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**Squash functions as the co-factor of Spindle-E
and ensures Argonaute 3 stability for germline
piRNA biogenesis in *Drosophila***

Ph.D. Thesis

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March 2026

The University of Osaka

Graduate School of Frontier Biosciences

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Abstract

PIWI-interacting RNAs (piRNAs) repress the expression of moveable transposons (TEs) and protect the integrity of genome in animal gonads. In *Drosophila* germline cells, piRNAs are produced in membraneless granules called nuage via the heterotypic ping-pong pathway performed by Aub and Ago3, known as PIWI family proteins. The Tudor-domain protein Tejas (Tej) promotes nuage assembly and piRNA precursor processing by recruiting the RNA helicases Vasa (Vas) and Spindle-E (Spn-E).

However, the function of another nuage component, Squash (Squ), which does not have any known domains/motifs, remains elusive. Here, we confirmed loss function of *squ* could lead to the upregulation of TEs and found the accumulation of piRNA precursors like *42AB* and *38C* on nuage, suggesting that Squ may function for the processing of piRNA cluster transcripts. Squ depletion also results in a severe reduction of Ago3 protein and its bound piRNAs, while Aub–Aub homotypic ping-pong occurs in *squ* mutants in an Ago3-dependent manner. Besides, we found that Squ interacted with Spn-E, and such the interaction is required for the TE repression and piRNA precursor processing. Based on these results, our findings identify Squ as a critical factor for heterotypic ping-pong amplification by maintaining Ago3 protein level and for piRNA precursor processing by modulating Spn-E helicase activity.

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1. Introduction

1.1 Transposons

Transposons, also known as transposable elements (TEs), are mobile DNA sequences that can move around the host genome almost across all eukaryotic organisms. In most organisms, TE contents correlate with their genomic sizes and in some species, the number of TE contents could be 85% of the genome (Wegrzyn et al., 2013; Wells and Feschotte, 2020). Based on the transcription mechanisms, TEs are generally divided into two major classes: Class I elements and Class II elements. Class I elements, also named retrotransposons, transpose through the RNA intermediates via the ‘copy-and-paste’ mechanism (Dombroski et al., 1994). Class I elements can be further divided into three subgroups: one long terminal repeat (LTR) retrotransposon and two non-LTR elements, long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs) (Wells and Feschotte, 2020). Class II elements, also called DNA transposons, move in genome by the ‘cut-and-paste’ mechanism (Feschotte and Pritham, 2007). Upon TE performs insertion in genome, a common feature is the generation of target site duplications and regulatory sequences that can influence the gene expression (Wells and Feschotte, 2020).

In *Drosophila melanogaster*, transposons occupy approximately 15–20% of the total genome, playing roles on the diversity of genome, chromatin organization and evolution (Kaminker et al., 2002; Rahman et al., 2015). Transposable elements in *D. melanogaster* can be classified into 49 LTR families, 27 LINE-like families, and 19 TIR families (Kaminker et al., 2002; Gleason et al., 2018). These elements exhibit different genomic distributions on the heterochromatin regions and euchromatin regions where they can influence the expression of neighboring genes through promoter interference or chromatin modification (Sentmanat and Elgin, 2012).

TE insertion in the host genome would lead to a broad range of consequences:

disrupting gene coding sequences or regulatory regions, causing chromosomal rearrangements through ectopic recombination process and modulating gene expression by providing alternative promoter or enhancers (Kazazian, 2004; Slotkin and Martienssen, 2007). In *Drosophila*, a well-characterized mutation caused by a DNA transposon insertion, *P-element*, has been used for mutagenesis. Firstly *P-element* was not found in the isolated laboratory strains in the early 20th century but after interbreeding between *P-element* carrying P strain males and *P-element* deficient lab stock M strain females, lead to a P-M hybrid dysgenesis and finally made *P-element* become a rare example of TE invasion in a eukaryotic species (Kidwell, 1983; Daniels et al., 1990).

TE mobilization in the host genome severely threatens genome integrity, to defend TE invasion, *Drosophila* has evolved a small-RNA mediated process known as PIWI-interacting RNA (piRNA) pathway (Brennecke et al., 2007; Sienski et al., 2012).

1.2 piRNAs

RNA silencing pathway protects the genome integrity against the TEs in plants and animals. Small RNAs ranging from 19 to 30 nucleotides (nts) can specify the targets to be repressed. Two well-known small RNA silencing pathways are small RNA (siRNA) and microRNA (miRNA) interference pathways (Vagin et al., 2006).

Distinct from siRNAs and miRNAs, another small-RNA named PIWI-interacting RNAs (piRNAs), which ranging from 23 to 29 nts and bearing 2'-O-methyl-modified 3' termini was found to bind PIWI-clade Argonaute proteins rather than AGO-clade proteins for siRNAs and miRNAs (Brennecke et al. 2007; Czech and Hannon, 2011; Ozata et al. 2019). piRNAs were first characterized by the role in suppressing the Stellate protein in the somatic germ cells and found to be the most abundant small RNAs in animal gonads (Aravin et al., 2001, 2004; Gleason et al., 2018). piRNAs were then reported in *Drosophila* ovaries and embryos but named as repeat-associated small RNAs called rasiRNAs (Vagin et al., 2006). Finally, they were renamed as piRNAs as

these small RNAs are associated with PIWI-family proteins Piwi, Aubergine (Aub), and Argonaute 3 (AGO3) to form the complex for TE repression (Brennecke et al. 2007; Gunawardane et al., 2007; Gleason et al., 2018).

1.3 PIWI-family proteins

In *Drosophila* gonads, piRNA pathway is active in both germline cells and somatic cells, represses the expression of transposons and protects the genome integrity through distinct processes (Gleason et al., 2018). piRNAs bind to PIWI-family proteins to form the piRNA-induced silencing complex (piRISC) and then silence transposon by the function of a post-transcriptional degradation for TE transcripts and a transcriptional silencing on transposon loci (Vagin et al., 2006; Rozhkov et al., 2013; Gleason et al., 2018).

Although different proteins would participate in germline and somatic piRNA biogenesis, these two cells share the core components as PIWI-family proteins, a subclade of Argonaute-family proteins (Cox et al., 1998; Carmell et al., 2002). The members of PIWI-family proteins are Piwi, Aubergine (Aub) and Argonaute3 (Ago3). Piwi, as the core member, functions in both germline and somatic cells. Aub and Ago3 are crucial for piRNA biogenesis in germline cells. These proteins harbor highly conserved functional domains with Argonaute-family proteins: PAZ (Piwi-Argonaute-Zwille), PIWI and MID domains (Song et al., 2004; Elkayam et al., 2012; Schirle and MacRae, 2012; Wei et al., 2012). PAZ domain contains a unique binding structure of oligonucleotide/oligosaccharide-binding-fold and a preference for 3' end modification of guide RNAs. The MID and PIWI domain contain a hydrophilic interface for 5' end phosphate of guide RNAs. Mature piRNAs function as guide RNAs to bring PIWI-family proteins to the targets for silencing (Lingel et al., 2003; Yan et al., 2003; Tian et al., 2011; Boland et al., 2011). Once piRNAs are anchored to PIWI-family proteins by the function of PAZ and MID domain, PIWI-proteins can directly target the RNAs which are complementary to the direction of piRNAs. PIWI domain structure is similar

to that of RNase H enzymes and functions on hydrolyzing the phosphodiester bond linker of nucleotides, cleaving its bound target RNAs (Yan et al., 2003; Song et al., 2004; Parker et al., 2004; Schwarz et al., 2004; Yuan et al., 2005). Recent studies revealed that not only these conserved functional domains are crucial for piRNA biogenesis, the disordered N-terminal regions of PIWI-family proteins are also important for the post-translational modification among piRNA biogenesis and TE repression process (Huang et al., 2021).

1.4 piRNA clusters

piRNA clusters were first identified by mapping analysis of the localization for piRNA positions in *Drosophila* genome and considered to be the main sources of piRNAs (Brennecke et al., 2007; Hiraoka and Siomi, 2016). Until now, genome-wide analysis for piRNA clusters have been performed in different species. Researchers identified 142 piRNA clusters in *Drosophila* genomes and 186 piRNA clusters in human genomes (Girard et al., 2006; Brennecke et al., 2007; Hiraoka and Siomi, 2016). piRNA clusters were found with a length from few kilobases to hundreds of kilobases among different animals. Transposons were found enriched in piRNA cluster region and with a diverse varieties and percentage among different species (Girard et al., 2006; Hirano et al., 2014; Hiraoka and Siomi, 2016). In *Drosophila*, piRNA clusters are found to be localized on the pericentric and telomeric regions of the boundaries between heterochromatin and euchromatin (Brennecke et al., 2007; Gleason et al., 2018).

Based on the transcription orientation, piRNA clusters are divided into two groups: uni-strand in somatic cells and dual-strand clusters in germline cells (Hiraoka and Siomi, 2016).

1.4.1 Transcription of dual-strand clusters

The transcription of dual strand piRNA clusters depends on the function of RNA polymerase II (RNA poly II), but different from the transcripts of canonical

transcription, most transcripts from piRNA clusters are uncapped in their 5' ends and lacked polyadenylated tails (Zhang et al., 2014; Mohn et al., 2014; Chen et al. 2016). Canonical transcription depends on the H3 lysine4 trimethylation (H3K4me3) and contains the specific sequence for RNA slicing and transcriptional termination. However, piRNA cluster transcription depends on another promoter named H3K9me3 and lacks the hallmarks of canonical transcription, implying two distinct mechanisms between canonical and piRNA cluster transcription (Li et al., 2009; Ozata et al., 2019). Several studies revealed that loss function of *dsetDB1* (*egg*), a crucial histone methyltransferase protein, leads to a significant reduction for piRNA cluster transcripts, supporting piRNA cluster transcription is associated with H3K9me3 (Rangan et al., 2011).

Due to the lack of canonical promoters in piRNA cluster regions, the transcription for such piRNA clusters is performed by a specific complex named RDC (Rhino-Deadlock-Cutoff) complex in germline cells (Mohn et al., 2014; Zhang et al., 2014; Gleason et al., 2018). The components of RDC complex are: H3K9me3-binding protein Rhino (Rhi), a homolog of the heterochromatin protein 1a (HP1a), harbors chromo- and chromo-shadow domains. Deadlock (Del) is a homolog of 5'-3' nuclease Rai1/DXO which functions on controlling the RNA qualities and transcription termination, but no conserved domains are found in Del. Cutoff (Cuff) is crucial for piRNA biogenesis (Xue et al., 2000; Volpe et al., 2001; Chen et al., 2007; Pane et al., 2011; Mohn et al., 2014; Zhang et al., 2014). The functional chromodomain of Rhino could recognize the H3K9me3 sites of piRNA clusters and then recruit Del and Cuff to form the RDC complex to license the transcription (Klattenhoff et al., 2009; Mohn et al., 2014; Zhang et al., 2014). Loss function of RDC complex proteins increases the splicing for piRNA cluster transcripts and may lead to the destabilization of these transcripts, support the importance of RDC complex in piRNA cluster transcription (Mohn et al., 2014; Zhang et al., 2014; Chen et al. 2016).

Recent studies revealed that many other components may also participate in the

process of piRNA cluster transcription. Moonshiner, a paralog of germline-specific transcription initiation factor II A subunit 1 (GTF2A1), was found to be recruited to RDC complex. Then, a TATA box-binding protein factor named TRF2 was recruited to RDC and initiates the transcription (Andersen et al., 2017). Previous study revealed that Transcription/Export (TREX) complex functions on the processing of pre-mRNAs and nuclear exporting of mRNAs in yeast and mammals (Masuda et al., 2005). And a recent study found that Tho5 and other THO subunits are recruited by Cuff and accumulated in nascent piRNA cluster transcripts (Hur et al., 2016). UAP56, a component of the nuclear pore complex, functions on the exportation of piRNA cluster transcript to nuage for further piRNA production by interacting with a nuage component named Vasa (Zhang et al., 2012).

1.4.2 Transcription of uni-strand clusters

In somatic cells, different from germline piRNA clusters, uni-strand piRNA clusters are transcribed in an RDC-complex independent manner and transcripts from uni-strand clusters contain polyadenylated tails (Gleason et al., 2018). The famous somatic piRNA cluster, *flamenco locus*, localized to the X-chromosome, was found to be transcribed by the function of a transcription factor named Cubitus interruptus (Ci). The transcripts from *flamenco locus* are found to be antisense to the active transposons like ZAM, *Idefix*, and *gypsy* (Prud'homme et al., 1995; Desset et al., 2003; Goriaux et al., 2014). After somatic piRNA cluster transcription, the unique cis-acting RNA elements on these transcripts are recognized by a protein named Yb for further slicing (Ishizu et al., 2015). The further details of uni-strand cluster transcription are still limited.

1.5 piRNA precursor export

1.5.1 Nuclear export of germline piRNA precursors

In *Drosophila* germline cells, piRNA biogenesis initiates with the transcription of dual strand piRNA precursor transcripts from discrete heterochromatic loci known as piRNA clusters. Once these piRNA precursors are transcribed, they need to be further exported from the nucleus to a perinuclear structure named ‘Nuage’ for mature piRNA production that loaded onto PIWI-family proteins: Aub, Ago3 and Piwi (Olivieri et al., 2010; Czech and Hannon, 2016).

Recent studies have revealed that the export of *Drosophila* germline piRNA precursors is distinct from the canonical Nxf1/Nxt1-dependent mRNA export pathway. This crucial pathway occurs by the function of a complex formed by UAP56-Nxf3-Boot (Mendel and Pillai, 2019; Suyama and Kai, 2019; Figure 1.1 A). UAP56, a DEAD-box helicase protein, is recruited to the nascent piRNA precursor transcripts by the function of Rhino protein and then transported to the nuclear periphery with the piRNA precursors. Nxf3, a germline-specific export factor, which is the paralog of Nxf1 (Czech et al., 2013), and its partner Bootlegger (Boot), have been confirmed to be coordinated with the UAP56 to direct them through the nuclear pore complex to the cytoplasmic nuage (ElMaghraby et al., 2019; Kneuss et al., 2019; Mendel and Pillai, 2019). Once these precursors are localized on nuage, they are captured by another DEAD-box helicase protein called Vas for further piRNA biogenesis (Zhang et al. 2012). Disruption of this export pathway causes nuclear retention and reduced cytoplasmic perinuclear localization of piRNA precursors, reduces the production of mature piRNAs, leads to the de-repression of transposons and instability of germline genome (Hur et al., 2016; Dennis et al., 2016; ElMaghraby et al., 2019; Kneuss et al., 2019).

1.5.2 Nuclear export of somatic piRNA precursors

In *Drosophila* somatic cells, transcription of somatic cluster like *flam* produces piRNA precursor transcripts with caps on their 5' end and polyadenylated tails on their 3' end. Once transcribed, somatic precursors needed to be exported from nucleus to the cytoplasmic perinuclear structure named Yb bodies, where primary piRNA biogenesis occurs (Saito et al., 2009; Mohn et al., 2014; Olivieri et al., 2016).

Distinct from that in germline cells, the export of somatic piRNA precursors is mediated by the components of Transcript/export complex (TREX), the nuclear export factor 1 (Nxf1) and its partner NTF2-related export protein 1 (Nxt1) (Suyama and Kai, 2024; Figure 1.1 B). Nxf1-Nxt1 complex is responsible for the transportation of somatic precursors through nuclear pore complex into the cytoplasm (Czech et al., 2013; Dennis et al., 2016; Kneuss et al., 2019; Ozata et al., 2019; Suyama and Kai, 2024). Once precursors are localized to Yb bodies, they are further processed by the function of an endonuclease named Zucchini (Zuc) and other somatic piRNA biogenesis components like Armitage (Armi), Vreteno (Vret) and Yb (Saito et al., 2010; Handler et al., 2011; Suyama and Kai, 2024). Like that of germline precursors, the disruption of somatic export pathway also causes the nuclear retention of *flam* precursors, a collapse of Piwi-bound piRNAs, and de-repression of transposons such as *gypsy*, *ZAM*, and *idefix* (Dennis et al., 2016).

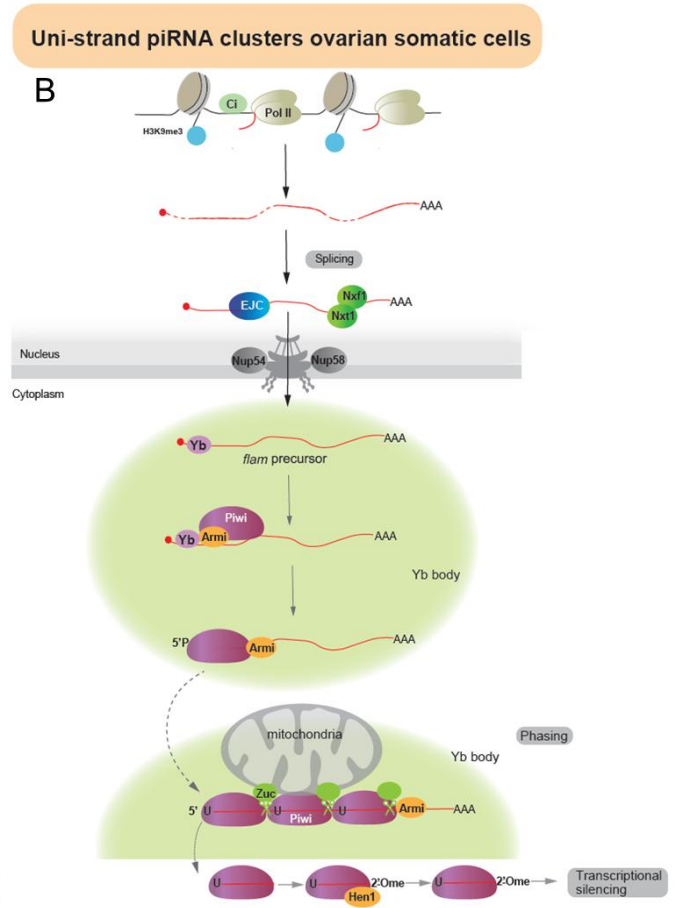
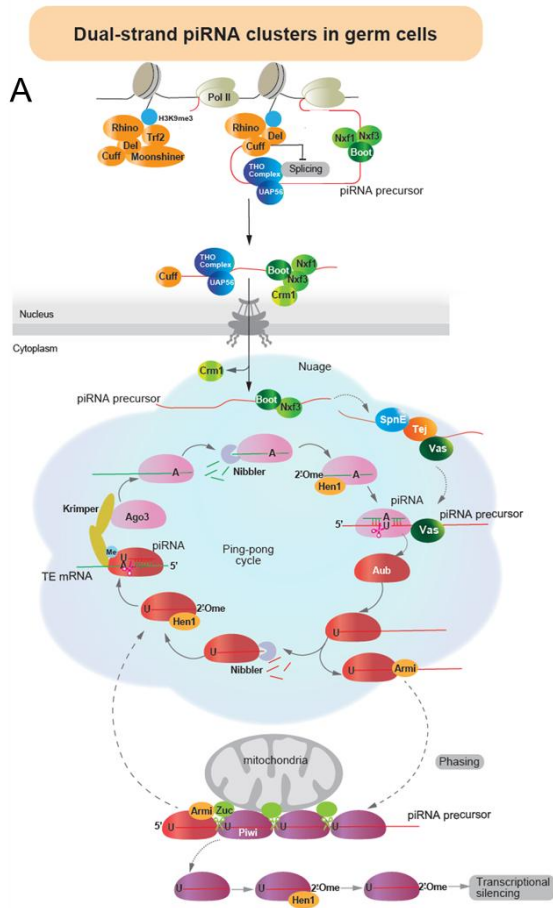


Figure 1.1. Transportation of dual-strand germline piRNA clusters and uni-strand somatic piRNA clusters

(A) Non-canonical piRNA precursors from the dual-strand piRNA clusters that are localized at the heterochromatin (H3K9me3) are transported via the Nxf3-Nxt1-Boot export pathway. Briefly, a H3K9me3-binding protein, Rhino (Rhi) recognizes H3K9me3 sites and then recruits Deadlock (Del) and Cutoff (Cuff) to license the transcription to produce piRNA precursor transcripts. After that, responding to the recruitment of Cuff, the transcription/export (TREX) complex formed by THO, UAP56, Nxf3 and Boot ensures the proper transportation of piRNA precursor transcripts. Nxf3 can also specifically bind to piRNA precursors and be recognized by the cellular exportin Crm1 and finish nuclear pore complexes (NPCs) translocation from nucleus to cytoplasm. After localized to nuage, these piRNA precursors are further processed for germline piRNA production named ping-pong cycle (explain later).

(B) Transcription and Transportation of Uni-strand clusters in somatic follicular cells. The uni-strand clusters transcription initiates through an RDC complex independent manner. A long noncoding RNA through the RNA polymerase II transcript mechanism activated by the transcription factor Cubitus interruptus (Ci) recognizing cis-acting RNA elements. The Uni-strand flamenco precursor will be capped and polyadenylated after alternatively spliced into multiple precursors. Further exportation relies on the cooperation of the TREX complex and the Nxf1-Nxt1 system. After localized to Yb body, these piRNA precursors are further processed for somatic piRNA biogenesis (explain later). This model is referred to by (Suyama and Kai, 2024).

1.6 Primary piRNA biogenesis in somatic cells

Somatic piRNA precursors are transported from nucleus to cytoplasm for primary piRNA biogenesis (Haase et al., 2010; Saito et al., 2010). Studies revealed that most components which function on primary piRNA biogenesis in somatic cells are localized to mitochondria, like Zucchini, Yb body and Piwi (Haase et al., 2010; Saito et al., 2010; Handler et al., 2011; Qi et al., 2011; Gleason et al., 2018; Sato and Siomi, 2020; Figure 1.2 A). Zucchini (Zuc), an endonuclease, localizes to the mitochondria by the mitochondrial targeting signal in the N-terminal and is responsible for the generation of 5' end of piRNAs (Ipsaro et al., 2012; Nishimasu et al., 2012). The 5' end of piRNAs generated by Zuc were not enriched with uridine (U), but most of the mature piRNAs have a strong bias for 1U at the 5' end (Girard et al., 2006; Brennecke et al., 2007; Ipsaro et al., 2012; Nishimasu et al., 2012). An in vitro study for Silkworm Siwi, a homolog of *Drosophila* Piwi, found that Siwi protein prefers to bind RNAs containing 1U, suggested that the 1U bias for mature piRNAs may depend on the process of piRNA loading to Siwi (Kawaoka et al., 2011). The 3' end of piRNAs are generated when piRNAs are loading to PIWI-family proteins (Gleason et al., 2018). The 3' ends of cleaved products from Zuc are several nucleotides longer than that of mature piRNAs and these extra nucleotides are proposed to be trimmed by another exonuclease named Nibbler (Feltzin et al., 2015). Another study revealed that a Tudor-domain protein PAPI may also function on the 3' end trimming of piRNAs, although it lacks any nuclease domain (Honda et al., 2013).

piRNAs are loaded to Piwi at Yb bodies on the mitochondria region and many other components are proposed to participate in this process. The complex formed by Piwi and other 3 Yb-body proteins Vreteno (Vret), Shutdown (Shu) and Armitage (Armi) is necessary for the piRNA loading on Piwi as a severe reduction of piRNAs together with the cytoplasmic localization of Piwi in these components' mutation were detected (Haase et al. 2010; Olivieri et al. 2010; Saito et al. 2010; Handler et al. 2011; Qi et al.

