

RNA-Based Technologies



SUMMARY

RNA is a versatile molecule. Not only is RNA an important intermediary in gene expression, but various RNAs are involved in many cell functions, including genomic integrity and protection, transcriptional regulation, RNA processing, translation, and enzymatic function (see Table 5.1). For RNA processing, snRNA, snoRNA, and gRNA are involved. RNAs with enzymatic capabilities are called ribozymes. Other noncoding RNAs include piRNA, siRNA, TERC, crRNA, and Xist, which all play roles in maintaining genomic integrity and protection. RNA transcriptional regulators include antisense RNAs, eRNAs, miRNA, circRNA, lncRNA, and sRNA. In addition to the well-known RNAs during translation (mRNA, tRNA, and rRNA), tmRNA, riboswitches, and dual-function RNAs also aid the process.

The ends of chromosomes, called telomeres, are difficult to replicate because DNA polymerase requires a 3'OH group, and the very ends simply do not have this functional group. Telomerase uses an RNA template called TERC to help synthesize the ends and maintain chromosome integrity. Without TERC and telomerase, the chromosome ends would shorten to the point of interfering with gene expression, leading to cancers and diseases.

Female insects and humans have two copies of the X chromosome, which can lead to gene dosage issues when compared with males. Both insects and mammals control the dosage of the X chromosome genes. Although the overall control mechanisms differ between insects and mammals, one common feature is the use of RNA molecules. In *Drosophila*, males double the expression of the genes on the X chromosome to match the female levels using two noncoding RNAs (*roX1* and *roX2*). In humans and mammals, a long noncoding RNA called *Xist* inactivates one of the X chromosomes in females.

Endogenous transposon movements in eukaryotic genomes are prevented with Piwi-interacting RNAs, or piRNAs. Members of the Argonaute protein family recognize piRNAs and cut them into smaller pieces, which then bind to complementary RNAs produced by transposons. This prevents the movement of the transposon and helps maintain genome structure.

RNAs are also involved in protecting cells from viruses. Eukaryotes use RNA interference to protect the cell from RNA viruses by targeting double-stranded RNA. In prokaryotes, the CRISPR system protects these cells from both RNA and DNA viruses, and also hostile plasmids and transposons. The CRISPR system relies on memory of foreign genetic sequences. This memory is supplied as sequences stored on the chromosome.

Antisense RNAs modulate transcription and help control translation by binding to complementary mRNA sequences and blocking translation. Antisense RNAs are synthesized using the coding strand as a template and are therefore complementary to the mRNA. They are useful in biotechnology as a method to interfere with protein synthesis and can even be produced artificially in a laboratory. Natural roles of antisense RNAs include control of circadian rhythms in the fungus *Neurospora*, bacterial iron metabolism, HIV-1 gene expression, control of eukaryotic transcription factors, control of RNA editing, alternative splicing of certain genes, control of the bacterial ColE1 plasmid replication, and induction of heterochromatin formation.

Antisense oligonucleotides can be constructed in two ways: *in vitro* and *in vivo*. In a laboratory setting, the method is similar to that for constructing DNA primers for PCR applications, except with the use of ribonucleotides, which are usually less stable than deoxynucleotides. Several modifications can be made to increase the stability of the RNA oligonucleotides. These modifications include the use of peptides with attached nucleic acid bases as the backbone of the molecule (peptide nucleic acids, or PNAs), or substitution of a sulfur for the oxygen in the phosphate groups, and several modifications to the five-carbon ribose. An RNA oligonucleotide is single-stranded and has the ability to fold back on itself to form secondary structures, like stem-loops or hairpins, which could be problematic in that the antisense RNA would have a difficult time binding to the target mRNA. A gene of interest can also be cloned



into a vector in such a way as to give an antisense RNA upon transcription. This cloned gene can then be transformed into target cells and expressed into antisense RNA. Since expression of the cloned gene can be controlled, the target antisense RNA is made only in the presence of specific signals or conditions.

Delivery of the antisense oligonucleotides into the cell and then to the appropriate location within the cell relies on the uptake of the oligonucleotides by endocytosis. Encapsulation of the oligonucleotides into liposomes, either within the liposome or attached to the surface, is also a method to deliver the RNA into the cell. Once it is inside the cell, however, there is a chance that the RNA oligonucleotides are degraded. To help prevent degradation, the oligonucleotides are attached to basic peptides. Attaching the oligonucleotides to basic peptides that function as transcription factors also has the advantage of delivering them straight to the nucleus.

RNA interference (RNAi) is present in many different organisms: plants, fungi, and many animals. RNAi involves the use of small, noncoding double-stranded segments of RNA (dsRNA) that target mRNA for degradation. The mechanism of RNAi begins by cutting a long dsRNA molecule into several shorter segments by an endonuclease named Dicer. The smaller fragments are called short-interfering RNAs (siRNAs). Dicer transfers the siRNAs to the RNA-induced silencing complex (RISC), which then becomes activated and unwinds the siRNAs into single-stranded RNA (ssRNA). The ssRNAs are then used to guide the RISC to complementary, target mRNAs, which ultimately results in their demise by degradation. In plants, this process is known as post-transcriptional gene silencing (PTGS). In fungi, the same mechanism of gene silencing is called quelling. Many other phrases exist for the same process: transcriptional gene silencing, co-suppression, and virus-induced silencing. GENE impedance (GENEi) is a new term that was coined to include all of these phenomena.

One application for RNAi in biotechnology is using this method to study the role of specific proteins in the organism, particularly those proteins involved in the development of the organism. RNAi has been used in *C. elegans* and *Drosophila* for this very purpose: to determine the role of specific proteins in the various stages of development. Another application in mammalian tissue is the investigation of the specific roles of human proteins in disease development. Perhaps a new direction for curing diseases can be sought by using RNAi. Additionally, construction of RNAi libraries aids in the identification of the role of unknown proteins in cells, where each clone in the library targets a specific protein in the cell and marks it for silencing.

Ribozymes are catalytic RNA molecules that may or may not be bound to proteins. Ribozymes are naturally occurring and have recently been exploited for medical and industrial applications. The functions of ribozymes include the self-removal of introns from an mRNA with the help of snRNAs, replication of some viruses and subviral agents (viroids and satellite viruses), cleavage plus ligation of single-stranded RNA molecules, and modification of rRNA ribonucleotides by snoRNAs (small nucleolar RNAs). Some snoRNAs are processed into smaller pieces that have an, as yet, unknown function in the cell although some studies suggest that they help regulate the cell cycle.

Riboswitches are sequences located on the 5' end of the mRNA for a gene that binds to small effector molecules; their purpose is to regulate protein expression by changing the stem loop structures on the mRNA molecule. In attenuated riboswitches, an effector molecule binds to the riboswitch as the mRNA for a gene is transcribed. This causes the transcription complex to fall apart and therefore prevents transcription of that gene. Some effector molecules bind to riboswitches to prevent translation of the mRNA transcript. Riboswitches have been discovered for a wide variety of natural processes in the cell. Thermal stress, synthesis of cell wall components, and thiamine biosynthesis are just a few of the processes that are known to be regulated by the use of riboswitches. In biotechnology, riboswitches working in



conjunction with ribozymes show promise to help modulate gene expression of targets, such as for the mRNA of a cancer-causing gene.

In biotechnology applications, ribozymes are used to suppress the expression of genes that are involved in cancer. Most naturally occurring ribozymes are specific to one target. The target RNAs for ribozymes are identified using RNA SELEX (Systematic Evolution of Ligands by Exponential enrichment). In this process, random single-stranded DNA oligonucleotides are produced and converted first to double-stranded DNA and then finally to single-stranded RNA. The ribozyme is bound to a label so that it may be isolated more easily at the end of the experiment. The labeled ribozyme is added to the mixture of random, single-stranded RNA molecules. Ribozymes bound to target molecules are isolated using the tag, and the ssRNA is converted back to cDNA for identification. Ribozymes can be combined with riboswitches and then controlled by a small effector molecule to trigger a riboswitch.

