Roles of piRNA Pathway Components Piwi and Aubergine In *Drosophila melanogaster* Female Germline Development

By

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Roles of piRNA Pathway Components Piwi and Aubergine In *Drosophila melanogaster* Female Germline Development

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Abstract

Recent studies have identified a new class of small RNAs with 24-30 nucleotides in length. This class of small RNAs is associated with the PIWI clade Argonaute proteins, thus named PIWI-interacting RNAs (piRNAs). piRNAs mediate silencing of transposable elements (TEs) by transcriptional silencing and transcript destruction. In this study, we investigated the roles of two piRNA pathway components Piwi and Aubergine (Aub) in *Drosophila melanogaster* female germline development. Our results demonstrate that Piwi functions in the differentiation niche, the escort cells (ECs), of the *Drosophila* ovary to repress the germline stem cell (GSC) self-renewal molecule *dpp* transcripts, thus ensuring proper germline differentiation. It has been proposed that Piwi functions in the somatic niche to control GSC self-renewal. Our study demonstrates the intrinsic role of Piwi in the germline to maintain primordial germ cells (PGCs) before adulthood and GSCs during adulthood. We have also revealed a new intrinsic role of Piwi in promoting germline differentiation. Our study demonstrates the requirement of Piwi in multiple cell types of the *Drosophila* ovary for both GSC maintenance and germ cell differentiation. Different from the expression of Piwi in all cell types in the ovary, Aub is specifically expressed in the germline. Our genetic results demonstrate that Aub is only required in the germline for GSC maintenance and germ cell differentiation, similar to the function of Piwi in the germline. A mutation of *lok*, the homolog of *chk2* in *Drosophila*, is sufficient to rescue the *aub* mutant GSCs, demonstrating that the *aub* mutant GSC loss is caused by DNA damage checkpoint activation. Removing one copy of *aub* significantly enhances the germ cell differentiation defect of *bam* heterozygous deletion. Aub is also capable of forming a protein complex with Bam in *Drosophila* S2 cells and co-localizes with Bam in the nuage, a perinuclear structure important for piRNA processing and TE silencing. These data together suggest that

Aub and Bam function cooperatively to promote germ cell differentiation. TE transcripts are upregulated in *bam* mutants as they are in *aub* mutants, further supporting the notion that Bam may interact with the piRNA pathway to repress TEs.

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List of Abbreviations

piRNA: PIWI-interacting RNA

TE: transposable element

TF: terminal filament

GSC: germline stem cell

CB: cystoblast

SGC: single germ cell

EC: escort cell

FSC: follicle stem cell

BMP: Bone morphogenetic protein

Dpp: Decapentaplegic

Gbb: Glass bottom boat

Aub: Aubergine

Armi: Armitage

Vret: Vreteno

Mael: Maelstrom

Zuc: Zucchini

Squ: Squash

Cuff: Cutoff

Rhi: Rhino

PGC: primordial germ cell

SGP: somatic gonadal precursor

Co-IP: co-immunoprecipitation

ACI: after clone induction

KD: knockdown

Nos: Nanos

nt: nucleotide

HSC: hematopoietic stem cell

miRNA: microRNA

HP1: Heterochromatin protein 1

List of Publications

Chen S, Kaneko S, **Ma X**, Chen X, Ip YT, Xu L, Xie T.
Lissencephaly-1 controls germline stem cell self-renewal through modulating bone morphogenetic protein signaling and niche adhesion.
Proc Natl Acad Sci U S A. **2010** Nov 16;107(46):19939-44. Epub 2010 Nov 1.

Ma X, Xie T. Stem cells: keeping BMP signaling local. Curr Biol. **2011** Oct 11;21(19):R809-11.

Ma X, Wang S, Do T, Song X, Inaba M, Nishimoto Y, Liu LP, Gao Y, Mao Y, Li H, McDowell W, Park J, Malanowski K, Peak A, Perera A, Li H, Gaudenz K, Haug J, Yamashita Y, Lin H, Ni JQ, Xie T. Piwi is required in multiple cell types to control germline stem cell lineage development in the *Drosophila* ovary. PLoS One. **2014** Mar 21;9(3):e90267. doi: 10.1371/journal.pone.0090267. eCollection 2014.

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Ma X, Do T, Song X, Xie T.
DNA Damage-Induced Checkpoint Activation Compromises Germline Stem Cell Self-Renewal and Differentiation in the *Drosophila* Ovary.

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Chapter 1: Introduction to Dissertation

1.1 *Drosophila* Female Germline Development: from Germ Plasm to GSCs

Germ cells are specialized cells that give rise to the gametes of sexually reproducing organisms and contribute genetically to subsequent generations. As the sole progenitors of eggs and sperms, germ cells have to undergo proper specification, migration and differentiation to ensure species survival and evolution. These cells are usually set aside during embryonic cleavage either by germ plasm segregation, a strategy employed by most animals, or by signaling induction from neighboring cells in mammals [1-3]. In *Drosophila,* germ plasm is germ cell specific cytoplasm that contains proteins and mRNAs for germ cell determination genes including *oskar, vasa, nanos (nos), pumilio (pum), tudor, aubergine* (*aub)* and *piwi*. The maternally synthesized germ plasm components are deposited into the posterior pole of the oocyte through directional transportation on polarized microtubule meshworks and stably anchored to the actin cytoskeleton. This actin-based anchoring mechanism maintains germ plasm localization and determines the localization of germ cells for the next generation [4, 5]. After fertilization and during early embryogenesis, the fly embryo undergoes 13 rounds of rapid nuclear division without cytokinesis before cellularization, during which each nucleus will be enclosed into an individual, membrane-bound cell. During nuclear cycle 8-10, the nuclei migrate to the cortex and continue to divide. The nuclei that have reached the posterior germ plasm initiate a process called pole cell budding, during which the plasm membrane incorporates the nuclei and the surrounding cytoplasm including the germ plasm to form 3-5 cytoplasmic buds. These buds will divide twice during nuclear cycle 9-13 before the buds pinch off to form 12-32 individual pole cells [6, 7]. These cells can divide 0-2 times to form ~40 pole cells while

somatic cells will be formed from the remaining cortical nuclei during cellularization at cycle 14 [6, 7]. Among the ~40 pole cells only less than half of them will survive the migration journey and eventually populate the germline, while the rest degenerate or become lost during migration [6, 8-12]. During gastrulation at embryonic stages 7-8, the pole cells initiate their active migration by penetrating an epithelium comprising endodermal cells into the future midgut where they remain immobile until stage 10. At this time, they pass across the posterior midgut primordium and move towards the gonadal mesoderm where they contact and adhere to the somatic gonadal precursor cells (SGP cells) to form two embryonic gonads, a compact structure containing ~10 pole cells lying in abdominal segment 5 [13]. The gonadal pole cells are generally called primordial germ cells (PGCs). Both PGCs and SGP cells divide but remain undifferentiated during larval development. At this stage, Dpp signaling, a homolog of BMP in *Drosophila* and also the most critical signaling for maintaining the undifferentiated status of PGCs and germline stem cells (GSCs), is active throughout the entire germ cell compartment as evidenced by pMad staining in all the PGCs [14]. Dpp signaling maintains the undifferentiated state of the PGCs until the GSC niche develops in the anterior of the gonad at the larval-pupal transition. During this transition, ovariole morphogenesis takes place, transforming the larval gonad into the functional adult ovary. Ovariole morphogenesis starts with the formation of the terminal filament (TF). It has been shown the Delta expression on newly formed TF cells activates Notch signaling in their neighboring SGP cells to induce cap cell formation during larval-pupal transition, which provides the major component and central signaling source of the GSC niche in the *Drosophila* ovary [15]. Once cap cells are established, they start to become the sole source of Dpp production and restrict Dpp diffusion to one cell diameter. The restricted Dpp signaling protects and converts 2-3 PGCs to GSCs, while the rest of the PGCs initiate

differentiation. The newly established niche and GSCs also further strengthen the cell fate commitment by expressing E-cadherin and β-catenin, to build the adherens junctions between cap cells and GSCs [16]. The adherens junctions serve at least three known purposes in this GSC system: anchoring GSCs to the central signaling source [16]; keeping Dpp molecules to only one cell diameter [17]; orienting GSC division so that one of the two daughter cells remains in contact with the niche while the other one leaves the niche and undergoes differentiation [18].

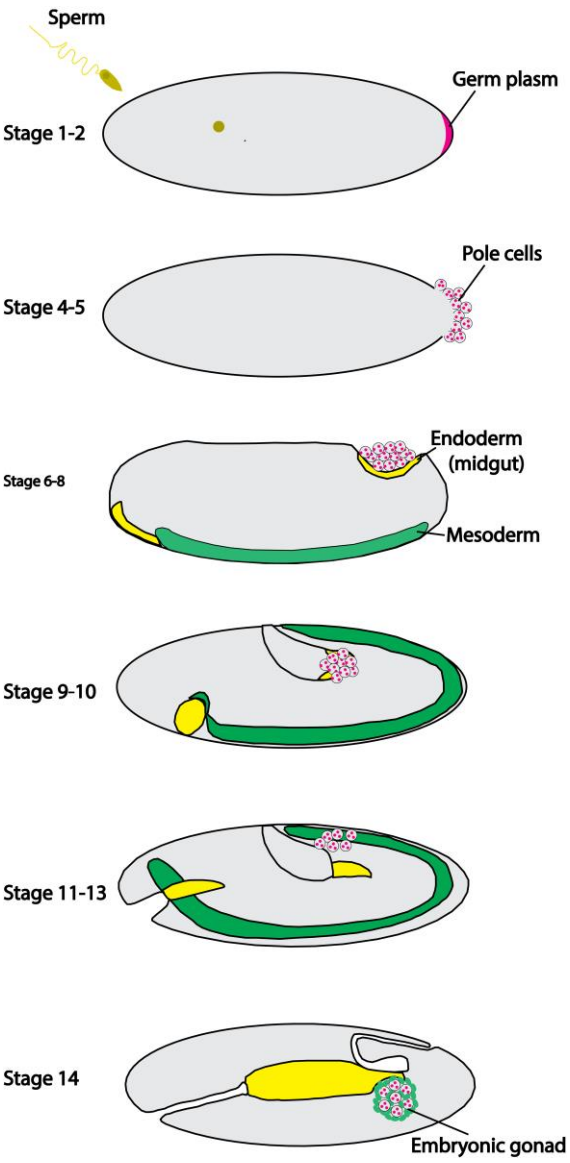

Figure 1.1. *Drosophila* pole cell formation and migration. Germ plasm (hot pink) is maternally deposited and anchored to the posterior of the egg. During pole cell budding, plasm membrane incorporate germ plasm and nuclei to form cytoplasmic buds which will further divide to form ~40 pole cells. Pole cells then penetrate an epithelium and enter the future midgut (yellow). Starting at stage 11, they pass across the posterior midgut primordium and move towards the gonadal mesoderm (green) where they coalesce with SGP cells to form two embryonic gonads. The gonadal pole cells are generally called PGCs. Both PGCs and SGP cells divide but remain undifferentiated during larval development. Adapted from Nakamura A and Seydoux G [19].

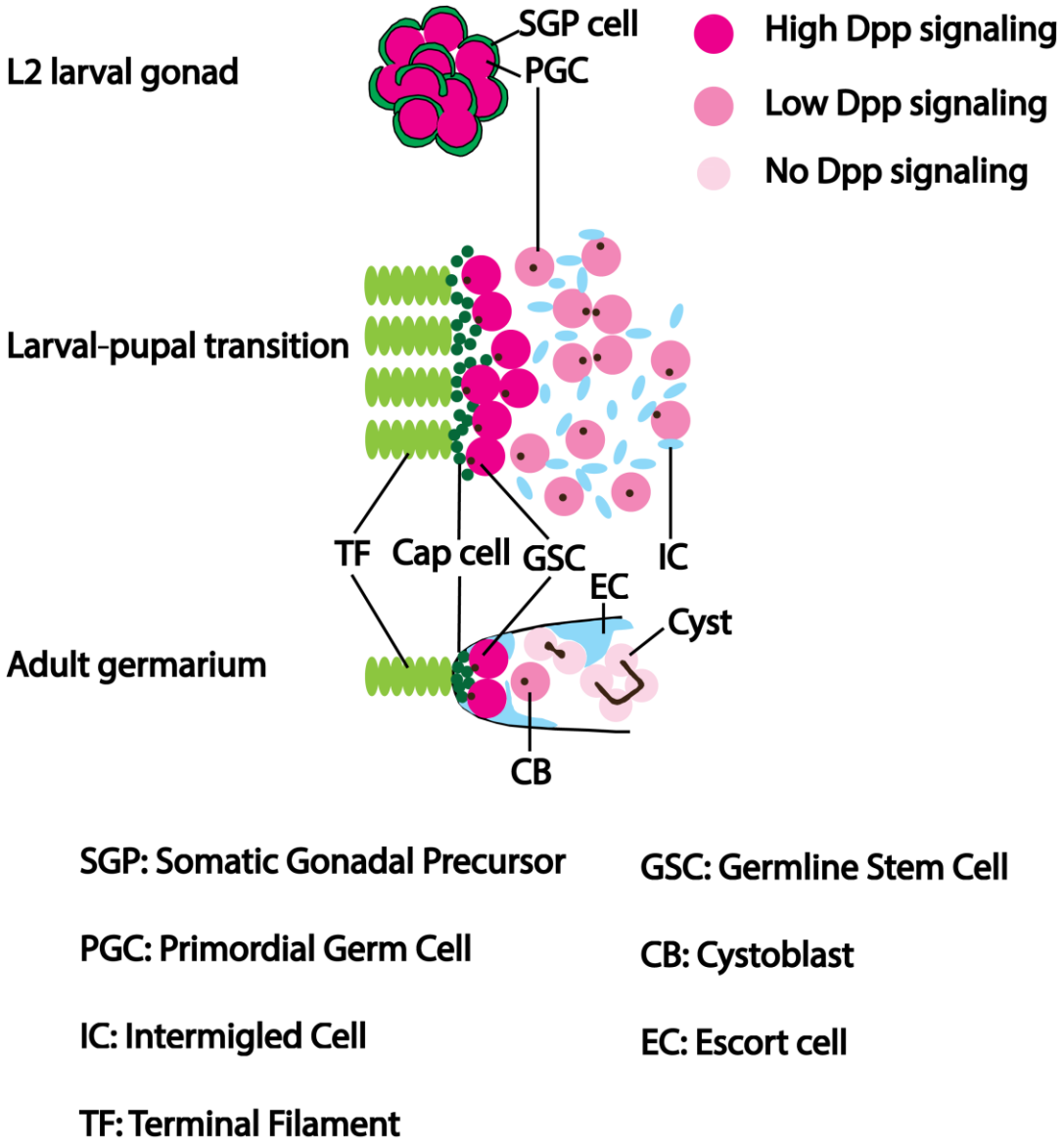

Figure 1.2. ***Drosophila*** **ovarian GSC-niche establishment.** High Dpp signalling activity maintains the undifferentiated state of PGCs and SGP cells during larval development. Cap cells are established during the larval-pupal transition by Notch signalling activation from surrounding TF cells. Cap cells then restrict Dpp signalling to one cell diameter and specify PGCs next to them as GSCs. Adult GSCs are anchored to the cap cells by adherens junctions and maintained by Dpp signalling from the cap cells.

1.2 Ovarian GSC Maintenance and Differentiation

The *Drosophila* ovarian GSC was identified in the 1970s by genetic and laser ablation analysis [20-23], and later it has become the most productive stem cell system due to the availability of reliable molecular and cellular markers. One female fly contains one pair of ovaries, each of which consists of 16-18 ovarioles. Each ovariole contains a germarium at the anterior tip followed by a string of progressively more differentiated egg chambers which will eventually develop into mature eggs over the course of approximately 5 days [20, 21, 24]. The anterior tip of the germarium resides a stack of 8-9 disc-shaped, postmitotic somatic cells called the TF cells. At the base of the TF is a group of 5-7 somatic cap cells, which constitute the major component of the GSC niche by forming adherens junctions with and providing self-renewal signaling to the GSCs. The germarium has been divided into 4 regions according to the germ cell developmental stages. Region 1 contains GSCs, cystoblasts (CBs) and mitotic 2-cell to 8-cell cysts; Region 2a and 2b harbor 16-cell cysts; Region 3 corresponds to a stage 1 egg chamber. GSCs are the most apically located germ cells and also form direct contact with cap cells. GSCs divide asymmetrically and in parallel to the germarial axis such that the daughter GSC remains contact with cap cells, whereas the differentiating daughter cell, the CB, is displaced one cell away from cap cells. The CB then undergoes 4 rounds of mitosis with incomplete cytokinesis to form a cyst of 16 germ cells interconnected by ring canals. Once the 16-cell cyst is formed, it enters the region 2a and starts directional deposit of oocyte determinant into 2 germ cells with 4 ring canals within the 16-cell cyst by polarized microtubule meshworks. The microtubule organizing center starts to form at the region 2a and becomes more readily visible at the region 2b. The differentiating 16-cell cyst continues to move towards the region 2b, where the oocyte is specified from one of the two germ cells with 4 ring canals and relocated to

the posterior of the 16-cell cyst and the remaining 15 germ cells will become highly polyploid nurse cells by default. In the region 2b, the 16-cell cyst becomes a lens-shaped structure that spans the whole width of the germarium. At the same time, follicle cells that are derived from follicle stem cells at the 2a/2b border start to migrate across the surface of the 16-cell cyst and enclose the cyst. When the 16-cell cyst continues to differentiate and reaches the region 3, it rounds up to become a stage 1 egg chamber enclosed by follicle cell with the oocyte lying at the posterior end, which defines the anterior-posterior axis of the egg chamber and of the future embryo. The stage 1 egg chamber eventually buds off but still is connected to the germarium by 5-7 stalk cells. Normally, it takes approximately 3.5 days for a CB to differentiate into a stage 1 egg chamber and 5 days from a stage 1 egg chamber to a mature egg [20, 24].

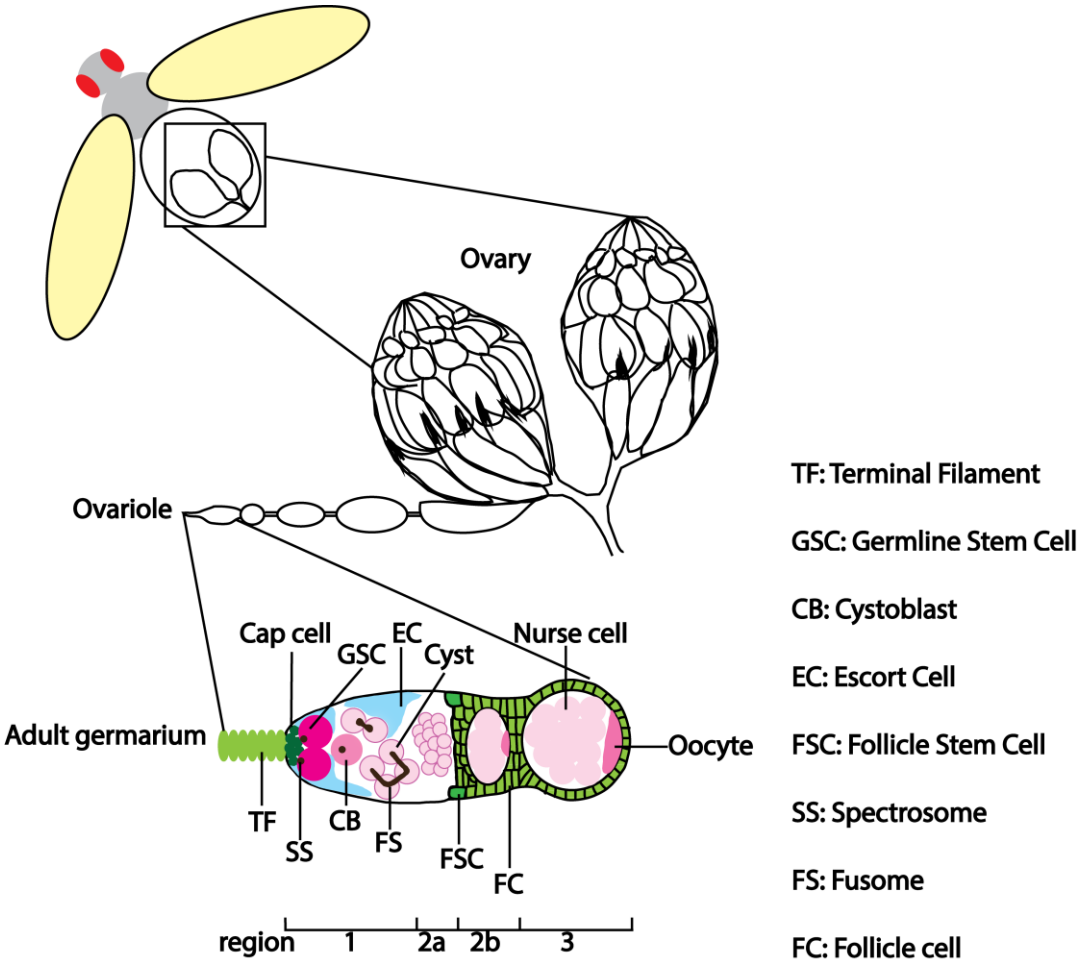

Figure 1.3. Schematic diagrams showing *Drosophila* oogenesis and germarial structure.

Extrinsic signals for GSC maintenance In the *Drosophila* ovary, GSC maintenance is achieved by the integration of extrinsic signals from the niche with intrinsic factors working within the stem cells. Cap cells are the major component of the GSC niche, whereas the influence of TF on GSCs is very limited in the adult ovary [25, 26]. A recent study with *c587-gal4* driven RNAi knockdown in the ECs shows that loss of ECs including GSC-contacting ECs leads to GSC loss, suggesting the involvement of GSC-contacting ECs in GSC maintenance [27]. Both daughter cells become GSCs if they are in direct contact with cap cells, demonstrating that the proximity to cap cells is essential for maintaining the GSC identity [25]. Cap cells produce both Dpp and

Gbb, two BMP homologs in *Drosophila*, to maintain GSC self-renewal by direct binding to the receptor complex on the GSC surface [25, 28, 29]. Cap cells also express E-cadherin and β-catenin to form adherens junctions with GSCs and keep the GSCs in direct contact with the signaling source. GSCs with E-cadherin mutations drift away from the niche and initiate differentiation [16]. Very interestingly, components of the somatic piRNA pathway Piwi and Yb have also been shown to function in the niche to maintain GSC self-renewal [30-33].

BMP signaling has been demonstrated to function in the niche to directly control GSC self-renewal by repressing differentiation [28, 29, 34]. In *Drosophila*, homodimers formed by two Dpp molecules or heterodimers formed by Dpp and Gbb can bind to type I and type II receptor complexes on the GSC surface to allow the constitutively active type II receptor kinase Punt to phosphorylate and activate the type I receptor Tkv or Sax. Phosphorylated Tkv or Sax then relays the signal by phosphorylating signaling transducer Mad. pMad then recruits Medea and translocates into the nucleus as a complex to regulate target gene transcription. *in situ* hybridization of *dpp* shows that *dpp* mRNAs are expressed in both the cap cells and ECs and overexpression of *dpp* in the somatic cells using hs-gal4/UAS system causes an accumulation of excess GSCs [25, 28], demonstrating that BMP signals from the niche to control the GSCs. Overexpression of a constitutively active Tkv (Tkv^{CA}) using hs-gal4/UAS system, however, does not produce germline tumor, whereas *nos-gal4* driven Tkv^{CA} overexpression in germ cells causes GSC hyperplasia [28, 35-37], demonstrating that BMP signaling directly activates the receptor complexes on the GSCs to control self-renewal. Bam has been shown to be the most potent differentiation factor in GSCs [38]. A silencer element has been found in the 5'UTR of *bam* transcript that is required for *bam* transcriptional silencing and this silencer element also binds to

Medea and Mad *in vitro* [34, 39]. These data together demonstrate that BMP molecules produced from the niche act directly on the GSCs to prevent differentiation.

The piRNA pathway components Piwi and Yb are expressed in cap cells to maintain GSCs. Both *piwi* and *Yb* mutant ovaries fail to maintain normal oogenesis due to GSC loss, and overexpression of Piwi or Yb in somatic cells using hs-gal4/UAS system increases the number of GSCs, demonstrating the role of Piwi and Yb in somatic cells for GSC maintenance [30-33]. Interestingly, Yb may regulate GSCs partially by modulating the function of Piwi in the somatic cells as Piwi expression and nuclear localization are disrupted in all the somatic cells including TF, cap cells, ECs and follicle cells in *Yb* mutants [33, 40]. In addition, *bam* transcription is upregulated in *piwi* mutant GSCs, which is similar to *dpp* and *gbb* mutants [29, 41]. In contrast to the molecular mechanism employed by BMP signaling, it remains unclear how Piwi represses *bam* transcription. Because the Mad-Medea binding site in the 5'UTR of *bam* is sufficient to silence *bam* transcription in the GSCs, the function of Piwi from the niche must integrate with BMP signaling [29, 34, 39]. An E3 ligase Smurf has been shown to suppress BMP signaling by targeting pMad for degradation [37, 42, 43]. Inactivation of Smurf also restores the GSCs in *piwi* mutants, indicating that Piwi silences *bam* transcription by repressing s*murf* in the GSCs [41]. Considering the expression of Piwi in both somatic cells and germ cells in the ovary, cell type specific requirement of Piwi in GSC maintenance needs to be determined. It will also be interesting to unravel the missing link between the function of Piwi in the cap cells and the repression of *smurf* in the GSCs. Alternatively, Piwi may modulate BMP signaling in the cap cells to silence *bam* transcription in the GSCs.

Intrinsic factors for GSC maintenance Extrinsic signals act in a coincident manner with intrinsic factors to maintain GSC self-renewal. Lis1, a WD40 repeat-containing protein, maintains the GSCs by regulating BMP signaling transduction and E-cadherin accumulation. Lis1 physically interacts with Mad to stabilize Mad protein and promote Mad phosphorylation [18]. The translation initiation factor eIF4A maintains GSC self-renewal and proliferation by directly inactivating Bam function and promoting E-cadherin expression [44]. Mutations of the chromatin remodeling factor ISWI also impair GSC self-renewal by increasing *bam* transcription in the GSCs [45]. Numerous studies have shown that GSCs can differentiate in a Bam-independent manner and GSCs assign large sums of intrinsic factors to repress the Bam-independent differentiation pathway. Pum and Nos, two translational repressors, were the first identified intrinsic factors to maintain GSC self-renewal by repressing the translation of differentiation factors as a Pum-Nos-mRNA complex [46-51]. Mutations of these two genes cause premature GSC loss via a Bam-independent pathway because *bam* transcription remains silenced in *pum* mutants and *pum bam* double mutant ovarioles contain germ cells differentiating as nurse cells [41]. Brat is another differentiation factor in the *Drosophila* ovary. Similar to Bam, overexpression of Brat causes GSC differentiation and *brat* mutant ovary accumulates more undifferentiated CBs [52]. Interestingly, Pum and Nos together down regulates Brat protein level in the GSCs by regulating the 3'UTR of *brat* [52], providing an alternative mechanism for GSC differentiation regulation. The homolog of the translation release factor 1α in *Drosophila*, Pelota, when mutated, also causes GSC loss very rapidly without upregulating *bam* transcription in the mutant GSCs [53]. Interestingly, microRNAs (miRNAs), which regulate gene expression by affecting mRNA stability or translation, are also required for GSC self-renewal by repressing a Bam-independent differentiation pathway as *dcr-1* mutant GSCs are

still lost from the niche even in *bam* mutant background [54]. Different from ISWI, some other epigenetic regulators including Stonewall, Scrawny and Eggless are required for GSC self-renewal but not for *bam* transcription repression [27, 55-58]. All together, intrinsic factors repress both Bam-dependent and Bam-independent differentiation pathways in the GSCs to prevent differentiation and thus maintain self-renwal. The requirement of various intrinsic factors with diverse biological functions in the GSCs shows the vital roles of both transcriptional and post-transcriptional regulations in maintaining GSC self-renewal. In the future, it will help further understand GSC self-renewal by identifying the mRNA targets of Pum and Nos in the germline. The discovery of functional miRNAs and their targeting mRNAs in GSC maintenance will also be insightful in stem cell biology. Lastly, unveiling the epigenetic regulation in the GSCs should help us understand how GSCs orchestrate transcriptional regulation with post-transcriptional regulation to maintain their self-renewal capacity.

Extrinsic signals for germ cell differentiation In parallel with GSC maintenance mechanisms, germline differentiation is also governed by extrinsic molecules and germline intrinsic differentiation factors. Type IV collagen encoded by *Vkg* in *Drosophila* localizes to the GSC membrane and binds to Dpp to prevent Dpp diffusion to outside the GSCs niche and ensure germ cell differentiation [59]. It has been shown that BMP activation is restricted to the adherens junctions at the GSC-niche interface in the *Drosophila* testis, raising the possibility that E-cadherin based adherens junctions might facilitate direct targeting of BMP to the receptor complex associated with the adherens junctions and prevent BMP diffusion to outside the niche [17]. *dpp* is also transcriptionally repressed in the ECs by histone lysine-specific demethylase 1

(Lsd1) to provide differentiation environment for germ cells [60]. EGFR-MAPK signaling activated by ligands produced in the germline functions in the ECs to ensure germ cell differentiation by repressing the transcription of *dally*, encoding a glypican family protein important for Dpp stabilization and diffusion [61, 62]. Two piRNA biogenesis regulators Eggless and Vreteno (Vret), and the Heterochromatin protein 1 (HP1), Su(var)205, also function in the ECs to promote germ cell differentiation via undefined mechanisms [27, 63, 64]. Type IV collagens and adherens junctions spatially restrict Dpp molecules within a short-range to maintain active BMP signaling in the GSCs and also provide a low Dpp differentiation environment for later germ cells. ECs actively repress the transcription of both *dpp* and *dally* to stop the production and diffusion of Dpp. In addition, piRNAs may also regulate germ cell differentiation in the ECs by direct or indirect modulation of BMP signaling. The enforcement of multiple layers of control over BMP signaling ensures proper germ cell differentiation.

Intrinsic factors for germ cell differentiation Localized BMP signaling activation imposed by type IV collagens and adherens junctions ensure low level BMP outside the niche and create CB differentiation environment [17, 59]. EGFR signaling and Lsd1 function in the ECs to inhibit *dally* and *dpp* transcription respectively [60, 62]. The piRNA pathway components Eggless and Vret act in the ECs to control germ cell differentiation via undefined mechanisms [27, 64]. In contrast to the limited knowledge about the extrinsic factors for germ cell differentiation, relatively more molecules have been identified to play roles intrinsically in the germline to regulate germ cell differentiation. Intrinsic factors induce germ cell differentiation by diminishing BMP response in the CBs or inhibiting intrinsic self-renewal factors. BMP

signaling receptors (Punt, Tkv and Sax) and transcription factors (Mad and Medea) are required in the GSCs to maintain self-renewal [28]. In the differentiating CB, however, multiple mechanisms are in place to destroy these BMP pathway components in order to initiate differentiation in full swing. The differentiation factor Bam may act redundantly with the Fused/Smurf complex to down regulate BMP signaling in the CB because Bam overexpression is sufficient but not necessary to diminish BMP response in the CB and removing *smurf* expands BMP gradient in a *bam* null mutant [37]. Mechanistically, Fused, a serine/threonine kinase and an essential component of the Hh signaling pathway, physically interacts with the E3 ligase Smurf to regulate the BMP type I receptor Tkv turnover by ubiquitination and proteolysis [65]. miR-184 might target the 3'UTR of *Sax* to translationally repress *Sax* to reduce BMP response in the CB [66]. The differentiation factor Brat interacts with Pum in the CB to repress Mad protein level via its 3'UTR [52]. Multiple intrinsic factors target BMP signaling at the receptor or signaling transduction level to diminish BMP response in the CBs to stop self-renewal and get the CBs ready for differentiation.