2011; Gleason et al., 2018). Loading of Zuc cleaved products to Piwi is proposed to be required for Shu and HSP90, but the precise mechanism is still elusive (Olivieri et al., 2012; Preall et al., 2012; Hirakata et al., 2016). Gasz and Minotaure (Mino) are localized to mitochondria and required for primary piRNA biogenesis in somatic cells. Gasz was found to be necessary for the localization of Armi (Czech et al., 2013; Handler et al., 2013).

1.7 piRNA biogenesis in germline cells

1.7.1 Primary piRNA biogenesis in germline cells

In *Drosophila* germline cells, piRNAs produced by primary piRNA biogenesis bind to Piwi and Aub to form the piRISCs. Piwi-piRISCs play the role of transposon silencing in nucleus and Aub-piRISCs function on repressing transposons in the cytoplasm by its slicer activity (Brennecke et al., 2007; Gunawardane et al., 2007; Hirakata et al., 2016). The mechanism of Aub-piRISC-mediated RNA cleavage is similar to that of Ago2-siRISC-mediated cleavage, but a difference between these two processes is that: the target RNAs of Ago2-siRISC-mediated cleavage are not necessary products but the target RNAs of Aub-piRISC-mediated cleavage are important for producing secondary piRNAs (Hirakata et al., 2016).

Although the precise mechanism of primary piRNA biogenesis in germline cells is still elusive, many piRNA components function on the somatic primary piRNA biogenesis like Zuc, Arim, Gasz, Shu, Mino and HSP90 are also expressed in germline cells and proposed to function on the germline primary piRNA biogenesis (Gleason et al., 2018).

1.7.2 Secondary piRNA biogenesis in germline cells: ping-pong cycle

The secondary piRNA biogenesis in germline cells also called ping-pong cycle is performed by two PIWI-family proteins, Aub and Ago3 (Sato and Siomi, 2020; Figure

1.2 B). piRNAs bound to Aub are antisense, so Aub-piRISCs can directly target the transposon transcripts with the sense orientation and cleave the target RNA transcripts by the slicer activity of Aub to generate the 5' end of sense piRNAs, which derived from transposons. Similar as that in somatic cells, the 3' end of piRNAs are generated by the slicer activity of Zucchini and further trimmed by another exonuclease named Nibbler to be the mature piRNAs with the correct size (Nishimasu et al., 2012; Hayashi et al., 2016; Gleason et al., 2018). Then these sense piRNAs bind to Ago3 to form the Ago3-piRISCs, target and cleave transposon or piRNA cluster transcripts with antisense orientation to generate more antisense piRNAs, and these antisense piRNAs will further load onto Aub or Piwi to make this processing in a feed-forward loop (Hirakata et al., 2016; Gleason et al., 2018).

piRNAs bind to Aub or Piwi show a strong bias for uridine (U) at their 1st nucleotide position, and piRNAs associated with Ago3 show a strong bias for adenine (A) at their 10th nucleotide position (Brennecke et al. 2007; Gunawardane et al. 2007). These features were found to be conserved among most animal species and named as ping-pong signatures (Houwing et al., 2007; Grimson et al., 2008; Ha et al., 2014; Hirakata et al., 2016). Analysis for Aub's intrinsic preference suggested that Aub prefers mRNA targets with an A on their 10th position which is opposite to the 1st nucleotide of Aub-bound piRNAs. After Aub-piRISC targeting and slicing, these mRNA targets become to sense piRNAs with 10A bias and further associate with Ago3 (Wang et al., 2014).

Besides PIWI-family proteins, recent studies revealed that many other components are also required for the ping-pong cycle. Notably, Tejas (Tej), a Tudor-domain protein, functions as a molecular scaffold that recruits the RNA helicases, Vas and Spn-E to nuage, thereby facilitating both piRNA precursor processing and the dynamic assembly of functional piRNA complexes (Lim and Kai, 2007; Nishida et al., 2007; Malone et al., 2009; Patil and Kai, 2010; Xiol et al., 2014; Sato et al., 2015; Lin et al., 2023; Adashev et al., 2024). Other nuage components, such as Krimper (Krimp) and Qin/Kumo, also act as regulators of PIWI-family protein interactions and strand bias production of

piRNA during ping-pong amplification (Zhang et al., 2011; Sato et al., 2015).

1.7.3 Secondary piRNAs-mediated phasing piRNAs in germline cells

Recent studies revealed that secondary piRNAs also trigger the production of Zuc-dependent, 3'-directed phased piRNAs (Han et al., 2015; Homolka et al., 2015; Mohn et al., 2015; Sato and Siomi, 2020; Figure 1.2 B). The secondary piRNAs involved in this process are so-called responder piRNAs, derived from Ago3-piRISC during the ping-pong cycle and normally processed to Aub. However, the piRNAs downstream of these responder piRNAs are associated with Piwi (not Aub) and named as trailer piRNAs. The responder piRNAs and trailer piRNAs present a marked phasing character of a 27-nt interval and a strong bias for U on the 1st nucleotide at the 5'-end. The production of trailer piRNAs depends on the function of Zuc and a degradome sequencing analysis also revealed that the production of Piwi-bound phased piRNAs also depends on Zuc. All these findings suggest that the piRNA 3'-ends rely on the endonucleolytic activity of Zuc (Han et al., 2015; Mohn et al., 2015).

Phased piRNA biogenesis extends piRNA production beyond the Aub-/Ago3-cleavage sites and enhances the diversity of piRNA sequences, which further functions on targeting transposon threat in a Transcriptional Gene Silencing (TGS) pathway or Post-Transcriptional Gene Silencing (PTGS) pathway (Han et al. 2015; Homolka et al., 2015; Mohn et al., 2015; Sato et al., 2015; Senti et al., 2015; Wang et al. 2015; Webster et al., 2015; Gleason et al., 2018). As the production of trailer piRNAs relies on secondary piRNAs, they can be considered tertiary piRNAs and further studies are needed to elucidate more details of phasing process (Hirakata and Siomi, 2016).

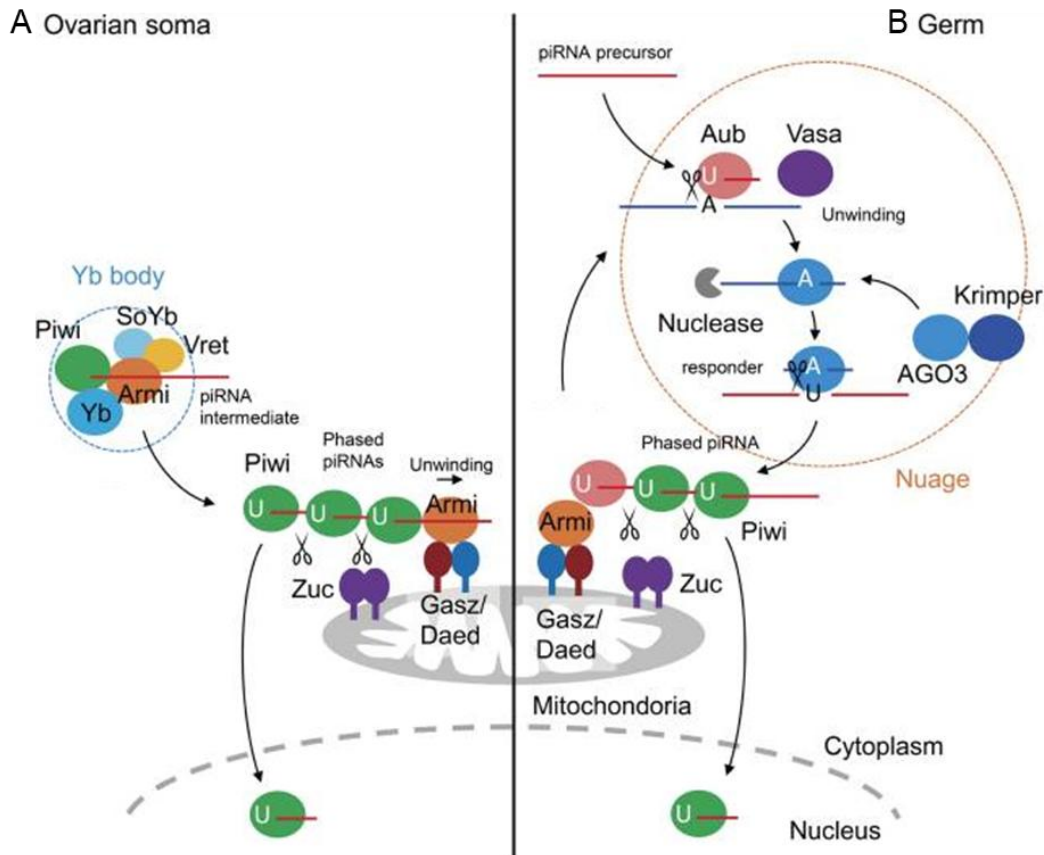


Figure 1.2. piRNA biogenesis in *Drosophila* ovary

(A) piRNA biogenesis in *Drosophila* ovarian somatic cells.

Yb body is assembled by Piwi, SoYb, Armi, Vret and Yb proteins. piRNA precursors derived from uni-strand clusters are recruited to Yb body and further converted into piRNA intermediates. Armi-RNA complexes are then recruited to mitochondria surface by Gasz/Daed, Piwi also translocate to mitochondria. Primary and phased piRNAs are produced here through Zucchini-dependent cleavage.

(B) piRNA biogenesis in *Drosophila* ovarian germline cells.

Once piRNA precursors are localized to the nuage, Aub and AGO3 recognize and cleavage precursor transcripts alternatively, aided by Vasa-dependent RNA unwinding and Krimper-mediated assembly, generating abundant Aub- and AGO3-bound piRNAs. As the responder piRNAs, the piRNA produced by Aub and AGO3 are fed into Zuc-dependent phased pathway on the mitochondria surface and support robust transposon repression in germline cells. The model is referred to by (Sato and Siomi, 2020).

1.8 Nuage, a crucial site for ping-pong cycle in germline cells

Nuage, is a germline-specific, electro-dense and non-membranous perinuclear organelle among different species including *Drosophila melanogaster* (Brennecke et al., 2007; Figure 1.3). Both PIWI-family proteins, Aub and Ago3, are localized on nuage and function on the ping-pong cycle as described above. Recent studies demonstrated that nuage also functions as a molecular hub that concentrates piRNA precursors, PIWI-family proteins and associated piRNA factors, promoting piRNA biogenesis and facilitating transposon repression in *Drosophila* germline cells (Xiol et al., 2014; Lim and Kai, 2015).

Besides Aub and Ago3, many studies have shown that a number of piRNA components are localized on nuage: Vasa (vas), a conserved RNA helicase protein containing DEAD-box domain, localize on nuage during early stages and accumulate on the polar plasm region during the later stages (Liang et al., 1994). Another RNA helicase protein containing DExH-box domain, Spindle-E (Spn-E), plays important roles during spermatogenesis and oogenesis (Gillespie and Berg, 1995). A set of Tudor-domain containing proteins: Krimper (Krimp), Tudor (Tud), Qin/Kumo, Tejas (Tej), Tapas (Tap), Vreteno (Vret) and Spn-E (Lim and Kai, 2007; Malone et al. 2009; Patil and Kai 2010; Handler et al., 2011; Zhang et al., 2011; Anand and Kai, 2012; Ott et al., 2014; Patil et al., 2014). Other proteins like Mealstrom (Meal) and Squash (Squ) (Pane et al., 2007; Sienski et al., 2012). Loss function of these piRNA components disrupted the piRNA production in different extents, suggesting that they may associate with the core ping-pong factors, Aub or Ago3 to function on piRNA biogenesis (Malone et al. 2009).

The molecular functions of many nuage proteins have been gradually identified by a series of recent studies. For example, Vas interacts with UAP56 and functions on the piRNA precursor processing from nuclear to nuage (Zhang et al., 2012). And ATP-dependent ribonucleoprotein (RNP) remodeling by Vasa facilitates transfer of 5' sliced

piRNA precursors between ping-pong partners, and loss of this activity causes sterility in *Drosophila* (Xiol et al., 2014). The Tudor-domain containing protein, Krimp, associating with Aub and Ago3 for proper piRNA biogenesis. Krimp harbors two distinct Tudor domains in its C-terminal region named Tud1 and Tud2. Tud1 shows a strong affinity to Ago3 even without piRNA loading or Arginine methylation, while the binding of Tud2 to Aub requires the arginine methylation at the N-terminal region of Aub. After mature piRNAs loading, a conformational change for Aub structure occurs and then exposes its N-terminal for Krimp binding. This binding regulated by arginine methylation is crucial for efficient ping-pong cycle (Webster et al. 2015; Wang et al. 2015; Sato et al. 2015; Huang et al. 2021). Another Tudor-domain-containing protein, Qin/Kumo, associating with both Aub and Ago3 for proper ping-pong cycle and preventing sense piRNAs loading to Piwi or Aub, thereby sustain the efficient heterotypic ping-pong by Aub and Ago3 (Zhang et al. 2011; Wang et al. 2015). Tap participates in the process for piRNA production and is essential for maintaining most core piRNA factors on nuage (Patil and Kai 2010; Patil et al. 2014). Tej, also as a Tudor-domain protein, functions as a molecular scaffold that recruits the RNA helicases Vas and Spn-E to nuage, thereby facilitating both piRNA precursor processing and the dynamic assembly of functional piRNA complexes (Lim and Kai, 2007; Nishida et al., 2007; Malone et al., 2009; Patil and Kai, 2010; Xiol et al., 2014; Sato et al., 2015; Lin et al., 2023; Adashev et al., 2024). However, the molecular functions of certain nuage components remain poorly understood, such as Squ. Loss of squ leads to the reduction for total amount of piRNAs and de-repression for TEs, suggests that Squ is involved in piRNA biogenesis.

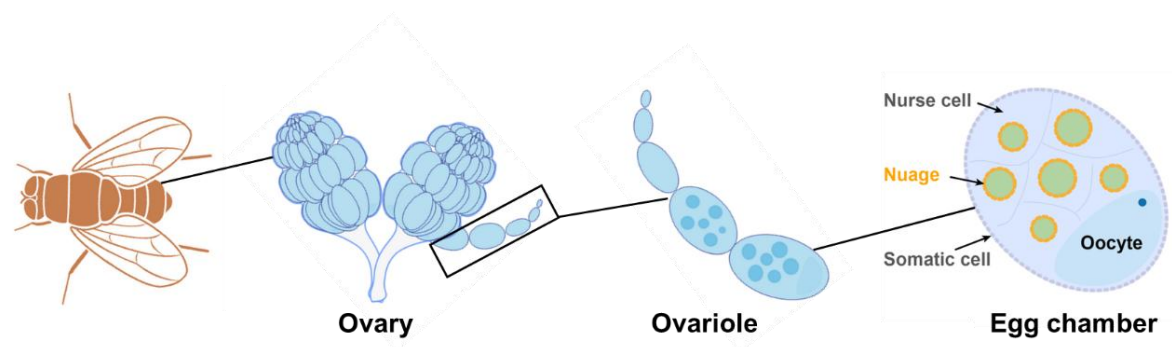


Figure 1.3. Schematic representation for germline cells of *Drosophila* female

Drosophila female has a pair of ovaries containing 16 – 20 ovarioles. Each ovariole consists of the germarium localized at the most anterior region and sequentially generated the egg chambers. Branches of the developing egg chambers will finally become a mature egg at the most posterior region. In each unmaturing egg chamber, somatic cells surround the oocyte and 15 nurse cells. Nuage, as the important site for ping-pong amplification process, is localized in the perinuclear region of nurse cells and somatic cells.

1.9 RNA helicase localized at nuage

RNA helicases are ATP-dependent enzymes that remodel RNA secondary structures and ribonucleoprotein (RNP) complexes (Jankowsky, 2011; Figure 1.4), functioning as key regulators of RNA metabolism in both somatic and germline tissues. Most RNA helicase proteins belonged to highly conserved DEAD-box (or DExH-box) families and characterized by tandem RecA-like domains and functional motifs that coordinate ATP-binding, ATP-hydrolysis and RNA-engagement. As ATP-binding drives conformational changes, RNA helicase proteins function on unwinding the short RNA duplexes, displacing RNA-binding proteins and re-organizing RNA-protein complexes to ensure proper RNA processing, translation and small RNA biogenesis for germline development and genome defense (Patel and Donmez, 2006; Jankowsky and Fairman, 2007; Linder and Jankowsky, 2011). In *Drosophila melanogaster*, RNA helicases Vas, Tej and Spn-E function as the core regulators during germline piRNA biogenesis by facilitating piRNA precursor processing, assembling nuage and RNA-protein complex (Xiol et al., 2014; Jeske et al., 2017; Lin et al., 2023; Izumi et al., 2024).

Vas, a DEAD-box RNA helicase, is highly conserved as a germline piRNA factor. Recent studies revealed that the helicase motifs of Vas ensure ATP-dependent RNA duplex unwinding and ribonucleoprotein (RNP) remodeling, facilitating the transfer of 5' sliced piRNA precursors between ping-pong partners, and loss of this activity causes sterility in *Drosophila* (Xiol et al., 2014). And the structural analysis revealed that Vas can 'clamp' RNA substrates and recruit Tudor-domain-containing proteins to assemble piRNA amplification complex for proper piRNA biogenesis (Liang et al., 1994; Sengoku et al., 2006; Xiol et al., 2014).

Tej contains a conserved helicase-like domain like RNA helicases, but in vitro assays revealed the limited enzymatic unwinding activity of Tej. However, Tej functions as a helicase-family protein contributes to RNA-dependent RNP remodeling, interacting with Vas through its lotus domain and enhances the ATPase activity of Vas in the

presence of single strand RNA (Jeske et al., 2017). Loss of Tej disrupts the assembly of helicase-containing piRNA complexes (Vas and Spn-E) and significantly reduces germline piRNA abundance (Patil and Kai, 2010; Lin et al., 2023).

Spn-E, a DEAH-box RNA helicase, is supposed to support ATP-driven unwind or remodeling of RNA substrates through its conserved helicase motifs. Spn-E interacts with both Aub and Ago3 to promote efficient ping-pong amplification by facilitating the transfer of cleaved RNAs among ping-pong partners (Malone et al., 2009). And a recent study in silkworm found Spn-E ATPase activity is essential for BmAgo3-dependent production of Siwi/Aub-bound piRNAs in the ping-pong cycle (Izumi et al., 2024).

Taken together, RNA helicases Vasa, Tej and Spn-E ensure the proper piRNA biogenesis through ATP-driven RNA remodeling, stabilization of RNP complexes, and coordination of multi-protein assemblies in the germline cells of *Drosophila*. However, whether the binding of RNA helicases with their binding factors enhance the helicase activities is still elusive, like such report for Tej binding to Spn-E is lacked.

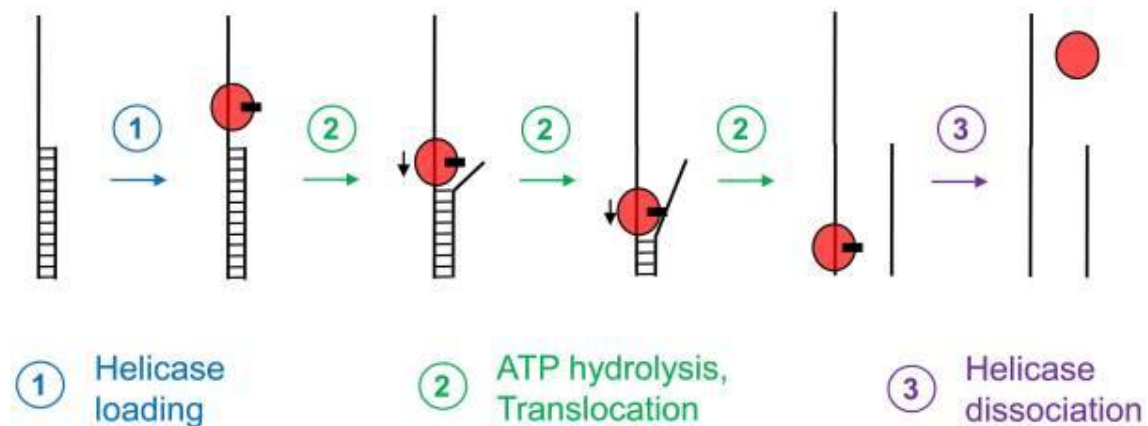


Figure 1.4. Schematic view of the main steps of translocation-based unwinding

Lines represent RNA strands, oval marks the RNA helicase proteins and the black rectangles indicate the ATP. Only a monomeric enzyme is displayed, but canonical helicases have also been shown to function as oligomers. The model is referred to by (Jankowsky, 2011).

1.10 Thesis overview

Through the current studies, mechanisms underlying the processing of piRNA precursors and mature piRNA production have been investigated by elucidating the functions of most core piRNA components. However, the molecular functions of co-factors for these core piRNA components and how the Aub-Aub homotypic ping-pong occurs in the mutation of some piRNA factors are still limited. A recent study working on the AF prediction of interactome within nuage revealed the potential binding among nuage factors (Figure 1.5 A). Among these predicted bindings, I decided to focus on Squash (Squ), a *Drosophila*-specific protein, harboring yet-unknown motifs/domains and may have an interaction with a crucial piRNA factor, RNA helicase Spindle-E (Spn-E) (Figure 1.5 B). Other studies also revealed some functions of Squ in TE repression and piRNA production (Pane et al., 2007; Malone et al., 2009), suggesting that Squ may function in piRNA biogenesis, but the details mechanisms are still elusive. So, during my PhD study, using *Drosophila melanogaster* as the research model, I focused on elucidating the molecular functions of one nuage component (Figure 1.5 C), Squash (Squ), which functions as the co-factor of Spn-E, ensures the proper piRNA biogenesis for TE repression. Besides, Squ sustains the Aub-Ago3 heterotypic ping-pong process by maintaining Ago3 protein level in an unknown mechanism.

To explore the molecular functions of Squ during the piRNA biogenesis, I performed Alphafold prediction for analyzing the binding sites between Squ and Spn-E, and I found that Squ binds to Spn-E through 4 conserved amino acids among *Drosophila* species. Moreover, I found that the interaction between Squ and Spn-E is crucial for precursor processing and TE repression by making the transgenic fly lines coding the mutation sequence for predicted binding sites. Besides, by analyzing the piRNA profiles in *squ* and *ago3* mutants, I found that the Aub-Aub homotypic ping-pong in *squ* mutant depends on the reduction of Ago3 protein level by performing small-RNA seq analysis.

Taking together, this study elucidates the functions of Squ and refines the current model of piRNA biogenesis in *Drosophila* germline cells. Additionally, structural characterization of the Squ - Spn-E complex could reveal how DEAD-box helicase activity is modulated by binding factors. Understanding these mechanisms will provide deeper insights into the mechanisms of piRNA precursor processing and the maintenance of genome integrity in germline cells.

