Multifunctional RNA-binding proteins mediate ecdysone-dependent germline stem cell self-renewal in *Drosophila*

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RUNNING TITLE

hnRNPs promote stem cell function

SUMMARY STATEMENT

Ecdysone signaling promotes expression of heterogeneous ribonucleoproteins to modulate BMP-dependent germline stem cell self-renewal in the *Drosophila* ovary.

ABSTRACT

Steroid hormones promote stem cell self-renewal in many tissues; however, the molecular mechanisms by which hormone signaling is integrated with niche-derived signals are largely uncharacterized. In the *Drosophila* ovary, the steroid hormone ecdysone promotes germline stem cell (GSC) self-renewal. Despite strong evidence that ecdysone modulates the reception of bone morphogenetic protein (BMP) signals in GSCs, transcriptional targets of ecdysone signaling that facilitate BMP reception are unknown. Here, we report that ecdysone signaling promotes the expression of distinct, multifunctional RNA-binding proteins in GSCs to support their self-renewal. We demonstrate that a group of heterogeneous nuclear ribonucleoproteins (hnRNPs), which regulate splicing, maturation, and localization of nascent mRNAs, are highly expressed in GSCs. In the absence of ecdysone signaling, expression of at least four hnRNPs (*squid*, *hephaestus*, *Hrb27C*, and *Hrb87F*) is decreased. HnRNPs are intrinsically required for proper GSC self-renewal, and functionally interact with both ecdysone signaling and BMP signaling to maintain GSC number. Our findings support the model that stem cells coordinate local and long-range signals at the transcriptional and post-transcriptional levels to maintain self-renewal in response to physiological demand.

INTRODUCTION

Stem cells are critical for tissue homeostasis and cellular diversity in developing and mature organs. Many stem cells divide asymmetrically, balancing long-term stem cell self-renewal with the production of progenitor cells that differentiate into functionally specialized cells (Chen et al., 2016; Ge and Fuchs, 2018; Gervais and Bardin, 2017). To ensure tissue integrity and proper organ function, stem cell self-renewal and proliferation must be tightly regulated and sensitive to changes in physiology over the lifetime of the organism. The molecular mechanisms connecting physiological signals to stem cell self-renewal, however, remain incompletely defined (Ables et al., 2012; Ghorbani and Naderi-Meshkin, 2016; Laws and Drummond-Barbosa, 2017). As stem cell decline contributes to age-related tissue degeneration (Keyes and Fuchs, 2018; Oh et al., 2014; Pan et al., 2007), understanding how physiological signals modulate stem cell activity may offer new strategies for optimization of tissue repair and regeneration *in vivo*.

Nuclear hormone receptors, which directly connect physiological signals to cellular responses, are well-suited to regulate stem cell self-renewal and proliferation. The levels of nuclear hormone receptor ligands (e.g. steroid hormones) fluctuate in response to nutritional status, stress, and sexual maturation, impacting cell fate, proliferation, and survival in a wide variety of tissues. In the hematopoietic system, estrogen- and 27-hydroxycholesterol-induced activation of Estrogen Receptor α is essential for hematopoietic stem cell proliferation and subsequent expansion of erythropoiesis during pregnancy (Chapple et al., 2018; Nakada et al., 2014; Oguro et al., 2017). Mammary stem cells are also responsive to ovarian hormones (Asselin-Labat et al., 2010; Fu et al., 2017; Joshi et al., 2010), while Retinoic Acid Receptor gamma promotes male spermatogonial differentiation (Gely-Pernot et al., 2012). In the intestinal epithelium, excess circulating lipids induce expression of Peroxisome Proliferator-activated receptor δ, promoting intestinal stem cell self-renewal and predisposing cells to tumorigenesis (Beyaz and Yilmaz, 2016). Despite the therapeutic significance of nuclear hormone receptor signaling in tissue-resident stem cells, the molecular mechanisms by which these important receptors achieve tight regulation of stem cell activity remain largely uncharacterized (Ables and Drummond-Barbosa, 2017; Rafalski et al., 2012).

The *Drosophila melanogaster* ovary is a robust model system with which to elucidate the molecular mechanisms connecting local and systemic regulation of stem cell function. Ovaries are

comprised of 14-16 ovarioles filled with maturing egg chambers or follicles, each of which will ultimately develop into a single egg (Spradling, 1993). Germline stem cells (GSCs) reside at the most anterior region of the ovariole (called the germarium; Fig. 1A-B) and are regulated by a complex network of paracrine and endocrine signaling mechanisms that maintain their self-renewal and proliferation (Xie, 2013). For example, GSCs are physically connected via adherens junctions to adjacent somatic cap cells (Fig. 1B), which secrete the bone morphogenetic protein (BMP) ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb) (Song et al., 2002; Xie and Spradling, 1998). Upon activation, BMP receptors Punt (Put) and Thickveins (Tkv) on GSCs suppress differentiation via activation of Mothers against decapentaplegic (Mad), which transcriptionally represses Bag of marbles (Bam), a primary differentiation factor (Chen and McKearin, 2003a; McKearin and Ohlstein, 1995; Song et al., 2004). Translational control of differentiation factors is also critical for regulating GSC self-renewal and cystoblast differentiation (Slaidina and Lehmann, 2014). Asymmetric division of the GSC perpendicular to the cap cells produces another GSC and a cystoblast committed to differentiation. The cystoblast divides to form an interconnected germline cyst of one oocyte and 15 nurse cells which load the oocyte with maternal factors essential for embryogenesis (Fig. 1B) (Spradling, 1993). GSCs, cystoblasts, and cysts in the anterior germarium can be reliably identified due to a specialized organelle called the fusome (de Cuevas and Spradling, 1998; Ong and Tan, 2010). Cysts are encapsulated in the posterior germarium by a monolayer of somatic follicle cells (which arise from follicle stem cells) that support the developing oocyte through 14 morphologically distinct stages of development.

We and others have demonstrated that GSC self-renewal and proliferation are also modulated by physiological signals, including the steroid hormone ecdysone (Ables and Drummond-Barbosa, 2017; Belles and Piulachs, 2015). In adult females, ecdysone is produced in ovarian follicles, where levels rise dramatically in response to mating cues (Ameku and Niwa, 2016). Ecdysone signals received by a heterodimeric combination of nuclear hormone receptors Ecdysone Receptor (EcR) and Ultraspiracle (Usp) are necessary for GSC self-renewal and proliferation, germ cell differentiation, and follicle formation (Fig. 1C) (Ables and Drummond-Barbosa, 2010; Ables et al., 2016; Ameku and Niwa, 2016; Konig et al., 2011; Morris and Spradling, 2012). In GSCs, EcR/Usp signaling promotes reception of BMP signals, indicating that paracrine and endocrine signals are functionally integrated to regulate GSC behavior (Ables

and Drummond-Barbosa, 2010). This integration is due, in part, to the activity of the ecdysone response gene *E74*, which is essential for GSC self-renewal; however, it is unclear how EcR and E74 modulate BMP signaling to predispose GSCs to an undifferentiated fate.

In a recent genetic mosaic screen, we identified *Heterogeneous nuclear ribonucleoprotein at 27C* (*Hrb27C*), which encodes an RNA binding protein homologous to human *DAZ Associated Protein 1* (*DAZAP1*) and *Heterogeneous Nuclear Ribonucleoprotein A3* (*HNRNPA3*), as a putative target of ecdysone signaling that regulates GSC self-renewal (Ables et al., 2016). *Hrb27C* is a member of the heterogeneous nuclear ribonucleoproteins (hnRNPs) family of RNA binding proteins, whose critical functions include alternative splicing, stabilization of newly formed mRNA, transport in and out of the nucleus, and localization of mRNA (Chaudhury et al., 2010; Piccolo et al., 2014). For example, hnRNPs stabilize *E-cadherin* mRNAs to promote stem cell adhesion (Ji and Tulin, 2012), spatially restrict mRNAs in the *Drosophila* oocyte to establish embryonic axes, and mediate oligodendrocytic and neuronal mRNA trafficking in mice and humans (Geuens et al., 2016; Piccolo et al., 2014). Dysregulation of hnRNP function in mammals is associated with several types of cancer and a variety of neurodegenerative diseases, highlighting the importance of these multifunctional proteins. Further, increasing evidence in mammals reveals that nuclear hormone receptor signaling can coordinately control transcription and pre-mRNA processing via recruitment of hnRNPs, perhaps as a mechanism to rapidly modify the cellular proteome in cells in response to physiological stimuli (Buoso et al., 2017; Curado et al., 2015; Dago et al., 2015; Zhou et al., 2015). Whether this mechanism controls stem cell behavior, however, has not been explored.

