc* or *vret* have rudimentary ovaries). Second, the genetic background of the analyzed flies was nearly identical, minimizing variations in TE load and piRNA cluster composition.

To benchmark this approach, we analyzed knockdowns for the three PIWI proteins. Immunofluorescence analysis indicated a near complete loss of germline Piwi/Aub/AGO3 levels in the respective knockdowns (Figure 5A), which was further supported by western analysis (Figure S1). As Aub antibody stainings were variable, we used a functional GFP tagged *aub* rescue construct. Loss of Piwi had no detectable impact on Aub or AGO3 localizations; loss of Aub did not affect Piwi, but resulted in cytoplasmic AGO3 bodies; and loss of AGO3 had no impact on localizations of Piwi or Aub (Figure 5A). These results are in line with reports showing that Piwi is dispensable for ping-pong, that Aub is an essential ping-pong factor and that loss of AGO3 permits auto ping-pong of Aub, though more pronounced effects of AGO3 loss on Piwi and Aub localization have been reported (Li et al., 2009; Malone et al., 2009).

We then performed *shu*-GLKD and, in agreement with our previous results, localization of all PIWI proteins was disrupted (Figure 5A). We sequenced 18–30 nt small RNAs from ovaries of two control knockdowns (control-GLKD [MTD x *w*[1118]strain] and *GFP*-GLKD) as well as from *piwi*, *aub* and *shu*-GLKDs. All libraries were normalized to one million miRNA reads and the levels of antisense 23–30 nt RNAs (piRNAs) were determined for the set of annotated Repbase elements (Figure 5B; Tables S1 and S2).

piRNA levels in the two control libraries were very similar (Figure 5B; Pearson correlation 0.97), arguing for the reproducibility of the approach. Knockdowns of Piwi or Aub resulted in significant reductions of piRNAs mapping to a subset of TEs, with defects being more pronounced in *aub*-GLKD ovaries (Figure 5B). As we only affected the piRNA pathway in germline cells, we divided TEs into soma dominant, intermediate and germline dominant (Malone et al., 2009). As expected, piRNA levels of soma dominant elements were unaffected and those of intermediate elements showed only slightly lower piRNA levels compared to controls (Figure 5B).

Knockdown of Shu resulted in a remarkably uniform reduction of piRNAs mapping to germline elements (Pearson correlation 0.86; see Figure S4 for the two outliers *rover* and *FB4*) and in a minor or no reduction of piRNAs mapping to intermediate or soma dominant elements, respectively (Figure 5B). The universal requirement of Shu for piRNA biogenesis allowed us to test the previous classification of TEs into soma-dominant, intermediate and germline-dominant (Malone et al., 2009). When sorted for the extent of piRNA loss in *shu*-GLKD ovaries, soma and inter-

mediate elements ranked at the end and a clear anti-correlation resulted with levels of OSC piRNAs (Figure 5C).

We next calculated ping-pong signatures for all TEs (Table S3). The two control libraries showed a strong correlation of 0.97 (Pearson). We sorted elements according to their classification and their ping-pong signature in control knockdowns and plotted the respective ping-pong signatures for the *piwi* and *aub* GLKD libraries (Figure 5D). In agreement with data from loss of function mutants (Malone et al., 2009), Piwi was not required for ping-pong (signatures were slightly enhanced), while Aub was uniformly required for it. In *shu*-GLKD, we observed a moderate to strong reduction in ping-pong signatures for nearly all elements (Figure 5D), arguing that the residual piRNA populations undergo low-level primary and secondary piRNA biogenesis. Knockdown of *shu* apparently affects primary and secondary piRNA biogenesis in a very uniform manner.

Primary and Secondary piRNA Biogenesis Require Distinct Factors and Feed into a Common Biogenesis Step

Numerous piRNA biogenesis factors have been identified. These include Armi, the Tdrd12 family (Yb, SoYb and BoYb), Zuc, Vas, Spindle-E (Spn-E), Krimp, Tej, Qin and Vret. We tested most of these in the GLKD assay for impacts on PIWI proteins and sequenced small RNAs from a subset of these knockdowns.

The localization patterns of PIWI proteins in these knockdowns defined three major groups (Figure 6A): Group I factors (Armi, Zuc) were required for Piwi levels and its nuclear accumulation, but had no or only limited impact on Aub and AGO3. Both factors are essential piRNA biogenesis factors in follicle cells supporting a role in primary piRNA biogenesis (Haase et al., 2010; Olivieri et al., 2010; Saito et al., 2010). Group II factors (Vas, Spn-E, Krimp) had no significant impact on Piwi levels, but were required for Aub/AGO3 biology; both proteins were delocalized from nuage, and AGO3 formed cytoplasmic foci (AGO3 foci were always positive for Krimp but were absent in *krimp*-GLKDs; Figure 6A). No group II factor is required for primary piRNA biogenesis in follicle cells (Malone et al., 2009; Olivieri et al., 2010), suggesting specific roles in germline specific secondary piRNA biogenesis. Group III factors (Shu, Vret, BoYb+SoYb) were required for all three PIWI proteins and resembled a combination of group I and II (Figure 6A). Consistent with their impact on Piwi, group III factors are also required for primary piRNA biogenesis in follicle cells (Handler et al., 2011; Zamparini et al., 2011). Our data support the proposed existence of factors that are specific for the primary (group I) or secondary (group II) biogenesis pathways (Malone et al., 2009) and further indicate that both branches feed into a common biogenesis step that requires group III factors.

To support this classification of biogenesis factors at a molecular level, we sequenced small RNAs from GLKDs of *armi*, *zuc* (group I) and *vas* (group II) and compared these to *shu* or *vret* knockdown libraries (group III). Consistent with the knockdowns being germline specific, piRNAs mapping to soma elements were not affected and those mapping to intermediate elements were mildly affected in all cases (Figure 6B). Knockdown of the group II gene *vas* led to decreases in

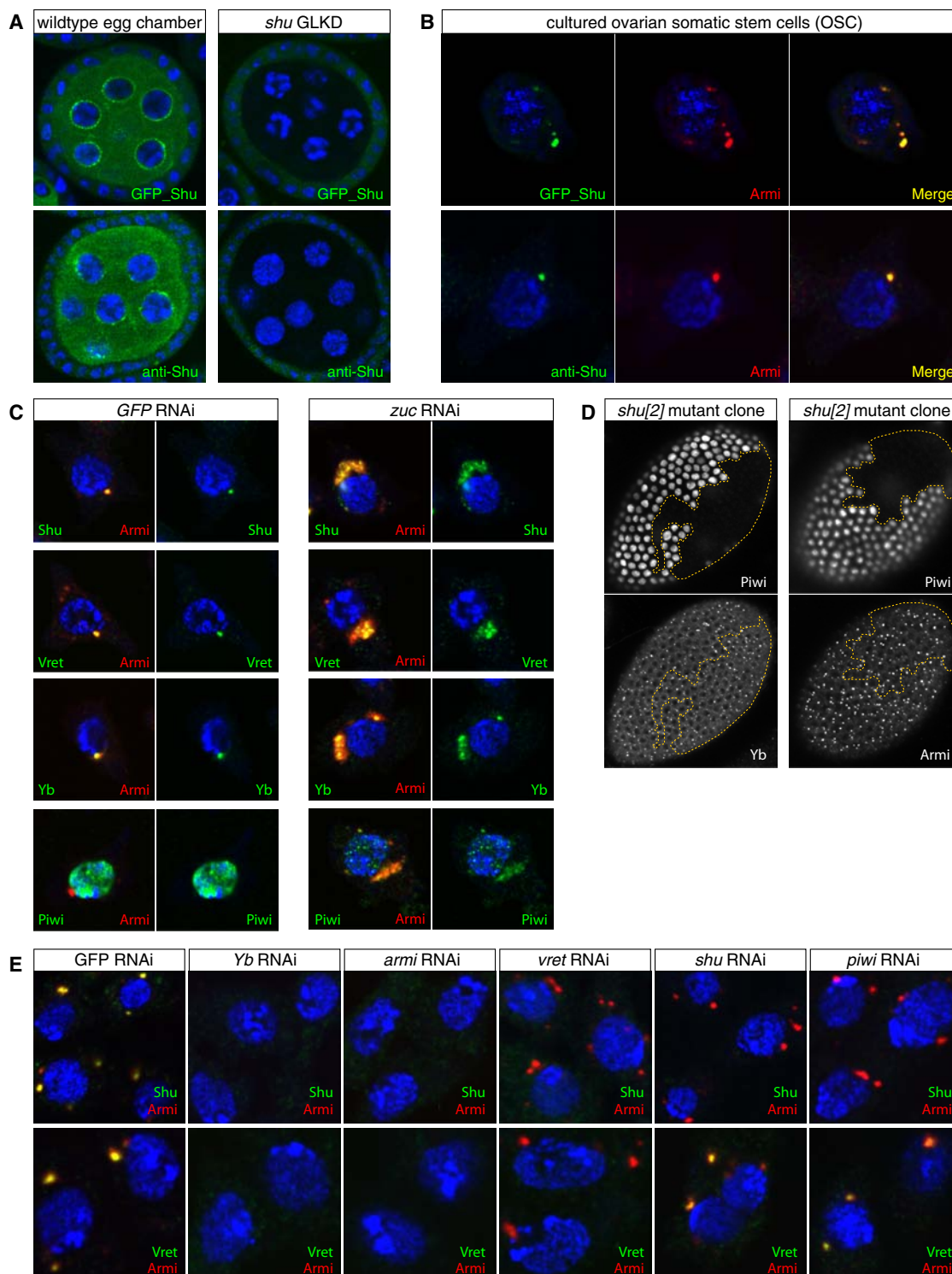


Figure 3. Shutdown Acts Downstream of Known piRNA Biogenesis Factors

(A) Shown are confocal sections of egg chambers expressing GFP-*shu* (top) or stained for endogenous Shu (bottom) from wild-type flies (left) or *shu*-GLKD. Signal in somatic follicle cells is unaffected.

(B) IF analysis of OSCs expressing GFP-Shu (top) or stained for Shu (bottom). Yb-bodies are identified via anti-Armi (red) and the right panels indicate colocalization of Shu and Armi in yellow. DNA in all panels stained with DAPI (blue).

(C) IF analysis of OSCs stained for DNA (blue), Armi (red) and indicated proteins (green) in control knockdowns (left) or *zuc* knockdowns (right). Colocalization of red and green appears yellow in the merge.

germline piRNA profiles that were highly correlated to the decreases in *aub* knockdowns (Figure 6B; Pearson correlation 0.93), arguing that Vas is tightly linked to Aub biology. In contrast, knockdown of *armi* resulted in a very different outcome. While many elements exhibited basically a collapse of piRNA levels, several elements were not or only mildly affected in the *armi*-GLKD library. A nearly identical impact on piRNA populations was observed in the *zuc*-GLKD library (Figure 6B; *armi/zuc* Pearson correlation: 0.93).

An even more striking contrast between *armi/zuc* knockdowns and *vas* knockdown was observed when ping-pong signatures were analyzed (Figure 6C). Consistent with previous findings (Malone et al., 2009), *vas* knockdowns resulted in a strong reduction of ping-pong signatures for nearly all elements. In contrast, knockdown of *armi* split the set of TEs essentially into two classes. While several elements lost ping-pong signatures, numerous elements exhibited strongly increased signatures (Figure 6C). Highly similar results were obtained from *zuc* knockdowns (Figure 6D; Pearson correlation: 0.92). Ping-pong signatures correlated with piRNA levels: While most elements with increased ping-pong signatures were only mildly affected in their piRNA levels (Figure 6D; elements with < 5-fold reductions in piRNAs colored blue), elements that lost ping-pong signals typically exhibited > 50-fold reductions in piRNA levels (orange). We specified elements with increased ping-pong signatures and moderate piRNA reductions in *armi/zuc*-GLKD as class-A elements and those with collapsed piRNA populations and ping-pong signatures as class-B elements (listed in Figure 6D).

We extended the analysis of class-A and B TEs to all libraries and calculated loss of piRNA populations as well as ping-pong signatures. Figures 6E and 6F show box-plots of this data and Figure S5 depicts the piRNA profiles of two representatives (*co-pia* for class-A and *l-element* for class-B) in detail. Several conclusions can be drawn from this analysis:

(1) Levels of class-A element piRNAs were remarkably resistant against knockdown of group I or group II biogenesis factors (Figure 6E). Strikingly, despite comparable piRNA levels, the ping-pong signatures (Figures 6F and S5) either doubled (group I) or collapsed (group II). Loss of group I or group II factors therefore split the population of primary and secondary piRNAs for class-A elements. In agreement with group III factors being required for both piRNA biogenesis branches, only *shu*- or *vret*-GLKD resulted in severely reduced piRNA levels (Figures 6E and S5).

(2) piRNA populations of class-B elements were highly sensitive toward loss of any biogenesis factor, with the group II factor Vas having the weakest impact (Figures 6E and S5). Similarly, ping-pong signatures essentially collapsed upon knockdown of any factor with Piwi being the only exception (Figures 6F and S5).

(3) The behavior of class-A elements indicated that group I factors are not required for ping-pong per se. As secondary piRNA biogenesis must be triggered by an input piRNA and

must therefore depend at some point on primary piRNA biogenesis, we speculate that maternally deposited piRNAs (Brennecke et al., 2008) allow class-A elements to engage in ping-pong in the absence of zygotic primary piRNA biogenesis. It is unclear why class-B elements do not behave similarly. Different temporal expression patterns of piRNA clusters with distinct TE content may lead to gaps in the availability of precursor RNA molecules that are required for continuous ping-pong.

Primary piRNA Biogenesis Factors Load Both Piwi and Aub Proteins, but Not AGO3

piRNA populations mapping to class-B elements are highly sensitive toward loss of the primary biogenesis (group I) factors Armi or Zuc. The only well established recipient of primary piRNAs in *Drosophila* is Piwi. Of all knockdowns, however, loss of Piwi had the lowest impact on class-B elements and ping-pong signatures were even slightly enhanced (Figures 6E and 6F). Thus, group I factors must also feed primary piRNAs into another PIWI protein. Indeed, loss of Aub had a pronounced impact on class-B element piRNAs (Figure 6E). However, due to Aub's involvement in ping-pong, this result was inconclusive.

We therefore turned to the OSC system that harbors a primary pathway feeding into Piwi. Importantly, OSCs do not express Aub or AGO3 (Lau et al., 2009; Saito et al., 2009). We expressed GFP- or FLAG/HA-tagged Piwi, Aub or AGO3 in OSCs and determined their subcellular localization and expression levels (Figures 7A–7C). As expected, tagged Piwi localized to nuclei and the faint cytoplasmic accumulations of Piwi colocalized with Armi in Yb-bodies (Figure 7A). Levels of tagged Aub were comparable to Piwi, but Aub localized to the cytoplasm (Figures 7A–7C). Aub was weakly enriched in Yb-bodies but interestingly accumulated in a discrete cytoplasmic dot that stained positive for Krimp, which is endogenously expressed in OSCs but not required for primary piRNA biogenesis (Malone et al., 2009). In contrast, tagged AGO3 (despite comparable mRNA levels; Figure 7B) was hardly detectable by western analysis (Figure 7C) and the residual AGO3 protein localized to Krimp bodies (Figure 7A) that never overlapped with Yb-bodies. We immunoprecipitated all three tagged PIWI proteins and tested for small RNA loading by CIP-Kinase labeling of copurified small RNAs (Figures 7D and 7E). Remarkably, this indicated that Aub was loaded efficiently with primary piRNAs.

To probe the identity of Aub bound OSC RNAs we sequenced small RNAs from FLAG-Piwi and FLAG-Aub immunoprecipitates. The two RNA populations were mirror images of each other in terms of library composition (Figure 7G), piRNA levels mapping to TEs (Figure 7J; Pearson correlation 0.995) as well as in terms of piRNA profiles mapping to clusters, TEs or genes (Figure 7K). The only slight differences were in terms of the uridine bias at the first nucleotide (Figure 7I; Piwi: 77%; Aub: 69%) and the length distribution (Figure 7H; Piwi: 26.2 nt; Aub: 26.0 nt on average). Both parameters also differ in piRNAs bound by Piwi

(D) Shown are confocal sections of the follicular epithelium with mitotic *shu[2]* clones stained for Piwi (top) and Yb or Armi (bottom). Clone borders shown with dashed yellow line.

(E) IF analysis of OSCs knocked down for indicated factors stained for DNA (blue), Armi (red) and Shu (green; top panels) or Vret (green; bottom panels). All panels show the merge (colocalization of red and green appears yellow).

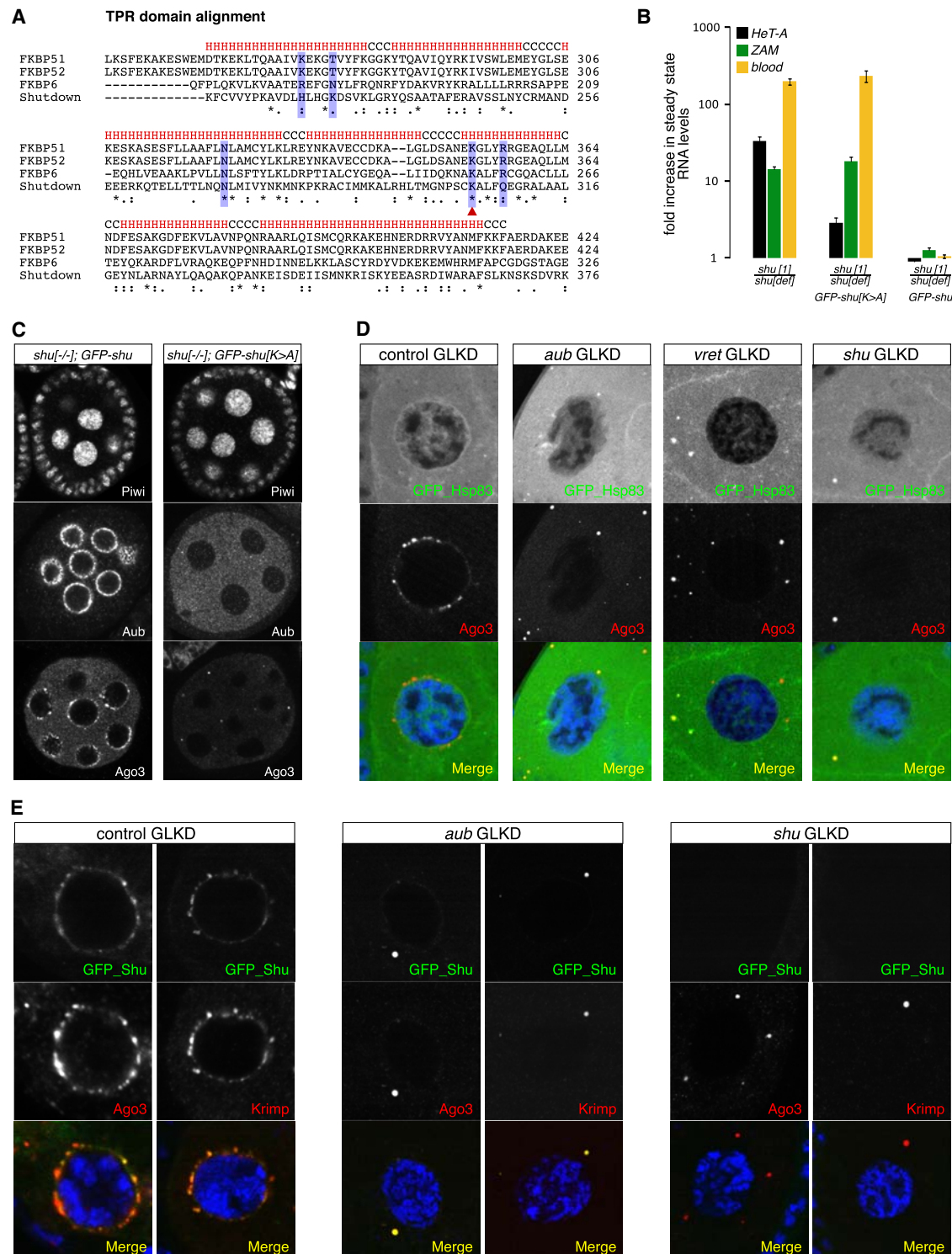


Figure 4. Shutdown Cooperates with Hsp83 and Colocalizes with Unloaded AGO3

(A) CLUSTAL alignment of the TPR domains from indicated proteins. α -helical segments indicated with 'H'; residues critical for an Hsp90 interaction highlighted with blue; the red arrowhead indicates the mutated Lys 304.