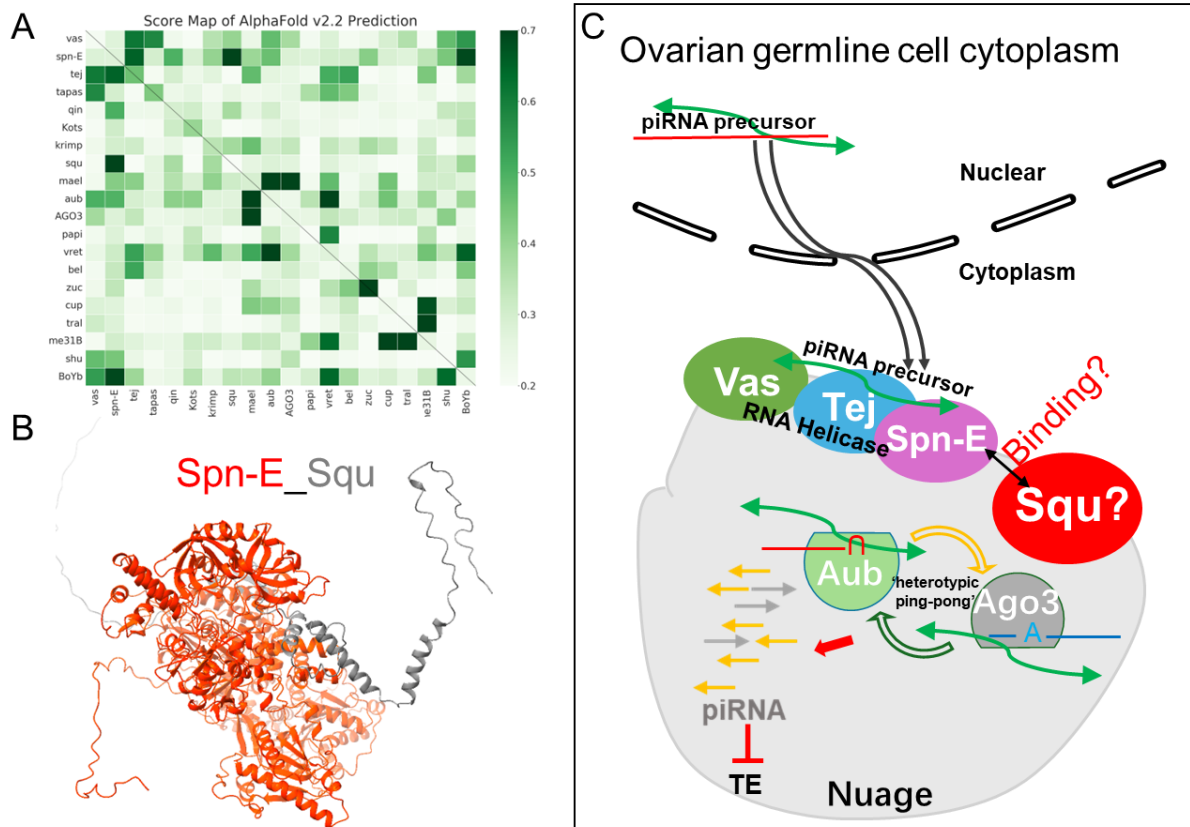


Figure 1.5. Modified model to elucidate Squ's role in piRNA biogenesis of *Drosophila* germline cells

(A) Heatmaps of the prediction confidence scores (ranking confidence, green) provided by AlphaFold2. The 20 types of proteins are aligned from top to bottom and left to right in the same order. Boxes on diagonal line represent homodimers (referred from elife paper, Kawaguchi et al., 2025).

(B) The predicted 3D structure for Squ–Spn-E heterodimers, the prediction confidence score is above 0.6 (referred from elife paper, Kawaguchi et al., 2025)..

(C) In *Drosophila* germline cells, piRNA precursors derived from dual-strand clusters are translocated from nuclear to cytoplasm through Nxf3-Nxt1 pathway (Figure 1.1 A). By the function of nuage localized RNA helicases, Vas, Tej and Spn-E, these piRNA precursors are further translocated at nuage and transferred to Aub and Ago3 for heterotypic ping-pong to produce piRNAs for TE repression. A *Drosophila*-specific protein, Squash (Squ), harboring unknown motifs/domains, is localized to nuage and crucial for TE repression and piRNA production (Pane et al., 2007; Malone et al., 2009; Kawaguchi et al., 2025). However, the detailed mechanisms of Squ are still elusive.

2. Results

2.1 Nuage component Squ is specifically required for Ago3 abundance

Previous studies reported that loss of *squ* reduces total piRNAs by ~50% without markedly altering the nuage localization of core ping-pong factors such as Aub and Ago3 (Pane et al., 2007; Malone et al., 2009). In this study, we first generated a Squ antibody which could be used for immunostaining and immunoblotting, I found that the Squ signal disappeared in *squ* mutant ovaries using the trans-heterozygous alleles, *squ*^{PP32/HE47} (hereafter, *squ* mutant) (Fig. 2.1 A and B). The immunostaining of the control ovaries showed that endogenous Squ was enriched in the perinuclear nuage granules of nurse cells (Fig. 2.1 A). This finding was consistent with previous observations using a transgene expressing HA-tagged Squ, confirming the localization of Squ to the nuage (Pane et al., 2007).

To further examine the molecular role of Squ, I analyzed the localization and abundance of major piRNA pathway components in *squ* mutant ovaries. In *squ* mutant ovaries, the perinuclear signals and protein levels of most core ping-pong factors (Spn-E, Aub, Vas, Tej and Krimp) were largely unaffected (Fig. 2.1 A and B). Notably, in the immunofluorescence observations of *squ* mutant ovaries, Ago3 signal is markedly weak (Fig. 2.1 A). Consistently, immunoblotting of *squ* mutant ovarian lysate revealed a reduction in Ago3 protein level by ~80% compared to those in wild-type condition (Fig. 2.1 B and C). Hence, this reduction is Ago3-specific. However, interestingly, ovarian transcriptome analysis confirmed that *ago3* mRNA level was essentially unchanged in *squ* mutant ovaries ($\log_2\text{FC} = -0.19$, $\text{padj} = 0.07$; Fig. 2.1 D), together with other core ping-pong factors (Spn-E, Aub, Vas, Tej and Krimp). Taken together, these data suggest that *squ* is critical in maintaining Ago3 protein abundance in germline cells.

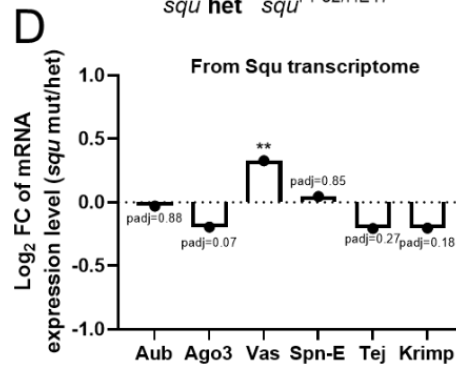
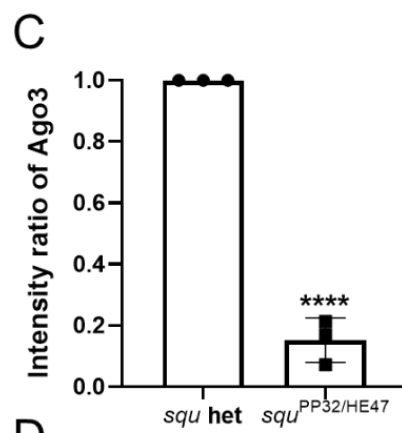
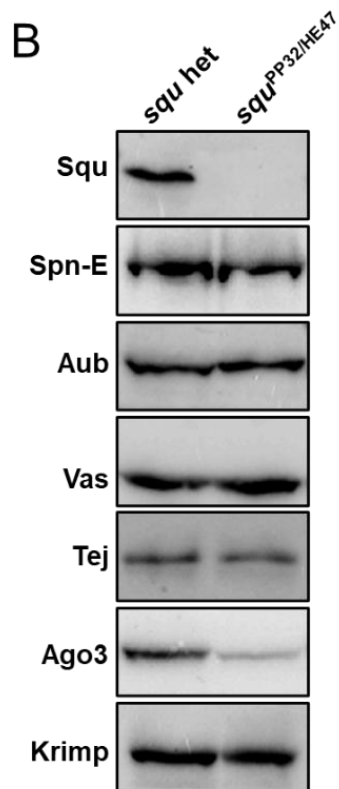
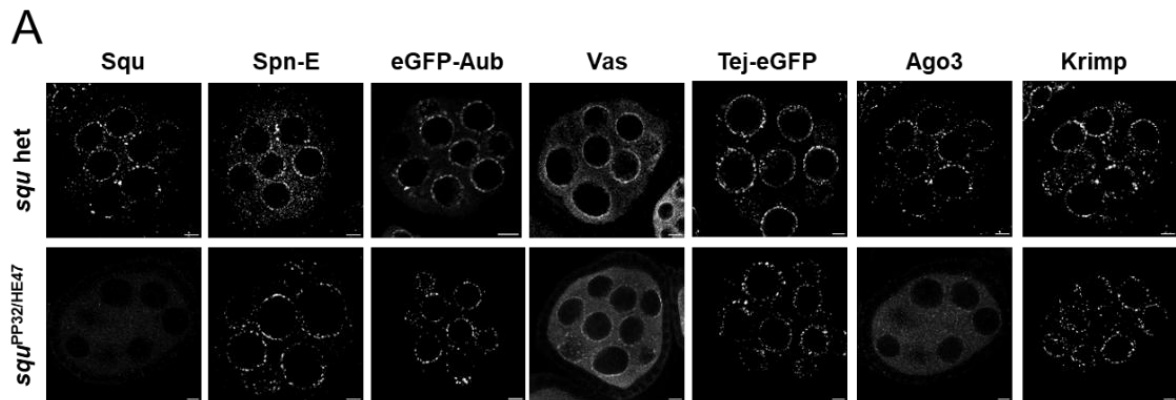


Figure 2.1. Squ is required for Ago3 abundance

- (A) Localization of indicated proteins in stage 4-5 egg chambers of *squ* heterozygous or transheterozygous mutants. Immunostaining; Squ, Spn-E, Vas, Ago3 and Krimp. Fluorescence; eGFP-Aub and Tej-eGFP. Scale bar is 5 μ m.
- (B) Immunoblotting of indicated proteins extracted from ovaries of *squ* heterozygous or mutants.
- (C) Quantification of Ago3 protein signal intensities. Error bars indicate standard deviation (3 biological replicates). Statistics: unpaired t-test, **** $p < 0.0001$.
- (D) Transcript levels of core ping-pong factors in control and *squ* mutant ovaries. Fold change given by differential expression analysis (DESeq2) is shown with adjusted p-value.

2.2 Squ's nuage enrichment depends on core ping-pong factors

To further detect the hierarchy of Squ with the core ping-pong factors described above, I further investigate the perinuclear signal and protein level of Squ in these factors' mutant ovaries. I found, in the mutant of three RNA helicases, *spn-E*, *vas*, or *tej*, Squ protein level was decreased and detected only faintly in the cytoplasm (Fig. 2.2 A, B and C), and interestingly, in *aub* mutant ovaries, only the dispersed signal in cytoplasm but not the reduced protein level of Squ was detected (Fig. 2.2 D), implies that Squ is not an indispensable factor for nuage formation but rather a factor recruited by these core ping-pong factors to nuage.

Notably, no defect for the nuage signal and protein abundance of Squ was detected in *ago3* and *krimp* mutant ovaries (Fig. 2.2 E and F), together with the previous result (Fig. 2.1 B), implies that Squ functions on the up-stream of Ago3. Take together, these results indicate that Squ is not a crucial factor for nuage formation but for Ago3 stability.

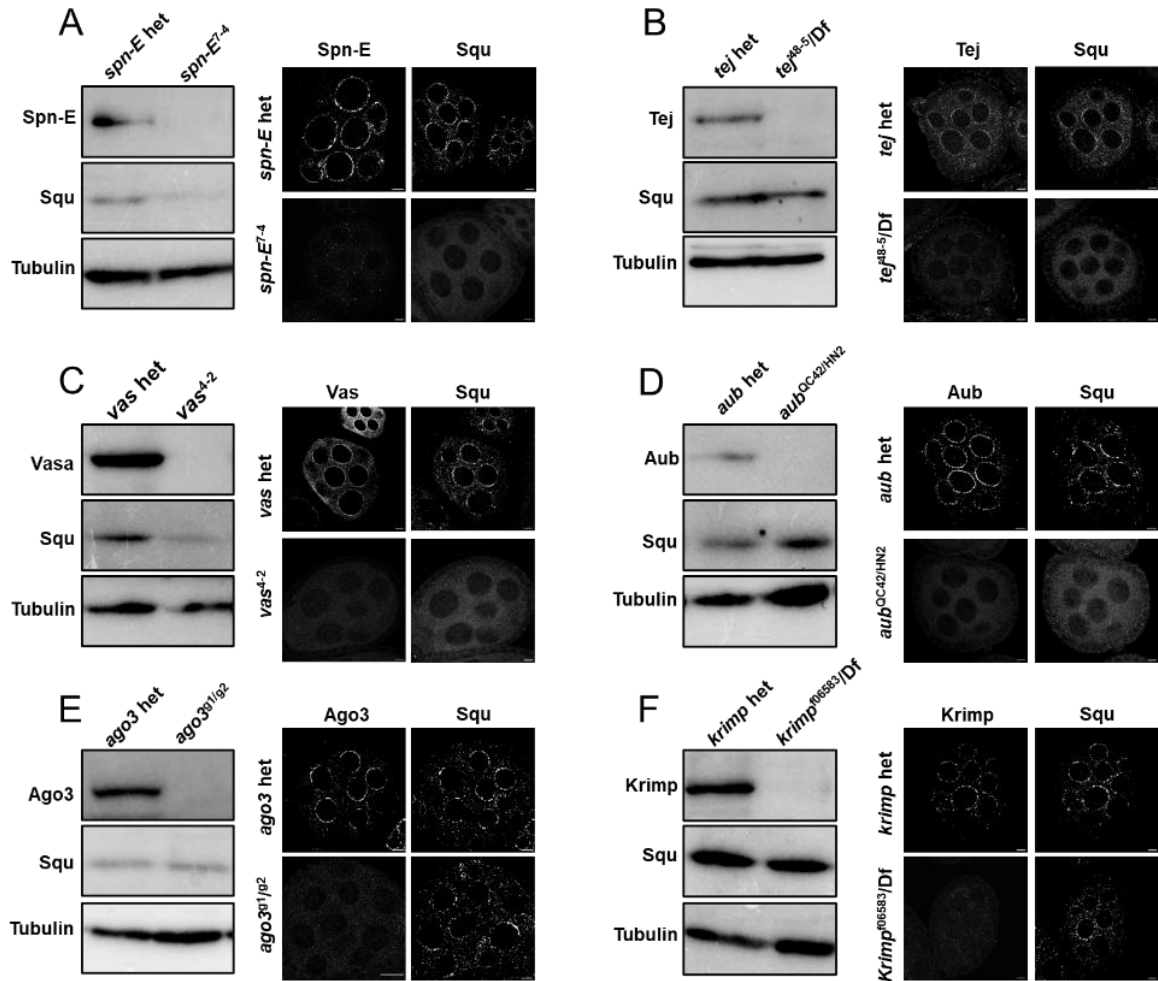


Figure 2.2. Squ's nuage enrichment depends on core ping-pong factors

(A-F) Protein level and Immunostaining of Squ in control and *spn-E* (A), *tej* (B), *vas* (C), *aub* (D), *ago3* (E) or *krimp* (F) mutant egg chambers.

2.3 *squ* loss derepresses germline-specific transposons

As one of the core ping-pong partners, the severe loss of Ago3 protein level detected in *squ* mutant ovaries (Fig. 2.1 B) may lead to the de-repression of transposable elements (TEs). To elucidate this question, I first performed RNA-seq in *squ* heterozygous control and mutant conditions. The RNA-seq analysis revealed that many TEs were significantly up-regulated in *squ* mutant ovaries (24 out of 119 TE families showed $\log_2FC > 1$, $p\text{-value} < 0.05$; Fig. 2.3 A). Consistent to the expression of Squ in germline cells (Fig. 2.1 A), I found that most of these up-regulated TEs belonged to germline-specific families (Fig. 2.3 B and C; Durdevic et al., 2018; Kneuss et al., 2019), implies that Squ may function on the repression of germline TEs.

To further detect whether Squ has an ability to repress such germline TEs, I checked the expression of several representative germline TEs, *HMS-Beagle*, *Burdock* and *blood*, in *squ* mutant ovaries by RT-qPCR. The results revealed that all of these TEs are significantly up-regulated in *squ* mutant ovaries (Fig. 2.3 D). As the independent protein-level readouts of de-repression, I examined two canonical piRNA-controlled targets, HeT-A in ovaries and Stellate in testis by immunostaining. The robust increase of HeT-A Gag protein in oocyte of *squ* mutant ovaries and Stellate protein in cytoplasm of *squ* mutant testis—which are both normally silenced by the piRNA pathway (Fig. 2.3 E and F; Shpiz et al., 2011). Together, these data indicate that *squ* is required to repress a broad set of germline TEs.

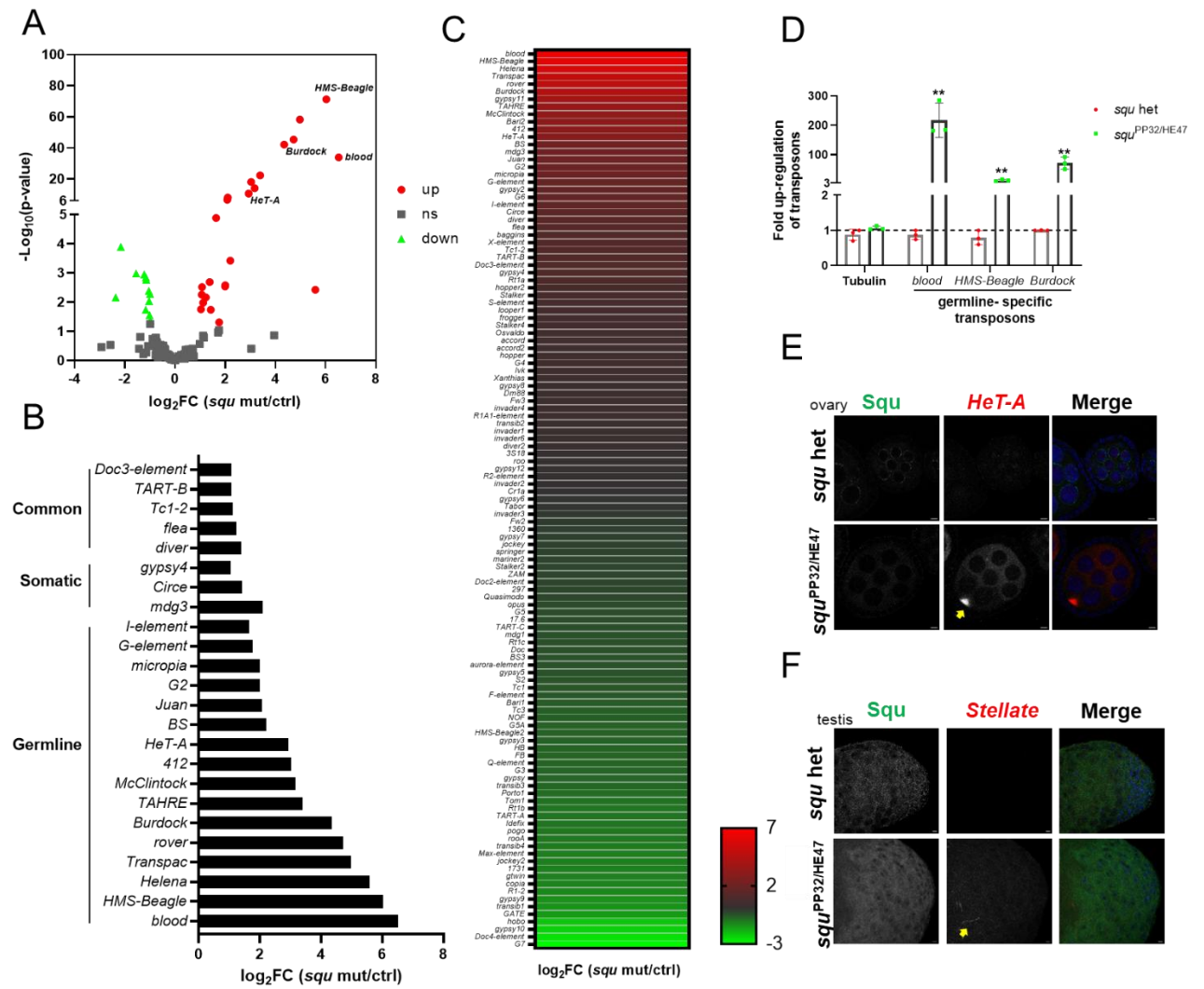


Figure 2.3. Loss of *squ* de-represses transposons

(A) Volcano plot shows fold changes of transposon transcript levels in ovaries (*squ* mutant compared to *squ* heterozygous condition). Red circles; significantly upregulated transposons ($\log_2FC > 1$, $p < 0.05$), green circles; significantly downregulated transposons ($\log_2FC < -1$, $p < 0.05$).

(B) Fold changes of TEs' expression upon *squ* loss. Transposons are categorized into germline, somatic, and common groups.

(C) The heatmap of fold changes of TEs' expression for all 119 canonical transposons in control and *squ* mutant ovaries.

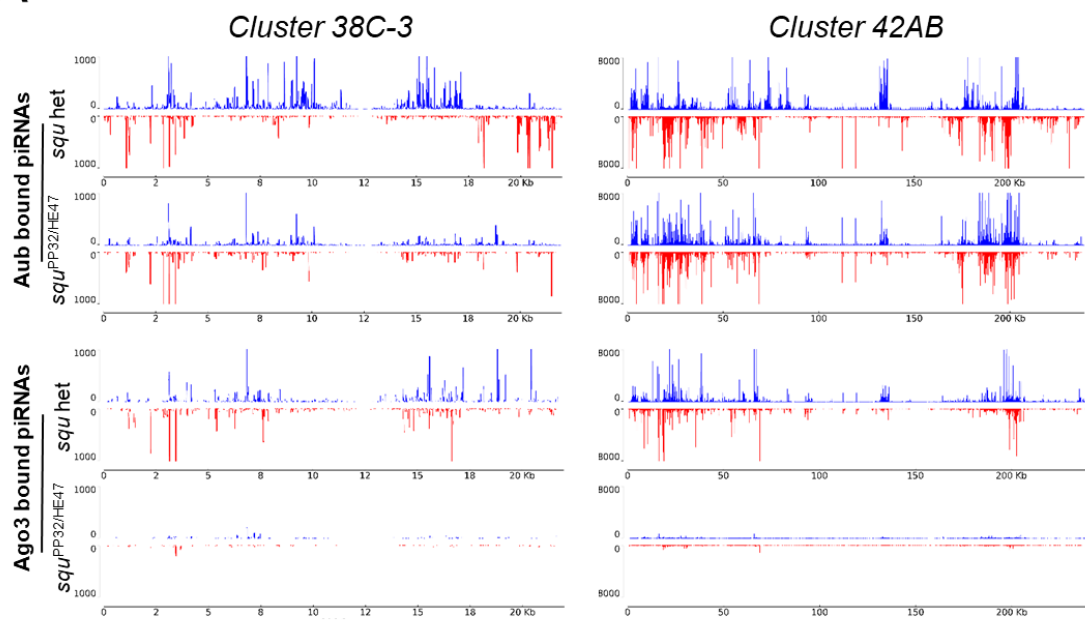
(D) RT-qPCR analysis of germline-specific transposons (*blood*, *HMS-Beagle*, *Burdock*). Relative transcript levels are calculated by $\Delta\Delta Ct$ using *rp49* as reference. Values represent fold changes in *squ* mutant relative to heterozygous controls. Error bars indicate standard deviation (3 biological replicates). Statistical analysis is performed using Student's t-test. ** $p < 0.01$.

