Doctoral Dissertation (Censored)
博士論文 (要約)

Requirements of Yb-driven multivalent phase separation
in production of transposon-repressible piRNAs

（トランスポゾン抑制性 piRNA の生合成には
多価性相互作用による Yb タンパク質の相分離が必要である）

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Contents

1. Abstract

1. Abstract

本章については、５年以内に雑誌等で刊行予定のため、非公開。

2. Abbreviations

2. Abbreviations

Ago3	Argonaute 3
Armi	Armitage
Aub	Aubergine
β-gal	Beta-galactosidase
BoYb	Brother of Yb
BSA	Bovine serum albumin
BSD	Blasticidin S deaminase
CLIP	Crosslinking and Immunoprecipitation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
eTud	Extended Tudor
FBS	Fetal bovine serum
flam	Flamenco
GFP	Green fluorescent protein
GST	Glutathione S-transferase
Hel-C	Helicase C-terminal
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IDR	Intrinsically disordered region
kb	Kilobase pairs
kd	Knockdown
kDa	Kilodaltone
LLPS	Liquid-liquid phase separation
Mino	Minotaur
miRNA	Micro RNA
mRNA	Messenger RNA
NA	Numerical aperture
n.i.	Non-immune immunoglobulin
NP40	Nonyl phenoxypolyethoxylethanol

nt	Nucleotide
OSC	Ovarian somatic cell
PBS	Phosphor buffered saline
PCR	Polymerase chain reaction
piRISC	piRNA-induced silencing complex
piRNA	PIWI-interacting RNA
qRT-PCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein
SDS	Sodium dodecyl sulfate
Shu	Shutdown
siRNA	Small interfering RNA
SoYb	Sister of Yb
tj	Traffic jam
T-PBS	0.1% Tween 20 in PBS
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride buffer
UTR	Untranslated region
Vret	Vreteno
WT	Wildtype
Yb	Female sterile (1) Yb
Zuc	Zucchini

3. Introduction

3. Introduction

3.1. RNA silencing and transposons

RNA silencing, or RNA interference (RNAi), is a gene-repression mechanism conserved among eukaryote (Aravin and Tuschl, 2005; Ghildiyal and Zamore, 2009; Hutvagner and Simard, 2008; Plasterk, 2002). The key components of RNA silencing are small RNA, which is 20-35 nucleotides (nt) in length, and RNA-binding protein belongs to Argonaute family (Figure 3.1A). They form complexes called RNA-induced silencing complex (RISC) in a stoichiometric manner. RISCs recognize and bind their targets using RNA – RNA base pairings between small RNAs and transcripts of target genes. Expression of target genes is repressed by small RNA-directed endonuclease (slicer) activity of Argonaute proteins and/or activities of accessory proteins bound to Argonaute.

In animals, small RNA-mediated gene silencing pathways are classified into three categories (Figure 3.1B) (Chu and Rana, 2007; Ghildiyal and Zamore, 2009; Hutvagner and Simard, 2008; Ipsaro and Joshua-Tor, 2015). micro RNAs (miRNAs) are derived from RNAs with imperfect hairpin structures and loaded onto AGO subfamily proteins. Small interfering RNAs (siRNAs) are

also loaded onto AGO proteins but processed from long double-stranded RNAs. PIWI-interacting RNAs (piRNAs) are processed from long single-stranded RNAs and loaded onto PIWI subfamily proteins. Each pathway has characteristic properties in its function and expression pattern. miRNAs are expressed ubiquitously and regulate mainly expressions of endogenous genes. siRNAs are also ubiquitous, but their major targets are viruses. piRNAs are expressed specifically in gonads and repress the expression of transposons.

Transposons, or transposable elements, are mobile deoxyribonucleic acid (DNA) elements that can transpose in the genome. Transposition of transposon is thought to be a driving force of evolution based on a long-term point of view. However, it can harm the essential genes and the host organisms have obtained various ways to repress the expression of transposons (Goodier, 2016; Slotkin and Martienssen, 2007). In *Drosophila* non-gonadal tissues, transposons are also targeted by siRNAs (Chung et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008). In *Drosophila* gonads, siRNAs and piRNAs cooperatively repress transposons and keep the genome integrity of germ cells, which are the only cells to be passed to the next generations (Czech et al., 2008; Lau et al., 2009). The contribution of each pathway for

silencing varies among transposons, however, the piRNA pathway is thought to be the dominant transposon-silencing mechanism in *Drosophila* gonads, because loss of piRNA functions reactivated expressions of most transposons (Vagin et al., 2006).

3.2. piRNAs in *Drosophila*

Among three categories of animal small RNA pathways, the piRNA pathway is the most recently discovered pathway. In 2006, piRNAs were found for the first time in flies and mammals (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2006). Since then, the studies of the piRNA pathway have been performed mainly using mouse testis and fly ovaries (Aravin et al., 2007; Ghildiyal and Zamore, 2009; Hirakata and Siomi, 2016; Iwasaki et al., 2015; Juliano et al., 2011; Malone and Hannon, 2009; Yamashiro and Siomi, 2018), even though piRNAs and piRNA-like small RNAs are found in many animal species including sponge, sea urchin, and planarian (Friedlander et al., 2009; Grimson et al., 2008; Wei et al., 2012).