(B) Shown are fold changes in RNA levels (normalized to *shu* het.) of indicated TEs in ovaries from indicated genotypes (n = 3; StDev).

(C) Confocal sections of *shu* mutant egg chambers expressing wild-type (left) or K304A mutant (right) GFP-Shu stained for Piwi, Aub or AGO3.

(D) Confocal sections of nurse cells from indicated GLKDs stained for GFP-Hsp83, (green), AGO3 (red) and DNA (blue).

(E) Confocal sections of nurse cells from indicated GLKDs stained for GFP-Shu (green), AGO3 or Krimp (red) and DNA (blue).

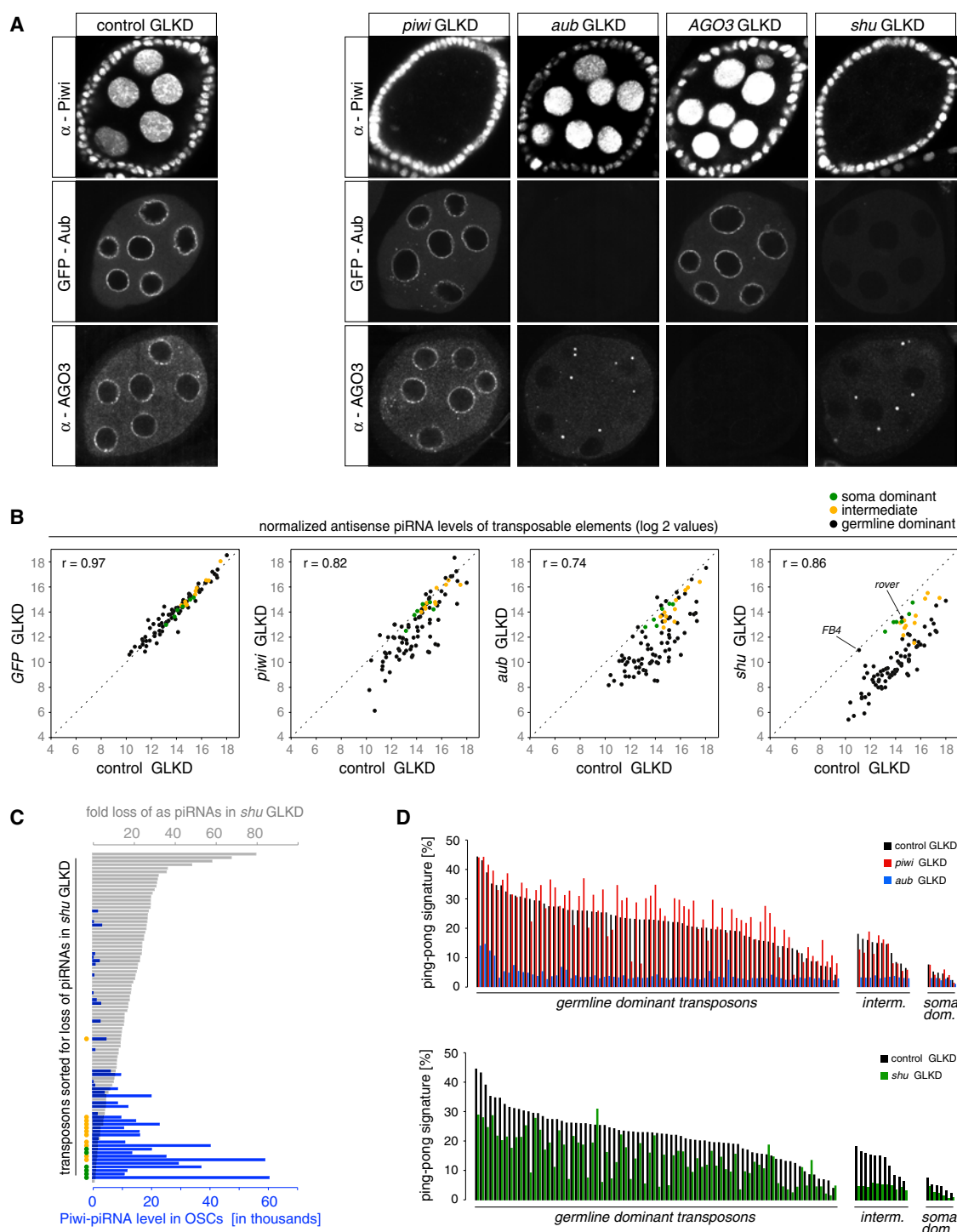


Figure 5. Probing the Importance and Role of Biogenesis Factors in the Germline

(A) Shown are confocal sections of egg chambers from indicated GLKDs stained for Piwi, Aub or AGO3.

(B) Scatter plots showing log2 values of normalized antisense piRNA levels mapping to soma dominant (green), intermediate (yellow) or germline dominant (black) TEs in indicated knockdowns (Pearson correlations based on germline dominant TEs).

(C) Shown is the fold-loss of piRNAs (gray bars) in *shu*-GLKD versus control-GLKD mapping to Repbase TEs (sorted for fold loss). Blue bars indicate levels of Piwi bound piRNAs for the same TEs. Bullets indicate soma-dominant (green), intermediate (yellow) and germline dominant (black) TEs.

(D) Shown are ping-pong signatures of germline dominant, intermediate and soma dominant TEs in control-GLKD (black bars) compared to *piwi*-, and *aub*-GLKDs (top) or *shu*-GLKD (bottom).

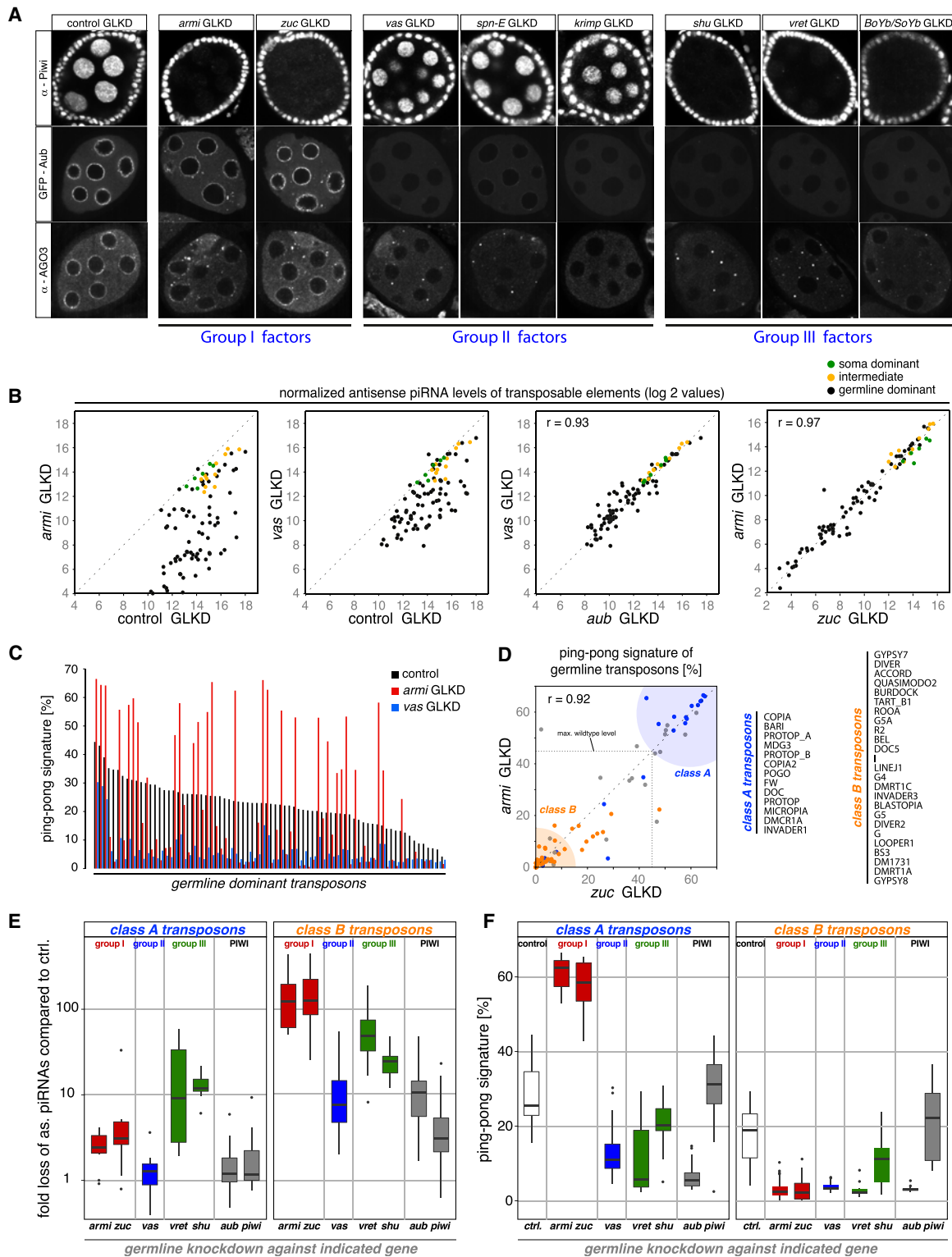


Figure 6. Three Groups of piRNA Biogenesis Factors

(A) Shown are confocal sections of egg chambers stained for Piwi, Aub or AGO3 from indicated GLKDs, which defines three groups of biogenesis factors. (B) Scatter plots showing log2 values of normalized antisense piRNA levels mapping to soma dominant (green), intermediate (yellow) or germline dominant (black) TEs in the indicated GLKDs (Pearson correlations based on all TEs). (C) Shown are ping-pong signatures of germline dominant TEs in control-GLKD (black bars) compared to *armi*- (red), and *vas*-GLKDs (blue).

or Aub in ovaries and follow the same trend (Brennecke et al., 2007). Thus, the rudimentary piRNA pathway in OSCs is fully capable of loading the germline specific Aub protein.

Ectopic expression of AGO3 did not yield efficient AGO3-piRISC formation (Figure 7C and 7E). Based on the following observations, we propose that primary piRNA biogenesis in OSCs is incompatible with AGO3 loading: (1) accumulating evidence suggests that PIWI proteins are degraded if not loaded with piRNAs. Indeed, AGO3 protein levels in OSCs were very low despite comparable mRNA levels. Moreover, GFP-Aub levels in OSCs were significantly reduced upon knockdown of any biogenesis factor including *shu* (Figure 7F) and Aub levels were nearly undetectable in *shu*-GLKD ovaries (Figure S1). (2) residual AGO3 colocalized with Krimp in a cytoplasmic body. This body also accumulated Shu, which was never found in Krimp bodies of un-transfected OSCs (not shown). siRNA mediated knockdown of Krimp in OSCs transfected with AGO3 did not result in higher AGO3 levels. Thus, AGO3 behaves in OSCs just as in germline cells that are defective in AGO3-piRISC formation. We conclude that the core genetic requirements for primary Piwi- and Aub-piRISC formation are likely identical and that the primary pathway is either incompatible with AGO3 loading or that AGO3 is actively prevented to be a substrate for primary piRISC formation.

Taken together, our analyses of piRNA populations from group I-III germline knockdowns as well as the analysis of PIWI protein biology in OSCs strongly argue for a very simple model (Figure S6): In somatic cells, a primary biogenesis machinery consisting of group I and group III factors loads Piwi with primary piRNAs. In germline cells, the same set of core factors is required to funnel primary piRNAs into Piwi and Aub but not AGO3. While it is unclear to which extent Piwi participates in ping-pong, Aub efficiently triggers secondary piRNA biogenesis via group II and group III factors and this is the only possibility to load AGO3 with piRNAs, which in turn triggers biogenesis of an Aub bound piRNA.

DISCUSSION

The outcome of this work is threefold: (1) The cochaperone Shutdown is essential for the biogenesis of all *Drosophila* piRNA populations. (2) Three major groups of piRNA biogenesis factors can be distinguished. (3) Piwi and Aub but not AGO3 are loaded with primary piRNAs, explaining how the cell maintains highly specific piRNA populations in the three PIWI proteins.

The Role of the Cochaperone Shutdown in piRNA Biogenesis

A remarkable feature of the *shu* mutant phenotype is that piRNA populations for every TE collapse. This already points to a common piRNA biogenesis step downstream of the primary

and secondary branches. Our epistatic analysis in somatic follicle cells is consistent with Shu acting at a late step in piRNA biogenesis: Shu is not required for the localization of any known biogenesis factor to Yb-bodies. On the other hand, Shu's localization to Yb-bodies depends on all other biogenesis factors and even on Piwi, arguing that unloaded Piwi recruits Shu to the Yb-body. Similarly, Shu colocalizes with nonloadable AGO3 in OSCs as well as in ovaries defective of ping-pong in discrete foci that also contain and are dependent on Krimp. Thus, in wild-type and in biogenesis mutants, Shu appears to colocalize with unloaded PIWI proteins.

Shu's C-terminal TPR domain falls into the class of Hsp90 binders (Scheufler et al., 2000) and Hsp90 is important for small RNA loading into Argonaute proteins (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). In addition, the plant cochaperone Cyp40 interacts with Hsp90 via its TPR domain and is a critical cofactor for small RNA loading into AGO1 (Iki et al., 2011). Our genetic and localization data support an analogous role for Shu and Hsp90 during small RNA loading into PIWI proteins. Clearly, in vitro assays will be crucial to dissect the precise order of events and the molecular role of Shu, especially its PPIase domain.

Three Groups of piRNA Biogenesis Factors in *Drosophila*

A major challenge in the field is to assemble piRNA biogenesis factors into pathways that explain the stereotypic populations of piRNAs in vivo. We took advantage of efficient germline specific knockdowns to study the impact of several factors on piRNA populations. Based on levels and localization of PIWI proteins as well as on piRNA populations obtained from several pathway factor knockdowns, we propose three major groups of piRNA biogenesis factors.

Group I factors are required for primary piRNA biogenesis but dispensable for secondary biogenesis. In fact, piRNAs that initiated ping-pong in group I knockdowns were amplified and ping-pong signatures of such TEs were strongly increased, presumably as primary piRNAs that do not feed into ping-pong were absent.

Group II factors are specific for ping-pong, as primary piRNA biogenesis feeding into Piwi was unaffected. An alternative explanation that we cannot exclude is that some or all group II genes are required specifically for Aub biology (primary and secondary) per se. This would similarly leave Piwi bound piRNAs intact and would lead to a collapse in ping-pong. Given our data on Aub loading in OSCs, we favor, however, a model where the primary biogenesis machineries that feed Aub and Piwi are very similar.

Finally, group III factors are required for the biogenesis of Piwi/Aub/AGO3 bound piRNAs. The prototypic member of this group is Shu. Loss of Shu affects essentially all piRNA populations to the same extent. We note that analysis of piRNA populations from *vret* mutants indicated a role for this group III factor

(D) Scatter plot showing the correlation (r = Pearson) of ping-pong signatures between *armi*- and *zuc*-GLKD libraries. The dashed line indicates the maximum value of any element in control GLKD. Elements with piRNA reductions < 5-fold are in blue, those with > 50-fold loss are in orange, all others in gray. Class A and class B TEs are listed to the right.

(E) Box plots showing the fold loss of antisense piRNAs mapping to class A (left) or class B (right) TEs in the indicated GLKD libraries compared to the control GLKD library.

(F) Box plots showing the ping-pong signatures of class A (left) or B (right) TEs in the control GLKD library (white) or the indicated GLKD libraries.

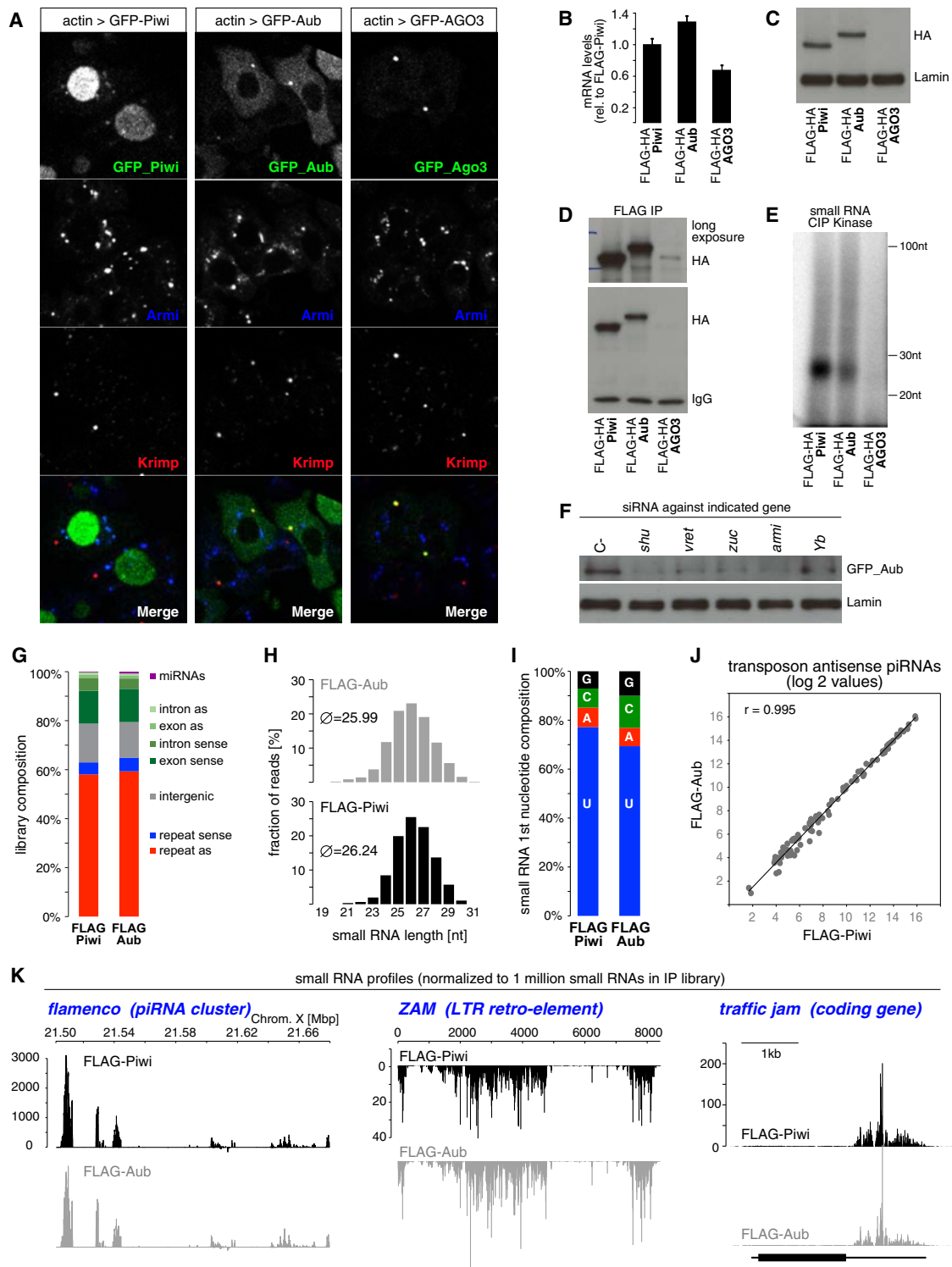


Figure 7. Piwi and Aub, but Not AGO3 Can Be Loaded by the Somatic Primary piRNA Biogenesis Pathway

(A) Shown are IF images of OSCs transfected with the indicated GFP-tagged PIWI proteins (green) stained for Armi (blue) and Krimp (red). The bottom row shows the overlay of all channels.