Bam, Bgcn and Sxl are required in the germ cell for differentiation by inhibiting the expression of the intrinsic self-renewal factor *nos*. Mutation of either *bam* or *bgcn* causes an accumulation of GSC-like cells while ectopic expression of Bam but not Bgcn causes premature differentiation of GSCs [38, 44, 67-70]. *bam* and *bgcn* genetically and physically interact with each other to drive germ cell differentiation by repressing the expression of the self-renewal factors including *nos* and *shg* (encoding E-cadherin) [44, 68, 70, 71]. Sxl, a RNA binding protein which controls gene expression via alternative splicing and/or translational repression, is required for germ cell differentiation by binding to the 3'UTR of *nos* and post-transcriptional downregulation of *nos* expression [72-74]. Mei-P26, a Trim-NHL containing protein, is also

essential for germ cell differentiation possibly by physical interaction with the core component of the RISC complex Ago1 and inhibiting the miRNA self-renewal pathway because *mei-p26* mutations cause both excess GSC-like cells and miRNA upregulation [75, 76]. Bgcn, Sxl and Mei-P26 are required for the differentiation function of Bam as ectopic Bam expression in any of these mutations fails to drive germ cell differentiation [71, 73, 76].

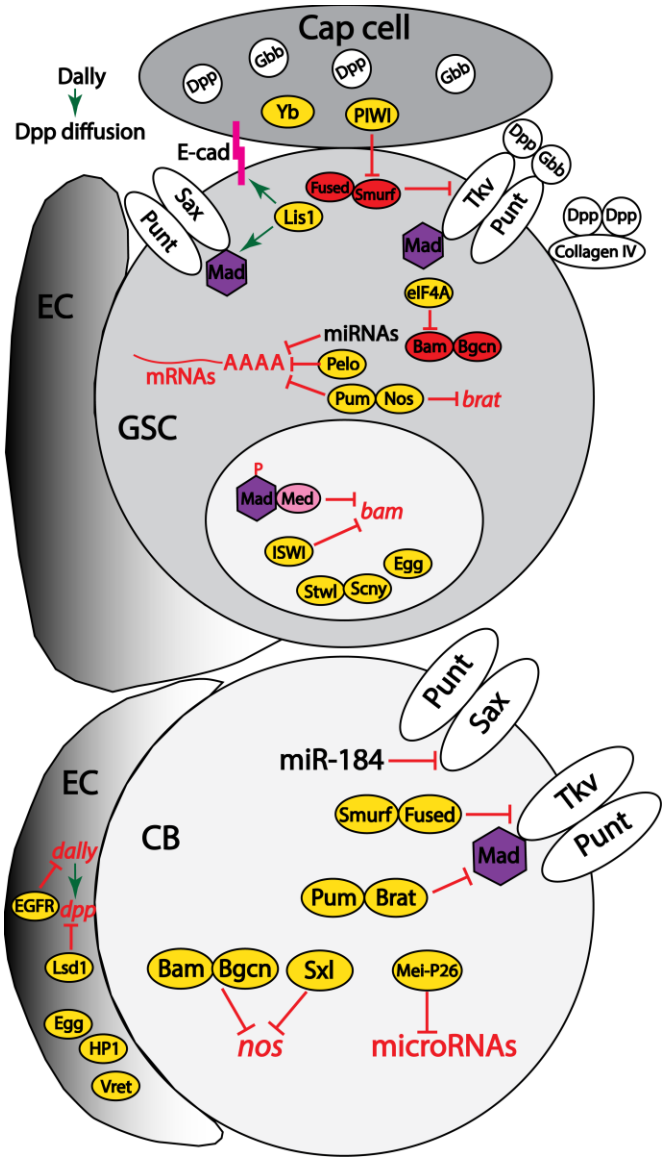

Figure 1.4. Extrinsic signals and intrinsic factors controlling *Drosophila* ovarian GSC maintenance and differentiation.

1.3 piRNA Biogenesis in *Drosophila*

PIWI-interacting RNAs (piRNAs) are a group of single-stranded non-coding RNAs with 24-30 nucleotides in length that associate with the PIWI clade Argonaute proteins. In general, Argonaute-RNA complexes use their RNA partners to recognize target RNAs by base pairing and destroy target RNAs by endonuclease-mediated cleavage by the Argonaute protein or inhibit mRNA translation by recruiting translation repressors [77-82]. Argonaute-RNA complexes can also recruit chromatin remodeling factors to modify transcription activity of their binding targets in nuclei [83-87]. The most abundant piRNAs are antisense to active TE trasncripts, and these antisense piRNAs preferentially associate with Piwi and Aub, two PIWI clade Argonautes. Sense-strand piRNAs, in contrast, prefer binding to Argonaute-3 (Ago3), the third PIWI clade Argonaute [88-90]. The major function of PIWI-piRNA complexes has been proposed to target selfish genomic elements which would otherwise move or multiply themselves within the genome and pose a threat to the genome integrity. The biogenesis of piRNAs is different from other non-coding small RNAs like miRNAs in several ways. First, miRNAs are derived from double-stranded precursors and depend on Dicer, which cleaves double-stranded RNAs, for processing. piRNAs, in contrast, are derived from long single-stranded RNA precursors of 20 to 90 kb and thus Dicer independent [91, 92]. Second, piRNAs can be grouped into two subclasses from the perspective of genomics. A subgroup of piRNAs, repeat-associated small interfering RNAs (rasiRNAs) are derived from repetitive regions of the genome such as transposons and heterochromatin and cannot map to specific chromosomal loci. The remaining piRNAs map to unique genomic loci called piRNA clusters in large pericentromeric or subtelomeric domains of up to 240 kb that are rich in TE fragments [88-90]. miRNAs, instead, are transcribed from their own genes or introns of their host genes. Third, piRNAs have no clear secondary structure while

primary miRNAs have hairpin loop secondary structures that can be recognized by the miRNA processing machinery. Instead, piRNA precursors are transported to the nuage to be processed into mature piRNAs.

Differentiating piRNA precursors from mRNAs One critical question to be answered is how piRNA precursors can be differentiated from mRNAs and delivered to the piRNA processing instead of the mRNA splicing machinery. Evidence exists that both cis- and trans-regulatory elements are utilized to distinguish piRNAs from mRNAs. In *C. elegans*, most piRNA species are encoded by individual genes and specified by a consensus motif ~40 bp upstream of the piRNA coding sequence. This motif is an octamer sequence that can be recognized by the forkhead family (FKH) transcription factors, which are required for the transcription of piRNAs in *C. elegans* [93-95]. In *Drosophila*, Rhino (Rhi), a fast evolving homologue of HP1, binds to and is required for the production of primary piRNAs from the germline-specific dual-strand clusters [96]. Rhino has been shown to colocalize with a DEAD box protein UAP56 and the Rhino-UAP56 foci on the nuclear side of nuclear pores are often flanked on the cytoplasmic side by the nuage marker Vasa, which is another DEAD box protein [97]. Importantly, Rhino functions with UAP56 and the Rai 1/DXO-related protein Cutoff (Cuff) to suppress piRNA precursor splicing. Rhino binding suppresses reporter gene splicing and is sufficient to drive piRNA biogenesis from reporters that express complementary transcripts from opposite orientations [98]. Thus, it has been proposed that cotranscriptional loading of Cuff through Rhino onto capped piRNA precursors competes with cap-binding complexes, which would otherwise promote pre-mRNA splicing. Cuff loading thus prevents piRNA precursors from

being spliced and differentiates piRNA precursor transcripts from pre-mRNAs. These Cuff-bound piRNA precursors will eventually be recognized by UAP56, transported through nuclear pores and delivered to the piRNA processing machinery [98].

Primary piRNA biogenesis in the soma Genetic analysis and deep sequencing suggest two distinct piRNA processing pathways in *Drosophila* [99]. The primary piRNA pathway is the only pathway that functions in both the soma and the germline to produce piRNAs. The primary pathway in the soma is Piwi-dependent and Aub/Ago3-independent, consistent with the expression of only one Argonaute protein Piwi in the soma. In the germline, however, primary piRNA processing is followed by the secondary piRNA pathway: also called the ping-pong cycle to amplify the piRNA pool as well as to destroy TE transcripts by Aub and Ago3 [100-102]. In the primary piRNA biogenesis pathway, long piRNA precursors are transcribed from piRNA clusters including *flamenco*. piRNAs generated from the *flamenco* cluster target transposons of the *gypsy* family of long terminal repeat transposons, including *gypsy*, *ZAM* and *idefix* [100]. Long piRNA precursors then are exported from the nucleus into the cytoplasm where they will be chopped into smaller fragments via undefined mechanisms. It has been speculated that Zucchini (Zuc), an outer mitochondrial membrane protein with single-strand-specific endonuclease activity *in vitro* [103], could cleave long piRNA precursors and produce smaller piRNA fragments based on the observations that Zuc is expressed in the cytoplasm of a somatic cell line derived from *Drosophila* ovary and is required for piRNA production in the soma [100, 101, 104-106]. The piRNA precursor fragments then enter the perinuclear Yb body, an ovarian soma-specific cytoplasmic structure rich in piRNA components Yb, Armitage (Armi) and Vret.

These components are critical for piRNA maturation and/or subsequent Piwi nuclear localization although the exact functions of Yb bodies are yet to be unraveled [40, 107-110]. Without Armi, Yb or Zuc, Piwi is loaded with fewer mature piRNAs and excluded from the nucleus. Thus, it has been proposed that mature and functional Piwi-piRNA complexes form and are then inspected in Yb bodies before its nuclear entry to exert its regulatory functions [108]. Antisense piRNAs from both soma and germline contain a characteristic 5' uridine (5'U) via unknown mechanisms. It is possible that PIWI proteins Piwi and Aub preferentially bind to RNAs with 5'U and differentiate piRNAs from mRNAs and also stabilize piRNA intermediates for further processing. An undefined protein trims the 3'end of piRNA intermediates and Hen1, a conserved methyltransferase, catalyzes 2'-O-methylation of mature piRNAs at their 3' ends, possibly to increase piRNA stability [111]. *hen1* mutants in *Drosophila*, however, are viable and fertile, suggesting that 3'end 2'-O-methylation is not essential for the functions of piRNAs [111].

Primary piRNA biogenesis in the germline Based on the limited experimental data on the primary piRNA pathway, redundant mechanisms might function in the germline to initiate piRNA production. According to the expression of Piwi in the germline and its requirement for primary piRNA biogenesis in the soma, Piwi has also been proposed to function in the germline primary pathway. However, *piwi* mutations reduce but do not completely eliminate piRNAs mapping to the germline specific clusters including 42AB [100], suggesting that Piwi might not be the major player in the germline primary pathway. In addition, long piRNA precursors could be simply cut by sequence-independent endonucleases into long RNA fragments, which will then be loaded onto Aub and trimmed to shorter and mature piRNAs, initiating the secondary

amplification cycle. Two candidate endonucleases Zuc and Squash (Squ) have been shown to express in the germline perinuclear nuage. Silimar to *piwi*, mutations in *zuc* or *squ* reduce but do not eliminate piRNA production [100, 112]. Therefore, a similar mechanism may be utilized by both somatic and germline primary piRNA pathways to generate fragmented piRNA precursors. The fact that piRNAs and PIWI proteins are maternally deposited into the germ plasm and incorporated into PGCs raises the possibility that these maternally deposited piRNAs may serve as the primary piRNAs for the next generation in the germline, which obviates the need for the primary piRNA pathway in the germline [88, 100, 113-115].

Germline-specific secondary piRNA biogenesis The three PIWI-family Argonaute proteins, Piwi, Aub and Ago3, are expressed in the *Drosophila* ovary with Aub and Ago3 only in the germline [31, 89, 114]. Thus, the secondary piRNA pathway involving the Piwi/Aub-antisense piRNA and Ago3-sense piRNA complexes is specific to the germline. Piwi and Aub prefer antisense piRNAs with a bias for a U at the 5'end while the Ago3-associated piRNAs have the bias towards sense strands, and show preference for adenine (A) at the 10^{th} nucleotide (nt) from the 5'end [88, 89, 116, 117]. In addition, Ago3-bound piRNAs have complementary partners in Piwi- or Aub-bound piRNAs with 10-nt overlap at their 5'ends [88, 89]. Therefore, a ping-pong model has been proposed for piRNA amplification in the germline in which antisense piRNAs derived from piRNA clusters guide the cleavage of sense transcripts from active TEs, generating sense piRNA fragments with an A at the 10^{th} position and 10-nt complementation to antisense piRNAs from the 5' terminus.

According to this model, Piwi and Aub loaded with antisense piRNAs generated from the primary pathway act as the initiator for the secondary pathway. Antisense piRNAs associated with Piwi or Aub bind to sense piRNA precursors or TE transcripts via sequence complementation to initiate the processing of sense piRNAs. For unknown reason, PIWI clade Argonaute proteins cleave target piRNA or TE transcripts between positions 10 and 11 of the guide piRNAs, generating a 10-nt 5'end overlap between the antisense and sense piRNA pair [89, 114, 116]. As noted above, antisense piRNAs from both soma and germline contain a characteristic 5'U via unknown mechanisms. As a consequence, sense piRNAs generated from the secondary pathway tend to have an A at the 10^{th} position. Sense piRNAs then are loaded onto Ago3, trimmed to the length of mature piRNAs by undefined 3' trimmers and modified by 2'-O-methylation at the 3'end. Mature sense piRNA-Ago3 complexes can then bind to and cleave antisense piRNA precursors to produce antisense piRNA fragments associated with Piwi and Aub. Trimming generates mature antisense piRNAs, fulfilling the ping-pong amplification cycle [118-120].

1.4. The Functions of PIWI Proteins and piRNAs in the Germline, Soma and Cancers

Germline functions of PIWI proteins and piRNAs Mutations in piRNA pathway components were first identified in genetic screens for genes required for oogenesis or embryonic axis specification in *Drosophila* [121, 122]. Accumulating evidence shows that disruption of the piRNA pathway including mutations of *aub, ago3, spn-E, armi, zuc, squ, maelstrom (mael)* and *krimper* correlates with GSC loss, axis specification defect of the egg and loss of fertility. These phenotypes are later found to be secondary consequences of increased TE activity, DNA damage

checkpoint kinase activation and thus localization defects of dorsal and posterior RNAs in the piRNA pathway mutants [30, 92, 102, 112, 121-128]. Three PIWI family proteins have been identified in mice, Miwi2, Mili and Miwi. They are highly expressed in the testis and required for male fertility [129-135]. All three PIWI proteins, Miwi2, Mili and Miwi bind to piRNAs and mutations of these genes reduce piRNA production and activate TEs [129-131, 133, 136-138]. Increased DNA damage has also been observed in *miwi2* mutants [137]. Similar mutant phenotypes of PIWI family genes in *Drosophila* and mice suggest that piRNAs may have an evolutionarily conserved role in repressing TEs and protecting genome integrity in the germline.

The functions of PIWI proteins outside the gonad In *Drosophila*, the three PIWI proteins are expressed as early as the embryonic stage and required for early somatic development of the embryo [139]. One of the possible molecular functions of PIWI proteins and piRNAs during embryonic development is to regulate maternal mRNA decay to ensure proper body patterning [140]. In the adults, PWI proteins function outside the gonad via epigenetic regulation and/or TE silencing. Piwi has both negative and positive regulations on position effect variegation (PEV) in the expression of the eye color gene *white*, depending on the insertion loci of the *white* reporter gene [90, 141-143]. Piwi associates with chromatin and interacts with the Heterochromatin protein 1a (HP1a), a key player in heterochromatic gene silencing [141]. The HP1a interaction motif of Piwi is required for the silcencing of *white* reporter genes [141]. On the contrary, in a subtelomeric heterochromatin region on the right arm of chromosome 3, *piwi* mutations lose euchromatic histone modifications, accumulate heterochromatin histone marks including HP1a and thus repress transcriptions of piRNAs and a *white* reporter gene in this

region [90]. Piwi might recruit HP1a in some genomic regions to establish or maintain heterochromatin status and exclude heterochromatin initiation factors in other genomic regions by interacting with different proteins in different chromatic context, and thus achieve both negative and positive regulations. Mutations of other piRNA pathway components including *aub* and *spn-E* have also been shown to disrupt heterochromatin status and thus derepress *white* reporter genes [143]. Although multiple components of the PIWI-piRNA pathway are required for heterochromatin establishment and/or maintenance to regulate PEV, no clear evidence shows that piRNAs or TE activity are involved in this process. TE activities, however, seem to be responsible for other phenotypes of PIWI depletion outside the gonad. A protein complex comprised of Hsp90, Piwi and Hop has been proposed to suppress phenotypic variation to ensure developmental robustness (also called canalization) [144]. Hsp90 mutations result in defective PIWI-piRNA mediated TE silencing and the resultant TE activity may be responsible for producing new phenotypes randomly [145], supporting the role of the PIWI-piRNA pathway in canalization. PIWI proteins Aub and Ago3 have been shown to express in the brain of *Drosophila* and mutations of *aub*, *ago3* or *armi* lead to TE upregulation [146]. Interestingly, during replicative cellular senescence or normal aging, the chromatin of TEs becomes relatively more open and both mRNA levels and transposition events are increased [147, 148]. The functions of PIWI proteins or piRNAs, if any, in repressing TEs during cellular senescence or normal aging, however, have not been identified. Instead, endogenous siRNAs corresponding to transposons and heterochromatic sequences generated via the Dicer-2/Ago2 RNAi pathway have been proposed to target transposons in the soma of *Drosophila* [142, 149-152] . This proposal is supported by the observation that siRNAs corresponding to the L1 retrotransposon have been detected in cultured human HeLa cells [153]. Despite the fact that PIWI proteins are expressed

and function in normal or diseased somatic tissues outside the gonad and TEs are derepressed in piRNA pathway mutants in these tissues, the existence of piRNAs outside the gonad is still a mystery. Further molecular and sequencing analysis is needed to solve this important biological question.

The functions of PIWI proteins and piRNAs in cancers Many studies have demonstrated that malignant tissues from invertebrates to vertebrates including human cancers gain the expression of PIWI proteins [154-160]. In *Drosophila*, mutations in the *lethal (3) malignant brain tumor* (*l(3)mbt*) gene cause formation of a brain tumor during laval development. More insightfully, PIWI proteins including Piwi and Aub are ectopically expressed in these tumors and inactivation of these genes suppresses *l(3)mbt* mutant malignant tumor growth [154], demonstrating the functional importance of ectopic PIWI protein expression in tumor growth. Hiwi, the human homolog of the PIWI protein, has been associated with gastric cancer, adenocarcinoma, soft-tissue sarcoma, seminomas and hepatocellular carcinoma. Knockdown of *hiwi* by antisense RNA or RNAi induces cell cycle arrest and inhibits proliferation of cultured gastric cancer cells [159]. Similarly, Knockdown of *hiwi* by RNAi also reduces proliferation and invasion of different hepatocellular carcinoma cell lines [157]. High Hiwi expression also correlates with low survival rates in hepatocellular carcinoma [157], soft-tissue sarcoma [158] and adenocarcinoma [160] patients. Another human PIWI protein Hili has been found in prostate, breast, gastrointestinal, ovarian and endometrial cancers [156]. Overexpression of Mili in a mouse fibroblast cell line increases cell proliferation [156]. Despite the interesting correlation between PIWI protein overexpression and low cancer survival rate, the lack of functional studies

in mouse cancer models or human cancers leaves the functions of PIWI proteins in cancer biology elusive and the question remains if PIWI protein overexpression is causative or secondary to tumor growth.

Biochemical functions of PIWI proteins and piRNAs PIWI proteins regulate the expression of target genes by heterochromatin formation, transcriptional repression, post-transcriptional transcript destruction and translational inhibition. In *Drosophila*, Piwi is predominantly localized to the nuclei [31] and co-localizes with epigenetic regulators including Polycomb group proteins and physically interacts with HP1a [141, 161]. In general, Piwi acts as a transcriptional silencer by establishing and maintaining a repressive chromatin state. Loss of Piwi decreases the repressive methylation mark on histone 3 lysine 9 (H3K9) [143, 162-165] and HP1a enrichment [166], and increases RNA Polymerase II occupancy [162-164] and transcription [142, 162, 165]. Ectopic insertions of piRNA complementary sequences into the genome are sufficient to recruit Piwi, HP1a and histone methyltransferase Su(var)3-9 to the ectopic sites and thus increase repressive histone mark H3K9me2/3 and reduce RNA Polymerase II occupancy [163]. These findings together demomstrate that Piwi-piRNA complexes are necessary to maintain and sufficient to establish a repressive chromatin state for transcriptional repression. In contrast to the general repressive function of Piwi, it can also act as an epigenetic activator as exemplified by the euchromatin and transcription promoting function of Piwi on chromosome 3R telomere-associated sequence (3R-TAS) [90]. HP1a enrichment decreases on piRNA clusters in a *piwi* mutant in general (80 out of 96 piRNA clusters investigated) as expected [166]. However, consistent with an active role of Piwi on transcriptions of some piRNA clusters, HP1a shows

increased enrichment on some piRNA clusters including 42AB and 3R-TAS [90, 166] and thus a *white* reporter gene is derepressed in these regions in *piwi* mutants [90, 167]. HP1a also enriches in the sub-telomeric regions of 2L, 2R, 3L and 3R in *piwi* mutants, further supporting an active role of Piwi in the sub-telomeric regions [166]. The mechanisms to distinguish the repressive and active roles of Piwi in epigenetic regulation might rely on the differential interacting partners of Piwi and/or different chromatic microenvironment that Piwi interacts with in different cell types and different developmental stages, which await more investigations to further delineate. In addition to the role of Piwi-piRNA complexes in histone modification and heterochromatin formation, Piwi-piRNA complexes have also been suggested to establish the DNA methylation and transcriptional silencing status on TEs by recruiting DNA methyltransferases in male fetal germ cells in mice [129, 130, 136, 137]. Piwi-piRNA complexes may direct heterochromatin formation and also establish DNA methylation status to repress transcription in a sequence complementarity manner.

Escapers of TEs from transcriptional repression will be destroyed by piRNA mediated homology-dependent target cleavage or translational inhibition. All three PIWI proteins in *Drosophila*, Piwi, Aub and Ago3, have been shown to associate with piRNAs and exhibit slicer activity *in vitro* [89, 114, 116]. The majority of piRNAs are mapped to TEs, and mutations of PIWI proteins lead to TE activation and thus increased DNA damage. The 10-nt 5'end overlap between sense and antisense piRNAs and the ability of PIWI proteins to cleave target RNAs between nucleotides 10 and 11 measured from the 5'end of the guide RNA [89] is consistent with previous observations with other Argonaute proteins [168]. These findings strongly support the functions of PIWI-piRNA complexes in transposon destruction. Some protein-coding genes might also be subjected to piRNA-mediated degradation. A subset of maternal mRNAs

including *nos* are targeted by the RNA binding protein Smaug and the deadenylase CCR4 to be degradated during maternal-to-zygotic transition. piRNA mutants including *piwi*, *aub*, *ago3* and *spn-E* delay the deadenylation process of *nos* mRNAs. Aub-associated *412* piRNA is complementary to the 3'UTR of *nos* mRNAs. In addition, PIWI proteins Aub and Ago3 are found to complex with Smaug, CCR4 and *nos* mRNAs in the *Drosophila* embryo [140], demonstrating the roles of PIWI-piRNAs in maternal mRNA decay. The slicer activity of PIWI proteins can cleave protein-coding gene transcripts in a similar manner to TE transcript destruction. The *Stellate* gene in the fly testis is targeted by the Aub-piRNA complexes by sequence complementarity between *Stellate* and the piRNA component in the complex [92, 114, 169]. *vasa* mRNAs can also be cleaved by Aub associated with two piRNAs AT-chX-1 and AT-chX-2 with strong complementarity to *vasa* mRNAs [114]. In mice, PIWI proteins Mili and Miwi co-fractionate with polysomes and associate with translation initiation factors in the testicular extracts [134, 170] and piRNAs have been found in polysome fractions from the same tissue [134, 171], raising the possibility of translational control by PIWI proteins and piRNAs.

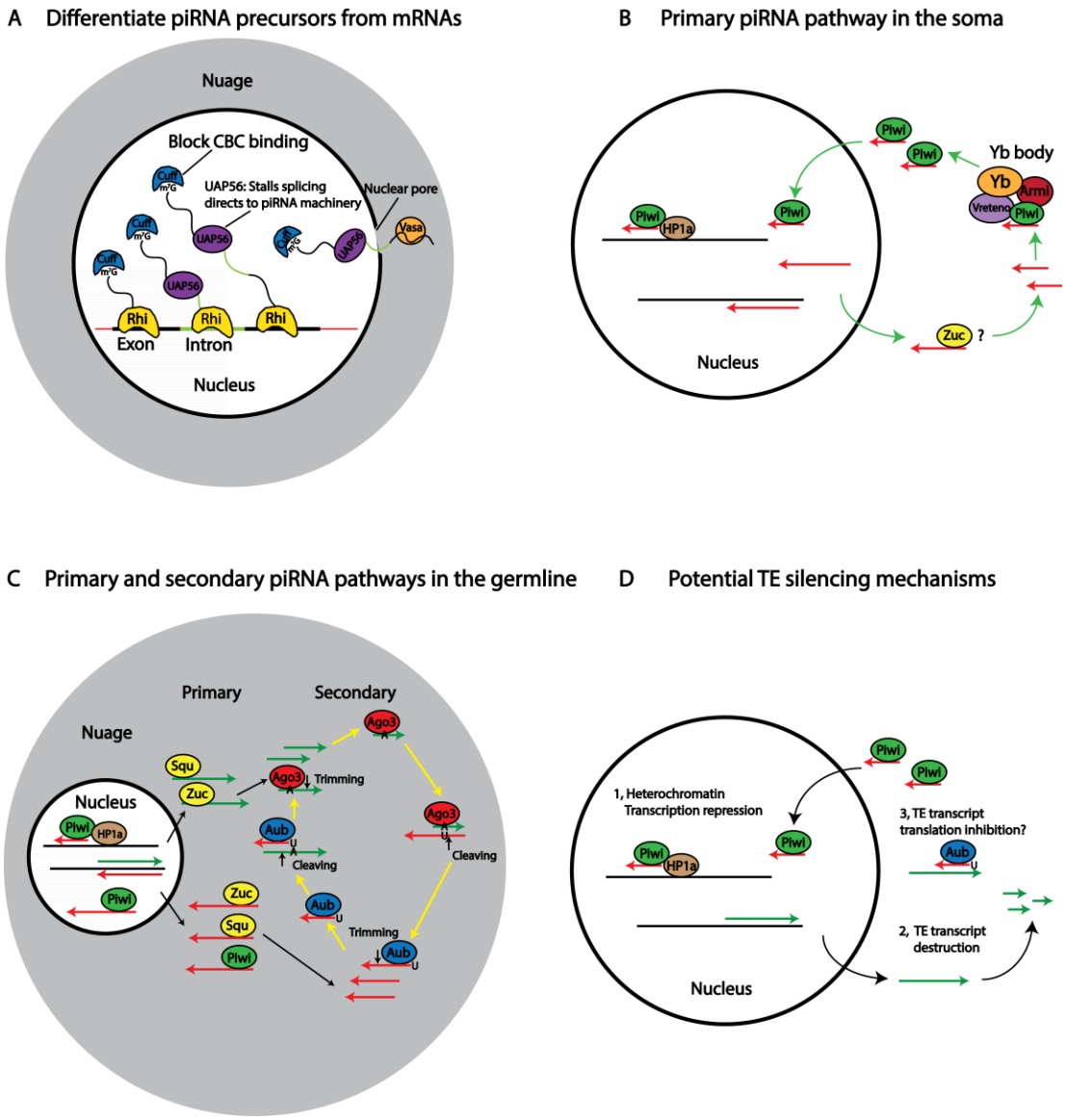

Figure 1.5. **piRNA biogenesis and TE silencing mechanisms.** (**A**) Rhi functions with UAP56 and Cuff to suppress piRNA splicing and direct piRNA precursor transcripts to piRNA processing machinery. (**B**) In the primary piRNA pathway of the soma, long piRNA precursors are chopped into smaller fragments possibly by Zuc and then transported to the perinuclear Yb body. (**C**) In the germline, there might be redundant primary piRNA pathways in addition to Zuc. The secondary piRNA pathway amplifies piRNAs via a Ping-pong mechanism. (**D**) Multiple levels of control over TEs are in place to protect genome integrity.

1.5. Purposes and Central Hypothesis of Dissertation

TEs comprise at least 45% of the human genome, which poses a potential threat to the genome stability on individuals and over generations if TE-induced mutations are carried on to the next generation [172-174]. The observations that PIWI family proteins are expressed in a wide range of human cancers led to the realization that TE activation might be responsible for various diseases [155-159, 175, 176]. The important correlation of TE activity with cellular senescence and tissue aging underlines the value of PIWI-piRNA research in age-related pathophysiology [147, 148]. Interest also remains if and how PIWI-piRNA complexes regulate stem cell self-renewal and differentiation pathways. About 15-22% of the *Drosophila* genome consists of TE sequences [174], which makes it a favor for studying the functions of piRNAs considering the variety of available genetic tools. The purpose of this project was to investigate the roles of piRNA pathway components in *Drosophila* ovarian germline development, especially in GSC maintenance and germ cell differentiation. Integrating piRNA regulation with stem cell self-renewal and differentiation programs shows important implications in tumorigenesis, tissue degeneration and aging.

The central hypothesis is that piRNA pathway components are required intrinsically and extrinsically for controlling GSC maintenance and differentiation. This study has shown that **t**he piRNA pathway component Piwi functions in the ECs to restrict BMP signaling in the niche and promote germ cell differentiation. Another PIWI protein Aub functions in the germline to regulate germ cell differentiation by interacting with the master differentiation factor Bam. Bam shows a novel regulatory role in repressing TEs. Piwi also functions with Aub in the GSCs to maintain GSCs intrinsically.

Chapter 2: Piwi Is Required in Multiple Cell Types to Control Germline Stem Cell Lineage Development in the *Drosophila* Ovary

2.1 Abstract

The piRNA pathway plays an important role in maintaining genome stability in the germ line by silencing TEs from fly to mammals. As a highly conserved piRNA pathway component, Piwi is widely expressed in both germ cells and somatic cells in the *Drosophila* ovary and is required for piRNA production in both cell types. In addition to its known role in somatic cap cells to maintain GSCs, this study has demonstrated that Piwi has novel functions in somatic cells and germ cells of the *Drosophila* ovary to promote germ cell differentiation. *piwi* knockdown in ECs causes a reduction in EC number and accumulation of undifferentiated germ cells, some of which show active BMP signaling, indicating that Piwi is required to maintain ECs and promote germ cell differentiation. Simultaneous knockdown of *dpp*, encoding a BMP, in ECs can partially rescue the germ cell differentiation defect caused by *piwi* knockdown, indicating that Piwi is required in ECs to repress *dpp*. Consistent with its key role in piRNA production, TE transcripts increase significantly and DNA damage is also elevated in the *piwi* knockdown somatic cells. Germ cell-specific knockdown of *piwi* surprisingly causes depletion of germ cells before adulthood, suggesting that Piwi might control PGC maintenance or GSC establishment. Finally, Piwi inactivation in the germ line of the adult ovary leads to gradual GSC loss and germ cell differentiation defects, indicating the intrinsic role of Piwi in adult GSC maintenance and differentiation. This study has revealed new germline requirement of Piwi in controlling GSC maintenance and lineage differentiation as well as its new somatic function in

promoting germ cell differentiation. Therefore, Piwi is required in multiple cell types to control GSC lineage development in the *Drosophila* ovary.