(E-F) Immunostaining of HeT-A Gag and Stellate proteins in control and *squ* mutant ovaries. A yellow arrow indicates HeT-A Gag and Stellate signals accumulating in oocyte and testis, respectively. Scale bar: 5 μm .

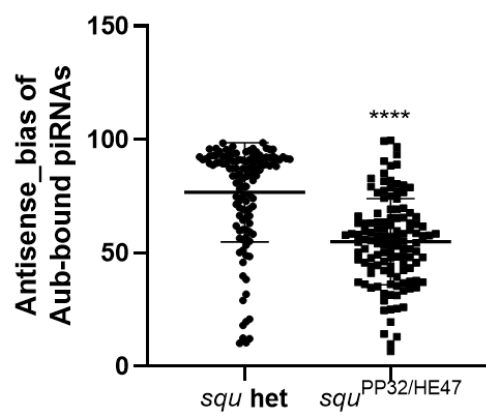
2.4 Squ is required for maintenance of Ago3-bound piRNAs

The de-repression of TEs always represents the reduction of piRNA production and previous study has already reported that the total number of piRNAs was reduced to half in *squ* mutant ovaries (Malone et al., 2009). However, the cause of the reduction in piRNAs remain unclear. To address this question, I analyzed 23-29 nt small RNAs bound to Aub and Ago3. NGS analysis revealed that the amount of Ago3-bound piRNAs from piRNA clusters *38C-3* and *42AB* were strongly depleted in *squ* mutant ovaries but that of Aub-bound piRNAs were not largely affected (Fig. 2.4 A). Although without significant reduction for Aub-bound piRNAs, the antisense bias of Aub-bound piRNAs mapping to transposons was reduced from ~77% to ~55% in *squ* mutant ovaries (Fig. 2.4 B). Of those, Aub-bound piRNAs mapping to severely upregulated TEs in *squ* mutant ovaries, such as *HMS-Beagle*, *blood*, *Burdock* and *HeT-A*, showed severe reduction of antisense bias (Fig. 2.4 C), implying that this reduction of antisense bias may further result in upregulation of corresponding TEs. Take together, these data suggest that Squ is required for efficient production for Ago3-bound piRNAs, which in turn maintains the antisense enrichment of Aub-bound piRNAs for effective transposon silencing.

A



B



C

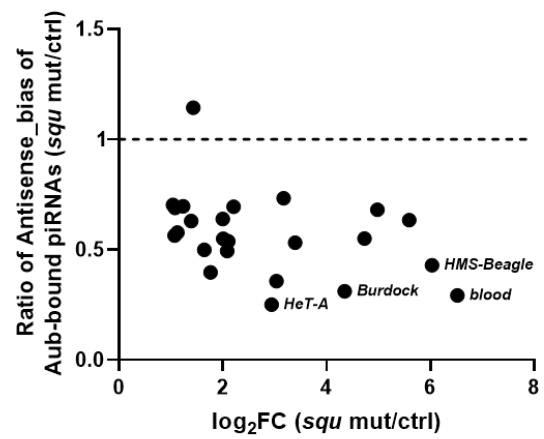


Figure 2.4. Squ is required for maintenance of Ago3-bound piRNAs

(A) Aub- (upper panels) and Ago3-bound (lower panels) small RNAs from control and *squ* mutant ovaries mapped to the major piRNA clusters 38C-3 and 42AB. Plus-strand (blue) and minus-strand (red) piRNAs are shown as upward and downward peaks, respectively.

(B) Antisense bias of Aub-bound piRNAs mapping to transposable elements (TEs) in control (*squ* het) and *squ* mutant ovaries. Each dot represents one TE; antisense piRNA ratios are calculated by dividing antisense-mapping piRNA reads by total mapping reads for each TE. Horizontal lines indicate the mean values. ****p < 0.0001 (unpaired t-test).

(C) Effect of loss of *squ* function on antisense ratio of Aub-bound piRNAs mapping to transposon consensus sequences. Dots indicate 24 up-regulated TEs (Fig. 2.3 A) in *squ* mutant relative to control ovaries. For each TE, the antisense bias in *squ* mutant is divided by that in *squ* heterozygous.

2.5 *Squ* is crucial for stalling piRNA precursor processing at nuage

As precursor processing disruption always results in piRNA reduction, I next examined whether the recruitment of piRNA precursors to perinuclear nuage was perturbed upon loss of *squ* function. Hybridization chain reaction–fluorescence in situ hybridization (HCR–FISH; Choi et al., 2018) targeting precursors from the major dual-strand clusters in germline cells, *38C* and *42AB*, showed accumulated perinuclear signals in *squ* mutant nurse cells compared with controls (Fig. 2.5 A). Quantification using a perinuclear-rim index (Lin et al., 2023) revealed that ~45% of the total precursor signal resided at the perinuclear rim in *squ* mutant nurse cells vs ~20% in heterozygous controls (Fig. 2.5 B). This perinuclear accumulation in *squ* mutant ovaries is comparable to that reported for *vas*, *tej*, and *spn-E* mutants, which function on recruiting precursors or TE transcripts to initiate the ping-pong amplification at nuage (Lin et al., 2023). Consistently, piRNA-cluster transcripts were increased in *squ* mutant ovaries by RT-qPCR (Fig. 2.5 C). Because signals remained predominantly cytoplasmic rather than nuclear, these observations argue against a primary nuclear-export defect and instead suggest inefficient precursor processing in perinuclear nuage upon loss of *squ* function. Taken together, these findings place *squ* as a factor required for Ago3-bound piRNA production and the efficient perinuclear processing of piRNA precursors.

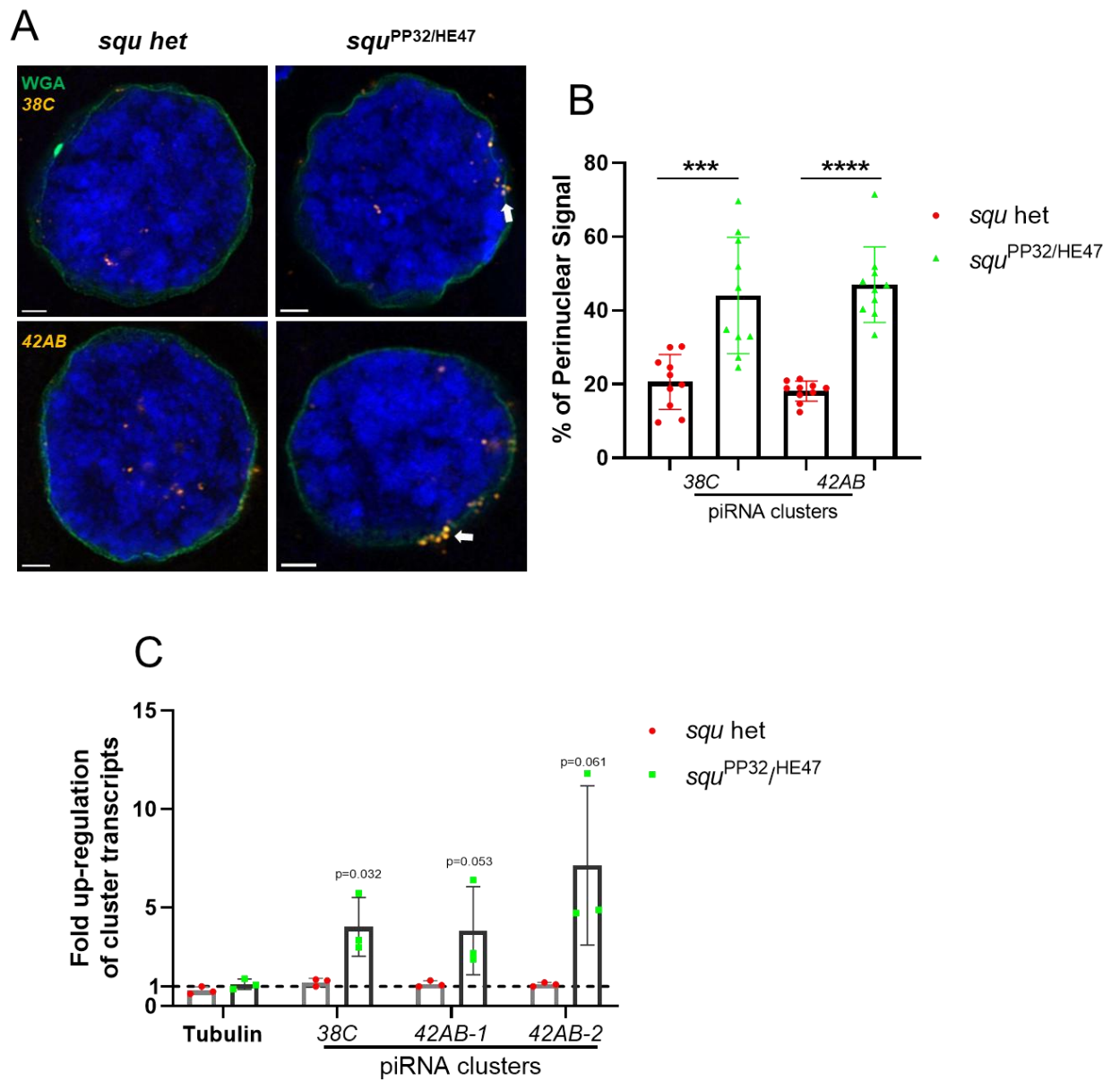


Figure 2.5. *Squ* is required for processing of piRNA precursors

(A) HCR-FISH detection of piRNA precursor transcripts from clusters *38C* and *42AB* in control and *squ* mutant ovaries. One representative nucleus for each sample is shown. A white arrow indicates precursor signals accumulating to perinuclear regions. Blue; DNA (DAPI), green; nuclear membrane (WGA). Scale bar: 2 μ m.

(B) Quantification of piRNA precursor signals accumulating in perinuclear regions. Fluorescence intensity of precursor foci within $\pm 5\%$ of nuclear diameter from the nuclear membrane is measured (n=10 nuclei per genotype). Signals are plotted as the percentage of total nuclear-associated intensity. Statistical analysis: *** $p < 0.001$, **** $p < 0.0001$.

(C) RT-qPCR analysis of transcripts derived from major germline piRNA clusters *38C* and *42AB*, with tubulin as a control. All values are normalized to *rp49* and are shown relative to expression levels in the presence of *squ* heterozygous. Error bars indicate standard deviation (3 biological replicates). Statistical significance is assessed by Student's unpaired t-test.

2.6 Low Ago3 protein level in *squ* mutant triggers Aub-Aub homotypic ping-pong

In *Drosophila* germline cells, the canonical ‘ping-pong’ amplification loop also known as ‘heterotypic ping-pong’ occurs between Aub (antisense, 1U bias for bound piRNA) and Ago3 (sense, 10A bias for bound piRNA), but in *kumo/qin* and *krimp* mutants, different extents of reduction for Ago3 protein level were detected together with a piRNA production step called ‘homotypic ping-pong’ occurred by Aub with itself harboring a ‘1U10A’ bias for bound piRNAs (Zhang et al., 2011; Sato et al., 2015). To test whether the low Ago3 level elicits Aub-Aub homotypic ping-pong, I first examined piRNA profiles in *squ* mutant ovaries. The 1U nucleotide bias of Aub-bound piRNAs and 10A bias of Ago3-bound piRNAs were detected in controls, as expected (Fig. 2.6 A). In contrast, Aub-bound piRNAs in *squ* mutant ovaries exhibited a strong ‘1U10A’ signature, a hallmark of Aub-Aub homotypic ping-pong (Fig. 2.6 A), whereas Ago3-bound reads were too few to assess.

To detect whether the Aub-Aub homotypic ping-pong occurs in *squ* mutant is directly by the loss of *squ* function or indirectly by the reduction of Ago3 protein level, I further check such piRNA profiles in *ago3* mutant ovaries like *squ* case. Notably, in *ago3* mutant ovaries, I also observed a strong ‘1U10A’ signature for Aub-bound piRNAs (Fig. 2.6 B), the characteristic for Aub-Aub homotypic ping-pong, together with the reduction for antisense bias in line with the previously reported up-regulation of transposons like *HMS-Beagle*, *blood*, *Burdock* and *HeT-A* (Fig. 2.6 C-E; Li et al., 2009). These data suggest that the role of Squ in maintaining Ago3 protein level is crucial for sustaining the heterotypic ping-pong cycle in *Drosophila* germline cells.

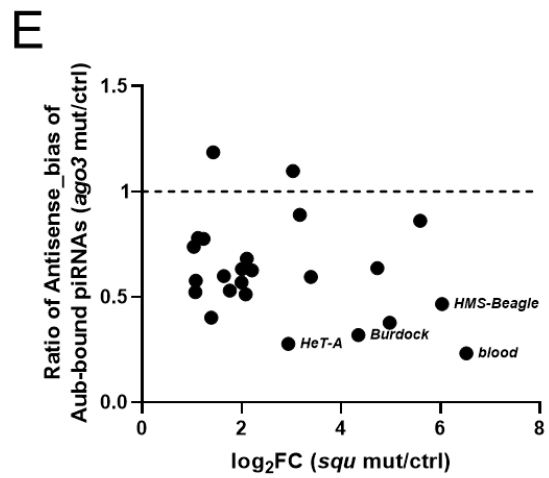
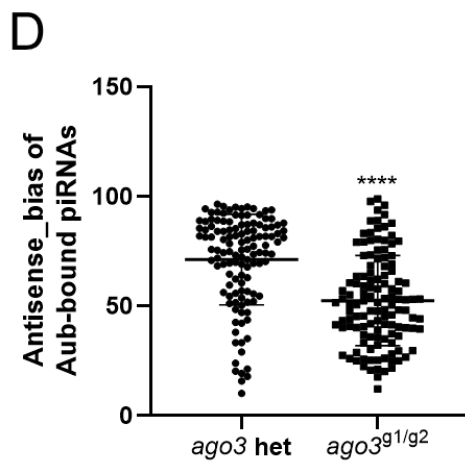
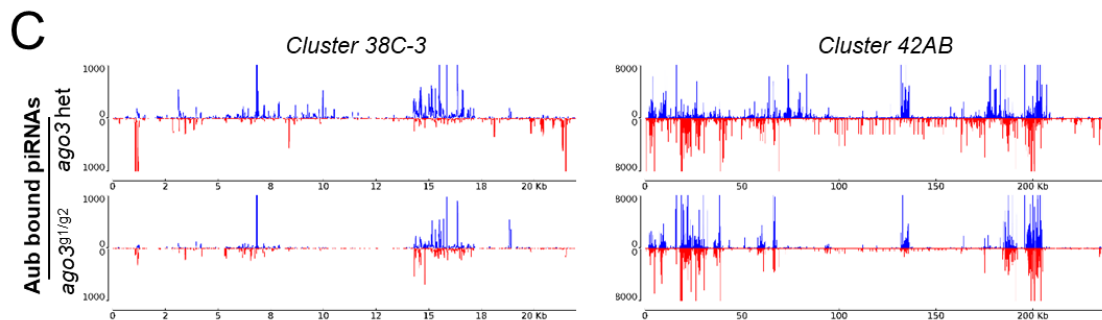
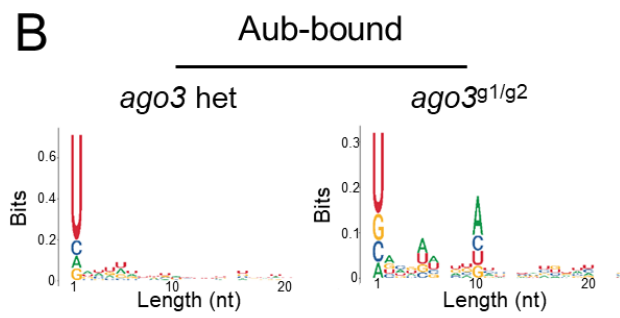
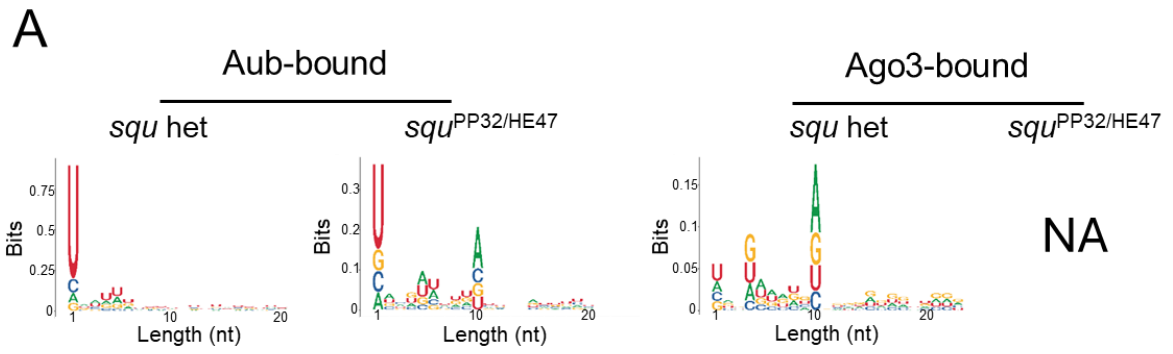


Figure 2.6. Low Ago3 protein level in *squ* mutant triggers Aub–Aub homotypic ping-pong

(A) Nucleotide bias of Aub-bound and Ago3-bound piRNAs mapped to piRNA cluster loci in control and *squ* mutant ovaries. Sequence logos indicate 1st to 20th of piRNAs.

10A signature of *squ* mutant condition reflects enhanced Aub–Aub homotypic ping-pong. NA (Not analyzed); Ago3-bound piRNAs are not detectable in *squ* mutant.

(B) Nucleotide bias of Aub-bound piRNAs in control and *ago3* mutant ovaries.

(C) Aub-bound piRNAs from control and *ago3* mutant ovaries mapped to the major germline piRNA clusters *38C-3* and *42AB*. Blue; genomic plus-strand reads, red; minus-strand reads.

(D) Antisense bias of Aub-bound piRNAs mapping to transposable elements (TEs) in control and *ago3* mutant ovaries. Each dot represents one TE; antisense piRNA ratios are calculated by dividing antisense-mapping piRNA reads by total mapping reads for each TE. Horizontal lines indicate the mean values. **** $p < 0.0001$ (unpaired t-test).

(E) Ratio of antisense bias of Aub-bound piRNAs for the 24 up-regulated TEs (detected in *squ* mutant) in *ago3* mutant versus control ovaries. Dots indicate 24 up-regulated TEs (Fig. 2.3 A) in *ago3* mutant relative to control ovaries. For each TE, the antisense bias in *ago3* mutant is divided by that in *ago3* heterozygous.

2.7 Squ associates with Spn-E through four conserved residues

Squash (Squ) was initially proposed as an RNase HIII-related protein by the previous study (Pane et al., 2007), yet structural prediction by the AlphaFold program suggested that it is an intrinsically disordered protein lacking any conserved or characterized motifs/domains (Kawaguchi et al., 2025). So, I speculate that Squ may function as the co-factor of other core ping-pong factors. In another study, Squ protein was detected, together with several piRNA factors, in immunoprecipitates of Spn-E from ovarian lysates, indicating that Squ and Spn-E interact either directly or indirectly (Andress et al., 2016). To further confirm the interaction between Squ and Spn-E, I performed the co-immunoprecipitation assays and immunostaining in ovaries. I found that Squ co-immunoprecipitated with Spn-E from ovarian lysates and formed the aggregate foci around the perinuclear nuage region, reflecting a specific interaction *in vivo* (Fig. 2.7.1 A and B). A faint signal with Ago3 was also observed (Fig. 2.7.1 A and C), whereas Aub, Vas, and Tej were undetectable in the Squ-immunoprecipitants, indicating that Squ preferentially associates with Spn-E, and only a minor potential interaction with Ago3.

To investigate the molecular basis and physiological relevance of the Squ–Spn-E interaction, we first examined the interface predicted by AlphaFold2. Four residues in Squ (E107, E109, R115, and K163), are predicted to form salt bridges with four corresponding residues in Spn-E (K834, R675, D640, and E1274) (Fig. 2.7.2 A and B). To validate whether these residues are crucial for Squ–Spn-E interaction, I generated Squ mutants substituting each residue (Squ^{E107A}, Squ^{E109A}, Squ^{R115A} and Squ^{K163A}) and all four residues (Squ^{4A}) with alanine. Co-immunoprecipitation assays in S2 culture cells expressing tagged proteins, Myc–Spn-E and Flag-Squ were performed to detect the interaction. The assay revealed that only the E107A single mutation did not affect the interaction, other single mutations mildly reduced the binding affinity of Squ to Spn-E (Fig. 2.7.2 C) and Squ^{4A} fully lost the binding with Spn-E (Fig. 2.7.2 D).

Furthermore, the localization of GFP-tagged Squ and mKate2 (mK2)-tagged Spn-E were also detected in S2 culture cells. When only Squ or that expressing the variants was expressed, the signal was dispersed in the cytoplasm (Fig. 2.7.2 E). On the other hand, when only expressed Spn-E in S2 culture cells, the signal was enriched in the nucleus as previous study (Fig. 2.7.2 E; Lin et al., 2023). When co-expressed Squ or that expressing Squ single mutants with Spn-E, I found that Spn-E translocated to the cytoplasm and formed granules with Squ or Squ single mutants (Fig. 2.7.2 E). However, Squ^{4A} completely lost the co-localization with Spn-E in cytoplasm (Fig. 2.7.2 E), implies that only mutating all these four residues will disrupt the interface of Squ and Spn-E. To further check whether these four residues from Spn-E region are also needed for the binding, I generated Spn-E mutant with alanine substitutions at the predicted interface residues (Spn-E^{4A}) and the co-immunoprecipitation assays in S2 culture cells reveal that Spn-E^{4A} lost the binding with Squ (Fig. 2.7.2 F). Take together, these results collectively confirmed that the four residues mediate the Squ–Spn-E interaction via salt bridges.

As a *Drosophila*-specific protein, Squ was found to be conserved only among *Drosophila* species by the phylogenetic analysis and vertebrate orthologs remain unidentified (Fig. 2.7.3 A). To further detect whether the binding residues of Squ and Spn-E are conserved or not, we performed the alignment of Squ and Spn-E amino acid sequence among *Drosophila* species. Importantly, I found both these four residues from Squ and Spn-E are conserved (Fig. 2.7.3 B and C), these results suggest that the four conserved residues are crucial for Squ–Spn-E interaction among *Drosophila* species.