In this study, we investigate the relationship between short and long-range signals and hnRNP function in the control of stem cell self-renewal. We identify a subset of hnRNPs expressed in GSCs and dividing cysts, and demonstrate that expression of hnRNPs encoded by *Hrb27C*, *squid* (*sqd*; human homolog *HNRNPA/B), hephaestus (heph;* human homolog *Polypyrimidine Tract Binding protein 1*), and *Hrb87F* (human homolog *HNRNPA2/B*) are modulated by ecdysone signaling. We use spatially and temporally controlled loss-of-function analyses to show that *Hrb87F* is necessary to maintain the proper numbers of GSCs in the niche and that *Hrb27C*, *sqd,* and *heph* are intrinsically required in GSCs for proper self-renewal. Our data support the hypothesis that hnRNPs regulate a diverse, but non-identical array of targets in GSCs that independently promote GSC behavior. We propose a model that ecdysone signaling

transcriptionally activates expression of a subset of hnRNPs, whose protein products modulate post-transcriptional processing of key regulatory nodes in the BMP signaling pathway to reinforce reception of BMP signals in GSCs. This positive feedback mechanism may stabilize GSC self-renewal following mating-induced ecdysone biosynthesis, thus promoting continued egg production. Our findings support a common mechanism, wherein nuclear hormone receptors regulate gene expression by transcriptionally regulating multifunctional RNA binding proteins, each with their own broad cohort of target mRNAs. Our study offers new insights into how tissue-resident stem cells are modulated by the endocrine environment.

RESULTS

HnRNPs are expressed at distinct, but overlapping, stages of ovarian germ cell differentiation.

Aberrant germline phenotypes, including dorsalized eggs and female sterility, were among the first biological processes attributed to mutations in *Drosophila* hnRNPs (Goodrich et al., 2004; Kelley, 1993; Matunis et al., 1994; Norvell et al., 1999). Yet while phenotypic reports of *Hrb27C* mutants hinted at potential function in the early germline (Yano et al., 2004), roles for hnRNPs in mitotically dividing germ cells have remained largely unexplored. We used available protein trap transgenes and antibodies to assess which of the 14 hnRNPs encoded in the *Drosophila* genome are expressed in the early germline (Fig. 2). Several distinct patterns of hnRNP expression emerged, suggesting independent roles in early oogenesis. For example, Sqd and Rumpelstiltskin (Rump) were expressed throughout the germarium at equivalent levels in both germline and somatic cells (Fig. 2A, B, J). In contrast, Smooth (Sm), No on or off transient A (NonA), and Heph were highly expressed in GSCs and early cysts, but at very low levels in 8- and 16-cell cysts (Fig. 2C, D, E, J), suggesting a switch in their function concomitant with cyst division. Strikingly, Heph is localized to GSC nuclei, but becomes exclusively cytoplasmic in 16-cell cysts (Fig. 2E, J). We also noted differences in the levels of Hrb27C and Hrb98DE across the germarium. Both displayed moderate expression in GSCs and 16-cell cysts, but higher expression in cysts in region 2A (4-cell and 8-cell) (Fig. 2F, G, J). Intriguingly, Hrb87F was consistently expressed in the germline, but at higher levels in adjacent somatic cells, including escort cells and early follicle cells (Fig. 2H, J). Unlike the other hnRNPs tested, Syncrip (Syp) was exclusively localized to somatic cells in the germarium (Fig. 2I, J). These distinct but similar

patterns of expression may indicate that, in addition to their roles in oocyte patterning in late oogenesis, hnRNPs are also important for the earliest stages of oocyte development.

Ecdysone signaling promotes expression of hnRNPs in GSCs.

We previously identified *Hrb27C* in a reverse genetic screen as a putative target of ecdysone signaling in GSCs (Ables et al., 2016). Since hnRNPs are expressed in response to steroid hormones in other cell populations (Beckstead et al., 2005; Gauhar et al., 2009; Stoiber et al., 2016; Syed et al., 2017), and hnRNP complexes are found at many transcriptionally active, ecdysone-responsive chromosome regions in salivary gland polytene chromosomes (Amero et al., 1991; Amero et al., 1993), we asked whether other ovary-enriched hnRNPs are downstream of ecdysone signaling. We used quantitative reverse-transcription PCR (qRT-PCR) to measure selected hnRNP mRNA levels in whole ovaries from EcR^{ts} mutant females, which display decreased ecdysone signaling (Ables and Drummond-Barbosa, 2010; Carney and Bender, 2000). We observed a statistically significant reduction in *Hrb27C, heph, sqd*, and *Hrb87F* levels in EcR^{ts} mutant ovaries (Fig. 3A), suggesting that ecdysone signaling is required for proper expression of specific hnRNPs.

Current models postulate that ecdysone signaling controls gene expression in a hierarchical transcriptional cascade (Ashburner, 1974; King-Jones and Thummel, 2005; Yamanaka et al., 2013). The EcR/Usp heterodimer binds directly to a small number of early-response gene targets that are themselves transcription factors; indirect responses to the hormone are generated as early-response factors activate or repress their own unique gene targets (Fig. 1C). We used publicly available Chromatin Immunoprecipitation sequencing (ChIP-seq) datasets to compare EcR and RNA Polymerase II (PolII) occupancy at the *Hrb27C, sqd*, *heph*, and *Hrb87F* loci to assess whether the transcriptional regulation of hnRNPs is directly modulated by EcR(Nègre et al., 2011; Roy et al., 2010) (Supplemental Fig. S1-S4). EcR and Pol II were bound to multiple sites along the first exon and intron of *Hrb27C* and *Hrb87F* (Fig. S1-S2), consistent with previously-reported regulation of other ecdysone-responsive genes (Bernardo et al., 2014; Shlyueva et al., 2014; Tourmente et al., 1993). The *sqd* locus was bound by Pol II in the first exon and intron, but we did not find strong evidence of EcR binding in the same region (Fig. S3). Similarly, we identified two regions of Pol II occupancy in the expansive *heph* locus, which encodes more than 20 predicted isoforms (Fig. S4). While the region contains many sites bound

by EcR binding, none overlap significantly with Pol II. Taken together, this suggests that ecdysone signaling both directly and indirectly regulates hnRNPs.

Many transcripts, including hnRNPs, are produced by ovarian nurse cells and maternally deposited into the oocyte to support early embryonic development. We therefore asked whether decreased hnRNP mRNA in *EcR*^{ts} ovaries resulted in a corresponding decrease in protein levels in the germarium. Using an anti-Sqd antibody that specifically recognizes a Sqd isoform expressed in early germ cells (Fig. S5) (Norvell et al., 1999), we observed decreased levels of Sqd throughout *EcR*^{ts} germaria, including GSCs (Fig. 3B-D). Similarly, levels of Heph protein were also decreased in ecdysone-deficient GSCs (Fig. 3E-G). Taken together, these results raise the possibility that a functional interaction between ecdysone signaling and hnRNPs mediates the effects of ecdysone on GSCs.

To specifically test the functional implications of ecdysone-dependent hnRNP expression, we examined genetic interactions between *sqd*, *heph*, *Hrb27C*, and ecdysone pathway components (Table 1), using the number of GSCs per germaria as an indicator of normal GSC self-renewal (Ables and Drummond-Barbosa, 2010). Single copy loss-of-function of *EcR*^{M554fs}, *heph*^{e2}, *sqd*^{ix50}, or *Hrb27C*^{rF680} in single heterozygous females resulted in a similar number of GSCs per germaria as age-matched wild-type females. As a negative control, we tested double heterozygous females for *EcR*^{M554fs} and the null *Insulin Receptor* (*InR*^{339}) allele, because insulin-like peptides and ecdysone control GSC self-renewal via distinct mechanisms (Ables and Drummond-Barbosa, 2010). As expected, we found no significant genetic interaction between the insulin and ecdysone pathways in controlling GSC number (Table 1). Similarly, *Hrb27C*^{rF680} and *InR*^{339} did not show a genetic interaction (Table 1). In contrast, we observed decreased GSC number in double heterozygotes for *Hrb27C*^{rF680} and *EcR*^{M554fs} relative to controls (Table 1). Further, we found evidence for strong genetic interactions between *Hrb27C*^{rF680} and *E74*$^{DL-1}$, a critical target of ecdysone signaling in GSCs (Ables and Drummond-Barbosa, 2010). We also detected strong interactions between *sqd* and *EcR*, but weak genetic interactions between *heph* and *EcR*. Taken together with the EcR chromatin occupancy data (Fig. S3-S4), we speculate that *sqd* is a direct transcriptional target of ecdysone, while *heph* functions as an indirect transcriptional target (Table 1). These observations strongly support the model that *sqd*, *heph*, and *Hrb27C* function downstream of ecdysone signaling to regulate GSCs.

HnRNPs are independently and autonomously required for GSC self-renewal.

Ecdysone signaling directly regulates GSC behavior; therefore, if hnRNPs are targets of ecdysone signaling, they should likewise be autonomously required in GSCs for their self-renewal. To test whether *Hrb27C*, *sqd*, and *heph* are essential for GSC self-renewal, we used *Flippase/Flippase Recognition Target* (*Flp/FRT*)-mediated mosaic recombination to inactivate their function in GSCs (Fig. 4). GSCs and their daughter cells carrying homozygous mutations in *sqd* (Fig. 4C), *heph* (Fig. 4D), or *Hrb27C* (Fig. 4E) were recognized by loss of GFP in mosaic germaria (Fig. 4A). In most control "mock mosaic" germaria, where all cells are wild-type, GFP-negative GSCs were accompanied by GFP-negative cystoblasts/cysts, indicating that these GSCs self-renew and produce differentiating progeny (Fig. 4B, H). In contrast, a significant percentage of *sqd*, *heph*, and *Hrb27C* mutant mosaic germaria contained GFP-negative cysts without an accompanying GFP-negative GSC, indicating that the mutant GSC failed to self-renew (Fig. 4C-E, H). Surprisingly, despite high levels of *Hrb27C* expression in 8-cell cysts, we did not observe any other obvious germline phenotypes in *Hrb27C* mutant mosaics. Cyst divisions were not interrupted, and we found no evidence of cyst death in mutant germaria. Outside of the germarium, however, we observed many phenotypes reminiscent of follicle formation defects, including dying cysts, fused cysts, and follicle cell overgrowth, indicating a critical role for *Hrb27C* in cyst encapsulation (Fig. S6A-C). Similarly, abnormal follicles were frequently observed in *heph* mutant mosaic ovarioles (Fig. S6D), though cyst mitotic divisions appeared to proceed normally (Fig. 4D).