2.2 Introduction

Small RNAs have received much attention in recent years because of their important and diverse roles in the regulation of various biological processes [84, 177-180]. In contrast to other small RNAs, piRNAs are abundantly expressed in germ cells of organisms ranging from *C. elegans* to human, and have emerged as an important class of small RNAs for maintaining genome stability in germ cells [99, 120, 181, 182]. Recent studies have shown that piRNAs also function in somatic cells to regulate gene expression and repress TEs [40, 101, 108, 144, 163, 183]. However, biological functions of piRNAs still remain poorly defined.

The *Drosophila* ovary is an attractive system for studying stem cell lineage development [184]. Two types of stem cells, GSCs and follicular stem cells (FSCs) are responsible for continuously producing differentiated germ cell cysts and follicle cells, respectively, which are assembled into egg chambers that eventually develop into mature oocytes. Two or three GSCs are situated in the tip of each ovariole, known as the germarium, and can be easily identified by their direct contact with cap cells and presence of an anteriorly localized spectrosome (Fig. 2.1A). Immediate GSC daughters, also known as CBs, move away from cap cells and undergo four rounds of synchronized cell division to form 2-cell, 4-cell, 8-cell and 16-cell cysts. CBs and cysts are tightly encased by cellular processes of escort cells (ECs), also known as inner germarial sheath cells (Fig. 2.1A). Genetic and cell biological studies have shown that TF/cap cells and

anterior ECs form the self-renewing niche for GSCs, which provides the essential BMP signal for repressing GSC differentiation and thereby maintaining their self-renewal [184].

Based on recent studies from us and others [62, 185], we have recently proposed that posterior ECs function as the microenvironment or niche for promoting germ cell differentiation [185]. One of the key functions of ECs is to prevent BMP signaling via two distinct strategies. First, EGFR-MAPK signaling has been proposed to directly repress expression of *dally*, encoding a proteoglycan facilitating BMP signal transduction and diffusion [62]. Rho signaling and Eggless have been shown to repress *dally* expression in ECs, thus promoting germ cell differentiation, but it remains unclear how they might regulate *dally* expression [185, 186]. The second strategy is direct repression of transcription of *dpp*, which encodes a BMP ligand essential for GSC self-renewal in *Drosophila*. Histone lysine-specific demethylase 1 (Lsd1, a chromatin regulator) and Rho signaling have been shown to repress *dpp* transcription in ECs [60, 185]. *dpp* knockdown can partially rescue the germ cell differentiation defects caused by inactivation of Lsd1 and Rho signaling in ECs, indicating that *dpp* upregulation contributes to the germ cell differentiation defects. Therefore, ECs have so far been demonstrated to promote germ cell differentiation by preventing the spreading of BMP signaling.

It is the *Drosophila* ovary in which the first piRNA regulator, *piwi*, was identified for its critical role in maintaining GSCs [30, 187]. Although it is expressed in all germ cells and somatic cells of the *Drosophila* ovary, it has been suggested to function in TF/cap cells for maintaining GSCs [30, 188]. In addition, Piwi is also required intrinsically to promote GSC division and PGC formation [189, 190]. In *Drosophila* ovarian somatic cells, Yb works with Piwi to control primary piRNA biogenesis [40, 108, 109], and is also suggested to work in

TF/cap cells to maintain GSC self-renewal [33]. In addition, recent studies have shown that Armi, Vret and Tdrd12 are also required in somatic cells to control primary piRNA biogenesis [64, 107, 110]. Inactivation of histone H3K9 trimethylase Eggless function in ECs leads to defective piRNA biogenesis, upregulation of transposons and germ cell differentiation defects, indicating that piRNAs are important for maintaining EC function by repressing transposons [63, 186]. Consistently, *vret* mutants also have a germ cell differentiation defect, which can be rescued by somatic cell-specific expression of *vret* [64, 110]. In this study, we show that *piwi* is required in ECs and germline to control germ cell differentiation.

2.3 Experimental Procedures

Drosophila strains and culture

The *Drosophila* stocks used in this study include: *c587-gal4* [29], *PZ1444* [191], *UAS-dcr2*, *UAS-dppRNAi* lines (TR00047P.1;HMS00011), *UAS-piwiRNAi* lines (VDRC101658, HMS00606 and THU00412), *UAS-armiRNAi* lines (GL00254; HMS00098), *UAS-aubRNAi* lines (GL00076; HMS00611) and *UAS-YbRNAi* (GL00053; GL00204). *Drosophila* strains were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified. To maximize the RNAi-mediated knockdown effect, newly eclosed flies were cultured at 29^{o}C for a week before the analysis of ovarian phenotypes.

Construction of UAS-RNAi and nos>mCherry SV40 polyA>gal4VP16 Strains

The new *THU UAS-RNAi* line targeting *piwi* was constructed using the pVALIUM20 vector according to the published procedure [192]. The targeting sequence for *piwi* is CCCGGTCATGCTGCAGACGAA, which was designed based on the algorithm of DSIR.

To construct *pnos-FRT-mCherry-SV40 polyA-FRT-gal4VP16-nos 3'UTR*, different components were assembled together by five steps. First, the coding region of *mCherry* was amplified from an *mCherry*-containing vector using 5'-acgctagctatggtgagcaagggcgaggag-3'and 5'-gactcgagttacttgtacagctcgtccat-3' primers (Nhe I and XhoI sites underlined), and was cloned into NheI-XhoI sites of the *pFRT-SV40 polyA-FRT* vector (a gift from Elizabeth R. Gavis). Then, the *FRT-mCherry* fragment amplified using 5'-atcatatgggggatcttgaagttcctatt-3' and 5'-gactcgagttacttgtacagctcgtccat-3' primers (Nde I and XhoI sites underlined) from the *pFRT-mCheery-SV40 polyA-FRT* was cloned into the pGEM-T vector (Promega) to generate *pFRT-mCherry*. Second, the *SV40 polyA-FRT* fragment amplified from the *pFRT-SV40 polyA-FRT* vector using 5'-gactcgagggtacctctagaggatctttgtga-3' and 5'-atgcggccgccatatgcaaaagcgctctgaagttcctatact-3' primers (XhoI and NotI NdeI sites underlined) was cloned into the XhoI-NotI sites of *pFRT-mCherry* to generate *pFRT-mCheery-SV40 polyA-FRT*. Third, the *EGFP* coding region amplified from *pEGFP-N3* (Clontech) using 5'-tcgaattccatcgccaccatggtgagcaa-3' and 5'-tacagatctcttgtacagctcgtccatgccga-3' primers (EcoR I and BglII sites underlined) was cloned into the BglII-EcoRI sites of *pUAST-attB* [193] to generate *pEGFP-attB*. Fourth, the NotI flanked 3.13 Kb fragment from *pCSpnosFGVP* (a gift from Elizabeth R. Gavis) containing *nos promoter-ATG (NdeI-start codon) gal4VP16-nos 3'UTR* was subcloned into two NheI sites of *pEGFP-attB* to generate *pnos-NdeI-gal4VP16-nos 3'UTR-attB*. Finally, the NdeI flanked *pFRT-mCherry-SV40 polyA -FRT* fragment from *pFRT-mCheery-SV40 polyA-FRT* was subcloned into the NdeI site of *pnos-NdeI-gal4VP16-nos 3'UTR-*

attB to generate *pnos-FRT-mCherry-SV40 polyA-FRT-gal4VP16-nos 3'UTR*, which was introduced into an *attP* site-containing fly strain (BL#24482) using PhiC31 integrase-mediated transgenesis by BestGene, Inc.

Immunohistochemistry

Immunohistochemistry was performed according to our previously published procedures [194, 195]. The following antibodies were used in this study: rabbit polyclonal anti-□-galactosidase antibody (1:100, Cappel), Guinea pig polyclonal anti-Piwi antibodies (1:100; produced by H. Lin), chicken polyclonal anti-GFP antibody (1:200, Jax), mouse monoclonal anti-Hts antibody (1:50, DSHB), mouse monoclonal anti-Yb antibody (1:200; kindly provided by Dr. H. Siomi), rabbit polyclonal anti-pS137 H2Av antibody (1:100, Rockland), rabbit monoclonal anti-pS423/425 Smad3 antibody (1:100, Epitomics), rabbit polyclonal anti-pERK antibodies (1:25, Cell Signaling) and rat monoclonal anti-Vasa antibody (1:50, DSHB). All images were taken with a Leica TCS SP5 confocal microscope.

Cell sorting and RNA sequencing

Drosophila ovaries were dissected and placed in Grace's medium (Sigma-Aldrich; G9771) and then washed twice by adding 1X DPBS and centrifuged at 700xg for one minute. The ovaries were incubated with prewarmed Collagenase (Worthington, Collagenase Type II, Lot# 50D11833) in 15ml conical tube at 37˚C water bath for three minutes with gentle shaking. Enzyme reaction was stopped after three minutes by adding cold 1X DPBS + 2% FBS.

Dissociated sample was washed by adding 1X DPBS and centrifuged at 700xg, 4 ˚C for five minutes. The cell pellet was resuspended in 1X DPBS and filtered with 70um Filcon (BD; 340605) in to 5ml flow tubes. Cells were centrifuged and then resuspended in 200ul of 1X DPBS for sorting at 45psi with 70um tip (BD; InFlux) immediately in to TRIzol (life technologies; 15596-018). Total RNAs were extracted with Trizol and purified by organic extraction followed by isopropanol precipitation.

Following manufacturer's directions and using the Illumina TruSeq library construction kits (Illumina, Cat. No. RS-122-2001/2), mRNA was isolated from 150ng of total RNA per sample and short fragment libraries were constructed. The resulting libraries were purified using Agencourt AMPure XP system (Backman Coulter, Cat. No. A63880), and were then quantified using a Bioanalyzer (Agilent Technologies) and a Qubit Fluorometer (Life Technologies). All libraries were pooled, re-quantified and run as 50 bp single-end lanes on an Illumina HiSeq 2000 instrument, using HiSeq Control Software 1.5.15.1 and Real-Time Analysis (RTA) version 1.13.48.0. Secondary Analysis version CASAVA-1.8.2 was run to demultiplex reads and generate FASTQ files.

For qRT-PCR, total RNAs were first treated by DNase I, and were then used for synthesis of cDNAs using mixed oligo dT and random primers and SuperScript III Reverse Transcriptase (Life Technologies). Fluorescence-based quantitative real-time PCR (qPCR) was performed to quantify *gypsy*, *zam*, *TART*, *gbb*, *dpp* and *dally* with *tbp*, *gapdh* and *rpl32* as internal controls using primers shown in Table S1. After cDNAs were diluted at 1:100, 2μl aliquots of each cDNA sample were added to 5μl of 2x power SYBR Green PCR Master Mix (Applied Biosysterms part No.: 4367659, Lot No. :1305403) , 0.5μl each of 10nm Forward &

Reverse primer and 2ul of water in a 384-well plate. The resulting reactions were run on an ABI 7900HT according to the instruments standard protocol. Analysis of the fluorescence curves was done using ABI's SDS2.4 software. The Ct values were analyzed using the Biogazelle qBase Plus version 2.4 software to generate normalized relative quantities using assays for endogenous controls.

Table 2.1. Primers for qRT-PCR

Gene Name	Fwd Primer	Reverse Primer
dpp	TCGGCCAACACAGTGCGAAGTTT	TTCACGTCGAAGTGCAGCCGAAA
gbb	AATGGTTCTGCTCATGTTCGTGGC	TCAGCACTCTGTGCATGATCGTCT
dally	GAGCAACAGCAGATGCACACGAAT	GTGCACTTCAAGGGTTTCACGGTT
gypsy	ATTATCAACGAAGCCGCAGCTCAC	AATTCAGAGCCGTTGATGGTTGCC
ZAM	AACGCTCGACCTAACTAGCGGTTT	AGATCGCCAAGAACGCTGTCCATA
tart	AGAGAGGGAAAGAAGGGAAAGGGA	ATTTCCTGCCTGGTTAGATCGCCA
gapdh	AGGGAGCCACCTATGACGAAATCA	AGACGAATGGGTGTCGCTGAAGAA
tbp	TCCAGACTGGCAGCGAGAAAGTAT	AACTTGACATCGCAGGAGCCG
Rpl32	AGCGCACCAAGCACTTCATC	GACGCACTCTGTTGTCGATACC

2.4 Results and Figures

Piwi is required in ECs to control germ cell differentiation and EC survival

To identify the genes that are required in ECs for controlling germ cell differentiation, we carried out a genetic screen using an EC-expressing *c587-gal4* driver and transgenic *UAS-RNAi* lines from the Vienna *Drosophila* RNAi Center (VDRC). These VDRC RNAi transgenic lines were designed based on the production of a long double-stranded RNA structure that can be further processed into small double-stranded RNAs degrading target mRNAs, and have been used to carry out genetic screens in various *Drosophila* tissue types [196-198]. *c587-gal4* is

expressed specifically in ECs and early follicle cell progenitors based on the expression of *UAS-GFP* [29](Fig. 2.1A). In our screen, *piwi* was identified for its requirement in ECs to control germ cell differentiation as *c587-gal4* mediated knockdown of *piwi* causes the accumulation of spectrosome-containing ill-differentiated single germ cells (SGCs) located distantly from cap cells in the knockdown germaria, which is in great contrast with the control germaria (Fig. 2.1B). Although GSCs and CBs contain a spherical spectrosome, only GSCs directly contact cap cells. Differentiated 2-cell, 4-cell, 8-cell and 16-cell cysts contain a branched fusome, and can be easily distinguished from GSCs and CBs. The spectrosome and fusome are the same germ cell-specific intracellular organelle with different morphologies, which can be reliably labeled with antibodies against their components such as Hu li-tai shao (Hts) [199] (Fig 1A and 1B). To verify the RNAi-mediated Piwi knockdown efficiency, we used polyclonal anti-Piwi antibodies to examine Piwi protein expression in the control and *piwi* knockdown germaria, in which cap cells and ECs are marked by the *PZ1444* enhancer trap line [191]. Two additional miRNA-based *UAS-RNAi* transgenic strains were also used in this study: one RNAi strain was generated by the Perrimon Laboratory at Harvard Medical School, HMS [200, 201], and the other RNAi line, THU, was generated to target a different *piwi* sequence by the Ni laboratory at Tsinghua University. The PZ1444 enhancer trap line expresses nuclear β-galactosidase protein in cap cells and ECs, which can be reliably distinguished by their location and morphology [191, 202](Fig. 2.1C). As previously reported [141, 188], Piwi is generally expressed in both somatic cells and germ cells of the control germaria, but ECs express higher levels of Piwi than cap cells (Fig. 2.1C). Indeed, all the RNAi lines can efficiently eliminate Piwi expression in PZ1444-positive ECs and cap cells, while Piwi expression in germ cells including GSCs remains unchanged in the *piwi* knockdown germaria (Fig. 2.1D-F). *c587-gal4* mediated expression of the HMS and THU

RNAi lines can also cause the accumulation of ill-differentiated SGCs outside the GSC niche, similar to the VDRC line (Fig. 2.1G and 2.1H). Because a wild-type germarium normally contains one or two CBs, a germarium containing three or more SGCs is considered to exhibit the germ cell differentiation defect [44]. To determine the severity of the germ cell differentiation defects, we classified the *piwi* knockdown germaria into three categories: normal (SGCs$\leq$2), moderate differentiation defect (3$\leq$SGCs$\leq$10) and severe differentiation defect (SGCs$\geq$11). Among the germaria in which *piwi* is knocked down in ECs by three independent RNAi lines, 40-60% of them have three or more SGCs, and approximately 20% of them have 11 or more SGCs (Fig. 2.1I). In addition, egg chambers are also often filled with undifferentiated spectrosome-containing SGCs (Fig. 2.1B and 2.1H). Quantitatively, the three RNAi lines produce similar degrees of germ cell differentiation defects (Fig. 2.1I). As shown in Fig. 2.1I, there are some variations on SGC numbers in the knockdown germaria by different RNAi strains, and Fig. 2.1B, 2.1G and 2.1H reflect the variations among the three RNAi strains. In addition, we have observed that 10-50% of the *piwi* knockdown germaria by the three independent RNAi lines completely lose GSCs and become agametic, suggesting that Piwi is required in somatic cells for maintaining GSCs (Fig. 2.1I-L). These results indicate that Piwi is indeed required in ECs to promote germ cell differentiation and in ECs, cap cells or both to maintain GSCs.

The germarial region of the *piwi* knockdown ovaries appears to be reduced in size, suggesting that the EC number may also be reduced as well (Fig. 2.1B and 2.1H). Our previous studies have suggested that the severity of EC loss is positively correlated with the severity of the germ cell differentiation defects [185, 186]. We then quantified EC numbers in the control and *piwi* knockdown germaria. In contrast with the control germarium containing an average of 35 ECs (Fig. 2.1M and 2.1N), the *piwi* knockdown germarium contains significantly fewer ECs (Fig.

2.1N-P). Because ECs rarely proliferate, the reduction in EC number could be due to apoptosis. To directly test this idea, we used TUNEL labeling to detect dying ECs identified by PZ1444 expression. Indeed, there is a consistent increase in apoptotic *piwi* knockdown ECs by the three independent RNAi lines (Fig. 2.S1). These results demonstrate that Piwi is also required for maintaining EC survival.

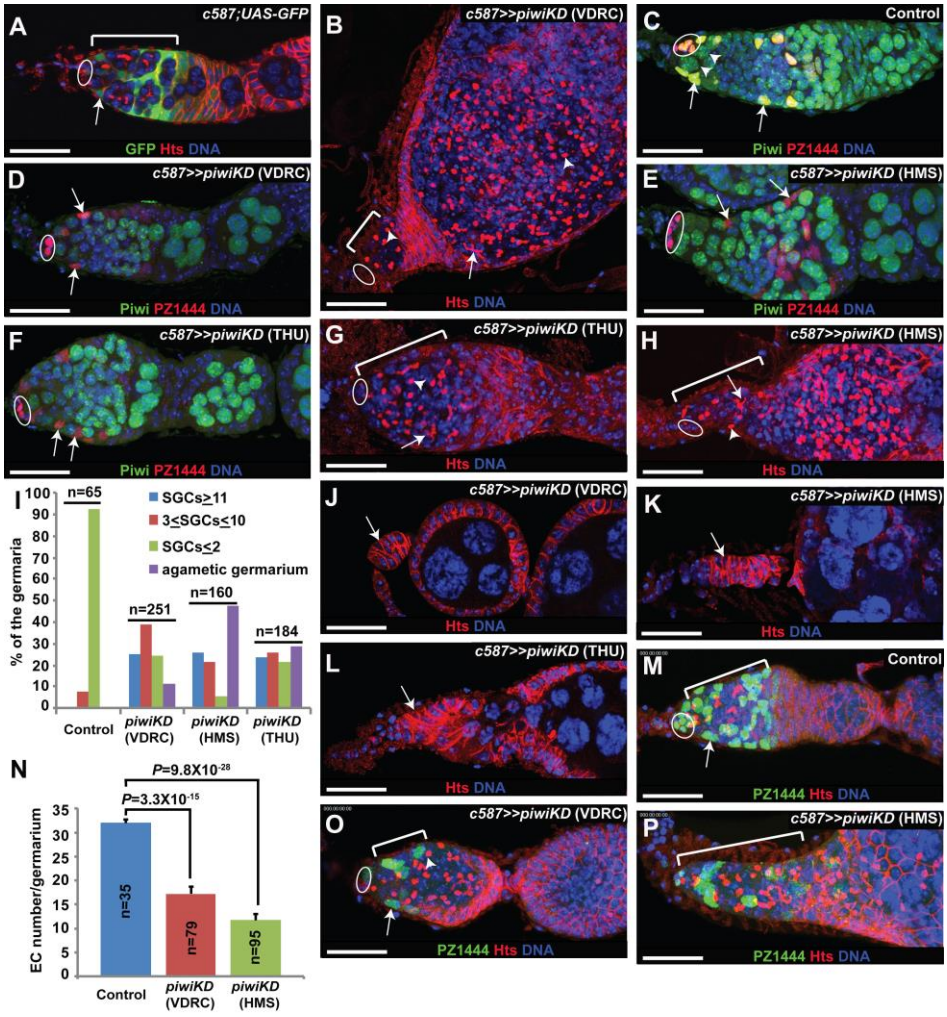

Figure 2.1. Piwi is required in ECs to promote germ cell differentiation and maintain EC survival. Ovals indicate cap cells, whereas brackets denote the germarial region covered by ECs. (**A**) *c587-gal4* drives GFP expression specifically in ECs (arrow). (**B**) *c587-gal4* mediated *piwi*

knockdown leads to an accumulation of many SGCs carrying a spectrosome (arrowhead) mixed with differentiated cysts containing a branched fusome (arrow) in the germarium and its associated egg chamber. (**C**) Piwi protein is expressed in *PZ1444*-positive cap cells and ECs (arrow) as well as in follicle cells and germ cells. (**D**-**F**) *c587-gal4* mediated *piwi* knockdown by three independent RNAi lines efficiently eliminates Piwi protein expression in *PZ1444*-positive cap cells and ECs (arrows), whereas Piwi protein expression in germ cells remains normal. (**G**-**I**) *c587-gal4* mediated *piwi* knockdown by THU and HMS lines leads to the accumulation of SGCs (arrowhead) mixed with differentiated cysts (arrow). **I** shows the quantitative results on the numbers of SGCs and agametic germaria (n indicates total germaria examined). (**J**-**L**) *c587-gal4* mediated *piwi* knockdown causes the formation of the germaria (arrows) containing no germ cells. (**M**-**P**) *c587-gal4* mediated *piwi* knockdown (**O**, **P**) results in a significant reduction in EC numbers in comparison with the control (**M**). **N** shows the quantitative results on EC numbers (n indicates total germaria examined). Scale bars: 25μm.

Piwi functions in adult ECs to promote germ cell differentiation

Since *c587-gal4* is known to be expressed by most, if not all, somatic precursor cells in the female gonad [203], the differentiation defects and the GSC loss phenotype caused by *piwi* knockdown could be due to its early requirement in somatic gonadal precursors. To definitively determine if Piwi is required in adult ECs to control germ cell differentiation, we carried out temperature shift experiments to inactivate Piwi function specifically in adult ECs. When the genetic crosses were carried out at 18^{o}C, which lowers *piwi* RNAi expression and thus its knockdown effect, the germaria show almost normal germ cell differentiation and GSC

maintenance because all the *piwi* knockdown germaria have normal SGC numbers and still retain two or three GSCs (Fig. 2.2A-D). After the adult females emerged at 18^{o}C, they were cultured at 29^{o}C for a week to increase RNAi expression and *piwi* knockdown efficiency and thus inactivate Piwi function in adult ECs. Interestingly, the number of the germaria carrying three or more SGCs drastically increases, indicating that Piwi is indeed required in adult ECs to promote germ cell differentiation (Fig. 2.2E-H). Similarly, the numbers of the germaria containing no GSCs also increase following Piwi knockdown by the three RNAi lines (Fig. 2.2H-K). In addition, Piwi expression is still reduced in cap cells, suggesting that *c587-gal4* is likely expressed at low levels in cap cells. Since the previous findings have shown that ECs also contribute to GSC maintenance [185, 186, 204], the GSC loss phenotype could come either from Piwi knockdown in cap cells, ECs or both. Taken together, these results indicate that Piwi is required in adult ECs to promote germ cell differentiation as well as in adult ECs, adult cap cells or both for GSC maintenance.

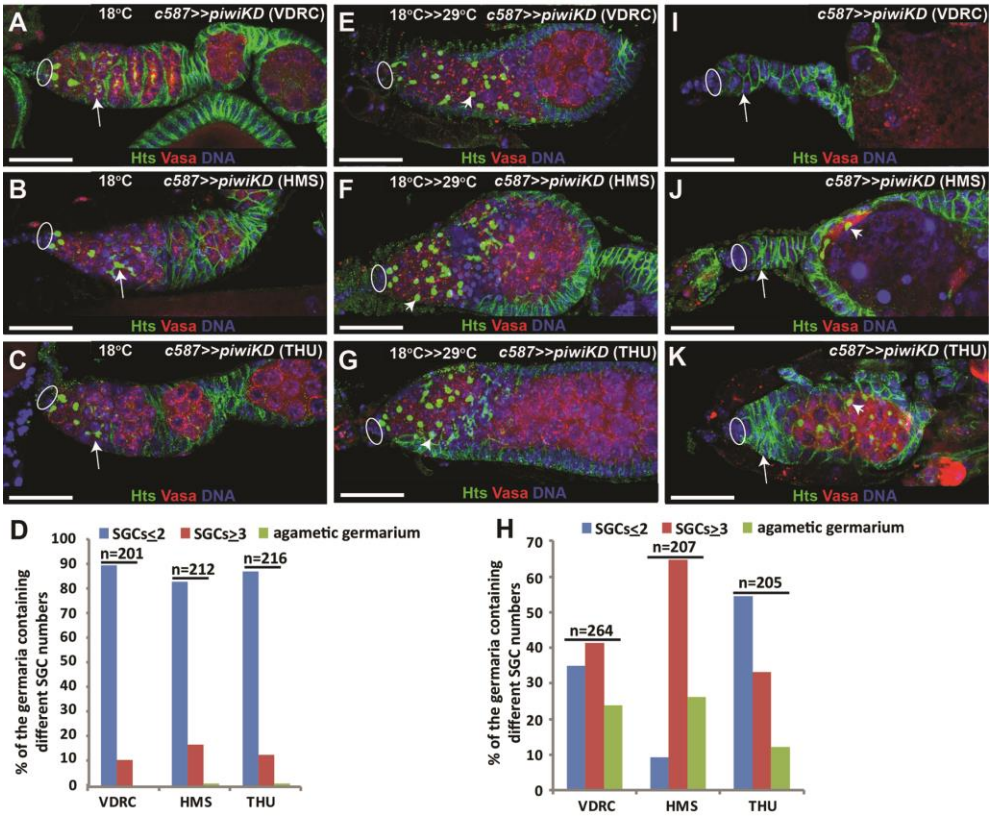

Figure 2.2. Piwi is required in adult ECs to maintain GSCs and promote germ cell differentiation. Ovals, arrows and arrowheads indicate cap cells, branched fusomes and spherical spectrosomes, respectively. Germaria in **A-C**, **E-G** and **I-K** are labeled for Hts (green, spectrosome/fusome), Vasa (red, germ cells) and DNA (blue). (**A-D**) At 18^{o}C, *c587-gal4* mediated *piwi* knockdown does not affect GSC and SGC numbers due to low RNAi expression. **D** represents quantitative results on the numbers of SGCs and germless germaria. (**E-H**) 1w after shifting to 29^{o}C, *c587-gal4* mediated *piwi* knockdown leads to an accumulation of excess SGCs in the germaria. **H** represents quantitative results on the numbers of SGCs and germless germaria. (**I-K**) 1w after shifting to 29^{o}C, *c587-gal4* mediated *piwi* knockdown causes some germaria to completely lose germ cells including GSCs. Scale bars: 25μm.

Piwi is required in ECs to prevent BMP signaling in differentiated germ cells

Previous studies have shown that the germ cell differentiation defects caused by defective EC function result from elevated BMP signaling [62, 185, 186]. To determine if BMP signaling activity is augmented in the germ cells of the *piwi* knockdown germaria, we examined the expression of pMad, *Dad-lacZ* and *bam-GFP*, three BMP signaling activity reporters in *Drosophila*, in the control and *piwi* knockdown germaria. Activation of BMP receptors (Tkv and Sax) upon BMP ligand binding leads to production of phosphorylated Mad (pMad), which translocates into the nucleus with Medea, a SMAD4 homolog, to activate *Dad* expression and repress *bam* expression in GSCs [29, 37, 205]. In contrast with the control germarium in which pMad accumulates primarily in GSCs (Fig. 2.3A), pMad is also expressed in some, but not all, SGCs outside the GSC niche of the *piwi* knockdown germaria, indicating that BMP signaling activity indeed spreads outside the GSC niche (Fig. 2.3B, 2.3C and 2.S3A). *bam-GFP* and *Dad-lacZ* can recapitulate the expression patterns of *bam* and *Dad* in the control germarium: *bam-GFP* is normally expressed in differentiated germ cells but is absent from GSCs [39], while *Dad-lacZ* is normally expressed in GSCs but is absent in differentiated germ cells [29, 37, 205] (Fig. 2.3D and Fig. 2.S3C). Although it is still expressed in GSCs of the *piwi* knockdown germaria (Fig. 2.3E, 2.3F and 2.S3B), *Dad-lacZ* reduces its expression by about 25% based on quantification results (Fig. 2.3G). Although *bam-GFP* remains repressed in the GSCs of the *piwi* knockdown germaria, it fails to be upregulated in some SGCs outside the GSC niche in the *piwi* knockdown germaria as in control CBs (Fig. 2.S3C-F). These results indicate that Piwi is required in ECs to prevent BMP signaling activity in differentiated germ cells.

Previous studies have revealed that the elevated transcription of *dpp*, which encodes a BMP ligand, in ECs can contribute to increased BMP signaling in differentiated germ cells [60, 185, 186]. In *Drosophila*, another BMP-encoding gene, *gbb*, is also expressed in the germarium and is required for maintaining GSCs [29]. In addition, *dally* upregulation in ECs has also been shown to be responsible for BMP signaling activity elevation [62, 206, 207]. To determine if the elevated BMP signaling activity in SGCs is due to upregulation of *dpp*, *gbb* or *dally* in ECs, we sequenced the mRNAs isolated from the purified GFP-labeled control and *piwi* knockdown ECs. Based on RNA sequencing and qRT-PCR results, *dpp* is significantly upregulated in the *piwi* knockdown ECs compared to the control ECs (Fig. 2.3H and 2.3I). Although RNA sequencing results show that *gbb* and *dally* are slightly upregulated in the *piwi* knockdown ECs (Fig. 2.3H), qRT-PCR results fail to confirm the finding (Fig. 2.3I). These results suggest that *dpp* upregulation in the *piwi* knockdown ECs might be responsible for germ cell differentiation defects.

To determine if *dpp* upregulation in the *piwi* knockdown ECs contributes to germ cell differentiation defects, we quantified SGCs outside the GSC niche in the germaria in which *piwi* and *dpp* are simultaneously knocked down in ECs. Here we used two different *piwi* (VDRC and HMS) and *dpp* RNAi (TRP and HMS) lines to knockdown *piwi* and *dpp* expression in ECs, respectively. Based on the numbers of the germaria carrying three or more SGCs, *c587-gal4* mediated *dpp* knockdown (TRP) can partially rescue the germ cell differentiation defects caused by *piwi* knockdown (VDRC) (Fig. 2.3J-L). *c587-gal4* mediated *piwi* knockdown by the HMS line yields stronger germ cell differentiation defects, which can be slightly and moderately repressed by *c587-gal4* driven expression of TRP and HMS *dpp* RNAi lines, respectively (Fig. 2.3L and Fig. 2.S4). *c587-gal4* driven expression of the *dpp* HMS line causes partial GSC loss,

but the expression of the *dpp* TRP line does not, suggesting that the HMS line might be stronger than the TRP line in knocking down *dpp* expression (Fig. 2.S4). Based on the finding that the germ cell differentiation defects caused by *piwi* knockdown can only be partially repressed by *c587-gal4* mediated *dpp* knockdown, the germ cell differentiation defects cannot be solely attributed to upregulated *dpp* expression in ECs (Fig. 2.S4). Taken together, we propose that *dpp* upregulation in *piwi* knockdown ECs contributes to, but is not one of the major causing factors, for germ cell differentiation defects.