(B) Shown are relative levels of indicated FLAG/HA-PIWI mRNAs isolated from transfected cells ($n = 3$; error bars indicate StDev).

(C) Shown are protein levels of FLAG/HA-PIWI proteins isolated from transfected cells in comparison to Lamin levels.

(D) IP-western analysis indicating efficient immunoprecipitation of tagged Piwi and Aub, but not AGO3 from lysate of transfected OSCs.

in primary biogenesis but not ping-pong (Handler et al., 2011; Zamparini et al., 2011). The distorted tissue composition of *vret* mutant ovaries coupled with perdurance of maternal Vret protein or RNA may underlie this discrepancy. The existence of group III factors predicts that primary and secondary piRNA biogenesis feed into a final piRISC maturation step that requires a set of common factors for all PIWI proteins. Given that piRNA biogenesis—irrespective of the source of the precursor RNA—requires an RNA loading step as well as a 3' trimming step, the existence of group III factors suggests itself.

The three proposed groups serve as a rough classification of biogenesis factors. Clearly, at a molecular level, the precise role of each factor within the biogenesis process will vary considerably. Of note, the classification of group I and group II genes extends to the mouse piRNA pathway. The *Armi* and *Zuc* orthologs *MOV10L1* and *PLD6* are required for primary piRNA biogenesis (Watanabe et al., 2011; Zheng et al., 2010), whereas mouse *VAS* and *TDRD9* (mouse *Spn-E*) were reported to be dispensable for primary biogenesis but are required for secondary biogenesis pathway (Kuramochi-Miyagawa et al., 2010; Shoji et al., 2009).

Wiring of PIWI Proteins into piRNA Biogenesis Pathways

Our data indicate that Aub is not only loaded via ping-pong, but also via primary piRNA biogenesis. We also postulate that Aub and Piwi proteins are wired into primary piRNA biogenesis processes in a very similar manner, meaning that they require the same or highly overlapping core factors (e.g., *Armi* or *Zuc*). In agreement with this, ectopically expressed Aub is loaded in OSCs that harbor a fully functional primary pathway but lack critical ping-pong factors such as *Vas*. We showed that the genetic requirements for Aub loading in OSCs are identical to those for Piwi. We extrapolate from this that the core primary biogenesis machinery that loads Piwi in the soma also loads Piwi and Aub in the germline. Analyses of piRNA populations from *armi* versus *piwi* or *aub*-GLKDs support a model where *Armi* and *Zuc* are required for the biogenesis of both Piwi and Aub bound primary piRNAs. We do not exclude the possibility that—despite a similar biogenesis machinery—populations of primary piRNAs in Aub and Piwi are different. For example, differences in subcellular localizations of PIWI proteins as well as piRNA precursor RNAs might result in such differences.

In contrast to Aub, AGO3 was unstable in OSCs. Coexpression of Aub or simultaneous knockdown of *krimp* had no impact on AGO3 stability. We therefore conclude that primary piRNA biogenesis is incompatible with AGO3. In fact, also in the germline genetic data indicated that AGO3 depends on secondary piRNA biogenesis for being loaded (Li et al., 2009; Malone et al., 2009). Blocking AGO3's access to the primary biogenesis machinery would allow the cell to load AGO3 with a unique

class of piRNAs if it couples AGO3 loading to a precursor RNA originating from Aub-slicer mediated cleavage of an active TE. This would explain the remarkable bias of AGO3 bound piRNAs being sense and carrying an Adenosine at position ten.

Interestingly, on a primary sequence level Aub—despite its significantly different biology—is more closely related to Piwi than to AGO3. A critical question emanating from this is to which extent Piwi is participating in ping-pong, and if it does not, why. A weak, yet statistically significant, ping-pong signature has been observed between Piwi and AGO3 bound piRNAs (Brennecke et al., 2007; Li et al., 2009). This could mean that there is indeed low level of Piwi-AGO3 ping-pong. An alternative explanation is that the Piwi-AGO3 signal is a misleading computational signal: If Piwi and Aub are loaded via the same primary biogenesis machinery, initiator piRNAs for ping-pong that end up in Aub also end up in Piwi. As primary piRNA biogenesis appears to be nonrandom and preferentially processed piRNAs likely trigger ping-pong more robustly, an “artificial” AGO3/Piwi ping-pong signature might result.

What could be the molecular basis of why Piwi does not or only moderately participate in ping-pong? Either, specific features of Aub (e.g., symmetric Arginine methylation) are funneling this protein into ping-pong and similar features are absent on Piwi. Or, the mere sequestration of Piwi into the nucleus prevents Piwi from participating in ping-pong. Notably, N-terminally truncated Piwi that is still loaded but that cannot translocate into the nucleus is enriched in nuage the proposed site of secondary piRNA biogenesis (Klenov et al., 2011). A simple difference in the subcellular localization of Aub and Piwi might thus contribute to the dramatic differences of piRNA populations residing in Aub or Piwi.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

Flies (stocks listed in Table S4) were kept at 25°C and were aged 5 days before analysis.

Immunocytochemistry

Rabbit (rb) α -Shu was raised against peptides C-SPIQEDVLTLSKPDVKFA (western and IP) and MEENFEPYTPQLKNP-C (IF). Other antibodies were: rb α -Piwi, α -Aub, α -AGO3 (Brennecke et al., 2007), rb α -Armi (Handler et al., 2011), mouse α -Armi (Saito et al., 2010), rb α -Vret and rb α -Yb (Handler et al., 2011), rb α -Krimp (Lim and Kai, 2007), mouse α -FLAG (SIGMA M2), mouse α -HA (16B12), mouse α -beta-Gal (Promega Z378B), mouse α -Lamin (DSHB ADL67.10). MitoTracker Red CMXRos was used according to the manufacturer's instructions.

Cell Culture

OSCs were cultured as described in Niki et al. (2006) and transfected with Nucleofector kit V (Amaxa; program T-029). siRNAs (Table S5) were transfected 2x and cells analyzed after 96 hr.

(E) CIP-Kinase experiment indicating efficient loading of transfected Piwi and Aub in OSCs.

(F) Western blot indicating that ectopically expressed GFP-Aub is destabilized in OSCs upon knockdown of piRNA biogenesis factors.

(G) Annotation of small RNAs isolated from indicated IPs from OSCs.

(H) Size profile of small RNAs isolated from indicated IPs.

(I) First nucleotide bias of small RNAs isolated from indicated IPs.

(J) Scatter plot of antisense piRNAs isolated from indicated IPs mapping to Repbase TEs.

(K) Profiles of small RNAs isolated from indicated IPs mapping uniquely to the *flamenco* cluster (left; 200 nt walking window) to the *ZAM* TE (middle) or to the *traffic jam* locus (right). All plots were normalized to one million small RNAs in the respective library.

act > *GFP_Shu*, *act* > *FLAG/HA_Piwi/Aub/AGO3*, *act* > *GFP_Piwi/Aub/AGO3* plasmids were produced with the Gateway Collection (DGRC).

Immunoprecipitation

IPs were performed as in Olivieri et al. (2010).

Small RNA Cloning

Total ovarian RNA was isolated with TRIzol and small RNAs from IPs with Phenol/Chloroform. Small RNA libraries were generated as described in Brennecke et al. (2007).

piRNA Labeling

piRNAs purified from IPs were dephosphorylated (CIP) and radioactively labeled with T4 PNK.

Transposon qPCR Analysis

qPCR analysis (primers in Table S6) was carried out according to Handler et al. (2011).

Bioinformatics Analysis

Raw libraries were demultiplexed, trimmed off linker sequences and mapped to the genome (100% match; release 5). For piRNA cluster mapping we considered genome-unique mappers, for TE mappings (Repbase; Jurka et al., 2005) all mappers (up to 3 MM). Libraries were normalized to 1 Mio miRNA reads. Small RNAs mapping to rRNAs, tRNAs and snoRNAs were excluded. The calculation of TE piRNA levels was based on antisense piRNAs. Ping-pong signatures were calculated as in Malone et al. (2009).

ACCESSION NUMBERS

Small RNA libraries were deposited at GEO (accession number GSE38728).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2012.07.021>.

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REFERENCES

- Anand, A., and Kai, T. (2011). The tudor domain protein kumo is required to assemble the nuage and to generate germline piRNAs in *Drosophila*. *EMBO J.* 31, 870–882.
- Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128, 1089–1103.
- Brennecke, J., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A., and Hannon, G.J. (2008). An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science* 322, 1387–1392.
- Crackower, M.A., Kolas, N.K., Noguchi, J., Sarao, R., Kikuchi, K., Kaneko, H., Kobayashi, E., Kawai, Y., Kozieradzki, I., Landers, R., et al. (2003). Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. *Science* 300, 1291–1295.
- Gangaraju, V.K., Yin, H., Weiner, M.M., Wang, J., Huang, X.A., and Lin, H. (2011). *Drosophila* Piwi functions in Hsp90-mediated suppression of phenotypic variation. *Nat. Genet.* 43, 153–158.
- Gunawardane, L.S., Saito, K., Nishida, K.M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., and Siomi, M.C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. *Science* 315, 1587–1590.
- Haase, A.D., Fenoglio, S., Muerdter, F., Guzzardo, P.M., Czech, B., Pappin, D.J., Chen, C., Gordon, A., and Hannon, G.J. (2010). Probing the initiation and effector phases of the somatic piRNA pathway in *Drosophila*. *Genes Dev.* 24, 2499–2504.
- Handler, D., Olivieri, D., Novatchkova, M., Gruber, F.S., Meixner, K., Mechtler, K., Stark, A., Sachidanandam, R., and Brennecke, J. (2011). A systematic analysis of *Drosophila* TUDOR domain-containing proteins identifies Vreteno and the Tdrd12 family as essential primary piRNA pathway factors. *EMBO J.* 30, 3977–3993.
- Iki, T., Yoshikawa, M., Nishikiori, M., Jaudal, M.C., Matsumoto-Yokoyama, E., Mitsuhashi, I., Meshi, T., and Ishikawa, M. (2010). In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. *Mol. Cell* 39, 282–291.
- Iki, T., Yoshikawa, M., Meshi, T., and Ishikawa, M. (2011). Cyclophilin 40 facilitates HSP90-mediated RISC assembly in plants. *EMBO J.* 31, 267–278.
- Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., and Tomari, Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell* 39, 292–299.
- Jurka, J., Kapitonov, V.V., Pavlicek, A., Klonowski, P., Kohany, O., and Walichiewicz, J. (2005). Repbase Update, a database of eukaryotic repetitive elements. *Cytogenet. Genome Res.* 110, 462–467.
- Kawaoka, S., Izumi, N., Katsuma, S., and Tomari, Y. (2011). 3' end formation of PIWI-interacting RNAs in vitro. *Mol. Cell* 43, 1015–1022.
- Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., Cook, H.A., and Theurkauf, W.E. (2007). *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev. Cell* 12, 45–55.
- Klenov, M.S., Sokolova, O.A., Yakushev, E.Y., Stolyarenko, A.D., Mikhaleva, E.A., Lavrov, S.A., and Gvozdev, V.A. (2011). Separation of stem cell maintenance and transposon silencing functions of Piwi protein. *Proc. Natl. Acad. Sci. USA* 108, 18760–18765.
- Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Takamatsu, K., Chuma, S., Kojima-Kita, K., Shiromoto, Y., Asada, N., Toyoda, A., Fujiyama, A., et al. (2010). MVH in piRNA processing and gene silencing of retrotransposons. *Genes Dev.* 24, 887–892.
- Lau, N.C., Robine, N., Martin, R., Chung, W.J., Niki, Y., Berezikov, E., and Lai, E.C. (2009). Abundant primary piRNAs, endo-siRNAs, and microRNAs in a *Drosophila* ovary cell line. *Genome Res.* 19, 1776–1785.
- Li, C., Vagin, V.V., Lee, S., Xu, J., Ma, S., Xi, H., Seitz, H., Horwich, M.D., Syrzycka, M., Honda, B.M., et al. (2009). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* 137, 509–521.
- Lim, A.K., and Kai, T. (2007). Unique germ-line organelle, nuage, functions to repress selfish genetic elements in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 104, 6714–6719.
- Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. *Cell* 136, 656–668.
- Malone, C.D., Brennecke, J., Dus, M., Stark, A., McCombie, W.R., Sachidanandam, R., and Hannon, G.J. (2009). Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. *Cell* 137, 522–535.

- Miyoshi, T., Takeuchi, A., Siomi, H., and Siomi, M.C. (2010). A direct role for Hsp90 in pre-RISC formation in *Drosophila*. *Nat. Struct. Mol. Biol.* **17**, 1024–1026.
- Munn, K., and Steward, R. (2000). The shut-down gene of *Drosophila melanogaster* encodes a novel FK506-binding protein essential for the formation of germline cysts during oogenesis. *Genetics* **156**, 245–256.
- Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., et al. (2011). A genome-scale shRNA resource for transgenic RNAi in *Drosophila*. *Nat. Methods* **8**, 405–407.
- Niki, Y., Yamaguchi, T., and Mahowald, A.P. (2006). Establishment of stable cell lines of *Drosophila* germ-line stem cells. *Proc. Natl. Acad. Sci. USA* **103**, 16325–16330.
- Nishida, K.M., Okada, T.N., Kawamura, T., Mituyama, T., Kawamura, Y., Inagaki, S., Huang, H., Chen, D., Kodama, T., Siomi, H., and Siomi, M.C. (2009). Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in *Drosophila* germlines. *EMBO J.* **28**, 3820–3831.
- Olivieri, D., Sykora, M.M., Sachidanandam, R., Mechtler, K., and Brennecke, J. (2010). An in vivo RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in *Drosophila*. *EMBO J.* **29**, 3301–3317.
- Patil, V.S., and Kai, T. (2010). Repression of retroelements in *Drosophila* germline via piRNA pathway by the Tudor domain protein Tejas. *Curr. Biol.* **20**, 724–730.
- Qi, H., Watanabe, T., Ku, H.Y., Liu, N., Zhong, M., and Lin, H. (2011). The Yb body, a major site for Piwi-associated RNA biogenesis and a gateway for Piwi expression and transport to the nucleus in somatic cells. *J. Biol. Chem.* **286**, 3789–3797.
- Robine, N., Lau, N.C., Balla, S., Jin, Z., Okamura, K., Kuramochi-Miyagawa, S., Blower, M.D., and Lai, E.C. (2009). A broadly conserved pathway generates 3'UTR-directed primary piRNAs. *Curr. Biol.* **19**, 2066–2076.
- Saito, K., Inagaki, S., Mituyama, T., Kawamura, Y., Ono, Y., Sakota, E., Kotani, H., Asai, K., Siomi, H., and Siomi, M.C. (2009). A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. *Nature* **461**, 1296–1299.
- Saito, K., Ishizu, H., Komai, M., Kotani, H., Kawamura, Y., Nishida, K.M., Siomi, H., and Siomi, M.C. (2010). Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in *Drosophila*. *Genes Dev.* **24**, 2493–2498.
- Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101**, 199–210.
- Schüpbach, T., and Wieschaus, E. (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**, 1119–1136.
- Senti, K.A., and Brennecke, J. (2010). The piRNA pathway: a fly's perspective on the guardian of the genome. *Trends Genet.* **26**, 499–509.
- Shoji, M., Tanaka, T., Hosokawa, M., Reuter, M., Stark, A., Kato, Y., Kondoh, G., Okawa, K., Chujo, T., Suzuki, T., et al. (2009). The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. *Dev. Cell* **17**, 775–787.
- Siomi, M.C., Sato, K., Pezic, D., and Aravin, A.A. (2011). PIWI-interacting small RNAs: the vanguard of genome defence. *Nat. Rev. Mol. Cell Biol.* **12**, 246–258.
- Specchia, V., Piacentini, L., Tritto, P., Fanti, L., D'Alessandro, R., Palumbo, G., Pimpinelli, S., and Bozzetti, M.P. (2010). Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. *Nature* **463**, 662–665.
- Vagin, V.V., Wohlschlegel, J., Qu, J., Jonsson, Z., Huang, X., Chuma, S., Girard, A., Sachidanandam, R., Hannon, G.J., and Aravin, A.A. (2009). Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. *Genes Dev.* **23**, 1749–1762.
- Watanabe, T., Chuma, S., Yamamoto, Y., Kuramochi-Miyagawa, S., Totoki, Y., Toyoda, A., Hoki, Y., Fujiyama, A., Shibata, T., Sado, T., et al. (2011). MITOPLD is a mitochondrial protein essential for nuage formation and piRNA biogenesis in the mouse germline. *Dev. Cell* **20**, 364–375.
- Zamparini, A.L., Davis, M.Y., Malone, C.D., Vieira, E., Zavadil, J., Sachidanandam, R., Hannon, G.J., and Lehmann, R. (2011). Vreteno, a gonad-specific protein, is essential for germline development and primary piRNA biogenesis in *Drosophila*. *Development* **138**, 4039–4050.
- Zhang, Z., Xu, J., Koppetsch, B.S., Wang, J., Tipping, C., Ma, S., Weng, Z., Theurkauf, W.E., and Zamore, P.D. (2011). Heterotypic piRNA Ping-Pong requires qin, a protein with both E3 ligase and Tudor domains. *Mol. Cell* **44**, 572–584.
- Zheng, K., Xiol, J., Reuter, M., Eckardt, S., Leu, N.A., McLaughlin, K.J., Stark, A., Sachidanandam, R., Pillai, R.S., and Wang, P.J. (2010). Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. *Proc. Natl. Acad. Sci. USA* **107**, 11841–11846.