Like *Hrb27C* and *heph* mutants, mosaic germaria containing *sqd* mutant GSCs frequently exhibited GSC loss (Fig. 4C, H). Loss of *sqd* function from germline cysts also caused a variety of other defects (Fig. S6E-H), including cystoblasts with thick nuclear membranes (Fig. S2F) and rounded cysts lacking branched fusomes (Fig. S6G). Other phenotypes in the posterior germarium and in developing follicles are likely due to loss of *sqd* in follicle cells (Fig. S6H), and suggest that *sqd* serves several other roles in early cyst development and encapsulation.

Levels of *Hrb87F* mRNA were severely compromised in ecdysone-deficient ovaries (Fig. 3A). Although technical limitations prevented us from specifically inactivating *Hrb87F* function in GSCs, a recent report demonstrating decreased female fecundity in *Df(3R)Hrb87F* mutants (Singh and Lakhotia, 2012) prompted us to investigate whether it is also necessary for GSC self-renewal. Female homozygous *Df(3R)Hrb87F* mutants (see Experimental Procedures) survived to

adulthood, but had multiple defects in oogenesis and were sterile (Singh and Lakhotia, 2012). Furthermore, we observed fewer GSCs per germarium as compared to heterozygous sibling controls (Fig. 4F-G, I). Taken together, these data support the model that individual hnRNPs are independently necessary for GSC self-renewal.

HnRNPs control GSC self-renewal via discrete molecular mechanisms.

HnRNPs frequently have overlapping targets (Blanchette et al., 2009; Brooks et al., 2015; Huelga et al., 2012; McMahon et al., 2016), at least in part because hnRNPs can form multi-protein complexes on RNA (Markovtsov et al., 2000). Because hnRNPs work in combination to promote mRNA splicing, transport, and stability (Matunis et al., 1992; Piccolo et al., 2014), we hypothesized that *Hrb98DE, sqd, heph,* and *Hrb27C* promote GSC self-renewal by binding a common target. *Hrb98DE* promotes GSC self-renewal by binding the 5' untranslated region of *E-cadherin*, promoting its translation and stabilizing the physical attachment of GSCs to the stem cell niche by adherens junctions (Ji and Tulin, 2012). E-cadherin localizes at the interface between cap cells and GSCs (Fig. 5) and is necessary for GSC self-renewal (Song et al., 2002). To test whether loss of *Hrb27C, heph,* or *sqd* abrogated E-cadherin expression, we compared E-cadherin mean fluorescence intensity at the cap cell-GSC interface between hnRNP mutant GSCs and adjacent wild-type GSCs (Fig. 5). We found no significant difference in E-cadherin expression in sqd^{ix50} (Fig. 5A-B), $heph^{e2}$ (Fig. 5C-D), or $Hrb27C^{rF680}$ mutant GSCs (Fig. 5E-F), indicating that these hnRNPs function independently of *Hrb98DE* in GSCs. Further, since attachment of GSCs to cap cells is essential for GSC self-renewal, these data suggest that the failure of *sqd, heph,* and *Hrb27C* mutant GSCs to self-renew was not due to loss of a physical attachment to the niche.

***Hrb27C, heph,* and *sqd* promote BMP signaling in GSCs.**

Loss of ecdysone signaling leads to a failure in GSC self-renewal due to a decreased ability of GSCs to receive BMP signals (Ables and Drummond-Barbosa, 2010). Failure of *sqd, heph,* and *Hrb27C* mutant GSCs to self-renew is not due to reduced E-cadherin levels. We therefore asked whether premature differentiation contributed to the progressive loss of *sqd, heph,* and *Hrb27C* mutant GSCs from the niche. BMP ligands, produced by somatic niche cells and received by GSCs, are essential for GSC self-renewal (Chen and McKearin, 2003a; Song et

al., 2004; Xie and Spradling, 1998). To test the ability of hnRNP mutant GSCs to respond to BMP signals, we measured the levels of phosphorylated Mothers against decapentaplegic (pMad), a well-characterized reporter of BMP pathway activation (Kai and Spradling, 2003; Song et al., 2004). *heph* and *sqd* mutant GSCs displayed lower levels of nuclear pMad than neighboring wild-type GSCs (Fig. 6B-C, F). Intriguingly, while *Hrb27C* mutant GSCs showed, on average, low levels of pMad in comparison to wild-type GSCs, a significant population of *Hrb27C* mutant GSCs had high levels of pMad (Fig. 6D, F), suggesting that Mad mRNA stability, translation, or protein phosphorylation are misregulated in the absence of *Hrb27C*. To confirm that *Hrb27C* functions with BMP signaling to promote GSC self-renewal, we assayed for genetic interactions between *Hrb27C* and components of the BMP signaling pathway. We found a strong interaction between $Hrb27C^{rF680}$ and the BMP ligand dpp^{hr56} and a weak interaction with the BMP receptor tkv^{7} (Table 1), further supporting the model that *Hrb27C* functions with BMP signaling to regulate GSC self-renewal. Taken together, these data indicate that GSCs do not respond properly to BMP signals in the absence of hnRNPs.

BMP signaling directly represses the transcription of the pro-differentiation factor *bam* to promote GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004). We therefore hypothesized that the low level of pMad in *Hrb27C* mutant GSCs was indicative of premature differentiation to the cystoblast fate. To test this hypothesis, we crossed a *bam* translational reporter (*bam::GFP*) (Chen and McKearin, 2003a; Chen and McKearin, 2003b) onto the $Hrb27C^{rF680}$ *FRT40A* background to assess the levels of Bam in adjacent wild-type and *Hrb27C* mutant GSCs. *bam::GFP* was well-expressed in wild-type and *Hrb27C* mutant 4- and 8-cell cysts; however, as in wild-type GSCs, *bam::GFP* was undetectable in *Hrb27C* mutant GSCs (Fig. 6E, E'). Since the reduction of BMP signaling in hnRNP mutant GSCs was not sufficient to derepress *bam*, we speculate that additional factors are required to promote differentiation in the absence of hnRNPs.

DISCUSSION

Stem cell self-renewal is regulated by a combination of paracrine and endocrine signals; however, the molecular mechanisms by which these critical signals converge to dictate stem cell fate has remained largely unclear. In this study, we demonstrate for the first time that distinct hnRNPs are transcriptionally regulated by the steroid hormone ecdysone in the *Drosophila*

ovary. We also show that conserved hnRNPs *sqd* (*hnRNPA/B*), *heph* (*PTBP1*), *Hrb87F* (*hnRNPA2/B*), and *Hrb27C* (*DAZAP1/hnRNPA3*) are essential for GSC self-renewal. Intriguingly, our data indicate that hnRNPs independently regulate GSC function, suggesting that hnRNPs modulate gene expression in GSCs by coordinating the post-transcriptional modification of distinct, yet overlapping sets of transcripts, perhaps as unique ribonucleoprotein (RNP) complexes. We propose a model (Fig. 7) in which ecdysone signaling promotes the expression of cell- or lineage- specific hnRNPs that regulate the stability, splicing, or nuclear transport of transcripts enabling GSCs to properly respond to BMP ligands. Such targets likely include the BMP receptor *tkv* or the signal transducers *Mad* or *Medea* (*Med*). Our model elucidates a key connection between paracrine and endocrine control of stem cell fate, and proposes a previously unidentified mechanism by which cell-specific responses are induced by a systemic steroid hormone.

hnRNPs are dynamically expressed in mitotic germ cells and surrounding somatic cells.

Gene expression involves the formation of distinct RNP complexes containing nascent transcripts, hnRNPs, and translation initiation factors (Bjork and Wieslander, 2017). Post-transcriptional regulation, translational repression, and the formation of RNP complexes are hallmarks of germ cell development, and are critical for meiosis in a variety of organisms (Jin and Neiman, 2016; Licatalosi, 2016; Nousch and Eckmann, 2013; Percipalle, 2014; Slaidina and Lehmann, 2014). It should perhaps come as no surprise then that individual hnRNPs are expressed in a spatially- and temporally-restricted manner in the *Drosophila* germarium. For example, our data demonstrate that while many hnRNPs are expressed in the germline and soma, Syp is expressed only in somatic cells. More intriguing, however, is our finding that hnRNPs exhibit distinct intracellular localization. HnRNPs shuttle between the nucleus and the cytoplasm to control the nuclear export of mature mRNAs (Bjork and Wieslander, 2017). Our results demonstrate that NonA and Hrb87F are both concentrated in the nucleus, while Hrb27C appears primarily cytoplasmic. In addition, Heph is primarily nuclear in mitotically dividing cysts, but becomes restricted to the cytoplasm coincident with the terminal mitotic division. The intracellular location of hnRNPs in germ cells likely reflects their diverse functions. For example, NonA preferentially binds introns of nascent RNAs to facilitate nuclear paraspeckle formation (Knott et al., 2016; McMahon et al., 2016), while Hrb27C binds 3'UTRs to control

alternative splicing, mRNA localization, and translation (Blanchette et al., 2009; Huynh et al., 2004; McMahon et al., 2016; Nelson et al., 2007). As the majority of the hnRNPs we identified in mitotically dividing germ cells localize to the nucleus, we speculate that they primarily regulate co- or post-transcriptional nascent mRNA processing steps. Future studies on the function of hnRNPs in GSCs and their dividing daughters will identify specific mRNA targets in these cells.