Defective EGFR-MAPK signaling in ECs causes germ cell differentiation defects by upregulating *dally* expression and thus increasing BMP signaling, and also prevents the formation of long cellular processes [62, 208]. Although our results show that Piwi knockdown does not lead to *dally* upregulation (Fig. 2.3H and 2.3I), we wanted to confirm if Piwi is required to maintain EGFR-MAPK signaling in ECs by examining the expression of pERK, a phosphorylated and active form of MAPK, in the *piwi* knockdown ECs. In the control, pERK is strongly and specifically expressed in all ECs, but not in cap cells and follicle cells (Fig. 2.S5A). pERK is expressed at low levels in the remaining *piwi* knockdown ECs (Fig. 2.S5A-E). Although pERK immunofluorescence intensity in the *piwi* knockdown ECs decreases by 25-65% in comparison with the control ECs (Fig. 2.S5E), overall pERK levels might increase instead because the *piwi* knockdown ECs are often larger (Fig. 2.S5A-D). To determine if increasing MAPK activity affects GSC maintenance and differentiation, we used *c587-gal4* to drive the expression of a kinase-active *rolled* (*rl*, encoding MAPK in *Drosophila*) mutant, rl^{SEM}, in ECs [209]. Increasing MAPK activity does not have any obvious effect on GSC maintenance and CB differentiation (Fig. 2.S5). Interestingly, following *c587-gal4* mediated *piwi* knockdown by the three RNAi lines, ECs lose their long cellular processes (Fig. 2.S6). These results align well

with our earlier finding of no expression changes for *dally* in the *piwi* knockdown ECs, and also suggest that EGFR signaling is not the only pathway for maintaining EC cellular processes.

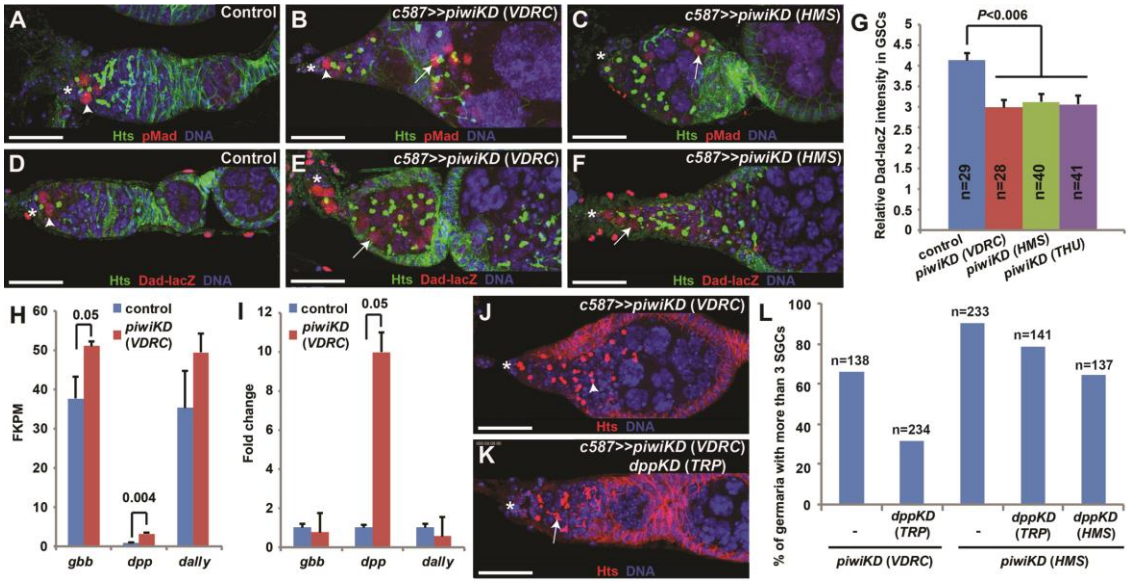

Figure 2.3. Piwi knockdown in ECs results in an elevation of BMP signaling in SGCs outside the GSC niche. Asterisks indicate the GSC niche. (**A**-**C**) Some SGCs (arrows) outside the GSC niche are positive for pMad in the *piwi* knockdown germaria (**B**, **C**) in addition to GSCs (arrowhead) in contrast with the control germarium in which only GSCs (arrowhead) are positive (**A**). (**D**-**G**) Some SGCs (arrows) outside the GSC niche are positive for *Dad-lacZ* in the *piwi* knockdown germaria (**E**, **F**) in contrast with the control germarium in which only GSCs (arrowhead) are positive (**D**). **G** shows quantification results on *Dad-lacZ* expression in GSCs. (**H**, **I**) RNA sequencing (**H**) and qRT-PCR (**I**) results show that mRNA expression levels for *dpp*, but not for *gbb* and *dally*, are significantly upregulated in the *piwi* knockdown ECs compared to the control ECs (FKPM stands for fragments per kilobase of exon per millions of reads). (**J**-**L**) *c587-gal4* mediated *dpp* knockdown can partially rescue the germ cell differentiation defects caused by *piwi* knockdown. **L** shows quantification results on percentages of germaria carrying

three or more SGCs among the *piwi* knockdown and *piwi dpp* knockdown germaria, which still contain at least one GSC. Scale bars: 25μm.

Piwi is required in ECs for repressing TE activity and preventing DNA damage

piRNAs have been shown to be required for silencing TE activity in both germ cells and somatic cells [120, 181, 182, 210]. One of the outcomes for elevated TE activity is DNA damage. Thus, we examined the expression of phosphorylated H2Av (γ-H2Av), a *Drosophila* equivalent of mammalian H2AX [211], in the control and *piwi* knockdown ECs, and quantified γ-H2Av-positive ECs. γ-H2Av has been shown to be associated with DNA double-strand breaks in *Drosophila* cells [211]. In the control germaria, less than 5% of the ECs are positive for γ-H2Av (Fig. 2.4A and 2.4C). In contrast, 8%-25% of the *piwi* knockdown ECs are positive for γ-H2Av depending on the RNAi lines (Fig. 2.4B and 2.4C). These results indicate that Piwi is required in ECs to prevent DNA damage.

To further determine if Piwi is required in ECs for silencing TE activity, we sequenced the RNAs from the purified GFP-labeled control and *piwi* knockdown ECs by fluorescence-activated cell sorting (FACS). In this study, we chose to examine two common somatic cell-specific TEs, *gypsy* and *zam*, and a germline-specific TE, *tart* [100]. Both *gypsy* and *zam* transcripts are drastically and significantly upregulated in the *piwi* knockdown germaria in comparison with the control (Fig. 2.4D and 2.4E). As expected, the germline-specific *tart* transcripts are not changed dramatically in the *piwi* knockdown germaria in comparison with the control (Fig. 2.4F). In addition, we also used the *gypsy-lacZ* reporter to verify the qRT-PCR

results. In the control germaria, *gypsy-lacZ* is not expressed (Fig. 2.4G). In contrast, it is dramatically upregulated in the *piwi* knockdown ECs by the three RNAi lines (Fig. 2.4H-J). These results further support the idea that Piwi is required in ECs to repress TE activity and prevent DNA damage.

Yb has been shown to regulate Piwi expression in TF and cap cells [33]. Indeed, in the *c587-gal4* mediated *Yb* knockdown germaria, Piwi protein expression in ECs and follicle cells is consistently downregulated (Fig. 2.S7A-C'). However, Yb protein expression in somatic cells, including cap cells, ECs and early follicle cells, remains unchanged in the *c587-gal4* mediated *piwi* knockdown germaria (Fig. 2.S7D-G). To further determine if Yb is also required in ECs to repress TE activity, we examined the expression of *gypsy-lacZ* in the *Yb* knockdown germarium. As previously reported, Yb is also expressed in all ovarian somatic cells, including ECs (Fig. 2.4K). c587-*gal4* driven expression of two independent *Yb* RNAi lines can efficiently eliminate Yb expression in cap cells, ECs and early follicle cells (Fig. 2.4L and 2.4M). Interestingly, *gypsy-lacZ* expression is upregulated in the *Yb* knockdown cap cells and ECs, indicating that Yb is also required in somatic cells to silence TEs (Fig. 2.4N and 2.4O). However, *gypsy-lacZ* expression appears to be lower in the *Yb* knockdown ECs than in the *piwi* knockdown ECs (Fig. 2.4H-J, 2.4N and 2.4O). Although most of the *Yb* knockdown germaria contain normal numbers of GSCs and SGCs (Fig. 2.4N and 2.4O), approximately 25% of the *Yb* knockdown germaria carry three or more SGCs (Fig. 2.S7H-K). These results suggest that Yb is also required in ECs to repress TEs and promote germ cell differentiation.

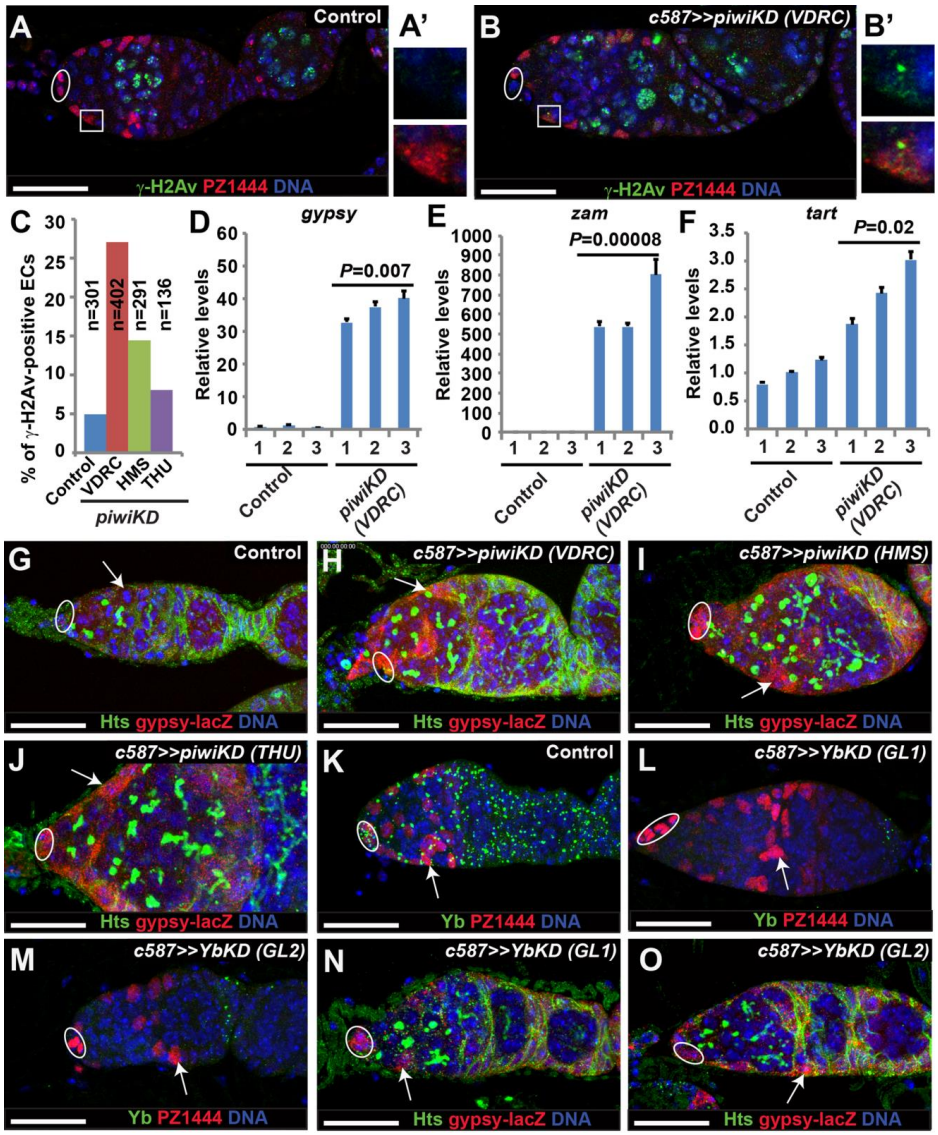

Figure 2.4. Piwi is required in ECs to repress transposon activity and thus prevent DNA damage. Ovals highlight cap cells. (**A**-**C**) Somatic *piwi* knockdown (**B**) causes an increase in γ-H2Av-positive and *PZ1444*-positive ECs in comparison with the control (**A**) in which *PZ1444*-positive ECs are negative for γ-H2Av. **A'** and **B'** highlight *PZ1444*-positive ECs in **A** and **B**, respectively. **C** represents quantitative results on γ-H2Av-positive ECs. (**D**-**F**) Quantitative RT-PCR results show that the transcripts for *gypsy* (**D**) and *zam* (**E**), but not *tart*, increase significantly in the *piwi* knockdown ECs in comparison with the control. (**G**-**J**) The *piwi*

knockdown ECs (arrows, **H**-**J**) elevate *gypsy-lacZ* expression in comparison with the control ECs (arrow, **G**). (**K**-**M**) The *Yb* knockdown ECs (arrows, **L** and **M**) lose Yb protein expression in comparison with the control ECs (arrow, **K**). (**N**, **O**) The *Yb* knockdown ECs (arrows) elevate *gypsy-lacZ* expression. Scale bars: 25μm.

Piwi is required intrinsically to maintain germ cells before adulthood

Since Piwi is expressed in all the germ cells, including GSCs, we then used *nanos-gal4VP16* (*nos-gal4*) to specifically knock down *piwi* in germ cells to determine if Piwi is also required intrinsically for GSC maintenance. The *nos-gal4* driver is expressed specifically in germ cells from PGCs to adult germ cells, including GSCs [36]. In contrast with the control third-instar female gonad (Fig. 2.5A), *nos-gal4* driven expression of the two independent *piwi* RNAi strains, HMS and THU, leads to a reduction in PGC numbers in the female gonads, indicating that Piwi is required for PGC proliferation, maintenance or both (Fig. 2.5B-D). Furthermore, germ cell-specific *piwi* knockdown germaria in newly emerged adults show a complete loss of all germ cells, including GSCs (Fig. 2.5E and 2.5F). The GSC establishment takes place at the transitional period from the third instar-larval stage to the pupal stage. These results indicate that Piwi is required intrinsically to control PGC maintenance and/or GSC establishment.

To further explore whether other piRNA components are also required for GSC maintenance before adulthood, we used *nos-gal4* driven expression of RNAi against *armi* and *aub* to inactivate their function throughout germ cell development. Germ cell-specific *armi* or

aub knockdown by two independent RNAi lines for each gene leads to a dramatic reduction in nuclear Piwi protein expression in germ cells, but does not affect nuclear Piwi expression in somatic cells (Fig. 2.5G-I). In addition, germline-specific *armi* or *aub* knockdown also causes the full penetrance of female sterility. These results suggest that both of them are efficiently knocked down in the germline because they are required for Piwi nuclear localization and to prevent the activation of meiotic cell cycle checkpoints caused by transposon-induced DNA damage [123]. However, the *armi* or *aub* knockdown germaria from newly eclosed females still contain two or three GSCs in their germaria, indicating that they are not required intrinsically for early germ cell development and GSC formation (Fig. 2.5G-I). Consistently, newly eclosed $armi^{72.1}/armi^{1}$ and aub^{HN2}/aub^{QC42} mutant females also maintain two or three GSCs in their germaria, and dramatically decrease nuclear Piwi expression in GSCs and their progeny (Fig. 2.5J-L). Since these mutants carry strong loss-of-function mutations in *armi* or *aub* [115, 123], these results further support that Armi and Aub are dispensable for germ cell development before adulthood. Taken together, our results indicate that Piwi controls early germ cell development, GSC formation or both independently of Armi and Aub.

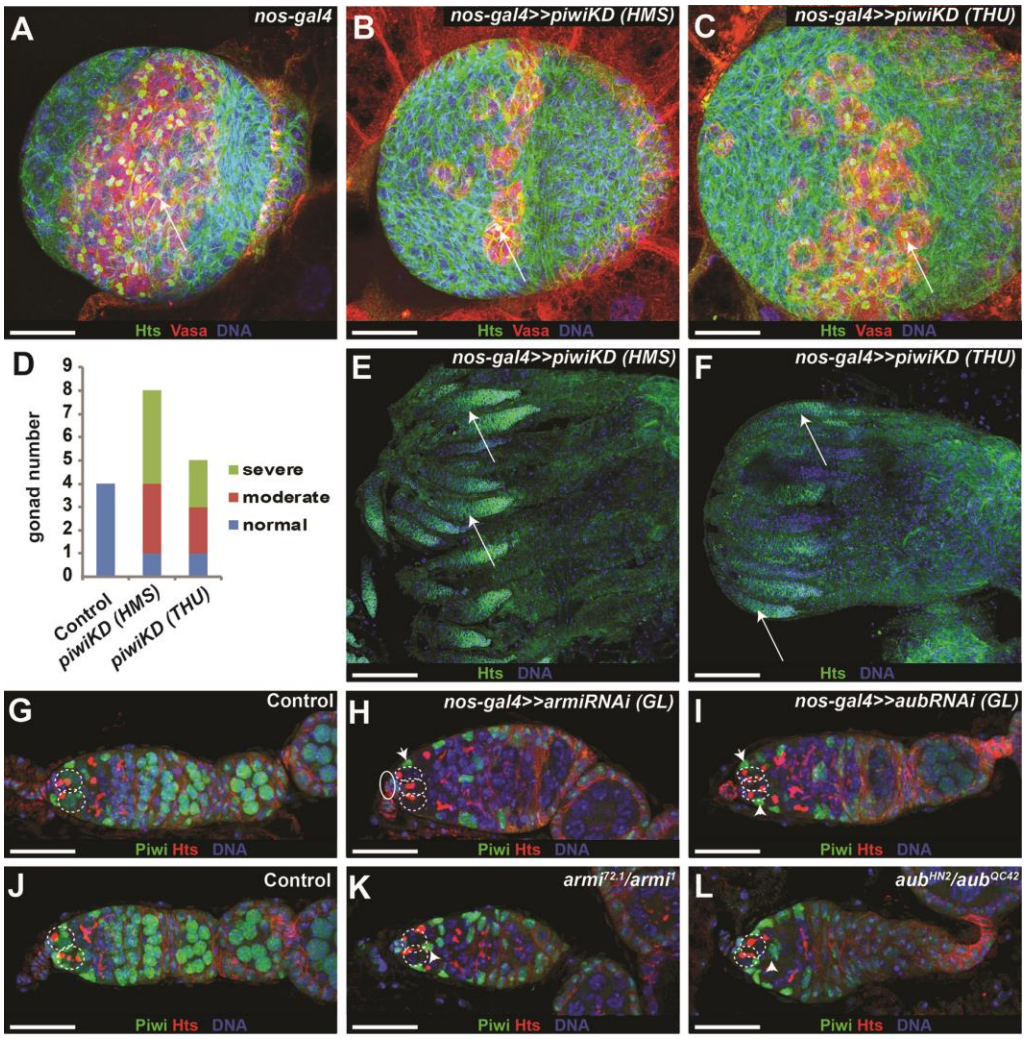

Figure 2.5. Piwi is required intrinsically to maintain PGCs or control GSC formation. (**A-D**) *nos-gal4* driven *piwi* knockdown (**B**, **C**) leads to a reduction in PGC number in the third-instar larval gonads in comparison with the control (**A**). PGCs (arrows, **A-C**) are positive for Vasa (red) and also carry a spectrosome). **D** quantifies normal (**A**), moderate (**C**) and severe (**B**) phenotypes based on PGC numbers. (**E**, **F**) *nos-gal4* driven *piwi* knockdown leads to complete germ cell loss in the germaria of the newly eclosed females, leaving empty germaria (arrows). (**G-I**) *nos-gal4* driven *armi* (**H**) or *aub* (**I**) knockdown decreases nuclear Piwi expression in germ cells, but does not affect GSCs because the germaria still contain two or three GSCs (broken lines) as the control germarium (**G**). Nuclear Piwi expression remains in ECs (arrowheads) of the

knockdown germaria (**H**, **I**). (**J-L**) *armi* (**K**) or *aub* (**L**) mutant germaria decrease nuclear Piwi expression in germ cells, but still have two or three GSCs as the control germarium (**J**). Nuclear Piwi expression remains in mutant ECs (arrowheads; **K**, **L**). Scale bars: 75μm (**E** and **F**); 25μm (**A-C** and **G-L)**.

Piwi is required intrinsically to control GSC maintenance and germ cell differentiation in the adult ovary

To determine if Piwi is required in the adult germline to maintain GSCs, we used the flip-out system, in which a transcriptional stop sequence flanked by two FRT sites is inserted between the *nos* promoter and *gal4VP16*, to activate the expression of RNAi lines along with the GFP reporter specifically in germ cells after heatshock treatments of adult females (Fig. 2.6A). In the control ovaries, GFP-positive GSCs detected 1 day after heatshock (1d AHS) remain in the niche for additional three weeks (Fig. 2.6B and 2.6C). The GFP-marked *piwi* knockdown GSCs can be readily detected in the germaria 1d AHS (Fig. 2.6D and 2.6E). In contrast, most of the GFP-marked *piwi* knockdown GSCs are lost three weeks AHS, and consequently over 30% of the *piwi* knockdown germaria have completely lost GSCs (Fig. 2.6F-H). In addition, more undifferentiated SGCs also accumulate in the *piwi* knockdown germaria three weeks AHS, indicative of germ cell differentiation defects (Fig. 2.6I and 2.6J). Interestingly, some SGCs outside the GSC niche are GFP-negative and also Piwi-negative, which is caused by the failure in *nos-gal4* driven GFP expression due to an unknown reason (Fig. 2.6I and 2.6J). These results demonstrate that Piwi is required in adult germline for GSC maintenance and germ cell differentiation.

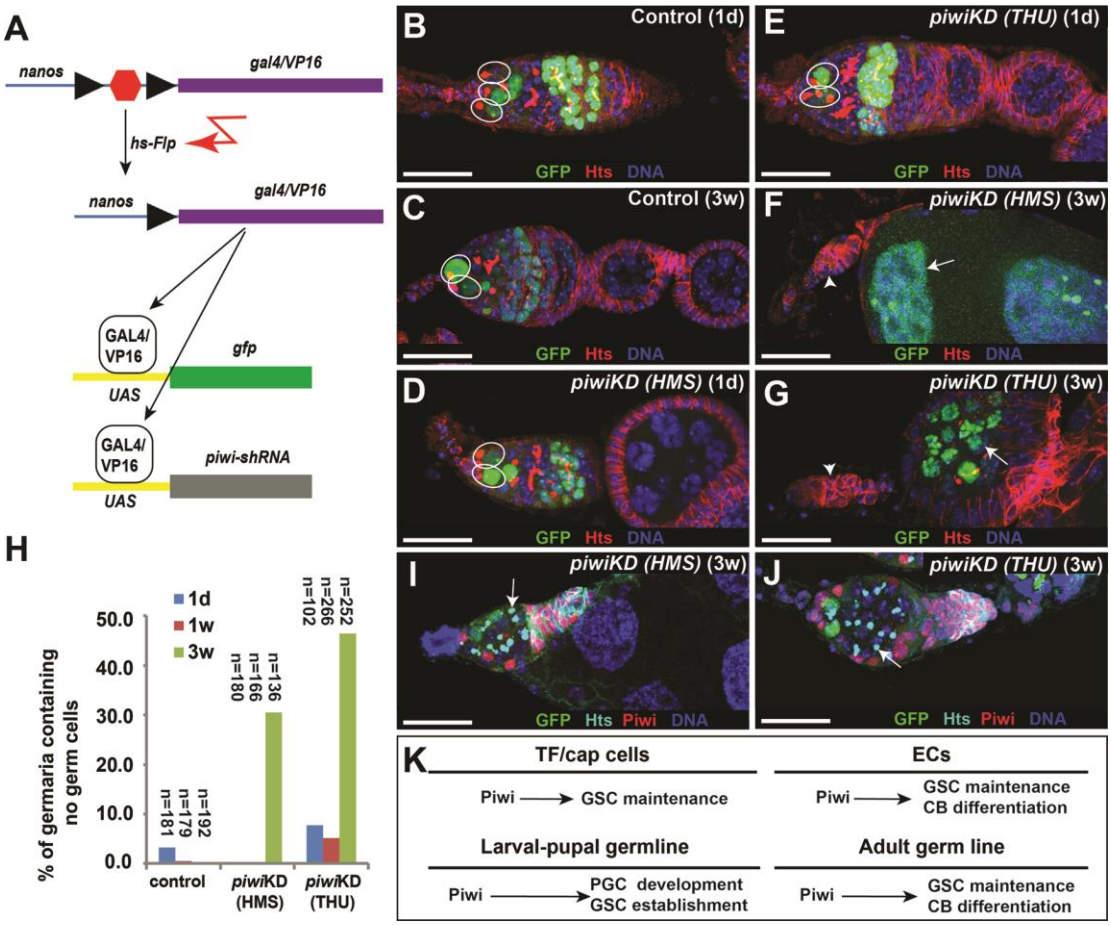

Figure 2.6. Piwi is required intrinsically to maintain GSCs and promote germ cell differentiation. (**A**) A flip-out strategy for *nos-gal4* driven *piwi* knockdown specifically in adult GSCs and their progeny, which are also labeled by GFP expression. (**B**, **C**) GFP-marked control GSCs (circles) detected 1d AHS (**B**) are still maintained 3 w AHS (**C**). (**D**-**H**) GFP-marked *piwi* knockdown GSCs (circles) detected 1d AHS (**D**, **E**) are lost 3 w AHS (**F**, **G**). Consequently, the *piwi* knockdown germaria (arrowheads) completely lose their germ cells, and some marked GSCs have developed into GFP-positive egg chambers (arrows). **H** represents the quantitative results on the germaria containing no germ cells. (**I**, **J**) *piwi* knockdown germaria accumulate excess SGCs (arrow), which are negative for Piwi protein though GFP-negative, outside the GSC niche 3w AHS. As expected, all somatic cells are still positive for Piwi. (**K**) A working model for the roles of Piwi in TF/cap cells, ECs and germ cells. Scale bars: 25μm.

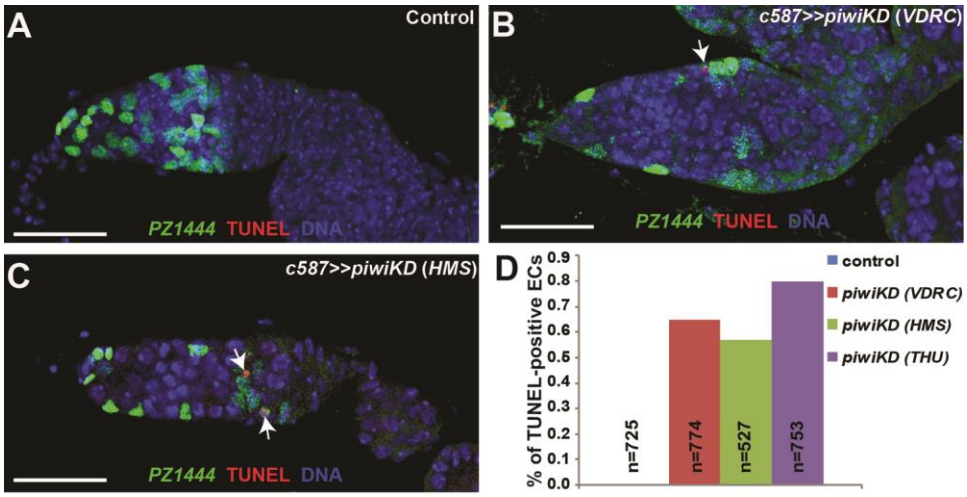

Figure 2.S1. Piwi knockdown increases apoptosis in ECs. (**A**) PZ1444-positive control ECs are negative for TUNEL labeling. (**B**, **C**) Apoptotic PZ1444-positive ECs (arrows) are detected in the *piwi* knockdown germaria by VDRC (**B**) and HMS (**C**) RNAi lines. The dying ECs appear to show low PZ1444 expression. (**D**) Quantification results of TUNEL-positive ECs in control and *piwi* knockdown germaria. Scale bars: 25μm.

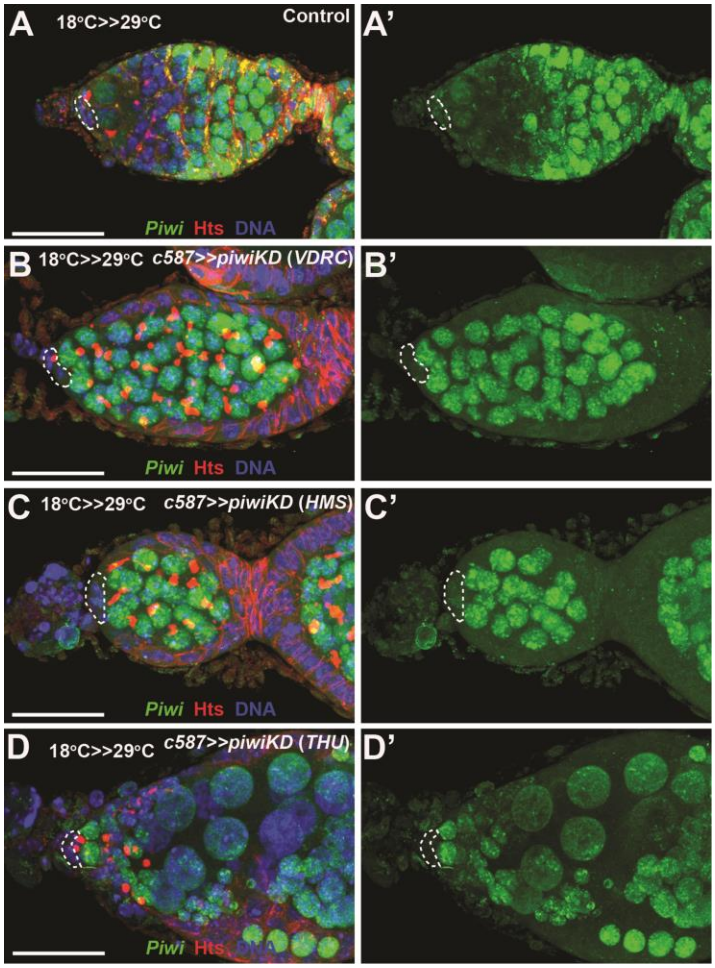

Figure 2.S2. *c587-gal4* drives expression of *piwi* RNAi in adult cap cells. (**A, A'**) Piwi is expressed in cap cells (broken lines) at low levels. (**B-D'**) *c587-gal4* driven expression of VDRC (**B, B'**), HMS (**C, C'**) and THU (**D, D'**) *piwi* RNAi lines reduces Piwi protein expression in adult cap cells as well as in ECs. Scale bars: 25μm.