Flies possess three *PIWI* genes: *Argonaute 3* (*Ago3*), *Aubergine* (*Aub*), and *Piwi*. A lack of any member of the PIWI

family in *Drosophila* causes severe defects in oogenesis and spermatogenesis, leading to infertility (Cox et al., 1998; Li et al., 2009; Lin and Spradling, 1997; Schmidt et al., 1999; Schüpbach and Wieschaus, 1991). Thus, the functions of PIWI proteins are not redundant and all are necessary for development of both ovaries and testes.

piRISCs implement transposon silencing at both the transcriptional and post-transcriptional levels, depending on the subcellular localization of the complexes (Aravin et al., 2007; Ghildiyal and Zamore, 2009; Iwasaki et al., 2015; Juliano et al., 2011; Malone and Hannon, 2009; Yamashiro and Siomi, 2018). Cytoplasmic PIWI proteins, such as Aub and Ago3 in *Drosophila*, repress transposons post-transcriptionally by cleaving RNA transcripts using slicer activity (Figure 3.2). By contrast, nuclear PIWI proteins, such as fly Piwi, repress transposons transcriptionally by inducing heterochromatinization at target loci. Target RNAs cleaved by cytoplasmic piRISCs can be used as substrates for producing secondary piRNAs. Thus, cytoplasmic PIWI proteins can also be considered piRNA biogenesis factors. Because of this, post-transcriptional silencing and secondary piRNA production are recognized as a coupled event.

Expression of PIWI proteins is regulated spatio-

temporarily in gonads (Bak et al., 2011; Brennecke et al., 2007; Cox et al., 2000; Gunawardane et al., 2007; Harris and Macdonald, 2001; Malone et al., 2009). In *Drosophila* ovaries, basically all PIWI proteins (Piwi, Aub and Ago3) are expressed in the germ cells, which are derived from germline stem cells (Figure 3.2). In contrast, follicle cells, which are somatic cells surrounding the germ cells, only express nuclear PIWI protein, Piwi. Therefore, secondary piRNAs are not produced in follicle cells. piRNAs in follicle cells are mainly targeting the *gypsy* family transposons, which may transpose from follicle cells to germ cells by making virus-like particles (Malone et al., 2009).

3.3. piRNA biogenesis in *Drosophila* ovarian somatic cells

piRNAs loaded on Piwi in *Drosophila* follicle cells are primary piRNAs, which are produced in a manner independent of slicer activity of PIWI. A cultured cell line composed of ovarian somatic cells (OSCs), established in 2009 (Saito et al., 2009), has been used as a powerful tool for researches to understand the mechanism underlying biogenesis of primary piRNAs. In OSCs, transposon-targeting piRNAs arise nearly exclusively from the *flamenco* (*flam*) locus, an intergenic region with a plenty of

transposon remnants whose orientations are mainly opposed against original active transposons (Figure 3.3A) (Brennecke et al., 2007; Lau et al., 2009; Li et al., 2009; Malone et al., 2009). Upon transcription, the *flam* RNA transcripts undergo splicing partially and are exported to the cytoplasm, where they accumulate into perinuclear structures Flam bodies/Dot COM for further processing (Dennis et al., 2016; Goriaux et al., 2014; Murota et al., 2014). The *flam* piRNA precursors may also be stored at nuclear Dot COM prior to nuclear export (Dennis et al., 2013). In addition to *flam*, some protein-coding genes serve as the sources of piRNAs in OSCs (Robine et al., 2009; Saito et al., 2009). The piRNAs derived from coding genes are called "genic piRNAs." Most of genic piRNAs do not possess the sequences complementary to transposons, and their targets and functions remain elusive.

piRNA processing in OSCs occurs in a manner depending on a number of piRNA factors including female sterile (1) Yb (Yb), Armitage (Armi), Sister of Yb (SoYb), Vreteno (Vret), Shutdown (Shu), Zucchini (Zuc), Gasz, and Minotaur (Mino) (Handler et al., 2013; Handler et al., 2011; Olivieri et al., 2012; Olivieri et al., 2010; Preall et al., 2012; Saito et al., 2010; Vagin et al., 2013; Zamparini et al., 2011). Not all, but some of the factors were analyzed previously, and models of, at least parts of, their roles in the piRNA

pathway were proposed. The precursors of piRNAs are selectively bound by Yb and funneled to the piRNA-processing pathway (Figure 3.3A) (Ishizu et al., 2015; Pandey et al., 2017). Single molecule of long precursor RNA is processed into multiple piRNAs in a sequential manner with 5' to 3' direction (phasing) (Figure 3.3B) (Han et al., 2015; Mohn et al., 2015). Cleavages of RNA during phasing are catalyzed by Zuc, an endo-ribonuclease (Ipsaro et al., 2012; Nishimasu et al., 2012).