Individual hnRNPs are essential for GSC self-renewal.

One of the first biological roles attributed to hnRNPs in *Drosophila* oogenesis was the establishment of anterior-posterior and dorsoventral axes in oocytes (Kelley, 1993; Matunis et al., 1994). In particular, *sqd*, *Hrb27C*, and *glo* are necessary for translational repression and localization of *gurken*, *nanos*, and *oskar* mRNAs, whose spatially-regulated translation establishes concentrated areas of asymmetrically distributed protein in the oocyte (Goodrich et al., 2004; Huynh et al., 2004; Kalifa et al., 2009; Kalifa et al., 2006). More recently, *Hrb98DE*, *Hrb27C*, and *Protein on ecdysone puffs* (*Pep*) were identified in genetic screens for novel regulators of GSC self-renewal (Ables et al., 2016; Yan et al., 2014). *Hrb98DE* promotes GSC self-renewal via regulation of E-cadherin translation (Ji and Tulin, 2012). Intriguingly, *sqd*, *heph*, and *Hrb27C* appear to promote GSC self-renewal independently of *Hrb98DE*. Moreover, even though many of the hnRNP-dependent factors that promote oocyte axes establishment (e.g. *nanos*, *vasa*) are also required for GSC self-renewal (Slaidina and Lehmann, 2014), we found no evidence that *sqd*, *Hrb27C*, or *heph* modulate Nanos or Vasa protein levels in GSCs or cystoblasts (data not shown). Taken together, our data suggests a broad role for these RNA binding proteins in the regulation of key events in oocyte development.

hnRNPs regulate discrete, overlapping sets of targets.

HnRNPs are a diverse family of RNA binding proteins that regulate post-transcriptional processing, maturation, and nuclear export of RNA polymerase II-dependent transcripts (Levengood and Tolbert, 2018). Attempts to identify transcripts bound by hnRNPs in *Drosophila* have yielded thousands of putative targets and illuminated extensive cross-regulatory interactions (Blanchette et al., 2009; Brooks et al., 2015; McMahon et al., 2016; Stoiber et al., 2015). Our data suggest that Sqd, Heph, and Hrb27C post-transcriptionally promote the expression of key

components of the BMP signaling pathway in GSCs. In support of this model, a recent study profiling Hrb27C targets in three different *Drosophila* neuronal cell lines observed specific protein-RNA interactions between Hrb27C and the BMP receptors *sax*, *tkv* and *put*, as well as the signal transducer *Mad* (McMahon et al., 2016). Consistent with the cell-autonomous requirement for *Hrb27C* in GSCs (Fig. 4), the BMP ligands *dpp* and *gbb* were not bound by Hrb27C in any of the cell lines tested (McMahon et al., 2016). Hrb27C is therefore capable of directly modulating the BMP singling pathway in GSCs. Multiple isoforms are encoded at the *sax*, *tkv*, and *put* gene loci, perhaps suggesting that splicing differences underlie decreased BMP signaling in *Hrb27C*, *sqd*, and/or *heph* mutants. Hrb27C may also impact *Mad* stability or transport, since pMad levels appear to be misregulated (both excess and depleted) in *Hrb27C* mutant GSCs. We cannot exclude the possibility, however, that Sqd, Heph, and/or Hrb27C bind other as-yet-unidentified transcripts critical for promoting BMP signal reception in GSCs.

Alternatively, hnRNPs could post-transcriptionally repress factors that normally repress BMP signaling and promote differentiation. Translational repression is critical for the initiation of cyst differentiation (Slaidina and Lehmann, 2014). RNA binding proteins Pumilio (Pum) and Brain tumor (Brat) post-transcriptionally repress *Mad* and *Med* mRNAs by recruiting the deadenylase complex CCR4-NOT (Joly et al., 2013; Newton et al., 2015). Destabilization of *Mad* and *Med* transcripts by CCR4-NOT aids in repressing BMP signaling in cystoblasts, permitting de-repression of *bam* and other pro-differentiation factors. Pum also forms complexes with Nanos to repress Brat and Mei-P26 in GSCs, suppressing differentiation (Joly et al., 2013; Li et al., 2013; Neumuller et al., 2008). Moreover, low levels of Mei-P26 promote GSC self-renewal by repressing Brat (Li et al., 2012), demonstrating that repressor levels are a key regulatory component of differentiation. Interestingly, loss of *Hrb27C*, *sqd*, or *heph* partially phenocopies *CCR4* mutants, which display decreased GSC self-renewal without an accompanying de-repression of *bam* (Joly et al., 2013). Since *CCR4* mutants also display reduced E-cadherin at the GSC/cap cell interface (Fu et al., 2015), our data does not at present support a direct link between the CCR4-NOT complex and Hrb27C, Sqd, or Heph in GSC self-renewal. A recent study in mammals demonstrated that hnRNPs A1 and A2/B1 bind 3' UTR motifs that promote transcript degradation via CCR4-NOT (Geissler et al., 2016); therefore, it remains a formal possibility that hnRNPs could coordinate GSC self-renewal in concert with CCR4-NOT-mediated translational stability.

The intersection between hormone signaling and translational control: a common mechanism promoting cell fate?

The cellular response to ecdysone signaling is complex, exhibiting both spatial and temporal specificity (King-Jones and Thummel, 2005; Li and White, 2003; Yamanaka et al., 2013). Ecdysone-dependent expression of hnRNPs, however, does not appear to be specific to ovarian cells (Beckstead et al., 2005; Gauhar et al., 2009; Stoiber et al., 2016; Syed et al., 2017). In the *Drosophila* central nervous system, ecdysone signaling temporally promotes expression of Syp to coordinate neuroblast terminal differentiation with organismal development (Doe, 2017; Syed et al., 2017). Further, a recent study profiling the transcriptome in ecdysone-responsive *Drosophila* cell lines demonstrated that hnRNP expression is frequently induced (Stoiber et al., 2016). We speculate that hormone signaling coordinates chromatin state, transcriptional regulation, and post-transcriptional processing to broadly regulate gene expression. We envision three possible models by which ecdysone may coordinate unique hnRNP-containing RNP complexes in germ cells to regulate differentiation. First, ecdysone signaling via EcR/Usp may transcriptionally promote hnRNP expression in specific cells or at specific stages of differentiation. Our data clearly demonstrates that some hnRNPs (*Hrb27C*, *Hrb87F*) are direct targets of EcR/Usp. Increased receptor concentration, co-activator or co-repressor activity, or EcR isoform specificity could bias RNP complex formation by up-regulating specific hnRNPs in specific cells. Second, ecdysone signaling could modify the chromatin landscape in specific cell lineages or stages of differentiation. We have previously demonstrated that ecdysone signaling functionally interacts with the nucleosome remodeler ISWI/NURF in GSCs (Ables and Drummond-Barbosa, 2010). EcR/ISWI activity could promote open regions of chromatin at hnRNP gene loci, such that other lineage-specific transcription factors have access promote transcription. Lastly, ecdysone signaling could be positively reinforced in specific cell types by the post-transcriptional regulation of early response genes by hnRNPs. Unique hnRNP complexes have been observed at sites of ecdysone-dependent transcription, including the *E74* and *E75* loci (Amero et al., 1993). Taken together, this presents an attractive model by which steroid hormone signals are uniquely interpreted by cells at different stages of development or differentiation, or in specific lineages within a given tissue. Given the recent interest in hnRNP regulation as a causative factor in neurodegenerative disorders (Geuens et al., 2016), our study may provide novel insight into the origins of complex human diseases.

MATERIALS AND METHODS

Drosophila strains and culture conditions

Flies were maintained at 22°-25°C on a standard medium containing cornmeal, molasses, yeast and agar (Nutrifly MF; Genesee Scientific) supplemented with yeast. For all experiments, unless otherwise noted, flies were collected 2 to 3 days after eclosion and maintained on standard media at 25°C. Flies were supplemented with wet yeast paste (nutrient-rich diet) 3 days before ovary dissection. Genes/alleles with multiple names are referenced using FlyBase nomenclature (www.flybase.org) for simplicity.

Protein expression of hnRNPs within the germaria were determined using GFP tagged protein trap transgenic lines including the following: *Sqd*^{CPTI000239}, *rump*^{CPTI004242}, *sm*^{CPTI002653}, *nonA*^{CPTI00309}, *and, Hrb98DE*^{CPTI000205,} (Kyoto) (Lowe et al., 2014), *PTB::GFP* (referred to as Heph::GFP) (Besse et al., 2009), *Hrb87F::GFP* (Singh and Lakhotia, 2015), and *Hrb27C*^{28387} (Sarov et al., 2016).