New ribozymes can be engineered *in vitro* by mixing random ribozyme sequences with known target RNA molecules for binding. *In vitro* evolution adds a mutagenesis step after each selection cycle, thus diversifying the outcome. Several engineered ribozymes are being used to inhibit replication of human immunodeficiency virus (HIV). The advantage of using an engineered ribozyme for the treatment for viruses, diseases, or cancer is that it is specific to a target mRNA. More traditional treatments often have unwanted side effects because they do not always adhere to the same specificity. So far, no survival advantage has been observed with the use of ribozymes for control of HIV.

Some researchers have investigated the possibility of catalytic DNA molecules. No natural DNA enzymes are known; however, *in vitro* selection has yielded artificial deoxyribozymes, or DNazymes. DNazymes are involved in RNA and DNA cleavage, DNA depurination, RNA ligation, DNA phosphorylation, and thymine dimer cleavage.



Case Study Yb Integrates piRNA Intermediates and Processing Factors into Perinuclear Bodies to Enhance piRISC Assembly

Yukiko Murota et al. (2014). *Cell Reports* 8, 1–11.

In animal genomes, PIWI-interacting RNAs (piRNAs) bind to PIWI proteins to form piRNA-induced silencing complexes (piRISCs), which repress transposons in germ cell lines. In addition to Piwi, other PIWI proteins include Aub and AGO3, although only Piwi is expressed in ovarian somatic cells of *Drosophila*. Aub and AGO3 cooperate to amplify piRNAs; therefore, piRNA is not amplified in ovarian somatic cells, and all piRNAs are loaded onto Piwi only. Once piRNAs are loaded onto Piwi, the Piwi-piRNA complex enters the nucleus and silences transcription. In previous research, perinuclear Yb bodies were identified as the sites for piRNA processing and piRISC formation in ovarian somatic cells. The protein components of Yb contain domains known to play roles in RNA metabolism. In fact, loss of Yb bodies prevents piRNA biogenesis. Additionally, Zucchini (Zuc) is a single-strand-specific endonuclease that converts piRNA intermediates into mature piRNAs. Loss of Zuc results in lack of mature piRNA, followed by dispersal of Yb bodies and failed nuclear localization of unloaded Piwi.

The authors of this research article investigate piRNA precursors in *Drosophila* ovarian somatic cells. Specifically, they investigated RNAs derived from the *flamenco* piRNA cluster, which is the main source of primary transcripts in *Drosophila* ovarian somatic cells and somatic follicle cells.

Where are *flam* transcripts localized in *Drosophila* ovarian somatic cells (OSCs)? What method was used to determine this localization? How did their results differ from other reports?

The authors used RNA fluorescence *in situ* hybridization (RNA-FISH) and electron microscopy *in situ* hybridization (EM-ISH) and a probe complementary to the 5' end of the *flam* transcripts to determine and visualize that *flam*-piRNA primary and intermediate transcripts localize to cytoplasmic bodies. Their results contradicted other reports that *flam* transcripts (called COM transcripts in the other report) were present in a single nuclear focus called Dot COM.

What is the relationship between Flam bodies and Yb bodies?

The perinuclear bodies where *flam* transcripts localized were called Flam bodies by the authors. Although Flam bodies and Yb bodies are similar in size and number, using RNA-FISH and immunofluorescence, the authors determined that Flam bodies localized in proximity to Yb bodies. Also, Flam bodies localized closer to the nucleus than Yb bodies.

Are Flam bodies the sites of mature piRNA accumulation? How did the authors investigate this question?

The authors examined whether or not mature *flam*-piRNAs or primary or intermediate piRNA transcripts were the major components of Flam bodies. They used RNA-FISH to visualize the localization of Flam bodies in Piwi-depleted ovarian somatic cells. In these cells, the levels of mature piRNAs are decreased because mature piRNAs are loaded onto Piwi. The Flam bodies were unaffected, suggesting that Flam bodies do not contain mature piRNA.

Since the authors determined that mature piRNAs do not accumulate in Flam bodies, what exactly does accumulate there? What role does Zuc play in Flam body formation?

Zuc is an endoribonuclease that converts intermediate piRNAs into mature piRNAs. Just like in Yb bodies, Zuc depletion causes dispersal of Flam bodies in ovarian somatic cells in a similar pattern to Yb bodies. Using Northern blot analysis and RNA-FISH, the authors determined that partial *flam* transcripts accumulated during Zuc depletion. Accumulation of partial *flam* transcripts was decreased if the cells were treated with a *flam* small-interfering RNA (siRNA), which targeted the probed area of the *flam* transcripts during Northern blot analysis. These results suggest that mostly intermediate *flam*-piRNAs accumulate in Flam bodies.

Does Yb affect the formation of Flam bodies? If so, what is the significance?

Yb bodies are made of Yb and other protein components that have known involvement in RNA metabolism. When Yb was depleted, neither Yb bodies nor Flam bodies formed. This suggests that Yb is the major component in piRNA biogenesis within *Drosophila* ovarian somatic cells. With respect to the piRNA pathway, Yb is necessary for the formation of Yb bodies and Flam bodies. Yb bodies are necessary for piRNA maturation, and Flam bodies are a storage structure for intermediate piRNAs.

Does Yb physically bind RNA? If so, what domain is responsible for binding RNA, and how did the authors determine this characteristic? Is there a piRNA preferentially bound by Yb?

Analysis of Yb revealed a conserved N-terminal RecA-like domain (NTD) with several motifs. Examination of these motifs and comparison to other RNA helicases suggest that two of these motifs bind ATP and a third motif binds RNA in an ATP-dependent manner. Mutagenesis of amino acids within those motifs of the NTD abolished the activity of Yb. Therefore, the conserved NTD of Yb is responsible for RNA binding. The authors further determined that *flam*-piRNAs, and not other highly expressed coding regions near the *flam* locus, were preferentially bound by Yb.

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Case Study **Yb Integrates piRNA Intermediates and Processing Factors into Perinuclear Bodies to Enhance piRISC Assembly—cont'd**

In this study, the authors determined that *flam*-piRNA intermediates localize to perinuclear Flam bodies within *Drosophila* ovarian somatic cells and follicle cells. Their original hypothesis was that the *flam* signals would occur within Yb bodies. However, this was not the case although it was determined that Yb bodies and Flam bodies are near each other within the cytoplasm. It is possible that *flam*-piRNAs are processed so quickly that the levels of *flam*-piRNA signals at Yb bodies are below detectable limits. In addition, the authors determined that upon Zuc depletion, intermediate piRNA transcripts accumulated, probably within Flam bodies. Therefore, Flam bodies are reservoirs for partial *flam*-piRNA transcripts and supply these transcripts to Yb bodies, which process the intermediate transcripts into

mature *flam*-piRNA. Yb protein binds *flam*-piRNAs at the NTD and aids in the formation of both Yb bodies and Flam bodies.

The authors proposed that since Yb is mostly a cytoplasmic protein, and the association of Yb with intermediate *flam*-piRNAs marks the initiation point of the cytoplasmic phase of piRNA biogenesis. This model was illustrated as Figure 4C. The authors also hypothesized that Yb captures *flam* transcripts as they are exported out of the nucleus and helps localize them as intermediates to Flam bodies. The spatial arrangement of Flam bodies, Yb bodies, and mitochondria with attached Zuc allows efficient piRISC assembly and nuclear import to silence transposon transcription.



Yb Integrates piRNA Intermediates and Processing Factors into Perinuclear Bodies to Enhance piRISC Assembly

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SUMMARY

PIWI-interacting RNAs (piRNAs) direct Piwi to repress transposons and maintain genome integrity in *Drosophila* ovarian somatic cells. piRNA maturation and association with Piwi occur at perinuclear Yb bodies, the centers of piRNA biogenesis. Here, we show that piRNA intermediates arising from the piRNA cluster *flamenco* (*flam*) localize to perinuclear foci adjacent to Yb bodies, termed Flam bodies. RNAi-based screening of piRNA factors revealed that Flam body formation depends on Yb, the core component of Yb bodies, while Piwi and another Yb body component, Armitage, are dispensable for formation. Abolishing the RNA-binding activity of Yb disrupts both Flam bodies and Yb bodies. Yb directly binds *flam*, but not transcripts from neighboring protein-coding genes. Thus, Yb integrates piRNA intermediates and piRNA processing factors selectively into Flam bodies and Yb bodies, respectively. We suggest that Yb is a key upstream factor in the cytoplasmic phase of the piRNA pathway in ovarian somatic cells.