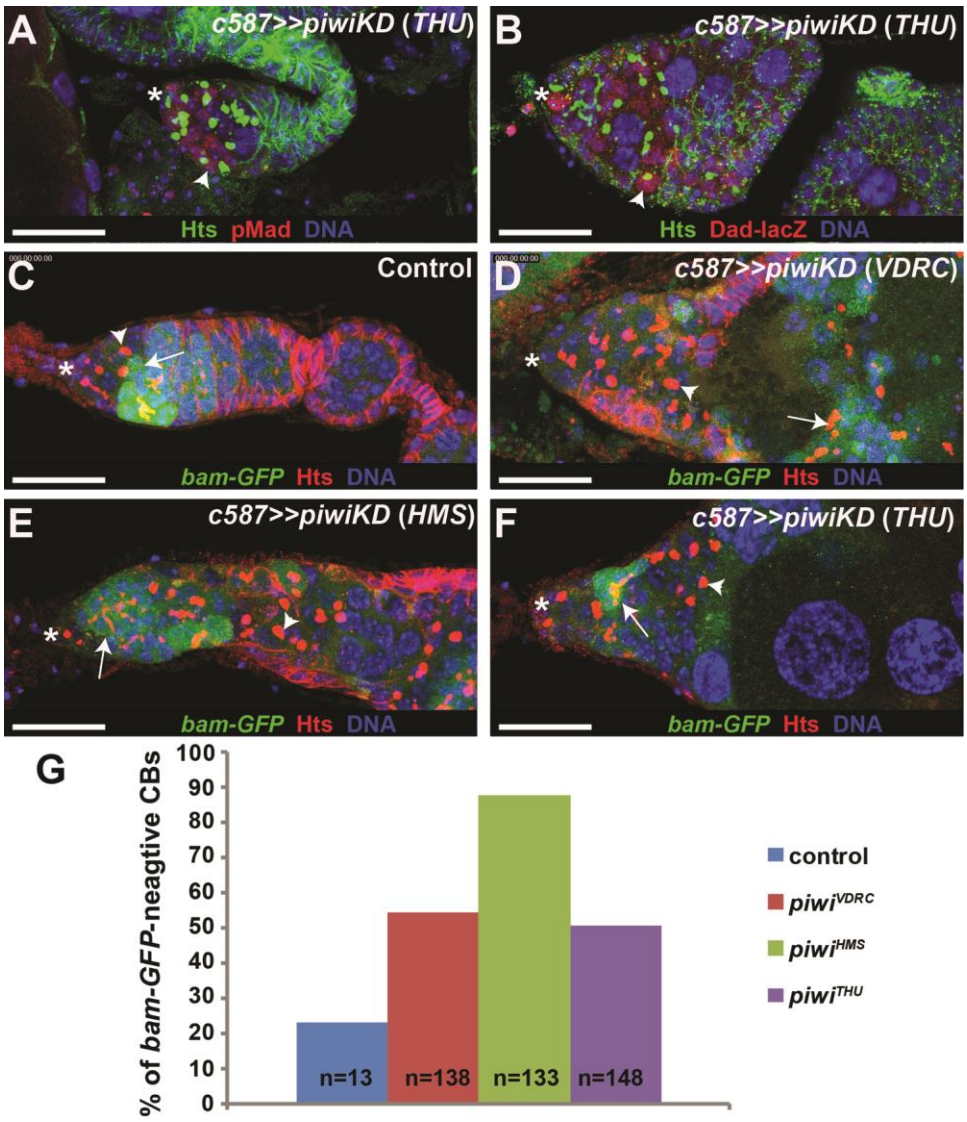

Figure 2.S3. Piwi is required in ECs to prevent BMP signaling in differentiated germ cells. Cap cells are highlighted by asterisks. (**A**, **B**) *c587-gal4* mediated *piwi* knockdown by the THU line results in upregulated pMad (**A**) and Dad-lacZ (**B**) expression in SGCs a few cells away from cap cells. (**C**) *bam-GFP* is repressed in GSCs and upregulated in differentiated germ cell cysts (arrow) of the control germarium. (**D**-**F**) *c587-gal4* mediated *piwi* knockdown by three *piwi* RNAi lines causes repression of *bam-GFP* expression in some SGCs (arrowheads) outside the

GSC niche. Differentiated cysts (arrows) still maintain high *bam-GFP* expression. **G** shows quantification results of *bam-GFP*-negative CBs. Scale bars: 25μm.

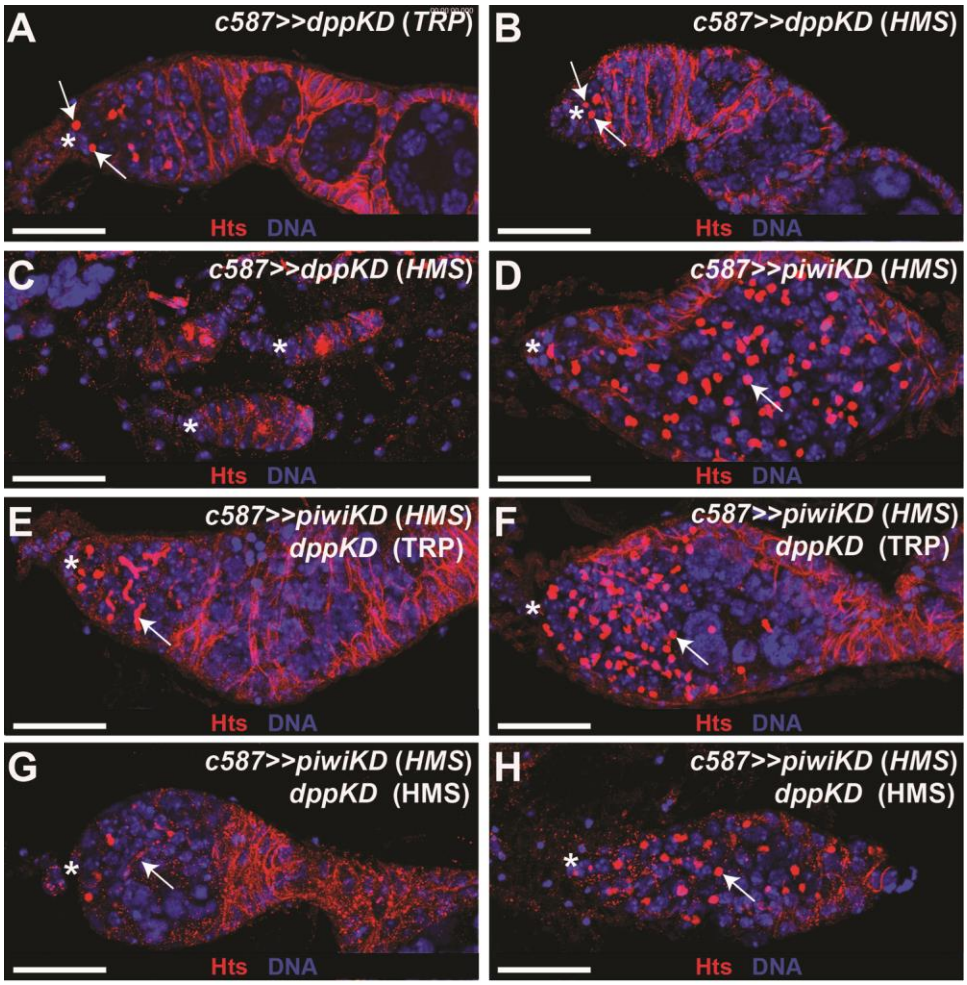

Figure 2.S4. *dpp* upregulation in *piwi* knockdown ECs might not be the major factor causing germ cell differentiation defects. Asterisks indicate the GSC niche. (**A-C**) *c587-gal4* mediated *dpp* knockdown by TRP (**A**) and HMS (**B**, **C**) lines does not affect GSC maintenance and differentiation because the knockdown germaria still maintain two GSCs (arrows). However, some *dpp* knockdown germaria (**C**) by the HMS line, but not by the TRP line, completely lose

their germ cells including GSCs. (**D-H**) *c587-gal4* mediated *dpp* knockdown suppresses the germ cell differentiation defects in some *piwi* knockdown germaria (**E**, **G**) but not in the other germaria (**F**, **H**) in comparison with the germ cell differentiation defects in the *piwi* knockdown germaria (**D**). Arrows in **D**, **F** and **H** point to spectrosomes, whereas those in **E** and **G** indicate branched fusomes. Scale bars: 25μm.

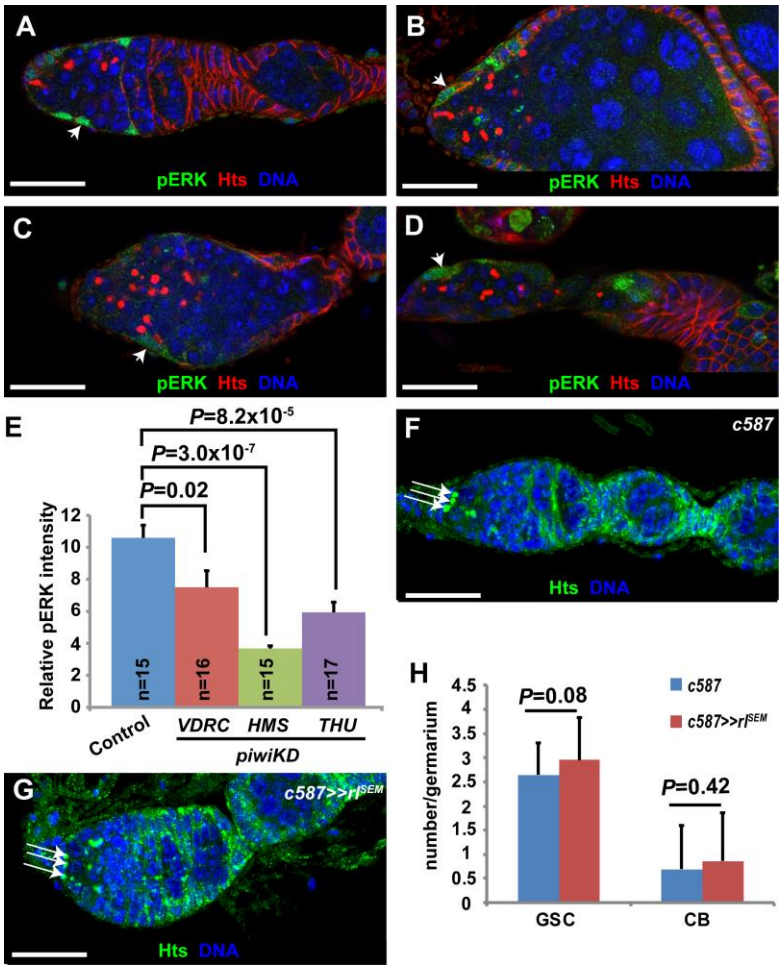

Figure 2.S5. pERK activity in *piwi* knockdown ECs. (**A**) pERK is specifically expressed in ECs (one by arrowhead) of the control germarium. (**B**-**E**) *c587-gal4* mediated *piwi* knockdown ECs (arrowheads) are often larger and show lower pERK fluorescence intensity. **E** shows quantification results on pERK intensity. (**F**-**G**) *c587-gal4* mediated rl^{SEM} expression does not affect GSC and CB numbers (arrows indicate GSCs). **H** shows that there are no significant differences in GSCs and CBs between control and rl^{SEM} -expressing germaria. Scale bars: 25μm.

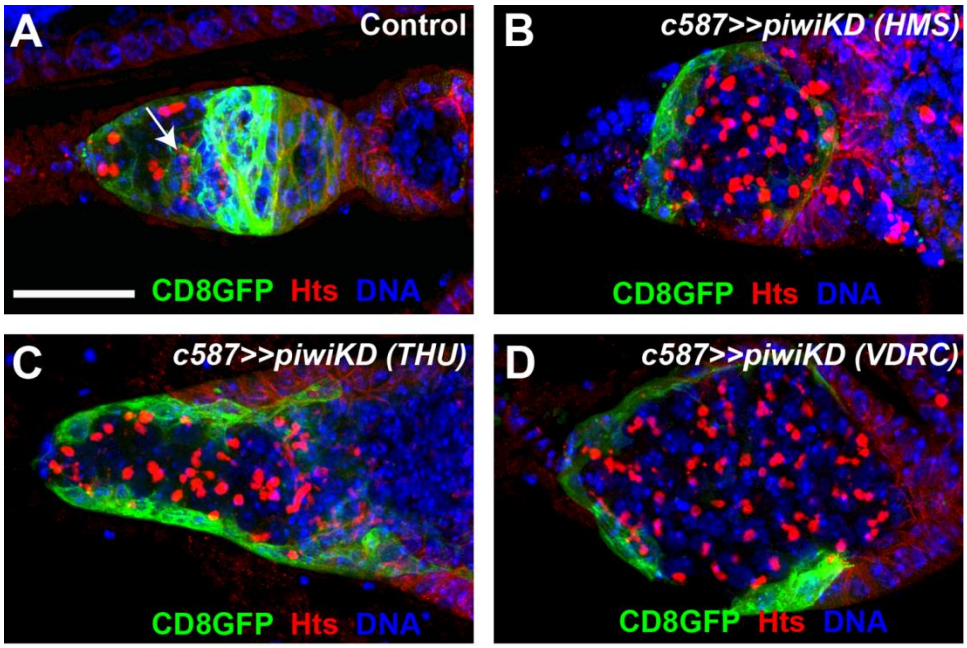

Figure 2.S6. Piwi knockdown in ECs disrupts the formation of their long cellular processes. (**A**) *c587-gal4* mediated *CD8GFP* expression highlights long EC cellular processes (arrows) wrapping CBs, mitotic cysts and 16-cell cysts in the control germarium. (**B-D**) In the *c587-gal4* mediated *piwi* knockdown germaria by three RNAi lines, *HMS* (**B**), *THU* (**C**) and *VDRC* (**D**), there are no long-GFP-positive cellular processes wrapping differentiated germ cells. Scale bars: 25μm.

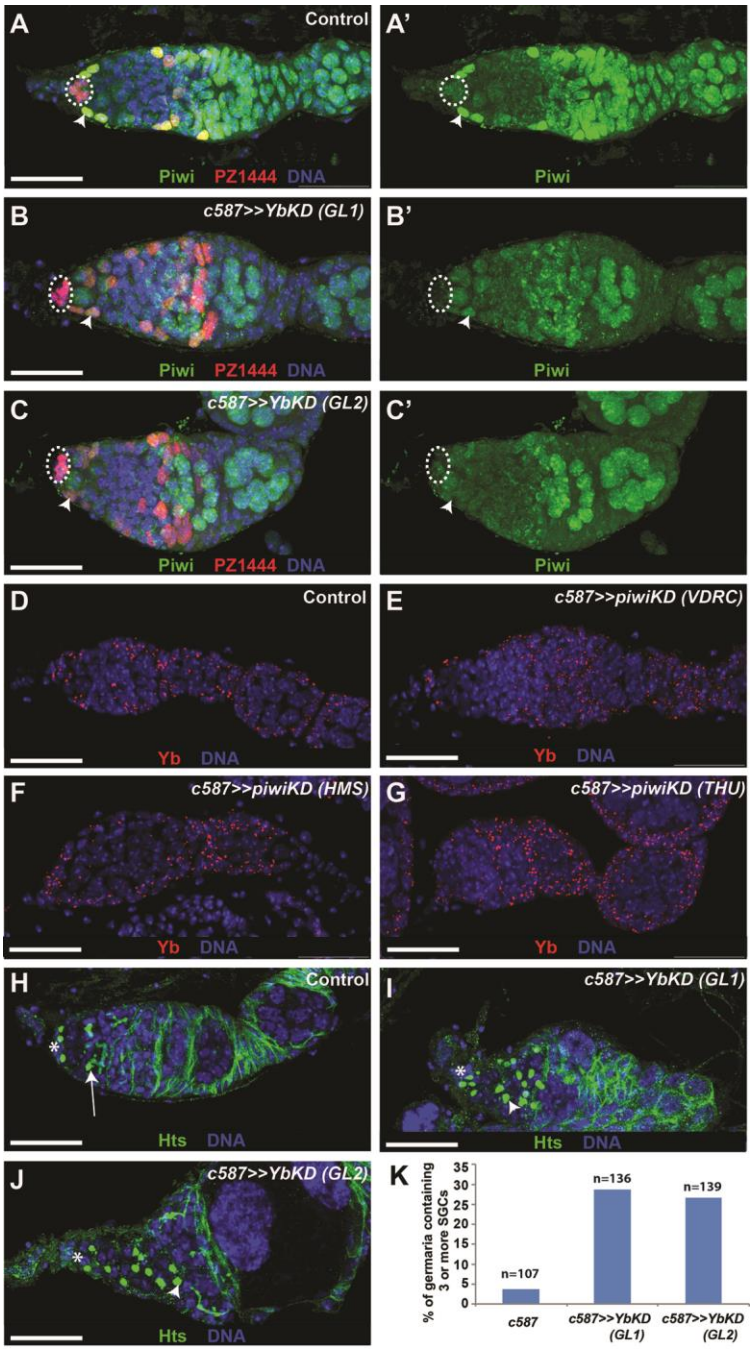

Figure 2.S7. Yb is required in ECs to promote germ cell differentiation. The GSC niche is highlighted by broken lines (**A-C'**) or the asterisk (**H-J**). (**A-C'**) *c587-gal4* mediated *Yb* knockdown by two RNAi lines, *GL1* (**B**, **B'**) and *GL2* (**C**, **C'**), leads to a Piwi protein expression reduction in cap cells (broken lines), ECs (arrowheads) and early follicle cells in comparison

with the control (**A**, **A'**). (**D-G**) *c587-gal4* mediated *piwi* knockdown by three RNAi lines, *VDRC* (**E**), *HMS* (**F**) and *THU* (**G**), has no effect on YB protein expression in cap cells, ECs and early follicle cells in comparison with the control (**D**). (**H**) The control germarium contains three GSCs and differentiated cysts (arrow). (**I-K**) *c587-gal4* mediated *Yb* knockdown causes an accumulation of excess SGCs (arrowheads) in the germarium. **K** represents the quantitative results on the germaria carrying three or more SGCs. Scale bars: 25μm.

2.5 Discussion

Although the primary piRNA pathway is known to operate in *Drosophila* ovarian somatic cells to repress TE activity, its biological importance in *Drosophila* oogenesis is not well understood. Piwi, one of the key components in the primary piRNA pathway, has been shown to function in TF/cap cells to control GSC maintenance [30, 188, 189]. In this study, we have revealed a novel role of Piwi in ECs to control GSC lineage differentiation and additional roles in the germline for GSC formation and maintenance (Fig. 2.6K). *piwi* knockdown in somatic cells results in defective GSC maintenance, defective germ cell differentiation as well as increased TE activity and DNA damage. The *dpp* upregulation contributes to the germ cell differentiation defects caused by somatic *piwi* knockdown. In addition, we have also shown that Piwi is required in PGCs to control PGC maintenance, GSC formation or both, and is also required in adult germline to maintain GSCs and promote germ cell differentiation (Fig. 2.6K). Therefore, our genetic results argue strongly that Piwi functions in germline to maintain PGCs and GSCs as well as to promote germ cell differentiation. Therefore, we have revealed new

functions of Piwi in multiple cell types to maintain GSCs and promote germ cell differentiation (Fig. 2.6K).

Piwi is required in ECs and germ cells to promote germ cell differentiation

Recent studies have shown that ECs play an important role in promoting germ cell differentiation by repressing BMP signaling [184]. Thus far, genes identified to be important in ECs for germ cell differentiation repress the expression of either *dally* or *dpp*, thereby preventing BMP signaling in ECs. EGFR signaling has been proposed to be responsible for directly repressing *dally* expression in ECs, but is dispensable for EC survival [62]. In addition, recent studies have shown that Rho signaling and Eggless are required in ECs for the repression of *dally* expression, and are also required for EC survival and the maintenance of long EC cellular processes [185, 186]. Lsd1, Rho signaling and Eggless have been shown to be required to repress *dpp* transcription [60, 185, 186]. In this study, we have shown that Piwi is required in ECs for *dpp* repression but is dispensable for *dally* repression. In addition, it is required in ECs for maintaining their survival and long cellular processes. Our genetic results suggest that *dpp* upregulation contributes to the germ cell differentiation defects caused by Piwi knockdown in ECs, but does not play a major role. In addition, Piwi is required in somatic ovarian cells to repress TE activity and prevent transposon-induced DNA damage. However, it remains unclear if *dpp* upregulation and the loss of ECs and their long cellular processes are caused by DNA damage, and how Piwi is involved in repressing BMP signaling activity in differentiated germ cells via repression of *dpp* expression in ECs.

Piwi has previously been demonstrated to be required intrinsically for promoting GSC division [33]. Piwi is expressed in GSCs and their differentiated progeny [33]. This study has shown that germline-specific knockdown of Piwi function in the adult ovary leads to the accumulation of undifferentiated single germ cells, revealing a new intrinsic role of Piwi in controlling germ cell differentiation (Fig. 2.6K). Piwi has been shown to be involved in the piRNA pathway and epigenetic regulation. In the future, it will be important to determine if the piRNA pathway, epigenetics or both play a role in the regulation of germ cell differentiation.

Piwi is required in both somatic cells and germ cells to maintain GSC lineage

Although Piwi is generally expressed in almost all somatic cells and germ cells of the *Drosophila* ovary, the previous studies proposed that Piwi acts in TF/cap cells to control GSC self-renewal [30, 188, 189]. In this study, we have confirmed the somatic role of Piwi in GSC maintenance, and have also revealed new roles of Piwi to maintain PGCs before adulthood and GSCs after adulthood. In addition, our temperature shift experiments have shown that Piwi is also required in adult somatic cells, TF/cap cells, ECs or both, to maintain GSCs. Interestingly, RNAi-mediated knockdown of Piwi function in adult somatic cells only produces a moderate GSC loss phenotype in comparison with the severe GSC loss phenotype of *piwi* mutants, suggesting that Piwi might also function in other cell types to maintain GSCs. In the future, it will be important to determine how Piwi functions in TF/cap cells and ECs to maintain GSC self-renewal. Therefore, our study has not only confirmed the somatic role of Piwi in controlling GSC self-renewal but also has suggested its function in additional cell types to maintain GSCs (Fig. 2.6K).

Although Piwi has been shown to be required to control PGC formation and GSC division [33, 190], it remains unclear if Piwi is required intrinsically to maintain PGCs and GSCs. In this study, we have revealed critical roles of Piwi in different developmental stages of germ cells. First, Piwi is required in the developing female gonad to control PGC proliferation, survival or both because germline-specific Piwi knockdown leads to a reduction in PGC number in third-instar female larval gonads. Second, Piwi is required in PGCs to control their survival or GSC formation because germline-specific *piwi* knockdown leads to a complete elimination of germ cells including GSCs in newly eclosed adult females. Interestingly, germline-specific knockdown of either *armi* or *aub*, two of which work with *piwi* to control piRNA biogenesis, fails to produce any GSC loss phenotype in newly eclosed adult females, suggesting that Piwi controls PGC proliferation and survival or GSC formation possibly independently of Armi and Aub, possibly piRNAs. Piwi has been shown to physically interact with HP1a to epigenetically control gene expression in somatic tissues [141]. In addition, *piwi* genetically interacts with *certo*, encoding a chromodomain-containing protein, to control GSC maintenance [212]. Our findings are consistent with the notion that Piwi controls early germ cell development perhaps via epigenetics. Third, Piwi is required in adult GSCs for their maintenance because germline-specific knockdown in the adult ovary also causes a moderate GSC loss phenotype. Therefore, we propose that Piwi functions in multiple stages of germline development to control PGC proliferation and survival, and GSC maintenance (Fig. 2.6K).

Chapter 3: Aubergine and Bam Cooperatively Control Germline Stem Cell Lineage Differentiation and Germline Genome Stability

3.1 Abstract

piRNAs are known to be important in germ cells for maintaining genome integrity by repressing TEs. In the *Drosophila* ovary, GSCs continuously divide to generate CBs, which then form mitotic cysts and eventually 16-cell cysts. However, it remains unclear if piRNA components have additional functions in the regulation of GSC lineage development. In this study, we show that the piRNA pathway component Aub works cooperatively with the master GSC differentiation factor Bam to promote GSC progeny differentiation and that Bam has a new role in repressing TEs. Our genetic results have demonstrated that Aub is required intrinsically to control GSC self-renewal by preventing DNA damage-induced checkpoint activation. In addition, our genetic results have also revealed a novel role of Aub in promoting Bam function and thus GSC differentiation. Moreover, Aub is required to maintain *bam* transcription and thus Bam protein expression in mitotic cysts. Bam and Aub physically interact with each other in yeast and *Drosophila* S2 cells, and they are co-localized in the nuage of mitotic germ cells, where piRNA biogenesis and TE repression take place. Finally, Bam is required for repressing the activities of TEs, but is dispensable for overall piRNA production. Therefore, this study has revealed a new role of Aub in promoting early germ cell differentiation and a novel role of Bam in repressing TEs in early mitotic germ cells.

3.2 Introduction

Germ cells are responsible for transmitting genomes from generation to generation in high fidelity. TEs, which are abundant in eukaryotic genomes, can generate double-stand DNA breaks and mutations during transposition, and thus it is important to silence TE activities in germ cells [118, 177, 213, 214]. The adult *Drosophila* ovary has been demonstrated to contain GSCs for continuous production of eggs throughout its lifetime as in other invertebrate and low vertebrate ovaries [215, 216]. PIWI-associated small RNAs or piRNAs are abundantly produced from the transcripts of TEs and repetitive elements to silence TEs [88, 99, 113, 217, 218]. Inactivation of the piRNA pathway leads to meiotic arrest and disruption of late germ cell development from *Drosophila* to mammals. However, the biological roles of piRNAs in the regulation of GSC maintenance and early GSC lineage differentiation remain poorly understood. In this study, we show that the piRNA pathway component Aub is essential for GSC maintenance by preventing DNA damage-induced checkpoint activation, and that it has a new role in GSC lineage differentiation.

The *Drosophila* piRNA pathway has recently been subjected to intense studies, and many piRNA pathway components have been identified from studies on *Drosophila* oogenesis and genome-wide RNAi-mediated screens [99, 118, 120, 219, 220]. *piwi* was identified for its role in somatic niche cells to maintain GSCs in the *Drosophila* ovary [30, 188, 218]. Similarly, many other piRNA pathway components were identified by their essential roles in the regulation of *Drosophila* oogenesis, including *aub*, *vasa*, *armi*, *spn-E*, *zuc*, *squ* and *cuff* [112, 115, 121, 124, 125, 221-225]. Tudor and tudor-domain containing proteins are also important for piRNA biogenesis [64, 110, 220, 226-229]. Recent genome-wide RNAi screens identified additional

piRNA components, including RNA splicing, mRNA transport and nuclear pore components [97, 230-232]. piRNA production in germ cells requires the Argonaute family members, Piwi, Aub and Ago3 [88-90, 116]. piRNAs in germ cells are mostly processed from the transcripts transcribed from both DNA strands of the piRNA clusters [88, 100]. Piwi and Aub mainly bind antisense piRNAs, while Ago3 binds sense piRNAs, leading to a feed-forward piRNA amplification loop, known as the "Ping-Pong" model [88, 89]. Interestingly, *Drosophila* ovarian somatic cells also produce piRNAs from only one DNA strand of other piRNA clusters, which requires Piwi, Yb, Mael, Vret and Eggless, but not Aub, Ago3 and Vasa [40, 63, 64, 88, 100-102, 107, 108]. In this study, we have revealed a novel function of the master GSC differentiation factor Bam in repressing TEs in early germ cells.

The adult *Drosophila* ovary is composed of 12-16 ovarioles, which each contains 2-3 GSCs at the tip, also known as the germarium [233]. GSCs continuously generate CBs, which divide synchronously without cytokinesis to form mitotic cysts (2-cell, 4-cell and 8-cell cysts) and eventually16-cell cysts. Each ovariole contains only two or three CBs and mitotic cysts, which represent a minor population of cells. *bam* is transcriptionally repressed by niche-activated BMP signaling in GSCs, but is then upregulauted in CBs and mitotic cysts [29, 34, 195]. Bam protein can be readily detected in mitotic germ cells [234]. A mutation in *bam* can completely block CB differentiation, while forced *bam* expression in GSCs causes their rapid differentiation, indicating that Bam is necessary and sufficient for GSC lineage differentiation [235, 236]. Bam works with Bgcn and Sxl to repress *nos* expression in mitotic cysts [70, 74]. In addition, Bam also interacts with eIF4A, regulating the balance between GSC self-renewal and differentiation [44]. Here, this study has also identified a novel function of Bam in TE repression in mitotic cysts.

3.3 Experimental Procedures

Drosophila strains and culture

The *Drosophila* stocks used in this study include: aub^{QC42}, aub^{HN2}, *Df(2L)BSC145* (a deficiency for *aub*), *hs-Flp, FRT40A arm-lacZ*, $bam^{\Delta 86}$, lok^{p6}, *bam-HA-bam3'UTR* (kindly provided by Dr. X. Chen), *UAS-aubRNAi* (HMS00611), *UAS-aubRNAi* (JF01390), *UAS-aubRNAi* (GL00076), $armi^{1}$, $armi^{72.1}$ (kindly provided by Dr. W. Theurkauf) $piwi^{1}$ and $piwi^{2}$ *(*kindly provided by Dr. H. Lin*)* The new EMS-induced bam^{E5} mutant was generated and kindly provided by J. Beeler, S. Hughes and S. Hawley. Flies were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified. To induce mitotic recombination, flies were heat shocked at 37°C twice for 1 hour daily for 3 consecutive days and then maintained at 25°C for 10 days, 21 days and 30 days before dissection and immunostaining.

Immunohistochemistry

Immunohistochemistry was performed according to our previously published procedures [16, 28]. The following antibodies were used in this study: mouse monoclonal anti-Hts antibody (1:50, DSHB), rabbit polyclonal anti-β-galactosidase antibody (1:100, Cappel), mouse monoclonal anti-Bam antibody (1:50, DSHB), rabbit polyclonal anti-pS137 H2AvD antibody (1:100, Rockland), rabbit polyclonal anti-HA antibody (1:100, Sigma), rabbit polyclonal anti-Myc antibody (1:300, sigma), rat polyclonal anti-Vasa antibody (1:50, DSHB), mouse monoclonal anti-Aub antibody (1:300, kindly provided by Dr. H. Siomi), mouse monoclonal anti-Mael antibody (1:100, kindly provided by Dr. H. Siomi), rabbit anti-Anillin (kindly

provided by Dr. C. Field) and chicken polyclonal anti-GFP antibody (1:200, Invitrogen, #A10262). All images were taken with a Leica TCS SP5 confocal microscope.

S2 cell transfection and immunoprecipitation

S2 cells were cultured in Hyclone SFX medium (Thermo Scientific) to $4x10^6$ cells/ml in 6-well plates, and were then transfected with 4μg DNA per well using cellfectin II reagent (Life Technologies). The cells were collected 2 days after transfection, and were then washed with cold PBS buffer before their lysis. Anti-Flag M2-agrrose affinity gel (Sigma) was added to the lysate, and was further incubated overnight at 4°C with rotation. After anti-flag M2-agrrose affinity gel was pelleted down, the pulldown proteins were eluted with 3xFlag peptides (Sigma). Total lysate and elute were denatured with in 2xSDS with 3% 2-Mercaptoethanol at 95°C for 10 minutes and loaded for western blotting assay.

Small RNA Sequencing and piRNA Analysis

Total RNAs from the wild-type and *bam* mutant ovaries were extracted with Trizol and further purified by organic extraction followed by isopropanol precipitation. Small RNAs were sequenced by Illumina Hiseq. After trimming adapters from 51-base single-end small RNA illumina reads and removing rRNA reads, the remaining reads longer than 15nt were aligned to the dm3 UCSC genome using tophat (2.0.9). Genomic locations of piRNA clusters were taken from the piRNABank (http://pirnabank.ibab.ac.in) and converted to the current genome assembly's coordinates using the UCSC liftOver tool. The counts of aligned reads corresponding

to putative piRNA (reads with lengths between 25 and 36 nt) overlapping individual piRNA clusters were computed in R using the BioConductor GenomicFeatures and GenomicRanges packages. Given the potential for dramatic and widespread effects on piRNA production due to the mutant phenotype, it was crucial to use the miRNA counts per library as normalization factors. Normalization and changes in gene expression were computed using the BioConductor edgeR package and significance was computing using the general linear model (glm) approach. False discovery rates (FDR) were calculated using the Benjamini–Hochberg method.