Upon processing, mature piRNAs form piRNA-induced silencing complexes (piRISCs) with Piwi. Piwi-piRISCs are then imported to the nucleus by Importin α (Yashiro et al., 2018), where they repress transposons cotranscriptionally with multiple cofactors (Brower-Toland et al., 2007; Dönertas et al., 2013; Iwasaki et al., 2016; Ohtani et al., 2013; Sato and Siomi, 2018; Sienski et al., 2015; Sienski et al., 2012; Yu et al., 2015).

Yb bodies are gonadal soma-specific membraneless organelles (Saito et al., 2010; Szakmary et al., 2009) to which Yb, Armi, SoY, Vret, and Shu are localized (Handler et al., 2011; Olivieri et al., 2012; Olivieri et al., 2010; Saito et al., 2010; Szakmary et al., 2009). Other processing factors, Zuc, Gasz, and Mino, are not localized to Yb bodies but are anchored on the surface of mitochondria through their own transmembrane signals

(Handler et al., 2013; Saito et al., 2010; Vagin et al., 2013). Yb bodies tend to be surrounded by mitochondria and adjacent to Flam bodies (Figure 3.3A) (Murota et al., 2014; Szakmary et al., 2009). This spatial arrangement of the organelles locally concentrates piRNA-processing factors and precursor RNAs; thus, Yb bodies were considered to be the site of piRNA production. The hierarchy of Yb body assembly has previously been examined (Handler et al., 2011; Olivieri et al., 2012; Saito et al., 2010). However, a comprehensive and systematic analysis including SoYb has not been performed, and the mechanism of Yb body formation remains elusive.

3.4. Yb protein

Yb was originally reported as a novel gene required for fertility in *Drosophila* female (Young and Judd, 1978). *Drosophila* Yb is expressed specifically in somatic cells of ovaries and testis (King and Lin, 1999; Szakmary et al., 2009). Expression of Yb in ovarian cap cells is necessary for stable expression of *piwi* and *hedgehog*, which are critical for maintenance of germline stem cells and division of somatic stem cells (Figure 3.4) (King et al., 2001). Although the mechanism(s) regulating *hedgehog* remain(s)

elusive, it is proposed that Yb may stabilize Piwi protein by piRISC formation (Qi et al., 2011). Also in follicle cells and cultured OSCs, Yb is required for primary piRNA biogenesis and repression of transposons (Olivieri et al., 2010; Saito et al., 2010). Lack of Yb barely affects the transcription of *flam* and possibly genic piRNA sources (Qi et al., 2011).

Yb is a member of TDRD12 family of proteins conserved from insects to mammals. Most animals possess one *TDRD12* gene, but only *Drosophila* possesses three *TDRD12* genes *Yb*, *SoYb*, and *Brother of Yb* (*BoYb*). In addition, *Drosophila TDRD12* genes are also unique in a view of their functions as summarized in Figure 3.5 (Handler et al., 2013; Handler et al., 2011; Pandey et al., 2013).

Yb contains three functional domains, Helicase-C terminal (Hel-C), RNA helicase, and extended Tud (eTud) domains (Figure 3.5) (Handler et al., 2011; Szakmary et al., 2009). The RNA helicase domain consists of P-loop NTPase and Hel-C domains. It was previously shown that alteration of Gln399 or Asp537 in the RNA helicase domain to alanine severely reduced the RNA-binding activity of Yb (Figure 3.6A) (Murota et al., 2014). Both mutants Q399A and D537A failed to form Yb bodies and barely restored the piRNA biogenesis and transposon silencing abrogated by loss of endogenous Yb in OSCs, suggesting that the association

of Yb with piRNA precursors via the RNA helicase domain is essential for Yb body formation and piRNA biogenesis.

The *cis*-elements that drive piRNA biogenesis were identified in *flam* transcripts and genic piRNA sources such as *traffic jam* (*tj*) messenger RNAs (mRNAs) (Homolka et al., 2015; Ishizu et al., 2015). Enforced tagging of the *cis*-element to the 5′ end, but not the 3′ end, of arbitrary RNAs induced artificial piRNA production from the downstream regions, which repressed genes highly complementary to the piRNAs (Homolka et al., 2015; Ishizu et al., 2015). Yb acts as the *trans*-acting factor, binding to the *cis*-element and triggering piRNA biogenesis (Ishizu et al., 2015; Pandey et al., 2017). RNA binding of Yb also determines the regions from which piRNAs are produced (Figure 3.6B) (Ishizu et al., 2015).

Both the Hel-C and eTud domains of Yb are necessary for *piwi* and *hedgehog* expression in ovarian cap cells, and for germarium development (Szakmary et al., 2009). However, their molecular functions in piRNA biogenesis remain elusive.