3.4 Supplemental information

**The Cochaperone Shutdown Defines
a Group of Biogenesis Factors Essential
for All piRNA Populations in *Drosophila***

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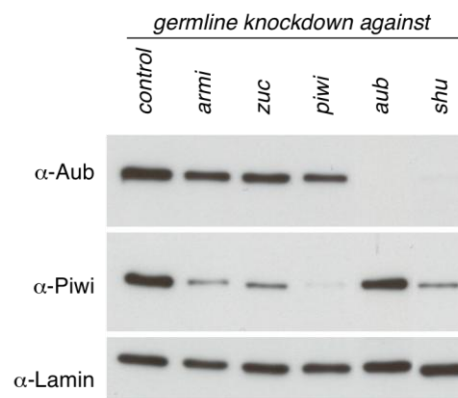


Figure S1.

Western blot analysis indicating protein levels of the PIWI members Piwi and Aub upon knockdown of the indicated genes. Lamin levels are shown for normalization purposes. While Aub levels were essentially undetectable in the *aub*-GLKD ovary lysate, low levels of Piwi were still observed in *piwi*-GLKD ovary lysate. This most likely reflects Piwi from somatic follicle cells that are unaffected by the GLKD.

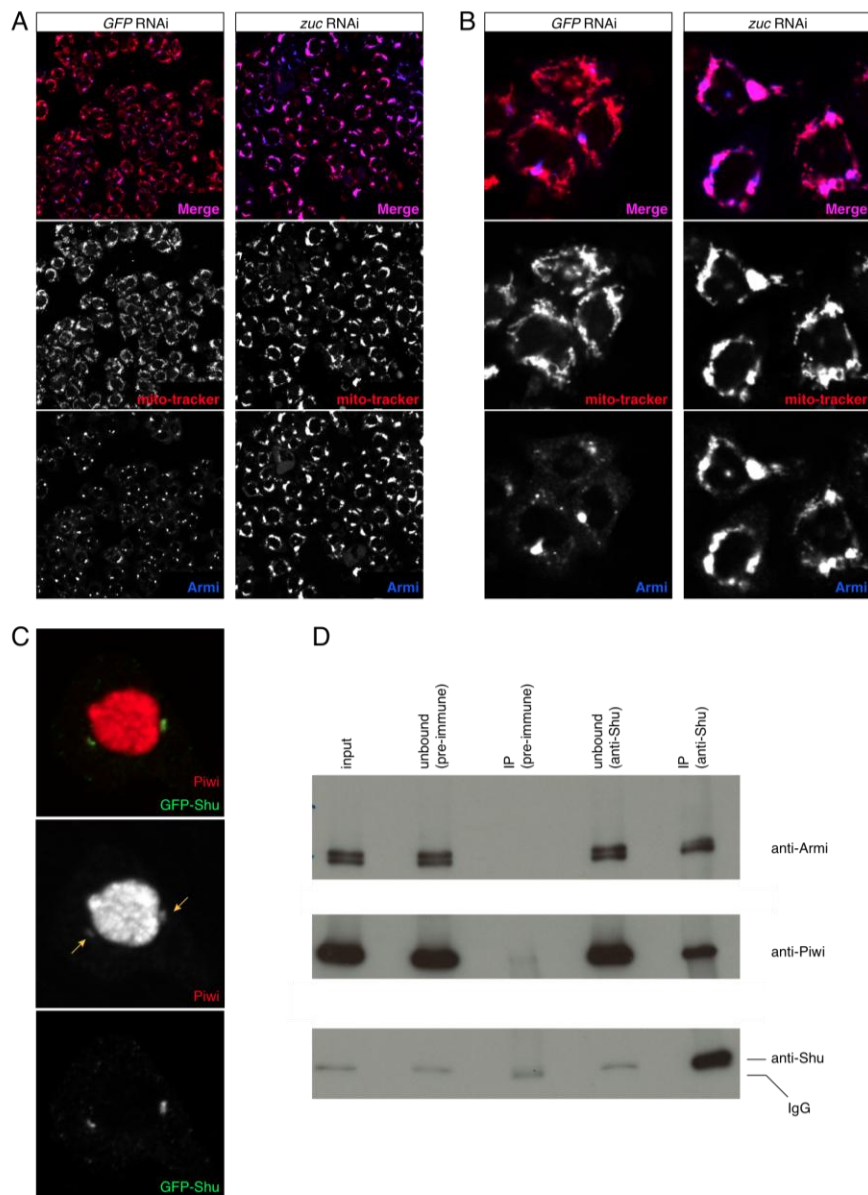


Figure S2.

(A) Shown are immuno fluorescence stainings of OSCs treated with GFP siRNAs (left) or *zuc* siRNAs (right). Mitochondria were visualized with Mito-tracker (red) and Yb-bodies with anti-Armi stainings (blue). In the merge image (top row), overlap of red and blue signal appears magenta. This illustrates the nearly complete co-localization of Armi and mitochondria in *zuc* knockdowns.

(B) Zoom in of an area shown in part (A).

(C) Immuno-fluorescence analysis of OSCs transfected with GFP-Shutdown (green) stained for Piwi (red). Yellow arrows indicate the faint but clearly visible accumulation of Piwi in peri-nuclear bodies that also accumulate Shu and represent therefore Yb-bodies.

(D) Western analysis of an anti-Shu IP experiment in comparison to a control IP experiment with pre-immune serum. Endogenous Armi and Piwi proteins are specifically co-purified with Shu from OSC lysate. The lower running and cross-reacting band in the Shu blot is likely IgG heavy chain, low levels of which are still present in the IP fraction despite the antibody being chemically cross-linked to the beads.

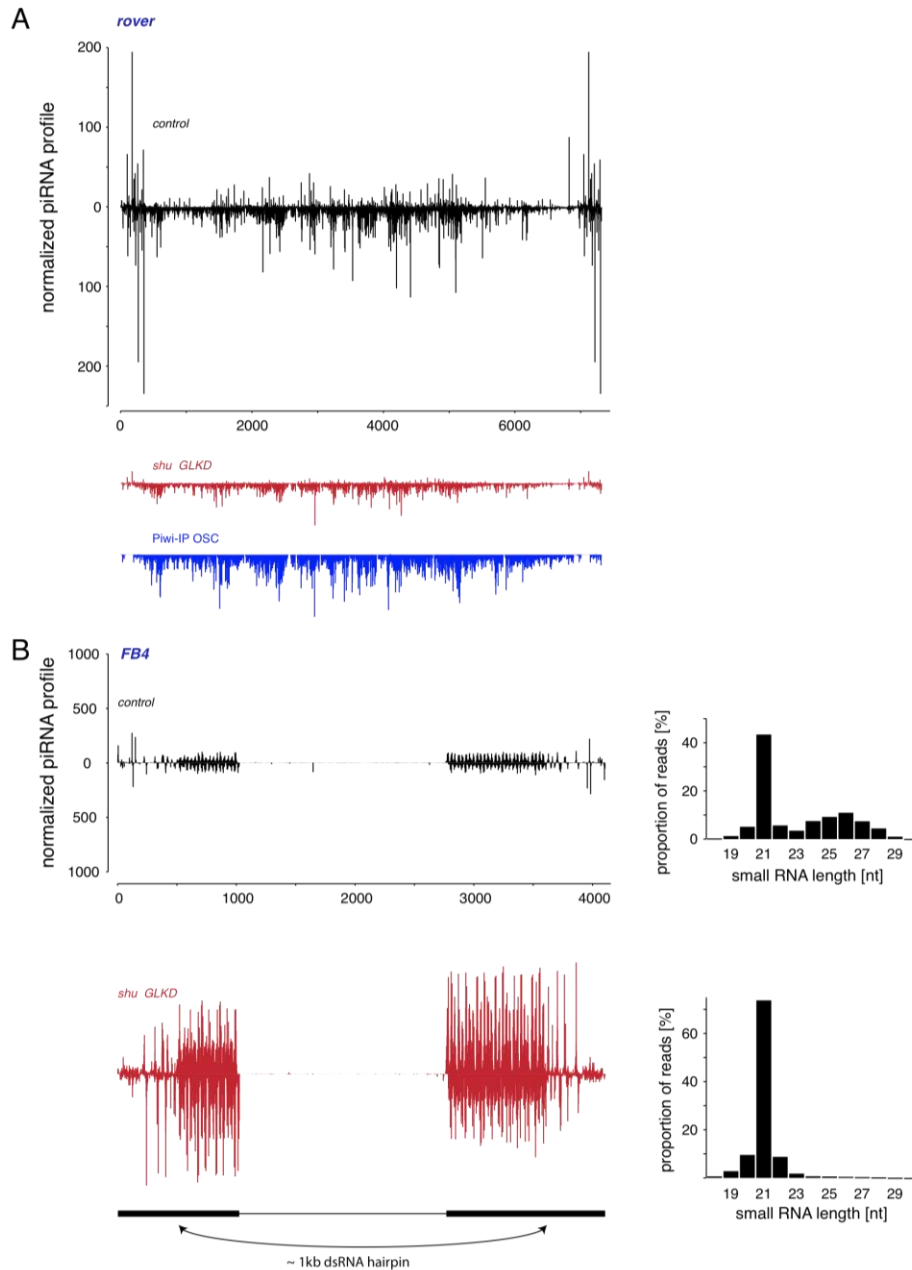


Figure S4.

(A) Shown are normalized piRNA profiles (23-30nt) mapping to the annotated “germline dominant” element *rover* in control GLKD (black) and *shu*-GLKD (red) libraries. Below, the piRNA profile from an OSC Piwi-IP library is shown, indicating that *rover* is a prototypical intermediate element.

(B) Shown are small RNA profiles (18-30nt) mapping to the DNA element *FB4* in control GLKD (black) and *shu*-GLKD (red) libraries. To the right, the length profiles of the corresponding small RNA populations are shown, indicating a complete shift to siRNAs in the *shu*-GLKD library. The cartoon below indicates the sequence stretches that are capable of folding into an extended dsRNA hairpin. For *shu*-GLKD we used the NGT > Dicer2 system and expressed the *shu* VDRC line (expression of an shRNA line resulted in distorted ovarian morphology); it is possible that the over-expression of Dicer2 contributes to the strong increase in siRNAs.

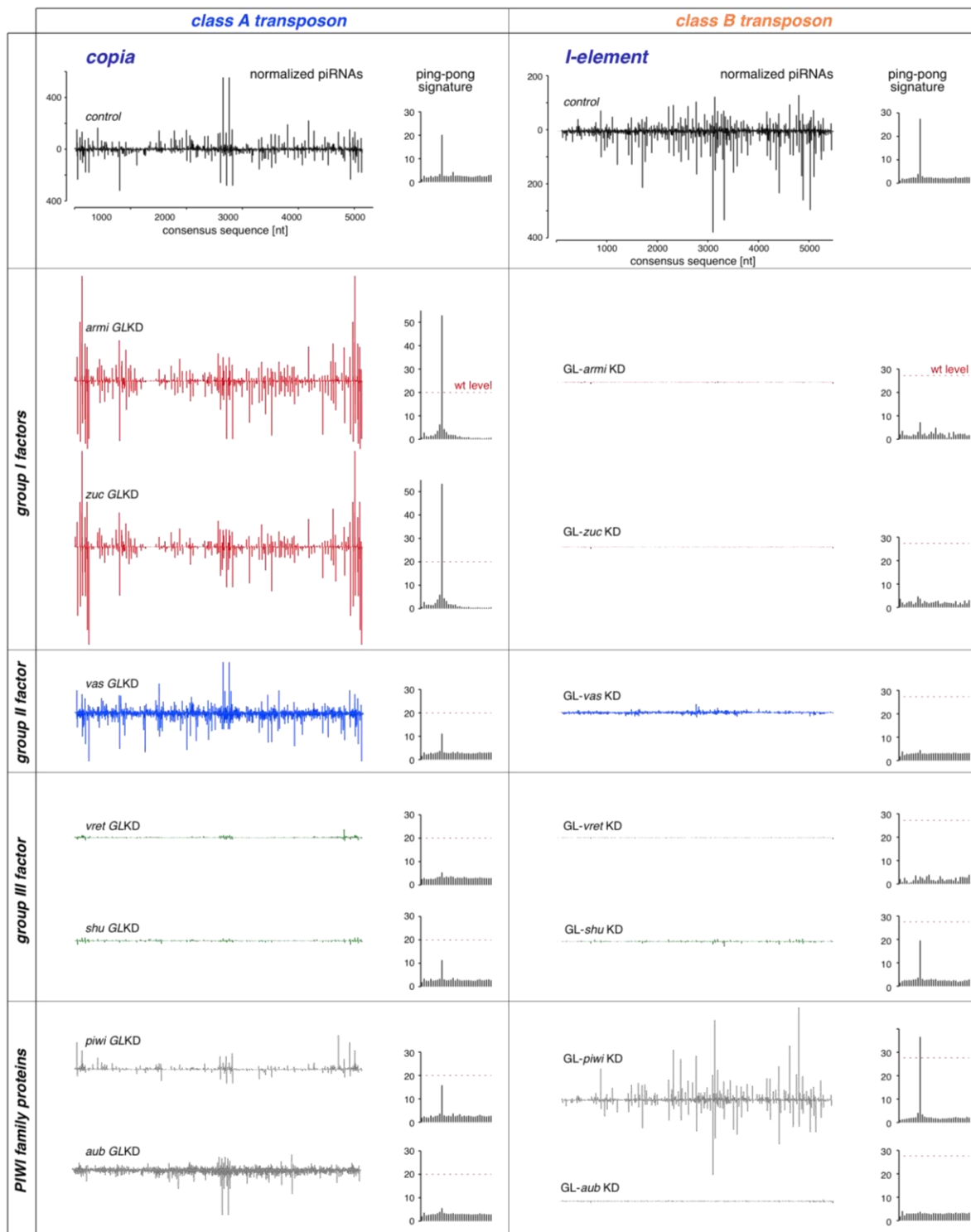


Figure S5.

Shown are normalized piRNA profiles mapping to the class A element *copia* (left column) and the class B element *I-element* (right column) in control-GLKD (top) and all other analyzed GLKD libraries that are classified into the biogenesis groups I-III. Also shown are the ping-pong signatures for each element in each library with the levels obtained from the control-GLKD library indicated with a red dashed line.

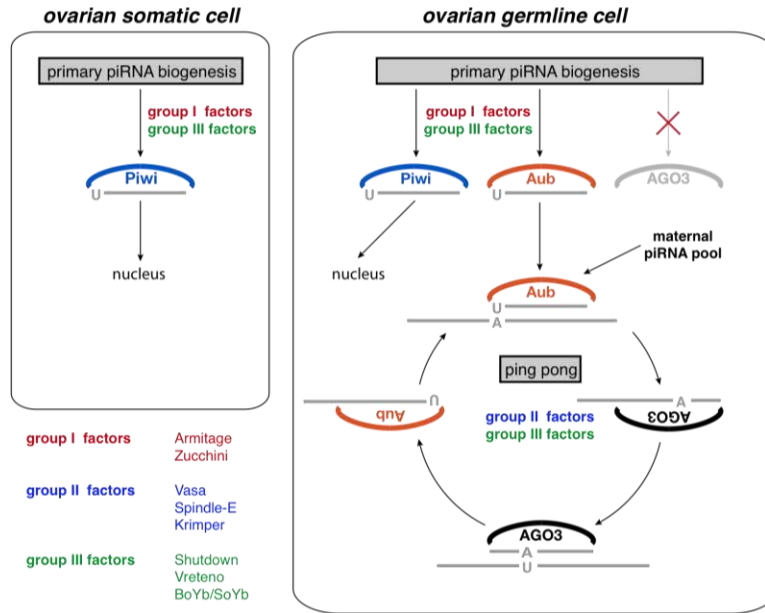


Figure S6.

Model of primary and secondary (ping-pong) piRNA biogenesis in *Drosophila*. Depicted are a somatic and a germline cell from the *Drosophila* ovary. The involvement of group I-III biogenesis factors as well as their identity is indicated.

Table S4 (used fly stocks)

<i>shu</i> ¹ /CyO (WQ41) and <i>shu</i> ² /CyO (WM40) (Schupbach and Wieschaus, 1991)
Df(2R)BSC665/SM6a (Bloom. # 26517);
<i>tj</i> -GAL4 (DGRC # 104055);
UAS- <i>Dcr-2</i> ; NGT (Bloom. # 25751);
P{GAL4::VP16- <i>nos</i> .UTR}CG6325 ^{MVD1} (Bloom. # 4937);
MTD-GAL4 (Ni et al., 2011);
<i>w</i> ¹¹¹⁸ (Bloom. # 3605);
<i>armi</i> shRNA, <i>vret</i> shRNA, <i>SoYb</i> shRNA, <i>BoYb</i> shRNA, <i>spn-E</i> shRNA (Handler et al., 2011);
<i>piwi</i> shRNA, <i>aub</i> shRNA (Ni et al., 2011);
<i>armi</i> RNAi (VDRC # 16205);
<i>zuc</i> shRNA (TRiP # GL00111);
<i>shu</i> RNAi (VDRC # 105832);
<i>shu</i> shRNA (TRiP # GL00379);
<i>vas</i> , <i>krimp</i> and <i>AGO3</i> shRNAs (sequences in Table S1) were integrated into attP2.
eGFP_ <i>Shu</i> , eGFP_ <i>Aub</i> , and eGFP_ <i>Hsp83</i> were cloned by inserting N-terminal eGFP via bacterial recombineering into genomic rescue constructs and integrated into the attP2 landing site.
<i>shu</i> ² mutant mitotic clones were obtained with <i>hsflp</i> ¹²² ; FRT42D <i>arm</i> -lacZ;
control GLKD was obtained from MTD x <i>w</i> ¹¹¹⁸ ;
<i>GFP</i> , <i>armi</i> , <i>zuc</i> , <i>piwi</i> , <i>vret</i> , <i>SoYb/BoYb</i> , <i>vas</i> , <i>spn-E</i> , <i>krimp</i> , <i>aub</i> and <i>AGO3</i> GLKDs were obtained from MTD-GAL4 and the respective shRNAs. <i>shu</i> GLKD was obtained from UAS- <i>Dcr-2</i> ; NGT; P{GAL4::VP16- <i>nos</i> .UTR}CG6325 ^{MVD1} and <i>shu</i> RNAi.
GLKDs of <i>shu</i> and <i>armi</i> for Fig. 1 were obtained with UAS- <i>Dcr-2</i> ; NGT; nosGAL4 and the respective VDRC lines.
All flies were aged 5 days at 25°C before analysis.

