The function of RNA Binding Protein 9 in germ cell differentiation in *Drosophila* ovary

BY

Min Yao

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Chairperson William H. Kinsey, Ph.D

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Ting Xie, Ph.D

_____

Susan Abmayr, Ph.D

_____

Joseph D. Fontes, Ph.D

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Kausik Si, Ph.D

Date defended: <Aug-29-2013>

The Thesis Committee of **Min Yao**

certifies that this is the approved version of the following thesis:

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Chairperson William H. Kinsey, PhD

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Abstract

Germ cell development is an essential process to ensure continuity of species. *Drosophila* oogenesis has long been served as a model system to study germ cell development. Previously, the Elav-Hu family protein *RNA-binding protein 9* (*rbp9*) has been reported important for germ cell differentiation in *Drosophila* ovary, but its mechanism of function is largely unknown. In this study, we aimed to further study the function of Rbp9 in germ cell differentiation. Firstly, we confirmed *rbp9* mutant phenotype by using *rbp9* germline specific RNAi knock-down. We further confirmed that Rbp9 is highly expressed in sixteen-cell cyst and early egg chambers by using BAC transgenic reporter. *Rbp9* mutant germarium contains accumulated early cysts. BMP signaling and Bam regulation is not affected in *rbp9* mutant, but early differentiation cysts expressing Nanos, Sxl and Bam are accumulated in *rbp9* mutant. Moreover, sixteen-cell cysts are failed to further differentiate in *rb9* mutant. Both Bruno and Orb expression cysts are present in *rbp9* mutant, but they are blocked before oocyte specification and begin to degenerate. And meiosis is also failed initiated in *rbp9* mutant. The function of *rbp9* in germ cell differentiation is required for both adult stage and before adult stage. Though Rbp9 is critical for cyst differentiation, over-expression of Rbp9 does not affect germ cell development in germarium region. But Rbp9 over-expression will affect number of cyst division times and mid stage egg chamber development. Moreover, we identified that *rbp9* genetically interacts with *bam* in regulating cystoblast differentiation. Rbp9 functions as downstream of Bam in germline differentiation, as Rbp9 over-expression can partially drive germ cell differentiation in *bam* mutant, but lack of Rbp9 blocks Bam driving germ cell differentiation. In all, this study will help better understand function of *rbp9* in germ cell differentiation in *Drosophila* ovary, and may also provide insights in general germ cell development.

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Chapter I: Background introduction

Germ cell development and *Drosophila* oogenesis

Germ cells are unique cell type that carries genetic information from one generation to the next and maintains continuity of species. Germ cell development is a complex process and tightly regulated. Many aspects of germ cell development are highly conserved (Lesch and Page, 2012). *Drosophila* oogenesis has long been served as a model system to study germ cell development (de Cuevas et al., 1997).

Drosophila female contains a pair of ovaries, in which oogenesis continues throughout their adult lifespan. Each ovary consists of about 15 units of ovarioles, each resembling a line of oocyte production chain. *Drosophila* oogenesis begins as germline stem cell located in gemarium goes through asymmetric cell division to generate one stem cell and one cystoblast. The cystoblast is located away from stem cell niche environment, and will go through differentiation process. During differentiation, cystoblast will divide to give interconnected two-, four-, eight- and finally sixteen-cell cysts through incompletely cytokinesis (de Cuevas et al., 1997). Within final sixteen-cell cysts, one germ cell will become the future oocyte and the remaining fifteen cells will become supporting nurse cells. Oocyte meiosis is initiated in 16-cell cysts in gemarium region and stay arrested in prophase of first meiosis division until fertilized by sperm (Lake and Hawley, 2012). The overall process of oogenesis takes about one week. Female germline stem cells and differentiation cystocytes can be easily identified by their location and a special organelle shape called fusome, thus providing a model system to study germ cell development (Yue and Spradling, 1992).

Drosophila female germline stem cell regulation

The maintenance of germline stem cells is achieved by both extrinsic environment and intrinsic factors. Extrinsically, cap cells serve as the main supporting cells of both by providing niche signaling and physical anchoring interaction. Specially, cap cells secret short ranged BMP ligands *dpp* and *gbb* to keep stem cell in undifferentiated state (Xie and Spradling, 1998; Song et al., 2004). Also, cap cells anchor stem cells by DE-cadherin junction to keep them in the niche environment to receive the BMP ligands (Song et al., 2002). Activated Smads from BMP signaling bind directly to *bam* promoter and repress its transcription (Chen and McKearin, 2003a; Song et al., 2004). Besides cap cells, escort cells have also been suggested to play a role in GSC self-renewal (Kirilly et al., 2011).

Intrinsically, germline stem cells also contain unique factors to maintain stem cell fate. The components for conducting of BMP signaling are essential for self-renewal, including BMP receptors (*tkv*, *punt* and *sax*) and signaling mediators (*mad*, *Med* and *shn*) (Xie and Spradling, 1998; Xie and Spradling, 2000). E-cadherin and cadherin junction components *β*-catenin/Amardillo are required for GSC anchorage to cap cells (Song et al., 2002). A few other factors acting on stabilizing and promoting cell adhesion and/or BMP signaling have also been showed to be important for GSC maintenance, such as microtubule moter protein regulator *lis1* (Chen et al., 2010) and chromosome remodeling factors *iswi* (Xi and Xie, 2005). Besides BMP signaling and adhesion regulation, there are also a group of regulation factors essential for GSC maintenance. *Nanos* and *pumillio* are two important factors for self-renewal (Forbes and Lehmann, 1998; Wang and Lin, 2004) . They are purposed to form a translational regulation complex, however their regulating targets are largely unknown (Forbes and Lehmann, 1998) . Other translational regulators *eIF4A* and *pelota* also have a function in stem cell self-renewal (Xi

et al., 2005; Shen et al., 2009). Additionally, miRNA pathway is also important in GSC self-renewal, as defects in miRNA pathway components *dcl-1* and *loqs* GSC also lead to GSC loss and reduced proliferation (Jin and Xie, 2007; Park et al., 2007).

Drosophila female germ cell differentiation regulation

Germ line differentiation begins by the generation of cystoblast from asymmetric division of germline stem cells. *Bag of marbles* (*bam*) is the most potent factor for promoting differentiation. *Bam* mutant blocks differentiation in the cystoblast stage, while overexpression of *bam* is sufficient to induce GSC differentiation (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). *Bam* transcription is repressed by BMP signaling in GSC, and it begins to express in the cystoblast when BMP signaling is reduced (Chen and McKearin, 2003a; Song et al., 2004).The expression of Bam reaches the highest in eight-cell cysts and decreases later (McKearin and Ohlstein, 1995). *Bam* functions with *bgcn* in a complex promoting differentiation, and they are proposed to function as a translational regulator (Lavoie et al., 1999; Ohlstein et al., 2000). Only a few regulatory targets have been identified for Bam/Bgcn complex so far, such as *shg* (Shen et al., 2009) and *nanos* (Li et al., 2009). *Sxl* has been recently reported to be required for Bam/Bgcn complex in regulation of *nanos* expression (Chau et al., 2012).

Besides the Bam/Bgcn complex, a few other genes have been identified to function in different steps of cystocytes differentiation. Mutations in *RNA-binding protein 9*(*rbp9*) (Kim-Ha et al., 1999), *sex lethal (sxl*) (Chau et al., 2009), *mei-P26 (Neumuller et al., 2008)* and *A2bp2 (Tastan et al., 2010)* arrest cystocytes differentiation mostly in single- to two-cell stages, while mutation in *aret (Parisi et al., 2001)* and *orb (Lantz et al., 1994)* arrest cystocytes mainly in the eight-cell cysts stage.

Sxl is a RNA binding protein and is required for *bam/bgcn* regulation of *nanos*, possibly by direct binding of *nanos* 3UTR (Chau et al., 2012). *A2bp2* also encodes a RNA binding protein, but its direct targets in germline are unknown (Tastan et al., 2010). Mei-P26 interacts with miRNA pathway components and functions to regulate cell proliferation and cell growth in cystocytes (Neumuller et al., 2008; Li et al., 2012).

In *rbp9* mutant ovary, a mixture of single, two- and fewer branched cystocytes are accumulated and their further differentiation are blocked (Kim-Ha et al., 1999). *Rbp9* expresses highly from sixteen-cell cysts to germ cells in stage two and stage three egg chambers (Kim-Ha et al., 1999; Tastan et al., 2010). Over-expression of Rbp9 has been reported to induce mid-stage egg-chamber degeneration (Jeong and Kim-Ha, 2004).

Both *orb* and *aret* begin expression in sixteen cell cysts, and regulate sixteen-cell cyst formation and later oocyte development (Lantz et al., 1994; Parisi et al., 2001). *Orb* encodes a RRM-containing protein homolog to CPEB protein, and functions in localization and translational regulation of mRNA targets involved in oocyte determination and polarity formation (Chang et al., 2001). *Aret* encodes RRM containing protein Bruno, which binds to mRNA 3UTR containing BRE site and represses their translation, functions in cystocytes differentiation, oocyte meiosis arrest, oocyte and embryo polarity maintenance (Kim-Ha et al., 1995; Parisi et al., 2001; Sugimura and Lilly, 2006).

Elav-Hu protein families

Rbp9 encodes a RNA-binding protein containing three-RRM domains, and belongs to a conserved *Elav/Hu* protein family (Kim and Baker, 1993). The *Elav/Hu* protein family are highly conserved RNA-binding proteins involved in a diverse range of functions. In mammalians, the

neural Hu genes (*HuB, HuC, HuD*) play an important role in neuron differentiation and plasticity, while the ubiquitous expressed *HuR* has been implicated in cell stress response, tissue differentiation and tumorigenesis (Pascale and Govoni, 2012). The function of r*bp9* in germ cell may not be unique, as other members of Elav/Hu have also been suggested function in the reproduction system in zebrafish and mice (Chi et al., 2011; Wiszniak et al., 2011), indicating a conserved role of the Elav/Hu family in reproduction system.

Structurally, proteins of Elav/Hu family members all contain three conserved RNA Binding Domains (RRM domain). Elav/Hu family members have been showed to directly bind to mRNA or pre-mRNA using their RRMs, and have functions in regulating mRNA splicing or mRNA translation (Pascale and Govoni, 2012). In translation regulation, this family of proteins have been showed to bind to U-rich ARE elements within 3UTR of mRNA targets, and promote target mRNA stability and increase translation, and in rare cases this binding can also affect translation independent of mRNA stability (Pascale and Govoni, 2012). Recently a genome wide HuR binding targets study has discovered that HuR binding sites are close to the miRNA binding site, suggesting HuR binding of mRNA targets may potentially protect them from miRNA mediated degradation (Lebedeva et al., 2011).

In *Drosophila*, three members of Elav/Hu family have been identified, consisting of *elav*, *fne* and *rbp9*. While all three family members are expressed in most mature neurons (Robinow et al., 1988; Samson and Chalvet, 2003), *rbp9* is also expressed in cystocytes in the ovary (Kim-Ha et al., 1999). *Elav* and *fne* both function in neuron differentiation and maintenance (Yao et al., 1993; Zanini et al., 2012), but *rbp9* mutant mostly affects females fertility (Kim-Ha et al., 1999; Kim et al., 2010). The three RRM domains of Rbp9 protein share high similarity (about 70%) with its *Drosophila* paralog Elav and human homolog HuD (Kim and Baker, 1993). A subsequence

study shows RRM1 and RRM3 of Rbp9 can partially replace Elav corresponding RRMs in function (Lisbin et al., 2000). *In vitro* binding assay using has identified consensus Rbp9 binding site as a stretch of poly U sequence (Park et al., 1998; Ray et al., 2013) , though functional *in vivo* targets have not been identified yet.

Significance of proposed research

Drosophila oogenesis has long been served as model system to study germ cell development. *Rbp9* has been reported before to be important for germ cell differentiation in female ovary. However careful characterization of the phenotype is lacking and the potential mechanism is largely unknown. In the proposed study we are planning further study of the function of *rbp9* in female germ cell differentiation. Our work will provide new insights in *rbp9* function in *Drosophila* oogenesis and help better understand germ cell regulation in general.

Chapter II: Materials and methods

Fly stocks and culture

Information about fly stocks used in this study are either available in Flybase (www.flybae.org) or specified here: *bam*$^{\Delta 86}$ (McKearin and Ohlstein, 1995), *bam*^{E5} (kindly provided by Dr. Scott Hawley at Stowers Institute, MO, USA) ,*bam-GFP* (Chen and McKearin, 2003b), *bam-HA* (kindly provided by Dr. Xin Chen from John Hopkins University (Eun et al., 2013)), *hs-bam* (Ohlstein and McKearin, 1997), *bgcn (bgcn*^{20093}*and bgcn*^{20915} (Jin et al., 2008)), *Dad-lacZ (Tsuneizumi et al., 1997), mei-P26* (mei-P26^{fs1} and mei-P26^{1} (Page et al., 2000)), *nos-gal4* (Van Doren et al., 1998), *nos-myc* (Verrotti and Wharton, 2000), *nos-gal4* conditional expression system (kindly provided by Dr. Yukiko Yamashita from University of Michigan and adapted by Xing Ma of Xie lab) , *rbp9* RNAi (attp2 *rbp9*^{TH00260} and attp40 *rbp9*^{TH00624} , kindly provided by Dr. Jianquan Ni from Tsinghua University, China (Ni et al., 2011)), *rbp9*$^{\Delta 1}$ (Kim-Ha, 2000).