For genetic interaction analyses, the following alleles were used: *Hrb27C*^{rF680}(Goodrich et al., 2004), *Hrb27C*^{02647} (Hammond et al., 1997), *InR*^{339} (LaFever and Drummond-Barbosa, 2005). *dpp*^{hr56} (Bloomington #36528) (Xie and Spradling, 1998), *tkv*^{7} (Bloomington #3242) (Tearle and Nusslein-Volhard, 1987), *EcR*^{M554fs} (Bloomington #4894) (Carney and Bender, 2000), *E74*^{neo24} (Bloomington # 10262) (Fletcher et al., 1995), *E74*$^{DL-1}$ (Bloomington #4435) (Fletcher et al., 1995), *heph*^{e2} (Dansereau et al., 2002), and *sqd*^{ix50} (Kelley, 1993). Average stem cell number of double heterozygote mutants were compared to average stem cell number of controls (single heterozygotes carrying a balancer). Data was analyzed using Student's t-test.

EcR-deficient ovaries (referred to as *EcR*^{ts}) were created using temperature-sensitive *EcR*^{A438T} mutants in trans with *EcR*^{M554s} (Ables and Drummond-Barbosa, 2010; Carney and Bender, 2000) null mutants. These flies were raised at a permissive temperature (18°C) until eclosion, then incubated at the restrictive temperature (29°C) for 3 days and supplemented with wet yeast paste prior to dissection (Ables and Drummond-Barbosa, 2010).

To visualize Bam expression in *Hrb27C* clones, *bam::GFP (Chen and McKearin, 2003b)* was recombined with *Hrb27C*^{rf680} *FRT40A* using standard crosses.

Genetic mosaic generation and stem cell analyses

Genetic mosaic analysis via *flipase/flipase recognition target* (*Flp/FRT*) (Xu and Rubin, 2012) used the following alleles on *FRT*-containing chromosomes: sqd^{ix50} and sqd^{ix77}*(Kelley, 1993)*, $heph^{e1}$*(Dansereau et al., 2002)*, $heph^{e2}$, $Hrb27C^{rF680}$, $Hrb27C^{K02814}$ (Kyoto #111072), $Hrb27C^{f04375}$ (Kyoto #114656) (Chen et al., 2005; Spradling et al., 1999). Other genetic tools are described in FlyBase (Ashburner and Drysdale, 1994). Genetic mosaics were generated using *FLP/FRT*-mediated recombination in 1-3 day old females carrying a mutant allele *in trans* to a wild-type allele (linked to a *Ubi-GFP* or *NLS-RFP* marker) on homologous *FRT* arms with a *hs-FLP* transgene, as previously described (Hinnant et al., 2017; Laws and Drummond-Barbosa, 2015). Flies were heat shocked at 37°C twice per day 6-8 hours apart for 3 days, then incubated at 25°C on standard media supplemented first with dry yeast, then with wet yeast 3 days prior to dissection. Flies were dissected 8 days after clone induction. Wild-type alleles (*FRT40A* or *FRT82B*) were used for control mosaics. GSCs were identified by the location of their fusomes adjacent to the cap cells (de Cuevas and Spradling, 1998). GSC loss was measured by the number of germaria that contain a GFP-negative cyst (generated from the original GFP-negative stem cell) but lack a GFP-negative GSC, compared to the total number of germaria containing a germline clone (Laws and Drummond-Barbosa, 2015). Results were analyzed by Chi-square tests using Microsoft Excel. To measure stem cell loss in $P\{w^+Tsr^+\}/P\{w^+Tsr^+\}$*; ry Df(3R)Hrb87F/ry Df(3R)Hrb87F* (referred to as *Df(3R)Hrb87F*) (Singh and Lakhotia, 2012; Zu et al., 1998), flies were raised at 25°C and dissected 8 and 12 days after eclosion. GSC loss was measured by the average number of GSCs per germarium in mutants compared to heterozygous sibling controls.

Quantitative RT-PCR

EcR^{ts} mutants (see above) were raised at a permissive temperature (18°C) until eclosion, then incubated at the restrictive temperature (29°C) for 3 days (Ables and Drummond-Barbosa, 2010). Ovaries were dissected in RNAlater (Ambion) and stored at -20°C. Samples were comprised of 10 whole ovary pairs. Total RNA was extracted using an RNAqueous Total RNA isolation kit (Thermo Fisher). Primers are listed in Supplementary Table 1. RNA was treated with a Turbo DNA-free kit (Thermo Fisher) following manufacturer's instructions. RNA quality was tested using agarose gel electrophoresis and concentration was quantified using a Nanodrop

Lite spectrophotometer (Thermo Fisher). Complementary DNA was created by reverse transcription using an iScript cDNA kit (Bio-Rad) following manufacturer's instructions and using 500 ng of input RNA for each sample. Quantitative PCR was performed using a CFX96 Touch Real-Time PCR detection system (Bio-Rad). Each analysis was performed in triplicate using iQ SYBR Green Supermix kit (Bio-Rad). Samples were standardized to a *RP49* control. Quantification was performed using the Bio-Rad CFX Manager program.

Immunofluorescence and microscopy

Ovaries were dissected, fixed, washed, and blocked as previously described (Ables et al., 2016; Hinnant et al., 2017). Briefly, ovaries were dissected and teased apart in Grace's media (Lonza or Caisson Labs) and fixed using 5.3% formaldehyde in Grace's media at room temperature for 13 minutes. Ovaries were washed extensively in phosphate-buffered saline (PBS, pH 7.4; Thermo Fisher) with 0.1% Triton X-100, then blocked for three hours in a blocking solution consisting of 5% bovine serum albumin (Sigma), 5% normal goat serum (MP Biomedicals) and 0.1% Triton-X-100 in PBS. The following primary antibodies were diluted in block and used overnight at 4°C: mouse anti-Lamin C (LamC) [LC28.26, Developmental Studies Hybridoma Bank (DSHB), 1:100], mouse anti-Hts (1B1, DSHB, 1:10), rabbit anti-GFP (ab6556, Abcam, 1:2000), chicken anti-GFP (ab13970, Abcam, 1:2000), guinea pig anti-Syncrip (a gift from I. Davis, 1:5000) (McDermott et al., 2012), rat anti-E-cadherin (DCAD2, DSHB, 1:20), rabbit anti-pMad [(Smad3) phospho S423 + S425, ab52903, Abcam/Epitomics, 1:50], rat anti-Heph (PTB, a gift from A. Ephrussi, 1:1000) (Besse et al., 2009), mouse anti-Sqd (Sqd S 1B11, DSHB, 1:7). Samples incubated with pMad were permeabilized with 0.5% Triton-X100 in PBS for thirty minutes before blocking. Samples were incubated with Alexa Fluor 488-, 568- or 633-conjugated goat-species specific secondary antibodies (Molecular Probes; 1:200) and counterstained with DAPI (Sigma 1:1000 in PBS). Ovaries were then mounted in 90% glycerol containing 20.0 µg/mL N-propyl gallate (Sigma). Data was collected using a Zeiss LSM 700 laser scanning confocal microscope. Images were analyzed using Zen Blue 2012 software and images were minimally and equally enhanced via histogram using Zen and Adobe Photoshop CS6.

AUTHOR CONTRIBUTIONS

D.S.F. and E.T.A. conceived and designed experiments, analyzed data, interpreted results, and wrote the manuscript. D.S.F. performed the experiments and managed the project. V.V.H. assisted with experiments and data collection (Figure 1 and Table 1). All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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COMPETING INTERESTS

The authors declare no competing conflicts of interest.

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Table 1. Genetic interactions between ecdysone pathway components, the hnRNPs *sqd*, *Hrb27C*, and *heph*, and the BMP pathway components *dpp* and *tkv*.