Table S5 (oligos used for shRNA cloning)

GENE	Primer	Sequence
<i>vas</i>	<i>vas_top</i>	ctagcagtAGCGATGTTCCACAACCTATAtagttatattcaagcataTATAGGTTGTGGAACATCGCTgcg
	<i>vas_bottom</i>	aattcgcAGCGATGTTCCACAACCTATAtatgcttgaatataactaTATAGGTTGTGGAACATCGCTactg
<i>krimp</i>	<i>krimp_top</i>	ctagcagtCAGATTGGGAGACTACGAATAtagttatattcaagcataTATTCGTAGTCTCCCAATCTGcg
	<i>krimp_bottom</i>	aattcgcCAGATTGGGAGACTACGAATAtatgcttgaatataactaTATTCGTAGTCTCCCAATCTGactg
<i>AGO3</i>	<i>AGO3_top</i>	ctagcagtCTGGTTGATCTTTATCAGCAAtagttatattcaagcataTTGCTGATAAAGATCAACCAgcg
	<i>AGO3_bottom</i>	aattcgcCTGGTTGATCTTTATCAGCAAtatgcttgaatataactaTTGCTGATAAAGATCAACCAgactg

Table S6 (used siRNAs for RNAi in OSCs)

GENE	siRNA	Sequence
eGFP	eGFP_guide	ACUUCAGGGUCAGCUUGCCTT
	eGFP_passenger	GGCAAGCUGACCCUGAAGUTT
<i>fs(1)Yb</i>	Fs(1)Yb_guide	UUGAAGAUCUUAUCGCAGCTT
	Fs(1)Yb_passenger	GCUGCGAUAAGAUCUUCAATT
<i>Armi</i>	Armi_guide	UAAACUUAGCUUGACAGCGTT
	Armi_passenger	CGCUGUCAAGCUAAGUUUATT
<i>Vret</i>	Vret1_guide	UUGUAGAGCACAAUUUGUCTT
	Vret1_passenger	GACAAAUUGUGCUCUACAATT
	Vret2_guide	UUCCUUGAUUAUUAUCCACGTT
	Vret2_passenger	CGUGGAUAAUAUCAAGGAATT
<i>Shu</i>	Shu1_guide	UAGUCGCUGUCGCUGUCGCTT
	Shu1_passenger	GCGACAGCGACAGCGACUATT
	Shu2_guide	UUCGCAUCUCCUAUGAGCGTT
	Shu2_passenger	CGCUCAUAGGAGAUGCGAATT
<i>Piwi</i>	Piwi_passenger	CACCUUCACGCCUGGGAGCTT
	Piwi_guide	GCUCCCAGGCGUGAAGGUGTT
<i>Zuc</i>	Zuc_passenger	UUGUUGUGCAUCAAGUUCGTT
	Zuc_guide	CGAACUUGAUGCACAACAATT
<i>Krimp</i>	Krimp_guide	UAUAUAUUUCCAAUCGUCCTT
	Krimp_passenger	GGACGAUUGGAAAUAUAUATT

Table S7 (primers for QPCR analysis)

GENE	Primer	Sequence
<i>rp49</i>	rp49_fwd	CCGCTTCAAGGGACAGTATCTG
	rp49_rev	ATCTCGCCGAGTAAACGC
<i>ZAM</i>	ZAM_fwd	ACTTGACCTGGATACACTCACAAC
	ZAM_rev	GAGTATTACGGCGACTAGGGATAC
<i>HeT-A</i>	HeT-A_fwd	CGCGCGGAACCCATCTTCAGA
	HeT-A_rev	CGCCGCAGTCGTTTGGTGAGT
<i>blood</i>	blood_fwd	AACAATAGAAAGAAGCCACCGAAC
	blood_rev	AGTCATGGACTATTGAGGGTGTTG
<i>FLAG HA tag</i>	TAG_fwd	GCCGCATCTTTTACCCATACGA
	TAG_rev	GAGCAGCGTAATCTGGAACGTCA

4. Discussion

At the beginning of my PhD thesis time very little was known about piRNA biogenesis. Several lines of evidence strongly pointed towards the existence of two different pathways, namely primary and secondary biogenesis. Mostly from genetic studies, a handful of factors with likely roles in piRNA biogenesis were known. But no systematic study existed that rigorously tested these predictions and that attributed their role to either primary or secondary piRNA biogenesis, or both. I believe that the key relevance of our work lies in providing such a systematic frame for the analysis of piRNA biogenesis processes. Throughout our work we established tools to analyze piRNA biogenesis at a genetic and molecular level, we defined criteria to assess a factor's biogenesis pathway specificity, and we looked into the PIWI protein specificity of the different biogenesis pathways.

In our first work, we established an *in vivo* piRNA pathway assay that could be coupled to the genome-wide RNAi resource of the VDRC (Vienna *Drosophila* RNAi Center). Based on this, we discovered several novel primary piRNA biogenesis factors. Their cell-biological and genetic study led us to assign a key role to the previously identified cytoplasmic Yb-body in primary piRNA biogenesis. The Yb-body is key in our current understanding of the relationship between primary biogenesis factors, as their Yb body localization follows a linear epistasis (FIG. 4).

Our conclusions about primary piRNA biogenesis in somatic follicle cells turned out to also be highly informative for the germline piRNA pathway. In our second work, we could systematically assign a role of known biogenesis factors to either primary or secondary piRNA biogenesis, or both. We believe this represents an important advancement in the way of thinking about primary and secondary piRNA biogenesis processes and we hope that it will be used as a framework for the analysis of further biogenesis factors.

4.1 An *in vivo* assay to identify somatic piRNA pathway genes

Genetic screens have been instrumental in assigning specific roles to genes in various biological processes. As only very few factors were known to be involved in the recently

discovered piRNA pathway, we reasoned that a robust assay system to probe a gene's function in piRNA pathway integrity would be a powerful tool in the further dissection of this pathway. We set up such an assay *in vivo* in the follicle cells of the *Drosophila* ovary, using a published transposon activity sensor (Sarot et al., 2004) and the *Drosophila* RNAi stocks from the VDRC (Dietzl et al., 2007), which were available in house.

The choice of the tissue was based on several reasons. 1) Follicle cells harbor a simplified piRNA pathway and were therefore the easiest system to dissect genetically. Follicle cells express only Piwi but not Aubergine and AGO3 and consequently they do not exhibit secondary piRNA biogenesis (Lau et al., 2009; Li et al., 2009a). This minimized potential pitfalls: as ping-pong is an amplification cycle we feared that it could compensate for deficiencies in primary biogenesis or for suboptimal gene knock down efficiencies. 2) We had established efficient transgenic RNAi conditions in follicle cells at the time but knockdowns in the germline were much less promising. 3) A highly sensitive and specific transposon activity reporter active in somatic cells had been published (Sarot et al., 2004).

A further key advantage of the somatic ovarian cell system is the availability of a robust cell culture system derived from ovarian somatic stem cells (the ovarian somatic cells, OSCs) (Saito et al., 2009).

Given the availability of the VDRC library our established *in vivo* piRNA pathway assay would make a genome wide screen for novel pathway factors possible. Such a screen was indeed performed in our lab by a colleague and the underlying assay proved to be very sensitive and specific at the same time. Among the strongest hits there is so far no evidence of any false positives and at the same time all but one of the known pathway members were identified.

As any RNAi screen, there will be limitations: RNAi lines might not be available for every gene and the efficiency of available lines has not been systematically assessed. Without a doubt, this will lead to a certain number of false negatives. In addition, redundantly acting genes but also genes with essential cell viability roles will not be identifiable. We note, however, that by using RNAi it might be possible to also recover some essential genes provided that the knock down is not complete.

Theoretically it is also possible that the employed *gypsy* sensor is not a general proxy for piRNA pathway activity. Indeed, at least in the germline it is rather likely that different TEs are repressed in different ways, making the choice of the transposon for the sensor very critical. We believe, however, that in the somatic piRNA pathway most if not all TEs are targeted by the same core machinery and that the *gypsy* sensor therefore should capture the majority if not all important pathway genes.

As shown in our second paper, efficient transgenic RNAi using the VDRC library is also possible in germline cells. This would allow an adaptation of the somatic assay to the germline, as strong germline specific GAL4 drivers do exist. In the case of the germline pathway, the choice of the transposon sequence for the sensor is expected to be critical. We know for example that the importance of primary biogenesis varies for piRNAs targeting different transposons (Olivieri et al., 2012). Furthermore, the biological diversity among germline transposons is greater than among somatic transposons, which are essentially all retrotransposons. Finally, a special case could be represented by the group of telomeric TEs (Pardue et al., 2005). Considering the physiological role of their activity, telomeric transposons might be regulated by the piRNA pathway in a special manner.

4.2 The identification of somatic piRNA biogenesis genes

Besides making an unbiased genetic screen possible, the somatic piRNA pathway activity assay had some immediate applications. First of all it allowed us to test candidate genes for their involvement in the somatic piRNA pathway. Before the establishment of our knock down system, all studies of piRNA genes were based on classic genetic alleles, making it difficult to assess the specific contribution of the different tissues to the final phenotype or if a gene had a role in the follicle cells at all.

We tested some genes based on the literature or on our observations and we assigned a role in the somatic piRNA pathway to *fs(1)Yb*, *armitage*, *zucchini*, *vreteno*, *SoYb*, and *shutdown*. Knocking down any of these genes in follicle cells leads to very similar molecular phenotypes: somatic transposons are upregulated and Piwi protein levels drop. All of these proteins besides Shutdown harbor domains predicted to interact with RNA. We therefore hypothesized that they would act as piRNA biogenesis factors, rather than

factors involved in Piwi expression. To support this hypothesis we profiled piRNAs upon knock down of these genes: in all cases piRNAs were strongly reduced. It is formally impossible at the current stage to exclude a role for these identified factors in Piwi protein stability per se. However, given that loss of any of these factors leads to very similar effects on Piwi and given also the published literature, we favor a model in which defects in primary piRNA biogenesis lead to a loss of primary piRNAs and a degradation of unstable empty Piwi. If correct, Piwi protein loss would be a very simple indicator of defects in primary piRNA biogenesis.

4.3 The Yb-body, the site for primary piRNA biogenesis

Given the sparse knowledge about the piRNA pathway at the time I started my thesis, it was at that point rather unclear where in the cell piRNA biogenesis processes take place. It was even unclear whether these processes occur in the nucleus and/or in the cytoplasm. Our finding that the previously discovered cytoplasmic Yb-body is the likely site where piRNA biogenesis in follicle cells occurs was therefore an important entry point in the understanding of the cellular compartmentalization of the pathway.

The Yb-body (Szakmary et al., 2009) is an electron-dense sphere associated with mitochondria and flanked by an RNA-rich body (possibly a P-body). It was defined based on the highly specific localization of the Fs(1)Yb RNA helicase to it. As not only Fs(1)Yb, but also Armitage, Vreteno, Shutdown, and Piwi localize to the Yb-body, it is the likely center of piRNA biogenesis in somatic ovarian cells. All Yb-body components co-immunoprecipitate other Yb-body components (Handler et al., 2011; Olivieri et al., 2012; Olivieri et al., 2010; Saito et al., 2010; Zamparini et al., 2011) hinting at a tight protein network. The participation in higher order structures might be the cause of the accumulation of these biogenesis factors in the Yb-body. In support of this hypothesis, Yb-body localization of the various factors obeys to a strict epistasis (FIG. 4): Fs(1)Yb determines the localization of all other factors; Armitage –while dispensable for Yb localization– is necessary for the localization of Vreteno, which in turn is required for proper Shutdown and Piwi localization (Handler et al., 2011; Olivieri et al., 2012; Olivieri et al., 2010; Saito et al., 2010; Zamparini et al., 2011). This Yb-body hierarchy might mirror the involvement of the various factors in subsequent biogenesis processes.

It is important to note that both Fs(1)Yb and the Yb-body are *Drosophila* and follicle cell specific (Szakmary et al., 2009). The only function of Fs(1)Yb might therefore be the formation of the Yb-body. If this hypothesis is true, then the formation of this organelle alone is an essential requirement for somatic piRNA biogenesis.

Based on electron microscopy, Yb-bodies are in contact with mitochondria (Szakmary et al., 2009). Given that Zucchini is an integral protein of the outer mitochondrial membrane also this essential biogenesis factor can essentially be considered a Yb-body factor, although it is important to note that Zucchini seemingly localizes to all mitochondria in the cell (Saito et al., 2010). Loss of Zucchini does not delocalize Yb-body factors, but rather causes their accumulation on the surface of mitochondria. This might indicate that as yet unknown piRNA biogenesis factors are associated with the outer mitochondrial membrane and recruit Yb body factors to the mitochondrial surface.

Similar to observations in mouse, loss of Zucchini causes mitochondrial aggregation (Olivieri et al., 2012). How this is related to the proposed nuclease activity of Zucchini is not understood. Maybe Zucchini's nucleolytic activity is required for the release of piRNA biogenesis factors from the Yb-body or mitochondria. If this is the case, the interaction between piRNA precursor molecules and RNA helicases on the mitochondrial surface might cluster mitochondria together.

The link between piRNA biogenesis and mitochondria is widely conserved and holds true also for the germline (Chuma et al., 2009; Mahowald, 1971). It can only be speculated about the function of this association. As mitochondria represent a prominent share of the intracellular membrane surface, one hypothesis is that some step in piRNA biogenesis needs to happen close to a cell membrane.

Based on our data, Yb-bodies are in close proximity to processing bodies (P-bodies). But as knocking down critical P-body components such as Lsm1 or Me31b (Eulalio et al., 2007) did not impact transposon silencing, we believe that P-bodies are not required for piRNA biogenesis. Nevertheless the physical proximity of these RNA-protein granules is rather striking, especially considering that it is mirrored by pi-bodies and piP-bodies in mouse gonocytes (Aravin et al., 2009b).

The localization of Yb-bodies might suggest an attractive hypotheses about the specific delivery of piRNA cluster transcripts to the biogenesis machinery. Yb-bodies are

very often juxtaposed to the outer nuclear membrane. Given that heterochromatin often associates with the inner nuclear membrane (Bank and Gruenbaum, 2011; Blobel, 2010) and given that piRNA clusters are often within heterochromatin, it is tempting to postulate a coupling of cluster transcription and processing across nuclear pores. It also might be that such a cell biological coupling serves an efficiency-enhancing role rather than defining piRNA precursor specificity, especially as Yb-bodies are not always in close contact with the nuclear envelope. If this association would be biologically so important, one would expect a more consistent interaction, for example like the one between nuage and the nuclear envelope.

4.4 piRNA biogenesis in the germline and the classification of biogenesis factors

As already stated our work was focused on the somatic follicle cells where only primary piRNA biogenesis takes place. Such gonadal cells that lack secondary biogenesis have been found so far only in *Drosophila*. It is therefore essential to study piRNA biogenesis processes also in germline cells where primary and secondary pathways occur in parallel.

Based on our data we postulate that the genetic architecture of primary biogenesis is essentially identical in follicle cells and in germline cells with the only obvious exception of *Fs(1)Yb*, which is essential in somatic but dispensable in germline cells.

Armitage, Zucchini, Vreteno, SoYb, and Shutdown are also expressed in soma and germline and loss of these factors (in the case of SoYb together with the paralog BoYb) causes loss of Piwi in both cell types, suggesting that they exert the same function in the two cell types. Strikingly, however, loss of these genes shows very different effects on the other PIWI proteins Aubergine and AGO3.

Loss of Vreteno, Shutdown or SoYb/BoYb leads to a severe reduction in Aubergine protein levels and triggers delocalization of AGO3 from nuage. In contrast, loss of Armitage or Zucchini has no or only marginal impacts on Aubergine and AGO3. Based on this, we propose that Armitage and Zucchini affect exclusively primary piRNA biogenesis. Upon loss of either factor Aubergine and AGO3 can still be loaded via secondary biogenesis (ping-pong). Instead, Vreteno, Shutdown and SoYb/BoYb are probably required for both primary and secondary biogenesis. Consequently, their loss

affects all PIWI proteins. This is in agreement with observations that several postulated secondary biogenesis factors are only required for Aubergine and AGO3 biology but have no clear role for Piwi.

Based on this, we propose a classification of piRNA biogenesis factors according to their PIWI protein phenotype (TAB. 2): factors specific for primary piRNA biogenesis affect only Piwi and belong to Class 1; factors specific for secondary piRNA biogenesis affect Aubergine and AGO3 and belong to Class 2; factors required for both biogenesis branches affect all three PIWI proteins and belong to Class 3; this classification allows to clearly predict the biogenesis pathway specificity of a given gene just by looking at the localization of PIWI proteins upon the respective knock down.

Observed changes in piRNA populations upon knock down of genes from each class are consistent with the predictions based on immuno-histochemistry: primary piRNAs are reduced in Class 1 and 3 gene knock downs and the ping-pong signature drops in Class 2 and 3 gene knock downs. Furthermore, the correlation among piRNA profiles within each class is much higher than between classes, supporting the proposed classification scheme.

Knocking down Class 1 genes results in distinct changes for piRNAs mapping to different transposons. While piRNAs mapping to some transposons show an increase in ping-pong piRNAs at the expense of primary piRNAs, piRNAs mapping to other transposons collapse nearly entirely. We interpret these differences by the different abilities of TEs to engage in ping-pong in the absence of primary biogenesis. This could depend on several factors: different levels of piRNAs could be maternally deposited against different transposons or the developmentally regulated expression of transposons and/or clusters could limit the availability of substrate RNAs for continuous ping-pong throughout oogenesis.

The most likely explanation for the observation that class 3 genes are necessary for all piRNA populations is that they are involved in biogenesis steps that are common between primary and secondary biogenesis, rather than having different functions in different contexts. Considering that primary and secondary biogenesis must diverge substantially in their early steps because of the different RNA substrates and the different PIWI proteins involved, the simplest model places class 3 genes downstream of primary or secondary biogenesis specific factors. This is in interesting agreement with the Yb-body

epistasis, which also places Class 3 genes in the most downstream steps. The available data that links Shutdown to PIWI protein loading would also be consistent with this hypothesis. Nevertheless, it cannot be excluded that different Class 3 genes could act at very distant biogenesis steps, maybe exerting the same action in primary and secondary biogenesis specific processes.

4.5 The molecular function of Shutdown

Shutdown was the first described factor with an essential function for the biogenesis of all piRNAs. Based on the biochemical data about the closely related protein CyP40 in plants (Iki et al., 2012), we formulated some specific hypotheses for Shutdown's molecular function.

Shutdown harbors a peptidylprolyl isomerase (PPIase) domain and a tetratricopeptide repeat (TPR) domain and thus belongs to the FK506-Binding Proteins (FKBPs) (Munn and Steward, 2000); nevertheless it is quite different from the better-known FKBPs that have a role in immunity, because it has only one PPIase domain rather than two. The best-studied protein that shares this domain composition is plant CyP40 that is involved in Hsp90 mediated miRISC formation (Iki et al., 2012). As there is accumulating evidence that Hsp90 might also be important for piRNA loading (Gangaraju et al., 2011; Specchia et al., 2010), we wondered if Shutdown could be the functional homolog of CyP40 in this process.

The association between TPR domains and Hsp90 has been characterized at the structural level (Scheufler et al., 2000). To probe for the functional importance of a putative Hsp90-Shutdown interaction we created *shutdown* alleles with specific point mutations in residues predicted to be essential for the interaction. These alleles cause transposon upregulation but not to the extent of a *shutdown* null allele. The likely reason for this is that these *shutdown* mutants exhibit Aubergine and AGO3 delocalization but they do show seemingly normal Piwi levels as if they would only impact secondary but not primary piRNA biogenesis. This is clearly in contrast with the fact that Shutdown is required for both processes. Different explanations can be envisioned: the alleles that we produced could fail in completely abolishing the putative Hsp90-TPR interaction and secondary piRNA biogenesis is more sensitive towards loss of Shutdown; or the Hsp90-

Shutdown interaction might still be bridged by other protein domains or by other factors; alternatively, the TPR domain of Shutdown has a function that is specific for secondary biogenesis. This last hypothesis could be in agreement with the mouse data (Xiol et al., 2012), as the mouse ortholog of Shutdown functions only in secondary biogenesis. On the other hand the hypothesis of additional interaction sites between Shutdown and Hsp90 has to be taken into serious consideration as there is evidence that an FKBP can also bind a site near the N-terminus of Hsp90 (Chadli et al., 2008).