To generate *rbp9* BAC transgenic reporter, the BAC clone CH322-140N12 from P[acman] library (Venken et al, 2009; http://www.pacmanfly.org/libraries.html) was ordered from CHORI. This BAC is about 20kb and contains *rbp9* gene (about 13kb) and flanking sequence in both ends. Previously, a 17kb genomic region within this BAC has been used to successfully rescue rbp9 mutant (Kim-Ha, 1999). The BAC is modified using BAC recombineering (Warming et al, 2005) to insert three repeats of Flag in front of ATG of Rbp9 coding region. The confirmed modified BAC is targeted to VK00033 site on chromosome three by phi31 mediated site-specific targeting (Venken et al, 2009) in Rainbow transgenic Inc service. Transformed flies were identified and analyzed for Rbp9 expression pattern.

To generate N-terminal Flag tagged *rbp9* over-expression transgenic fly, *rbp9* coding sequence (NM 057589, variant I) are cloned into pPFW vector by gateway clone. Correct clone is confirmed by sequencing and sent to Rainbow Transgenic Inc. to generate random inserted transgenic flies. Transformed flies stocks are established, balanced, and chromosome insertion sites are determined before they are used for this study.

Fly lines are maintained in room temperature, and crossings are carried out in 25^{o}C. To enhance RNAi effect, adult *nos-gal4 rbp9* RNAi flies are maintained in 29 ^{o}C. Adult flies of one week old are used for analysis unless specified. To analyze newly enclosed fly phenotype, 1-2 days old adult *nosgal4 rbp9* RNAi and control flies are used.

To analyze *rbp9* function in adult germline stem cells, one week old adult conditional gene activation flies (see text in next chapter) and control flies are heat shocked for one hour in 37^{o}C water bath, and cultured for another one week or two weeks before analyzed.

To test whether Bam over-expression can drive germ cell differentiation in *rbp9* mutant, one week old flies from control (*rbp9*$^{\Delta 1}$*/+;hs-bam/+*) and *rbp9* mutant (*rbp9*$^{\Delta 1}$*;hs-bam/+*) are heat shocked in 37^{o}C water bath one hour once or twice (with eight hour interval). Non heat shocked flies are used as control. Heat shocked flies are further cultured for one week before analysis.

Immunostaining

Drosophila ovary staining is performed according to previously published procedure (Xie and Spradling, 1998; Song et al., 2002). Briefly, ovaries from about 25 adult female flies are dissected and teased apart in Grace's medium, then fixed in 4% paraformaldehyde (Sigma) for 15 min. The fixed samples are then washed in 0.1% Trition-X100 containing PBS solution for 1h, blocked with 5% goat serum (Jackson lab) for 1h and stained with primary antibody overnight.

The samples are then washed for six times in 0.1% Trition-X100 containing PBS solution for total of two hours and stained with secondary antibody overnight. The next day the samples are washed again and stained with DAPI and then are put into slides in mounting medium. Pictures are taken under confocal microscope (Leica SP5) and counting of samples is done under a fluorescent microscope (Leica).

Primary antibodies used in this study include: mouse monoclonal anti-Hts (1:50, Developmental Studies Hybridoma Bank (DSHB)), rabbit polyclonal anti-beta-galactosidase antibody (1:100, Promega), chicken anti-GFP (1:100, Invitrogen), mouse anti-Sxl M114 (1:4, DSHB), mouse anti-Orb (1:30, DSHB), rabbit anti-myc (1:100, Sigma), rabbit anti-HA (1:100, Sigma), mouse anti-Flag (1:100, Sigma). Antibody rabbit anti-Bruno (1:3000) is kindly provided by Dr. Mary Lilly from National Institute of Health in Maryland. Antibody rabbit anti-C(3)G (1:500) is kindly provided by Dr. Scott Hawley in Stowers Institute.

Quantification and Statistical analysis

For quantification and statistical analysis, around 25 flies are dissected and stained for each sample. Germline stem cells and cystoblast from about 50 ovarioles are counted under fluorescent microscope from each sample. Average and standard derivation are calculated and student t-test are used for comparison, with P value ($p<0.05$) as the threshold of significant difference.

Chapter III: Rbp9 functions in germline differentiation in *Drosophila* ovary

Rbp9 mutant germarium contains accumulated early cysts

Previously, *rbp9* mutant has been reported to block female germ cell differentiation (Kim-Ha et al., 1999). We aimed to further characterize the phenotype. First we confirmed the *rbp9* mutant phenotype using two *rbp9* RNAi lines under *nanos-gal4* germ line specific knockdown. The *rbp9* knockdowns show similar phenotype as previously reported *rbp9* mutant (Figure 1). Based on spectrosome morphology (revealed by Hts staining), *rbp9* mutant germarium regions contain accumulated cysts, and majority of those cysts are one-cell cystoblast and two-cell cysts. A small portion of eight-and sixteen-cell cysts are also found in germarium (Figure 1B, 1C and 1F) in two independent RNAi lines. In early egg chambers, germ cell begins to degenerate as indicated by condensed nuclei and reduced cell numbers. No normal late stage egg chambers or mature oocytes form in *rbp9* mutants (Figure 1A and 1B).

To further characterize *rbp9* mutant phenotype, we first tried to check whether BMP signaling is up-regulated in *rbp9* mutant. As previous reports suggested that up-regulation of BMP signaling can lead to cystoblast accumulated phenotype (Liu et al, 2010). BMP signaling is an important signaling in controlling GSC maintenance (Xie and Spradling, 1998) and its activity can be revealed by Dad-lacZ reporter (Tsuneizumi et al., 1997). In wildtype GSC, LacZ is highly expressed in GSC and one-cell cystoblast (Figure 2A; (Song et al., 2004)). In *rbp9* RNAi mutant, high expression of LacZ is still restricted to GSC (Figure 2B). In wildtype GSC, *bam* transcription is repressed by BMP signaling in GSC, as indicated by bam-GFP reporter (Figure 2C; (Chen and McKearin, 2003b)). Bam-GFP expression gradually increases from cystoblast to eight-cell cysts and decreases in sixteen-cell cysts (Figure 2C). In *rbp9* RNAi mutant, bam-GFP

is also not detected in GSCs and expressed normally in differentiation cysts (Figure 2D). These results suggest that *rbp9* mutant does not affect BMP signaling and Bam regulation.

To further check early steps of differentiation, a few markers in different step of differentiation were examined in *rbp9* mutant. Sxl is expressed highly in cytoplasm in wildtype GSC, cystoblast and two-cell cysts, and then it is translocated into few nuclei foci in four- to sixteen cell cysts (Figure 3A; (Bopp et al., 1993)). In *rbp9* RNAi mutant, cytoplasmic Sxl is detected in many germ cells throughout germarium region while few nuclei located Sxl germ cells present (Figure 3B). Nanos protein is expressed highly in GSC and cystoblast, but the expression is diminished in four- and eight-cell cysts, and then it expresses highly again in sixteen-cell cysts (Figure 3C; (Li et al., 2009)) as revealed by Nanos-myc reporter (Verrotti and Wharton, 2000). In *rbp9* mutant, majority of germ cells are positive for Nanos-myc expression, constaining of Hts reveals they are mostly early differentiation cysts (Figure 3D). The down-regulation of Nanos in four- and eight-cell is also present in *rbp9* mutant (Figure 3D). Lastly, Bam protein level is examined by Bam-HA reporter (Eun et al., 2013). Bam protein express highly in eight-cell cysts in similar level between wildtype control and *rbp9* RNAi mutant (Figure 3E and 3F). Together, those results indicate *rbp9* mutant cystoblast, two-cell cyst, four- and eight-cell cyst differentiation can oocure with normal expression marker. However, the differentiation process is comprised and cystoblasts and early cysts are accumulated in *rbp9* mutant.

Rbp9 is required for sixteen-cell cyst differentiaiton

To further determine which step of germ cell differentiation is defective, a few sixteen-cell cyst differentiation markers were examined in *rbp9* RNAi mutant. Bruno begins to express in sixteen-cell cysts in wildtype ovariole and concentrates in oocyte in sixteen-cell cysts and early

egg chambers (Figure 4A; (Parisi et al., 2001)). In *rbp9* RNAi mutant, Bruno expressing cysts are present and they begin to degenerate in egg chambers; no Bruno concentrated oocytes can be observed in egg chambers or in germariums (Figure 4B). C(3)G is protein component of synaptic complex, thus functions as marker of meiosis initiation. In wildtype germarium, nuclei located C(3)G is highly expressed in about four cells within sixteen-cell cyst, and then is gradually restricted to oocyte (Figure 4C; (Lake et al, 2012)). In contrast, no strong nuclei located C(3)G signal is observed in *rbp9* RNAi mutant (Figure 4D). Lastly, Orb also begins to express in sixteen-cell cysts in wildtype, and its expression is concentrated in oocytes in sixteen-cell cysts in later sixteen-cell cysts and all later egg chambers (Figure 4E and 4G; (Lantz et al., 1994)). In *rbp9* RNAi mutant, Orb expressing-cysts are present but begin to degenerate and no Orb concentrated oocytes are observed (Figure 4F). In a weaker *rbp9* RNAi ($rbp9^{TH00624}$), some egg chambers are able to develop and contain condensed nurse-cell like nuclei. Staining with Orb indicates no oocyte is specified in those egg chambers (Figure 4H).

These results suggest sixteen-cell cyst can be initially generated in *rbp9* mutant, but they are failed to initiation of meiosis and develop into oocyte-specified later stage sixteen-cell cyst. Thus, Rbp9 is required for proper differentiation of sixteen-cell cyst.

Rbp9 is highly expressed in sixteen-cell cyst and early egg chambers

To better correlate *rbp9* function with its mutant phenotype, we would like to determine its expression pattern. Previously Rbp9 has been reported to express highly in posterior germarium and early egg chamber (Kim-Ha et al, 1999). Due to unavailability of this antibody, we decided to generate a *rbp9*-BAC transgenic reporter to confirm its expression pattern. A BAC clone containing *rbp9* gene and flanking sequence from P[acman] library (Venken et al, 2009) was used and was engineered to insert Flag tag in N-terminal in frame with Rbp9 coding region. The

BAC transgenic reporter fly was generated by targeting the modified vector to a defined docking site in chromosome three (Figure 5A). Examination of Rbp9 expression reveals that Rbp9 is highly expressed in sixteen cell cysts (Figure 5C and 5C') and stage two and stage three egg chambers, and gradually decreased later on (Figure 5B). And it also is slightly enriched in oocyte in sixteen-cell cysts and early egg chambers (Figure 5B and 5C').

***Rbp9* is required for female germ cell differentiation both before and in adult stage**

To determine the time window when *rbp9* begins to function importantly for germ cell differentiation, *rbp9* RNAi mutant ovaries were examined at an early time. Germ cells first begin to differentiation in pupa stage in some primordial germ cells. We checked indirectly on 1-2 days old newly enclosed *rbp9* mutant RNAi flies and discovered that germ cell differentiation defect is already apparent (Figure 6G and 6H). This suggests that *rbp9* begins to function in germ cell differentiation before adult stage.

To determine whether *rbp9* is also required for germ cell differentiation in adult ovary, a conditional *nos-gal4* expression system was used to knock down *rbp9* in adult ovary (Figure 6A; generously provided by Dr. Yukiko Yamashita). In this system, *rbp9* knockdown germarium is also marked by histone GFP expression (Figure 6C, 6D and 6E). Two weeks after induction of *rbp9* knockdown, cystoblasts are accumulated in GFP positive clones (Figure 6B and 6D). In addition, germ cells in later stage egg chambers are degenerated as *rbp9* mutant (Figure 6E and Figure 1B). This suggests *rbp9* is also required for adult germ cell differentiation in the ovary.

Rbp9 over-expression affects cyst division and germ cell development in mid-stage egg chamber

To gain further insights of how *rbp9* functions in germline differentiation, Rbp9 was over-expressed in germ cell to test whether it affects germ cell development. N-terminal 3X Flag tagged UASp driving Rbp9 transgenic fly lines were generated by P-element mediated random insertion, and different insertion lines were used for the study. *Nanos-gal4* driving over-expression of *rbp9* in wildtype germ cells does not affect GSCs and cysts differentiation (Figure 7A and 7B). And this Flag tagged Rbp9 is able to full rescue *rbp9* mutant (Figure 7C). We observed that some percentage of germ cell degeneration in mid-stage egg-chambers as reported before (Figure 7E; (Jeong and Kim-Ha, 2003)). The degree of germ cell degeneration is variable between different transgenic lines, and possible indicates different transgene expression levels (Figure 7F). Interestingly, we also observed that a small portion of egg chambers containing 32-cell , 8-cell or 4-cell cysts as contrast to normal 16-cell cysts in *rbp9* over-expression ovaries (Figure 7D). This phenotype was also consistent in different transgenic fly lines (Figure 7F). These results suggest Rbp9 over-expression affects cyst division and germ cell development in mid-stage egg chamber.