3.5. Membraneless organelles and liquid-liquid phase separation

Some of the membraneless organelles, including

ribonucleoprotein (RNP) granules such as P bodies and P granules, are reported to show liquid-like propensities like fusion and fission events in cells (Banani et al., 2017; Boeynaems et al., 2018; Gomes and Shorter, 2018) (Figure 3.7A). P bodies sequester translationally repressed mRNAs to increase their stability (Luo et al., 2018). P granules are germline-specific structures necessary for germline development in *C. elegans* (Strome, 2005). These granules were reported to be formed by liquid-liquid phase separation (LLPS), where excess components in one liquid phase spontaneously accumulate to new liquid phase (Figure 3.7B). Components of organelles formed by LLPS tend to interact each other weakly but in a multivalent manner. Treatment of cells permeabilized by digitonin with 1,6-hexanediol, a chemical that inhibits weak hydrophobic interactions, often disrupt the granules. Thus, 1,6-hexanediol is widely used as chemical probe to detect LLPS.

In ovarian germ cells, piRNA factors are localized in nuage, germ cell-specific RNP granules. A mouse homolog of Vasa, the germ cell-specific RNA helicase localized in nuage, undergoes LLPS *in vivo* and *in vitro* (Nott et al., 2015). However, the involvement of LLPS to the formation of Yb bodies, somatic counter part of nuage, was not tested.

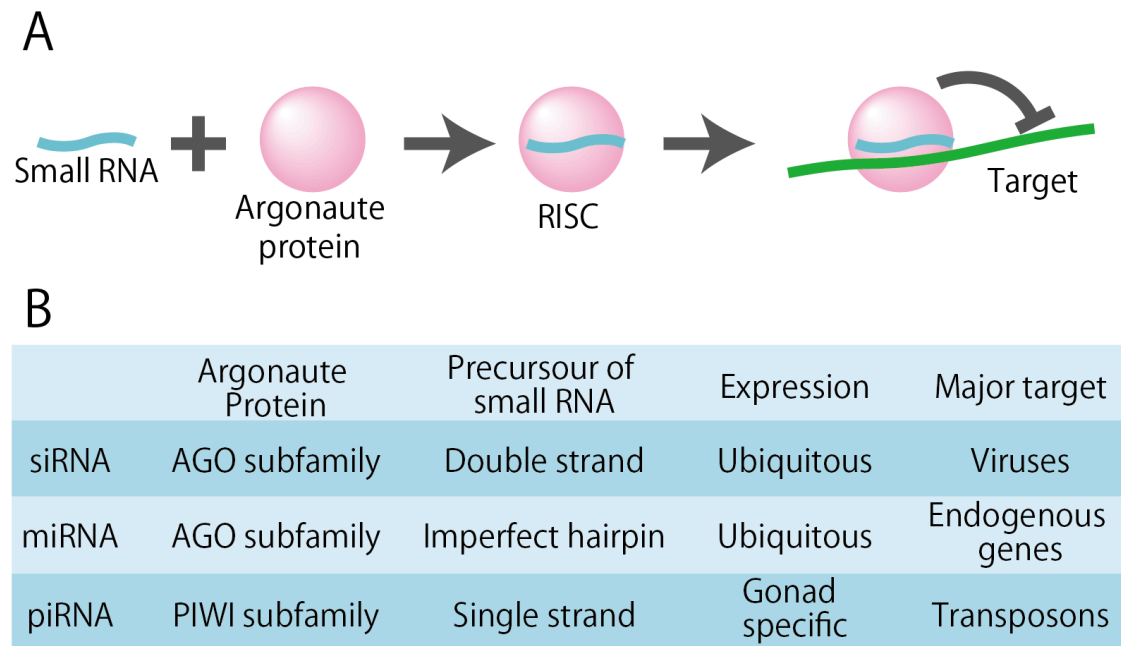## 3.6. Summary of this study

本節については、5年以内に雑誌等で刊行予定のため、非公開。

A

B

	Argonaute Protein	Precursour of small RNA	Expression	Major target
siRNA	AGO subfamily	Double strand	Ubiquitous	Viruses
miRNA	AGO subfamily	Imperfect hairpin	Ubiquitous	Endogenous genes
piRNA	PIWI subfamily	Single strand	Gonad specific	Transposons