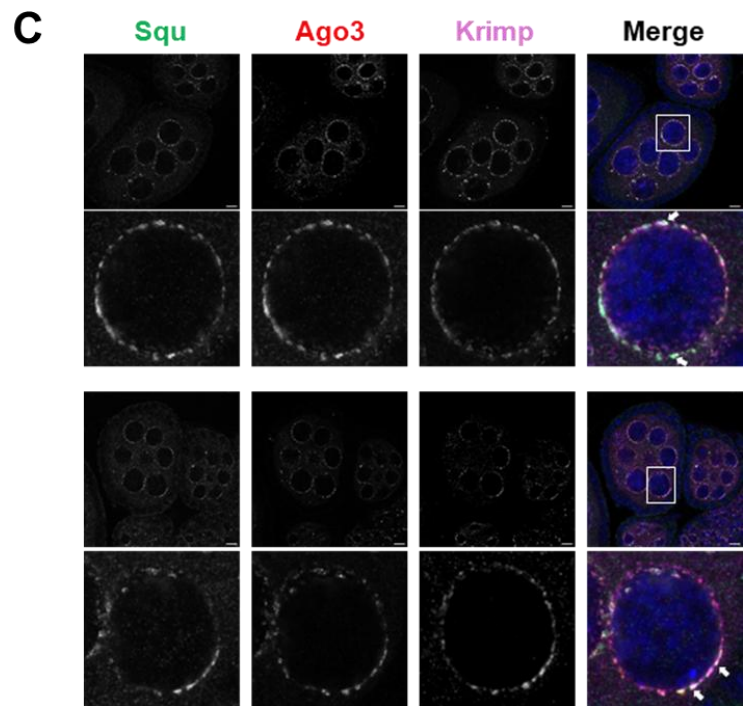
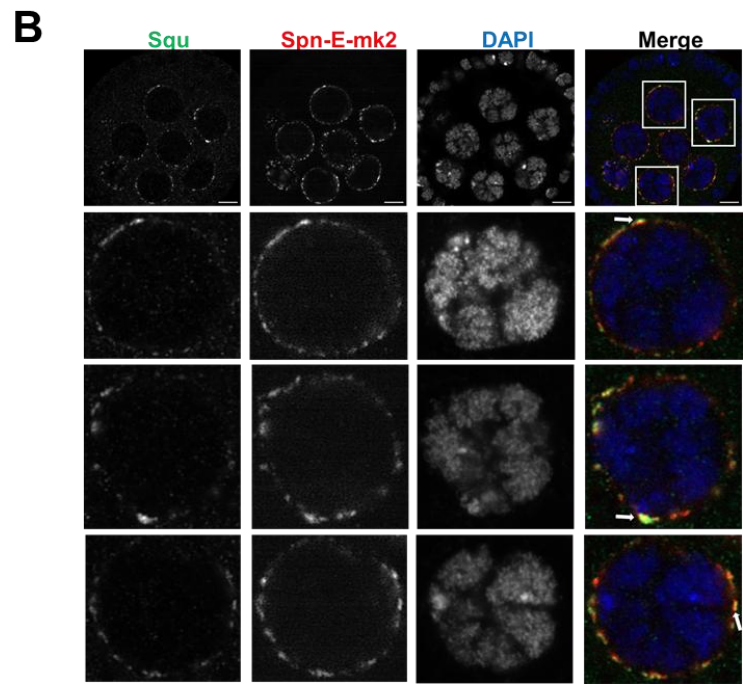
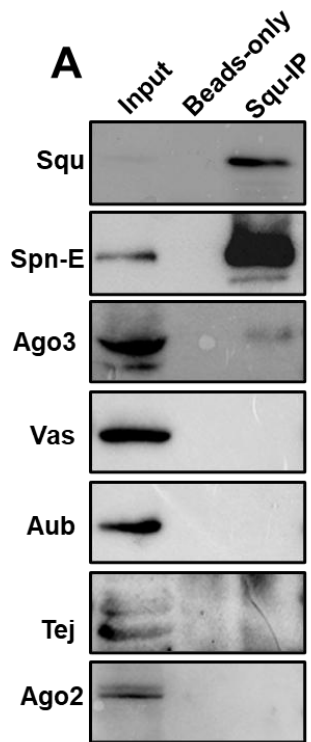


Figure 2.7.1 Squ associates with Spn-E and mildly with Ago3

(A) Immunoblotting analysis of Squ interactors in ovaries. Squ is immunoprecipitated from the control *y w* ovaries using anti-Squ antibodies. Precipitation process without anti-Squ antibodies serves as negative control. piRNA biogenesis factors; Spn-E, Tej, Vas, Ago3, and Aub. Ago2, not involved in piRNA biogenesis, serves as a negative control.

(B) Localization of Squ and mk2-tagged Spn-E in the *squ* heterozygous control germline cells. (upper 1st panels: stage 4–5 egg chambers; lower three panels: magnified nuclei indicated by squares in the upper panels). White arrows represent the co-localized granules of Squ and Spn-E. Scale bar is 5 μ m.

(C) Localization of Squ, Ago3 and Krimp in the *squ* heterozygous control germline cells. (upper panels: stage 4–5 egg chambers; lower panels: magnified nuclei indicated by squares in the upper panels). White arrows represent the co-localized granules of Squ and Ago3. Scale bar is 5 μ m.

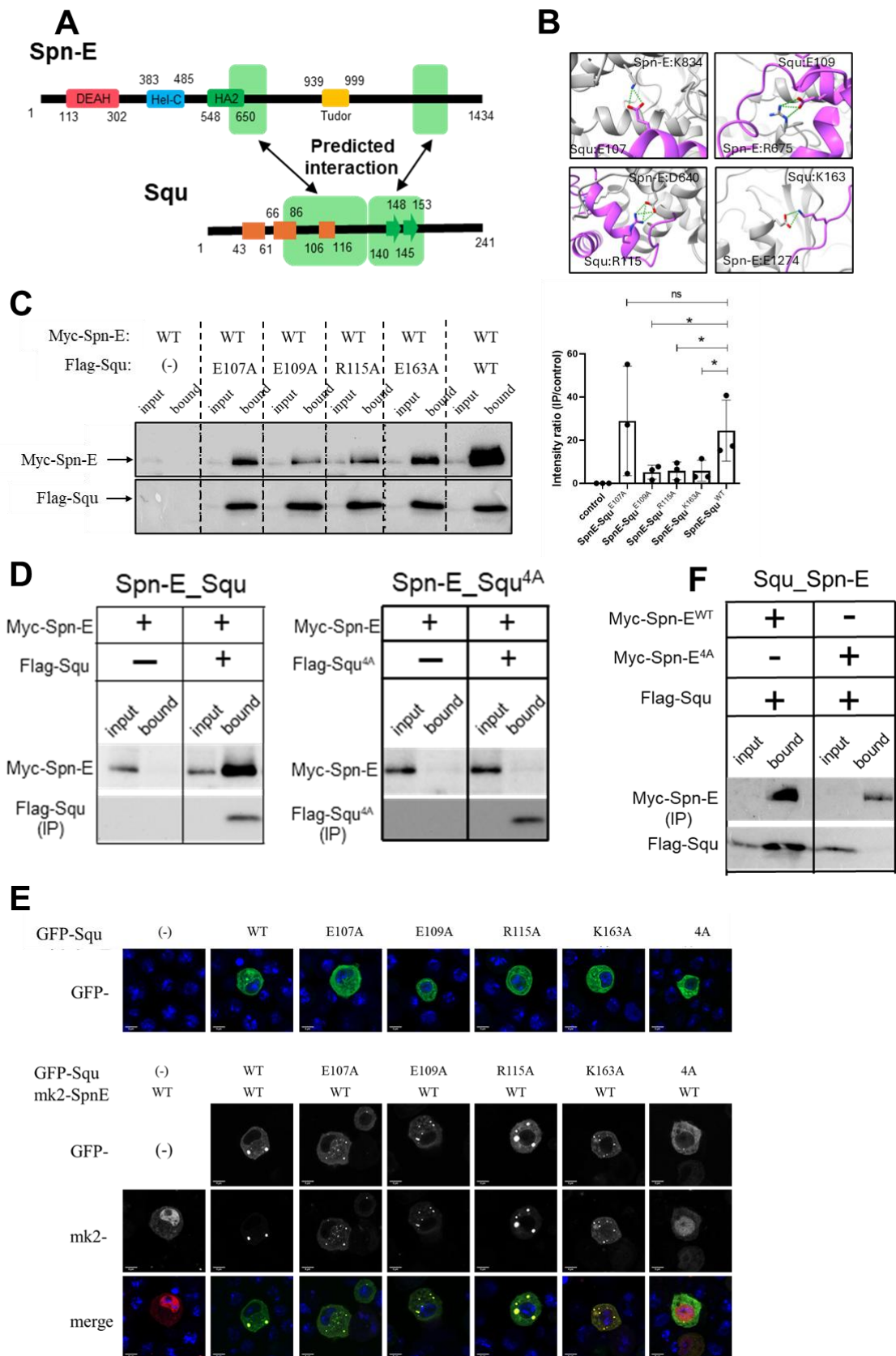


Figure 2.7.2 Squ associates with Spn-E through four residues

(A) Schematic of Spn-E domain structures defined in SMART44. Boxes (α -helix: orange) and arrow (β -sheet: green) for Squ structure. The predicted interacting regions between Spn-E and Squ are indicated in gray boxes. Tej interaction site of Spn-E is also shown (Lin et al., 2023).

(B) The predicted salt bridges at the interface, with Spn-E in gray and Squ in magenta. The residues forming salt bridges are depicted in stick model.

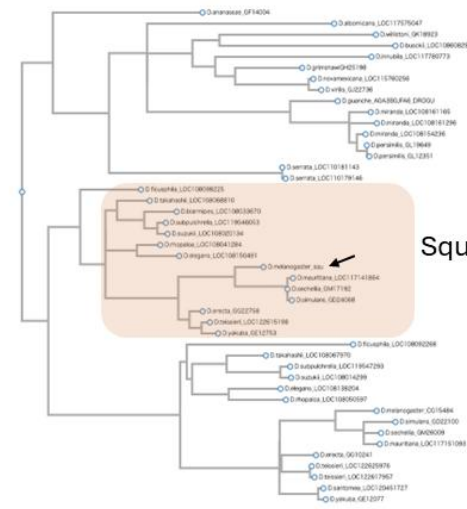
(C) Co-immunoprecipitation of Myc-Spn-E and Flag-Squ expressed in S2 culture cells. In addition to the wildtype, Squ mutants containing amino acid residues predicted to form salt bridges altered to Alanine were also examined. The right panel shows quantifications of the intensity ratio (IP/input) with error bars indicating s.d. (n=3). ns: not significant. *: p-value < 0.10.

(D) Co-immunoprecipitation assay using S2 cell lysate to examine the interaction between Myc-Spn-E and Flag-Squ^{WT/4A}. S2 cells expressing Myc-Spn-E alone is used as a control.

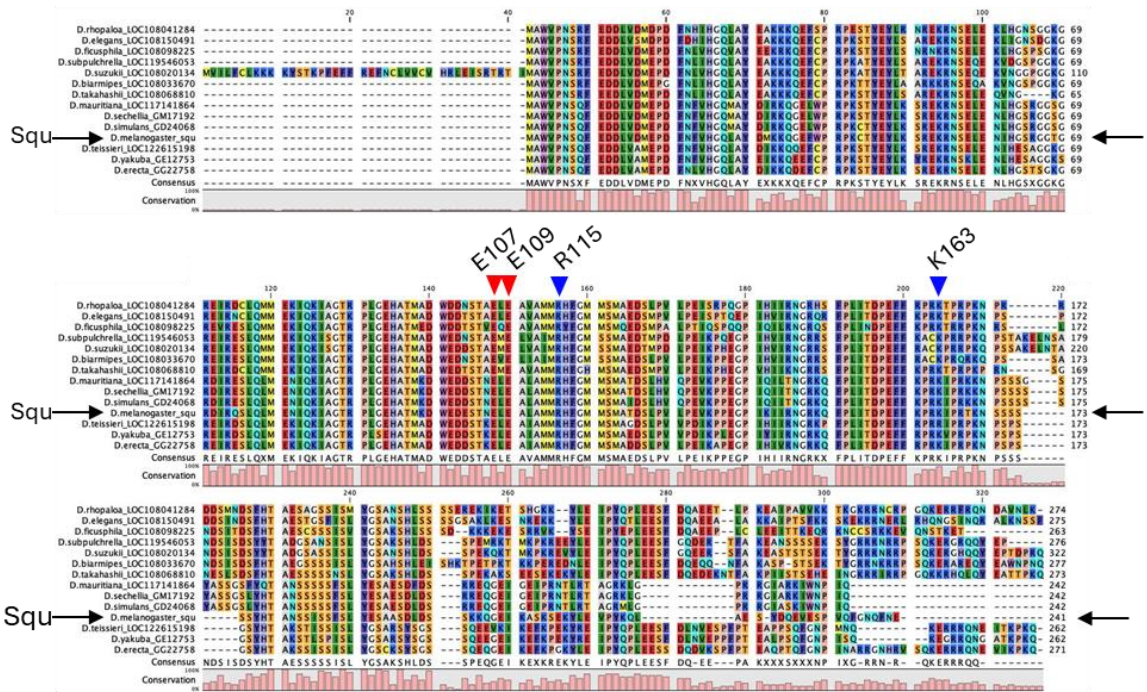
(E) Localization of GFP-Squ wildtype and mutants in S2 cells (upper panels). Scale bars: 5 μ m. Co-localization of mK2-Spn-E and GFP-Squ wildtype or mutant proteins (except for the 4A mutant) are shown in lower panels. Scale bars: 5 μ m.

(F) Immunoprecipitation of Myc-tagged Spn-E^{WT} and Spn-E^{4A} using anti-Myc antibodies with the co-expression of Flag-Squ in S2 culture cells. Flag-Squ can be detected in Spn-E^{WT} but not in Spn-E^{4A} immunoprecipitates.

A



B



C

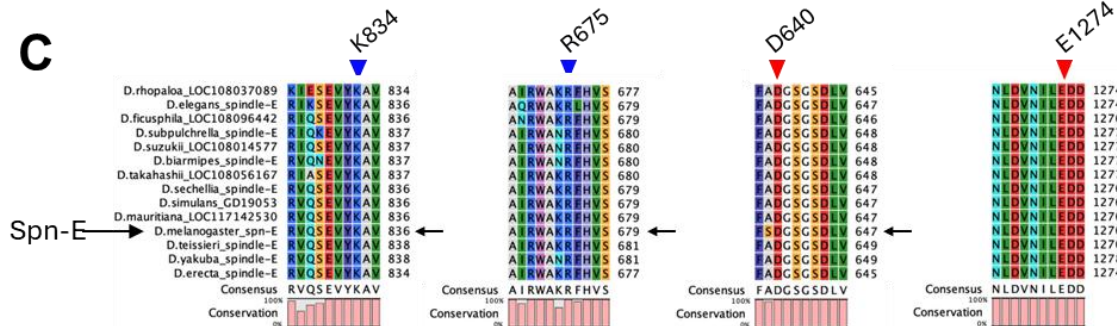


Figure 2.7.3 Four binding residues are conserved among *Drosophila* species

- (A) Phylogenetic tree of Squ homologs across various *Drosophila* species.
- (B) Multiple sequence alignment of Squ orthologs from different *Drosophila* species, highlighting residues predicted to form salt bridges with Spn-E.
- (C) Multiple sequence alignment of Spn-E orthologs in *Drosophila* species focusing on regions around residues predicted to interact with Squ. The legend was shown in the original pdf, but the legends have been removed during the process

2.8 Squ–Spn-E interaction is essential for piRNA precursor processing and TE repression

To assess the physiological consequences of disrupting this Squ–Spn-E interaction *in vivo*, Squ^{4A} or wild-type Squ (Squ^{WT}) was expressed under the native promoter in ovaries lacking endogenous *squ*. Co-immunoprecipitation revealed markedly reduced binding of Squ^{4A} to Spn-E compared with Squ^{WT} in ovaries (Fig. 2.8.1 A). Besides, Squ^{4A} showed detached aggregates in the cytoplasm in addition to the perinuclear foci and was expressed at lower levels than Squ^{WT} (Fig. 2.8.1 B and C). These results suggest that Squ interacts with Spn-E through four conserved residues *in vivo* and this interaction is crucial for the nuage enrichment and protein stability of Squ in *Drosophila* ovaries.

To further elucidate whether this interaction is crucial for piRNA biogenesis, rescue experiments that over-express are Squ^{4A} or Squ^{WT} were performed in ovaries lacking endogenous *squ*. I found that Squ^{WT} restored the repression of TEs and suppression of HeT-A Gag protein and Stellate accumulation in germline cells (Fig. 2.8.2 A and B), the proper localization of major germline piRNA clusters, *38C* and *42AB*, and processing of these piRNA cluster transcripts as reflected by the reduction of their levels (Fig. 2.8.2 C and D). Moreover, Squ^{WT} restored *Drosophila* female fertility as measured by the hatching rates (Fig. 2.8.2 E). In contrast, Squ^{4A} failed to rescue any of these defects despite with a higher expression than endogenous Squ (Fig. 2.8.1C and 2.8.2 A-E). Collectively, these results indicate that the four conserved residues in Squ are critical for direct binding to Spn-E *in vivo* and this interaction is essential for proper piRNA biogenesis, TEs' repression, and female fertility in *Drosophila*.

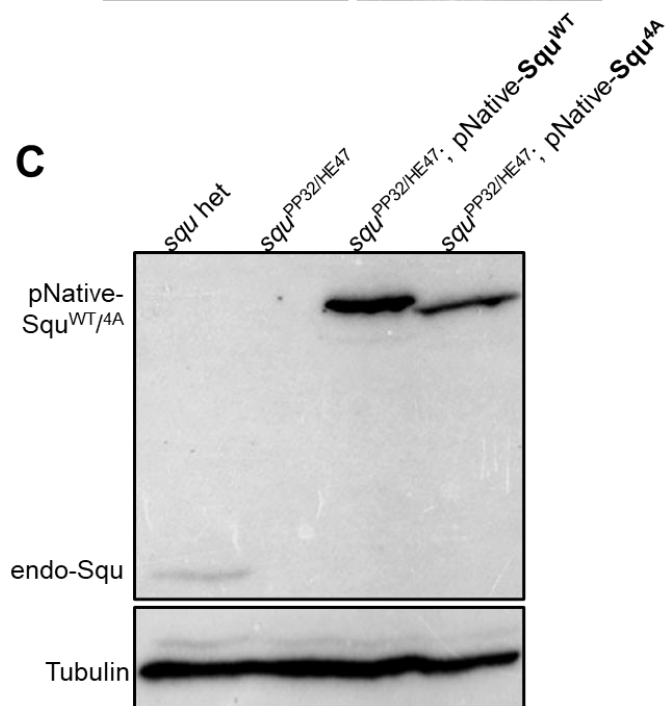
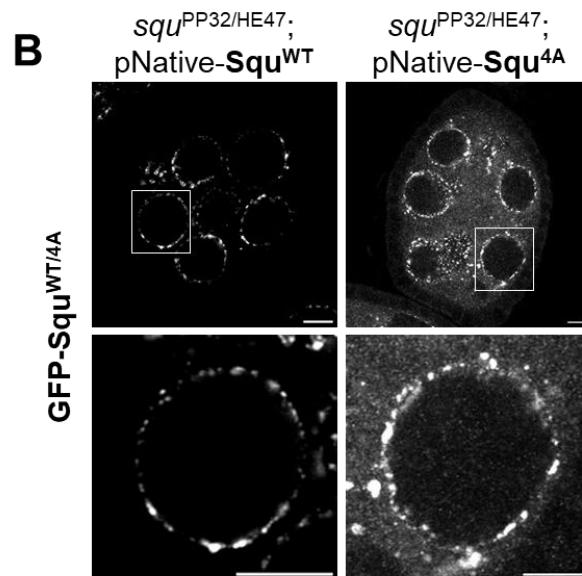
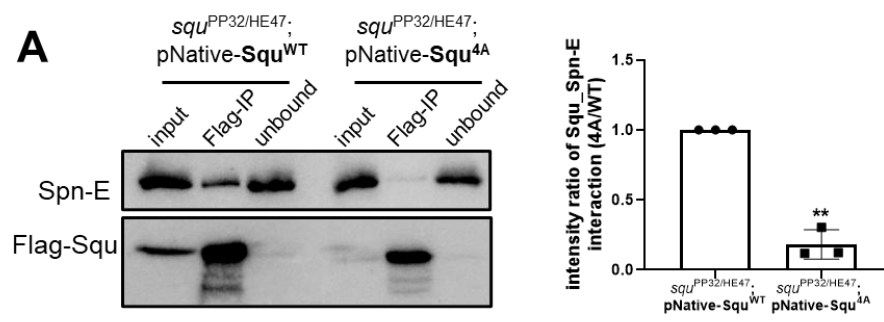


Figure 2.8.1 Squ–Spn-E interaction is crucial for nuage enrichment and protein stability of Squ

(A) Immunoprecipitation of GFP–3×Flag–Squ and the variants expressed under the native promoter (pNative-Squ^{WT} and pNative-Squ^{4A}) using anti-3FLAG antibodies. The right panel shows the quantification of the Squ–Spn-E interaction, plotted as the intensity ratio relative to Squ^{WT} (3 biological replicates).

(B) Localization of Squ^{WT} and Squ^{4A} expressed under the native promoter in *squ* mutant germline cells (upper panels: stage 4–5 egg chambers; lower panels: magnified nuclei indicated by squares in the upper panels). Squ^{WT} is enriched in perinuclear nuage, whereas Squ^{4A} shows detached aggregates in the cytoplasm in addition to the perinuclear foci. Scale bar, 5 μm.

(C) Immunoblotting of proteins extracted from the heterozygous control, *squ*^{PP32/HE47} and *squ*^{PP32/HE47} expressing Squ^{WT} or Squ^{4A} ovaries.

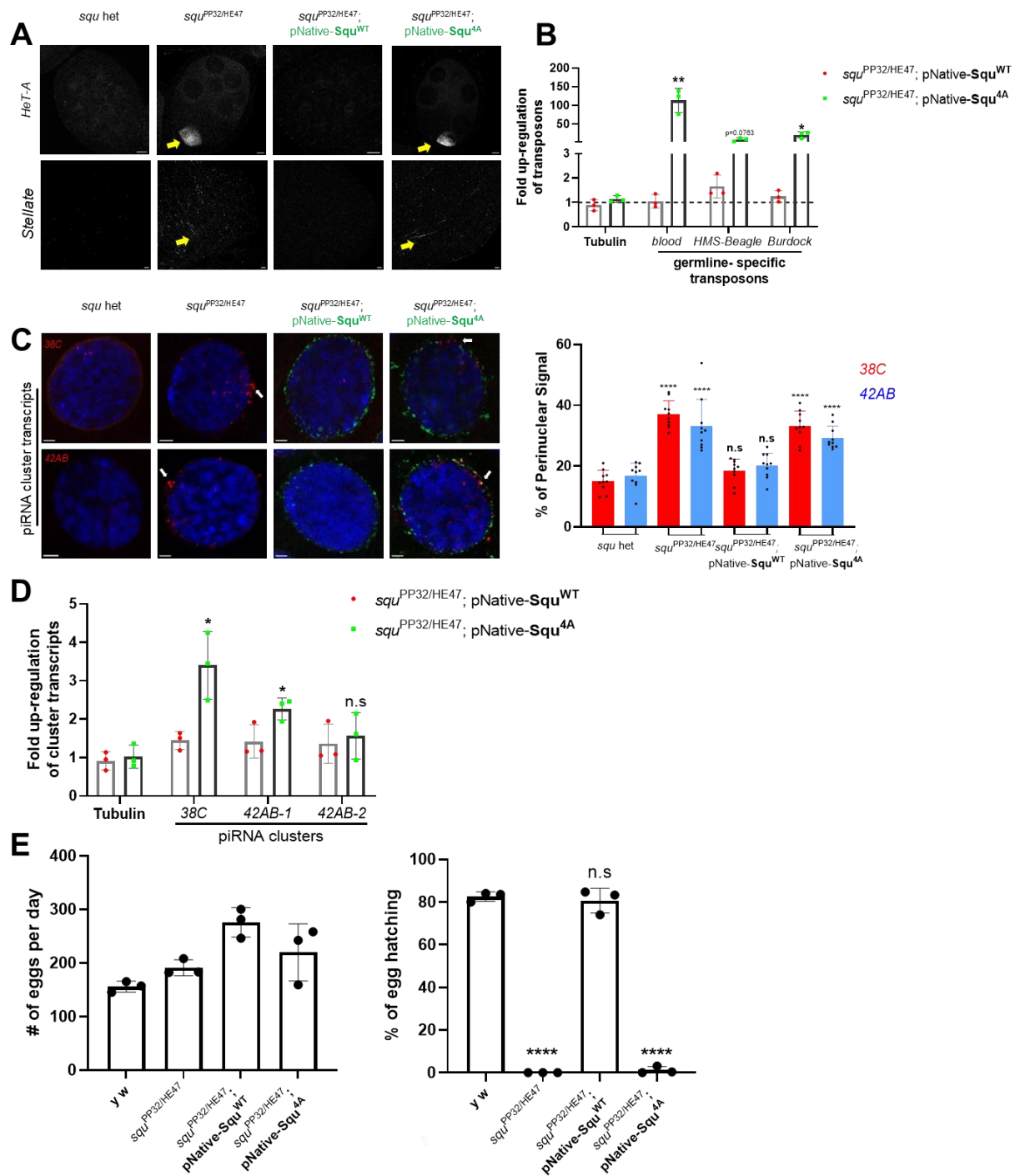


Figure 2.8.2 Interaction between Squ and Spn-E is crucial for piRNA biogenesis, transposon silencing, and oogenesis

(A) Immunostaining of HeT-A Gag and Stellate protein in the heterozygous control, *squ*^{PP32/HE47} mutant, and *squ*^{PP32/HE47} expressing Squ^{WT} or Squ^{4A} ovaries. Yellow arrows indicate HeT-A Gag and Stellate signals, respectively. Scale bar, 5 μ m.