***Rbp9* genetically interacts with *bam* in regulating germ cell differentiation**

Germ cell differentiation defect in *rbp9* mutant is similar to a few other reported mutants such as *bam*, *sxl* and *mei-P26* (McKearin and Ohlstein, 1995; Neumuller et al., 2008; Chau et al., 2009). Genetic interactions were tested to determine whether *rbp9* functions with these factors in regulating differentiation process. Interestingly, removal of one copy of both *rbp9* and *bam* delays germ cell differentiation by accumulating cystoblasts and cysts (Figure 8A,8B, 8C and 8F). This interaction seems to be specific to *bam*, as there is no such synergistic effect between

rbp9 and *sxl* or *rbp9* and *mei-p26* (Figure 8D,8E and 8G). This suggests *rbp9* genetically interacts with *bam* in regulating germ cell differentiation.

Over-expression of Rbp9 can partially drive germ cell differentiation in *bam* mutant

To understand how *rbp9* and *bam* interacts, we hypothesized that Rbp9 may function as downstream of Bam in germline differentiation process. As we reasoned that Rbp9 high expression comes later than Bam expression (Figure 5C and 3E). To test this, Rbp9 was over-expressed in *bam* mutant to check whether it can rescue germ cell differentiation defect. In bam mutant, germ cell differentiation is blocked in cystoblast stage (Figure 9A, 9D and 9G; (Mackearin et al, 1995)). Interestingly, over-expression is able to drive germ cell further differentiated into four-, eight- and even sixteen-cell cyst stage (Figure 9B, 9C, 9H and 9I). And this is confirmed by different rbp9 over-expression transgenic fly line (Figure 9C and 9F). These results suggest over-expression of Rbp9 can partially drive germ cell differentiation in *bam* mutant.

Lack of Rbp9 blocks Bam-driven germ cell differentiation

Lastly, we wished to test whether Bam over-expression can drive germ cell differentiation in *rbp9* mutant. In wildtype ovary, heat-shocked induced over-expression of Bam is sufficient to induce germline stem cells and germ cell differentiation (Ohlstein et al, 1997). About 77% percent control gemariums contain no germ cell one week after one hour heat shock (Figure 10A,10B and 10E). In contrast, majority of germariums from *rbp9* mutant still contain accumulated cysts and germline stem cells after heat shock (Figure 10C, 10D and 10E). We quantified the Bam-driven differentiation process by measuring GSC numbers. Bam over-expression significantly reduce GSC numbers, while in *rbp9* mutant GSC are present after *bam* over-expression (Figure 10D and 10F). Those germ cells are still present even by increased heat

shock time (data not shown). These results suggest that lack of Rbp9 blocks Bam-driven germ cell differentiation.

Figure 1. *Rbp9* is required for germ cell differentiation in *Drosophila* ovary.

Ovarioles and gemariums were stained for Hts (green) and DNA (blue). Images from B-F are overlay of serial sections of confocal images, and image in A is single section. Scale bar represents 15um.

(A) Wildtype ovariole with normal oogenesis from single confocal section.

(B) *Rbp9* RNAi knockdown by *nos-gal4/rbp9TH*^{00260} shows germ cell differentiation defect in one week old adult ovary. It contains accumulated cysts in gemarium and egg chambers.

(C) *Rbp9*^{A1} mutant germarium shows similar phenotype as *rbp9* RNAi knockdown.

(D) Wildtype gemarium contains germline stem cells and differentiation cysts.

(E) *Rbp9* knockdown gemarium contains germline stem cells and accumulated cysts.

(F) *Rbp9* knockdown in a different RNAi line *nos-gal4/rbp9*^{TH00624} shows similar phenotype as *nos-gal4/rbp9TH*^{00260} and *rbp9* mutant.

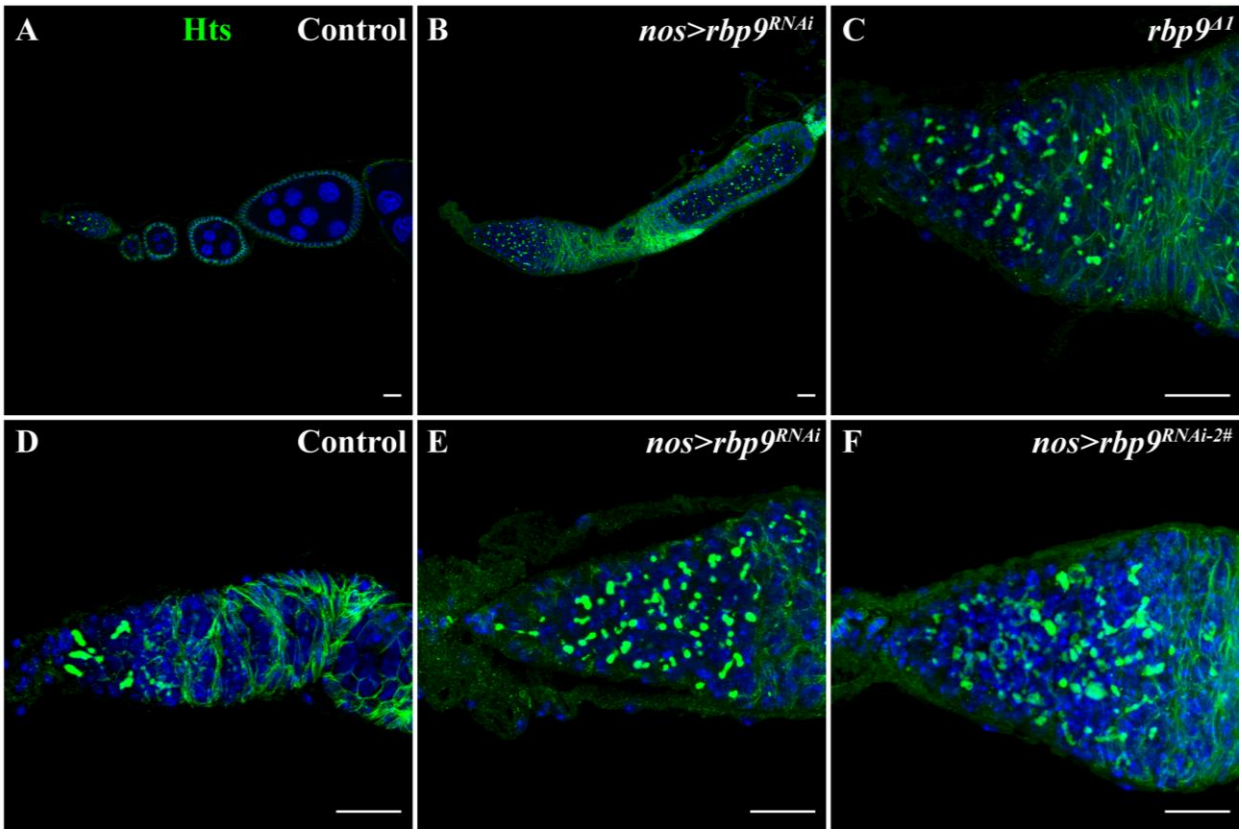

A
Hts
Control
B
nos>rbp9RNAi
C
rbp9Δ1
D
Control
E
nos>rbp9RNAi
F
nos>rbp9RNAi-2#

Figure 2. BMP signaling and *bam* regulation is normal in *rbp9* mutant.

Comparison of BMP signaling and Bam expression in adult germariums between wildtype control (A and C) and *rbp9* RNAi (B and D). All samples were co-stained DNA (blue). All images are presented as single confocal section. Scale bar represents 15um.

(A) Dad-lacZ expression pattern in wildtype control germarium (*nos-gal4, Dad-lacZ/+*) stained by LacZ (green) and Hts (red)..

(B) Dad-lacZ expression in *rbp9* RNAi (*nos-gal4, Dad-lacZ/rbp9*^{TH00260}) germarium. Note that LacZ expression is restricted to germline stem cells as in wildtype.

(C) Bam transcription pattern in wildtype control germarium (*nos-gal4, bam-GFP/+*) stained by GFP (green) and Hts (red).

(D) Bam transcription pattern in *rbp9* RNAi (*nos-gal4, bam-GFP/rbp9*^{TH00260}) germarium. Note that germline stem cells are negative for GFP and differentiation cysts are positive for GFP.

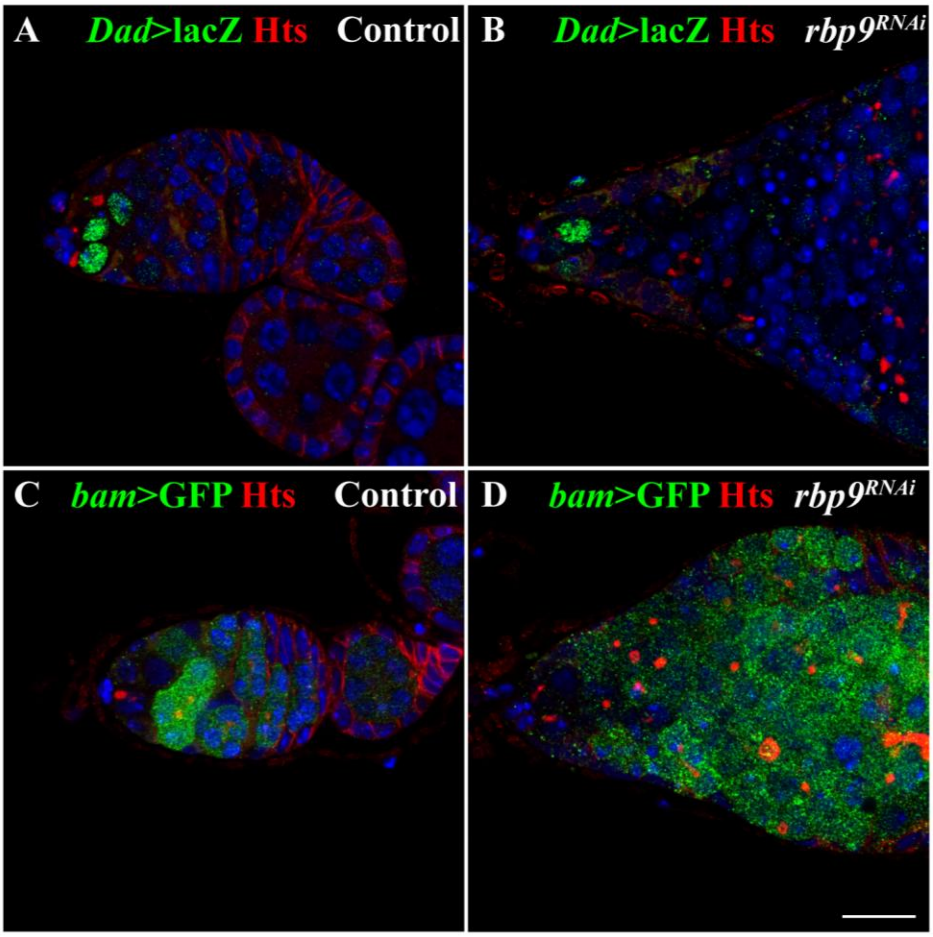

A
Dad>lacZ Hts
Control
B
Dad>lacZ Hts
rbp9RNAi
C
bam>GFP Hts
Control
D
bam>GFP Hts
rbp9RNAi

Figure 3. *Rbp9* mutant accumulates cysts in early differentiation stages.

Comparison of early cyst differentiation markers in adult germariums between wildtype control (A, C and E) and *rbp9* RNAi (*nos-gal4/rbp9TH*^{00260} in B, D and F). All samples were co-stained DNA (blue). All images are presented as single confocal section. Scale bar represents 15um.

(A) Sxl protein level in wildtype control germarium (*nos-gal4/+*) stained with Sxl (green). Note the Sxl is expressed highly in cytoplasm in early germ cells, and then its expression is decreased and the protein is translocated into nuclei in later germ cells.

(B) Sxl protein level in *rbp9* RNAi (*nos-gal4/rbp9*^{TH00260}) germarium. Note cytoplasmic Sxl is detected in many germ cells throughout the germarium.

(C) Nos-myc expression in wildtype control gemarium (*nos-gal4,nos-myc/+*) stained with Myc (green) and Hts (red). Nos-myc is highly expressed in all germ cells except four- or eight-cell cysts.