Figure 3.1. RNA silencing and its classification
(A) Outline of RNA silencing. Silencing of target genes may involve other factors. (B) Classification of RNA silencing pathways in animals.
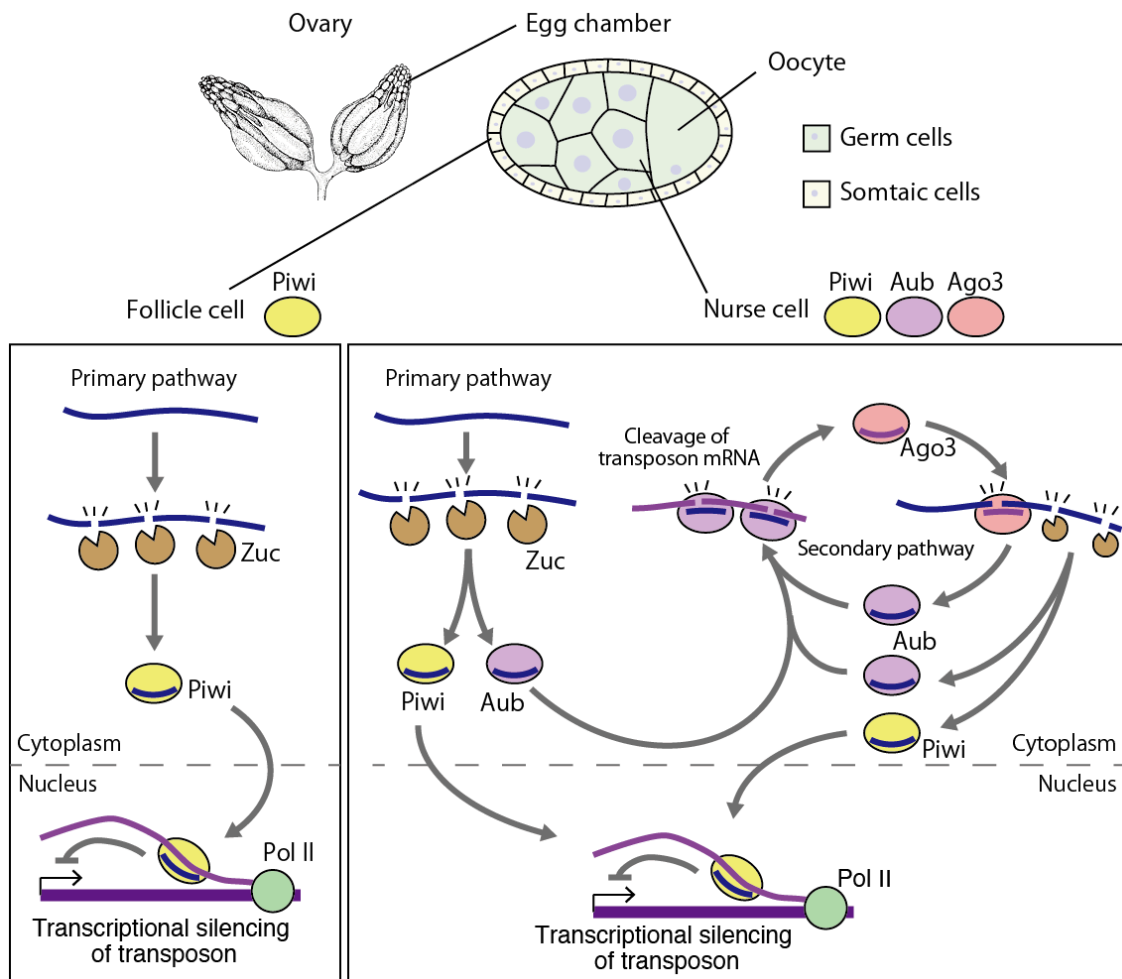
Figure 3.2. piRNA pathway in *Drosophila* ovary.

Germ cells in *Drosophila* ovaries possess three PIWI proteins and complicated piRNA pathway. In contrast, somatic cells produce only primary piRNAs loaded onto Piwi proteins. Therefore, somatic cells are suitable for analysis of the primary pathway.
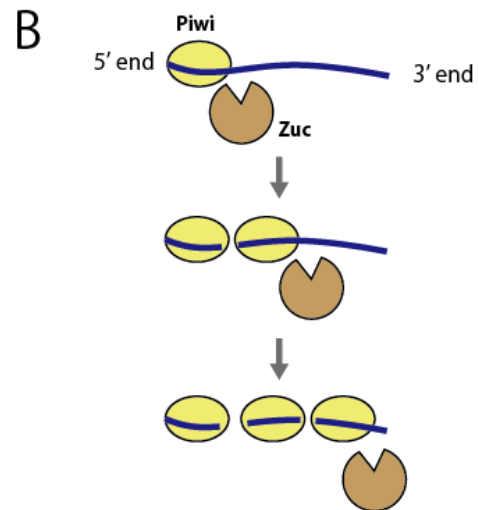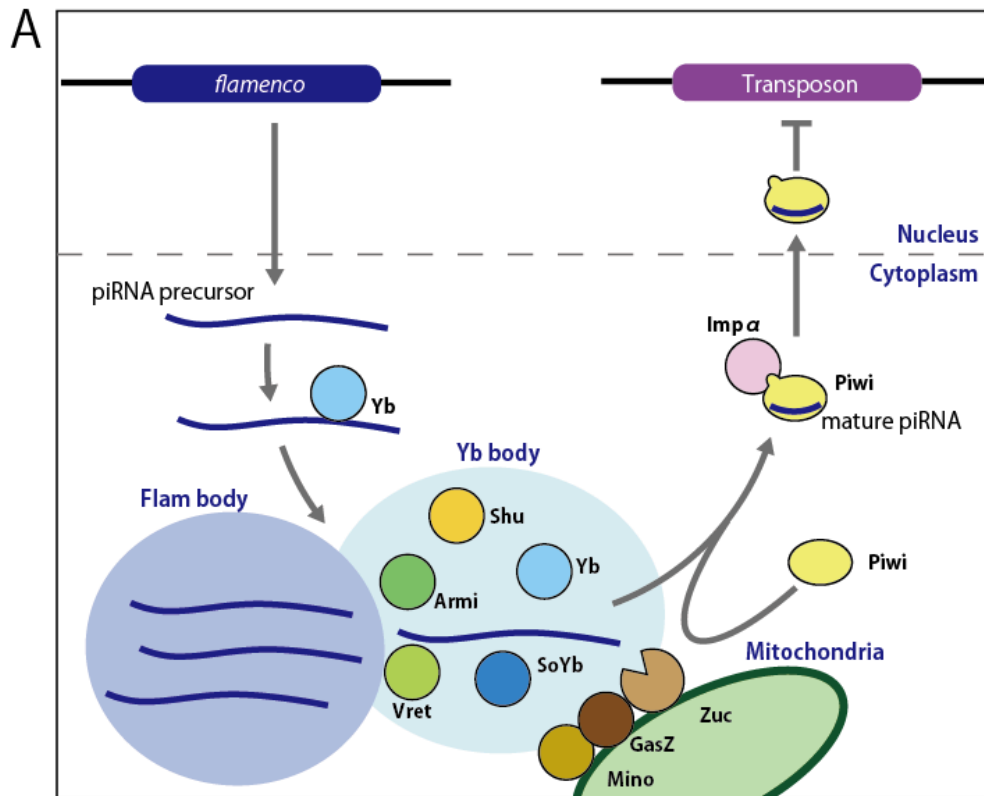
Figure 3.3 (legend on next page).