(B) RT-qPCR analysis of transcripts derived from the germline-specific transposons *blood*, *HMS-Beagle*, and *Burdock*, with tubulin as a control. All values are normalized to *rp49* and are shown relative to expression levels in *squ*^{PP32/HE47}; pNative-Squ^{WT} ovaries. Error bars indicate standard deviation (3 biological replicates). Statistical significance is assessed by Student's unpaired t-test. **p* < 0.05, ***p* < 0.01.

(C) HCR in situ hybridization of the major germline piRNA precursors derived from clusters *38C* and *42AB* in germline cells of heterozygous control, *squ*^{PP32/HE47}, and *squ*^{PP32/HE47} expressing Squ^{WT} or Squ^{4A} under the native promoter. DNA is stained with DAPI (blue); the nuclear membrane is labeled with WGA (green). White arrows indicate precursor signals accumulating to perinuclear regions. Scale bar, 2 μ m. Right, quantification of perinuclear precursor signals. Fluorescence intensities of foci located within $\pm 5\%$ of the nuclear diameter inside and outside the nuclear membrane are summed, normalized to the total nuclear signal, and plotted as the percentage of perinuclear signal.

(D) RT-qPCR analysis of transcripts derived from major germline piRNA clusters *38C* and *42AB*, with tubulin as a control. All values are normalized to *rp49* and are shown relative to expression levels in *squ*^{PP32/HE47}; pNative-Squ^{WT} ovaries. Error bars indicate standard deviation (3 biological replicates). Statistical significance is assessed by Student's unpaired t-test. **p* < 0.05.

(E) Analysis of egg laying and hatching rate. Infertility of *squ* mutant is rescued by the pNative-promoter-Squ^{WT} but not Squ^{4A} mutant. Daily egg production by five females is recorded (3 biological replicates). T-test is performed for the statistics analysis. (*****p* < 0.0001).

2.9 Squ contributes to Ago3 protein maintenance independently of Spn-E

In *squ* mutant ovaries, both Ago3 immunostaining signals and total protein levels were severely reduced (Fig. 2.1). And this reduction was efficiently rescued by expression of Squ^{WT}, whereas the Squ^{4A} mutant protein restored Ago3 only weakly (Fig. 2.9.1 A and B), suggesting that Squ may contribute to the maintenance of Ago3 protein levels in a Spn-E-independent manner.

To further examine whether the Squ–Spn-E complex is required for the Ago3 stability or Squ has a direct effect on maintaining Ago3 protein, Ago3 localization and protein levels were analyzed by expressing Squ^{WT} or Squ^{4A} in the *spn-E* mutant background. Previous studies have reported a strong reduction of Ago3 protein in *spn-E* mutant ovaries (Ryazansky et al., 2016; Andress et al., 2016), which I also confirmed with our newly generated loss of function allele, *spn-E⁷⁻⁴*, in this study. Upon the loss of *spn-E* function, Ago3 protein level was strongly reduced together with a pronounced decrease in endogenous Squ (Fig. 2.9.2 A). Notably, the expression of Squ^{WT} substantially increased Ago3 abundance in *spn-E* mutant ovaries, suggesting that Squ^{WT} stabilizes Ago3 protein in a Spn-E-independent manner. On the other hand, the Squ^{4A} mutant had only a minor effect on the level of Ago3, which may be due to its lower expression level and/or the impaired functional activity of the mutant protein (Fig. 2.9.2 A). To test whether Squ associates with Ago3 independently of Spn-E, I performed co-immunoprecipitation assays and immunostaining in the *spn-E* mutant background. Both Squ^{WT} and Squ^{4A} were found to associate with Ago3 even in the absence of Spn-E (Fig. 2.9.2 B and C), indicating that Squ can interact with Ago3 independently of Spn-E. In contrast, overexpression of Flag-Ago3 by a strong germline driver, NGT40 and nosGal4, in *squ* mutant ovaries failed to restore Ago3 protein levels (Fig. 2.9.2 D), indicating that Squ is required for Ago3 protein stabilization rather than for Ago3 expression itself. Together, these results suggest that although proper Squ–Spn-E

function is required for robust maintenance of Ago3 protein, Squ also exerts a Spn-E–independent effect on Ago3 protein stability through an as-yet-unknown mechanism.

To determine whether the defects in piRNA precursor processing and transposon repression in *spn-E* mutant ovaries are primarily caused by Ago3 reduction, I analyzed the expression of piRNA cluster transcripts from 38C and 42AB, as well as the retrotransposon *HeT-A* in *spn-E* mutant ovaries (Fig. 2.9.3 A and B). Despite the substantial restoration of Ago3 protein levels by Squ^{WT} expression, the upregulation of cluster transcripts and *HeT-A* remained largely unchanged in *spn-E* mutant ovaries (Fig. 2.9.3 A and B). Similar results were observed with Squ^{4A} (Fig. 2.9.3 A and B). These results indicate that recovery of Ago3 protein alone is not sufficient to rescue the defects in piRNA precursor processing or transposon repression in *spn-E* mutant ovaries. Instead, it implies that Spn-E acts upstream of both Squ and Ago3 in the piRNA pathway, supporting the notion that Spn-E is a core nuage factor essential for piRNA pathway activity.

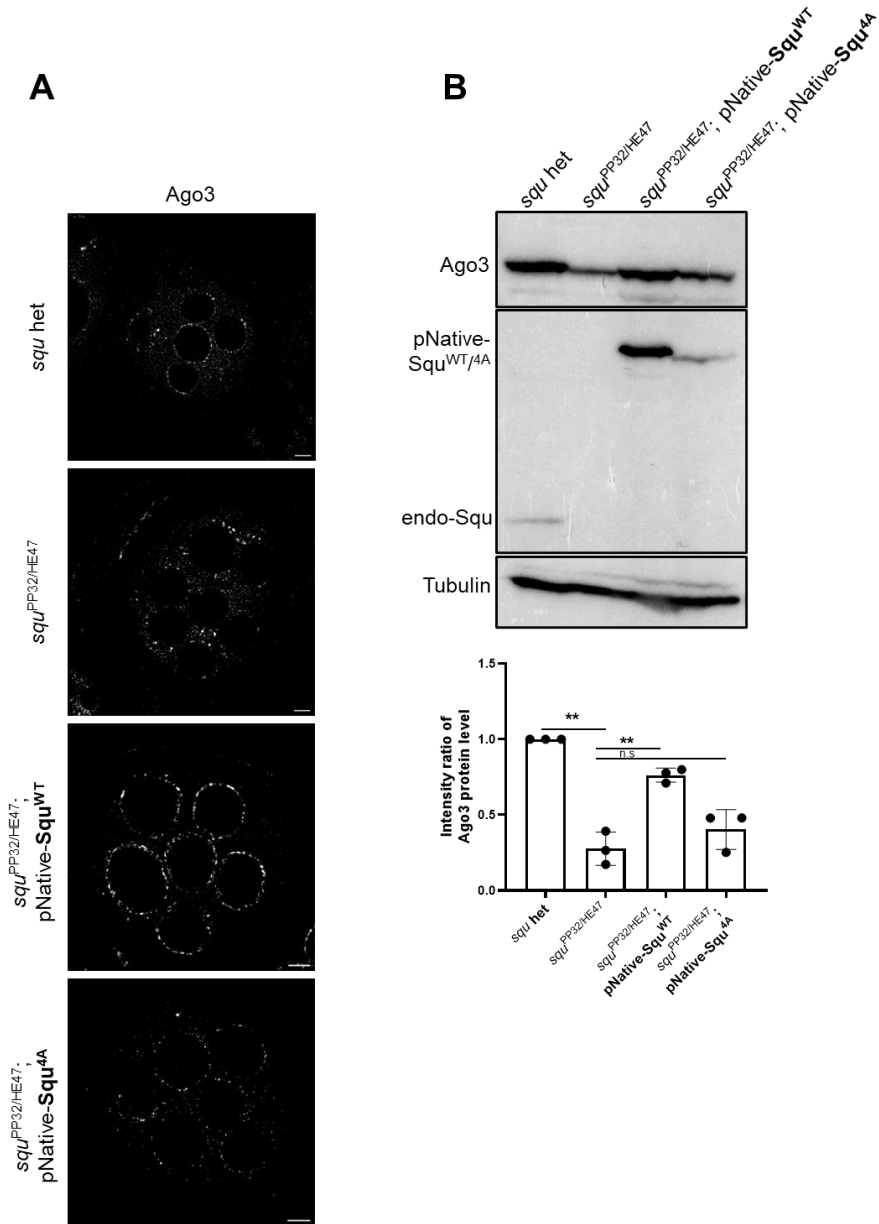


Figure 2.9.1 Squ contributes to Ago3 stability

(A) Immunostaining of mid-stage egg chambers from the heterozygous control, *squ*^{PP32/HE47}, and *squ*^{PP32/HE47} expressing Squ^{WT} or Squ^{4A} under the native promoter.

Scale bar is 5 μ m.

(B) Immunoblotting of proteins extracted from the heterozygous control, *squ*^{PP32/HE47} and *squ*^{PP32/HE47} expressing Squ^{WT} or Squ^{4A} under the native promoter. Lower panel shows the quantification of signal intensities (3 biological replicates). Statistical significance is assessed by Student's unpaired t-test. **p < 0.01.

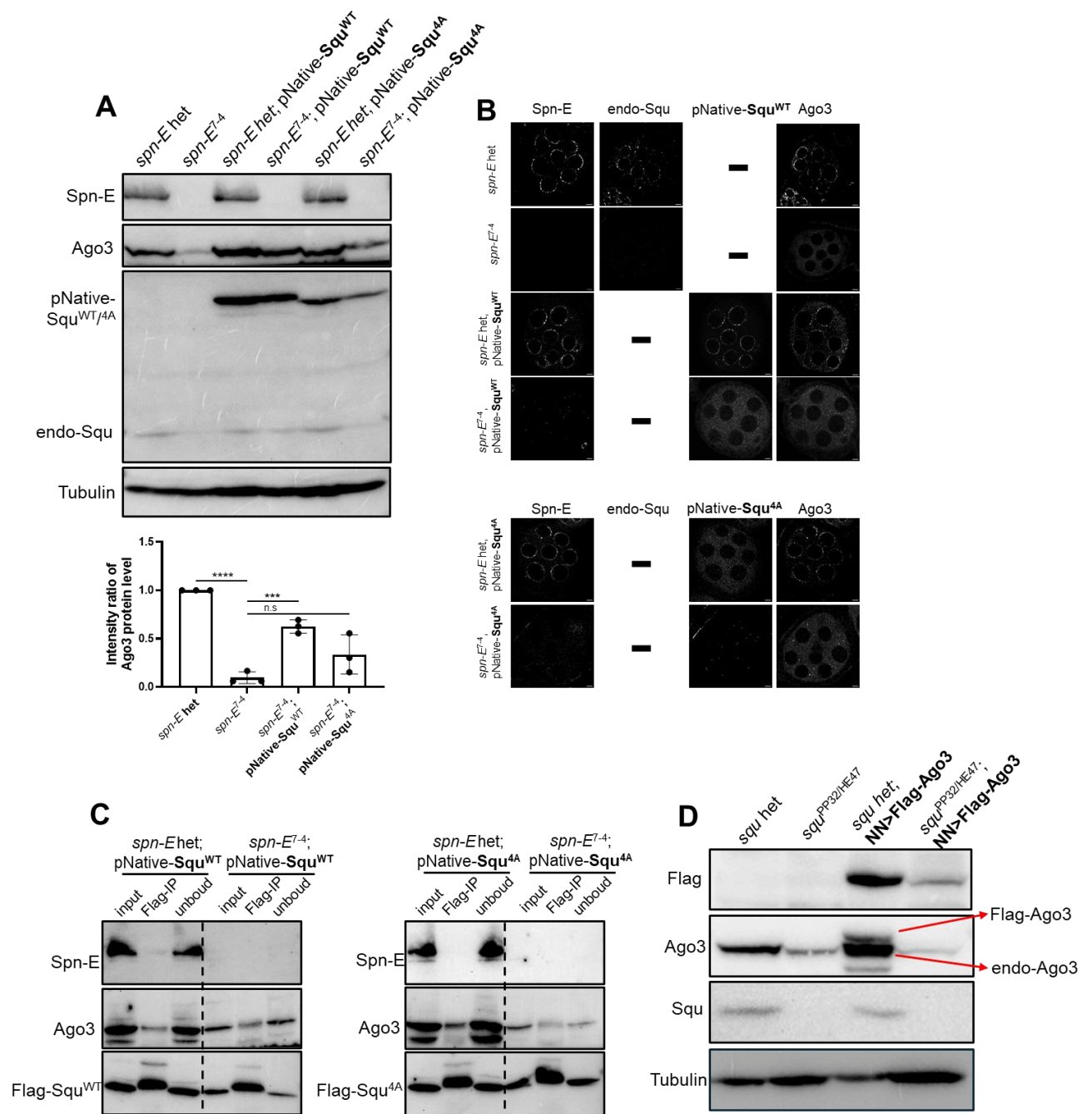


Figure 2.9.2 Squ contributes to Ago3 stability in a Spn-E-independent manner

(A) Immunoblotting of proteins extracted from the ovaries of the heterozygous control, *spn-E*⁷⁻⁴, and *spn-E*⁷⁻⁴ expressing Squ^{WT} or Squ^{4A} under the native promoter. Lower panel shows the quantification of signal intensities (3 biological replicates). Statistical significance is assessed by Student's unpaired t-test. ***p < 0.001, ****p < 0.0001.

(B) Immunostaining of mid-stage egg chambers from the heterozygous control, *spn-E*⁷⁻⁴, and *spn-E*⁷⁻⁴ expressing Squ^{WT} or Squ^{4A} under the native promoter. Scale bar is 5 μm.

(C) Immunoprecipitation of GFP-3×Flag-Squ and the variants expressed under the native promoter (pNative-Squ^{WT} and pNative-Squ^{4A}) using anti-3FLAG antibodies in the ovaries expressing pNative-Squ^{WT} and - Squ^{4A} under the loss of endogenous *spn-E* condition.

(D) Immunoblotting of proteins extracted from the heterozygous control, *squ*^{PP32/HE47}, *squ* heterozygous and *squ*^{PP32/HE47} expressing NGT40; nosGal4 driven Flag-Ago3 protein. Two bands are detected using anti-Ago3 antibodies (upper bands are the NGT40; nosGal4 driven Flag-Ago3 and lower bands are the endogenous Ago3).

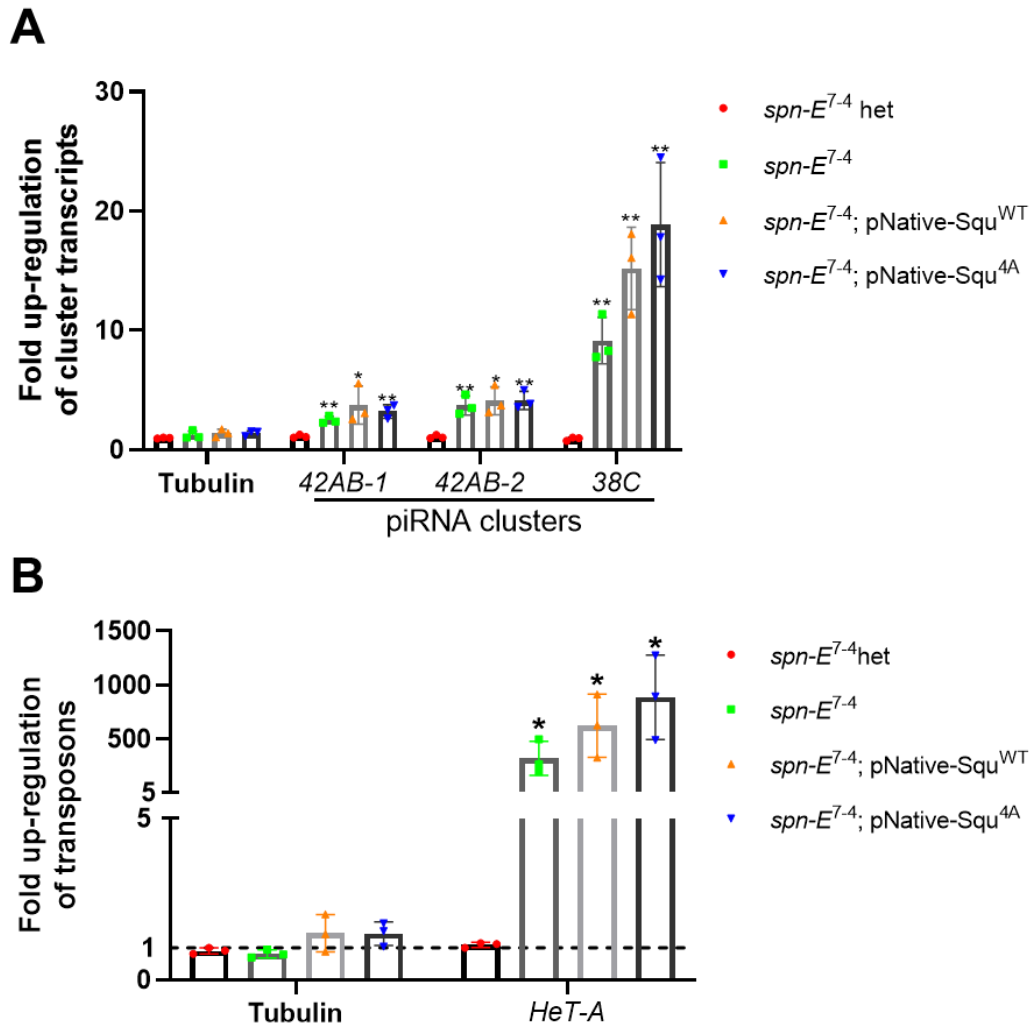


Figure 2.9.3 piRNA cluster disruption and TE repression are not rescued by only Ago3 recovery

(A and B) RT-qPCR measurement of transcripts from piRNA cluster loci, *38C* and *42AB*, those from *HeT-A* and the control tubulin. Relative expression values are obtained by $\Delta\Delta C_t$ using *rp49* as reference. Error bars; standard deviation of biological triplicates. Statistics; unpaired T-test (* $p < 0.05$, ** $p < 0.01$).

2.10 Squ facilitates Spn-E–mediated RNA processing at Aub

A recent study in silkworm demonstrated that Spn-E is recruited to the Siwi (the silkworm ortholog of *Drosophila* Aub)–piRNA–target RNA complex formed with GTSF1 and Meal (referred to as Siwi* or Aub*) (De et al., 2025). To examine whether the formation or dynamics of the Aub*–Spn-E complex is affected by Squ, I analyzed the interaction between Aub and Spn-E by immunoprecipitation. When Aub was immunoprecipitated from control ovary lysates, Spn-E was barely detectable in the bound fraction. In contrast, the amount of Spn-E co-precipitated with Aub was increased in *squ* mutant ovaries (Fig. 2.10.1 A). These results indicate that Spn-E remains more stably associated with Aub* in the absence of Squ. To further examine the nature of this Aub*–Spn-E complex, I analyzed the RNA species recovered in the Spn-E immunoprecipitants. The levels of piRNA cluster transcripts derived from 38C and 42AB, which serve as major target RNAs for Aub, were significantly increased in *squ* mutant ovaries compared with the heterozygous controls, despite a similar amount of Spn-E being present in the IP fractions (Fig. 2.10.1 B and C). These data indicate that, in the absence of Squ, Spn-E is retained on the Aub* complex together with piRNA precursor transcripts, which are not efficiently processed further.

As the interaction between Squ and Spn-E has been predicted by AlphaFold program and verified by the mutational analysis, and importantly, the predicted Squ-binding interface on Spn-E is located on the surface opposite to the Aub*-binding interface (Fig. 2.10.2 A), suggesting that Squ does not directly compete with the Spn-E–Aub* interaction. As an RNA helicase protein, Spn-E is expected to possess RNA-binding and ATPase activities (Ott et al., 2014), so I next examined whether Squ directly affects the ATPase activity of Spn-E. Purified wild-type Spn-E protein (Spn-E^{WT}) exhibited basal ATP hydrolysis activity *in vitro*, whereas the ATPase-dead mutant, Spn-E^{DQVH}, showed no detectable ATPase activity (Fig. 2.10.2 B). Squ protein alone also did not exhibit ATPase activity (Fig. 2.10.2 B). Notably, the addition of Squ did not

significantly alter the ATP hydrolysis activity of Spn-E^{WT} in these *in vitro* conditions (Fig. 2.10.2 B). Together, these results demonstrate that, although Squ does not disrupt Aub*–Spn-E binding directly and affect the basal ATPase activity of Spn-E *in vitro*, loss of Squ leads to persistent retention of Spn-E on the Aub* complex together with piRNA precursor transcripts *in vivo*. This aberrant accumulation of the Aub*–Spn-E–RNA intermediate provides a mechanistic explanation for why piRNA precursor processing and downstream piRNA pathway function remain defective in conditions where Ago3 protein levels can be partially restored by Squ in the *spn-E* mutant background.