The involvement of Shutdown in Hsp90 mediated PIWI protein loading is supported by the observation that unloaded AGO3, which differently from empty Aubergine and empty Piwi is not being degraded, co-localizes with Hsp90 and Shutdown as if these three proteins would form a pre-loading complex when AGO3 cannot be loaded. An interesting feature of this putative complex is that it is found in large cytoplasmic spheres that are easily and unambiguously recognizable. Localization to these spheres might be a powerful assay to identify components of the PIWI protein pre-loading complex. On the other hand the only other protein that has been found in these spheres so far is Krimper, which is a Class 2 factor. Considering that Krimper is a Class 2 factor (secondary biogenesis specific) it could be the first ping-pong factor that recruits unloaded AGO3 rather than a general PIWI loading factor.

The fact that Shutdown only impacts piRNAs and that plant CyP40 is required for miRISC formation sparks the hypothesis that there might be other FKBP in *Drosophila* that are necessary for miRISC and maybe siRISC formation. Indeed, the *Drosophila* genome encodes two proteins that share the same domain composition with Shutdown (CG5482 and CG1847). By postulating redundant or overlapping functions for these factors, one might even be able to explain why no such factor has been identified in screens for miRNA or siRNA biogenesis genes.

Our interest in Hsp90 and FKBP drew our attention to a biological process very distant from the piRNA pathway but with striking analogies to small RNA loading. Hsp90 and FKBP are essential proteins in the biology of steroid receptors (Smith, 2004). Steroid receptors are indeed bound to the same chaperones and cochaperones that are also involved in miRNA/siRNA loading. In the absence of the ligand the chaperone machinery is necessary for the stabilization of the receptor and after ligand binding it is

essential for the receptor's biological activity (Galigniana et al., 2010). FKBP52, a homolog of Shutdown, is indeed thought to be necessary for the nuclear translocation of the ligand-bound receptor (Echeverría et al., 2009). These molecular parallels are very suggestive and spark the hypothesis that the chaperones and cochaperones of steroid receptors might constitute a chaperone machinery with a more general function that is involved in the stabilization of proteins that undergo significant structural changes (like hormone binding or small RNA binding) and that regulates them through the modular interaction between HSP90 and TPR domains. We therefore believe that the knowledge from the field of the steroid receptors could benefit the research about small-RNA loading, at least in terms of molecular concepts.

4.6 PIWI protein specificity of primary and secondary piRNA biogenesis

Based on sequenced piRNA populations associated with each individual PIWI protein, every PIWI protein binds a different and highly unique set of piRNAs. While some PIWI proteins bind mostly sense piRNAs (e.g. AGO3) others bind mostly antisense piRNAs (e.g. Piwi or Aubergine). Furthermore some PIWIs bind mostly piRNAs from primary biogenesis (e.g. Piwi) and others bind mostly piRNAs from secondary biogenesis (e.g. Aubergine and AGO3).

This implies that primary and secondary biogenesis processes are able to select the PIWI protein identity prior to loading. Based on the different impact of Class 1 and Class 2 genes on the different PIWI proteins and on the ectopic expression of Aubergine and AGO3 in follicle cells, we think that Aubergine and Piwi, but not AGO3, are compatible with primary piRNA biogenesis. Instead, Aubergine and AGO3 (but not Piwi) are most compatible with secondary piRNA biogenesis.

What is the molecular basis for this selectivity? It is possible that Piwi does not participate significantly in ping-pong because it is sequestered to the nucleus right after loading. This hypothesis is supported by the observation that Piwi lacking its nuclear localization signal (NLS) accumulates in nuage (Klenov et al., 2011), as if, when kept in the cytoplasm, Piwi could interact with the secondary biogenesis machinery. This hypothesis would have to be confirmed by sequencing the piRNAs bound by the NLS deficient Piwi. Our data suggests that additional aspects are also important: when

Aubergine and AGO3 are expressed in follicle cells that lack secondary biogenesis, they still accumulate to spheres that contain Krimper, a secondary biogenesis specific factor. This is never observed for Piwi, as if it wouldn't be able to engage in this interaction. It is therefore possible that Piwi lacks some feature that would enable it to interact with some secondary factors and that in the end this leads to incompatibility with secondary biogenesis.

This hypothesis is quite similar to our interpretation of the fact that AGO3 cannot be loaded in the follicle cells: AGO3 could be inherently incompatible with primary biogenesis.

We believe therefore that the main determinant for the biogenesis pathway specificity of a PIWI protein is the structure of the PIWI protein itself, which allows or prevents the interaction with key factors of primary or secondary biogenesis.

Why would the compartmentalization of piRNAs in different PIWI proteins be important? The fact that AGO3 and Aubergine bind sense and antisense piRNAs respectively is clearly necessary to yield proper ping-pong and produce a higher proportion of antisense piRNAs. If secondary biogenesis couldn't distinguish between Aubergine and AGO3 there would be no way that it could bias the production of piRNAs towards antisense. The fact that Aubergine and AGO3 bind ping-pong piRNAs makes post-transcriptional silencing more efficient, because ping-pong piRNAs reflect the expression levels of transposons. Piwi instead binds mainly primary piRNAs, which are mainly antisense and are required for transcriptional silencing of transposons (Sienski et al., 2012).

4.7 Outlook

It is apparent that our understanding of piRNA biogenesis is still very limited. We still don't know how some of the fundamental steps of this process happen and our mechanistic insight is limited to a few molecules. There are a few key questions that we should try to answer in order to understand the key molecular and biological concepts underlying piRNA biogenesis. These questions define three lines of research:

- 1) How are transcripts processed to mature piRNAs?

As we mentioned in the introduction, one of the most challenging concepts in piRNA

biogenesis is how the right transcripts are selected for biogenesis. There is still no convincing model for this and most probably there is still a lot of work to do to gather enough data to come to a reasonable hypothesis. Potentially genetics will highlight the role of an unknown factor in this process. Otherwise the chromatin characterization of piRNA clusters and the biochemical analysis of the known chromatin factors might provide some answers towards this question.

There is evidence that piRNA clusters are initially very long transcripts (Brennecke et al., 2007; Klattenhoff et al., 2009). When, where and by which factors are these long RNAs fragmented? Is Zucchini the only endonuclease responsible for this process? Furthermore, within these transcripts piRNA production hot spots are present. Understanding why some regions produce more piRNAs than others would be a key advancement in the understanding of precursor processing. At the moment, we can just speculate that, for example, some transcript regions might be more readily available to the piRNA biogenesis machinery than others, because of the RNP structure or of certain protein-RNA interactions. Identifying and localizing RNA intermediates and analyzing these upon gene knock down might be a powerful approach to answer these questions.

Last but not least, an enzyme for the 3' exonuclease activity that is known to generate the piRNA 3' end still has to be identified. A targeted genetic screen or further biochemical purification of the active fractions might help in this case.

2) How are PIWI proteins regulated?

PIWI proteins have some extremely interesting features such as biogenesis pathway specificity and regulated degradation of the unloaded pool.

Based on our data, we made the hypothesis that the biogenesis pathway specificity of a PIWI protein is determined by the structure of the PIWI protein itself, which allows or prevents the interaction with key factors of primary or secondary biogenesis. To test this hypothesis one could clone chimeric PIWI proteins and assay their biogenesis pathway specificity using the OSCs system, for example. If this succeeds and yields a loaded and functional protein, one could try to identify its binding partner(s) that are determining specificity.

Accumulating evidence suggests that unloaded PIWI proteins are being actively degraded. This must of course be a highly regulated mechanism to avoid degradation of

newly synthesized proteins. What are the factors that are regulating PIWI protein stability or inducing their degradation? Considering that these processes must happen when the PIWI proteins are empty, the chaperones and other factors involved in PIWI protein loading might play a role there. Identifying all proteins that are in complex with empty PIWIs could be a fundamental step to gain more insight into PIWI protein loading, stabilization, and degradation.

Identifying the mechanism of protein degradation, looking at poly-ubiquitylation for example, and the responsible factors could be also useful for technical development. If the degradation of PIWI proteins could be inhibited, this could be a useful tool to study intermediate complexes in piRNA biogenesis, whenever a knock down would lead to PIWI degradation.

As Shutdown seems to bind empty PIWI proteins and probably has a role in their loading, further and more mechanistic studies of Shutdown might help to answer some of the questions above. It would be very interesting to clarify why the mutant alleles that we produced are impacting only secondary biogenesis and what the function of the elusive PPIase domain is.

3) How are piRNA biogenesis factors organized and regulated?

Assigning a precise biochemical function to all biogenesis factors is the bold final objective. But before that, we might want to understand some of the basic concepts of piRNA biogenesis by looking at it with lower resolution. For example, we only have a rough idea of how the known biogenesis factors interact with each other. The organization of these factors in different complexes and their recruitment to different places in the cell are probably essential for their regulation. We could start to look into these issues by quantitatively probing the interactors of each factor. As far as the cell compartmentalization of biogenesis is concerned, we made a lot of progress in the understanding of the Yb-body, but our understanding of nuage is still very limited. The ectopic expression of germline factors in follicle cells has the potential to be a powerful tool to understand the minimal genetic requirements of secondary biogenesis or at least of the cytological features of this process.

One of the most interesting issues raised by the cell-biological analysis of piRNA biogenesis is for sure the function of mitochondria in this process. A prerequisite to

clarify this point is to know all piRNA biogenesis factors that are associated to mitochondria. Once that these have been achieved it might be possible to test if piRNA biogenesis can also take place on other cellular membranes or whether it does require specifically the mitochondrial membrane.

5. Tables and Figures

TAB. 1 The PIWI family of Argonaute proteins

All known PIWI family proteins are listed and indicated is their ability to accept primary piRNAs as well as their function in secondary biogenesis as trigger and/or acceptor of secondary piRNAs. Note that only PIWI proteins that are both trigger and acceptor of secondary piRNAs are able to trigger a ‘ping-pong’ amplification cycle.

TAB. 2: The *Drosophila* and mouse piRNA biogenesis factors.

Shown are all known piRNA biogenesis factors and their domain composition as well as their biogenesis pathway specificity (modified from Handler et al., 2011).

ZnF: zinc finger; RRM: RNA recognition motif; DEAD: DEAD-Box RNA Helicase; Hel-C: Helicase C-terminal; HA2: Helicase associated domain; OB: oligo-nucleotide binding; CS: HSP20-like domain; TM: trans-membrane domain; KH: K homology; UBA: ubiquitin-associated domain; PLD: phospholipase-D domain; Ank: ankyrin repeat; bZIP: basic Leucine zipper domain; SAM: sterile alpha motif; Tud: Tudor domain; TPR: tetratricopeptide repeat domain; PPIase: peptidylprolyl isomerase domain.

The number within parentheses indicates how many times a domain is present in a protein.

TAB. 1 The PIWI family of Argonaute proteins

<i>Drosophila</i>	Acceptor of primary piRNAs	Acceptor of secondary piRNAs	Trigger of secondary piRNAs
Piwi	Yes	maybe	No
Aub	Yes	Yes	Yes
AGO3	No	Yes	Yes
<i>Mouse</i>			
MILI	Yes	Yes	Yes
MIWI	Yes	No	No
MIWI2	No	Yes	No
<i>Zebrafish</i>			
Ziwi	Yes	Yes	Yes
Zili	No	Yes	Yes

->ping-pong

->ping-pong

->ping-pong

->ping-pong

->ping-pong

TAB. 2 piRNA biogenesis factors in flies and mice

<i>Drosophila</i>	Domains	Primary (I) or secondary (II) biogenesis	Class	Mouse	Domains	Primary (I) or secondary (II) biogenesis
Fs(1)Yyb	Hel-C like, DEAD, Hel-C, ZnF, Tud	I	1	<i>D.mel.</i> specific		
Arm	DEAD	I	1	MOV10L1	DEAD	I
Zuc	PLD	I	1	MITOPLD	PLD	I
CG2183	Ank (4), SAM, bZIP	unknown	unknown	GASZ	Ank (4), SAM, bZIP	unknown
Kumo	ZnF-RING, B-box (2), Tud (5)	II	2	TDRD4	ZnF-RING, Tud (5)	unknown
Vasa	DEAD	II	2	MVH	DEAD	II
Tejas	LOTUS, Tud	II	2	TDRD5	LOTUS, Tud	II
Spn-E	DEAD, Hel-C, HA2, OB, RRM, Tud, ZnF	II	2	TDRD9	DEAD, Hel-C, HA2, OB, RRM, Tud, ZnF	II
Krimp	Coiled coil, Tudor, ZnF, Tud	II	2	<i>D.mel.</i> specific		
Papi	TM, KH (2), Tud	II	2	TDRD2/TDRKH	TM, KH (2), Tud	unknown
Vret	RRM, ZnF-MYND, Tud (2)	I and II	3	TDRD1	ZnF-MYND, Tud (4)	II
SoYb	Tud, DEAD, Hel-C, ZnF, Tud, CS	I and II	3	TDRD12	Tud, DEAD, Hel-C, ZnF, Tud, CS	unknown
BoYb	DEAD, Hel-C, ZnF, Tud, CS	I and II	3			
Shu	PPIase, TPR	I and II	3	FKBP6	PPIase, TPR	II

FIG. 1: The *Drosophila* ovariole and germarium (from Bastock and St Johnston, 2008).

Here is shown a confocal image of an ovariole, the constituent subunit of the *Drosophila* ovary; germline cells are marked in green by anti-Staufen, surrounding somatic support cells are outlined by an actin staining (red); the left-right axis is also the developmental axis, going from the germarium, where the germline stem cells are located, to the developing oocyte. Above each egg chamber the developmental stage is indicated (stage 1 is still in the germarium).

The cartoon represents an enlarged view of the germarium, which contains several types of somatic cells besides the germline stem cells and the cystoblasts. All somatic cells represented (terminal filament cells, cap cells, follicle cells) express Piwi (Saito et al., 2006).

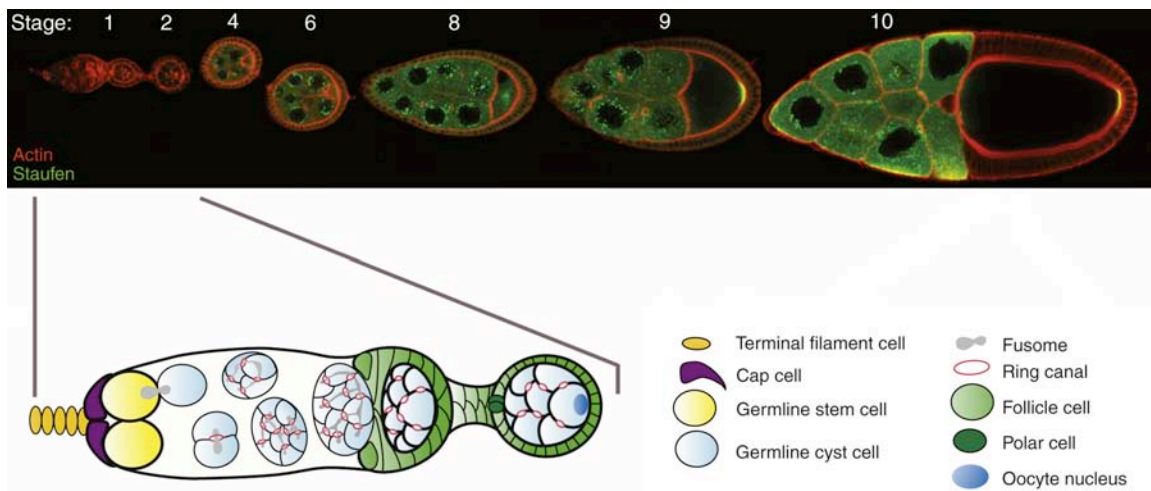
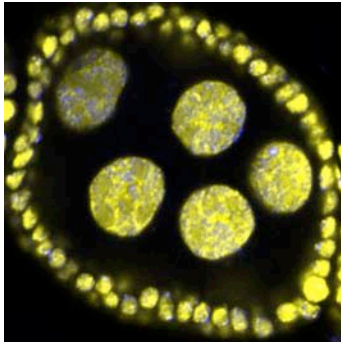
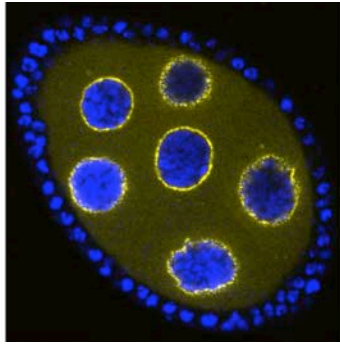


FIG. 2: The expression pattern of PIWI proteins in the *Drosophila* ovary.

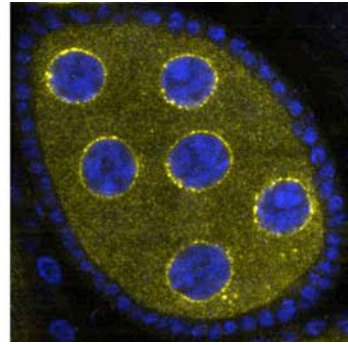
Here are shown confocal sections of *Drosophila* egg-chambers stained for DNA (blue) and the individual PIWI proteins (yellow; as indicated). While Piwi is a nuclear protein expressed in germline and somatic cells of the ovary, Aub and AGO3 are germline specific cytoplasmic proteins that accumulate in nuage, an electron dense structure around the nucleus.



Piwi



Aub



AGO3

FIG. 3: *flamenco*, the main piRNA cluster of the follicle cells (from Brennecke et al., 2007).

A) Shown is a cartoon of the *Drosophila melanogaster* X-chromosome with euchromatin in light grey, pericentromeric heterochromatin in grey and non-assembled highly repetitive heterochromatin in black. The plot above indicates genome uniquely mapping piRNAs. *flamenco* is localized at the boundary between peri-centromeric heterochromatin and euchromatin and is just downstream of piRNA cluster #2. The black bar indicates the density of annotated transposons for *flamenco* and cluster #2.

B) Shown is a detailed annotation of the transposon content of the *flamenco* cluster. *flamenco* mainly consists of transposon fragments in antisense orientation (shown in red), and harbors fragments from the transposons *gypsy*, *idefix*, and *ZAM*, all of which have been shown to be regulated by *flamenco*.

C, D) Shown are detailed views of two *flamenco* regions indicating the annotated transposon fragments, uniquely mapping piRNAs (green) and the location of P-elements in the *flamenco* promoter region that are known to disrupt *flamenco* function. *flamenco* is probably transcribed in only one direction (from left to right), giving rise to piRNAs that are able to silence the transposons that are present in the cluster.