Genotype[a]	3+ GSCs	2 GSCs	1 GSC	0 GSCs	Average # GSCs[b]	*p* value[c]
***InR^{339}*/+ or *EcR^{M554fs}*/+ sibling controls[d]**	206	58	5	0	2.944(269)[e]	
***InR^{339}*/+; *EcR^{M554fs}*/+**	114	47	1	1	2.859(163)	0.238
***EcR^{M554fs}*/+ or *dpp^{hr56}*/+ sibling controls**	166	107	11	1	2.632(285)	
***EcR^{M554fs}*/+;*dpp^{hr56}*/+**	61	172	46	1	2.054(280)	**9.58E-22**
***EcR^{M554fs}*/+ or *Hrb27C^{rF680}*/+ sibling controls**	153	25	0	0	3.096(178)	
***EcR^{M554fs}*/+; *Hrb27C ^{rF680}*/+**	141	48	5	1	2.856(195)	**0.001**
***EcR^{M554f}*s/+ or *sqd^{ix50}*/+ sibling controls**	157	26	8	1	3.083(192)	
***EcR^{M554fs}*/+; *sqd^{ix50}*/+**	109	55	16	3	2.612(183)	**3.00E-07**
***EcR^{M554fs}*/+ or *heph^{e2}*/+ sibling controls**	142	50	6	0	2.984(198)	
***EcR^{M554f}*s/+; *heph^{e2}*/+**	129	44	11	0	2.712(184)	**0.0168**
***Hrb27C ^{rF680}*/+ or *E74^{neo24}*/+ sibling controls**	121	59	6	1	2.733(187)	
***Hrb27C ^{rF680}*/+; *E74^{neo24}*/+**	135	49	20	2	2.704(206)	0.730
***Hrb27C ^{rF680}*/+ or *E74^{DL-1}*/+ sibling controls**	89	12	1	1	3.301(103)	
***Hrb27C ^{rF680}*/+; *E74^{DL-1}*/+**	141	58	24	1	2.696(224)	**6.45E-08**
***Hrb27C ^{rF680}*/+ or *InR^{339}*/+ sibling controls**	92	20	0	0	4.779(113)	
***Hrb27C ^{rF680}*/+; *InR^{339}*/+**	134	16	2	0	3.289(152)	0.771
***Hrb27C ^{rF680}*/+ or *dpp^{hr56}*/+ sibling controls**	115	41	10	0	2.771(166)	
***Hrb27C ^{rF680}*/+;*dpp^{hr56}*/+**	86	80	34	3	2.302(203)	**5.53E-07**
***Hrb27C ^{rF680}*/+ or *tkv^{7}*/+ sibling controls**	96	11	4	0	3.286(112)	
***Hrb27C ^{rF680}*/+; *tkv^{7}*/+**	70	25	1	0	2.990(98)	**0.016**

[a]Females kept at 22°C–25°C were analyzed at 8 days of age.

[b]Average number of germline stem cells (GSCs) per germarium ± SEM.

[c]*p* values relative to sibling controls. $p < 0.05$ was considered statistically significant (shown in bold).

[d]All heterozygous sibling controls carry balancer chromosomes containing wild-type alleles (+).

[e]The number of germaria analyzed is shown in parentheses.

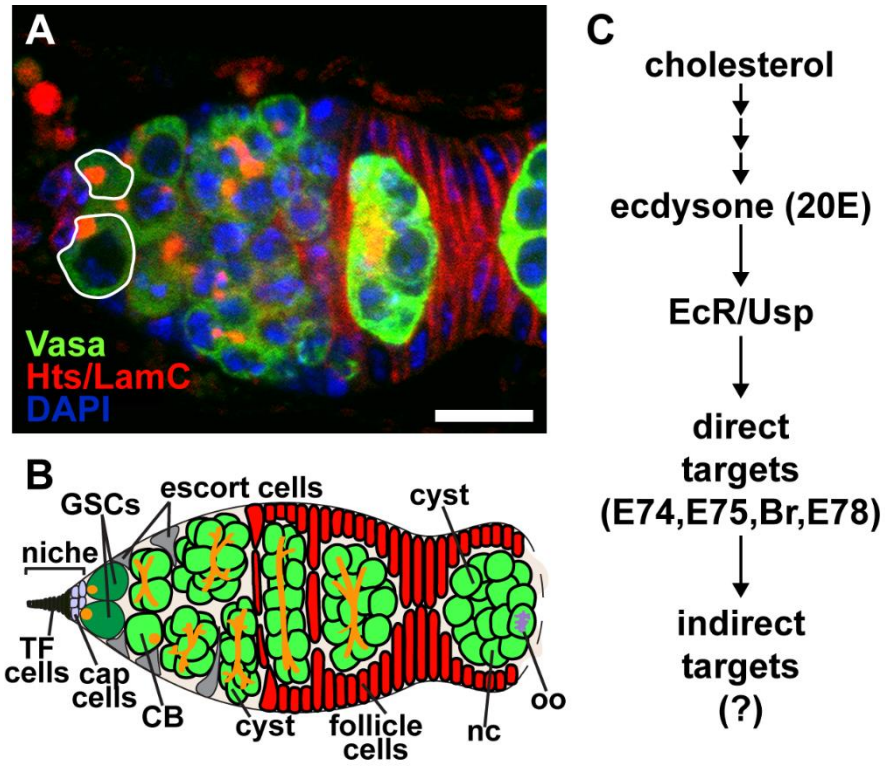

Fig. 1. *Drosophila* oocyte development begins in the germarium. (A) *Drosophila* germarium labeled with anti-Vasa (green; germ cells) anti-Hts (red; fusomes and follicle cell membranes), and anti-LamC (red; nuclear envelope of cap cells). Dotted lines demarcate GSCs. (B) GSCs (dark green) are anchored to a niche (composed of cap cells and terminal filament cells) at the anterior tip of each ovariole. Germ cells are characterized by the presence of a fusome (orange), which extends as germ cells divide. Escort cells (grey) signal to germ cells to promote differentiation. Follicle cells (red), surround the 16-cell germline cyst, giving rise to an egg chamber or follicle that leaves the germarium. (C) Diagram of the ecdysone signaling pathway depicting components with known roles in *Drosophila* oogenesis.

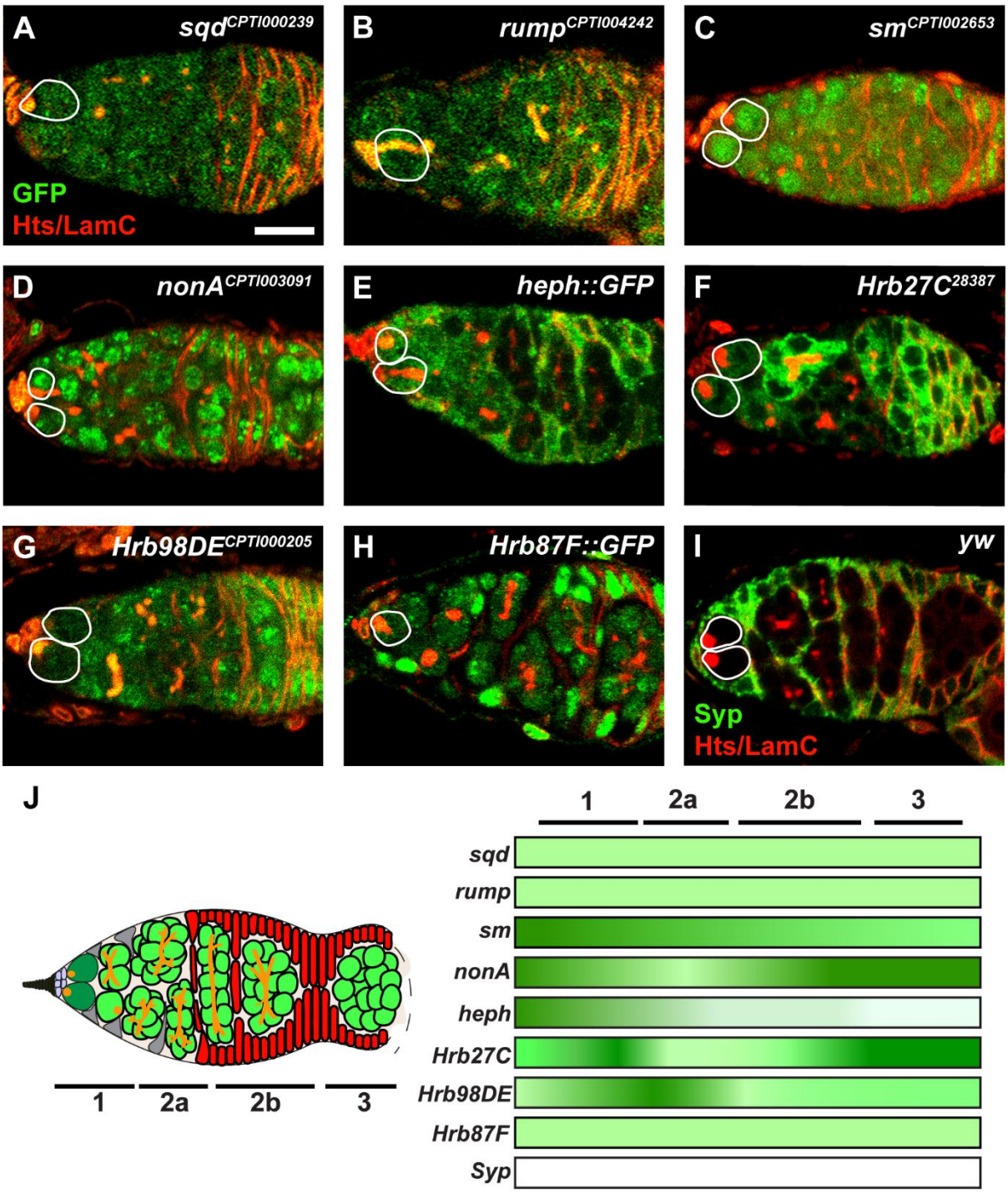

Fig. 2. hnRNPs are expressed in distinct patterns in the germarium. (A-I) Representative germaria from GFP-tagged hnRNP transgenic flies labeled with anti-GFP (A-H) or wild-type flies labeled anti-Syncrip (I) and counterstained with anti-Hts (red; fusomes and follicle cell membranes), and anti-LamC (red; nuclear envelope of cap cells). Dotted lines demarcate GSCs. Scale bar = 10 μm. (J) Summary of hnRNP expression in the early germline. Regions (1, 2a, 2b and 3) correspond to anterior-posterior locations in the germarium. Region 1 contains GSCs and mitotically dividing cysts.