Figure 3.3. piRNA biogenesis in *Drosophila* ovarian somatic cells. (A) Current model of piRNA biogenesis pathway in *Drosophila* ovarian somatic cells. Most of transposon-repressible piRNAs are derived from intergenic loci, *flam*. mRNAs of some protein coding genes including *traffic jam* (*tj*) are also used as piRNA precursors and processed into "genic piRNAs" (not shown). (B) Phased piRNA biogenesis by Zuc.
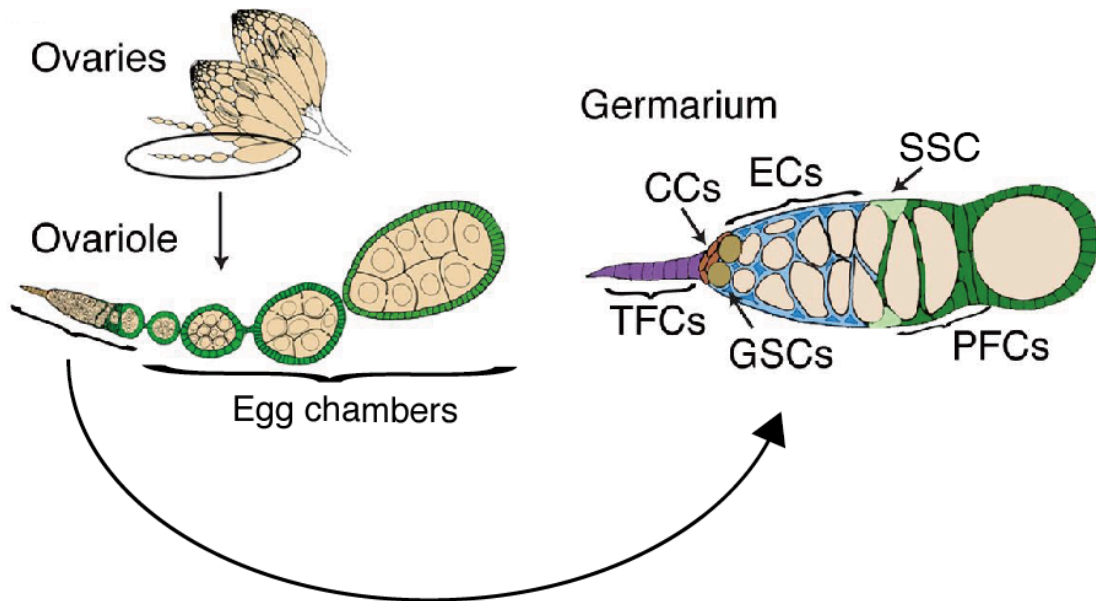
Figure 3.4. Cartoon of *Drosophila* germarium.