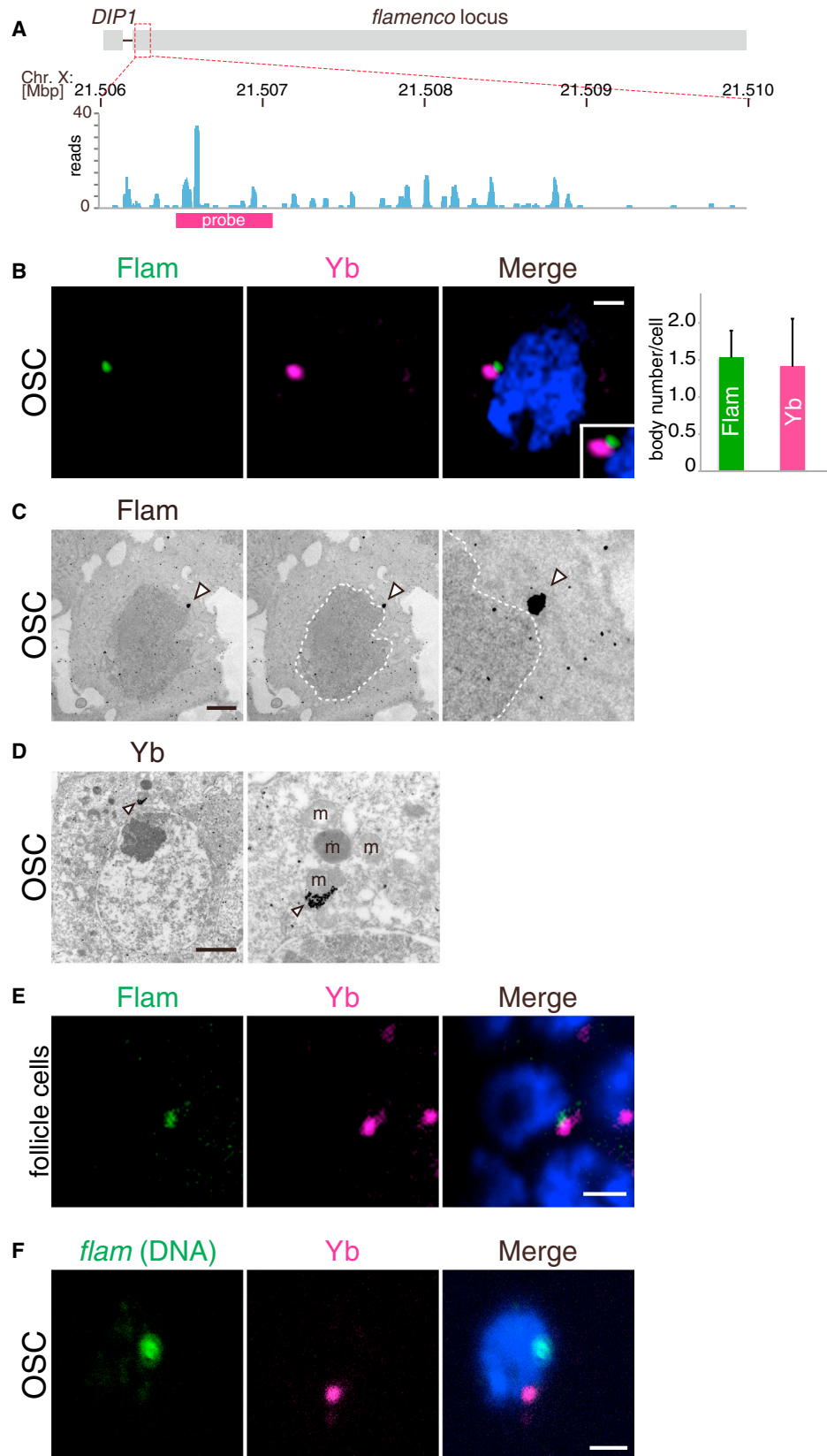
INTRODUCTION

PIWI-interacting RNAs (piRNAs) are small noncoding RNAs of 23–30 nt that are enriched in animal germlines. piRNAs specifically interact with PIWI proteins to form piRNA-induced silencing complexes (piRISCs) and direct them to repress transposons and thus maintain genome integrity in the gonads (Ishizu et al., 2012; Juliano et al., 2011; Siomi et al., 2011). Loss-of-function mutations of PIWI proteins or piRNA biogenesis impairment cause derepression of transposons, leading to defects in gametogenesis and sterility (Aravin et al., 2007; Khurana and Theurkauf, 2010).

Drosophila express three PIWI proteins: Piwi, Aubergine (Aub), and AGO3 (Siomi et al., 2011). In germ cells in the ovaries, primary piRNAs originate from intergenic piRNA clusters through the primary piRNA processing pathway in a Dicer-independent fashion and are loaded onto Piwi and Aub. Following this, Piwi localizes to the nucleus to mediate transposon silencing. In contrast, Aub localizes to the cytoplasm, where it plays a role in the piRNA amplification cycle cooperating with AGO3 through reciprocal RNA cleavage that depends on PIWI-Slicer (endonuclease) activity (Brennecke et al., 2007; Gunawardane et al., 2007; Ishizu et al., 2012; Malone et al., 2009). In this system, transposon transcripts in both sense and antisense orientations are consumed as piRNA precursors; thus, transposon silencing and piRNA production occur simultaneously, enabling a constant supply of piRNAs in cells.

Somatic cells in ovaries express Piwi, but not Aub or AGO3; therefore, they fail to amplify piRNAs. Thus, all piRNAs are primary and are specifically loaded onto Piwi (Ishizu et al., 2012). The Piwi-piRNA complex is then translocated to the nucleus, in which it implements transcriptional silencing in cooperation with cofactors such as Maelstrom and DmGTSF1/Asterix (Döntert et al., 2013; Muerdter et al., 2013; Ohtani et al., 2013; Sienski et al., 2012). Whether Piwi in germ cells analogously collaborates with Maelstrom and DmGTSF1/Asterix to achieve transposon repression remains unknown.

Investigation of the primary piRNA pathway using a cultured *Drosophila* ovarian somatic cell (OSC) line and fly ovaries has revealed that perinuclear Yb bodies are the centers for piRNA processing and piRISC formation in ovarian somatic cells (Olivieri et al., 2010; Saito et al., 2010). Protein constituents of Yb bodies include Yb, Armitage (Armi), Shutdown (Shu), Sister of Yb (SoYb) and Vreteno (Vret), all of which contain domains associated with RNA metabolism; for instance, Yb shows significant similarity to DEAD-box proteins and contains a Tudor (Tud) domain, whereas Vret contains two Tud domains and an RNA-recognition motif (Ishizu et al., 2012). Armi belongs to the Upf1p family of ATP-dependent RNA helicase (Cook et al., 2004). Loss of Yb body components prevents accumulation of primary piRNAs in the



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soma; thus, they are all required for primary piRNA biogenesis and gonadal development (Haase et al., 2010; Handler et al., 2011; Olivieri et al., 2010, 2012; Qi et al., 2011; Saito et al., 2010; Szakmary et al., 2009; Zamparini et al., 2011).

Nascent, piRNA-unloaded Piwi in OSCs interacts with Armi and Yb, and the resultant complex associates with piRNA intermediates in Yb bodies (Olivieri et al., 2010; Saito et al., 2010). Depletion of Yb disrupts Yb bodies, liberating other components into the cytosol. Under these conditions, Piwi and Armi still associate, although the heterodimer does not contain piRNA intermediates. As a result, piRISC formation fails. It is likely that Yb is at the top of the hierarchy for Yb body formation and piRISC formation and that Armi, although categorized as an RNA helicase based on peptide sequence similarity, binds RNA substrates (piRNA intermediates) upon localization to Yb bodies.