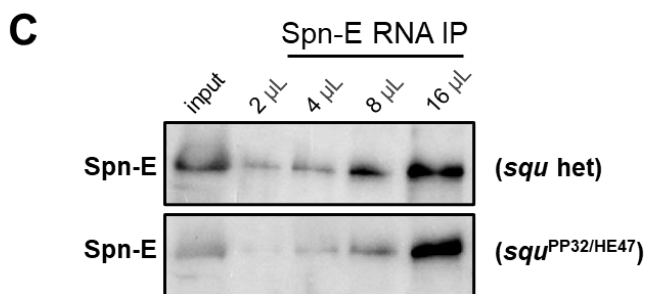
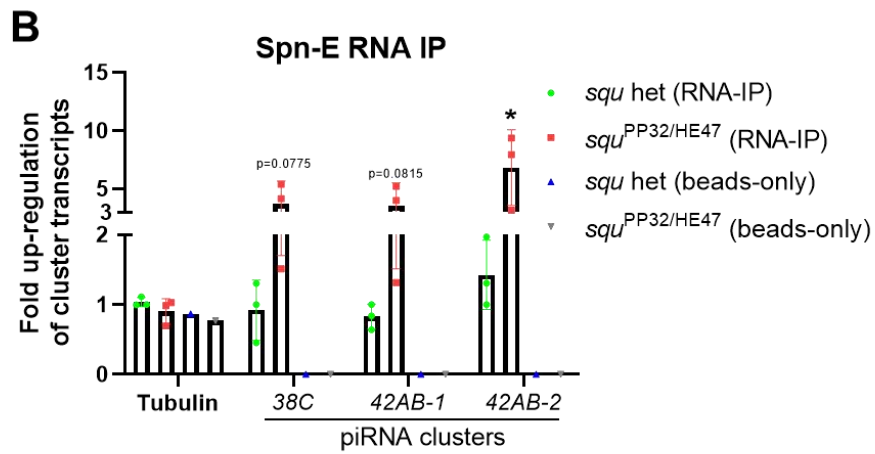
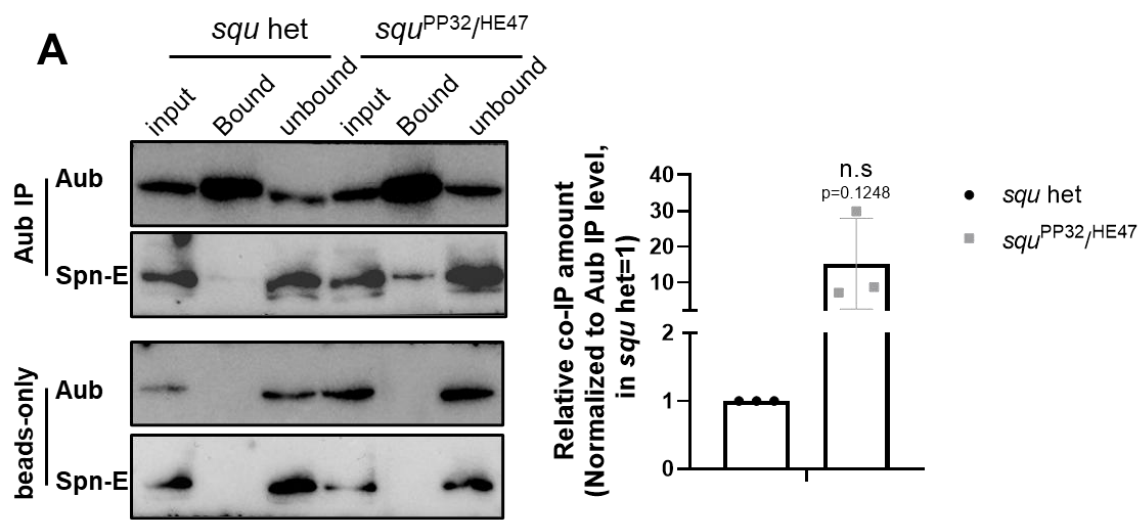


Figure 2.10.1 The effect of *squ* loss on Spn-E functions

- (A) Immunoprecipitation of Aub in the heterozygous control or *squ*^{PP32/HE47} mutant ovaries. Immunoprecipitation without anti-Aub antibodies is shown as a negative control. Right panel: Quantification of intensity ratio for Aub–Spn-E interaction in the presence and absence of *squ*. Relative values are obtained by $\Delta\Delta$ Ct using *rp49* as reference. The signal is further normalized by the Aub protein levels in IP fractions. Error bars indicate standard deviation (3 biological replicates). The beads only controls are also shown as a negative control. Statistics; unpaired T-test.
- (B) RT-qPCR measurement of Spn-E-bound piRNA cluster transcripts from *38C* and *42AB*, and the control *tubulin*. RNAs extracted from Spn-E immunoprecipitates from the heterozygous control or *squ*^{PP32/HE47} mutant ovaries are analyzed. Relative values are obtained by $\Delta\Delta$ Ct using *rp49* as reference. The signal is further normalized by the Spn-E protein levels in IP fraction (Fig. 2.10.1 C). Error bars indicate standard deviation (3 biological replicates). Beads-only samples are included as a negative control. Statistics; unpaired T-test (*p < 0.05).
- (C) Immunoblotting of the input and Spn-E IP fraction samples in the presence and absence of *squ*. 2, 4, 8 and 16 μ l of the IP samples are loaded to perform the comparison of IP efficiency.

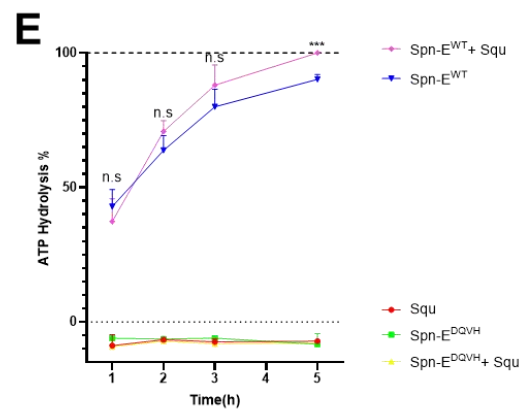
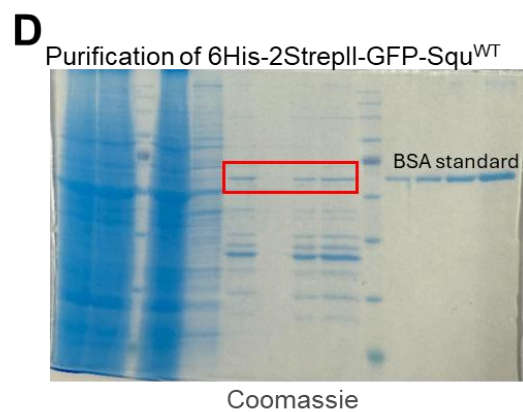
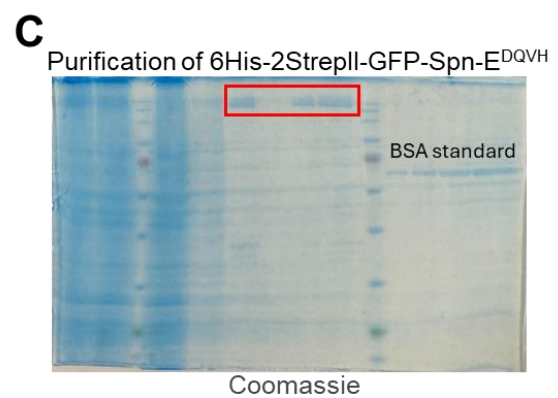
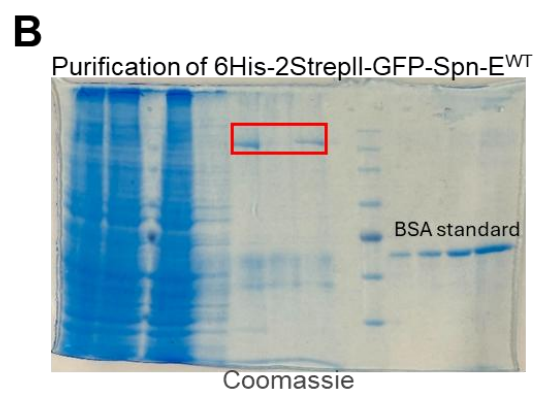
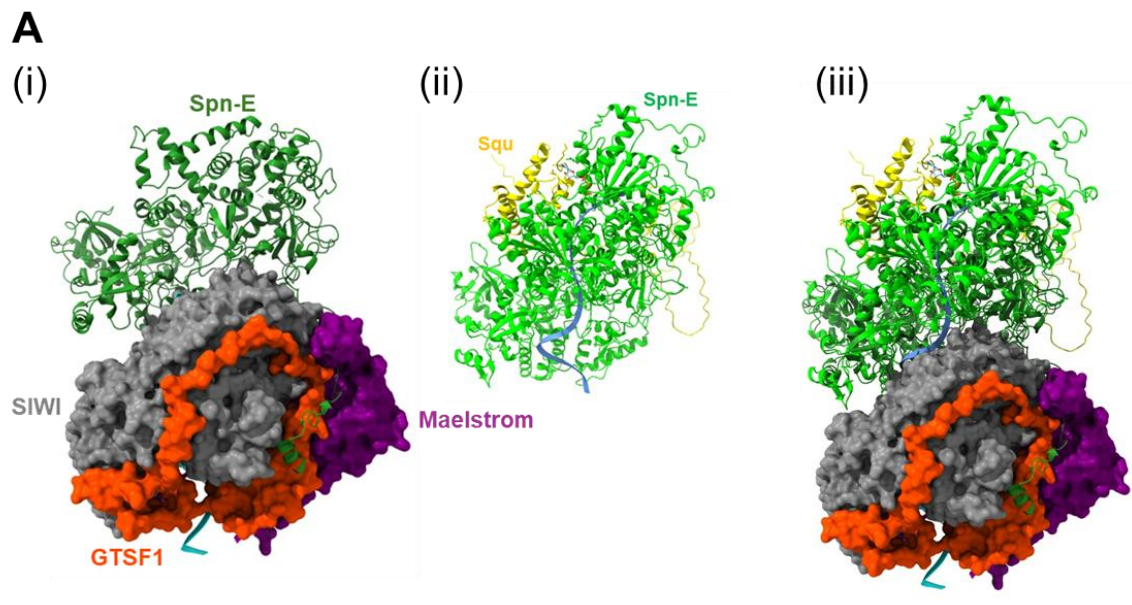


Figure 2.10.2 Structural modeling and in vitro characterization of Spn-E and Squ

(A) Predicted structure of the SIWI*/Aub* complex with Spn-E and Squ.

(i) Cryo-EM structure of the *Bombyx mori* SIWI/Aub–piRNA–target RNA–GTSF1–Maelstrom–Spn-E complex (PDB: 9NHS). SIWI is shown in gray, GTSF1 in orange, Maelstrom in purple, and Spn-E in dark green. The target RNA is depicted as a cyan ribbon.

(ii) Predicted *Drosophila melanogaster* Spn-E–Squ complex generated using AlphaFold3. Spn-E is shown in green and Squ in yellow. A single-stranded RNA is displayed as a blue ribbon, and ATP is shown in stick representation.

(iii) Structural superposition of the two models based on alignment of their Spn-E subunits.

(B-D) Coomassie staining of purified recombinant proteins used for in vitro assays:

B: 6His-2StrepII-GFP–Spn-E^{WT}

C: 6His-2StrepII-GFP–Spn-E^{DQVH}

D: 6His-2StrepII-GFP–Squ^{WT}

Red boxes indicate bands corresponding to the purified proteins.

(E) ATPase activity of purified Spn-E^{WT}, Spn-E^{DQVH}, and Squ. ATP hydrolysis is measured up to 5 h, and released inorganic phosphate is quantified. Spn-E^{WT} shows robust ATPase activity, whereas Spn-E^{DQVH} and Squ alone exhibit minimal ATP hydrolysis. Error bars indicate standard deviation (3 biological replicates). Statistics; unpaired T-test (***) $p < 0.001$.

3. Discussion

The piRNA pathway, which represses transposable elements, is well conserved across virtually all animals examined to date, including *Drosophila melanogaster*. In *Drosophila* germline cells, piRNA amplification occurs in the membrane-less perinuclear organelle known as nuage, where multiple piRNA pathway components localize (Aravin et al., 2006; Hirano et al., 2014; Lim and Kai, 2015; Suyama and Kai, 2024). However, the precise mechanisms underlying piRNA biogenesis and the molecular functions of individual nuage components remain elusive. In this study, I systematically characterized the molecular role of Squ, a *Drosophila*-specific nuage protein, and uncovered its dual functions in piRNA pathway: facilitating Spn-E-dependent RNA processing and maintaining Ago3 protein stability (Fig. 3; Model).

3.1 Squ is crucial for maintaining Ago3 stability for proper piRNA biogenesis

In previous studies, it has been reported that TEs were de-repressed and total piRNAs were reduced to ~50% in *squ* mutant ovaries (Pane et al., 2007; Malone et al., 2009), implies that Squ may function on the piRNA biogenesis, but the details are elusive. In this study, I confirmed that several germline-specific TEs are de-repressed in *squ* mutant ovaries (Fig. 2.3), consistent to the nuage enrichment of Squ, suggesting that Squ may function on repression of germline TEs. Also, small-RNA analysis revealed that the reduced piRNAs in *squ* mutant ovaries are from the Ago3-bound piRNAs but not Aub-bound piRNAs (Fig. 2.4 A). Consistent to the reduced piRNAs, I confirmed that the processing of germline-specific piRNA clusters is disrupted in *squ* mutant ovaries (Fig. 2.5). These results suggest that Squ functions on piRNA biogenesis in *Drosophila* germline cells.

In this study, I detected a severe loss of Ago3 protein level in *squ* mutant ovaries which is different from previous study (Fig. 2.1B; Malone et al., 2009). Consistent to

this reduction, the Ago3-bound piRNAs are also reduced in *squ* mutant, whereas Aub protein level and Aub-bound piRNAs were not affected (Fig. 2.1B). As the heterotypic ping-pong between Aub and Ago3 was severely compromised and Aub-Aub homotypic ping-pong activity was clearly detected in *squ* mutant ovaries, I propose that, when Ago3 is non-functional, sliced RNAs are transferred from one Aub molecule to another to sustain this alternative cycle (Fig. 3; Model). This homotypic pathway, although active, is less efficient in piRNA production and transposon repression than the heterotypic pathway. Aub-Aub homotypic ping-pong has also been reported in *krimp* and *qin*, also known as *kumo*, mutant ovaries; Krimp promotes Aub-Ago3 coupling through its Tudor-domain-mediated dimerization, and Qin/Kumo is likewise required for Aub-Ago3 association (Zhang et al., 2011; Anand and Kai, 2012; Sato et al., 2015). These observations suggest that efficient heterotypic ping-pong is supported by the coordinated actions of multiple regulatory factors, whereas the Aub-Aub cycle can become prominent when this regulatory network is perturbed.

Our results demonstrate that Squ is essential for maintaining Ago3 protein stability, while *ago3* transcript level was not affected (Fig. 2.1 D). These results suggest that Squ regulates Ago3 via a post-transcriptional mechanism. It has been reported that unloaded Piwi protein is destabilized and degraded (Sienski et al., 2012; Kina et al., 2025), and I therefore propose that the piRNA-free, “empty” form of Ago3 may likewise be subjected to proteasome-dependent turnover. However, ectopic expression of Squ in *spn-E* mutant ovaries restored Ago3 protein abundance despite the severe reduction in piRNA production caused by loss of *spn-E* function, implying that Squ may possess an additional, direct role on stabilizing Ago3 protein (Fig. 2.9.2 A). Notably, restoration of Ago3 protein level in *spn-E* mutant did not rescue piRNA precursor processing defects and transposon de-repression, indicating that *spn-E* is genetically epistatic to *squ* and *ago3* function. These results uncover a functional hierarchy among nuage components, where Squ contributes to transposon repression by supporting Ago3 stability via an unresolved post-transcriptional mechanism.

3.2 Squ–Spn-E interaction is crucial for proper piRNA biogenesis

As a *Drosophila*-specific protein, Squ is only conserved among *Drosophila* species and without known motifs/domains by AlphaFold prediction (Fig. 2.7.2 A and 2.7.3), so I speculate Squ may function as the co-factor of any core ping-pong components. In this study, I reported that Squ and Spn-E expressed in S2 cells, a somatic cell line that lacks germline-specific piRNA factors, can directly bind each other (Fig. 2.7.2 D). Such interaction can also be detected in ovaries (Fig. 2.7.1 A), suggesting the specific binding between Squ and Spn-E. Four interacting residue-pairs between Squ and Spn-E which are conserved in *Drosophila* species and mutation of four conserved interacting residues in Squ (Squ^{4A}) abolishes this interaction in S2 cells (Fig. 2.7.2 D). I further confirmed that Squ^{4A} also fails to properly interact with Spn-E *in vivo* using transgenic expression in the ovary, and that reciprocal mutation of the corresponding residues in Spn-E (Spn-E^{4A}) similarly abolishes its interaction with Squ in S2 culture cells (Fig. 2.7.2 F and 2.8.1 A). Importantly, rescue experiments revealed that Squ^{4A} failed to rescue any piRNA-deficient phenotypes of the *squ* mutant ovaries (Fig. 2.8.2). Nevertheless, a substantial fraction of Squ^{4A} remained detectable in the perinuclear nuage region (Fig. 2.8.1 B). A similar discrepancy between physical interaction and nuage localization has been reported for Tej: although Tej-^ΔSRS loses its ability to interact with Spn-E in S2 culture cells, it does not disrupt the nuage localization of Spn-E or Vas *in vivo*, whereas a bit larger, extended SRS deletion is required to abolish their interaction (Lin et al., 2023). These observations suggest that the highly condensed molecular environment of nuage and/or additional protein interactions can maintain nuage localization even when direct interactions with Spn-E are weakened or disrupted.

Our results suggest that Squ functions as a co-factor of Spn-E, an RNA helicase and facilitates the efficient transfer of sliced RNA intermediates from Aub to Ago3 during the ping-pong amplification cycle. RNA helicases generally utilize their ATPase activity to unwind RNA structures or release a single-stranded RNA from the duplexes

(Jankowsky and Fairman, 2007; Jankowsky, 2011). Considering this general property, Spn-E–dependent RNA remodeling is likely required for efficient heterotypic ping-pong between Aub and Ago3. In support of this concept, the ATPase activity of another nuage-localized RNA helicase, Vas, is stimulated by the nuage factor Tejas (Tej) through its Lotus domain in the presence of single-stranded RNA (Xiol et al., 2014; Jeske et al., 2017). Notably, Tej, like Squ, also binds Spn-E (Lin et al., 2023), suggesting that multiple nuage factors may cooperate to regulate Spn-E activity. In this study, I detected the intrinsic ATPase activity of Spn-E *in vitro*; however, Squ did not significantly enhance Spn-E ATPase activity (Fig. 2.10.2 B). I speculate that Tej and/or other yet-identified Spn-E interacting proteins may serve as primary activators of Spn-E *in vivo*, and these may associate with the Aub*–Spn-E–Squ complex (Fig. 2.10.2 A). Further experiments are still necessary to elucidate the enzymatic activity of Spn-E and its functional relevance *in vivo*.

Taking together, our results refine the current model of piRNA biogenesis in *Drosophila* germline cells. Squ functions on stabilizing Ago3 protein for heterotypic ping-pong between Aub and Ago3. Squ also acts as a crucial co-factor of Spn-E in the nuage, promoting piRNA precursor turnover or facilitating transfer of sliced transcripts between ping-pong partners (Fig. 3; Model). Further studies are needed to elucidate the molecular mechanism for Squ to perform these functions. Understanding these mechanisms will provide deeper insights into the piRNA production mechanism cooperated by many regulatory proteins.

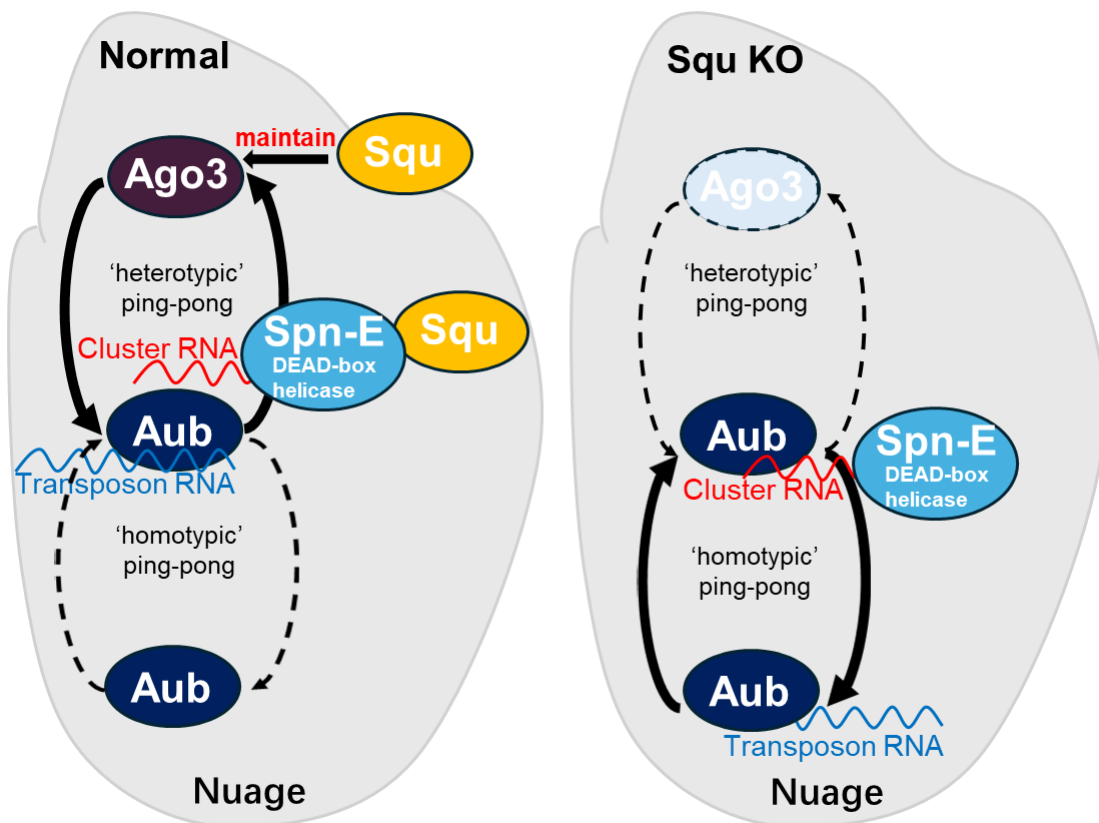


Fig.3 Model for Squ functions in piRNA biogenesis in nuage.

(Left panel) Squ contributes through two mechanisms of Squ: (1) Squ directly binds Spn-E, ensuring efficient heterotypic ping-pong between Aub and Ago3 and promoting processing of interacting transcripts including piRNA precursors. (2) Squ stabilizes Ago3 by unknown mechanisms, supporting proper piRNA production.

(Right panel) Upon loss of *squ* function, Spn-E remains bound to the Aub* complex at nuage together with the cleaved transcripts which are normally transferred to Ago3. Reduced Ago3 protein is not efficient for proper heterotypic ping-pong, leading a part of Aub to substitute for Ago3 and engage Aub-Aub homotypic ping-pong, which is not effective for piRNA production and TE repression.