(D) Nos-myc expression in *rbp9* RNAi gemarium (*nos-gal4,nos-myc/rbp9*^{TH00260}). Note that Nos-myc is highly expressed in most germ cells in *rbp9* RNAi germarium, but is not expressed in the four-cell cyst in the image.

(E) Bam protein level in wildtype control germarium (*Bam-HA/+; nos-gal4/+*) stained by HA (green) and Hts (red). Bam is highly expressed in eight-cell cyst in control.

(F) Bam protein pattern in *rbp9* RNAi (*Bam-HA/+; nos-gal4/ rbp9*^{TH00260}) germarium. Note that *rbp9* RNAi germarium contains multiple Bam- expression eight-cell cysts.

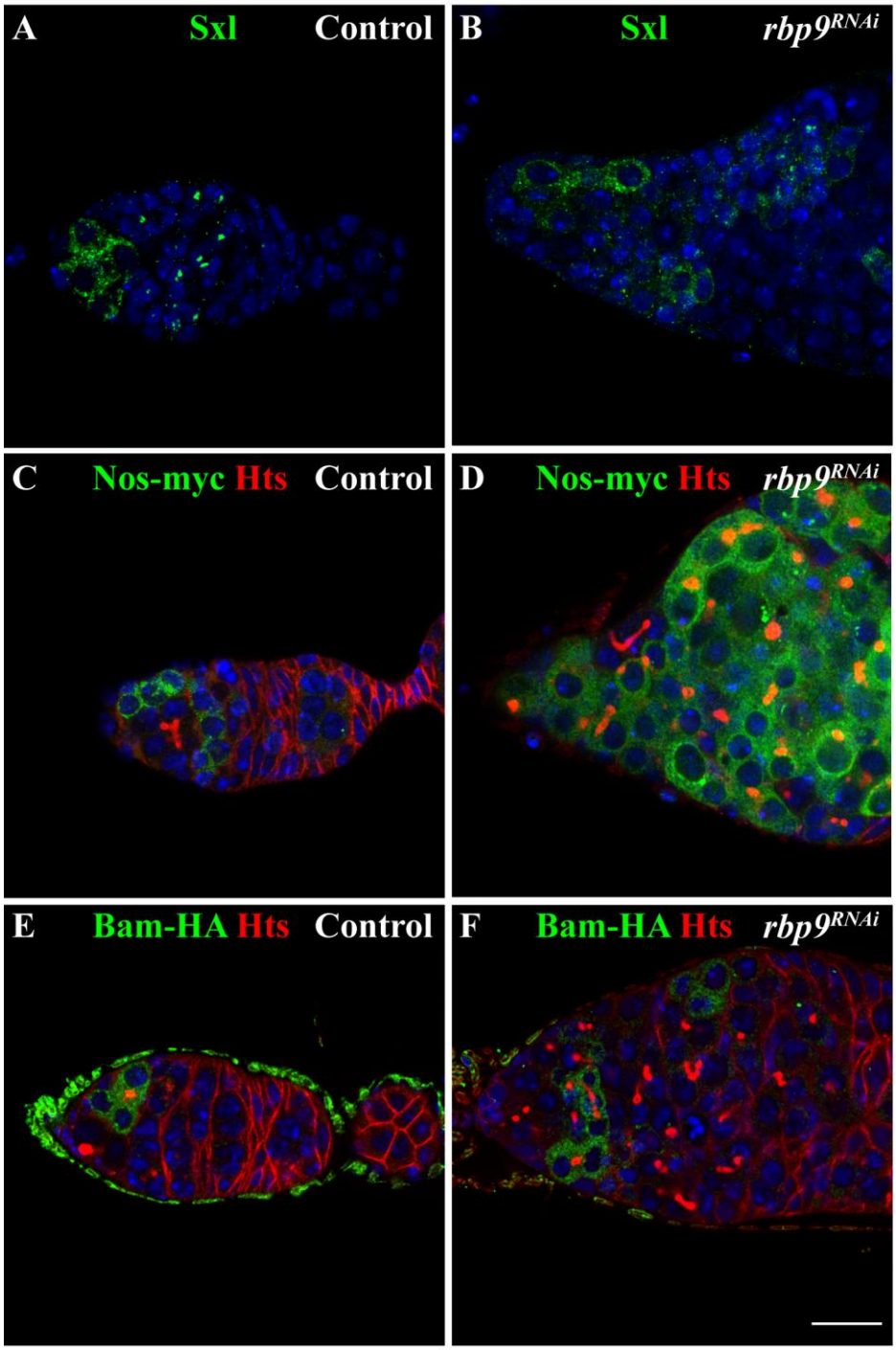

A Sxl Control
B Sxl rbp9RNAi
C Nos-myc Hts Control
D Nos-myc Hts rbp9RNAi
E Bam-HA Hts Control
F Bam-HA Hts rbp9RNAi

Figure 4. Germ cell differentiation is defective in sixteen-cell cyst stage in *rbp9* mutant.

Examination of sixteen-cell cysts markers in adult germariums between wildtype control (A, C, E and G) and *rbp9* RNAi (*nos-gal4/rbp9TH*^{00260} in B, D, F and *nos-gal4/rbp9*^{TH00624} in H). All samples were co-stained DNA (blue). All images are presented as single confocal section. Images in A-F are in same scale, and images in G and H are in same scale. Scale bar represents 15um.

(A) Wildtype control germarium stained with Bruno (green) and Hts (red). Note that Bruno is highly expressed in sixteen-cell cysts and begins to concentration in future oocyte within in later sixteen-cell cyst.

(B) *Rbp9* RNAi germarium stained with Bruno (green) and Hts (red). Note Bruno is expressed in sixteen-cell cysts, but the cysts begin to degenerate and are able to further differentiate.

(C) Wildtype germarium stained with meiosis marker C(3)G (green) and Vasa (red). Note that C(3)G begins to highly expressed in a few cells within sixteen-cell cysts and then is restricted to future oocyte in later stage sixteen-cell cysts.

(D) *Rbp9* RNAi germarium stained with C(3)G, Note the expression of C(3)G is very weak.

(E) Wildtype germarium stained with Orb (green). Note that Orb begins to express in sistenn-cell cysts and concentrate in future oocyte in later stage sixteen-cell cysts and egg chambers.

(F) *Rbp9* RNAi germarium stained with Orb. Note that rare Orb expression cysts are present, but no presence of oocyte-concentrated Orb expressing cysts.

(G) Wildtype ovariole stained with Orb (green). Note that Orb is concentrated in oocytes in all egg chambers.

(H) *Rbp9* RNAi ovariole (*nos-gal4/rbp9*^{TH00624}) stained with Orb (green). Note that no Orb concentrated oocytes are observed in egg chambers.

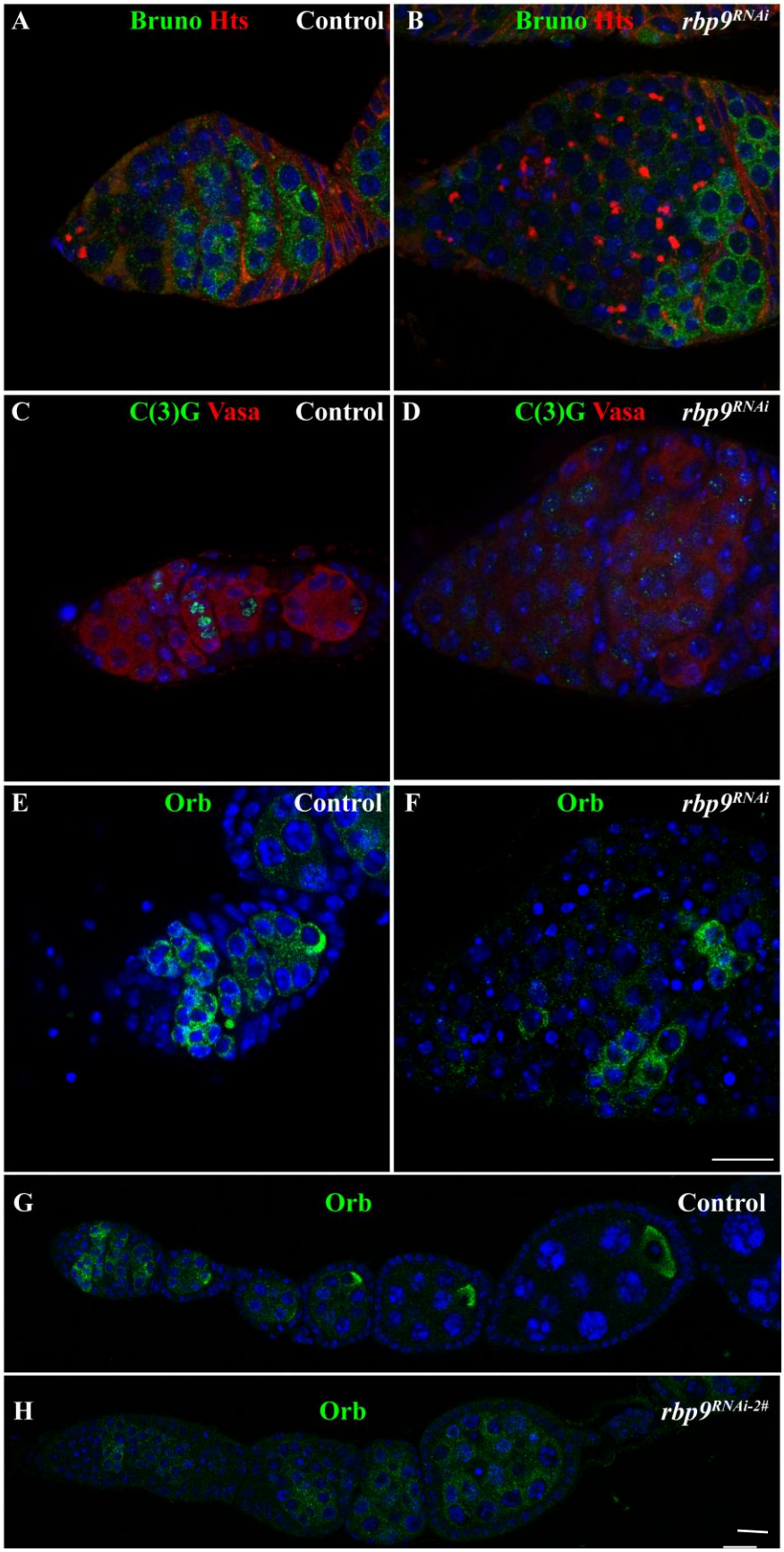

A
Bruno Hts
Control
B
Bruno Hts
rbp9RNAi
C
C(3)G Vasa
Control
D
C(3)G Vasa
rbp9RNAi
E
Orb
Control
F
Orb
rbp9RNAi
G
Orb
Control
H
Orb
rbp9RNAi-2#

Figure 5. Rbp9 is highly expressed in sixteen-cell cysts and early egg chambers.

Examination of *rbp9* expression pattern by generating rbp9 BAC transgenic reporter. Scare bar in B, C and C' represent 15um.

(A) Overview of scheme of generating *rbp9* BAC transgenic fly. BAC clone CH322-140N12 containing *rbp9* gene an d flanking sequence (about 20kb in length) is engineered to insert three copies of Flag tag in frame in N-terminal of Rbp9 protein. The modified BAC is inserted in the VK33docking site in chromosome 3 by attp mediated site specific integration.

(B) Rbp9 expression pattern in ovariole revealed by Flag staining (green) and DNA (blue). Note that Rbp9 is highly expressed in posterior of germarium regions and early egg chambers.

(C) Rbp9 expression pattern in germarium region. Image in C is overlay of DNA staining and Flag staining, while C' shows Flag staining only. Note that Rbp9 is highly expressed in sixteen-cell cysts and slightly enriched in later oocyte. Weak background staining in escort cells is observed, this is probably due to of mouse secondary antibody.