RNA Isolation and RT-qPCR Assays

Total RNAs from the cultured GSCs mixed with somatic cells were extracted with Trizol and purified by organic extraction followed by isopropanol precipitation. After DNase I treatment, complementary DNA (cDNA) was synthesized from 400 ng of RNAs with oligo dT primers and random hexamers using SuperScript III Reverse Transcriptase (Life Technologies). qPCR was performed to assay levels of *TART*, *HetA*, *gypsy*, *bam*, *tbp*, *gapdh* and *rpl32*.

Primers for SYBR qPCRs	
Name	Sequence
tbp-F	TCCAGACTGGCAGCGAGAAAGTAT
tbp-R	AACTTGACATCGCAGGAGCCG
gapdh-F	AGGGAGCCACCTATGACGAAATCA
gapdh-R	AGACGAATGGGTGTCGCTGAAGAA
Rpl32-F	AGCGCACCAAGCACTTCATC
Rpl23-R	GACGCACTCTGTTGTCGATACC

bam-F	TTGCTAATTGGTCTGCGCGATTGG
bam-R	AGTAGCGGTGCTCCAGATCCATTT
TART-F	AGAGAGGGAAAGAAGGGAAAGGGA
TART-R	ATTTCCTGCCTGGTTAGATCGCCA
HetA-F	GGCCTTGCACAACTATCAACGCTT
HetA-R	TAAATCATCCTGAGCGGAAGGGCA
gypsy-F	ATTATCAACGAAGCCGCAGCTCAC
gypsy-R	AATTCAGAGCCGTTGATGGTTGCC

3.4 Results and Figures

Aub Maintains GSCs and Promotes Early Germ Cell Differentiation

Although Aub is known to be important in late oogenesis to control the dorsal-ventral polarity establishment of egg chambers [115, 123, 124], it remains unclear whether it is also required for GSC self-renewal and differentiation in the adult ovary. To investigate the role of *aub* in the regulation of early GSC lineage development, we labeled the ovaries of wild-type and *aub* mutant ovaries for Hu-li tai-shao (Hts) and Vasa, and quantified the numbers of GSCs and CBs in the ovaries. Hts protein labels both spherical spectrosomes in GSCs and CBs and branched fusomes in mitotic cysts and 16-cell cysts, whereas Vasa protein specifically identifies germ cells, including GSCs and their differentiated progeny [199, 224, 225]. Two strong loss-of-function *aub* mutations, aub^{QC42} and aub^{HN2} (abbreviated as aub^{QC} and aub^{HN}), were used to delineate its function in GSC lineage development [115, 237]. As the control, the 3-day and 20-day old wild-type germaria contain two or three GSCs (Fig. 3.1A, 3.1B and 3.1F). Interestingly,

most of the 3-day old *aub* mutant germaria still contain two GSCs (Fig. 3.1C and 3.1F). In contrast, most of the 10-day old *aub* germaria contain one GSC and accumulate spectrosome-containing CBs (Fig. 3.1D and 3.1F). Surprisingly, almost all the 20-day old *aub* mutant germaria completely lose their GSCs (Fig. 3.1E and 3.1F). Our quantification results indicate that *aub* ovaries gradually lose their GSCs within 20 days in contrast with the control ovaries where GSCs are reliably maintained during the same period (Fig. 3.1F). These results indicate that Aub is required for GSC maintenance.

Wild-type germaria normally contain one CB on average. In contrast, *aub* mutant germaria accumulate 3-4 CBs on average during the 3-20 day period (Fig. 3.1C, Fig. 3.1D and Fig. 3.1G). To further define the germ cell differentiation defect of the *aub* mutants, we also quantified the percentages of the germaria containing three or more CBs in the aub^{HN}/ *Df(2L)BSC145* and aub^{QC}/ *Df(2L)BSC145* mutant ovaries, in which *Df(2L)BSC145* is a deficiency deleting the entire *aub* region and nearby genes. The germaria containing three or more CBs are considered to have a germ cell differentiation defect because a wild-type germarium rarely contains three or more CBs [44]. Consistent with the idea that aub^{HN} and aub^{QC} are strong loss-of-function mutations, the aub^{HN}/*Df* and aub^{QC}/*Df* mutant ovaries show similar percentages of the germ cell differentiation-defective germaria to that of the aub^{HN}/aub^{QC} mutant ovaries (Fig. 3.1H and 3.1I). These results indicate that Aub is also required for germ cell differentiation.

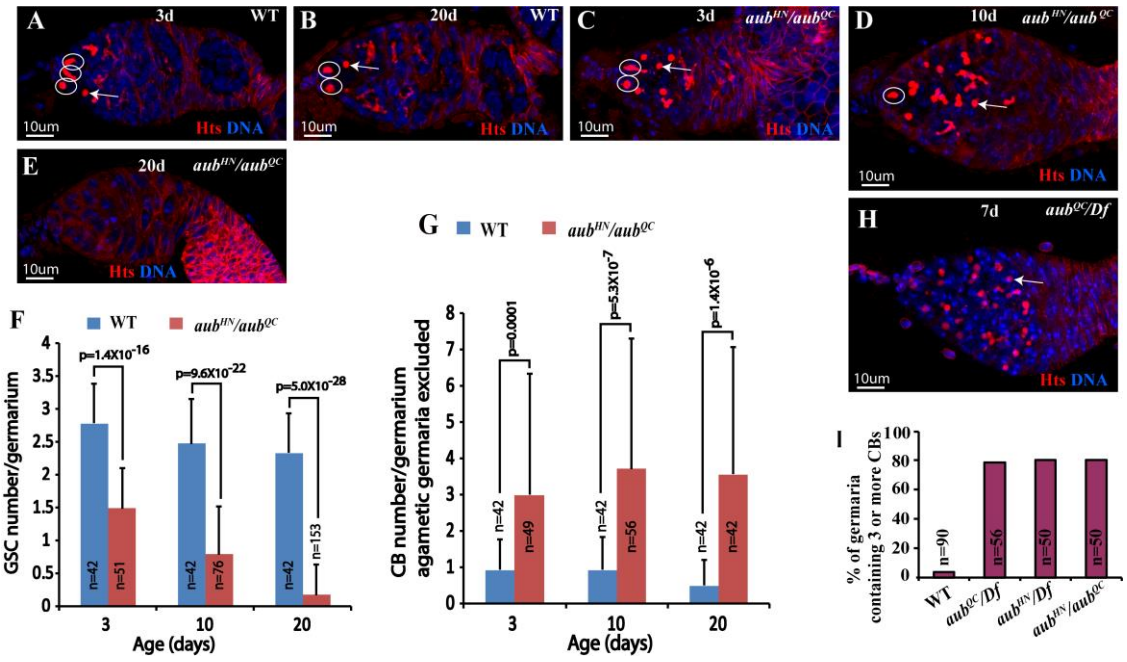

Figure 3.1. Aub is required for GSC maintenance and germ cell differentiation. GSCs in **A-D** are highlighted by ovals, whereas spectrosome-containing CBs are indicated by arrows in **A-D** and **H**. (**A**, **B**) 3-day (**A**) and 20-day (**B**) old wild-type ovaries contain three and two GSCs, respectively. (**C**-**E**) 3-day (**C**), 10-day (**D**) and 20-day (**E**) old *aub* mutant ovaries carry two GSCs, one GSC and zero GSC, respectively. The *aub* mutant germaria in **C** and **D** show excess spectrosome-containing CBs, and that in **E** completely loses all the germ cells including GSCs. (**F**) Quantification results show that GSCs in *aub* mutant ovaries are lost significantly faster with age than wild-type GSCs. (**G**) Quantification results show CB accumulation in the *aub* mutant germaria with agametic germaria excluded. (**H**) The aub^{QC}/Df mutant germarium contains excess CBs. (**I**) Quantification results show that aub^{QC}/Df, aub^{HN}/Df and aub^{HN}/aub^{QC} mutant ovaries contain similar percentages of differentiation-defective germaria.

Aub Is Required Intrinsically to Control GSC Self-Renewal by Preventing DNA Damage-Induced Checkpoint Activation

To further determine if Aub is required intrinsically to maintain GSCs and promote differentiation, we used FLP-mediated FRT recombination to generate marked LacZ-negative control and *aub* mutant GSCs and then study their maintenance and differentiation as reported previously [195]. Most of the marked control GSC clones detected 10 days after clone induction (ACI) can be maintained for up to 30 days ACI (Fig. 3.2A, 3.2B and 3.2E). In contrast, the marked *aub* GSCs are lost faster from the niche than the marked control GSCs, and most of the marked *aub* mutant GSCs detected 10 days ACI are lost from the niche 30 days ACI, indicating that Aub is required intrinsically for GSC maintenance (Fig. 3.2C-E). Also, some germaria containing marked *aub* mutant GSCs harbor many undifferentiated spectrosome-containing CBs, indicating that Aub is also required intrinsically for germ cell differentiation (Fig. 3.2F and 3.2G). These results indicate that Aub is indeed required intrinsically to control GSC maintenance and differentiation.

Because the piRNA pathway is important to repress TE activities and prevent DNA damage in germ cells, one of the possibilities for the loss of *aub* mutant GSCs is that accumulated DNA damage leads to their loss via activation of apoptosis. To test this idea, we used TUNEL-based ApopTag labeling to determine if two-week old *aub* mutant GSCs are apoptotic. Like wild-type GSCs, the *aub* mutant GSCs are also negative for ApopTag labeling (Fig. 3.2H). It is worth noting that apoptotic germ cells are indeed detected in the *aub* mutant germaria (Fig. 3.2I). These results suggest that *aub* mutant GSCs are lost unlikely due to

apoptosis. However, we could not completely rule out the possibility that *aub* mutant GSCs die via other death mechanisms.

DNA damage in piRNA pathway mutants also leads to DNA damage-induced cell cycle checkpoint activation, which is often mediated through Chk2 [120, 178, 214]. In *Drosophila*, Chk2 is encoded by *loki* (*lok*), and the lok^{P6} used in this study is a null mutant deleting the translation start codon [238]. To determine if the self-renewal defect of *aub* mutant GSCs are caused by checkpoint activation, we examined the number of GSCs and CBs in *aub* mutant germaria, which are also *lok* mutant. ϒ-H2AvD is a phosphorylated form of the H2A variant, and is commonly used as a DNA damage marker in *Drosophila* [239]. In the wild-type and *lok* mutant germaria, GSCs are negative for ϒ-H2AvD expression, but meiotic germ cells are positive, indicating that those GSCs do not accumulate DNA damage (Fig. 3.2J and 3.2K). Similarly, wild-type and *lok* mutant germaria have similar numbers of GSCs and CBs, indicating that Chk2 itself does not play an important role in the regulation of GSC maintenance and differentiation (Fig. 3.2J-L). As expected, the remaining *aub* mutant GSCs are positive for ϒ-H2AvD, indicating that *aub* mutant GSCs accumulate DNA damage (Fig. 3.2M). Surprisingly, the *lok* homozygous mutation can significantly and almost fully suppress the GSC loss phenotype of the *aub* mutants (Fig. 3.2L-N). Although the *lok* mutation rescues the GSC loss phenotype of the *aub* mutant ovaries, DNA damage is still persistent in the *aub lok* double mutant GSCs based on ϒ-H2AvD expression, indicating that inactivation of checkpoint helps relieve checkpoint activation-induced GSC loss in *aub* mutants not via DNA damage repair (Fig. 3.2N). These results suggest that Chk2-dependent checkpoint activation is largely responsible for the GSC loss phenotype of *aub* mutants.

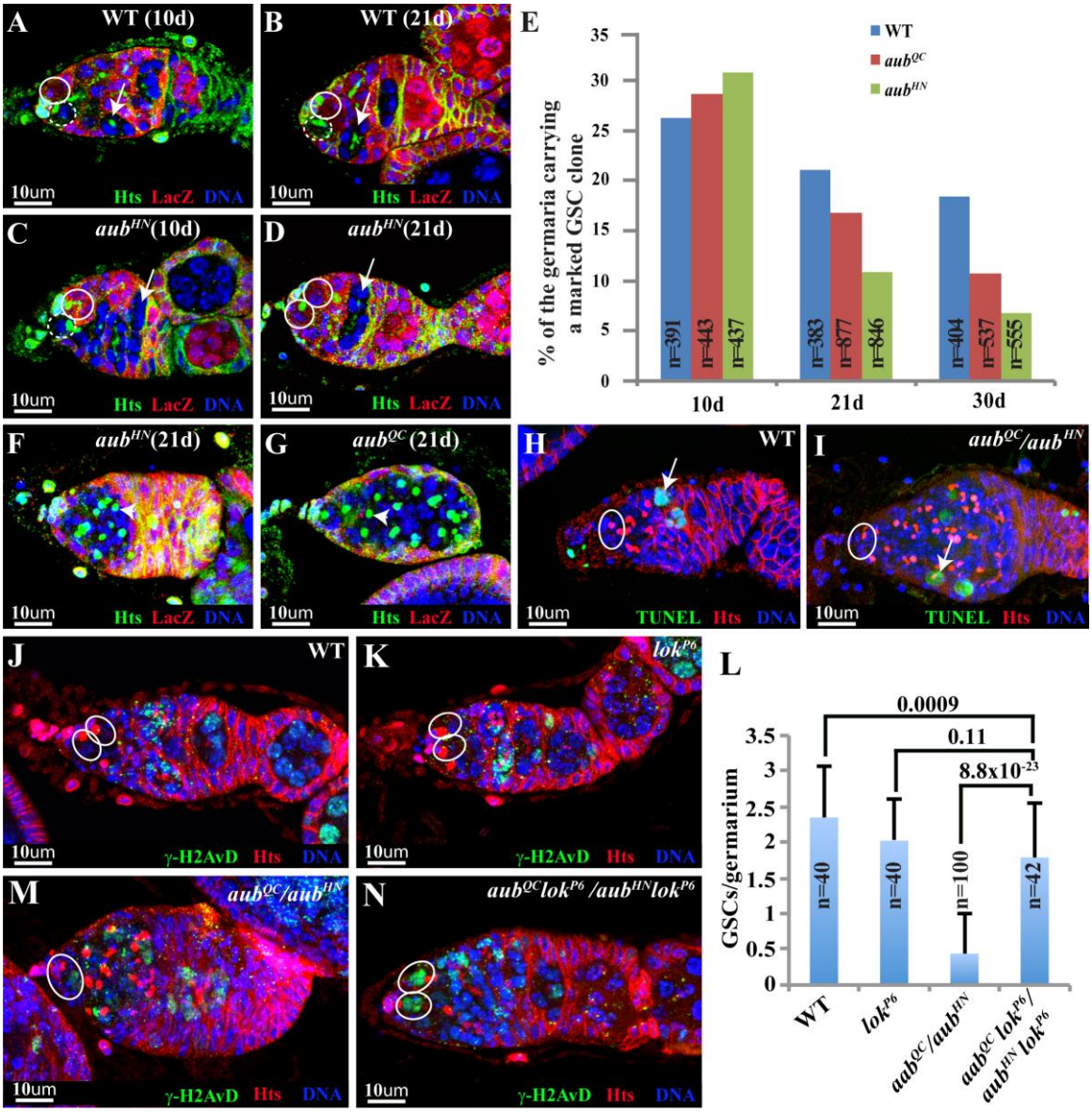

Figure 3.2. Aub is required for GSC maintenance by preventing transposon-induced DNA damage. In **A-D**, LacZ-negative marked GSCs and marked cysts are indicated by broken circles and arrows, respectively; LacZ-positive unmarked GSCs are highlighted by circles. (**A**, **B**) The marked control GSCs remain 10 days (**A**) and 21 days (**B**) ACI. (**C**) The marked *aub* mutant GSC remains 10 days ACI. (**D**) The marked *aub* mutant cyst (arrow) indicates the loss of a marked *aub* mutant GSC 21 days ACI. (**E**) Quantification results show that marked *aub* mutant

GSCs are lost faster with time than marked control GSCs. (**F**, **G**) Germaria are filled with LacZ-negative marked spectrosome-containing single germ cells 21 days ACI. Arrowheads indicate spectrosomes. (**H**, **I**) GSCs are negative for TUNEL labeling in the *aub* mutant germarium (**I**) as in the control germarium (**H**). TUNEL-positive germ cells (arrows) away from the germarial tip are observed in both control and *aub* mutant germaria. (**J**, **K**) *lok* mutant (**K**) and control (**J**) germaria contain two GSCs (ovals), which are negative for ϒ-H2AvD. (**L**) Quantification results show that a mutation in *lok* can drastically and significantly rescue the GSC loss phenotype of the *aub* mutant ovaries. (**M**) The *aub* mutant germarium contains one GSC at the tip, which is positive for ϒ-H2AvD. (**N**) The *lok aub* double mutant germarium contains two ϒ-H2AvD-positive GSCs (ovals).

Aub Works with Bam to Promote CB Differentiation

Recent studies have shown that ECs are important for promoting germ cell differentiation [62, 185, 186]. To determine if Aub is also required in ECs to promote germ cell differentiation, we used *c587-gal4* to knock down the expression of *aub* in ECs, and examine the accumulation of GSC-like single germ cells. The *c587-gal4* line is expressed in ECs and early follicle cells, and has been widely used to drive gene expression in ECs [29, 185]. As in the control ovaries, *aub* knockdown ovaries have the normal number of GSCs and CBs, indicating that *aub* is indeed dispensable in ECs for maintaining GSCs and promoting germ cell differentiation (Fig. 3.S1). This is also consistent with its expression in germ cells [99, 124].

Next, we then investigated if Aub and Bam function together to regulate germ cell differentiation. We used the two *aub* mutants to test if inactivating one copy of *aub* could

enhance the germ cell differentiation defect of the *bam* heterozygous mutant $bam^{\Delta 86}$, in which most of the coding region of *bam* is deleted [235]. The $bam^{\Delta 86}$ heterozygous mutant germaria contain two CBs on average in comparison with the control germaria with one CB on average, which is consistent with our previous published results [44] (Fig. 3.3A-C). The heterozygous mutant germaria for the two *aub* mutations also carry one CB on average, behaving similarly to the control (Fig. 3.3B and 3.3D). Interestingly, the two a*ub* heterozygous mutations can significantly enhance the germ cell differentiation defect of the *bam* heterozygous mutant, causing the accumulation of excess GSC-like cells (Fig. 3.3B and 3.3E). Since Aub works with Piwi and Armi in the piRNA pathway to repress TEs [99, 120, 178, 182, 219, 240], we then tested if heterozygous mutations in *armi* and *piwi* could also enhance the germ cell differentiation defect of the *bam* heterozygous mutant. In contrast, heterozygous mutations in either *armi* or *piwi* do not enhance the differentiation defect of the $bam^{\Delta 86}$ heterozygous mutant, suggesting that Aub promotes germ cell differentiation by enhancing Bam function likely in a piRNA-independent manner (Fig. 3.S2). These results show that Aub enhances Bam function, thereby promoting CB differentiation.

To further investigate the epistatic relationship between *bam* and *aub* in the regulation of germ cell differentiation, we examined *aub bam* double mutant ovaries, which are labeled for Hts and Vasa, for the germ cell differentiation defect. Here, an EMS-induced *bam* mutant bam^{E5}, which behaves like a null mutant, was used in combination with the deletion allele $bam^{\Delta 86}$. The *aub bam* double mutant germaria containing two GSCs are filled with spectrosome-containing GSC-like single germ cells, which are reminiscent of *bam* mutant germaria [235] (Fig. 3.3F). The *aub bam* double mutant germaria containing one GSC have fewer spectrosome-containing GSC-like single germ cells than *bam* mutant germaria (Fig. 3.3G and 3.3G'). Some double

mutant germaria completely lose their GSCs (Fig. 3.3H). These results suggest that *bam* is epistatic to *aub* in the regulation of germ cell differentiation.

Bam was previously shown to work with Bgcn and Sxl to repress Nos protein expression in mitotic cysts at the post-transcriptional level [70, 74]. Here, the Myc-tagged *nos* genomic transgene, *nos-myc*, carries all the regulatory elements and is sufficient to rescue various *nos* mutants [241]. In the wild-type ovaries, Nos-Myc protein is indeed repressed in mitotic 2-cell, 4-cell and 8-cell cysts, but is expressed in GSCs and 16-cell cysts, indicating that this transgene can recapitulate the endogenous Nos expression in germ cells (Fig. 3.3I-J'). In contrast, Nos-Myc expression is upregulated in the mitotic *aub* mutant 2-cell, 4-cell and 8-cell cysts, indicating that Bam-mediated repression of Nos in mitotic cysts requires Aub function (Fig. 3.3K-L'). These results further support the notion that Aub enhances Bam function in promoting germ cell differentiation.

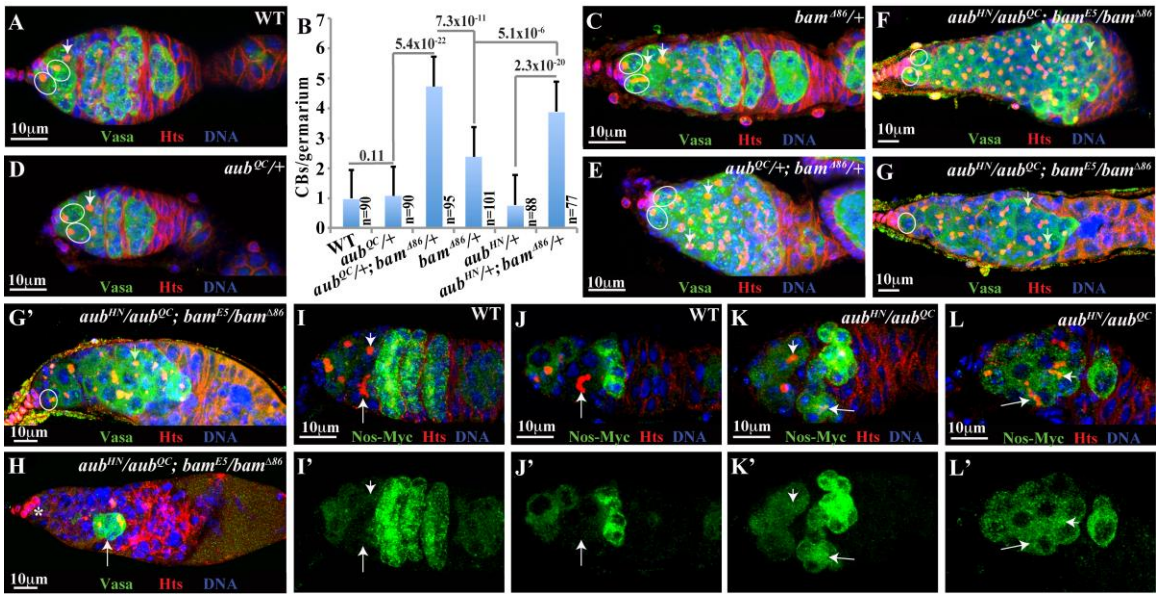

Figure 3.3. Aub enhances Bam function in promoting germ cell differentiation and is also required for Nos repression in mitotic cysts. (**A**) Wild-type germarium carries two GSCs

(ovals) and one CB (arrowhead). (**B**) Quantification results show that heterozygous mutations in *aub* enhance the germ cell differentiation defect of the *bam* heterozygous mutant, resulting in the accumulation of significantly more CBs. (**C**) The *bam* heterozygous mutant germarium carries two GSCs (ovals) and two CBs (arrowheads). (**D**) The *aub* heterozygous mutant germarium contains two GSCs (ovals) and one CB (arrowhead). (**E**) The *bam* and *aub* transheterozygous germarium contains two GSCs (ovals) and excess CBs (two by arrowheads). (**F**) The *aub bam* double mutant germarium contains two GSCs (ovals) and many CBs (two by arrowheads). (**G**, **G'**) The *aub bam* double mutant germaria contain one GSC (oval) and fewer CBs (arrowheads) than the one in **F**. (**H**) The *aub bam* double mutant germarium contains no GSCs at the tip (indicated by *) with the remaining germ cells (arrow) in the middle. (**I**, **I'**) Nos-Myc expression is repressed in 2-cell (arrowhead) and 8-cell (arrow) cysts in the wild-type germarium in comparison with that in GSCs and 16-cell cysts. (**J**, **J'**) Nos-Myc expression is repressed in the 4-cell cyst (arrow) in the wild-type germarium. (**K**, **K'**) Nos-Myc expression is upregulated in the *aub* mutant 2-cell (arrowhead) and 8-cell (arrow) cysts. (**L**, **L'**) Nos-Myc expression is upregulated in the *aub* mutant 2-cell (arrowhead) and 4-cell (arrow) cysts.

Aub Is Required in Mitotic Cysts to Sustain High Bam Expression

To further understand how Aub might regulate Bam function in mitotic cysts, we examined Bam protein expression in the mitotic cysts of the control and *aub* mutant ovaries, which are labeled for Anillin and Bam proteins. Anillin protein accumulates in the nuclei of somatic and germ cells in the ovary, and is also localized to ring canals in mitotic cysts [242] (Fig. 3.4A and 3.4B). Here, Anillin protein expression was used to identify mitotic cysts based

on ring canal and cell numbers. In the control germaria, Bam proteins can be detected in 2-cell and 4-cell mitotic cysts (Fig. 3.4A' and 3.4B'). In contrast, 2-cell and 4-cell mitotic cysts in the *aub* mutant germaria show much lower levels of Bam protein expression (Fig. 3.4C-D'). These results suggest that Aub is required for germ cell differentiation by promoting Bam protein accumulation in mitotic cysts.

To understand how Aub regulates Bam accumulation in mitotic cysts, we used quantitative RT-PCR to determine *bam* mRNA expression levels in the control and *aub* mutant ovaries. In comparison with the wild-type control ovaries, *aub* mutant ovaries show a significant reduction in *bam* mRNA levels, indicating that Aub is required to maintain *bam* mRNA levels (Fig. 3.4E). To determine if Aub regulates *bam* transcription, we examined the expression of *bam-GFP*, in which GFP expression is under the control of the *bam* promoter in control and *aub* mutant mitotic cysts. The *bam-GFP* transgene has been extensively used to examine *bam* transcription in germ cells [39, 184]. In the control germaria, *bam-GFP* increases its expression levels in 2-cell, 4-cell and 8-cell cysts (Fig. 3.4F-I). Surprisingly, *bam-GFP* expression levels are significantly lower in *aub* mutant 2-cell, 4-cell and 8-cell cysts than those in the control counterparts (Fig. 3.4I-L). Taken together, these results suggest that Aub is required to sustain *bam* mRNAs in mitotic cysts at least at the transcriptional level, thereby maintaining Bam protein expression and promoting germ cell differentiation.

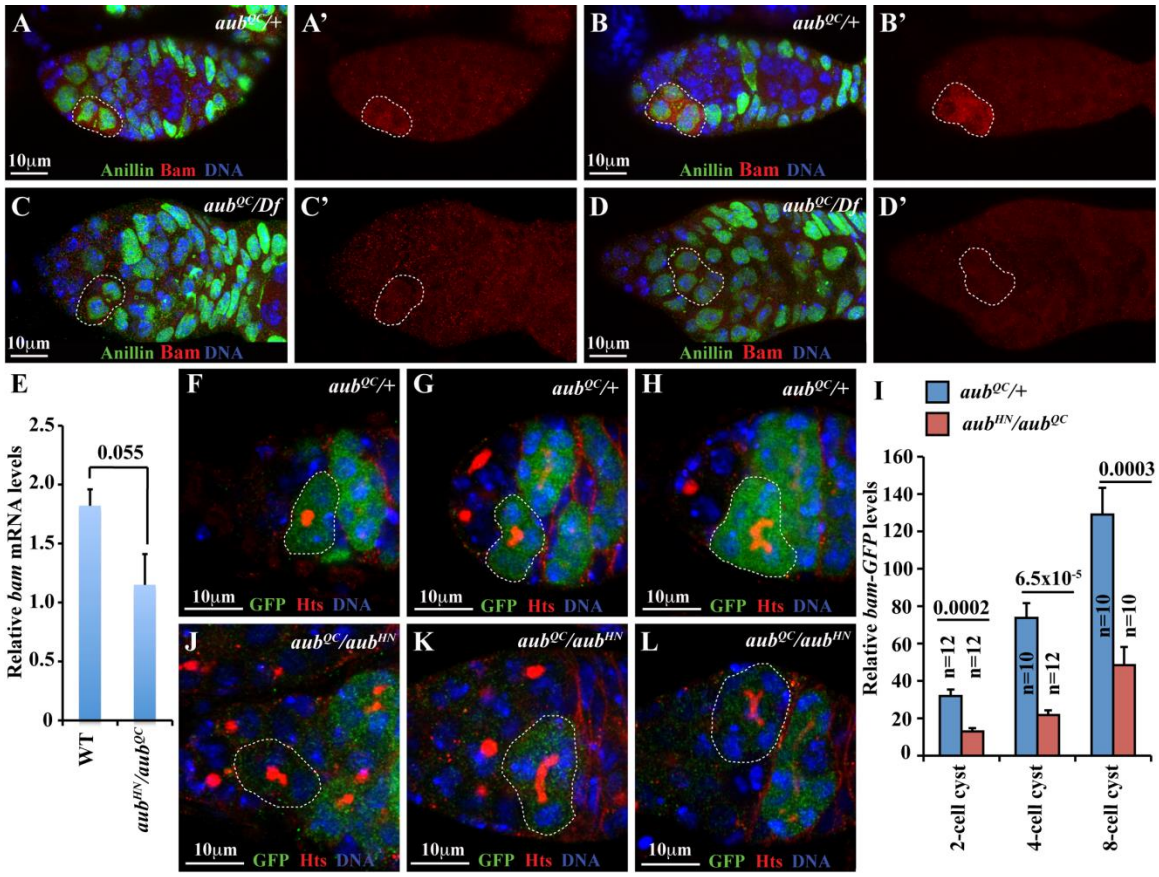

Figure 3.4. Aub is required to maintain Bam protein expression in mitotic cysts. (**A**, **B**) 2-cell (**A**) and 4-cell (**B**) cysts in the *aub* heterozygous ovaries express Bam protein. **A'** and **B'** show Bam protein expression only. (**C**, **D**) 2-cell (**C**) and 4-cell (**D**) cysts in the *aub* homozygous mutant ovaries express less Bam protein than the heterozygous control (**A**, **B**). **C'** and **D'** show Bam protein expression only. (**E**) Quantitative RT-PCR results show that *bam* mRNA expression levels are reduced in *aub* mutant ovaries in comparison with wild-type control ovaries. (**F**-**H**) Control germaria show *bam-GFP* expression in 2-cell (**F**), 4-cell (**G**) and 8-cell (**H**) cysts. (**I**) Quantitative results show that *bam-GFP* expression levels increase from control 2-cell to 8-cell cysts, which are significantly downregulated in *aub* mutant 2-cell, 4-cell and 8-cell cysts. (**J**-**L**) *aub* mutant germaria exhibit reduced *bam-GFP* expression in 2-cell (**J**), 4-cell (**K**) and 8-cell (**L**) cysts.

Aub and Bam Form a Protein Complex and Are Enriched in the Nuage of Mitotic Germ Cells

In the yeast two-hybrid screen as previously described [44], we identified the C-terminal 75aa residue of Aub for its interaction with Bam (Fig. 3.5A and 3.5B). To verify if Aub and Bam are indeed capable of forming a protein complex in *Drosophila* S2 cells, we generated and expressed N-terminal Flag-tagged full-length Bam (Flag-Bam) and C-terminal HA-tagged full-length Aub (Aub-HA) proteins in S2 cells, and performed co-immunoprecipitation (co-IP) experiments (Fig. 3.5B). The Flag-tagged Bam can pull down the HA-tagged Aub, indicating that Bam and Aub are capable of forming a protein complex (Fig. 3.5C). Aub contains two functional domains, PAZ and Piwi domains [115](Fig. 3.5B). Consistent with the yeast two-hybrid screen result, the C-terminal Piwi domain is indeed capable of interacting with Bam (Fig. 3.5C). Surprisingly, the PAZ-containing N-terminal half is also capable of interacting with Bam (Fig. 3.5C). Thus, Bam can interact independently with at least two regions of Aub to form a protein complex.