Zucchini (Zuc), a phospholipase D superfamily member, is a single-strand-specific endonuclease required for converting piRNA intermediates to mature piRNAs (Ipsaro et al., 2012; Nishimasu et al., 2012). Depletion of Zuc in OSCs results in dispersal of Yb bodies, because piRNA-unloaded Piwi is stalled and fails to localize to the nucleus (Saito et al., 2010). Without Zuc, an excess of unprocessed piRNA intermediates accumulate in OSCs as ribonucleoprotein (RNP) complexes with Armi, Yb, and Piwi; as a result, few piRNAs are produced (Haase et al., 2010; Nishimasu et al., 2012; Saito et al., 2010). These defects can be rescued by ectopic expression of wild-type (WT) Zuc, but not by ectopic expression of endonuclease-deficient Zuc mutants (Nishimasu et al., 2012). Zuc has a mitochondrial targeting signal at the N terminus, and indeed mouse Zuc (also known as MitoPLD) localizes to the outer membranes of mitochondria, facing into the cytosol in mammalian cells (Choi et al., 2006). Zuc signals in OSCs can be detected in close proximity to Yb bodies (Saito et al., 2010), as pi-bodies and piP-bodies, which are considered to be the sites of piRNA biogenesis in mouse testis, are located in intermitochondrial regions (Pillai and Chuma, 2012). This type of intracellular architectural arrangement might raise the rates of Zuc-mediated conversion of piRNA intermediates to mature piRNAs in the cells.

The functions of piRNA protein factors have been well studied. In contrast, the cell biology of piRNA precursors is poorly understood. Therefore, in this study, we performed RNA-fluorescence in situ hybridization (RNA-FISH) in OSCs to visualize RNA transcripts arising from the *flamenco* (*flam*) piRNA cluster. *flam* is the main source of primary piRNAs in OSCs and somatic follicle cells in *Drosophila* ovaries (Brennecke et al., 2007; Malone et al.,

2009; Saito et al., 2009). A P-element insertion in *flam* causes derepression of transposons such as *gypsy* and piRNA loss in mutant follicle cells (Brennecke et al., 2007; Mével-Ninio et al., 2007). RNA-FISH showed that *flam* transcripts are condensed into perinuclear foci adjacent to Yb bodies, which we termed Flam bodies. Recently, a similar type of body named “Dot COM” was reported (Dennis et al., 2013). The similarities and differences between Flam bodies and Dot COM will be described below. RNAi-based screening of piRNA factors revealed that Flam body formation depends on Yb, the core component of Yb bodies, while Piwi and another Yb body component, Armi, are dispensable for the assembly. Depletion of Zuc, which causes excessive accumulation of unprocessed piRNA intermediates (Haase et al., 2010; Nishimasu et al., 2012), results in the dispersion of Flam bodies, which overlap considerably with Yb bodies. Abolishing the RNA-binding activity of Yb disrupts both Flam bodies and Yb bodies. Yb directly binds *flam* transcripts, but not the transcripts of a neighboring protein-coding gene, *DIP1*. Thus, Yb integrates piRNA intermediates and piRNA processing factors selectively into Flam bodies and Yb bodies, respectively. We propose that Yb is a key upstream factor in the cytoplasmic phase of the piRNA pathway in the ovarian soma.

RESULTS

flam Transcripts Are Localized to Perinuclear Flam Bodies in Ovarian Soma

To examine the cellular localization of *flam* transcripts in OSCs, we performed RNA-FISH using a *flam*-specific riboprobe complementary to the 5' region of the *flam* transcript (Figure 1A). This region produces a substantial amount of primary piRNAs in OSCs (Saito et al., 2010) (Figure 1A). By RNA-FISH, perinuclear, punctate signals were observed, suggesting that the *flam*-piRNA precursors/intermediates concentrate at the cytoplasmic bodies (Figures 1B and S1A). A recent report showed that *flam*/COM transcripts (*flam* is also known as COM) are enriched in a single nuclear focus, termed Dot COM (Dennis et al., 2013). However, using electron microscopy in situ hybridization (EM-ISH), we detected *flam* signals in the cytoplasm (Figures 1C and S1B). Fluorescence quantification analysis revealed that the average number of Flam bodies per cell was 1.5 (Figure 1B). Signals from RNA-FISH riboprobes that were used originally by Dennis et al. (COM 508 and 527) (Dennis et al., 2013) coincided with the *flam* signals (Figure S1C). Hereinafter,

Figure 1. Flam Bodies Localize Adjacent to Yb Bodies in *Drosophila* Ovarian Soma

(A) Schematic drawing of the genomic *flam* locus and an upstream protein-coding gene *DIP1* on chromosome X. Mature piRNAs uniquely mapping to the 5' region of the *flam* locus corresponding to 21,506,000 to 21,510,000 on chromosome X (Saito et al., 2009) are shown by blue bars. A 583 nt riboprobe for RNA-FISH complementary to the *flam* transcripts is shown by a magenta box (probe) (chrX: 21,506,472–21,507,054).

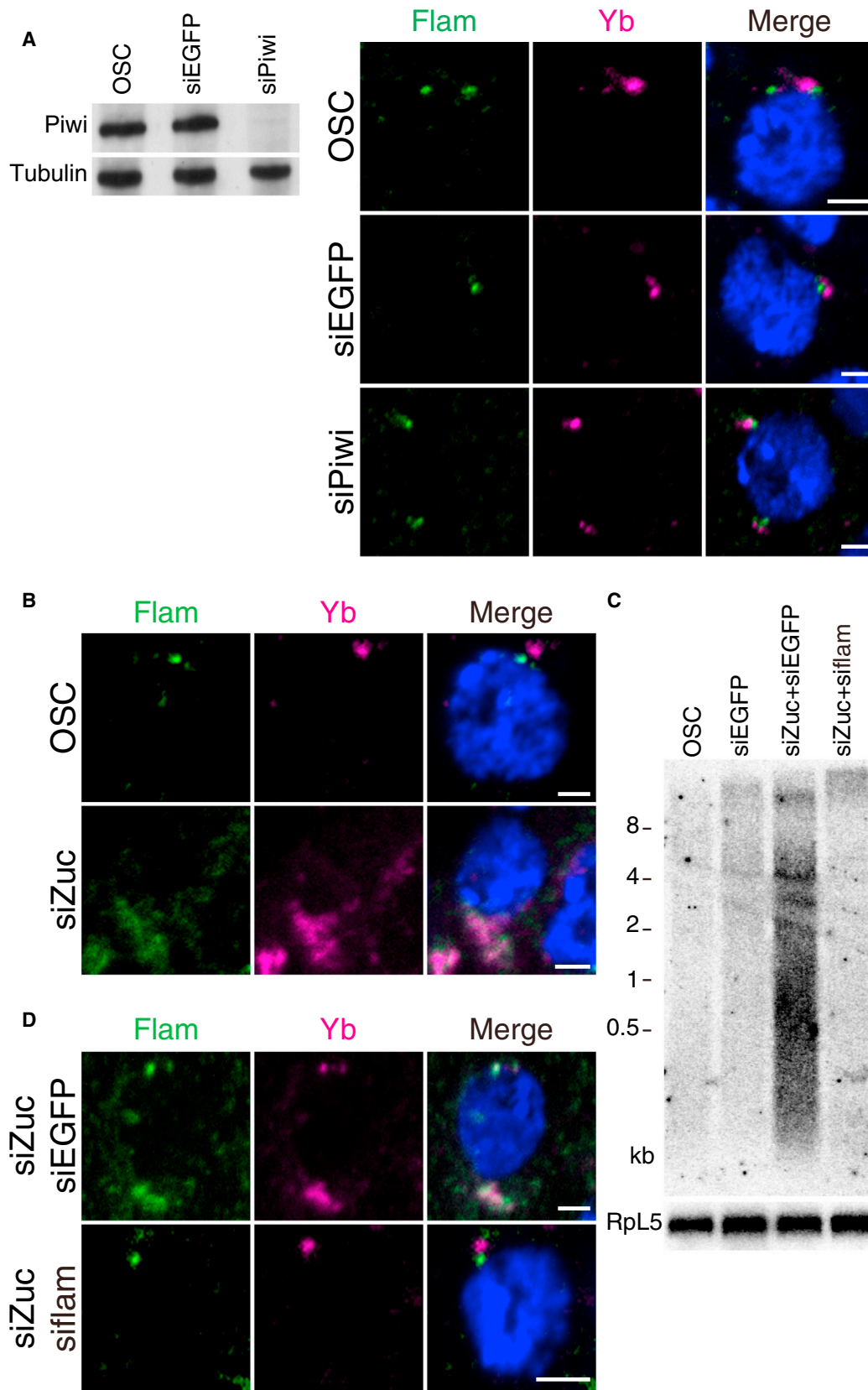
(B) Flam body (green) and Yb body (magenta) in OSCs visualized by RNA-FISH and immunofluorescence using anti-Yb antibody, respectively. The nucleus is stained with DAPI (blue). Scale bar, 2 μ m. The numbers of Flam bodies and Yb bodies were determined by quantification analysis of *flam* and Yb signals in OSCs (n = 20).