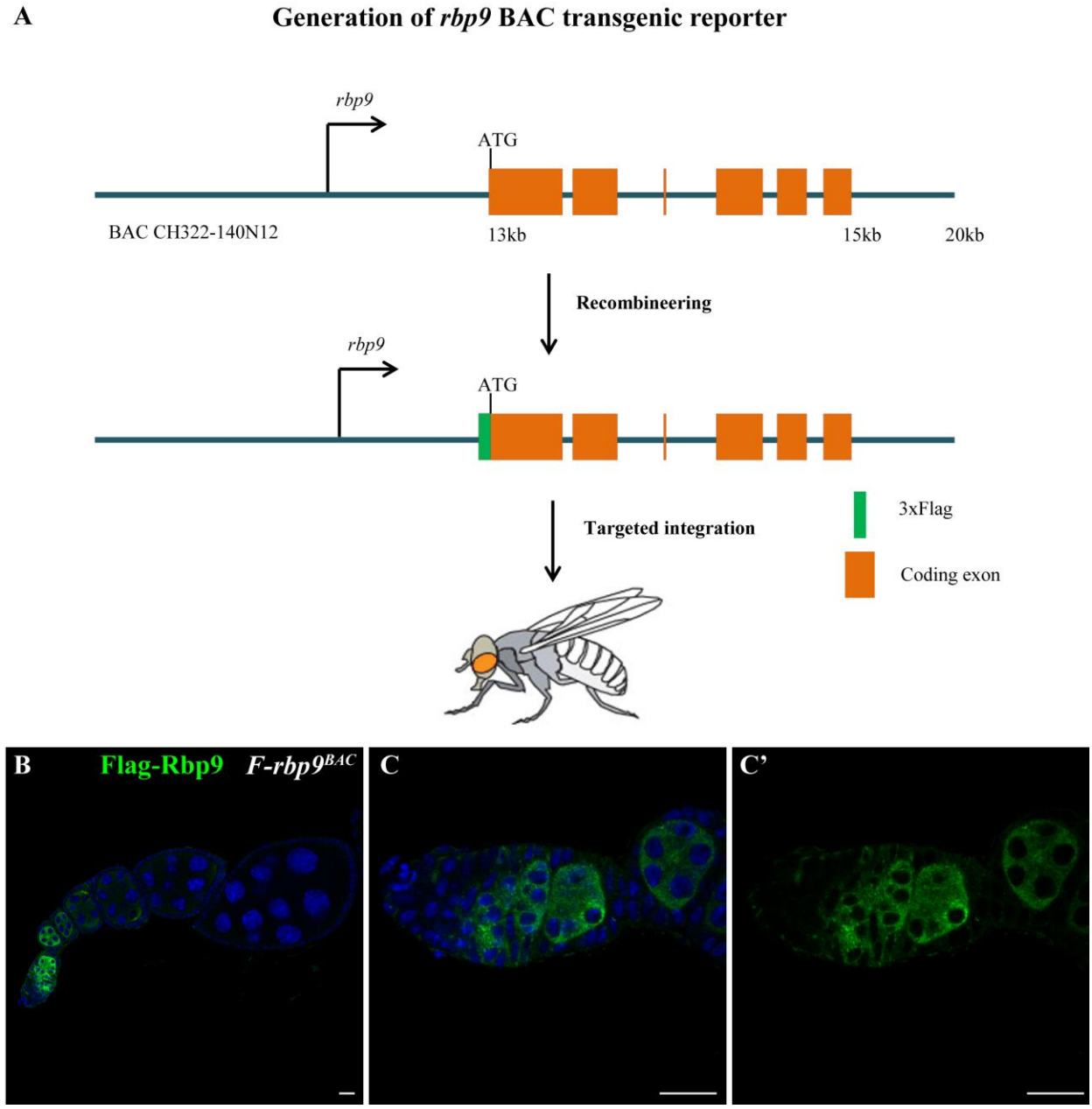

A
Generation of rbp9 BAC transgenic reporter
rbp9
ATG
BAC CH322-140N12
13kb
15kb
20kb
Recombineering
rbp9
ATG
Targeted integration
3xFlag
Coding exon
B
Flag-Rbp9
F-rbp9BAC
C
C'

Figure 6. *Rbp9* is required for germ cell differentiation both in adult and before adult stage.

Rbp9 function in germ cell development was examined in adult conditional knockdown and newly enclosed ovaries. For conditional knock down *rbp9* in adult ovary (B-E), one week old adult control flies (*hs-Flipase/+; nos->stop->gal4, UAS-H4-GFP/+* in C) and *rbp9* RNAi flies (*hs-Flipase/+; nos->stop->gal4, UAS-H4-GFP/+; rbp9*^{TH00260}*/+* in D,E and F) were heat shocked for 1h to induce *nos-gal4* expression, and further cultured for 2 weeks before analysis. Samples in C-F were all stained with GFP (green) and Hts (red). Pictures in C,D,F,G and H are overlay of serial confocal section images, with same scale. E is a single section image. DNA is stained blue in C-H. Scale bar represents 15um.

(A) Scheme of conditional knockdown *rbp9*. After induction of heat shock, nuclear GFP colonies mark *nos-gal4* expression and *rbp9* RNAi knockdown.

(B) Quantification of germline stem cells (GSC) and cystoblast (CB) number in control and *rbp9* RNAi flies two weeks after heat shock. Note there is significant increase ($p<0.05$) of cystoblast numbers in *rbp9* RNAi compared with control.

(C) Example of GFP positive clone from control sample two weeks after heat shock (AHS). Note the gemarium is normal.

(D) Example of GFP positive clone from *rbp9* RNAi sample two weeks after heat shock. Note the gemarium contains accumulated cystoblasts.

(E) Example of GFP positive ovariole from *rbp9* RNAi sample two weeks after heat shock. Note that later stage egg chambers containing GFP positive germ cells are degenerated.

(F) Gemarium from *rbp9* RNAi fly without heat shock is normal.

(G) Gemarium from 2 days old control fly (*nos-gal4/+*) stained with Hts (green) shows normal oogenesis.

(H) Gemarium from 2 days old *rbp9* RNAi fly (*nos-gal4/ rbp9*^{TH00260}) stained with Hts (green) accumulates cysts.

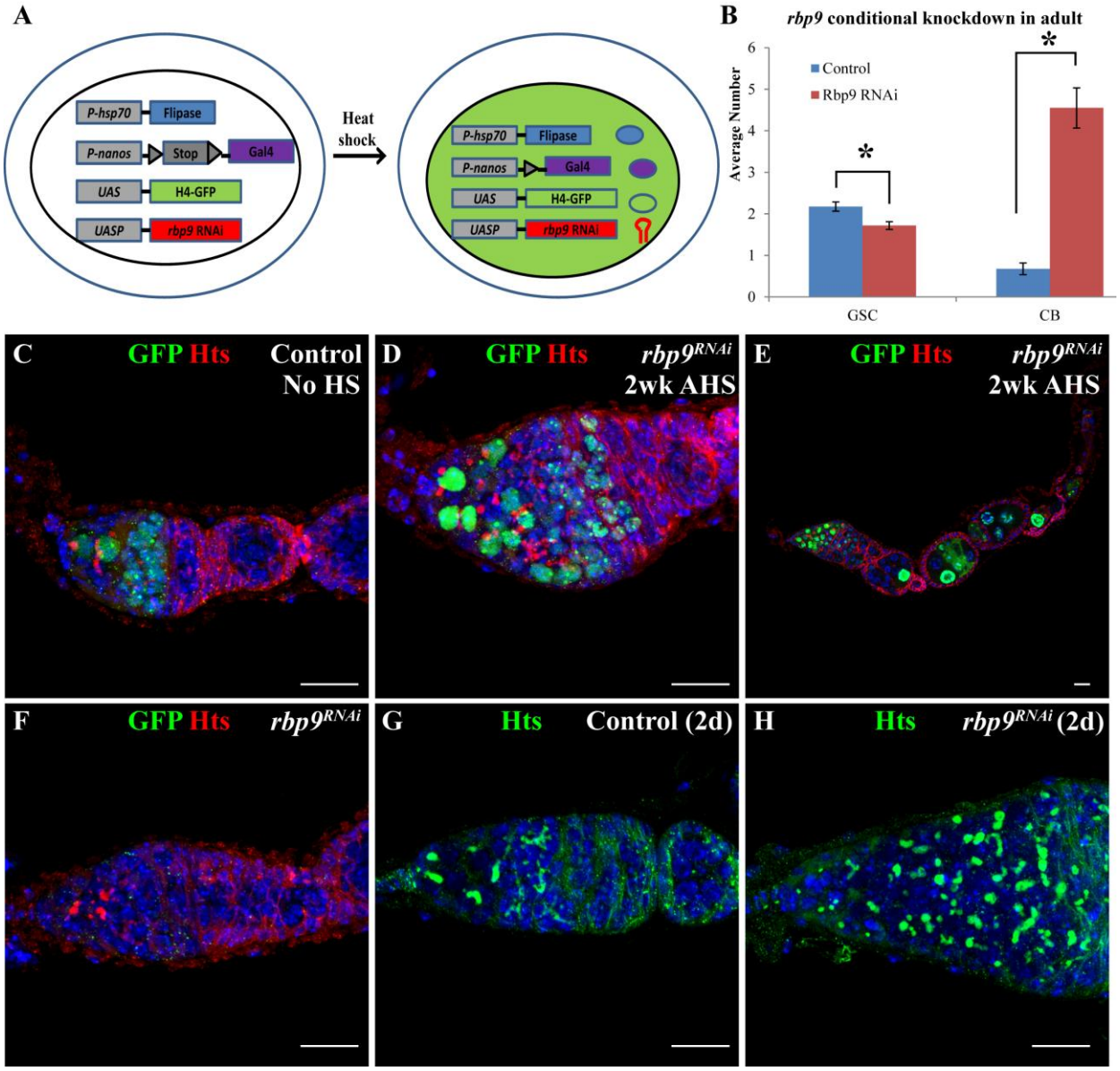

A
P-hsp70
Flipase
P-nanos
Stop
Gal4
UAS
H4-GFP
UASP
rbp9 RNAi
Heat shock
B
rbp9 conditional knockdown in adult
Control
Rbp9 RNAi
Average Number
GSC
CB
C
GFP Hts
Control
No HS
D
GFP Hts
rbp9RNAi
2wk AHS
E
GFP Hts
rbp9RNAi
2wk AHS
F
GFP Hts
rbp9RNAi
G
Hts
Control (2d)
H
Hts
rbp9RNAi (2d)

Figure 7. Over-expression of Rbp9 affects cyst division and egg chamber development.

Effects of Rbp9 over-expression in all germ cells in ovary. N-terminal Flag tagged Rbp9 CDS was cloned into pUASp vector, and the construct was random inserted to fly genome. All images were co-stained with DNA (blue). Scare bar represents 15um.

(A) Gemarium from Rbp9 over-expression fly line (*UASp-F-rbp9-7#/+; nos-gal4/+*) stained with Hts (green) and DNA (blue). Picture is overlay from whole gemarium sections. Note normal oogenesis in the germarium region.

(B) Rbp9 protein expression from transgenic line 7# over expression. Gemarium was stained with Flag (green) and DNA (blue).

(C) Rescue of Rbp9 over-expression in *rbp9* mutant from fly line (*UASp-F-rbp9-7#/+* $rbp9^{\Delta 1}$/ $rbp9^{\Delta 1}$*; nosgal4/+*). Note that normal oogenesis occurs with normal egg chambers appeared.

(D) Example of egg chambers containing cyst with abnormal number of germ cells from fly line (*UASp-F-rbp9-7#/+; nos-gal4/+*)). Picture is from single confocal section. Yellow arrow indicates a cyst containing 32-cell (17 cells can be seen from the single section); white arrow indicates a cyst containing 4-cell cyst.

(E) Example of germariums from Rbp9 over-expression (*UASp-F-rbp9-12#/+; nos-gal4/+*) cause germ cells degeneration in mid-stage egg chambers. Note white arrows point to degenerated egg chamber with condensed nuclei.

(F) Quantification ratio of ovarioles containing degeneration or non-16 cell cysts egg chambers. Note that Rbp9 over-expression from different transgenic lines has variable but consistent phenotype.