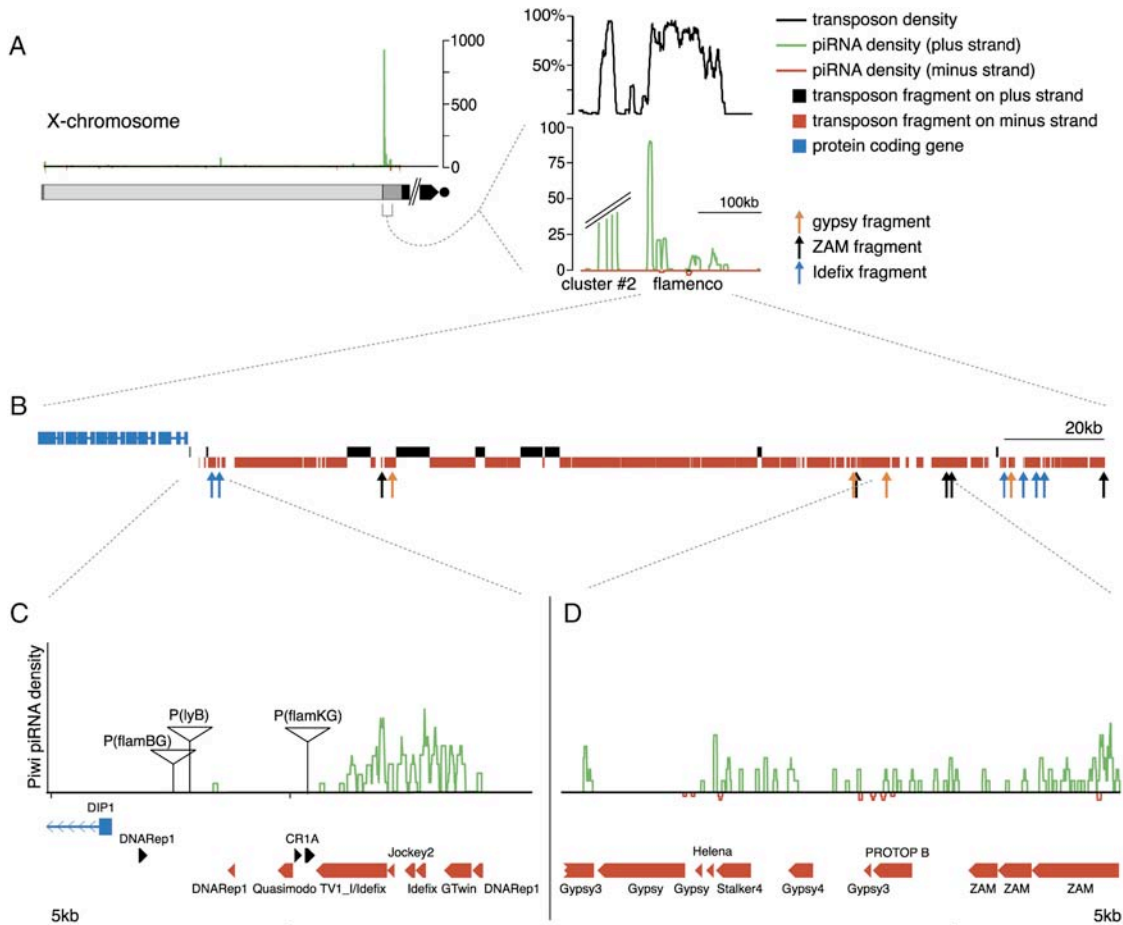


FIG. 4: The Yb-body and nuage localization epistasis of piRNA biogenesis factors.

The two main piRNA biogenesis cellular structures in the *Drosophila* ovary are the Yb-body (in follicle cells) and nuage (in germline cells). In this cartoon a single egg-chamber is shown, with the germline surrounded by the epithelial monolayer of the somatic follicle cells.

In the germline it is possible to distinguish nurse cells (containing large, polyploid nuclei) from the oocyte (small, diploid nucleus), which is located at the posterior end (right). Only the nurse cells, which are transcriptionally active, possess nuage, a speckled structure that surrounds their nuclei (shown in red). So-called 'ring canals' connect the cytoplasm of all the germline cells (black rings). On the right side (in red) the almost linear nuage localization epistasis is depicted. It links most secondary piRNA biogenesis factors and Aubergine and AGO3, the PIWI proteins acting in ping-pong.

The follicle cells build a continuous epithelium and envelope the germline. Each cell contains at least one Yb-body (in green), a small accumulation of primary piRNA biogenesis factors in close proximity to the nucleus. On the left side (in green) the almost linear Yb-body localization epistasis is shown. It depicts most primary piRNA biogenesis factors and Piwi, the only PIWI protein that is expressed in follicle cells.

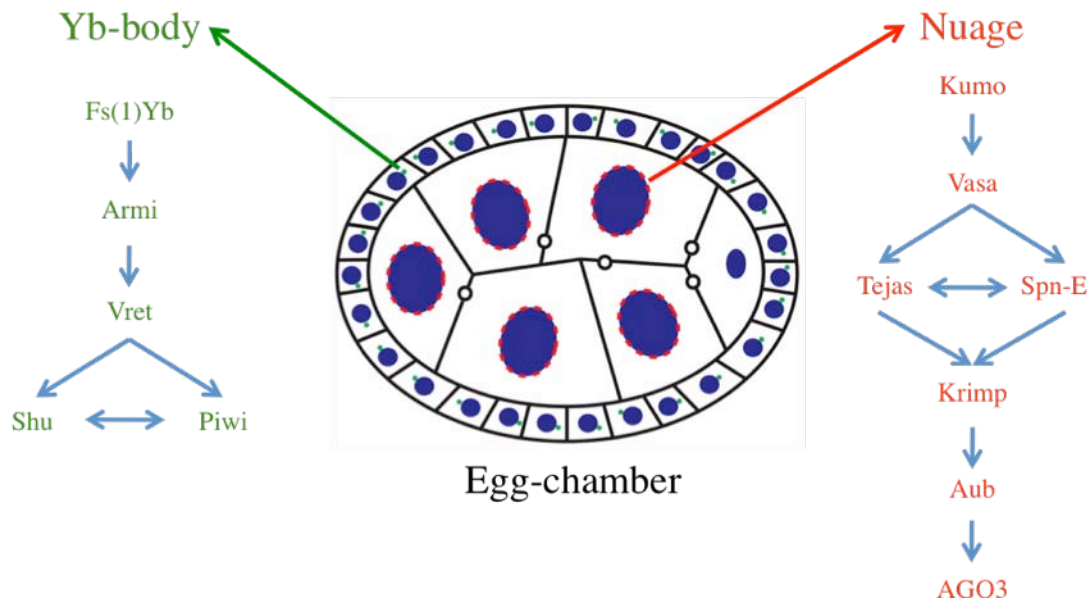
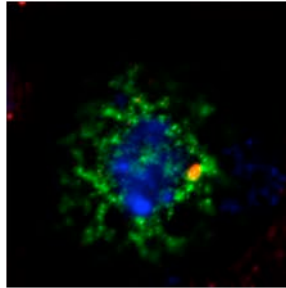


FIG. 5: The Yb-body and Zucchini.

Here is shown a confocal image of a cultured ovarian somatic cell (OSC) expressing the fusion protein Zucchini-GFP (green). The Yb-body is visualized via an antibody staining against Armitage (red; DAPI labels DNA in blue). The image illustrates the tight association between Yb-bodies and mitochondria, as Zucchini is an integral protein of the mitochondrial outer membrane and marks the whole surface of all mitochondria.



6. References

- Ameres, S.L., Horwich, M.D., Hung, J.-H., Xu, J., Ghildiyal, M., Weng, Z., and Zamore, P.D. (2010). Target RNA-directed trimming and tailing of small silencing RNAs. *Science* 328, 1534-1539.
- Anand, A., and Kai, T. (2012). The tudor domain protein kumo is required to assemble the nuage and to generate germline piRNAs in *Drosophila*. *EMBO J* 31, 870-882.
- Aravin, A.A., and Hannon, G.J. (2008). Small RNA silencing pathways in germ and stem cells. *Cold Spring Harb Symp Quant Biol* 73, 283-290.
- Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T., and Hannon, G.J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* 31, 785-799.
- Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G.J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* 316, 744-747.
- Aravin, A.A., van der Heijden, G.W., Castaneda, J., Vagin, V.V., Hannon, G.J., and Bortvin, A. (2009a). Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet* 5, e1000764.
- Aravin, A.A., van der Heijden, G.W., Castañeda, J., Vagin, V.V., Hannon, G.J., and Bortvin, A. (2009b). Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet* 5, e1000764.
- Ashe, A., Sapetschnig, A., Weick, E.-M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.-L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., *et al.* (2012). piRNAs Can Trigger a Multigenerational Epigenetic Memory in the Germline of *C. elegans*. *Cell* 150, 88-99.
- Assis, R., and Kondrashov, A.S. (2009). Rapid repetitive element-mediated expansion of piRNA clusters in mammalian evolution. *Proc Natl Acad Sci USA* 106, 7079-7082.
- Bank, E.M., and Gruenbaum, Y. (2011). The nuclear lamina and heterochromatin: a complex relationship. *Biochem Soc Trans* 39, 1705-1709.
- Bastock, R., and St Johnston, D. (2008). *Drosophila* oogenesis. *Curr Biol* 18, R1082-1087.
- Blobel, G. (2010). Three-dimensional organization of chromatids by nuclear envelope-associated structures. *Cold Spring Harb Symp Quant Biol* 75, 545-554.
- Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128, 1089-1103.
- Chadli, A., Bruinsma, E.S., Stensgard, B., and Toft, D. (2008). Analysis of Hsp90 cochaperone interactions reveals a novel mechanism for TPR protein recognition. *Biochemistry* 47, 2850-2857.
- Chen, C., Jin, J., James, D.A., Adams-Cioaba, M.A., Park, J.G., Guo, Y., Tenaglia, E., Xu, C., Gish, G., Min, J., *et al.* (2009). Mouse Piwi interactome identifies binding mechanism of Tdrkh Tudor domain to arginine methylated Miwi. *Proc Natl Acad Sci USA* 106, 20336-20341.
- Chen, C., Nott, T.J., Jin, J., and Pawson, T. (2011). Deciphering arginine methylation: Tudor tells the tale. *Nat Rev Mol Cell Biol* 12, 629-642.

Choi, S.-Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J., and Frohman, M.A. (2006). A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nat Cell Biol* 8, 1255-1262.

Chuma, S., Hosokawa, M., Tanaka, T., and Nakatsuji, N. (2009). Ultrastructural characterization of spermatogenesis and its evolutionary conservation in the germline: germinal granules in mammals. *Mol Cell Endocrinol* 306, 17-23.

Cook, H.A., Koppetsch, B.S., Wu, J., and Theurkauf, W.E. (2004). The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* 116, 817-829.

Czech, B., Malone, C.D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., Perrimon, N., Kellis, M., Wohlschlegel, J.A., Sachidanandam, R., *et al.* (2008). An endogenous small interfering RNA pathway in *Drosophila*. *Nature* 453, 798-802.

De Fazio, S., Bartonicek, N., Di Giacomo, M., Abreu-Goodger, C., Sankar, A., Funaya, C., Antony, C., Moreira, P.N., Enright, A.J., and O'Carroll, D. (2011). The endonuclease activity of Mili fuels piRNA amplification that silences LINE1 elements. *Nature* 480, 259-263.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblaue, S., *et al.* (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448, 151-156.

Earley, K.W., and Poethig, R.S. (2011). Binding of the cyclophilin 40 ortholog SQUINT to Hsp90 protein is required for SQUINT function in *Arabidopsis*. *J Biol Chem* 286, 38184-38189.

Echeverría, P.C., Mazaira, G., Erlejman, A., Gomez-Sanchez, C., Piwien Pilipuk, G., and Galigniana, M.D. (2009). Nuclear import of the glucocorticoid receptor-hsp90 complex through the nuclear pore complex is mediated by its interaction with Nup62 and importin beta. *Mol Cell Biol* 29, 4788-4797.

Eulalio, A., Behm-Ansmant, I., Schweizer, D., and Izaurralde, E. (2007). P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol* 27, 3970-3981.

Frost, R.J.A., Hamra, F.K., Richardson, J.A., Qi, X., Bassel-Duby, R., and Olson, E.N. (2010). MOV10L1 is necessary for protection of spermatocytes against retrotransposons by Piwi-interacting RNAs. *Proc Natl Acad Sci USA* 107, 11847-11852.

Galigniana, M.D., Echeverría, P.C., Erlejman, A.G., and Piwien-Pilipuk, G. (2010). Role of molecular chaperones and TPR-domain proteins in the cytoplasmic transport of steroid receptors and their passage through the nuclear pore. *Nucleus* 1, 299-308.

Gangaraju, V.K., Yin, H., Weiner, M.M., Wang, J., Huang, X.A., and Lin, H. (2011). *Drosophila* Piwi functions in Hsp90-mediated suppression of phenotypic variation. *Nat Genet* 43, 153-158.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. *Nat Rev Genet* 10, 94-108.

Girard, A., and Hannon, G.J. (2007). [The role of piRNAs in mouse spermatogenesis.]. *J Soc Biol* 201, 411-418.

Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B.J., Chiang, H.R., King, N., Degnan, B.M., Rokhsar, D.S., and Bartel, D.P. (2008). Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* 455, 1193-1197.

Grivna, S.T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev* 20, 1709-1714.

Gunawardane, L.S., Saito, K., Nishida, K.M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., and Siomi, M.C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. *Science* 315, 1587-1590.

Hackett, J.A., Zyllicz, J.J., and Surani, M.A. (2012). Parallel mechanisms of epigenetic reprogramming in the germline. *Trends Genet* 28, 164-174.

Handler, D., Olivieri, D., Novatchkova, M., Gruber, F.S., Meixner, K., Mechtler, K., Stark, A., Sachidanandam, R., and Brennecke, J. (2011). A systematic analysis of *Drosophila* TUDOR domain-containing proteins identifies Vreteno and the Tdrd12 family as essential primary piRNA pathway factors. *EMBO J* 30, 3977-3993.

Houwing, S., Berezikov, E., and Ketting, R.F. (2008). Zili is required for germ cell differentiation and meiosis in zebrafish. *EMBO J* 27, 2702-2711.

Houwing, S., Kamminga, L.M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D.V., Blaser, H., Raz, E., Moens, C.B., *et al.* (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* 129, 69-82.

Huang, H., Gao, Q., Peng, X., Choi, S.-Y., Sarma, K., Ren, H., Morris, A.J., and Frohman, M.A. (2011a). piRNA-associated germline nuage formation and spermatogenesis require MitoPLD profusogenic mitochondrial-surface lipid signaling. *Dev Cell* 20, 376-387.

Huang, H.-Y., Houwing, S., Kaaij, L.J.T., Meppelink, A., Redl, S., Gauci, S., Vos, H., Draper, B.W., Moens, C.B., Burgering, B.M., *et al.* (2011b). Tdrd1 acts as a molecular scaffold for Piwi proteins and piRNA targets in zebrafish. *EMBO J* 30, 3298-3308.

Iki, T., Yoshikawa, M., Meshi, T., and Ishikawa, M. (2012). Cyclophilin 40 facilitates HSP90-mediated RISC assembly in plants. *EMBO J* 31, 267-278.

Iki, T., Yoshikawa, M., Nishikiori, M., Jaudal, M.C., Matsumoto-Yokoyama, E., Mitsuhashi, I., Meshi, T., and Ishikawa, M. (2010). In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. *Mol Cell* 39, 282-291.

Ipsaro, J.J., Haase, A.D., Knott, S.R., Joshua-Tor, L., and Hannon, G.J. (2012). The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. *Nature*.

Ishizu, H., Siomi, H., and Siomi, M.C. (2012). Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines. *Genes Dev* 26, 2361-2373.

Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., and Tomari, Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell* 39, 292-299.

Ji, L., and Chen, X. (2012). Regulation of small RNA stability: methylation and beyond. *Cell Res* 22, 624-636.

Juliano, C., Wang, J., and Lin, H. (2011). Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms. *Annu Rev Genet* 45, 447-469.

Kamminga, L.M., Luteijn, M.J., den Broeder, M.J., Redl, S., Kaaij, L.J.T., Roovers, E.F., Ladurner, P., Berezikov, E., and Ketting, R.F. (2010). Hen1 is required for oocyte development and piRNA stability in zebrafish. *EMBO J* 29, 3688-3700.

Kawamura, Y., Saito, K., Kin, T., Ono, Y., Asai, K., Sunohara, T., Okada, T.N., Siomi, M.C., and Siomi, H. (2008). *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature* 453, 793-797.

Kawaoka, S., Izumi, N., Katsuma, S., and Tomari, Y. (2011). 3' end formation of PIWI-interacting RNAs in vitro. *Mol Cell* 43, 1015-1022.

Khurana, J.S., Wang, J., Xu, J., Koppetsch, B.S., Thomson, T.C., Nowosielska, A., Li, C., Zamore, P.D., Weng, Z., and Theurkauf, W.E. (2011). Adaptation to P element transposon invasion in *Drosophila melanogaster*. *Cell* 147, 1551-1563.

Kirino, Y., Kim, N., de Planell-Saguer, M., Khandros, E., Chiorean, S., Klein, P.S., Rigoutsos, I., Jongens, T.A., and Mourelatos, Z. (2009). Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. *Nat Cell Biol* 11, 652-658.

Kirino, Y., Vourekas, A., Kim, N., de Lima Alves, F., Rappsilber, J., Klein, P.S., Jongens, T.A., and Mourelatos, Z. (2010a). Arginine methylation of vasa protein is conserved across phyla. *J Biol Chem* 285, 8148-8154.

Kirino, Y., Vourekas, A., Sayed, N., de Lima Alves, F., Thomson, T., Lasko, P., Rappsilber, J., Jongens, T.A., and Mourelatos, Z. (2010b). Arginine methylation of Aubergine mediates Tudor binding and germ plasm localization. *RNA* 16, 70-78.

Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., Cook, H.A., and Theurkauf, W.E. (2007). *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev Cell* 12, 45-55.

Klattenhoff, C., Xi, H., Li, C., Lee, S., Xu, J., Khurana, J.S., Zhang, F., Schultz, N., Koppetsch, B.S., Nowosielska, A., et al. (2009). The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. *Cell* 138, 1137-1149.

Klenov, M.S., Sokolova, O.A., Yakushev, E.Y., Stolyarenko, A.D., Mikhaleva, E.A., Lavrov, S.A., and Gvozdev, V.A. (2011). Separation of stem cell maintenance and transposon silencing functions of Piwi protein. *Proc Natl Acad Sci U S A* 108, 18760-18765.

Kojima, K., Kuramochi-Miyagawa, S., Chuma, S., Tanaka, T., Nakatsuji, N., Kimura, T., and Nakano, T. (2009). Associations between PIWI proteins and TDRD1/MTR-1 are critical for integrated subcellular localization in murine male germ cells. *Genes Cells* 14, 1155-1165.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Takamatsu, K., Chuma, S., Kojima-Kita, K., Shiromoto, Y., Asada, N., Toyoda, A., Fujiyama, A., et al. (2010). MVH in piRNA processing and gene silencing of retrotransposons. *Genes Dev* 24, 887-892.

Lau, N.C., Robine, N., Martin, R., Chung, W.J., Niki, Y., Berezikov, E., and Lai, E.C. (2009). Abundant primary piRNAs, endo-siRNAs, and microRNAs in a *Drosophila* ovary cell line. *Genome Res*.

Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P., and Kingston, R.E. (2006). Characterization of the piRNA complex from rat testes. *Science* 313, 363-367.

Lécher, P., Bucheton, A., and Péliçon, A. (1997). Expression of the *Drosophila* retrovirus gypsy as ultrastructurally detectable particles in the ovaries of flies carrying a permissive flamenco allele. *J Gen Virol* 78 (Pt 9), 2379-2388.

Lee, H.-C., Gu, W., Shirayama, M., Youngman, E., Conte, D., and Mello, C.C. (2012). *C. elegans* piRNAs Mediate the Genome-wide Surveillance of Germline Transcripts. *Cell* *150*, 78-87.

Li, C., Vagin, V.V., Lee, S., Xu, J., Ma, S., Xi, H., Seitz, H., Horwich, M.D., Syrzycka, M., Honda, B.M., *et al.* (2009a). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* *137*, 509-521.

Li, C., Vagin, V.V., Lee, S., Xu, J., Ma, S., Xi, H., Seitz, H., Horwich, M.D., Syrzycka, M., Honda, B.M., *et al.* (2009b). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* *137*, 509-521.

Lim, A.K., and Kai, T. (2007). Unique germ-line organelle, nuage, functions to repress selfish genetic elements in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* *104*, 6714-6719.

Liu, L., Qi, H., Wang, J., and Lin, H. (2011). PAPI, a novel TUDOR-domain protein, complexes with AGO3, ME31B and TRAL in the nuage to silence transposition. *Development* *138*, 1863-1873.

Ma, J.-B., Yuan, Y.-R., Meister, G., Pei, Y., Tuschl, T., and Patel, D.J. (2005). Structural basis for 5'-end-specific recognition of guide RNA by the *A. fulgidus* Piwi protein. *Nature* *434*, 666-670.

Ma, L., Buchold, G.M., Greenbaum, M.P., Roy, A., Burns, K.H., Zhu, H., Han, D.Y., Harris, R.A., Coarfa, C., Gunaratne, P.H., *et al.* (2009). GASZ is essential for male meiosis and suppression of retrotransposon expression in the male germline. *PLoS Genet* *5*, e1000635.

Mahowald, A.P. (1971). Polar granules of *Drosophila*. 3. The continuity of polar granules during the life cycle of *Drosophila*. *J Exp Zool* *176*, 329-343.

Malone, C.D., Brennecke, J., Dus, M., Stark, A., McCombie, W.R., Sachidanandam, R., and Hannon, G.J. (2009). Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. *Cell* *137*, 522-535.

Mathioudakis, N., Palencia, A., Kadlec, J., Round, A., Tripsianes, K., Sattler, M., Pillai, R.S., and Cusack, S. (2012). The multiple Tudor domain-containing protein TDRD1 is a molecular scaffold for mouse Piwi proteins and piRNA biogenesis factors. *RNA*.

Muerdter, F., Olovnikov, I., Molaro, A., Rozhkov, N.V., Czech, B., Gordon, A., Hannon, G.J., and Aravin, A.A. (2012). Production of artificial piRNAs in flies and mice. *RNA* *18*, 42-52.

Munn, K., and Steward, R. (2000). The shut-down gene of *Drosophila melanogaster* encodes a novel FK506-binding protein essential for the formation of germline cysts during oogenesis. *Genetics* *156*, 245-256.

Nagao, A., Mituyama, T., Huang, H., Chen, D., Siomi, M.C., and Siomi, H. (2010). Biogenesis pathways of piRNAs loaded onto AGO3 in the *Drosophila* testis. *RNA* *16*, 2503-2515.

Nishida, K.M., Okada, T.N., Kawamura, T., Mituyama, T., Kawamura, Y., Inagaki, S., Huang, H., Chen, D., Kodama, T., Siomi, H., *et al.* (2009). Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in *Drosophila* germlines. *EMBO J* *28*, 3820-3831.

Nishimasu, H., Ishizu, H., Saito, K., Fukuhara, S., Kamatani, M.K., Bonnefond, L., Matsumoto, N., Nishizawa, T., Nakanaga, K., Aoki, J., *et al.* (2012). Structure and function of Zucchini endoribonuclease in piRNA biogenesis. *Nature*.

Okamura, K., Chung, W.-J., Ruby, J.G., Guo, H., Bartel, D.P., and Lai, E.C. (2008). The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. *Nature* **453**, 803-806.

Olivieri, D., Senti, K.-A., Subramanian, S., Sachidanandam, R., and Brennecke, J. (2012). The Cochaperone Shutdown Defines a Group of Biogenesis Factors Essential for All piRNA Populations in *Drosophila*. *Mol Cell* **47**, 954-969.

Olivieri, D., Sykora, M.M., Sachidanandam, R., Mechtler, K., and Brennecke, J. (2010). An in vivo RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in *Drosophila*. *EMBO J* **29**, 3301-3317.

Pan, J., Goodheart, M., Chuma, S., Nakatsuji, N., Page, D.C., and Wang, P.J. (2005). RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development* **132**, 4029-4039.

Pane, A., Jiang, P., Zhao, D.Y., Singh, M., and Schüpbach, T. (2011). The Cutoff protein regulates piRNA cluster expression and piRNA production in the *Drosophila* germline. *EMBO J* **30**, 4601-4615.

Pane, A., Wehr, K., and Schüpbach, T. (2007). *zucchini* and *squash* encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. *Dev Cell* **12**, 851-862.

Pardue, M.-L., Rashkova, S., Casacuberta, E., DeBaryshe, P.G., George, J.A., and Traverse, K.L. (2005). Two retrotransposons maintain telomeres in *Drosophila*. *Chromosome Res* **13**, 443-453.

Patil, V.S., and Kai, T. (2010). Repression of retroelements in *Drosophila* germline via piRNA pathway by the Tudor domain protein Tejas. *Curr Biol* **20**, 724-730.

Pélisson, A., Teyssset, L., Chalvet, F., Kim, A., Prud'homme, N., Terzian, C., and Bucheton, A. (1997). About the origin of retroviruses and the co-evolution of the gypsy retrovirus with the *Drosophila* flamenco host gene. *Genetica* **100**, 29-37.

Rangan, P., Malone, C.D., Navarro, C., Newbold, S.P., Hayes, P.S., Sachidanandam, R., Hannon, G.J., and Lehmann, R. (2011). piRNA production requires heterochromatin formation in *Drosophila*. *Curr Biol* **21**, 1373-1379.

Reuter, M., Berninger, P., Chuma, S., Shah, H., Hosokawa, M., Funaya, C., Antony, C., Sachidanandam, R., and Pillai, R.S. (2011). Miwi catalysis is required for piRNA amplification-independent LINE1 transposon silencing. *Nature* **480**, 264-267.

Reuter, M., Chuma, S., Tanaka, T., Franz, T., Stark, A., and Pillai, R.S. (2009). Loss of the Mili-interacting Tudor domain-containing protein-1 activates transposons and alters the Mili-associated small RNA profile. *Nat Struct Mol Biol* **16**, 639-646.

Robert, V., Prud'homme, N., Kim, A., Bucheton, A., and Pélisson, A. (2001). Characterization of the flamenco region of the *Drosophila melanogaster* genome. *Genetics* **158**, 701-713.

Robine, N., Lau, N.C., Balla, S., Jin, Z., Okamura, K., Kuramochi-Miyagawa, S., Blower, M.D., and Lai, E.C. (2009). A broadly conserved pathway generates 3'UTR-directed primary piRNAs. *Curr Biol* **19**, 2066-2076.

Saito, K., Inagaki, S., Mituyama, T., Kawamura, Y., Ono, Y., Sakota, E., Kotani, H., Asai, K., Siomi, H., and Siomi, M.C. (2009). A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. *Nature* **461**, 1296-1299.

Saito, K., Ishizu, H., Komai, M., Kotani, H., Kawamura, Y., Nishida, K.M., Siomi, H., and Siomi, M.C. (2010). Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in *Drosophila*. *Genes Dev* *24*, 2493-2498.

Saito, K., Nishida, K.M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H., and Siomi, M.C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes Dev* *20*, 2214-2222.

Sarot, E., Payen-Groschêne, G., Bucheton, A., and Péliisson, A. (2004). Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* flamenco gene. *Genetics* *166*, 1313-1321.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* *101*, 199-210.

Schüpbach, T., and Wieschaus, E. (1989). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. *Genetics* *121*, 101-117.

Schüpbach, T., and Wieschaus, E. (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* *129*, 1119-1136.

Shigenobu, S., Kitadate, Y., Noda, C., and Kobayashi, S. (2006). Molecular characterization of embryonic gonads by gene expression profiling in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* *103*, 13728-13733.

Shirayama, M., Seth, M., Lee, H.-C., Gu, W., Ishidate, T., Conte, D., and Mello, C.C. (2012). piRNAs Initiate an Epigenetic Memory of Nonself RNA in the *C. elegans* Germline. *Cell* *150*, 65-77.

Shoji, M., Tanaka, T., Hosokawa, M., Reuter, M., Stark, A., Kato, Y., Kondoh, G., Okawa, K., Chujo, T., Suzuki, T., *et al.* (2009). The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. *Dev Cell* *17*, 775-787.

Sienski, G., Dönertas, D., and Brennecke, J. (2012). Transcriptional silencing of transposons by piwi and maelstrom and its impact on chromatin state and gene expression. *Cell* *151*, 964-980.

Simon, B., Kirkpatrick, J.P., Eckhardt, S., Reuter, M., Rocha, E.A., Andrade-Navarro, M.A., Sehr, P., Pillai, R.S., and Carlomagno, T. (2011). Recognition of 2'-O-methylated 3'-end of piRNA by the PAZ domain of a Piwi protein. *Structure* *19*, 172-180.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* *8*, 272-285.

Smith, D.F. (2004). Tetratricopeptide repeat cochaperones in steroid receptor complexes. *Cell Stress Chaperones* *9*, 109-121.

Specchia, V., Piacentini, L., Tritto, P., Fanti, L., D'Alessandro, R., Palumbo, G., Pimpinelli, S., and Bozzetti, M.P. (2010). Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. *Nature* *463*, 662-665.

Spradling, A., Fuller, M.T., Braun, R.E., and Yoshida, S. (2011). Germline stem cells. *Cold Spring Harb Perspect Biol* *3*, a002642.

Szakmary, A., Reedy, M., Qi, H., and Lin, H. (2009). The Yb protein defines a novel organelle and regulates male germline stem cell self-renewal in *Drosophila melanogaster*. *J Cell Biol* 185, 613-627.

Theurkauf, W.E., Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., and Cook, H.A. (2006). rasiRNAs, DNA damage, and embryonic axis specification. *Cold Spring Harb Symp Quant Biol* 71, 171-180.

Tomari, Y., Du, T., Haley, B., Schwarz, D.S., Bennett, R., Cook, H.A., Koppetsch, B.S., Theurkauf, W.E., and Zamore, P.D. (2004). RISC assembly defects in the *Drosophila* RNAi mutant armitage. *Cell* 116, 831-841.

Vagin, V.V., Klenov, M.S., Kalmykova, A.I., Stolyarenko, A.D., Kotelnikov, R.N., and Gvozdev, V.A. (2004). The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of *Drosophila melanogaster*. *RNA Biol* 1, 54-58.

Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V., and Zamore, P.D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313, 320-324.

Vagin, V.V., Wohlschlegel, J., Qu, J., Jonsson, Z., Huang, X., Chuma, S., Girard, A., Sachidanandam, R., Hannon, G.J., and Aravin, A.A. (2009). Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. *Genes Dev* 23, 1749-1762.

Vermaak, D., and Malik, H.S. (2009). Multiple roles for heterochromatin protein 1 genes in *Drosophila*. *Annu Rev Genet* 43, 467-492.

Watanabe, T., Chuma, S., Yamamoto, Y., Kuramochi-Miyagawa, S., Totoki, Y., Toyoda, A., Hoki, Y., Fujiyama, A., Shibata, T., Sado, T., *et al.* (2011). MITOPLD is a mitochondrial protein essential for nuage formation and piRNA biogenesis in the mouse germline. *Dev Cell* 20, 364-375.

Williams, R.W., and Rubin, G.M. (2002). ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos. *Proc Natl Acad Sci U S A* 99, 6889-6894.

Xiol, J., Cora, E., Koglgruber, R., Chuma, S., Subramanian, S., Hosokawa, M., Reuter, M., Yang, Z., Berninger, P., Palencia, A., *et al.* (2012). A Role for Fkbp6 and the Chaperone Machinery in piRNA Amplification and Transposon Silencing. *Mol Cell* 47, 970-979.

Yabuta, Y., Ohta, H., Abe, T., Kurimoto, K., Chuma, S., and Saitou, M. (2011). TDRD5 is required for retrotransposon silencing, chromatoid body assembly, and spermiogenesis in mice. *J Cell Biol* 192, 781-795.

Zamparini, A.L., Davis, M.Y., Malone, C.D., Vieira, E., Zavadil, J., Sachidanandam, R., Hannon, G.J., and Lehmann, R. (2011). Vreteno, a gonad-specific protein, is essential for germline development and primary piRNA biogenesis in *Drosophila*. *Development* 138, 4039-4050.

Zheng, K., Xiol, J., Reuter, M., Eckardt, S., Leu, N.A., McLaughlin, K.J., Stark, A., Sachidanandam, R., Pillai, R.S., and Wang, P.J. (2010). Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. *Proc Natl Acad Sci USA* 107, 11841-11846.

7. Curriculum Vitae

Personal information

Name	Olivieri Daniel
Address	Hainburger Strasse 48/42, 1030 Vienna, Austria
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Nationality	Italian
Date and place of birth	29 th October 1985, Bolzano (BZ), Italy

Education

Dates	From September 1999 to June 2004
Institute	Liceo Scientifico E. Torricelli, via Rovigo 42, 39100 Bolzano (BZ), Italy <i>Scientific College E. Torricelli</i>
Title awarded	Diploma Superiore Scientifico <i>Scientific Superior Diploma</i>
Final grade	100/100 (<i>Highest possible grade</i>)

Dates	From October 2004 to September 2007
Institute	Università degli Studi di Trieste – C.d.L. in Biotecnologie <i>University of Trieste – BSc in Biotechnology</i>
Grade Point Average	29,7/30
Title awarded	Laurea di Primo Livello in Biotecnologie <i>BSc in Biotechnology</i>
Final grade	110/110 e Lode <i>110/110 with Honours (Highest possible grade)</i>

“Bovedani Price” Winner – Best BSc Graduate at the Faculty of Medicine in 2007

Dates	From October 2007 to July 2009
Institute	Università degli Studi di Trieste – C.d.L.S. in Biotecnologie mediche <i>University of Trieste – MSc in Medical Biotechnology</i>
Grade Point Average	30/30 e Lode <i>30/30 with Honours (Highest possible grade)</i>
Title awarded	Laurea di Secondo Livello in Biotecnologie mediche <i>MSc in Medical Biotechnology</i>
Final grade	110/110 e Lode <i>110/110 with Honours (Highest possible grade)</i>

“Prelog Price” Winner – Best MSc Graduate at the Faculty of Medicine in 2009

Dates From October 2009 to January 2013 (**expected**)
 Institute University of Vienna – Vienna Biocenter PhD-Program

**Research
 Experience**

Dates August 2006 and from February to September 2007
 Supervisor Prof. MD Mauro Giacca
 Laboratory Molecular Medicine Laboratory
 International Centre for Genetic Engineering and Biotechnology
 (I.C.G.E.B.)
 Trieste, Italy

Dates From July 2008 to September 2008 and from March to June 2009
 Supervisor Prof. Marco Milán
 Laboratory Development and Growth Control Laboratory
 Institute for Research in Biomedicine – IRB Barcelona
 Barcelona, Catalunya, Spain

Dates From July 2009 to January 2013
 Supervisor Dr. Brennecke Julius
 Laboratory The piRNA Pathway Laboratory
 IMBA, Institute of Molecular Biotechnology of the Austrian Academy
 of Sciences
 Vienna, Austria

Publications

2010 The miRNA machinery targets Mei-P26 and regulates Myc protein levels in the Drosophila wing.
 Herranz H, Hong X, Pérez L, Ferreira A, **Olivieri D**, Cohen SM, Milán M.
 EMBO J. 2010 May 19;29(10):1688-98.

2010 An in vivo RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in Drosophila.
Olivieri D, Sykora MM, Sachidanandam R, Mechtler K, Brennecke J.
 EMBO J. 2010 Oct 6;29(19):3301-17.

2011 A systematic analysis of Drosophila TUDOR domain-containing proteins identifies Vreteno and the Tdrd12 family as essential primary piRNA pathway factors.
 Handler D, **Olivieri D**, Novatchkova M, Gruber FS, Meixner K, Mechtler K, Stark A, Sachidanandam R, Brennecke J.
 EMBO J. 2011 Aug 23;30(19):3977-93.

2012 The co-chaperone Shutdown defines a group of biogenesis factors essential for all piRNA populations in Drosophila.
Olivieri D, Senti KA, Subramanian S, Sachidanandam R, Brennecke J.
 Mol Cell. 2012 Sep 28;47(6):954-69.

Meetings & Seminars

Event	SymBioSE (Symposium of Biology Students in Europe) 2008
Dates and place	From 30 th July to 9 th August 2008, Coimbra and Aveiro, Portugal
Description	Symposium of Biology students from all over Europe, with university lectures, workshops and field trips.
Event	10th EMBL International PhD Student Symposium
Dates and place	From 23 rd to 25 th October 2008, Heidelberg, Germany
Description	Symposium of PhD students from all over the world, with lectures, poster sessions and discussions.
Event	EMBO symposium about non-coding RNAs
Dates and place	From 13 th to 16 th October 2010, EMBL, Heidelberg, Germany
Description	International Symposium about non-coding RNAs. I was a selected speaker .
Event	RNA society 2011 meeting
Dates and place	From 14 th to 18 th June 2011, Kyoto, Japan
Description	Institutional meeting of the RNA society, very broad conference.
Event	Advanced Practical Microscopy Course
Dates and place	From 6 th to 11 th November 2011, IMP, Vienna, Austria
Description	Lectures and practical sessions addressing methods like FRAP, FRET, TIRF, 2-Photon microscopy, live-cell imaging, basic and advanced image analysis.
Event	International Course of Developmental Biology
Dates and place	From 5 th to 17 th January 2012, CIMARQ, Quintay, Chile
Description	Intensive laboratory and lecture course for advanced graduate students, introducing to zebrafish, sea urchin, drosophila, chicken, frog and planaria.
Event	EMBO symposium “Germline - Immortality through Totipotency”
Dates and place	From 13 th to 16 th October 2012, EMBL, Heidelberg, Germany
Description	International Symposium about germline development and totipotency. I was a selected speaker .

Languages

Italian *Mother tongue*

German

Reading skills	Excellent
Writing skills	Excellent
Verbal skills	Excellent

English

Reading skills	Excellent
Writing skills	Excellent
Verbal skills	Excellent

Spanish

Reading skills	Good
Writing skills	Elementary
Verbal skills	Elementary

The Italian grading system is based on thirtieths for the exams (sufficiency is 18/30 and the best note is 30/30) and on hundred tenths for the degrees (sufficiency is 60/110 and the best note is 110/110). Honours (Lode) can be obtained both for exams and for degrees.

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