(C) Electron microscope in situ hybridization using the probe shown in (A) detects a *flam* signal (white arrowhead) in the cytoplasm of OSC. The far-right image is an enlarged image of the middle image (partial). A white dot line shows where the nucleus is. Scale bar, 2 μ m.

(D) Immunoelectron microscopy using anti-Yb antibody shows a perinuclear Yb body in an OSC (white arrowhead). The right-hand image is an enlarged image of the left-side image (partial). m, mitochondria. Scale bar, 2 μ m.

(E) Flam body (green) and Yb bodies (magenta) in follicular cells of *Drosophila* ovaries. The nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

(F) The *flam* loci (green) in the nucleus and Yb body (magenta) in the cytoplasm. The nucleus is stained with DAPI (blue). Scale bar, 2 μ m.



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we refer to the *flam*-positive, perinuclear bodies as Flam bodies.

Flam bodies appear to be similar to Yb bodies, both in size and number. Therefore, we set out to understand the spatial relationship between Flam bodies and Yb bodies in OSCs by combining *flam* RNA-FISH and immunofluorescence using an anti-Yb antibody that we raised in this study (Figure S1D). Immunoelectron microscopy using the antibody confirmed the perinuclear localization of Yb bodies (Figure 1D). Flam bodies were frequently located adjacent to Yb bodies and tended to be closer to the nucleus than Yb bodies (Figures 1B and S1A). The average number of Yb bodies was similar to that of Flam bodies (1.4 per cell) (Figure 1B). Both signals were also detected in the somatic follicle cells of *Drosophila* ovaries (Figures 1E and S1E). Thus, they are not specific for cultured cells. In *flam* mutant follicle cells, Flam bodies were not detected, confirming the specificity of the RNA-FISH probe (Figure S1F). EM-ISH confirmed the cytoplasmic localization of Flam bodies in follicle cells (Figure S1G). DNA-FISH (Figure S1H) was conducted concomitantly with immunofluorescence using the anti-Yb antibody. No spatial correlation was found between *flam* nuclear foci and Yb bodies (Figures 1F and S1I).

Flam Bodies Are Not the Sites of Mature piRNA Accumulation

It is possible that mature *flam*-piRNAs, rather than their intermediates and/or full transcripts, might be the major components of Flam bodies. To examine this, we visualized Flam bodies and Yb bodies in Piwi-depleted OSCs. Loss of Piwi decreased the level of mature piRNAs drastically (Saito et al., 2009, 2010). This was because Piwi is the sole protein loaded with mature piRNAs in OSCs and, thus, piRNAs are destabilized without Piwi. If mature piRNAs were the major RNA components of Flam bodies, Piwi depletion would cause disappearance of these bodies. However, they were unaffected by Piwi depletion (Figure 2A). These results indicate that Piwi is dispensable for Flam body formation and that Flam bodies are not the sites of mature piRNA storage.

piRNA Intermediates Concentrate in Flam Bodies

Depletion of Zuc caused Yb body dispersion and stalling of Piwi at Yb bodies (Saito et al., 2010). This particular fraction of Piwi was associated with few or no mature piRNAs, although the Armi complex containing Yb and Piwi still bound piRNA intermediates (Saito et al., 2010). These phenomena correlate well with our recent finding that Zuc is the endoribonuclease necessary for converting piRNA intermediates to mature piRNAs (i.e., piRNA maturation) (Nishimasu et al., 2012). We asked if Flam body for-

mation is affected by loss of Zuc. RNA-FISH in Zuc-depleted OSCs showed that Flam bodies were dispersed similarly to Yb bodies (Figure 2B). Interestingly, the two fluorescent signals were superimposed (Figure 2B). We speculated that the increased level of piRNA intermediates might have caused the superimposition. To examine this, northern blotting was performed to detect *flam* transcripts using probes designed to recognize the 5' end region of *flam* (the "probe" region in Figure 1A). In naive OSCs, a smeary signal for *flam* transcripts was only slightly detected, although Flam bodies were clearly visible by RNA-FISH (Figures 2B and 2C). However, partial *flam* transcripts, being several hundred to 4,000 nt in length, were aberrantly accumulated upon Zuc depletion (Figures 2C and S2A) in agreement with our earlier observation (Nishimasu et al., 2012). The corresponding signal decreased after treatment of the cells with *flam* small interfering RNAs (siRNAs) targeting the probed *flam* region (Figures 2C and S2A). These results suggest that the smeared northern blot signal mostly reflects *flam*-piRNA intermediates. After transfection of cells with *flam* siRNAs, the fuzzy fluorescent signals of Yb bodies and Flam bodies returned to normal, as in naive OSCs (Figure 2D). We suggest that Flam bodies are where *flam*-piRNA intermediates concentrate and that piRNA intermediates originating from loci other than *flam* would be minor. Treatment of normal OSCs with *flam* siRNAs did not disrupt Flam bodies (Figure S2B) and barely affected the levels of *flam* transcripts (Figure S2C), although *flam* siRNAs effectively downregulated *flam* transcripts in Zuc-depleted cells (Figure 2C). *flam* siRNAs were designed to target the region corresponding to the *flam* RNA-FISH probe (Figure 1A). Thus, it is plausible that *flam*-piRNA intermediates at Flam bodies would not be accessible to the RNAi machinery under normal conditions likely due to the compactness of the bodies.

Yb Is Necessary for Formation of Both Yb Bodies and Flam Bodies

Depletion of Yb, but not other Yb body components, disrupts Yb body formation (Handler et al., 2011; Saito et al., 2010). We examined if depletion of Yb would affect Flam body formation. Interestingly, neither Flam bodies nor Yb bodies were detected in the cells (Figures 3A and S3A). Expression of siRNA-resistant Yb (Yb WT-r) restored the formation of both structures (Figures 3A and S3A). Thus, Flam bodies depend on Yb for their formation as do Yb bodies. Yb mutant follicle cells contain neither Flam bodies nor Yb bodies (Figure S3B). Depletion of Armi and Vret, other components of Yb bodies (Olivieri et al., 2010), affected neither Flam nor Yb body formation (Figure S3C and data not

Figure 2. Depletion of Zuc, but Not of Piwi, Affects Yb Body and Flam Body Formation

(A) Western blotting shows the efficiency of Piwi depletion. Piwi siRNA (siPiwi) has little effect on Flam body (green) and Yb body (magenta) formation. Enhanced GFP siRNA (siEGFP) was used as a negative control. Nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

(B) Transfection of OSCs with Zuc siRNA (siZuc) led to superimposition of Flam bodies (green) with Yb bodies (magenta). Nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

(C) Northern blotting shows that transfection of OSCs with Zuc siRNA (siZuc) induces aberrant accumulation of *flam*-piRNA intermediates in OSCs. Transfection of Zuc-depleted OSCs with *flam* siRNAs (siflam) targeting the probed *flam* region downregulates the expression of *flam*-piRNA intermediates. Enhanced GFP siRNA (siEGFP) was used as a negative control. *RpL5* mRNA was visualized as a loading control.