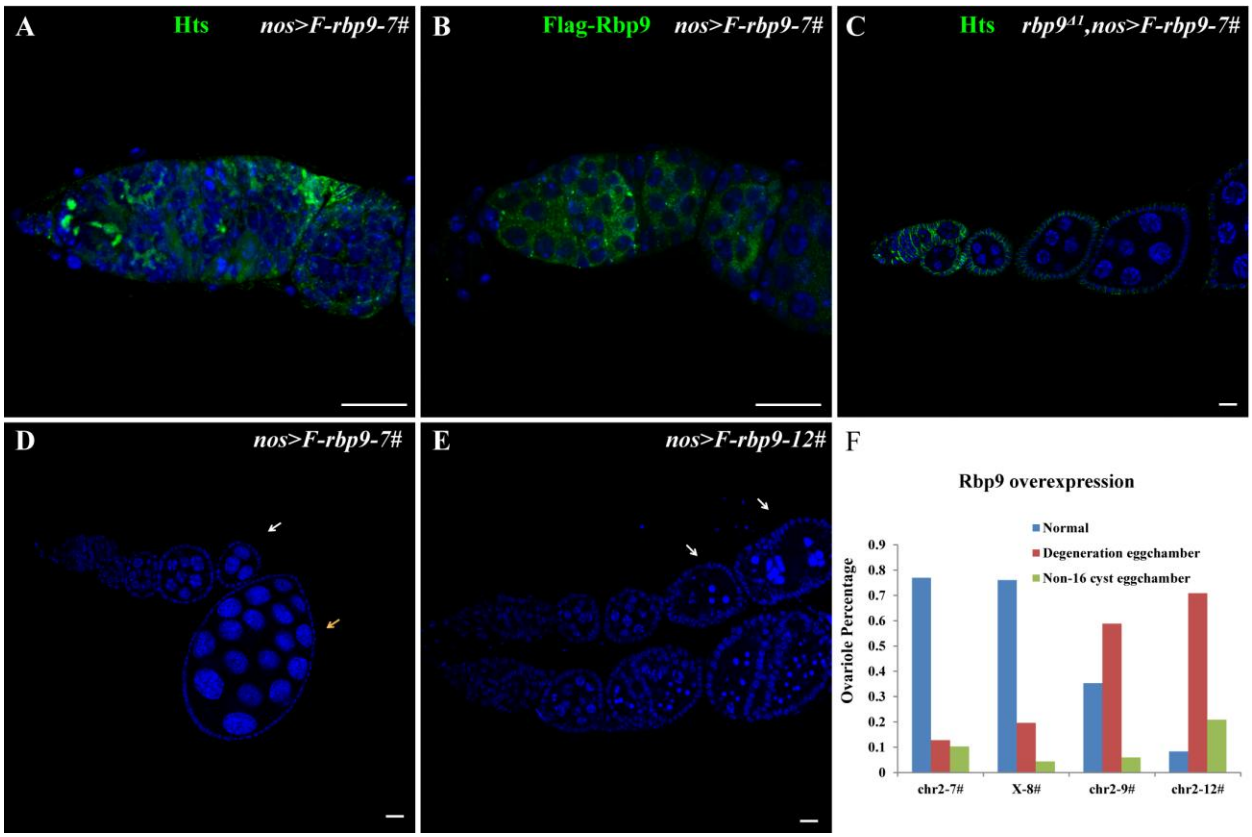

A
Hts
nos>F-rbp9-7#
B
Flag-Rbp9
nos>F-rbp9-7#
C
Hts
rbp9Δ1,nos>F-rbp9-7#
D
nos>F-rbp9-7#
E
nos>F-rbp9-12#
F
Rbp9 overexpression
Normal
Degeneration eggchamber
Non-16 cyst eggchamber
Ovariole Percentage
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
chr2-7#
X-8#
chr2-9#
chr2-12#

Figure 8. *Rbp9* interacts with *bam* in regulating germ cell differentiation.

Test of genetic interaction between *rbp9* and other differentiation factors. One week old ovaries samples were stained with Hts (green) and DNA (blue). Cystoblasts were quantified for each sample (n>=50, results is average±SEM), and student t-test is used for statistical analysis. Pictures in A-E are overlays of serial confocal sections of whole gemarium. Scale bar represents 15um.

(A) Gemarium from $bam^{\Delta 86}$/+ fly contains normal number of germline stem cells and cysts.

(B) Gemarium from $rbp9^{\Delta 1}$/+ fly contains normal number of germline stem cells and cysts.

(C) Gemarium from $rbp9^{\Delta 1}$/+ ;$bam^{\Delta 86}$/+ fly contains accumulated cystoblast and cysts.

(D) Gemarium from $rbp9^{\Delta 1}/bgcn^{20915}$ fly contains normal number of germline stem cells and cysts.

(E) Gemarium from $mei\text{-}P26^{fs1}$/+; $rbp9^{\Delta 1}$/+ fly contains normal number of germline stem cells and cysts.

(F) Comparison of cystoblast number from interactions between *rbp9* and different alleles of *bam/bgcn* complex. Note *rbp9* only shows strong interaction with *bam* alleles ($bam^{\Delta 68}$and bam^{E5}). Asterisk marker (*) shows statistical significance with $p<0.05$.

(G) Comparison of cystoblast number from interactions between *rbp9* and different alleles of *bam*, *sxl* and *mei-P26*. Note that *rbp9* only shows strong interaction with *bam*, but not with *sxl*, and *mei-P26* alleles. Asterisk marker (*) shows statistical significance with $p<0.05$.

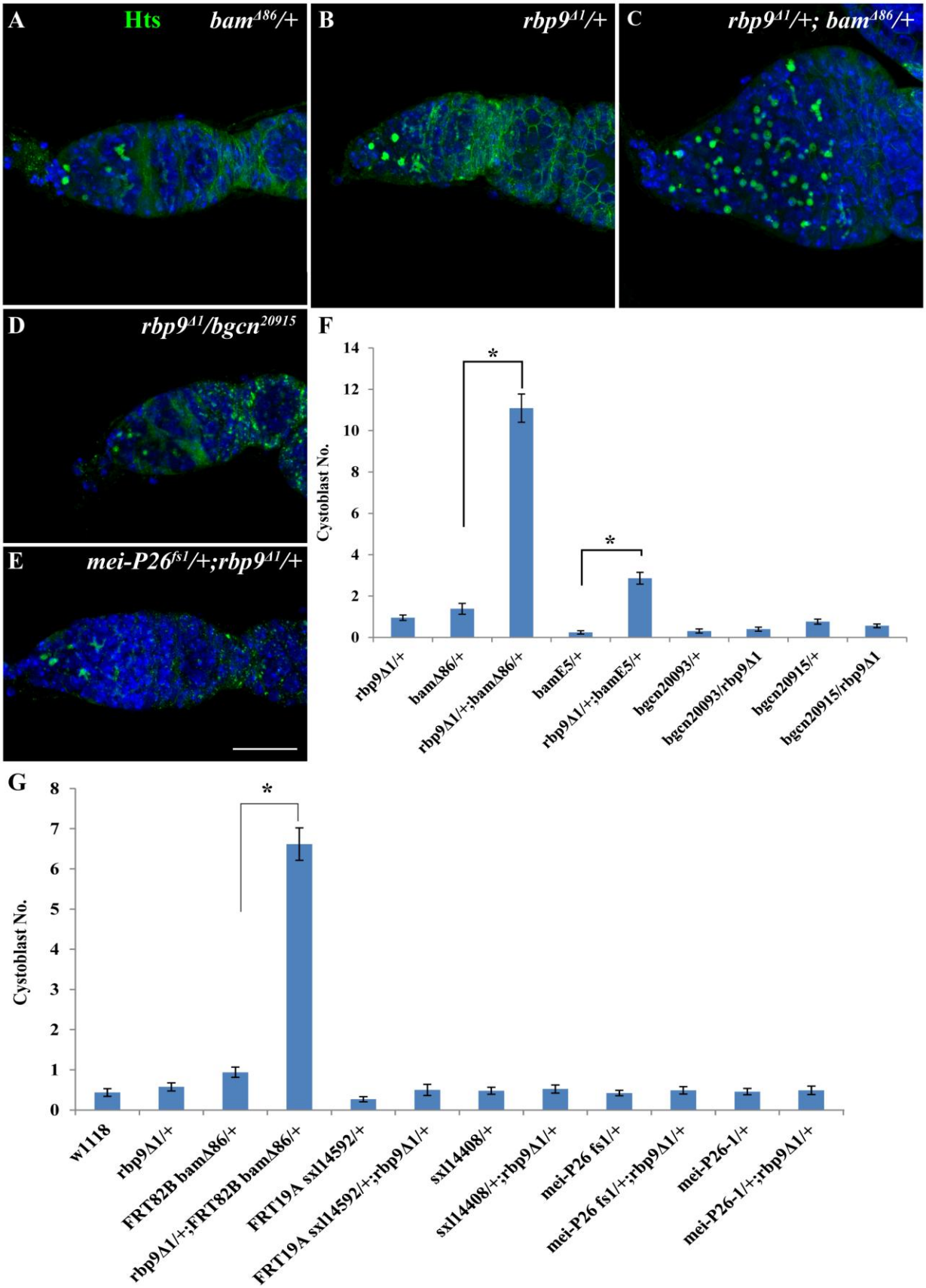

A
Hts
bam^Δ86/+
B
rbp9^Δ1/+
C
rbp9^Δ1/+; bam^Δ86/+
D
rbp9^Δ1/bgcn^20915
E
mei-P26^fs1/+;rbp9^Δ1/+
F
Cytoblast No.
14
12
10
8
6
4
2
0
*
*
rbp9Δ1/+
bamΔ86/+
rbp9Δ1/+;bamΔ86/+
bamE5/+
rbp9Δ1/+;bamE5/+
bgcn20093/+
bgcn20093/rbp9Δ1
bgcn20915/+
bgcn20915/rbp9Δ1
G
Cytoblast No.
8
7
6
5
4
3
2
1
0
*
w1118
rbp9Δ1/+
FRT82B bamΔ86/+
rbp9Δ1/+;FRT82B bamΔ86/+
FRT19A sxl14592/+
FRT19A sxl14592/+;rbp9Δ1/+
sxl14408/+
sxl14408/+;rbp9Δ1/+
mei-P26 fs1/+
mei-P26 fs1/+;rbp9Δ1/+
mei-P26-1/+
mei-P26-1/+;rbp9Δ1/+

Figure 9. Rbp9 over-expression can partially drive germ cell differentiation in *bam* mutant.

Analysis phenotype of over-expression of Fbp9 in *bam* mutant. All samples were stained with Hts (green) and DNA(blue). Images from A-C are in single confocal section, while images from D-I are overlays of several sections. Images in A-F are in same scale, and images in G-I are in same scale. Scale bars represent 15um.

(A) Germarium example from control *bam* mutant germarium without Rbp9 over-expression from fly line (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that the germarium contains acumulated single-cell cystoblasts.

(B) Germarium example from Rbp9 over-expression in *bam* mutant germarium (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that the germarium contains differentiated cyst later than single-cell cystoblast stages.

(C) Germarium example from different Rbp9 over-expression line in *bam* mutant germarium (*UASp-F-rbp9-7#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that in the germarium similar differentiated cyst later than single-cell cystoblast stages are observed.

(D) Germarium example of overlays sections from control *bam* mutant germarium without Rbp9 over-expression from fly line (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that the germarium contains only single-cell cystoblasts.

(E) Germarium example of overlays sections from Rbp9 over-expression in *bam* mutant germarium (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that the germarium contains many differentiated cyst.

(F) Germarium example of overlays sections from Rbp9 over-expression in *bam* mutant germarium (*UASp-F-rbp9-7#/+; bam$^{\Delta 86}$/bam^{E5}*). Note that the germarium contains many differentiated cyst.

(G) High magnification of single cell cystoblasts example from germarium of *bam* mutant germarium without Rbp9 over-expression from fly line (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*).

(H) High magnification of eight-cell cyst example from germarium of Rbp9 over-expression in *bam* mutant germarium (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*).

(H) High magnification of sixteen-cell cyst example from germarium of Rbp9 over-expression in *bam* mutant germarium (*UASp-F-rbp9-12#/+; bam$^{\Delta 86}$/bam^{E5}*).

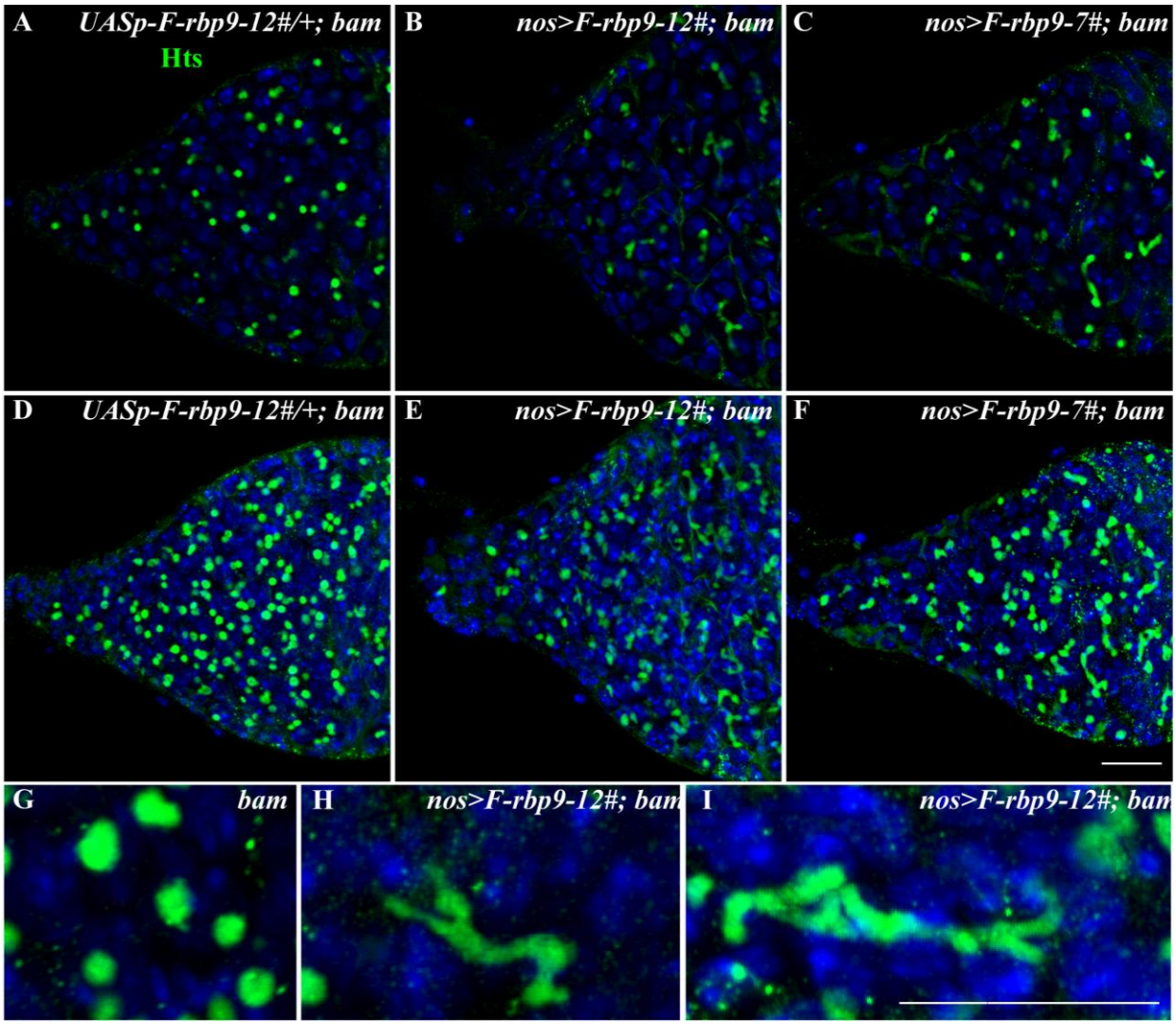

A
UASp-F-rbp9-12#/+; bam
Hts
B
nos>F-rbp9-12#; bam
C
nos>F-rbp9-7#; bam
D
UASp-F-rbp9-12#/+; bam
E
nos>F-rbp9-12#; bam
F
nos>F-rbp9-7#; bam
G
bam
H
nos>F-rbp9-12#; bam
I
nos>F-rbp9-12#; bam

Figure 10. Lack of Rbp9 blocks Bam driving germ cell differentiation.