Next we examined if Bam and Aub are co-localized in mitotic germ cells. A C-terminal HA-tagged *bam* transgene under its endogenous promoter and 3'UTR (*bam-HA*), which recapitulates its endogenous Bam expression and is fully functional, was used to examine the co-localization with Aub in mitotic cysts [243]. As expected, HA-Bam protein is also detected in 2-cell, 4-cell and 8-cell cysts [234] (Fig. 3.5D-F). Aub is generally and specifically expressed in germ cells, including GSCs, CBs, mitotic cysts and 16-cell cysts [115] (Fig. 3.5D-F). In mitotic cysts, both Bam and Aub proteins are distributed throughout the cytoplasm (Fig. 3.5D-F). Interestingly, they are also co-localized in the perinuclear nuage (Fig. 3.5D'-F"'). These results

support the idea that Bam and Aub can function together to promote germ cell differentiation *in vivo.*

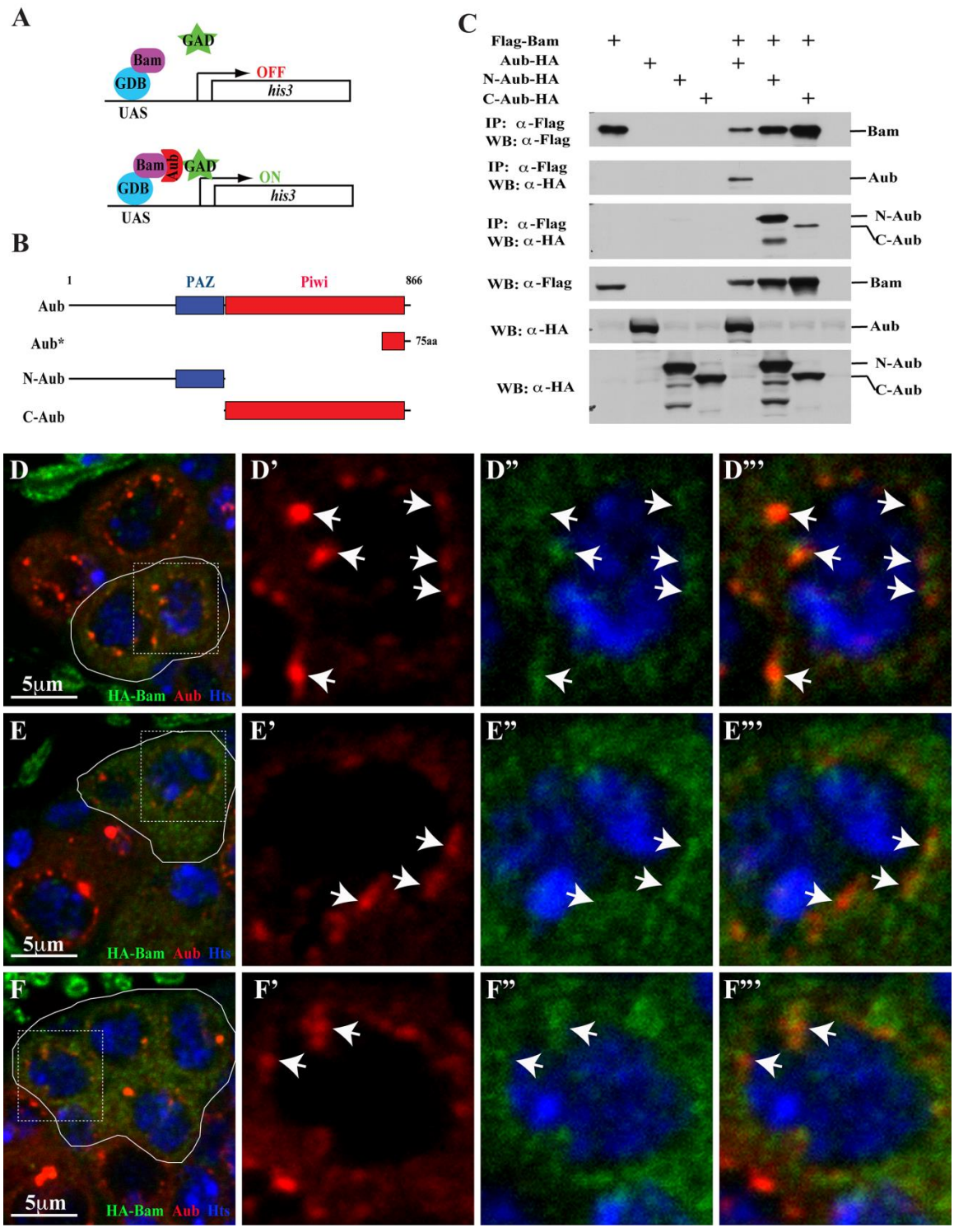

Figure 3.5. Bam and Aub physically interact with each other, and are enriched in the peri-nuclear foci in mitotic cysts. (**A**) The Bam-GDB (GAL4 DNA-binding domain) fusion protein

interacts with the C-terminal Aub fused with GAD (GAL4 activation domain) to activate the expression of the reporter gene *his3*. (**B**) Aub protein contains two domains, PAZ and Piwi. Aub* is the fragment identified to interact with Bam in yeast cells. N-Aub and C-Aub represent N-terminal and C-terminal halves, which are truncated between PAZ and Piwi domains. (**C**) Co-IP experimental results in S2 cells show that Flag-tagged Bam pulls down full-length Aub protein as well as both C-terminal and N-terminal halves. (**D**-**F**) HA-Bam is expressed in 2-cell (**D**), 4-cell (**E**) and 8-cell (**F**) cysts, in wild-type germaria where Aub protein is enriched in the perinuclear structure- the nuage. **D'-D"'**, **E'-E"'** and **F'-F"'** represent the areas highlighted in broken squares in **D**, **E** and **F**, respectively. Arrowheads indicate the Aub-positive foci, where HA-Bam is also enriched.

Bam Is Required in Germ Cells to Repress Transposon Expression

In the *bam* heterozygous control germaria, 16-cell cysts in the meiotic prophase are positive for ϒ-H2AvD, but GSCs, CBs and mitotic cysts are negative for ϒ-H2AvD (Fig. 3.6A). Surprisingly, the $bam^{\Delta 86}$ homozygous germaria contain many ϒ-H2AvD-positive CBs, but not ϒ-H2AvD-positive GSCs, indicating that Bam is required for preventing DNA damage in the accumulated CBs (Fig. 3.6B). To further determine if TE transcriptional activities increase in *bam* mutant ovaries as in *aub* mutant ovaries, we used qRT-PCR to quantify the mRNA levels for germline-specific transposons, *TART* and *HetA*, and a soma-specific transposon, *gypsy*. As expected, *TART* and *HetA* transcripts, but not *gyspy* transcripts, are significantly upregulated in *aub* homozygous ovaries in comparison with wild-type ovaries (Fig. 3.6C-E). Consistent with the idea that *bam* mutant germ cells are positive for ϒ-H2AvD, *TART* and *HetA* are significantly

upregulated in comparison with the control (Fig. 3.6C and 3.6D). Surprisingly, *gyspy* transcripts are also significantly upregulated in *bam* mutant ovaries, suggesting that Bam might have a non-cell autonomous role in repressing TEs in the ovarian soma (Fig. 3.6E). These results demonstrate that Bam is indeed required for repressing TEs in germ cells and somatic cells of the *Drosophila* ovary.

To further determine how extensive TE upregulation in *bam* mutant ovaries is, we performed deep sequencing of polyA-containing TE transcripts in *bam* mutant and wild-type ovaries. Based on total TE transcripts detected by sequencing, *bam* mutant ovaries exhibit a 7-fold increase in TE transcripts over wild-type ovaries (Fig. 3.6F). Among the TEs detected, over half of them are upregulated by more than four folds (Fig. 3.6G). These results have further confirmed that many TEs are upregulated in *bam* mutant ovaries.

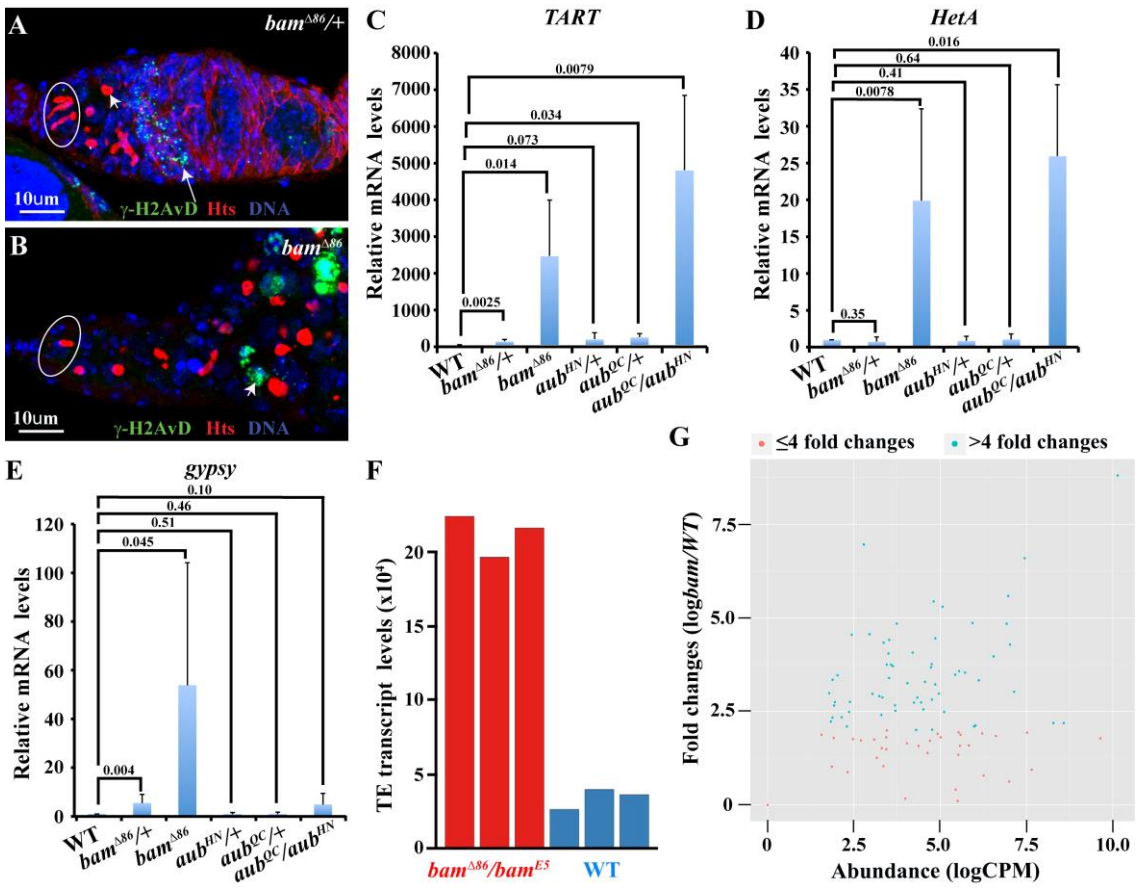

Figure 3.6. Bam is required in germ cells to repress TE activities and prevent DNA damage. (**A**) A *bam* heterozygous germarium lack ϒ-H2AvD-positive foci in GSCs (circles) and mitotic cysts (arrowheads), but are present in meiotic germ cells (arrows). (**B**) The *bam* homozygous mutant germarium contains ϒ-H2AvD-positive foci in CBs (arrowheads), but not in GSCs. (**C**-**E**) Quantitative RT-PCR results show that germline-specific transposons *TART* (**C**) and *Het-A* (**D**) significantly upregulate their expression levels in both *aub* and *bam* homozygous mutant ovaries in comparison with wild-type and heterozygous ovaries. Soma-specific transposon *gypsy* significantly upregulates its expression levels in *bam* homozygous mutant ovaries, but not in *aub* mutant ovaries, in comparison with wild-type and heterozygous ovaries (**E**). (**F**) RNA sequencing results show that *bam* mutant ovaries drastically increase the expression of TE

transcripts in comparison with wild-type ovaries. (**G**) A dot plot shows that most TEs drastically upregulate their expression.

Bam Is Dispensable for General piRNA Production

Because Bam and Aub physically interact and are co-localized in the nuage of mitotic cysts, we then examined if Aub expression or localization in *bam* mutant germ cells are changed. Here, the *bam* mutant ovaries used for examining Aub localization in germ cells carry transhomozygous $bam^{E5}/bam^{\Delta 86}$ mutations. Normally, Aub and Vasa proteins are present in the cytoplasm and enriched as granules in the nuage of GSCs, CBs and mitotic cysts (Fig. 3.7A-A'"). Aub-positive and Vasa-positive granules in the nuage remain similar in *bam* mutant GSCs and CBs (Fig. 3.7B-B'"). In addition, Aub expression and localization in marked *bam* mutant clones are also comparable with neighboring germ cells (Fig. 3.7C-C"). Mael is required for piRNA-mediated TE silencing in the nuage of germ cells [123, 229, 244, 245]. Mael localization in the nuage remains normal in *bam* mutant GSCs and CBs (Fig. 3.7D-E"). These results indicate that Bam is dispensable for the nuage localization and expression of piRNA pathway components Aub, Vasa and Mael.

To investigate if Bam is required for piRNA production, we sequenced small RNAs in the wild-type and *bam* mutant ovaries. piRNAs are identified based on the length and mapping to the known piRNA clusters. Based on the total count of piRNAs, general piRNA production remains unchanged in the wild-type and *bam* mutant ovaries (Fig. 3.7F). In addition, the expression levels for the majority of known piRNA clusters remain unchanged in the wild-type and *bam* mutant ovaries (Fig. 3.7G). Only 7 clusters and 12 clusters are downregulated and

upregulated in the *bam* mutant ovaries in comparison with the wild-type ovaries, respectively, which might reflect different genetic backgrounds or different cell compositions of the wild-type and *bam* mutant ovaries (Fig. 3.7G). For example, the most abundantly expressed piRNA cluster 42AB show similar expression levels on both strands between the wild-type and *bam* mutant ovaries (Fig. 3.7H). These results indicate that Bam is dispensable for general piRNA production.

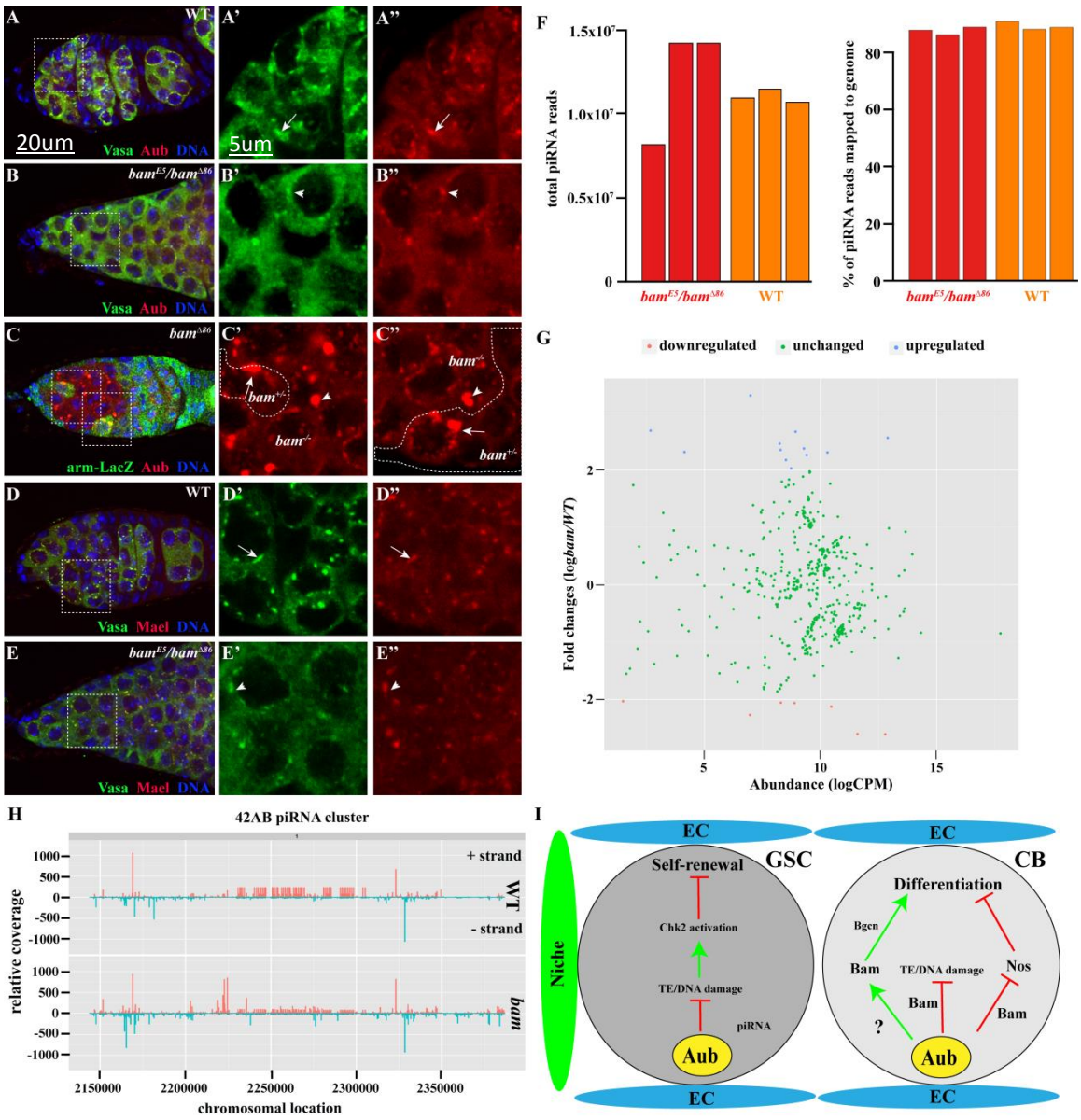

Figure 3.7. Bam is dispensable for nuage localization of Aub and Vasa proteins and for overall piRNA production. (**A-A"**) Vasa and Aub proteins are co-localized in the perinuclear foci (arrows) of control early differentiating germ cells. **A'** and **A"** represent three-times magnification of the area highlighted by the broken rectangle in **A**. (**B-B"**) Vasa- and Aub-positive foci (arrowheads) in *bam* mutant germ cells remain unchanged. **B'** and **B"** represent three-times magnification of the area highlighted by the broken rectangle in **B**. (**C-C"**) LacZ-

negative *bam* mutant (highlighted by broken lines in **C'** and **C"**) and LacZ-positive control germ cells contain similar perinuclear Aub foci (arrows and arrowheads point to Aub foci in *bam* mutant and control germ cells, respectively). (**D-E"**) Mael- and Vasa-positive foci in *bam* mutant germ cells remain similar to those (arrows) in comparison with control early differentiating germ cells. (**F**) Total piRNAs mapped to known piRNA clusters remain unchanged in *bam* and wild-type ovaries. (**G**) Most of individual piRNA clusters do not show expression changes in *bam* mutant ovaries in comparison with wild-type ovaries, but only a few are upregulated or downregulated in *bam* mutant ovaries in comparison with wild-type ovaries ($P<0.05$). (**H**) piRNA expression in the 42AB cluster remains unchanged in *bam* mutant and wild-type ovaries. (**I**) A working model showing that Aub is required to maintain GSCs by preventing DNA damage-induced checkpoint activation and to promote germ cell differentiation. In addition, Bam is involved in repressing TEs.

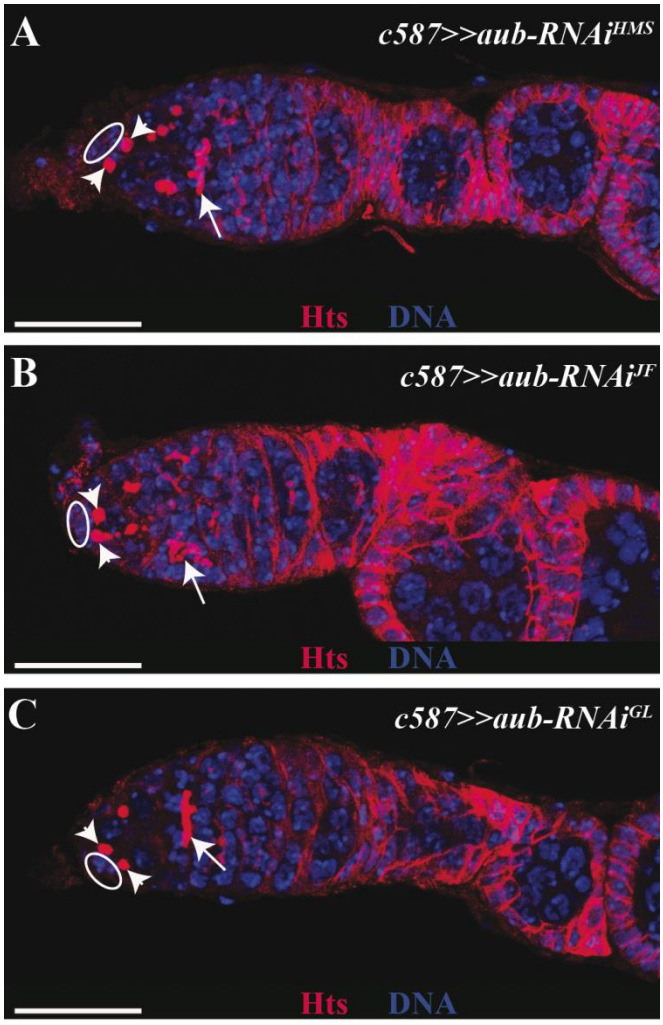

Figure 3.S1. Knockdown of *aub* in ECs does not affect early germ cell differentiation. (**A-C**) Germaria, in which *aub* is knocked down in ECs by three RNAi lines, still retain two GSCs (arrowheads) close to cap cells (oval) and differentiated germ cell cysts containing a branched fusome (arrow).

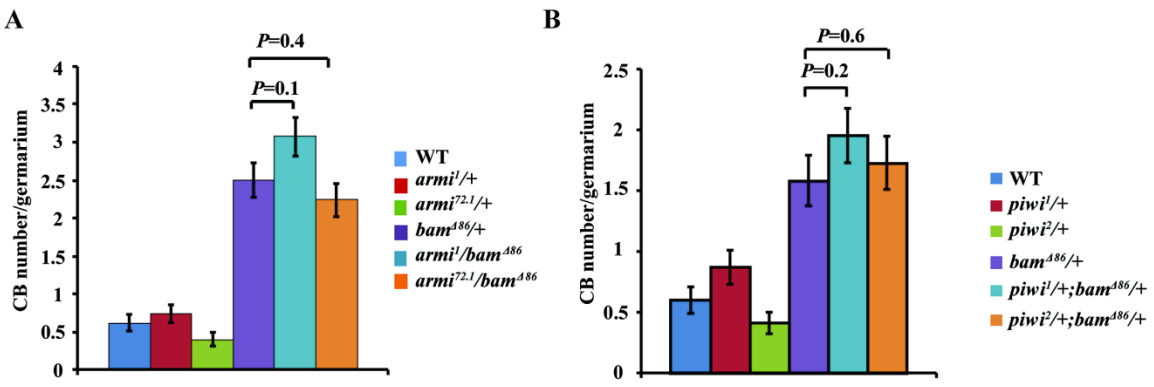

Figure 3.S2. Quantitative results on CB numbers show that heterozygous mutations in either *armi* (A) or *piwi* (B) do not enhance the germ cell differentiation defect of the *bam* heterozygous mutant.

3.5 Discussion

During *Drosophila* ovary development, Bam is a master regulator for driving germ cell differentiation [235, 236], where Aub is an essential piRNA pathway component for silencing TEs in germ cells [123, 124]. Because almost all the piRNA studies are focused on mature 16-cell cysts, which are much more abundant than GSCs, CBs and mitotic cysts in the ovary [107, 232], it remains unclear whether piRNA-mediated repression of TEs in those early germ cells are regulated differently from late germ cells. In this study, we demonstrate that Aub is required intrinsically to maintain GSCs and promote germ cell differentiation in the *Drosophila* ovary, and that Bam is required for TE repression in mitotic germ cells (Fig. 3.7I). *aub* is required in GSCs to maintain self-renewal by preventing DNA damage-evoked checkpoint activation because dismantling checkpoint by inactivating Chk2 function can effectively rescue the GSC loss phenotype of *aub* mutant ovaries. Aub promotes CB differentiation by maintaining Bam

expression partly at the transcriptional level. Bam is also required for repressing TEs in early germ cells because *bam* mutant germ cells increase TE transcripts and consequently DNA damage. Mechanistically, Bam is also localized to Aub-positive foci in the nuage possibly via protein-protein interaction. However, Bam is dispensable for overall piRNA production. Therefore, this study has revealed a new role of Aub in promoting germ cell differentiation by regulating *bam* transcription and a novel role of Bam in repressing TEs in early germ cells (Fig. 3.7I).

Aub is required intrinsically to maintain GSCs by preventing DNA damage-evoked checkpoint activation

Although the piRNA pathway has been well studied for its role in preventing DNA damage in meiotic germ cells, its role in GSC regulation has not been well studied. Cuff transcriptionally controls the expression of piRNA clusters and thus piRNA biogenesis, and it is required for maintaining GSCs [124, 221]. Similarly, Eggless, a H3K9 trimethyltransferase, is required for GSC maintenance and differentiation as well as for piRNA biogenesis [63, 186]. Because other key piRNA components have not been directly linked to GSC maintenance, the findings on Cuff and Eggless can also be interpreted as their additional role besides a role in the piRNA pathway. This study shows that Aub is intrinsically required to maintain long-term GSC self-renewal. We first used two strong loss-of-function *aub* alleles to demonstrate that GSCs are gradually lost within 20 days after eclosion. Interestingly, the ovaries of newly eclosed *aub* mutant females still contain the average of two GSCs, which are close to those in wild-type females. We then used FLP-mediated mitotic recombination to demonstrate that the marked *aub*

mutant GSCs are lost faster from the niche than the marked control GSCs. Interestingly, *aub* mutant GSCs are not lost due to apoptosis despite of the elevated DNA damage shown by ϒ-H2AvD staining. These results are consistent with an important role of Aub in piRNA-mediated TE repression. Dorsal-ventral polarity defects of the *aub* mutant egg chambers can be drastically rescued by inactivation of checkpoint regulators Chk2 and ATR [123]. Consistently, the GSC loss phenotype of *aub* mutants can also be rescued almost fully by Chk2 inactivation. These results demonstrate that the GSC loss phenotype of *aub* mutants is caused primarily by DNA damage-induced checkpoint activation. Therefore, Aub is indeed required in GSCs for their long-term maintenance by preventing DNA damage-induced checkpoint activation.

Aub Is Required Intrinsically to Promote GSC Lineage Differentiation At Least In Part By Maintaining Bam Expression

Aub was initially identified to enhance *oskar* translation in the *Drosophila* ovary, and was later shown to be a polar granule component in the oocyte [115, 223]. Subsequently, Aub is shown to be required for TE repression and piRNA production [89, 112, 123, 124]. Mechanistically, Aub is localized in the perinuclear nuage, where it binds to anti-sense piRNAs and regulates the "Ping-Pong" piRNA amplification cycle [88, 89]. In addition, Aub is present in the protein complex regulating *nos* mRNA localization in the oocyte [246]. Finally, Aub also forms a protein complex with Smaug and CCR4, which controls the deadenylation and decay of maternal mRNAs in the early *Drosophila* embryo, including *nos*, in cooperation with piRNAs [140]. In this study, we show that Aub is required in early germ cells of the adult *Drosophila*

ovary to control *bam* transcription and thus maintain Bam expression and promote early germ cell differentiation.

In this study, we show that *aub* mutant ovaries accumulate undifferentiated CBs during the 3-day to 20-day age period before GSCs are completely lost. In contrast with control germaria containing one CB, *aub* mutant germaria can accumulate 3-4 CBs on average, indicating that Aub indeed promotes early germ cell differentiation. Although ECs are known to control germ cell differentiation [62, 185], we have also ruled out the possibility that *aub* is required in ECs to control early germ cell differentiation because *aub* knockdown in ECs fails to interfere with early germ cell differentiation in the adult *Drosophila* ovary. Consistent with its germline requirement, we show that the germaria carrying marked mutant *aub* GSCs can also accumulate excess undifferentiated *aub* mutant CBs. Bam is a master regulator for driving CB differentiation [235, 236]. Interestingly, *aub* heterozygous mutations genetically enhance the germ cell differentiation defect of the *bam* heterozygous mutant, suggesting that Aub and Bam function in the same pathway or parallel pathways to control germ cell differentiation. At the molecular level, we show that Bam protein is lower in *aub* mutant mitotic cysts than in the control ones. Additionally, *bam* mRNA levels are significantly lower in *aub* mutant ovaries than the control one. Finally, we used the *bam* transcriptional reporter *bam-GFP* to demonstrate that *bam* transcription is significantly lower in the *aub* mutant mitotic cysts than in the control mitotic cysts, indicating that Aub is required to maintain *bam* transcription in mitotic cysts. This is in contrast with the previous finding on the requirement of piRNA component Mael for promoting germ cell differentiation by alleviating miRNA-mediated *bam* repression [245]. Although how Aub controls *bam* transcription at the molecular level remains unknown, it likely controls *bam* transcription indirectly because Aub is a cytoplasmic protein shown to bind to piRNAs and

mRNAs [89]. So far, no *bam* transcriptional activators have been identified, making it difficult to test this possibility directly. Another possible way for Aub to regulate Bam-dependent germ cell differentiation is through the formation of Aub-Bam protein complexes. In this study, we show that Bam and Aub can physically interact in yeast and S2 cells. More importantly, they are both also co-localized to the cytoplasm of mitotic cysts. Therefore, Bam is localized to the nuage possibly via its interaction with Aub.

Bam Is Required to Repress TEs in Early Germ Cells

The *Drosophila* ovary has been one of the premier systems for genetically identifying new regulators and studying biological functions of the piRNA pathway [99, 120, 182, 219, 230]. The studies are almost exclusively focused on 16-cell cysts in the germarium and egg chambers, but it remains unclear how piRNAs repress TEs in early germ cells, including GSCs, CBs and mitotic cysts. Bam has recently been shown to be a translational regulator by interacting with other RNA binding proteins, including Bgcn, Sxl and eIF4A [44, 70, 74]. In addition, it has recently been shown to bind directly to the 3' UTR *of mei-P26* [247]. In this study, we have, for the first time, shown that Bam functions as an effector of the piRNA pathway to repress TEs in early germ cells. As in the *aub* mutant ovaries, germ cell-specific transposons, *Het-A* and *TART*, are significantly upregulated in *bam* mutant ovaries in comparison with wild-type control ones. In addition, we used RNA sequencing to show that *bam* mutant ovaries significantly upregulate more than half of the transposons by more than four folds. Consistent with the idea that Bam is required to repress TEs in germ cells, there is an increase in DNA damage in *bam* mutant germ

cells. Taken together, this study provides the first experimental evidence that Bam is required for repressing TEs in germ cells.

In this study, we show that Bam is localized to the nuage of mitotic cysts (2-cell, 4-cell and 8-cell cysts). In addition, Bam and Aub interact with each other. Interestingly, Bam is dispensable for the nuage localization of Aub and other piRNA pathway components, including Mael and Vasa. Furthermore, our small RNA sequencing results indicate that piRNA production in wild-type and *bam* mutant ovaries is comparable. Therefore, we propose that Bam might function as an effector for piRNA-mediated TE repression in early germ cells, but is dispensable for piRNA production. This study also raises an interesting possibility that distinct cellular factors work with piRNAs to repress transposons in different germ cell developmental stages.