4. Materials and methods

4.1 Fly stocks

All fly stocks were maintained at 25°C on standard food (5% (w/v) dry yeast, 5% (w/v) corn flower, 2% (w/v) rice bran, 10% (w/v) glucose, 0.7% (w/v) agar, 0.2% (v/v) propionic acid, and 0.05% (w/v) p-hydroxy butyl benzoic acid). The mutant alleles used in this study were *squ*^{PP32/HE47} (Pane et al., 2007), *aub*^{QC42/HN2} (BL #4968; BL #8517; Schüpbach and Wieschaus, 1991), *ago3*^{g1/g2} (VDRC #313531; VDRC #313532; Senti et al., 2015), *spn-E*⁷⁻⁴ (this study), *tej*⁴⁸⁻⁵/Df(2R) Exel7131 (Patil and Kai, 2010; BL #7876), *vas*⁴⁻² (this study) and *krimp*^{r06583}/Df(2R) Exel6063 (BL #18990, Lim and Kai, 2007; BL #7545). The heterozygotes of individual mutants were used as the control conditions. Knock-In fly line *aub*^{EGFP.KI} (DGRC #118621, Kina et al., 2019) and transgenic fly line UASp-3xFlag.AGO3.WT (DGRC #118699) were obtained from the *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology, Japan. *tej*^{EGFP.KI} was generated in our laboratory (Lin et al., 2023). NGT40-Gal4; *nos*-Gal4-VP16 (Grieder et al., 2000) was used as germline driver line. The genotypes of all fly strains are listed in Table 1.

4.2 Generation of knock-out flies

Loss of function alleles, *spn-E*⁷⁻⁴ and *vas*⁴⁻², were generated using the CRISPR/Cas9 system. Guide RNAs (gRNAs) targeting the *spn-E* and *vas* loci were designed and cloned into pDCC6 vector, respectively (Gokcezade et al., 2014). The sequences of the gRNAs were: Spn-E gRNA: 5' -GTGCCCAACCGCGAGTTGAT-3' and Vasa gRNA: 5' -GGGGATACAATCACTACCTG-3'. Obtained gRNA expression plasmids (120 ng/μl) were injected into *y w* embryos. PCR-amplified genomic fragments from candidate lines were subjected to Sanger sequencing, which revealed that *spn-E*⁷⁻⁴ contains an 8-bp deletion, GCGAGTTG, at 15837616-15837623 (release=r6.53), together with a 4-bp insertion, AACA, causing a frameshift and

premature stop codon. Similarly, *vas*⁴⁻² carries a 4-bp deletion, GTAG, at 15072945-15072948 (release=r6.53), causing a frameshift leading to premature termination. Loss of the corresponding protein products was confirmed by western blotting and immunostaining using anti-Spn-E and anti-Vas antibodies, respectively.

4.3 Generation of transgenic flies

The transgenic fly lines carrying native *squ* promoter-GFP-3xFlag-Squ^{WT} (pNative-Squ^{WT}) or -Squ^{4A} (pNative-Squ^{4A}) were generated using PhiC31-mediated integration system. The DNA fragments encoding Squ^{WT} or Squ^{4A} were amplified from pENTR-Squ^{WT} and Squ^{4A} plasmids, respectively (Kawaguchi et al., 2025). The fragments of promoter region, 5'UTR and 3'UTR were amplified by PCR from genomic DNA. GFP-3xFlag fragment was amplified from the plasmid containing GFP-3xFlag-tag sequence. All the fragments are combined and cloned into pUAS-attB vector (Brand and Perrimon, 1993) by the reaction of In-Fusion® Snap Assembly Master Mix (Takara). The generated constructs were introduced into attP2 site (BDSC#25710).

4.4 Antibodies

Polyclonal rabbit anti-mKate2 antibody was generated in this study. His-tagged full-length mKate2 was expressed in *Escherichia coli* BL21(DE3) from the pDEST17 (Thermo Fisher Scientific) plasmid carrying the *mkate2* coding sequence. His-tagged *mkate2* protein was solubilized with 6 M urea in PBS and purified using Nickel Sepharose beads (GE Healthcare) according to the manufacturer's protocol. The antibodies used in this study were rat anti-Squ (1:1000, Kawaguchi et al., 2025), guinea pig anti-Aub (1:1000, Lim et al., 2022), mouse monoclonal anti-Ago3 (1:500, National Institute of Genetics, Japan), guinea pig anti-Vas (1:2000, Patil and Kai, 2010), rat anti-Spn-E (1:500, Patil and Kai, 2010), rat anti-Tej (1:500, Lin et al., 2023), guinea pig anti-Krimp (1:1000, Lim and Kai, 2007), guinea pig anti-Ago2 (1:100, Lin et al., 2023),

rabbit anti-mKate2 (1:1000, this study), rabbit anti-HeT-A Gag polyclonal antibody (1:1000, Lin et al., 2023), rabbit anti-Stellate (1:1000, gift from Dr. William Theurkauf), mouse monoclonal anti-Tubulin (1:1000, DM1A, sc-32293, Santa Cruz Biotechnology). The secondary antibodies used in this study were HRP-conjugated goat anti-guinea pig (1:1000, Dako, Cat. #P0141), HRP-conjugated goat anti-rat (1:1000, Dako, Cat. #P0450), HRP-conjugated goat anti-mouse (1:3000, BioRad, Cat. # 1706516) and HRP-conjugated goat anti-rabbit (1:3000, BioRad, Cat. #1706515). ANTI-FLAG[®] M2-Peroxidase (1:1000, Sigma, Cat. #A8592) and HRP-conjugated anti-Myc-tag antibody (1:1000, MBL, Cat. # M192-7) were also used.

4.5 Western blotting

Ovaries were homogenized in the ice-cold PBS and denatured in the presence of 1×SDS sample buffer at 95°C for 5 min. The protein samples were subjected to SDS-PAGE and transferred to ClearTrans SP PVDF membrane (Wako). Primary and secondary antibodies described above were diluted in the Signal Enhancer reagent HIKARI (Nacalai Tesque). Chemiluminescence was induced by the Chemi-Lumi One reagent kit (Nacalai Tesque) and detected by ChemiDoc Touch (Bio-Rad). The immunoblotting signals were quantified using ImageJ (Schneider et al., 2012) or Image Lab software (Bio-Rad).

4.6 RNA immunoprecipitation

To analyze RNAs interacting with proteins, we followed the methods described previously (Lin et al., 2023). To extract Aub- and Ago3-bound piRNAs, and Spn-E-bound RNAs, 300 ovaries (Aub), 500 ovaries (Ago3) and 400 ovaries (mKate2-Spn-E) were dissected from 1-day-adult females in cold PBS buffer. Ovaries were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2 mM DTT, 10% (v/v) glycerol, 2 mM MgCl₂, 1% (v/v) Triton X-100, 1× cOmplete protease inhibitor cocktail (Roche), and 1% (v/v) RNaseOUT recombinant ribonuclease inhibitor

(Invitrogen). The lysates were cleared by centrifugation at $20,000 \times g$ for 10 min at 4°C three times to remove cell debris. Dynabeads Protein G/A (Invitrogen) pre-equilibrated with lysis buffer were added to the cleared lysates and rotated at 4°C for 1 h for pre-clearing. After clearance, guinea pig anti-Aub (1:10, Lim et al., 2022) or mouse monoclonal anti-Ago3 (1:500, NIG) or rabbit anti-mKate2 (1:500, this study) was added to the lysates and incubated for 3 h with gentle rotation at 4°C . Dynabeads Protein G/A were then added to the mixture and incubated at 4°C for 1 h with rotation. After incubation, magnet beads were collected and washed 5 times by wash buffer containing 20 mM Tris-HCl (pH7.4), 400 mM NaCl, 2 mM DTT, 10% (v/v) glycerol, 2 mM MgCl_2 , 1% (v/v) Triton X-100, $1 \times$ cOmplete protease inhibitor cocktail (Roche), and 1% (v/v) RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). After washing, beads were resuspended in 100 μl wash buffer. Ten μl was used for western blotting to check the efficiency of protein immunoprecipitation and the remaining 90 μl solution was used for RNA extraction using TRIzol LS (Invitrogen) following standard manufacturer's protocol. Extracted RNA were stored at -80°C until analysis. For mKate2-Spn-E RNA IP, negative control was performed by adding equal amount of Dynabeads Protein G/A with IP groups but without antibody.

4.7 Small RNA sequencing and analysis

To compare Aub- and Ago3-bound piRNAs in two conditions, the amount of Aub or Ago3 proteins in IP samples were equalized using the signals in the immunoblotting analysis. Libraries from Aub- and Ago3-bound RNAs were prepared and sequenced on an Illumina HiSeq 2500 platform (Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University). After adaptor trimming (5'-AGATCGGAAGAGCACACGTCT-3'), reads mapping to miRNA, rRNA, snRNA, snoRNA and tRNA sequences were removed. Remaining reads were size-selected with 23-29 nt and mapped to major piRNA clusters or the consensus sequences of transposable elements using Bowtie allowing up to 3 mismatches (Langmead et al.,

2009). piRNA cluster definition was according to previous paper (Brennecke et al., 2007) and TE sequences were from Flybase (Release 6.32). Read counts were normalized with the counts for 21nt-spike RNA (ACATGGCATGGATGAACTATA) which were added equally to individual RNA samples prepared for library construction (Lutzmayer et al., 2017). piRNA cluster-mapping reads were visualized by pyGenomeTracks (Ramírez et al., 2018). Nucleotide preference was visualized by sequence logo using Ggplot2 R ggseqlogo (Wagih, 2017).

4.8 Crosslinking immunoprecipitation (CL-IP)

200 ovaries were dissected from 1-day-adult *y w* females in ice-cold PBS and fixed in PBS containing 0.1% (w/v) paraformaldehyde for 20 min on ice. Crosslinking was quenched in 125 mM glycine for 20 min at room temperature. Ovaries were homogenized in CL-IP lysis buffer containing 50 mM Tris-HCl (pH 8.5), 150 mM KCl, 5 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5 mM DTT, and 1× cOmplete protease inhibitor cocktail. The lysate was incubated at 4°C for 20 min with rotation and then sonicated with Bioruptor (Lin et al., 2023). After centrifugation at 20,000 ×g for 10 min at 4°C, the supernatant was collected and diluted with an equal volume of CL-IP wash buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 0.5 mM DTT, and 1× cOmplete protease inhibitor cocktail. Pre-washed Dynabeads Protein G/A mixture (1:1) were added for pre-clearance at 4°C for 1 h with rotation. Anti-Squ antibody was added to pre-cleared supernatant with 1:500 dilution and incubated at 4°C overnight. The pre-equilibrated Dynabeads Protein G/A mixture (1:1) were added for binding and incubated at 4°C for 3 h with rotation. After washing three times with CL-IP wash buffer, beads were collected and resuspended in 50 µl of CL-IP wash buffer containing 1× SDS sample buffer. The beads were then boiled at 95°C for 5 min and subjected to SDS-PAGE for western blotting analysis. Negative control was performed as the description above.

4.9 Immunostaining of ovaries

Ovaries were dissected, fixed with 5% (w/v) paraformaldehyde containing PBS for 20 min and permeabilized in PBX (PBS with 0.2% [v/v] Triton X-100). The primary antibodies used in this study were described above. Secondary antibodies were Alexa Fluor 488-, 555-, and 633-conjugated goat antibodies at a 1:200 dilution (Thermo Fisher Scientific, United States) and CF®633 goat antibodies at a 1:1000 dilution (Biotium, Fremont, CA, United States). Ovaries expressing endogenous fluorescent-tagged proteins were fixed with 0.1% (w/v) paraformaldehyde containing PBS for 20 min and washed with PBX for 15 min, twice. DAPI was diluted for 1:1000 to stain DNA. Ovaries were equilibrated in Fluoro-KEEPER Antifade Reagent (Nacalai Tesque) and stored at 4°C before mounting. Images were taken by ZEISS LSM 900 with Airy Scan 2 using 63x oil NA 1.4 objectives and processed by ZEISS ZEN 3.0 and ImageJ (Schneider et al., 2012).

4.10 RNA in situ hybridization chain reaction (HCR)

Probes (Molecular Instruments, *42AB*-LOT: PRJ124; *38C*-LOT: PRJ125) targeting piRNA cluster transcripts from specific regions of cluster *42AB* (Chr2R: 6322410-6323756) and *38C* (Chr2L: 20104896-20213637) were generated and used in this experiment. Commercial reagents were used for this reaction (Molecular Instruments, Inc.) according to the manufacturer's instructions and previously described protocols (Lin et al., 2023).

4.11 Quantification of in situ-HCR signal for piRNA precursors

Fluorescence intensities for piRNA clusters *38C* and *42AB* were quantified using ImageJ (Fiji) after background subtraction. WGA was used to stain the nuclear envelope, and a region extending $\pm 5\%$ of the nuclear diameter inside and outside the envelope was defined as the perinuclear region, as previously described (Lin et al.,

2023).

4.12 RT-qPCR

Flies were fattened up with moist yeast paste for 2 days and then dissected for RNA extraction using TRIzol LS (Invitrogen) following manufacturer's protocol with DNase I (Invitrogen) treatment. Reverse-transcription was performed by Super Script III system (Invitrogen) using primers of a mixture for d(T)20 and randomized hexadeoxyribonucleotide. KAPA SYBR Fast qPCR Master Mix (KAPA biosystems) was used for qPCR reactions and primer sequences used in this study were listed in Table 2. The relative expression levels were calculated by $\Delta\Delta C_t$ method using *rp49* as reference. All the experiments were performed by 3 biological replicates.

4.13 mRNA-seq

Total RNAs were extracted from *squ* heterozygous and mutant ovaries using TRIzol LS (Invitrogen) following the manufacturer's instruction. Poly(A)+ RNA was enriched using oligo(dT) beads with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, USA). The enriched RNA was fragmented, reverse-transcribed, adapter-ligated, and PCR-amplified to prepare cDNA libraries using the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, USA). Library preparation and sequencing were outsourced to Rhelixa (Japan), yielding approximately 35 million reads per sample. Low quality sequences and adapters were removed by fastp (Chen et al., 2018). Paired end reads were mapped to *Drosophila melanogaster* reference genome r6.32 and the canonical sequences of TEs by STAR version 2.7 with default setting. (Dobin et al., 2013). Reads mapped to exons of individual genes and the canonical TEs were counted using featureCounts (Liao et al., 2014). Differential expression analysis was performed by DESeq2 (Love et al., 2014) with significant up-regulation ($\text{Log}_2\text{FC} > 1$, $\text{p-adj} < 0.05$) and down-regulation ($\text{Log}_2\text{FC} < -1$, $\text{p-adj} < 0.05$). Two biological replicates were performed for the mRNA-seq

analysis.

4.14 Co-immunoprecipitation in S2 cells

The *Drosophila* S2 cell line (S2-DRSC) was cultured at 28°C in Schneider's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin and streptomycin). Protein-coding sequences of *squ* and *spn-E* were amplified and cloned into the pENTR vector (Thermo Fisher Scientific) and subsequently transferred into pAFW or pAMW destination vectors for plasmid construction. S2 cells ($0.2\text{--}2 \times 10^6$ cells/ml) were pre-cultured in 12-well plates overnight before transfection with Hilymax (Dojindo Molecular Technologies, Japan). After 36–48 h of transfection, S2 cells were collected for co-immunoprecipitation (co-IP) experiments as previously described (Kawaguchi et al., 2025).

4.15 *Drosophila* fertility test

Female fertility was assessed by pairing three *y w* males with five females of each genotype in an enclosed environment on apple juice plates supplemented with a small amount of moist yeast paste. The number of eggs laid was recorded after 24 h of mating, and the hatching rate was calculated 24 h after egg laying. Egg counts and hatching rates were recorded for at least 3 consecutive days.

4.16 Purification of recombinant proteins in Sf9 cells

Spodoptera frugiperda Sf9 cells were cultured using ExpiSf™ CD Medium (Thermo Fisher Scientific/Gibco) in 27°C on an orbital shaker platform at 120 rpm. The fragment of DNA product for 6His-2StrepII-GFP-Spn-E^{WT}, -Spn-E^{DQVH} or -Squ^{WT} was amplified and inserted into pFastBac1 vector (Thermo Fisher Scientific). Recombinant baculoviruses expressing 6His-2StrepII-GFP-Spn-E^{WT}, -Spn-E^{DQVH}, or -Squ^{WT} were generated using the NucleoBond® Xtra BAC kit (Clontech/Takara) and used to infect Sf9 cells according to the manufacturer's protocol (Thermo Fisher Scientific). After

several days, infected Sf9 cells were resuspended in 10 ml lysis buffer: 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% TritonX-100, 1 mM DTT, 1x cOmplete protease inhibitor cocktail (PIC, Roche) and 1 mM PMSF, sonicated for total 10 min with 1 min on/1 min off on the ice and centrifuged for 1 h at 15,000 ×g to collect the supernatant. 1 ml Strep-Tactin® Sepharose® resin beads (IBA Lifesciences) were added to the Poly-Prep Chromatography Column (Bio-rad) and followed with 10 ml wash buffer: 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM DTT and 1 mM PMSF. All supernatant was then loaded into columns and washed with 10 ml wash buffer, protein was eluted by 10 ml elution buffer: 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 5 mM desthiobiotin and 1x PIC. Recombinant proteins were concentrated by vivaspin turbo 15 (Nippon Genetics Co., Ltd) and stored in -80°C after rapid freezing by liquid nitrogen. SDS-PAGE and CBB staining were performed to check the purification and quantity for protein.

4.17 ATPase assay

ATP hydrolysis reactions were performed at 25°C for 1, 2, 3, or 5 h with 25 μM recombinant protein (6His-2StrepII-GFP-Spn-E^{WT}, Spn-E^{DQVH}, or Squ^{WT}) in ATPase assay buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 200 μM ATP (Sigma)). To examine the effect of Squ on Spn-E ATPase activity, Squ protein was added at 25 μM to the Spn-E ATPase reaction mixtures. Inorganic phosphate was quantified using BIOMOL® Green Reagent (Enzo Life Sciences) in 96-well plates according to the manufacturer's protocol. Absorbance at 620 nm was measured with an SH 9000 microplate reader (Corona Electric), background values were subtracted, and phosphate concentrations were normalized to calculate ATP hydrolysis (%) using a phosphate standard curve.

4.18 Tables

Table 1. *Drosophila* genotypes used in this study.

Genotypes	Source
<i>w¹-l</i> ; <i>squ</i> ^{PP32} /CyO; <i>TM3, Sb/ TM6, Tb</i>	Gift from (Pane et al., 2007)
<i>w¹-l</i> ; <i>squ</i> ^{HE47} /CyO; <i>TM3, Sb/ TM6, Tb</i>	Gift from (Pane et al., 2007)
<i>w¹-l</i> ; <i>Pin</i> /CyO; <i>spn-E⁷⁻⁴</i> /TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>tej</i> ⁴⁸⁻⁵ /CyO	Patil and Kai, 2010
<i>w¹-l</i> ; <i>Df(2R)Exel7131</i> /CyO (Df stock for <i>tej</i> mutant)	BL#7876
<i>w¹-l</i> ; <i>vas</i> ⁴⁻² /CyO	This study
<i>w¹-l</i> ; <i>PBac{WH}krimp</i> ^{f06583} /CyO	BL #18990 (Lim and Kai, 2007)
<i>w¹-l</i> ; <i>Df(2R)Exel6063</i> /CyO (Df stock for <i>krimp</i> mutant)	BL#7545
<i>w¹-l</i> ; <i>ago3</i> ⁹¹ /TM3, <i>Sb</i>	VDRC#313531
<i>w¹-l</i> ; <i>ago3</i> ⁹² /TM3, <i>Sb</i>	VDRC#313532
<i>aub</i> ^{HN2} <i>cn</i> ¹ <i>bw</i> ¹ /CyO	BL #8517
<i>w</i> ¹¹¹⁸ ; <i>aub</i> ^{QC42} <i>cn</i> ¹ <i>bw</i> ¹ /CyO, <i>P{sevRas1.V12}FK1</i>	BL #4968
<i>w¹-l</i> ; <i>squ</i> ^{HE47} /CyO; native-promoter-GFP-3xFlag-Squ ^{WT} /TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>squ</i> ^{HE47} /CyO; native-promoter-GFP-3xFlag-Squ ^{4A} /TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>Pin</i> /CyO; <i>spn-E⁷⁻⁴</i> , native-promoter-GFP-3xFlag-Squ ^{WT} /TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>Pin</i> /CyO; <i>spn-E⁷⁻⁴</i> , native-promoter-GFP-3xFlag-Squ ^{4A} /TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>squ</i> ^{PP32} , <i>NGT40</i> -Gal4/ CyO; <i>nos</i> -Gal4 VP16	This study
<i>w¹-l</i> ; <i>squ</i> ^{HE47} /CyO; UASp-3xFlag.AGO3.WT / TM3, <i>Sb</i>	This study
<i>w¹-l</i> ; <i>squ</i> ^{PP32} /CyO; <i>Spn-E-mk2</i>	This study

Table 2. List of primers used for qRT-PCR in this study.

Primer label	Primer sequence	Source
Rp49_Fw	ATGACCATCCGCCCAGCATAC	(Lin et al., 2023)
Rp49_Rv	CTGCATGAGCAGGACCTCCAG	(Lin et al., 2023)
Tubulin_Fw	GGTAACCGTCGAAATCAGTGTT	(Lin et al., 2023)
Tubulin_Rv	TGGCTTTTCTGCTATACGTGTC	(Lin et al., 2023)
38C_Fw	TCCGTGACGGTTTAGCCCA	(Lin et al., 2023)
38C_Rv	AGGTTTCAAACCTTCCAG	(Lin et al., 2023)
42AB #1_Fw	CGTCCCAGCCTACCTAGTCA	(ElMaghraby et al., 2019)
42AB #1_Rv	ACTTCCCGGTGAAGACTCCT	(ElMaghraby et al., 2019)
42AB #2_Fw	CGCTGTTGAAAGCAAATTGA	(ElMaghraby et al., 2019)
42AB #2_Rv	GAGACCTTCGCTCCAGTGTC	(ElMaghraby et al., 2019)
blood_Fw	CCAACAAAGAGGCAAGACCG	(Zhang et al., 2021)
blood_Rv	TCGAGCTGCTTACGCATACTGTC	(Zhang et al., 2021)
HMS-Beagle_Fw	CTGTTGACCCTTATTCGCCG	This study
HMS-Beagle_Rv	CCGAGAATCCGAGCACTTTG	This study
Burdock_Fw	AGGGAAATATTTGGCCATCC	(Czech et al., 2013)
Burdock_Rv	TTTTGGCCCTGTAAACCTTG	(Czech et al., 2013)

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Publication and Contribution

Publication

1. Kawaguchi S, **Xu X**, Soga T, Yamaguchi K, Kawasaki R, Shimouchi R, Date S, Kai T. 2025. In silico screening by AlphaFold2 program revealed the potential binding partners of nuage-localizing proteins and piRNA-related proteins. *Elife*. 13:RP101967. doi: 10.7554/eLife.101967. PMID: 40259744. **(Co-first author)**.
2. **Xin Xu**, Taichiro Iki, Shinichi Kawaguchi, Toshie Kai. Squash ensures Spindle-E–dependent heterotypic ping-pong amplification of piRNAs in the *Drosophila* ovary. **(Under submission, first author)**.

Conference Presentation

Xin Xu, Taichiro Iki, Shinichi Kawaguchi, Toshie Kai. Squash functions as the co-factor of Spindle-E and ensures Argonaute 3 stability for germline piRNA biogenesis in *Drosophila* (Poster presentation). The 47th Annual Meeting of the Molecular Biology Society of Japan, Fukuoka. November 26th – 29th, 2024.

Xin Xu, Taichiro Iki, Shinichi Kawaguchi, Toshie Kai. Squash functions as the co-factor of Spindle-E and ensures Argonaute 3 stability for germline piRNA biogenesis in *Drosophila* (Poster presentation). The 48th Annual Meeting of the Molecular Biology Society of Japan, Yokohama, December 3rd – 4th, 2025.