(D) Flam bodies (green) and Yb bodies (magenta) in Zuc-depleted OSCs. Either enhanced GFP siRNA (siEGFP) or *flam* siRNA (siflam) was transfected into OSCs. Nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

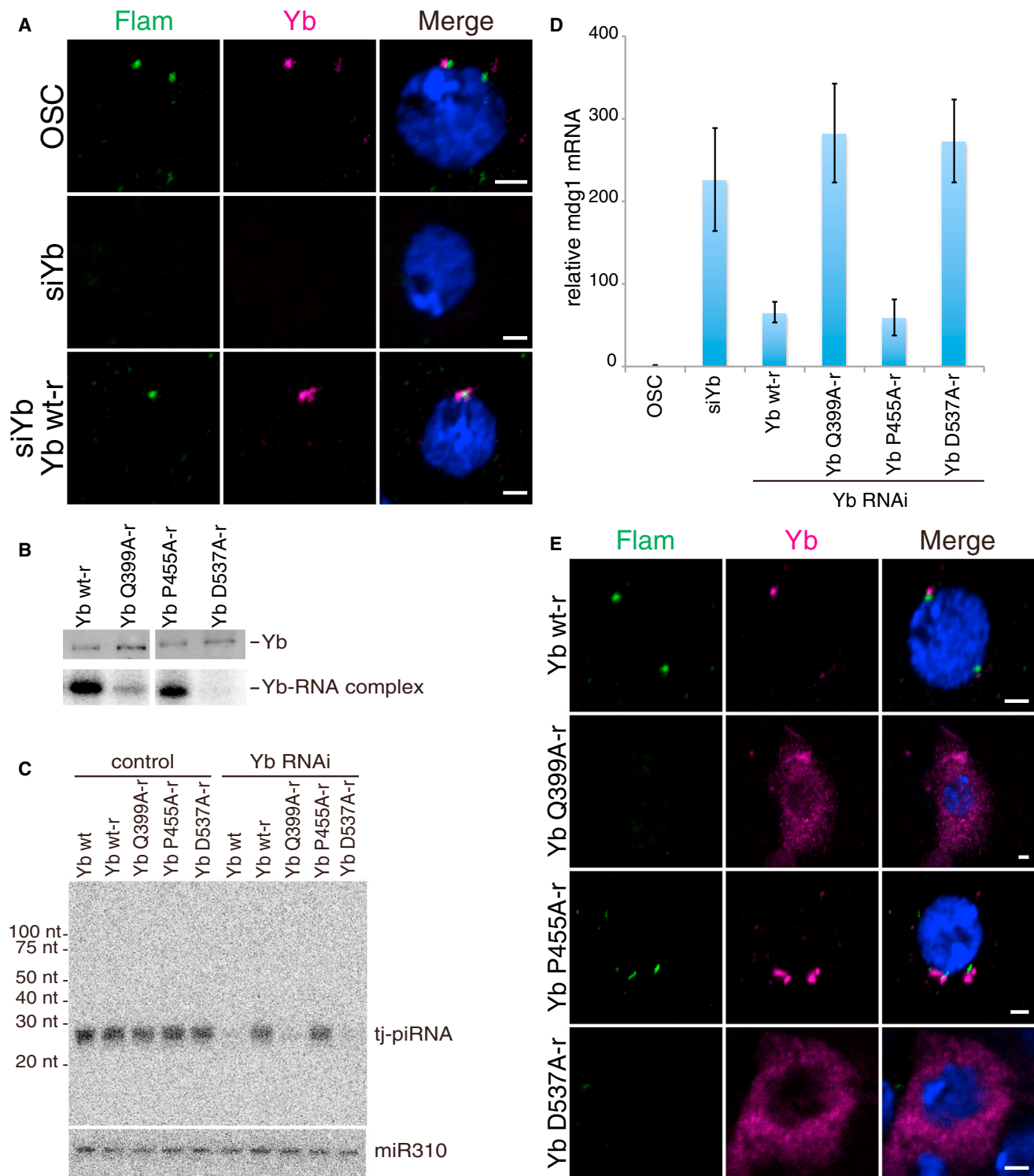


Figure 3. Yb Requirement for Flam Body and Yb Body Formation

(A) Depletion of Yb in OSCs abolishes formation of Flam bodies (green) and Yb bodies (magenta). Expression of siRNA-resistant Yb (Yb WT-r) restored the formation of both structures. Nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

(B) The RNA-binding activity of Yb is abolished by either the Q399A or the D537A mutation in the Yb NTD. Western blotting using the anti-Yb antibody shows that an approximately equal amount of Yb was obtained through Yb-CLIP (top). However, the intensity of the Yb Q399A- and D537A-RNA complexes was much lower than that of the Yb P455A- and Yb WT-r-RNA complexes (bottom).

(legend continued on next page)

shown). These observations support the notion that Yb is the central player in piRNA biogenesis in OSCs; namely, Yb triggers the fabrication of two perinuclear structures, Yb bodies and Flam bodies, the hubs for piRNA maturation and piRNA intermediate concentration, respectively, in the piRNA pathway.

RNA Binding of Yb through the N-Terminal RecA-like Domain Is Required for Yb Body and Flam Body Formation

Yb shows significant similarity to DEAD-box RNA helicases such as Vasa in *Drosophila* and DDH5 and DDX18 in humans, especially in the N-terminal RecA-like domain (NTD) consisting of a Q motif and motifs I, Ia, Ib, II, and III (Figure S3D). To examine the functional involvement of the NTD of Yb in the piRNA pathway, we individually mutated highly conserved residues in Yb, Gln399, Pro455, and Asp537, to Ala. Gln399, Pro455, and Asp537 reside within the Q motif, motif Ia, and motif II, respectively (Figure S3D). In Vasa, Gln272 in the Q motif and Asp399 in motif II, which correspond to Gln399 and Asp537 in Yb, are involved in ATP binding, while Pro326 in motif Ia, which corresponds to Yb-Pro455, contributes to RNA substrate binding (Sengoku et al., 2006). ATP binding by Vasa is necessary for RNA binding (Banroques et al., 2010). Thus, we expected that the alteration of Gln399, Pro455, and Asp537 in Yb to Ala would abolish the RNA-binding function of Yb. Indeed, crosslinking and immunoprecipitation (CLIP) showed that mutation of Gln399 and Asp537 to Ala (Q399A and D537A, respectively) severely decreased the RNA-binding activity of Yb (Figure 3B). Thus, Yb is a bona fide RNA-binding protein and binds RNA substrates through the conserved NTD.

To determine if the RNA-binding activity of Yb is required in the piRNA pathway, three mutants of siRNA-resistant Yb (Q399A, P455A, and D537A), as well as Yb WT, were expressed in Yb-depleted OSCs (Figure S3E). Northern blotting showed that while the WT control Yb WT-r rescued the defect in piRNA accumulation caused by loss of Yb function, the Q399A and D537A mutants (Q399A-r and D537A-r) failed to rescue the defective phenotype (Figure 3C). The P455A mutant (P455A-r) behaved similarly to the WT control (Figure 3C), suggesting that Pro455, despite its high conservation (Figure S3D), is dispensable for piRNA biogenesis. We also examined the expression level of the *mdg1* transposon in the transfected cells, and found that Yb WT-r and P455A-r rerepressed *mdg1*, but Q399A-r and D537A-r failed to do so (Figure 3D). Q399 and D537, but not P455, in the Yb NTD are necessary for both piRNA production and piRNA-mediated transposon silencing.

We then asked if Yb WT-r and the three Yb mutants in Yb-depleted OSCs were able to restore formation of Flam bodies and Yb bodies. Yb WT-r and P455A-r were able to form Yb bodies, while Q399A-r and D537A-r were dispersed in the cytosol and did not accumulate at specific foci (Figures 3E and S3F). Correlating with this, Flam bodies appeared when

Yb WT-r and P455A-r were expressed (Figures 3E and S3F). However, the expression of Q399A-r and D537A-r did not result in the formation of Flam bodies (Figures 3E and S3F). Quantitative RT-PCR (qRT-PCR) detected *flam*-piRNA intermediates in Q399A-r- and D537A-r-expressing OSCs, where endogenous Yb had been depleted by RNAi (Figure S3G). Thus, the substitution of endogenous Yb with Q399-r or D537A-r mutant did not interfere with *flam* expression.

Yb Directly Binds *flam*-piRNA Intermediates, but Not Neighboring Protein-Coding *DIP1* Transcripts

Does endogenous Yb in OSCs indeed bind *flam* transcripts that serve as piRNA intermediates in piRNA biogenesis? To address this question, HITS-CLIP was performed in OSCs using an anti-Yb antibody. Illumina HiSeq2000 sequencing resulted in a total of 72,464,026 reads, consisting of 353,894 unique reads. We mapped these unique reads to the *Drosophila* genome, and 220,197 reads (62.2%) were mapped to a unique position. Annotation of mapped Yb-CLIP tags was similar to that of Piwi-associated piRNAs (Saito et al., 2009); over half of the reads (50.3%) were mapped to transposon regions (Figure S4A).