This figure is modified from Huang et al., 2014. Terminal filament cells (TFCs; purple), cap cells (CCs; red), escort cells (ECs; blue), somatic stem cells (SSCs; light green), prefillicle cells (PFCs; green), and follicle cells (green) are somatic cells. Germline stem cells (GSCs; brown) and germline cysts (beige) are germ cells.
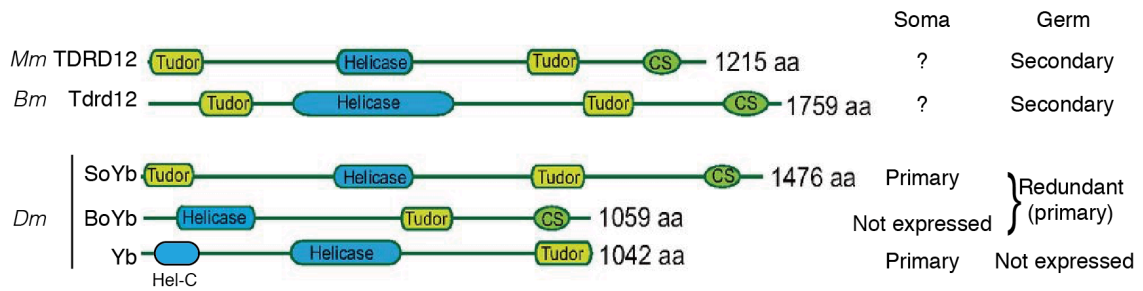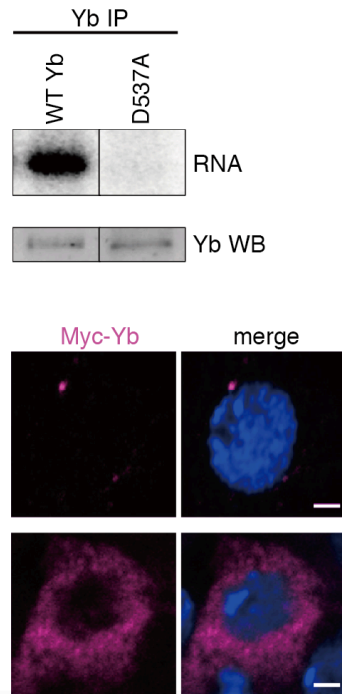
Figure 3.5. Comparison of TDRD12 family genes.
TDRD12 family genes of mouse (*Mm*), fruit fly (*Dm*), and silkworm (*Bm*) are compared. Extended Tudor (eTud) domains are shown as "Tudor." This figure is modified from Pandey et al., 2013.
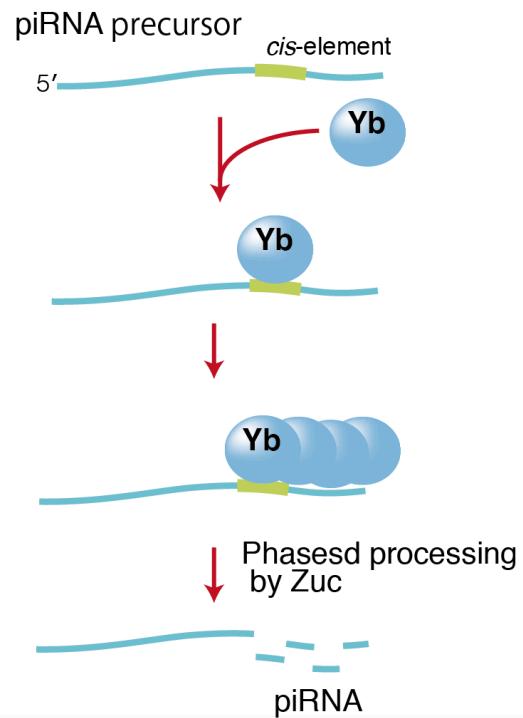
Figure 3.6. Molecular function of Yb in piRNA biogenesis.
(A) Asp537 in the RNA helicase domain of Yb is necessary for RNA binding (upper) and Yb body formation (lower) of Yb. D537A mutant failed to produce piRNAs (Murota et al., 2014). This figure is modified from Murota et al., 2014. Scale bar; 2 μm. (B) RNA binding of Yb triggers piRNA biogenesis from downstream region of the RNA by spreading towards 3' end and inducing the processing.
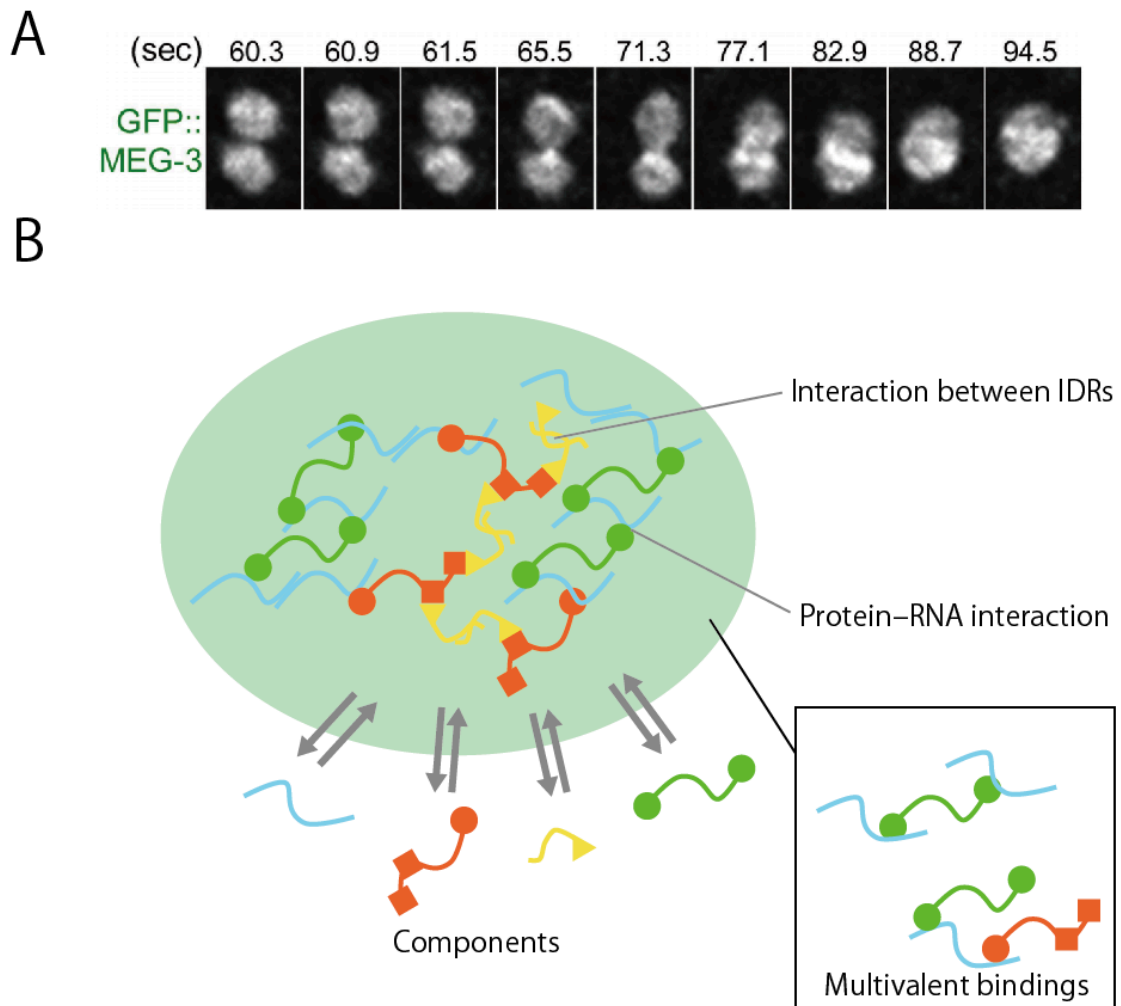
Figure 3.7. Liquid-liquid phase separation triggers granule formation.