Effect of Bam over-expression in *rbp9* mutant. Samples in A-D were stained with Hts (green) and DNA (blue), and the images were presented in overlay of whole germarium sections. Scale bar represents 15um.

(A) Control germarium from fly line (*rbp9$^{\Delta 1}$/+;hs-bam/+*) without heat-shock induced Bam over-expression (No HS)contains normal germline stem cells and cysts.

(B) Germarium from *rbp9* mutant fly line (*rbp9$^{\Delta 1}$;hs-bam/+*) without heat-shock induced Bam over-expression (No HS) contains accumulated cysts.

(C) Germarium from control fly line (*rbp9$^{\Delta 1}$/+;hs-bam/+*) with over-expression of Bam by one hour heat shock. One week after 1h heat shock (AHS), germ cells from most germariums (78%) in *rbp9$^{\Delta 1}$/+;hs-bam/+* fly are all differentiated, leaving a gemarium without any germ cells left.

(D) Germarium from *rbp9* mutant fly line (*rbp9$^{\Delta 1}$;hs-bam/+*) with over-expression of Bam by one hour heat shock. One week after 1h heat shock (AHS), accumulated germ cells from most germariums (84%) in *rbp9$^{\Delta 1}$;hs-bam/+* fly are still present.

(E) Quantification of ratio of gemariums containing germ cells in control fly (*rbp9$^{\Delta 1}$/+;hs-bam/+*) and *rbp9* mutant fly (*rbp9$^{\Delta 1}$;hs-bam/+*) one week after no heat shock (No-HS) or 1h heat shock (1h-HS). Note that in control fly most gemariums loss germ cell after Bam over-expression by heat shock, while in *rbp9* mutant germ cells are still present in most germariums after Bam over-expression.

(H) Quantification of germline stem cells numbers (average $\pm$SEM) in germariums of control fly (*rbp9$^{\Delta 1}$/+;hs-bam/+*) and *rbp9* mutant fly (*rbp9$^{\Delta 1}$;hs-bam/+*) one week after no heat shock (No-HS) or 1h heat shock (1h-HS).. Asterisk marker (*) shows statistical significance with $p<0.05$. Note that in control fly germline germ cells are lost significantly after Bam over-expression by heat shock, while in *rbp9* mutant germline stem cells number does not change significantly after Bam over-expression.

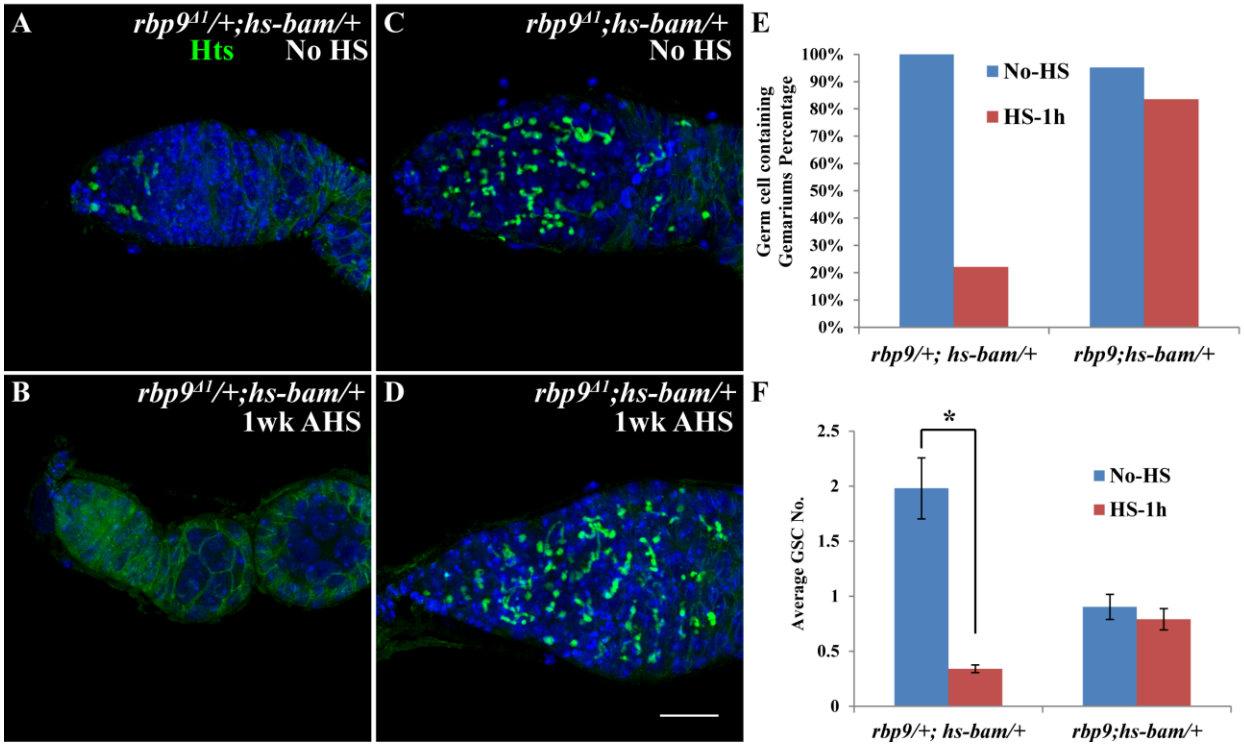
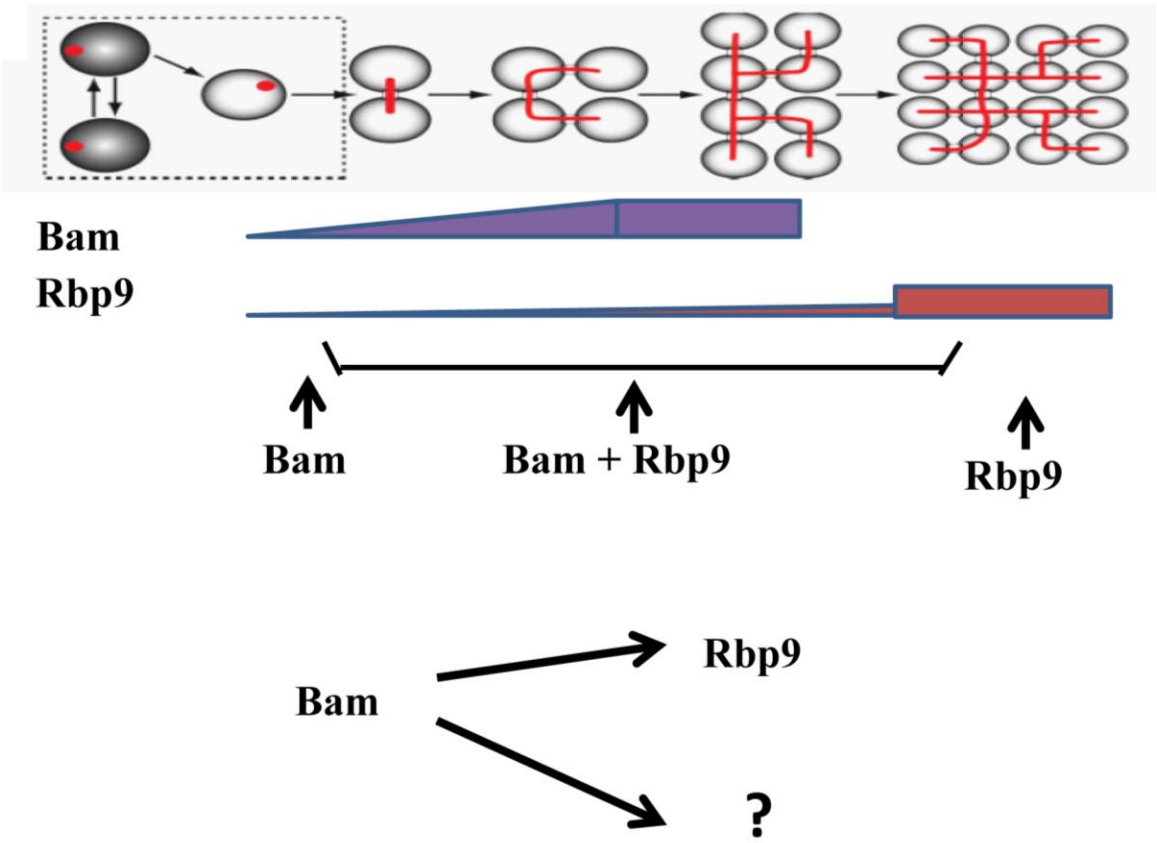

A
rbp9Δ1/+;hs-bam/+
Hts
No HS
C
rbp9Δ1;hs-bam/+
No HS
B
rbp9Δ1/+;hs-bam/+
1wk AHS
D
rbp9Δ1;hs-bam/+
1wk AHS
E
Germ cell containing Gemariums Percentage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
No-HS
HS-1h
rbp9/+; hs-bam/+
rbp9;hs-bam/+
F
Average GSC No.
2.5
2
1.5
1
0.5
0
*
No-HS
HS-1h
rbp9/+; hs-bam/+
rbp9;hs-bam/+

Figure 11. Model of Rbp9 function in germ cell differentiation in *Drosophila* ovary.

The function of Rbp9 in germ cell differentiation in *Drosophila* ovary is summarized in our model as following: Rbp9 is required to modulate early steps of cyst differentiation, and is essential for sixteen-cell cyst differentiation. Rbp9 is highly expressed in sixteen-cell cyst, consistent with its critical role in sixteen-cell cyst. We also hypothesize that a low expression of Rbp9 in early differentiation cyst accountable to *rbp9* mutant phenotype. Rbp9 genetically interacts with Bam in regulating germ cell differentiation. And Rbp9 acts as one downstream of Bam in this regulation, though other targets of Bam is also required for fully germ cell differentiation.


Bam
Rbp9
Bam
Bam + Rbp9
Rbp9
Bam
Rbp9
?

Chapter IV: Discussion

Rbp9 has been previously reported to be required for germ cell differentiation in *Drosophila* ovary (Kim-Ha et al., 1999), but the detailed phenotype characterization and mechanism of function are largely unknown. In this study, we aimed to further study the function of Rbp9in germ cell differentiation. Firstly, we confirmed *rbp9* mutant phenotype by using *rbp9* germline specific RNAi knockdown. We further confirmed Rbp9 is highly expressed in sixteen-cell cyst and early egg chambers by using BAC transgenic reporter. *Rbp9* mutant germarium contains accumulated early cysts. BMP signaling and Bam regulation is not affected in rbp9 mutant, but early differentiation cysts expressing Nanos, Sxl and Bam are accumulated in *rbp9* mutant. Moreover, sixteen-cell cysts are failed to further differentiate in *rb9* mutant. Both Bruno and Orb expression cysts are present in *rbp9* mutant, but they are blocked before oocyte specification and begin to degenerate. The function of *rbp9* in germ cell differentiation is required for both adult stage and before adult stage. And meiosis is also failed initiated in *rbp9* mutant. Though Rbp9 is critical for cyst differentiation, over-expression of Rbp9 does not affect germ cell development in germarium region. But Rbp9 over-expression will affect number of cyst division times and mid stage egg chamber development. Moreover, we identified that *rbp9* genetically interacts with *bam* in regulating cystoblast differentiation. Rbp9 functions as downstream of Bam in germline differentiation, as Rbp9 over-expression can partially drive germ cell differentiation in *bam* mutant, but lack of Rbp9 blocks Bam driving germ cell differentiation. In all, this study will help better understand function of *rbp9* in germ cell differentiation in *Drosophila* ovary, and may provide insights in general germ cell development.