Further analysis of the Yb-CLIP tags showed that Yb most preferably binds transcripts from the *flam* locus among all piRNA clusters (Figure S4B). We then precisely analyzed the Yb-CLIP tags mapped to the *flam* locus. The distribution of the Yb-CLIP tags on the *flam* locus revealed that Yb in OSCs indeed associates with *flam* transcripts (Figure 4A). The Yb-CLIP tags significantly overlapped with *flam*-piRNAs associated with Piwi in OSCs (Figure 4A). By contrast, none of the Yb-CLIP tags were mapped to a neighboring coding gene, *DIP1* (Figures 4A and 4B), which is highly expressed in OSCs (Sienski et al., 2012; Cherbas et al., 2011). Yb-CLIP tag mapping to protein-coding genes that produce genic piRNAs (Saito et al., 2009) revealed that Yb almost exclusively binds the 3' UTR, but not the protein coding sequence (CDS) or 5' UTR of the transcripts (Figure S4C). The 3' UTR, but not the CDS or the 5' UTR, of genic piRNA-producing mRNAs serves as the piRNA sources (Robine et al., 2009; Saito et al., 2009). These results suggest that Yb directly, and somewhat selectively, binds piRNA intermediates in OSCs.

DISCUSSION

In this study, we visualized *flam*-piRNA intermediates in OSCs and follicle cells using RNA-FISH and EM-ISH and revealed that they concentrate at perinuclear Flam bodies. Flam bodies locate in very close proximity to Yb bodies, the sites of piRNA maturation and piRISC formation. We postulated that *flam* signals might also be detectable within Yb bodies. However, this was not the case (Figures 1B and S1A). The simplest explanation for this observation is that piRNA processing at Yb bodies occurs so quickly, and the processed piRNAs localize to the nucleus as

(C) Expression of Yb WT-r and P455A-r, but not Q399A-r and D537A-r, rescued the defect in piRNA accumulation in Yb-depleted OSCs. A piRNA arising from a *traffic jam (tj)* gene (Saito et al., 2009) was visualized by northern blotting using a specific DNA probe. miR310, loading control.

(D) Expression of Yb WT-r and P455A-r rerepressed the *mdg1* transposon in Yb-depleted OSCs, but expression of Q399A-r or D537A-r could not.

(E) Mutation of Q399A and D537A in Yb abolished Flam body (green) and Yb body (magenta) formation in OSCs. Endogenous Yb was depleted by RNAi. Nuclei are stained with DAPI (blue). Scale bar, 2 μ m.

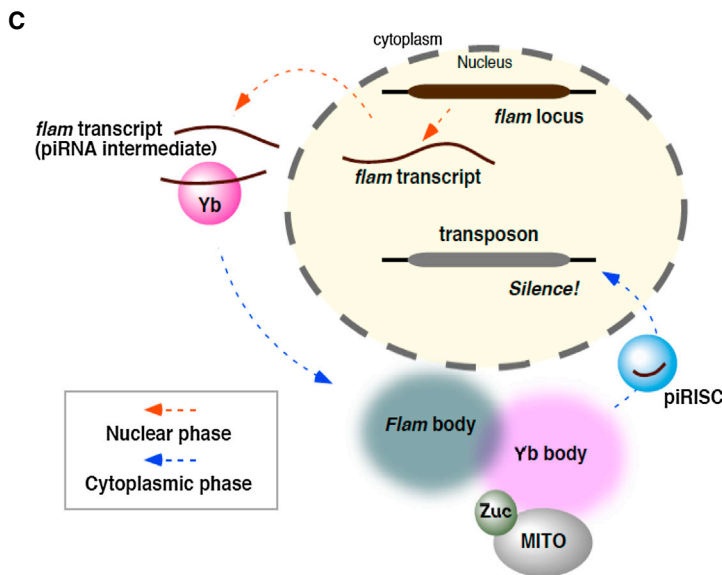
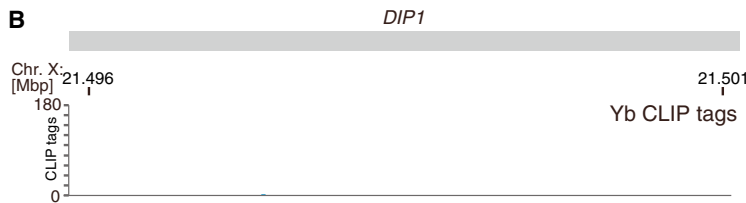
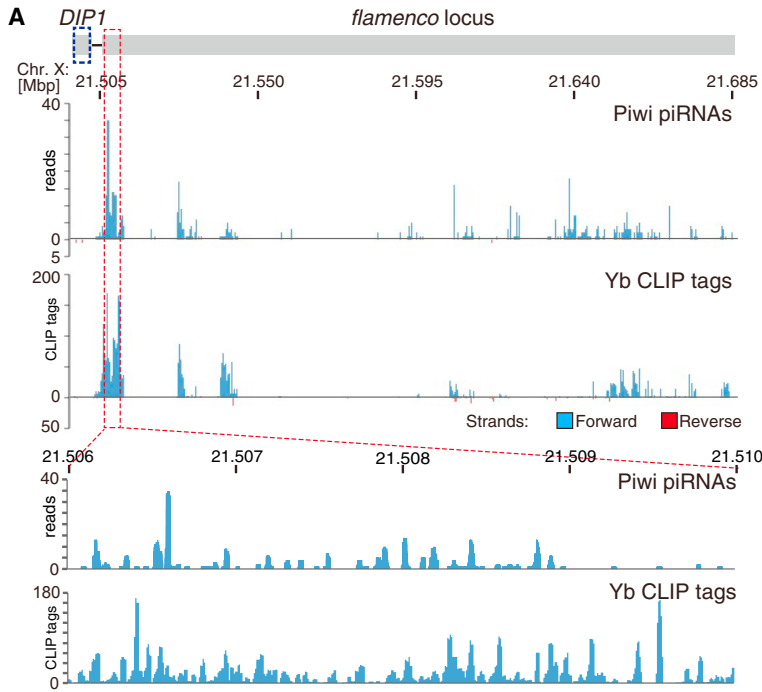


Figure 4. Yb Binds *flam* Transcripts, but Not *DIP1* mRNAs, In Vivo

(A) Mapped Piwi-piRNA reads and Yb-CLIP tags on the *DIP1* coding and *flam* cluster region. Forward reads are shown in blue, and reverse reads are shown in red. The number on the side of tracks denotes the number of reads in each position. Genomic coordinates are marked above the tracks. The bottom panel shows a further zoom-in view of sequences around the region where the RNA-FISH probe was designed to bind.

(B) A further zoom-in view of sequences of the *DIP1* gene in (A) (boxed by a dotted blue line).

(C) A new model for primary piRNA biogenesis in OSCs. The transcription and nuclear export of the transcripts are considered to be the “nuclear phase” of piRNA biogenesis. The *flam* transcripts become *flam*-piRNA intermediates by “shortening” in either the nucleus or the cytoplasm. The mechanism underlying the shortening process remains unknown. Yb binds piRNA intermediates in the cytoplasm and locates to Flam bodies. piRNA intermediates are further processed to mature piRNAs at Yb bodies with the help of Zuc on mitochondria (MITO) and loaded onto Piwi, giving rise to the piRISC. The piRISC is imported to the nucleus, where the RNP complex exerts its function: transcriptional silencing of transposons. Yb-piRNA intermediate association, piRNA maturation, and piRISC assembly are considered to be the “cytoplasmic phase” of piRNA biogenesis. Components of Yb bodies include Yb, Armi, Vret, SoYb, and Shu, all of which are required for piRNA biogenesis and piRISC formation in OSCs.

piRISCs so immediately, that the *flam* signal at Yb bodies was below the level of detection at Yb bodies.