(A) P granules in *C. elegans* fuse in a liquid-like manner. GFP-fused MEG-3, one of the P granule components, was expressed in embryo. This figure is modified from Wang et al., 2014. (B) Cartoon of liquid-liquid phase separation. Some examples of interactions typical for liquid-liquid phase separation are indicated. Intrinsically disordered regions (IDRs) are regions of polypeptide chains that do not form unique stable three-dimensional structures.

4. The aim of this study

4. The aim of this study

The main goal of this study was to elucidate the molecular mechanism of Yb body formation and function of both Hel-C and eTud domains of Yb in piRNA biogenesis pathway. To this end, I conducted biochemical analyses to determine the hierarchy of protein components in Yb body assembly. In addition, to understand the functions of the Hel-C and eTud domains of Yb, I produced two deletion mutants, ΔHel-C and ΔeTud, and examined their biological properties. Furthermore, I postulated that Yb bodies might be formed by phase separation. To test this, I assessed the properties of Yb bodies *in vivo*.

Elucidation of both the molecular mechanism of Yb body formation and the function of Yb is essential for comprehensive understanding of piRNA pathway, especially of Yb-driven precursor selection, which is a critical step for silencing of correct targets and hence for fertility.

5. Materials and methods

5. Materials and methods

本章については、５年以内に雑誌等で刊行予定のため、非公開。

6. Results

6. Results

本章については、5年以内に雑誌等で刊行予定のため、非公開。

7. Discussion

7. Discussion

本章については、５年以内に雑誌等で刊行予定のため、非公開。

8. Conclusion

8. Conclusion

In this study, I revealed that Hel-C is necessary for self-association of Yb while eTud is essential for Yb to interact with piRNA precursors and Armi. All three domains of Yb are necessary for Yb body assembly and transposon silencing. In addition, my results suggest that Yb bodies are multivalent RNA−protein condensates whose assembly depends both on homotypic interaction of Yb, and on Yb binding *flam* RNAs. Surprisingly, Yb body formation is not absolutely required for piRNA biogenesis. My current study provides a new insight about function of Yb bodies; that is Yb bodies are the elaborative system specialized for facilitation of production of piRNAs functional in transposon silencing.

Determinants of Hel-C dependencies of individual precursors remain ambiguous. However, this is the first report to show the effect of loss of Yb bodies under the condition where all of their components are expressed, and the biological relevance of Yb body assembly has now emerged. I believe that this study contributes to profound understanding about how animals specifically repress the transposon to maintain fertility.

9. References

9. References

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