Rbp9 regulates multiple steps of germ cell differentiation in *Drosophila* ovary

Germ cell differentiation in gemarium goes through four rounds of mitotic cell division and switches to meiosis (de Cuevas et al., 1997). In *rbp9* mutant, a mixture of cysts are accumulated in gemarium regions, without forming normal mature oocyte with fifteen-nurse cells (this study and (Kim-Ha et al., 1999)). This indicates *rbp9* has a critical role in transition from mitotic division to meiosis initiation in six-teen cell cysts. In addition, majority of the cysts are one-cell cystoblast and two-cell cysts, indicating rbp9 functions in early cyst differentiation, especially transition from cystoblast to two-cell cyst and two-cell cyst to four-cell cysts. This is further confirmed that by the accumulation of cystoblasts in *rbp9/+;bam/+* gemarium. The mixture of cysts in *rbp9* mutant or *rbp9* RNAi is unlikely due to non-complete reduction of endogenous Rbp9 protein, because the *rbp9* mutant used in this study is a reported null allele (Kim-Ha, 2000). This suggests some cystoblasts can bypass the requirement of Rbp9 protein and produce some two-, four-, eight- and sixteen-cell cysts, but cannot further differentiation beyond six-teen cell cysts.

When carefully examination differentiation markers in *rbp9* mutant, the accumulated cysts express many normal marker correlated with their differentiation stage. In *rbp9* mutant, the germline stem cells contain BMP activity and *bam* regulation is still present. Germline stem cells and early cysts express Nanos and Sxl. Bam protein is correctly expressed in four- and eight-cell cysts, and Nanos is repressed in those as reported (Li et al., 2009). Bruno and Orb are expressed in few sixteen-cell cysts. While in wildtype Bruno and Orb proteins concentrate in future oocytes in later sixteen-cysts and egg chambers, those specified oocytes are not observed in *rbp9* mutant. Additionally, sixteen-cell cysts expressing Bruno and Orb in *rbp9* mutant ovary also begin to

degenerate possible through apoptosis. These results suggest Rbp9 play critical role in oocyte specification and germ cell survival.

These detailed phenotype characterization and later interaction with Bam all confirm that Rbp9 functions in both early cyst differentiation and later sixteen-cell cyst differentiation in our model (Figure 11) . While previously Rbp9 expression has been highly showed in germarium 2A region (corresponding to 16-cell cysts) and egg chamber stage 2 and 3 (Lavoie et al., 1999; Tastan et al., 2010), those results correlate well with the requirement of high level of Rbp9 protein in those steps. In those antibody staining, they also suggested a possible low level of Rbp9 expression in early cyst. In this study, we generated *rbp9* BAC reporter as an alternative way to try to further confirm *rbp9* expression pattern. The resulted transgenic reporter also confirmed high expression of Rbp9 in sixteen-cell cyst and early egg chambers, but was not able to detect low expression of Rbp9 in early cysts. It is possible that the BAC may miss some regulatory elements that makes not it able to show low level of expression in early stage, though previously a genome region about 17kb within the BAC genomic sequence has been used for rescue of rbp9 mutant (Kim-Ha et al, 1999) . We still need to further confirm this by test whether the BAC reporter can rescue *rbp9* mutant. It is also possible that a low level of Rbp9 expression is hard to detect by immuno-staining. Alternative method such as detecting *rbp9* transcription by RNA in situ or transgenic reporter may help provide indirect evidence. Also, test rescue of *rbp9* mutant by over-expression of Rbp9 only in sixteen-cell cyst using sixteen-cell cyst expressing gal4 may help clarify this.

Lastly, over-expression of Rbp9 thorough germ line supported previously antibody staining rOver-expression of Rbp9 in all stages of germline using *nos-gal4* results in mid stage egg chamber degeneration (this study and (Kim-Ha t iset al., 1999)), suggesting Rbp9 needs to be

properly turned off during normal oogenesis. It will be interesting to determine in the normal situation whether this regulation comes from transcriptional or translational level.

Rbp9 interacts with *bam* in regulating germ cell differentiation

Bam is critical to induce germ cell differentiation from germline stem cells to cystoblast. *Bam* or *bgcn* mutant blocks germ cell differentiation in one-cell cystoblast stage (McKearin and Ohlstein, 1995; Ohlstein et al., 2000). Though *rbp9* phenotype is distinct from *bam* mutant, interestingly, our study identified that removal one copy of *rbp9* and *bam* (*rbp9/+;bam/+)* leads to strong cystoblast accumulation. While Bam and Bgcn are purposed to function in a complex (Ohlstein et al., 2000), there is no synergistic effect between *rbp9* and *bgcn*, nor synergistic effect between *bam* and *bgcn* (data not shown). This is possibly due to high expression level of Bgcn even in heterozygous background.

To examine the possible mechanism of *rbp9* and *bam* interaction, we first tested whether *rbp9* regulated Bam expression. This hypothesis is favored by previous report that Rbp9 can bind to *bam* mRNA 3UTR (Kim-Ha et al., 1999). Bam transcription and protein level are examined by bam-GFP and Bam-HA reporters in *rbp9* mutant, and these results suggest relatively normal expression. In addition, over-expression of Bam cannot drive germ cell differentiation in *rbp9* mutant. All these results suggest that *rbp9* does not regulate *bam* expression.

Interestingly, we found that over-expression of Rbp9 can partially drive germ cell differentiation in *bam* mutant. While *bam* mutant blocks germ cell differentiation in cystoblast stage, over-expression of Rbp9 can drive some cyst differentiate into four-, eight- or even sixteen-cell cyst stage. But those cysts are not able to full differentiate into normal egg chambers.

These genetic interactions of Bam and Rbp9 suggest Rbp9 may serve as downstream of Bam in function of germline differentiation (Figure 11).

So what would be the molecular mechanism of this genetic interaction? We are considering a few possibilities. Firstly, Bam and Rbp9 can potentially form a protein complex. Recently, there is a report shows that Bam, Bgcn, Sxl and Mei-P26 can be identified in same gel fraction (Li et al., 2013), indicating they may function in a complex. Additionally *nanos* mRNA can be detected from Mei-P26 or Sxl immune-precipitation (Chau et al., 2012; Li et al., 2013). We examined whether Rbp9 can interact with Bam or Bgcn in cultured S2 cells, though could not detect any interaction. It is still possible that Rbp9 may interact with Bam/bgcn complex *in vivo*. Rbp9 and Sxl share similar RRM domain, and their RNA binding site also similar (Samuels et al., 1994; Park et al., 1998). And the *nanos* mRNA regulatory site by Sxl is also potential a target site of rbp9 (our observation). Therefore we further tested genetic interaction between *rbp9* and *sxl* or *mei-P26*, though found they did not have such synergistic interaction as *rbp9* and *bam*. And examination of Nanos expression in *rbp9* mutant suggests Nanos expression is not regulated by *rbp9*. A further study use double mutant of *rbp9* and sxl may help further determine whether those two RNA binding protein may share other targets.

Secondarily, it is also possible rbp9 is a downstream target of Bam regulation. This regulation possibly comes from translational regulation, as Bam and Bgcn are purposed to function as a translational regulator. Examination of *rbp9* transcription and protein expression, as well as a rescue experiment by over-expression of Rbp9 in *bam* mutant, may help find an answer to this hypothesis.

The regulatory targets of Rbp9 in germ cell differentiation

What is the potential molecular mechanism of Rbp9 in regulating germ cell differentiation? Rbp9 belongs to Elav-Hu protein family, contains three RRM domains, and is potentially involved in RNA binding. The Elav-Hu family proteins have been showed to function in mRNA stability regulation or splicing (Pascale and Govoni, 2012). Rbp9 expression has been detected in the cytoplasm (our study and (Kim-Ha et al., 1999)), potentially suggesting its role in mRNA stability regulation. Members of Elav-Hu family can bind to mRNA targets and enhance their stability, thus promoting translation ((Pascale and Govoni, 2012)). Elav-Hu family binding RNA targets include cell cycle regulator, cell survival and apoptosis genes (Lebedeva et al., 2011). Previously, the binding sequence of Rbp9 protein has been identified through *in vitro* binding assay, and a census sequence of UUUXUUUU is the potential binding site (Park et al., 1998). And a similar consensus site has been recently reported by another independent group (Ray et al., 2013). The direct RNA targets of Rbp9 have not been identified yet in germ cell differentiation process.

Previously, Rbp9 has been showed to bind to *bam* mRNA 3UTR (Kim-Ha et al., 1999). However, our current data with Bam protein expression pattern and Bam over-expression results suggest *bam* is not a direct target of *rbp9* regulation. *Nanos* also contains a potential Rbp9 binding site, but its regulation is not altered in *rbp9* mutant (this study). Therefore the *rbp9* regulatory targets in early cysts differentiation are still unknown.

In this study, we found out that cyst differentiation is blocked before oocyte specification. While sixteen-cell cyst markers Bruno and Orb are normally expressed, they fail to concentrate into future oocyte. Oocyte specification begins early in sixteen-cell cyst formed, and one of the two germ cells in the middle of the cyst with four ring canals will become future oocyte. The differentiated oocyte can be marked by polarized localized proteins, such as BicD, Egl, Bruno

and Orb and mRNAs such as *osk*, *BicD* and *orb* (Huynh and St Johnston, 2004). BicD/Egl complex are the earliest proteins to concentrate in future oocyte, and they interact with microtubules motor complex to help transport other oocytes specific proteins and mRNAs to oocytes (Swan et al., 1999; Navarro et al., 2004). Previously *osk* mRNA localization was checked in weak *rbp9* mutant allele ovary and the *osk* mRNA localized is disrupted (Kim-Ha et al., 1999). Our new results are consistent with this report and suggest *rbp9* mutant has defect in oocyte specification. It will be interesting to check whether *rbp9* mutant affect early step of oocyte specification, such as ring canals organization, early localization of BicD/Egl, microtubules organization and related motor complex.

Oocyte specification defect alone cannot account for the all *rbp9* mutant phenotypes. In *BicD* or *Egl* mutant, the oocyte fails to specific and the sixteen-cell cyst all become nurse cells, and the sixteen nurse cells containing egg chambers can still be able to survive up to stage eight (Ran et al., 1994; Oh and Steward, 2001). However in *rbp9* mutant, the cysts begin to degenerate in germarium region before oocyte specification stage, indicating *rbp9* also functions importantly for cell survival directly or indirectly. The mammalian homolog HuR protein has been showed to translational regulate anti-apoptosis genes such as *bcl-2* and *XIAP* (Abdelmohsen et al., 2007; Durie et al., 2011). It will be interesting to determine whether *rbp9* directly regulate anti-apoptosis factor during oogenesis.

In addition, over-expression of Rbp9 has produced 32-cell, 8-cell and 4-cell cysts in this study. This change of germ cells number within final cyst reflects a change in cyst division times. It has been previously reported that a few cell cycle regulators mutation or over-expression can cause such altered cyst numbers (Lilly et al., 2000; Mata et al., 2000). Interestingly, Elav-Hu family member *HuR* has been showed to regulate cell cycle regulator such as *CycB* and *p21* through

binding with their mRNAs (Wang et al., 2000a; Wang et al., 2000b). This suggests some of the cell cycle regulators may be direct Rbp9 regulatory targets.

Finally, to comprehensively identify Rbp9 binding targets, a RNA immunoprecipitation based method may be needed.

Is *rbp9* a conserved germ cell regulator?

A few reports suggest Elav-Hu family proteins may also function in germ cell development. The *Xenopus* Elav protein ElvB has been reported to repress *Vg1* mRNA translation during oogenesis (Colegrove-Otero et al., 2005). In mouse, there are four members in Elav-Hu family, including *HuR*, *HuB*, *HuC* and *HuD*. *HuR* is ubiquitous expressed in most tissue, including testis. Knockout of *HuR* in testis suggests it is essential for germ cell meiosis and post-meiosis development (Chi et al., 2011). *HuB* is also very interesting because it is expressed only in brain and testis (Atasoy et al., 1998), though its function in germ cell has been studied. In zebrafish, *Hub* has been showed to be strongly expressed in primordial germ cells and brain (Wiszniak et al., 2011). Preliminary study of Hub function by morpholino knockdown in zebrafish suggests it is not required for primordial germ cells formation and migration (Mickoleit et al., 2011). However its function in later germ cell differentiation has not been studied. There is also possible functional redundant between HuB and HuR in germ cell development. It will be interesting to further study function of Elav-Hu family member in germ cell development in mammalian system.

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