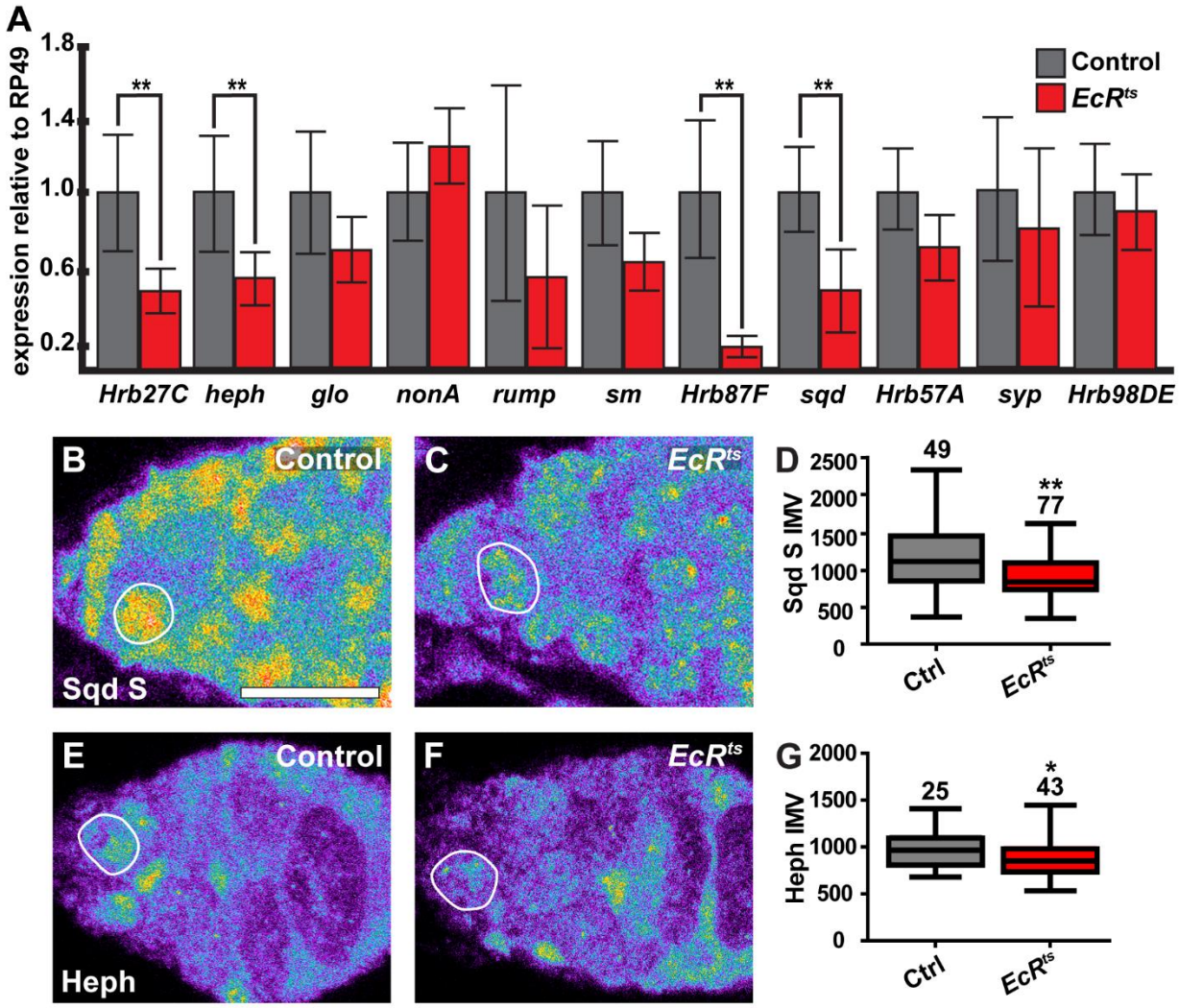

Fig. 3. Ecdysone signaling transcriptionally regulates hnRNP expression in the ovary. (A) qRT-PCR of various hnRNPs in heterozygous sibling control (gray bars) and *EcR*^{ts} (red bars) ovaries. Relative expression of three biological replicates normalized to *RP49* control. Error bars, mean ± SEM. ***p* < 0.001; Student's two-tailed t-test. (B-C, E-F) Heat maps (purple < yellow < red) depicting Sqd (B-C) or Heph protein levels (E-F) in sibling control (B, E) and *EcR*^{ts} (C, F) germaria. GSC nuclei are circled in white. Scale bar = 10 µm. (D, G) Fluorescence intensity mean value (IMV) of anti-Sqd (D) or Heph (G) antibody labeling. Number of GSCs analyzed is above bars. ***p* < 0.001; **p* < 0.05; Student's two-tailed t-test.

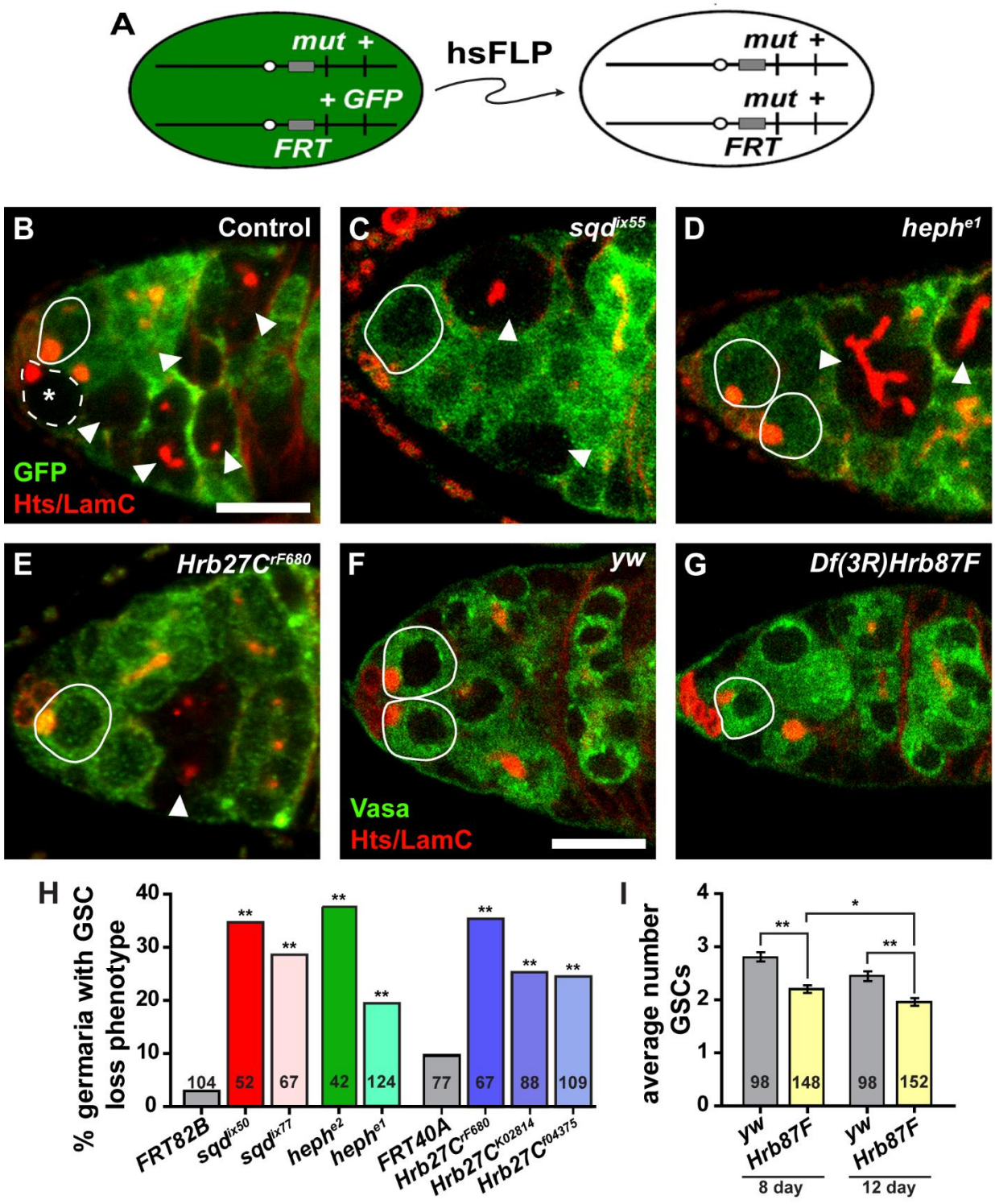

Fig. 4. HnRNPs are required for GSC self-renewal. (A) The *FLP/FRT* technique was used to generate genetic mosaics. Mitotic recombination is mediated by heat-shock-induced expression of *flippase* (*hsFLP*). Homozygous mutant (mut) cells are identified by the absence of a GFP marker, which is linked to the wild-type allele. (B-E) Representative control (B), sqd^{ix50} (C), $heph^{e1}$ (D), or $Hrb27C^{rF680}$ (E) mutant mosaic germaria labeled with anti-GFP (green;

wild-type cells), anti-Hts (red; fusomes and follicle cells), and anti-LamC (red; nuclear envelope of cap cells). GSCs are outlined in white (wild-type = solid line; mutant = dotted line). Arrowheads indicate GFP-negative cysts. (F-G) Sibling control (F) or *Df(3L)Hrb87F* mutant germaria labeled with anti-Vasa (green; germ cells), anti-Hts (red), and anti-LamC (red). GSCs are outlined. Scale bars = 5 μm. (H) Percentage of germline-mosaic germaria with a GSC loss event 8 days after clone induction. Numbers in bars represent the number of germline-mosaic germaria analyzed. $^{**}p < 0.001$; Chi-squared test. (I) Average number of GSCs per germarium at 8 and 12 days after eclosion. $^{**}p < 0.001$; $^{*}p < 0.05$; Student's two-tailed t-test. Error bars, mean ± SEM. Numbers in bars represent number of germaria analyzed.