In Zuc-depleted cells, *flam* transcripts were detected predominantly as *flam*-piRNA intermediates, being several hundred to 4,000 nt in length (Figures 2C and S2A), while the full transcriptional unit of *flam* is estimated to be over 180 kb (Brennecke et al., 2007; Malone et al., 2009). Both Yb body and Flam body formation require Yb, or more precisely, its RNA-binding activity through its NTD (Figures 3B, 3E, and S3F). Yb binds *flam*-piRNA intermediates directly (Figure 4A). Based on these findings, we propose a new model for primary piRNA biogenesis in ovarian soma (Figure 4C), in which the association of Yb with piRNA intermediates, which most likely occurs in the cytoplasm because Yb is a cytoplasmic protein (Olivieri et al., 2010; Saito et al., 2010), is the initiation point of the cytoplasmic phase of piRNA biogenesis. This follows the nuclear phase of piRNA biogenesis: *flam* transcription and nuclear export of *flam* transcripts through the nuclear pores. *flam* transcription is initiated by RNA polymerase II and requires the transcriptional factor Cubitus interruptus (Goriaux et al., 2014). However, it is not known by which export factors and in what lengths the *flam* transcripts are exported from the nucleus. Further investigation will be required for a detailed understanding of the nuclear phase of piRNA biogenesis.

The locations of the genomic *flam* loci in the nucleus and Flam bodies do not seem to be arranged to be close to each other, meaning that the *flam* transcripts move a long distance to arrive at Flam bodies (Figure 4C). Do the *flam* transcripts move within the nucleus to get closer to Flam bodies before export to the cytoplasm? Alternatively, does nuclear export occur first and then *flam* transcripts are localized to Flam bodies? Yb localization in the cytoplasm seems to be so dynamic that a point mutation in Yb that disrupts the RNA-binding capacity of Yb drastically changes the subcellular localization of Yb, causing it to be scattered evenly in the cytosol (Figures 3E and S3F). Thus, the latter scenario appears more likely, in which Yb plays a crucial role; upon nuclear export, Yb captures *flam* transcripts through direct binding and localizes them, as *flam*-piRNA intermediates, to Flam bodies. Flam body formation depends on the RNA-binding activity of Yb, a cytoplasmic protein (Olivieri et al., 2010; Saito et al., 2010); this notion further supports the idea that Flam bodies are cytoplasmic structures.

Unlike *flam* transcripts, *DIP1* mRNAs were virtually undetectable in Yb-CLIP tags (Figures 4A and 4B), although the *DIP1* protein-coding gene and the *flam* piRNA cluster are neighbors on chromosome X (Figure 1A) and *DIP1* is expressed in OSCs. We looked carefully at the sequences of Yb-CLIP tags but found no obvious consensus sequences. Yb may recognize binding substrates owing to higher-order structures.

Immunoelectron microscopy using an anti-Yb antibody showed that Yb bodies are often attached to mitochondria, to which Zuc endoribonuclease, the piRNA intermediate processor, anchors on the surface to face into cytoplasmic Yb bodies (Figure 1D). This peculiar spatial arrangement of Zuc and Yb bodies, along with Flam bodies, integrates all the ingredients necessary for primary piRNA production locally, enhancing the rates of piRISC assembly. Another virtue of this perinuclear

arrangement is that it enables assembled piRISCs to be immediately imported into the nucleus, where the RNP complex (i.e., the PIWI-piRNA complex) exerts its nuclear-specific function of silencing transposon transcription (Sienski et al., 2012). How does Yb decide where within the perinuclear region to integrate all the materials necessary for primary piRNA biogenesis? Reconstitution of the whole machinery in, for instance, nongonadal somatic Schneider2 cells, in which no primary piRNAs are otherwise expressed, might address this fundamental question.

EXPERIMENTAL PROCEDURES

Detailed procedures are provided in [Supplemental Experimental Procedures](#).

Drosophila Strains

Yellow white (*y w*) and the *flam* [*KG00476*] and the *fs(1)Yb[72]* alleles were used. Fly stocks were maintained at 25°C.

Cell Culture and RNAi

OSCs were grown in OSC medium prepared from Shields and Sang M3 Insect Medium (Sigma) supplemented with 0.6 mg/ml glutathione, 10% fetal bovine serum, 10 mU/ml insulin, and 10% fly extract. RNAi was performed using RNA oligos shown in [Table S1](#).

Production of Anti-Yb Antibodies

Monoclonal antibodies against Yb were raised primarily as described previously (Ohtani et al., 2013). A recombinant protein consisting of glutathione S-transferase and the N-terminal region of Yb (200 amino acids) was purified from *E. coli* and injected into mice.

RNA-FISH

The fluorescein isothiocyanate (FITC)- and digoxigenin-labeled RNA probes were prepared using RNA labeling mixture (Roche) and SP6RNA polymerase (Roche) according to the manufacturer's instructions. To prepare a probe specific for the *flam* locus, OSC genomic DNA was used as a template for PCR. The primers used for PCR are indicated in [Table S1](#). In situ hybridization was carried out essentially as described previously (Sone et al., 2007).

DNA-FISH

Digoxigenin- or biotin-labeled DNA probes were prepared using Nick Translation Mix (Roche) according to the manufacturer's instructions. To prepare the probes, bacterial artificial chromosome clones DME1-021J16 (upstream of the *flam* locus) and DME1-014M21 (downstream of the *flam* locus), were used as templates. OSCs were treated with ice-cold 0.75 M KCl for 5 min and then, after resuspending in acetic acid-methanol (1:3), spread onto slides. The slides were treated essentially according to the procedures of Masumoto et al. (Masumoto et al., 1989).

Immunofluorescence

Immunofluorescence was performed primarily as described previously (Ohtani et al., 2013; Saito et al., 2009).

Body Counting

Confocal images of immunofluorescence were transferred to the Columbus System (PerkinElmer Japan) and analyzed by Building Block (PerkinElmer Japan). Nuclei were masked and then perinuclear signals for Yb bodies and Flam bodies were detected.

Electron Microscopy In Situ Hybridization

OSCs were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4) overnight and then washed with RNase-free PBS. The samples were hybridized with FITC-conjugated specific RNA probe, except that 0.5% Triton X-100 was used for 5 min. Samples were incubated with a primary rabbit anti-FITC (1:500) antibody and then washed in 0.1 M phosphate buffer (PB) containing

0.005% saponin. Samples were incubated for 24 hr at 4°C with nanogold-conjugated anti-rabbit secondary antibody (1:100, Invitrogen).

Immunoelectron Microscopy

OSCs were fixed with 0.1% glutaraldehyde and 4% PFA in 0.1 M PBS, followed by incubation with 5% Block Ace containing 0.1% saponin in 0.1 M PB. The cells were stained with anti-Yb antibody (1:250) and nanogold-conjugated anti-mouse secondary antibody (1:100, Invitrogen).

Western Blot Analysis

Western blotting was performed primarily as described previously (Miyoshi et al., 2005).

Northern Blot Analysis

For *flam* transcript detection, total RNAs were isolated from OSC using ISOGEN (Nippon Gene). Hybridization was performed with random-labeled antisense oligodeoxynucleotide probe. Small RNAs were detected essentially as previously described (Saito et al., 2009).

Plasmid Construction

An expression vector for myc-Yb WT was generated by inserting the WT Yb coding region into the pAcM vector (Saito et al., 2009). myc-Yb-r was constructed as essentially described previously (Saito et al., 2010). Primers are listed in Table S1.

qRT-PCR Analysis

Reverse transcription was performed using a PrimerScript RT Master Mix (TaKaRa). The resulting cDNAs were amplified using a LightCycler 480 Real-Time PCR Instrument II (Roche) with SYBR Premix Ex Taq (TaKaRa). The primers used are listed in Table S1.

CLIP

CLIP was performed primarily as described previously (Jaskiewicz et al., 2012). Anti-Yb was used to immunopurify Yb from OSCs after irradiation by UV (254 nm) for crosslinking.

Bioinformatic Analysis

The Yb-CLIP library was sequenced using the Illumina HiSeq2000 platform according to the manufacturer's instructions. The average base-wise quality was checked, and those that passed quality control were subjected to analyses.

ACCESSION NUMBERS

Yb-CLIP tag sequencing data have been deposited in the Gene Expression Omnibus under the accession number GSE54875.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.043>.

AUTHOR CONTRIBUTIONS

M.Y., H.I., S.N., Y.W.I., S.S., M.K.K., and K.S. designed and performed experiments and helped write the manuscript. H.O. supervised electron microscopic experiments. H.S. and M.C.S. conceived the study and wrote the manuscript.

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