Chapter 4: Dissertation Conclusions and Discussion

4.1 The Functions of Piwi, Aub and Bam in GSC Maintenance and Differentiation

Although it has been 16 years since *piwi* was identified as an important factor for stem cell self-renewal [30], the biological consequences of *piwi* depletion at different developmental stages and in specific cell types are still not well defined. In this study, we take advantage of the newly developed miRNA based RNAi lines by the TRiP project combined with tissue specific promoters to perform *piwi* knockdown at specific developmental stages and in specific cell types to pinpoint the biological functions of Piwi. Using *nos-gal4* driven *piwi* RNAi specifically in the germline, we show that Piwi functions in the germline during early development to maintain the PGCs probably their survival and/or GSC establishment (Fig. 2.5A-F). Previous studies concluded that Piwi functions in the TF/cap cells to control GSC self-renewal [30, 31]. Our study, however, reveals a previously unidentified role of Piwi in the adult GSCs to control the same process (Fig. 2.6A-G), in addition to confirming the requirement of Piwi in the somatic cells. By inducing *piwi* knockdown only at the adult stage, we were also able to show the intrinsic requirement of Piwi in germ cell differentiation. More importantly, with *c587-gal4* driven *piwi* knockdown in ECs, we define a new role of Piwi in ECs extrinsically for germ cell differentiation partially by repressing the transcript level of the self-renewal signaling molecule *dpp*, consistent with the general repressive role of Piwi in transcription regulation. Making our discovery more interesting is the observation that knockdown of *eggless*, encoding a histone H3K9 trimethylase [27], *Su(var)205*, encoding the HP1 [27], or histone *lysine-specific demethylase 1* (*Lsd1*), required for heterochromatin formation [60], in ECs leads to the same

germ cell differentiation defect as *piwi* knockdown [27]. These data suggest that a repressive heterochromatin status in ECs established and maintained by Piwi, Eggless, HP1 and Lsd1 is important for the survival and proper functions of ECs, which in turn is critical for germ cell differentiation.

The functions of Aub and other piRNA pathway components in later oogenesis have been shown to repress TE activity, maintain genome stability, and thus ensure proper localization and translation of axis specification factors [123, 124]. However, the role of Aub in early germ cell development including GSC maintenance and germ cell differentiation has not been defined. Our study clearly shows that Aub is required intrinsically in the GSCs for self-renewal (Fig. 3.1 and Fig. 3.2). DNA damage is increased in *aub* mutant GSCs and inactivation of *chk2/lok* is sufficient to rescue the GSC loss caused by *aub* mutations (Fig. 3.2). Importantly, the *chk2/lok* mutation does not suppress either the TE activation or increased DNA damage in the *aub* mutant ovary [123] or the increased DNA damage in the *aub* mutant GSCs (Fig. 3.2N), suggesting that TE silencing or DNA damage repression is not directly required for GSC self-renewal and Chk2 activity and downstream events are the direct cause for GSC loss in *aub* mutants.

From the similar germ cell differentiation defect of both *aub* and *bam* mutants, we reasoned that functional interaction may exist between these two proteins. The data we presented in this study support both physical interaction and functional enhancement of Aub and Bam. More surprisingly, *bam* mutant ovaries significantly upregulate over half of TE transcripts (Fig. 3.6) without affecting the piRNA production profile (Fig. 3.7), indicating that Bam functions downstream of piRNA production to regulate TE silencing, possibly at the stage of TE transcript slicing by recruiting Aub-piRNA complexes. A question of interest remains if Bam binds to TE transcripts directly or indirectly by interacting with RNA binding partners such as

Bgcn. Bgcn protein contains a DExH domain and is related to the DExH family of RNA helicase [68] while no conserved functional domain has been identified in Bam. An alternative scenario is that the Bam-Bgcn complex binds to TE transcripts via the Bgcn subunit and Bam recruits Aub-piRNA complexes by protein-protein interaction. Stronger interaction between Bam, Bgcn and Aub in the complex might result from the complementarity of the piRNA subunit and the TE transcript followed by slicing of the TE transcript by Aub. The transient interaction between Bam and Aub may not lead to slicing if it does not get reinforced by piRNA-TE transcript complementarity. This model is only highly speculative without support from experimental data. One of our ongoing experiments is to identify Bam and Bgcn binding TE transcripts to further search for the molecular mechanisms.

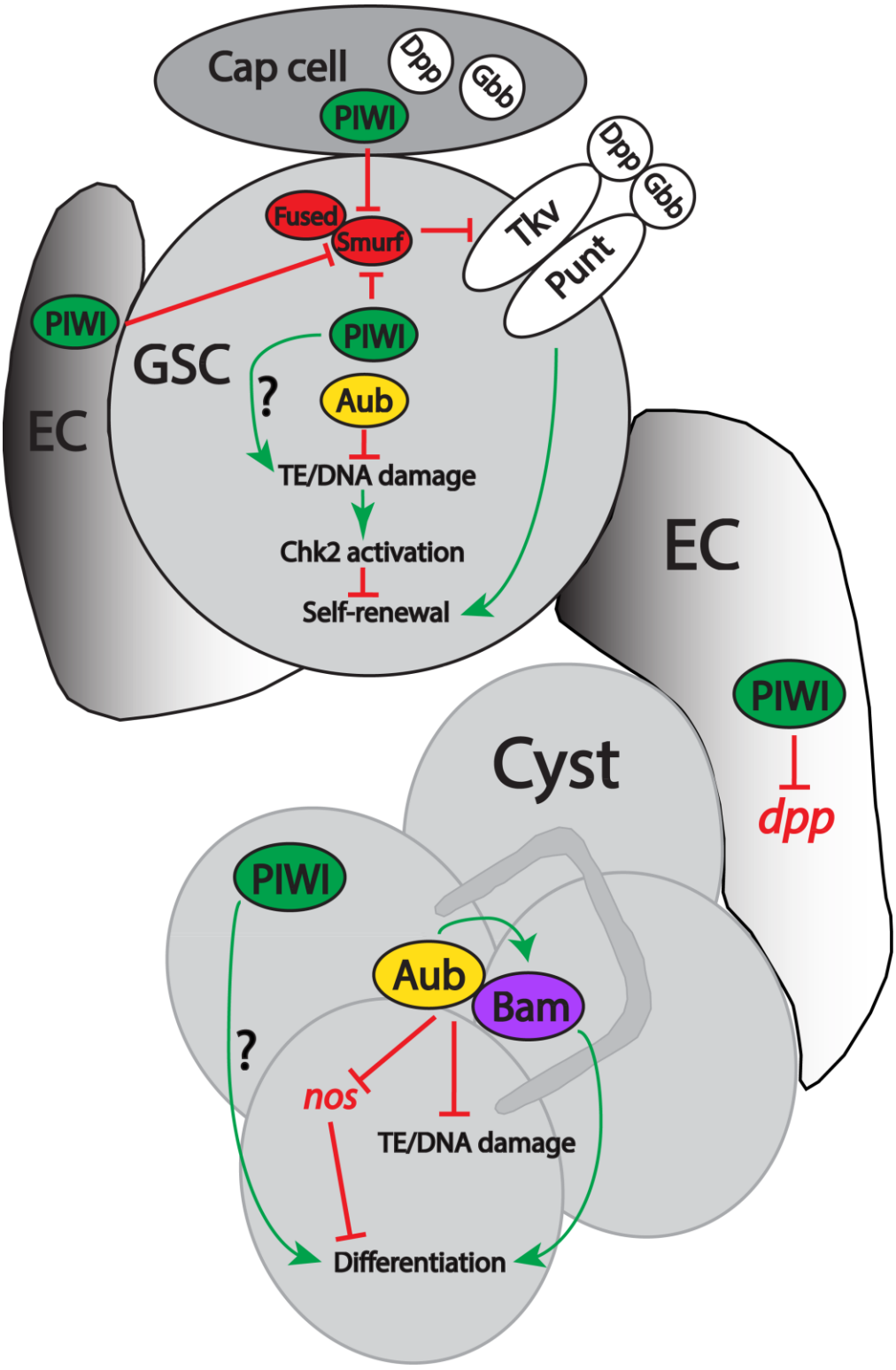
Cap cell
Dpp
Gbb
PIWI
Fused
Smurf
Dpp
Gbb
Tkv
Punt
PIWI
GSC
?
Aub
EC
TE/DNA damage
Chk2 activation
Self-renewal
EC
PIWI
dpp
Cyst
PIWI
Aub
Bam
?
nos
TE/DNA damage
Differentiation

Figure 4.1. The functions of Piwi, Aub and Bam in GSC maintenance and germ cell differentiation. GSC maintenance requires concerted actions of Piwi in the cap cells and/or TF, GSC-contacting ECs and Piwi and Aub in the GSCs. Piwi functions in the cap cells and ECs to maintain GSCs possibly by repressing the E3 ligase Smurf-dependent Tkv degradation pathway. Aub maintains GSCs probably by repressing TE-induced DNA damage checkpoint activation while the molecular function of Piwi in GSCs is unclear. Piwi functions in the ECs for germ cell differentiation partially by repressing *dpp*. Aub switches its function from GSC maintenance to germ cell differentiation by physically interacting with and functionally enhancing the differentiation factor Bam.

4.2 Potential Impact on the Field

Piwi is the founding member of the PIWI clade Argonautes and has been shown to be involved in various biological processes [248]. Prior to this study, Piwi has been shown to be required for heterochromatin formation and transcriptional repression of TEs [143, 162-166]. Biologically, Piwi has been related to tumor growth in flies [154] and mammals [155-160].

Piwi has also been proposed to function in the soma to maintain GSCs and in the germline to control GSC division [30, 31]. The developmental stage- and cell type-specific functions of Piwi, however, are not clear. In addition to confirming the requirement of Piwi in the soma for GSCs maintenance, this study unveils an intrinsic requirement of Piwi during PGC development and GSC maintenance. More strikingly, *piwi* depletion in either ECs or germ cells is sufficient to block germ cell differentiation. In ECs, *piwi* knockdown leads to elevation of *dpp* transcripts as presented by both RNA sequencing and RT-qPCR results (Fig. 2.3H-I). To our knowledge, this is the first experimental evidence showing the repressive role of Piwi in purified ECs on *dpp*

transcriptional or post-transcriptional regulation regardless of the direct or indirect involvement. However, *dpp* upregulation only partially contributes to the germ cell differentiation defects caused by *piwi* knockdown in the ECs. In addition, *dally* transcript level is not affected. These data strongly suggest that other factors besides Dpp signaling pathway have to be actively regulated in the ECs for proper germ cell differentiation.

Although several studies have shown the requirement of Aub in later oogenesis to repress TEs and maintain genome stability to protect normal oogenesis [123, 124], little is known about its functions in the GSCs. Our study shows a very similar role of Aub in the GSCs of the *Drosophila* ovary as in later oogenesis to protect genome stability and thus repress the DNA damage response pathway. More importantly, a *chk2*/*lok* mutation rescues the GSC loss caused by *aub* mutations, demonstrating that activation of DNA damage response leads to *aub* mutant GSC loss. Previous study has shown that an *ATR/mei-41* mutation resuces the later oogenesis defect of *aub* mutants [123]. Our experimental results (data not included in the dissertation), however, show that an *ATR*/*mei-41* mutation does not rescue *aub* mutant GSCs as the *chk2*/*lok* mutation. These results suggest the differences of DNA damage response between later oogenesis and GSCs and place Chk2/Lok in the most important position in the DNA damage response pathway of the GSCs. Surprisingly, *aub* mutant GSCs do not undergo apoptosis (Fig. 3.2 H-I). Instead, about 22% of *aub* mutant GSCs are negative for the BMP signaling reporter and the GSC marker Dad-lacZ and about 16% of *aub* mutant GSCs are positive for the differentiation marker Bam-GFP (data not included in the dissertation). These data together lead to a very important implication that *aub* mutant GSCs activate Chk2/Lok dependent DNA damage response, which results in GSC premature differentiation instead of DNA damage induced cell death. Age-related DNA damage accumulation has been linked to adult somatic

stem cell depletion and age-associated pathophysiology. Depletion of the ATR DNA damage checkpoint kinase in adult mice leads to premature appearance of age-related phenotypes including hair graying and these phenotypes are associated with dramatic reductions of tissue specific stem cells and progenitor cells [249]. Also in mice, DNA damage caused by ionizing radiation abrogates melanocyte stem cell renewal by inducing premature differentiation into mature melanocytes rather than apoptosis or senescence, thus leading to hair graying [250]. More importantly, loss of Batf, an AP-1 superfamily transcription factor that induces differentiation in cells of lymphoid and myeloid lineages [251], confers a selective advantage to hematopoietic stem cells (HSCs) in response to telomere dysfunction or ϒ-irradiation [252]. Interestingly, Batf depleted HSCs keep renewing with evident DNA damage accumulated from telomere shortening or ϒ-irradiation [252]. There are similarities between the DNA damage response in the *Drosophila* ovarian GSCs and the somatic stem cell models in mice from three different aspects. First, they both undergo premature differentiation rather than apoptosis or cellular senescence, which leads to a depletion of adult stem cells and consequently the loss of tissue homeostasis. Second, mutations of a DNA damage response gene *chk2/lok* in the ovarian GSC model and a differentiation gene *Batf* in HSCs in mice, dramatically rescue DNA damage induced premature stem cell differentiation. Third, the rescued stem cells still carry persistent DNA damage, indicating that interfering the downstream events of DNA damage may have beneficial effects on age-related disease treatment and cancer therapy. With the successfully cultured *Drosophila* ovarian GSC lines [104], the identification of Chk2/Lok targets in these cell lines in response to DNA damage will have tremendous impact on the stem cell field.

In addition to the role in GSC renewal, this study also shows the requirement of Aub in germ cell differentiation by enhancing the differentiation factor Bam. Aub and Bam show both

physical and genetic interactions, and additionally, *aub* mutations also affect the transcription and protein level of Bam. This is the first evidence showing the crosstalk between the piRNA pathway and the germline differentiation pathway. Even more intriguing is the observation that TEs are derepressed in *bam* mutants, similar to many piRNA pathway mutants. In contrast to most piRNA pathway mutants, *bam* mutations do not affect piRNA production in general, raising the possibility that Bam functions downstream of piRNA production to silence TEs. Considering the distinct expression pattern of Bam in only the 2-cell, 4-cell and 8-cell mitotic cysts during germ cell differentiation, this study has identified a novel role of Bam in TE silencing, which may differentiate TE regulation in the early differentiating germ cells from later germ cells.

4.3 Caveats and Discrepancies

As with any large body of research, the experiments and methods of this project present several potential caveats that need to be considered when interpreting the presented data.

c587-gal4 driven CD8GFP expression we used to purify ECs in Chapter 2 is not exclusively expressed in the ECs. This *gal4* line also drives expression in early follicle cells [64] (reference 66, Fig. 2.1A and Fig. S2A), although the GFP level is lower than that in ECs in general. The GFP expression level within the EC population can also be divided into two groups with lower GFP expression in the ECs in the anterior of the germarium and higher in those in the posterior region. Although we tried to avoid contamination from follicle cells by setting the gate for fluorescence sorting to eliminate cells with lower GFP expression so that the number of ECs from cell sorting matches that from *in vivo* quantification with other EC markers such as PZ1444, the method is rather arbitrary and does not completely separate ECs with lower GFP expression

from early follicle cells. When taking into consideration the observation that *c587-gal4* driven *piwi* knockdown reduces EC number (Fig. 2.1M-P) and maybe also change GFP expression level (Fig. 2.S6), the setting based on the control *c587-gal4* driven CD8GFP expression might not be perfectly applied to *piwi* knockdown groups to separate the ECs from early follicle cells. In consequence, the purified ECs using this method may contain a small portion of early follicle cells and the RNA sequencing and RT-qPCR results of these cells might reflect changes in the early follicle cells.

Our genetic results show that *dpp* knockdown only partially rescues the differentiation defects caused by *piwi* knockdown in ECs (Fig. 2.3 J-L), suggesting that BMP signaling elevation is not a major cause of the *piwi* knockdown phenotype. In contrast, one recent study shows that *dpp* knockdown during adult stage substantially rescues the germ cell differentiation defect in the $piwi^{1}/piwi^{\Delta 37}$ transheterozygote [253]. However, although two *dpp* RNAi lines (BSC #31172 and #33767) were listed in the section of experimental procedures, only one quantification figure was shown in the supplementary data (Fig. S3I [253]). To make a convincing case that *dpp* upregulation is responsible for the tumor phenotype in *piwi* mutants, the rescue effect of multiple *dpp* RNAi lines are needed to test on different heteroallelic combinations of *piwi* mutants. In addition, further experiments need to determine the knockdown efficiency of different *dpp* RNAi lines used in both studies to exclude the possibility that different *dpp* RNAi knockdown efficiency causes the discrepancy.

In the same study [253], the authors also claimed that adult-specific knockdown of *piwi* does not significantly induce GSC tumor (Fig. 3E, [253]) by using the temperature sensitive Gal80 to control Gal4 activity. This statement is also contradictory to our observation that the temperature shift experiment to specifically knockdown *piwi* with three independent RNAi lines

in the adult produces consistent tumor phenotypes. In that study [253], *tub-Gal80[ts];c587-gal4>UAS-piwi-RNAi* flies were used in the temperature shift experiment and *c587-gal4>UAS-piwi-RNAi* flies were analyzed in our study. Although *Gal80* is a temperature sensitive allele, the authors did not rule out the possibility that it is still partially functional at 29°C. The partially functional Gal80 at 29°C may lead to better suppression of Gal4, lower knockdown efficiency of Piwi and thus weaker tumor phenotype when flies were shifted to 29°C at adult stage compared to our experimental setup which completely lacks Gal80. Piwi immunostaining is needed to further confirm the repressive effect of Gal80 on Gal4 and the knockdown efficiency of *piwi* RNAi lines.

Comparing Fig. 3.1F and Fig. 3.2E, one can easily see that GSCs are lost more rapidly in *aub* mutants than mitotic recombination induced *aub* mutant GSC clones. These two experiments are different in two aspects. First, mitotic recombination was induced at the adult stage, meaning that Aub was fully functional during early germline development in *aub* mutant GSC clones while Aub was completely eliminated through development to the adult stage in *aub* mutants. Second, *aub* was fully functional in the soma in the mitotic recombination induced *aub* mutant GSC clones. One possibility is that Aub is required for the PGC development and/or GSC establishment, which is supported by the observation that 3-day old *aub* mutant germaria contain lower number of GSCs than that of the wild type on average (Fig. 3.1F). Second possibility is Aub protein perdurance in the mutant GSC clones and a longer period of time ACI may be needed to reveal more severe *aub* mutant GSC clone loss phenotype. Alternatively, Aub might also function in the soma to maintain the GSCs, which is highly unlikely because Aub is exclusively expressed in the germline of the *Drosophila* ovary and testis [114] and *c587-gal4*

mediated *aub* knockdown in the soma does not produce any detectable effect on the soma or the germline in the ovary (Fig. 3.S1).

The diffuse ϒ-H2AvD staining in the *aub* mutant GSCs is different from the punta staining in the 2a/2b region where DNA double-strand breaks initiate meiotic recombination (Fig. 3.2J-N). This expression pattern of ϒ-H2AvD might be specific to the germ cells with exogenous insult-induced DNA breaks in contrast to the highly regulated endogenous DNA breaks required for meiotic recombination. Similar diffuse ϒ-H2AvD staining has also been observed in *dacapo* mutant nurse cells in the *Drosophila* ovary [254], supporting the notion that this pattern might be specific to the germline. In addition, diffuse ϒ-H2AX, the homolog of H2AvD in mammals, has been shown in different mammalian cell lines after different DNA damage reagent treatments [255-257]. Thus, ϒ-H2AX as a DNA damage marker, might not specifically label DNA double-strand breaks. Instead, it might label different types of DNA damage with distinct localization patterns. It is possible that *aub* mutations induce various types of DNA damage with DNA double-strand breaks being the majority, which results in diffuse ϒ-H2AvD staining in *aub* mutant GSCs. Alternatively, pannuclear ϒ-H2AX has been attributed to the overactivation and diffusion of the ATR kinase in response to replication stalling in a human cell line [257]. Because ATR/Mei-41 is the major checkpoint kinase in response DNA damage in *Drosophila* [258-263], *aub* mutation induced DNA damage may lead to activation and diffusion of the ATR/Mei-41 kinase and thus pannuclear ϒ-H2AvD in the germ cells.

4.4 Future Directions

Several important conclusions have been made from the data presented in this study and many interesting questions still remain to be explored.

As presented in chapter 2, *piwi* knockdown in the ECs leads to germ cell differentiation defect, which coincides with the EC knockdown phenotypes of *eggless* [27], *Su(var)205* [27] and *Lsd1* [60]. These proteins have been associated with heterochromatin formation and transcriptional repression, strongly suggesting the importance of maintaining a repressive heterochromatin state in ECs for their proper functions and/or survival. Future experiments need to differentiate the chromatin status of the ECs and other ovarian somatic cell types such as the follicle cells and determine if this is functionally related to the ECs. It is also equally important to determine the developmental stage at which the repressive chromatin status, if any, is established in the ECs and if this developmental stage is correlated with the repression of *dpp* transcription in the somatic cells of the germarium other than the cap cells and TF cells. Further investigation is also needed to establish the molecular mechanism by which *dpp* is repressed in normal ECs and derepressed in *piwi* knockdown ECs. In principle, *dpp* repression in ECs might be regulated by a Piwi-piRNA mediated heterochromatin formation and/or piRNA-mediated transcript destruction. The repressive role of Piwi over *dpp* in the ECs appears to be opposite to its positive regulation of Dpp signlaing in the cap cells for GSC renewal [41, 264]. Future experiments need to delineate the molecular functions of Piwi in different cell populations in the ovary.

As it is shown in Fig. 2.4, TE activity and DNA damage are increased in *piwi* knockdown ECs, consistent with the general view of piRNAs in TE repression and genome protection. TE silencing is disrupted in mutations of most piRNA pathway components including Armi, Aub and Spn-E [92, 169] and transcription of LINE retrotransposons in mammalian cells induces DNA damage and DNA damage signaling response [265, 266]. Loss of TE silencing in *piwi* knockdown ECs could therefore cause DNA damage. DNA damage, however, can also cause

TE derepression [267-269]. *piwi* knockdown in the ECs therefore could compromise DNA repair and thus increase DNA damage, which, in turn, leads to TE derepression. Future experiments need to determine which event comes first, TE activation or DNA damage, in *piwi* knockdown and mutations of other piRNA pathway components. In addition, it remains unclear if TE activation and DNA damage have any causative role in *dpp* derepression in the *piwi* knockdown ECs. Future experiments also need to explore these possibilities.

This study confirmed TE activation in the ECs by knockdowning Yb, a partner of Piwi in the somatic piRNA pathway (Fig. 2.4G-O). *Yb* knockdown in the ECs with two independent RNAi lines shows similar but much weaker germ cell differentiation defect than *piwi* EC knockdown (Fig. 2.S7), although the TE reporter *gypsy-lacZ* is increased to a comparable level in both *Yb* and *piwi* knockdown. This observation suggests that TE activation in the ECs is not sufficient to induce the dramatic germ cell differentiation defect we observed in *piwi* EC knockdown germaria. Since TE activation almost always associates with DNA damage, it remains of interest if inducing DNA damage specifically in the ECs could cause germ cell differentiation defect and how it would regulate germ cell differentiation in a cell non-autonomous manner.

Presented in Fig. 3.2 is the drastic rescue of *aub* mutant GSCs by a *chk2/lok* mutant. Considering the intrinsic requirement of both PIWI proteins, Piwi and Aub, in GSC self-renewal, it would be of great interest to test the effect of *chk2*/*lok* mutations on *piwi* germline-specific knockdown. The major function of Piwi-piRNA complexes have been assigned to heterochromatin formation and maintenance in the nucleus [143, 162-166], while Aub-piRNA complexes function in the cytoplasm to amplify the piRNA pool and destroy TE transcripts. Both Piwi and Aub have been shown to predominantly bind to antisense piRNAs [88-90], but it

is not clear how many overlapping TE targets they share and if their mutations or knockdown initiate similar downstream DNA damage response. Neither is it clear if DNA damage response is the most significant consequence of *piwi* depletion in the ovarian GSCs. The universal expression of Piwi in both somatic and germline tissues adds more layers of complexity to this issue. Germline-specific knockdown of *piwi* and *chk2*/*lok* will answer the questions if DNA damage checkpoint activation is responsible for the *piwi* knockdown GSC loss and if Piwi and Aub share the same mechamism in maintaining the ovarian GSCs.

Aub binds to *nos* mRNAs in both *Drosophila* embryos [140] and ovaries [246] possibly through the 3'UTR of *nos*. The Bam-Bgcn complex has also been shown to negatively regulate the GSC renewal factor *nos* to promote germ cell differentiation via the 3'UTR of *nos* [70]. Supporting the idea that Aub and Bam form a complex to promote germ cell differentiation, we showed that *nos-Myc* genomic reporter is increased in *aub* mutant differentiating germ cell cysts (Fig. 3.3I-L'). Future experiments will be needed to test if Aub binds to *nos* mRNAs in the cultured GSCs with and without heat shock induced Bam expression to determine if Aub-*nos* mRNA interaction is Bam-dependent. Because *aub* mutations dramatically induce DNA damage in the germ cells, future experiments need to answer if Nos-Myc downregulation is the direct consequence of Aub depletion or the general effect of DNA damage response by comparing Nos-Myc levels in different piRNA pathway mutants and the ionizing radiation induced DNA damage background.

Both *bam-GFP* transcriptional reporter and Bam protein are downregulated in *aub* mutants (Fig. 3.4). Thus, *bam* can be regulated indirectly by Aub through transcription. Alternatively, Aub can stabilize *bam* mRNAs or enhance *bam* mRNA translation through *bam* 3'UTR or stabilize Bam protein via physical interaction. Future experiments need to distinguish

between these alternatives. Similar to the circumstance of Nos-Myc, the effect of *aub* mutations on Bam might be caused indirectly by TE activation and DNA damage. Future experiments also need to compare *bam-GFP* and Bam protein levels in different piRNA pathway mutants and the ionizing radiation induced DNA damage background to distinguish between the direct effect of Aub depletion and the general effect of DNA damage.

This study shows the physical (Fig. 3.5C) and genetic (Fig. 3.3A-E) interaction between a PIWI protein, Aub and a stem cell differentiation factor Bam. Taking into consideration the intrinsic requirement of another PIWI protein, Piwi, in germ cell differentiation (Fig. 2.6I-J), it is very attempting to speculate that Bam may interact with multiple PIWI proteins and even different components of the piRNA pathway to regulate various aspects of piRNA biology. Mass spectrometry is needed to be performed in the cultured GSCs with heat shock induced Bam expression to identify the interaction partners of Bam in the germline. Further *in vivo* immunoprecipitation with ovary lysate should be completed to follow up on the candidates from this large scale screen. Interestingly, Bam is also required for hematopoietic progenitor maintenance in *Drosophila* [270]. A human PIWI protein Hiwi is expressed in HSCs but not in their differentiated progeny [271] although the functional requirement is not clear [272]. The counterpart of Bam in mammals might interact with PIWI proteins to regulate the HSC lineage. Future experiments need to identify Bam homolog in mammals and determine if Bam-PIWI protein interaction is conserved in higher organisms. If Bam-PIWI interaction can be applied to mammals, follow up experiments need to determine the function of this interaction in stem cell regulation.

In this study, we show that *bam* mutant ovaries upregulate TEs (Fig. 3.6) without affecting piRNA production (Fig. 3.7), indicating that Bam functions downstream of piRNA

production to regulate TEs. This observation is similar to that of *mael* deficiency, which increases TEs in both soma and germline in the ovary without affecting piRNA biogenesis [162]. In addition, Mael is dominantly expressed in the cytoplasm, particularly the perinuclear nuage, which coincides with the expression domain of Bam. Future experiments should be performed to determine if Bam and Mael function together in the germline to regulate TEs. One possible function of Bam in TE regulation is to bring TE transcripts and Aub together so that Aub can exert its slicing activity on the TE transcripts. Future experiments are needed to test if Bam directly binds to TE transcripts or indirectly binds to TE transcripts via its RNA-binding partners such as Bgcn.

As determined by RT-qPCR in Fig. 3.6C-E, germline-specific TEs including *TART* and *Het-A* are significantly upregulated in *bam* mutants. More surprisingly, soma-specific TEs including *gypsy* also increase expression levels in *bam* mutants, which is contradictory to the traditional view of the germline specific expression and function of Bam in the *Drosophila* ovary. It has been well studied that the somatic specific *gypsy* transposon is post-transcriptionally silenced by the *flamenco* piRNA cluster. The *flamenco* piRNA cluster is located in the centromeric heterochromatin of the X chromosome that is exclusively transcribed from one strand in comparison with most piRNA clusters that produce piRNAs from both genomic strands including the typical germline specific piRNA cluster 42AB. The *flamenco* locus contains fragments of active TEs that are located throughout the genome including *gypsy* and *ZAM* elements and mutations in this locus lead to overexpression of these dispersed TEs in the genome [88, 273]. The *flamenco* locus functions primarily in the ovarian somatic cells, with mutations of which upregulating *gypsy* and *ZAM* transcripts in the soma of the ovary [273]. However, the transcriptional and/or post-transcriptional regulation of the gypsy TE in the germline is not well

understood. The increased transcript level of *gypsy* in *bam* mutant ovaries can be attributed to either unidentified cell non-autonomous function of Bam if any in the soma to modify *gypsy* repression or cell autonomous function of Bam in the germline to regulate *gypsy* transcription or transcript silencing. Further experiments are needed to quantify *gypsy* RNA levels in purified *bam* mutant somatic cells and germ cells to determine which cell population contributes to the increased *gypsy* transcript level. More detailed molecular studies might be needed to determine if and how *gypsy* is transcriptionally and post-transcriptionally repressed in the germline and the role of Bam in these processes. Chromatin immunoprecipitation with several heterochromatin markers including Piwi, HP1 and H3K9me3 followed by sequencing may be needed to determine the chromatin status of *gypsy* and other somatic specific TEs in the purified germ cells.

As presented in Fig. 3.6 and Fig. 3.7, *bam* mutations lead to TE activation without affecting the levels of piRNAs, indicating that TE activation is not the result of compromised piRNA production. Instead, *bam* mutant undifferentiated CBs stay healthy and keep proliferating in the germarium as long as the fly lives with an abnormally high level of TE activities. This observation raises the possibility that TEs might have physiological functions in the GSCs and/or CBs without inducing massive DNA damage. It has been more than 20 years since TEs were suggested to be selfish genomic elements that create "junk DNA" and destabilize host genomes [274, 275]. In the meantime, as more and more genomes are being sequenced, our understanding of the beneficial aspects of TEs increases. Examples from the literature illustrate how TEs can shape the genome of both somatic and germline cells in many different aspects. These include creating new coding or non-coding genes, modifying cis regulatory elements and even changing the epigenetic regulation of genes proximal to the TE insertion sites as a by-product of the defense mechanism from the host against the TEs [276]. TE activity in the soma

may diversify gene regulation and expression in a tissue-specific manner at the level of single individuals. TE activity induced genome modification in the germline may be passed on to the next generation, which may act as the template for natural selection and drive evolution. Future experiments need to be targeted to the innovative functions of the TEs in both the soma and the germline in the *bam* mutant *Drosophila* ovary and to determine if these TE activities are related to stem cell renewal and differentiation.

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