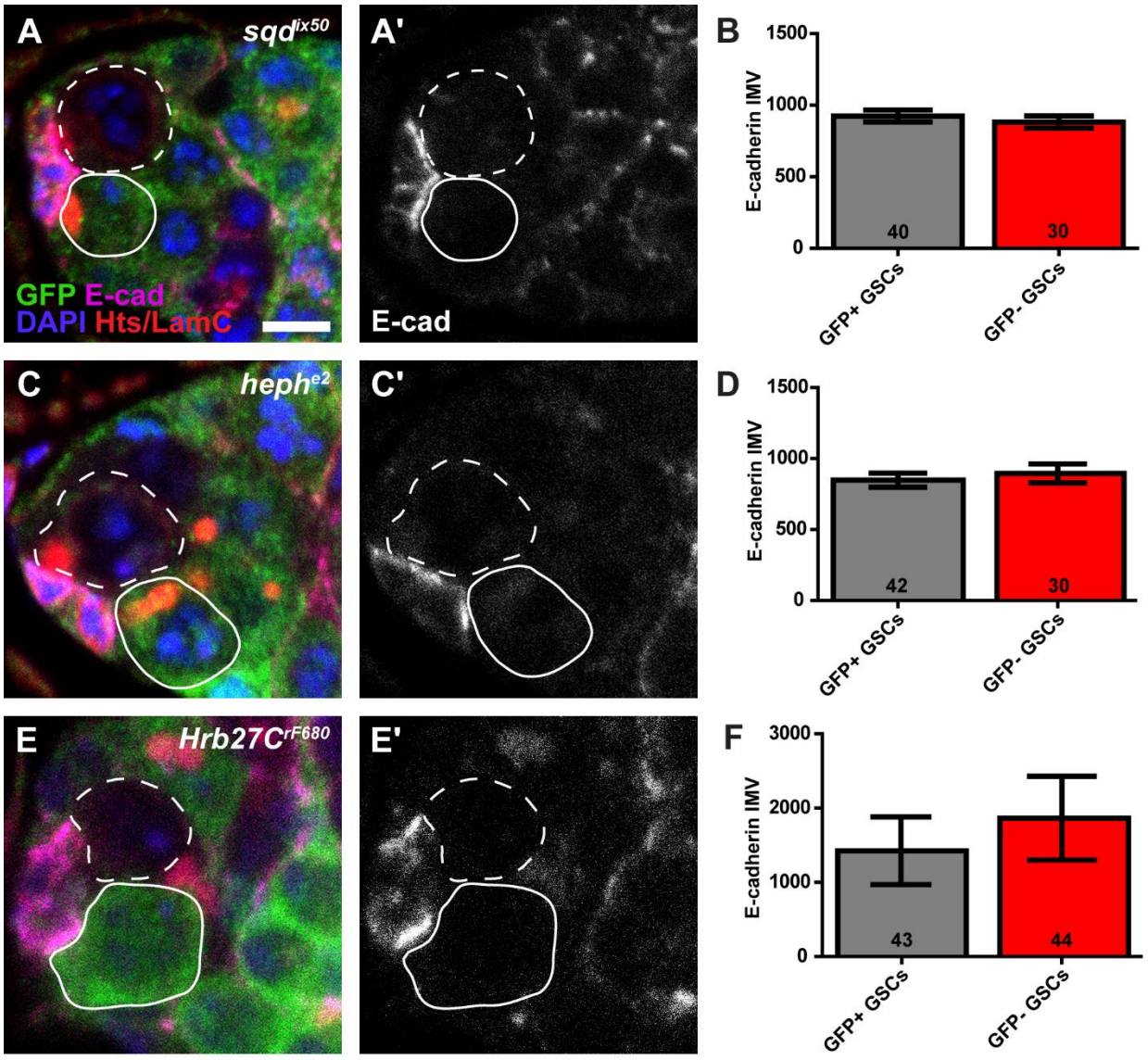

Fig. 5. Loss of *Hrb27C*, *sqd*, or *heph* does not abrogate E-cadherin levels at the GSC/Cap cell interface. (A, C, E) *sqd*^{ix50} (A), *heph*^{e2} (C), and *Hrb27C*^{rF680} (E) mosaic germaria labeled with anti-GFP (green, wild-type cells), anti-E-cadherin (magenta), anti-Hts (red; fusomes, and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA). Greyscale images of E-cadherin alone are shown in A', C', and E'. GSCs are outlined in white (wild-type = solid line; mutant = dotted line). (B, D, F). Fluorescence intensity mean value (IMV) of E-cadherin antibody labeling in adjacent control (wild-type; GFP+) and mutant (GFP-) GSCs. Error bars, mean ± SEM. Number of germaria analyzed within bars. Scale bar = 5 μm.

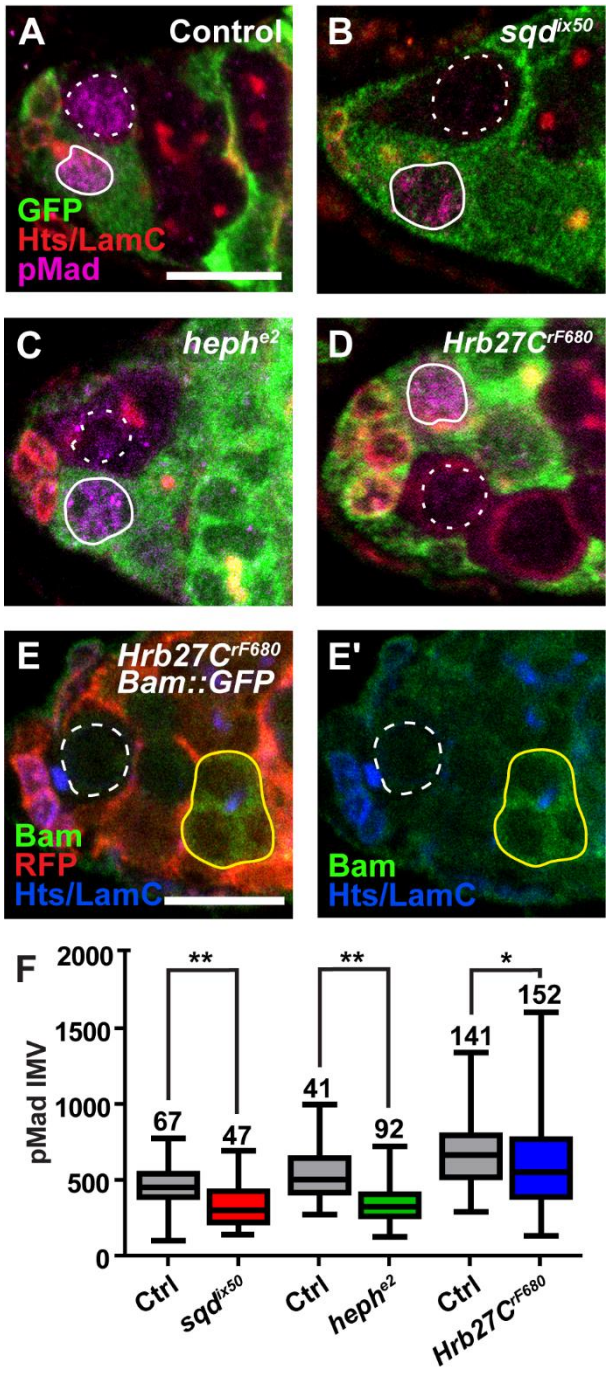

Fig. 6. HnRNPs are necessary in GSCs to receive BMP signaling. (A-D) Control (A), *sqd*^{ix50} (B), *heph*^{e2} (C), and *Hrb27C*^{rF680} (D) mosaic germaria labeled with anti-GFP (green, wild-type cells), anti-pMad (magenta), anti-Hts (red; fusomes, and follicle cell membranes), and anti-LamC (red; nuclear envelope of cap cells). (E) *Hrb27C*^{rF680} mosaic germarium labeled with anti-RFP (red; wild-type cells), anti-Bam (green), anti-Hts (blue), and anti-LamC (blue). (Red channel removed in E' for clarity.) GSC nuclei are outlined in white (wild-type = solid line; mutant = dotted line); cysts are outlined in yellow. Scale bars = 5 μm. (F) Fluorescence intensity mean value (IMV) of anti-pMAD antibody labeling in control and mutant GSCs. $^{**}p < 0.001$; $^{*}p$

< 0.05; Student's two-tailed t-test. Error bars, mean ± SEM. Number of germaria analyzed above bars.

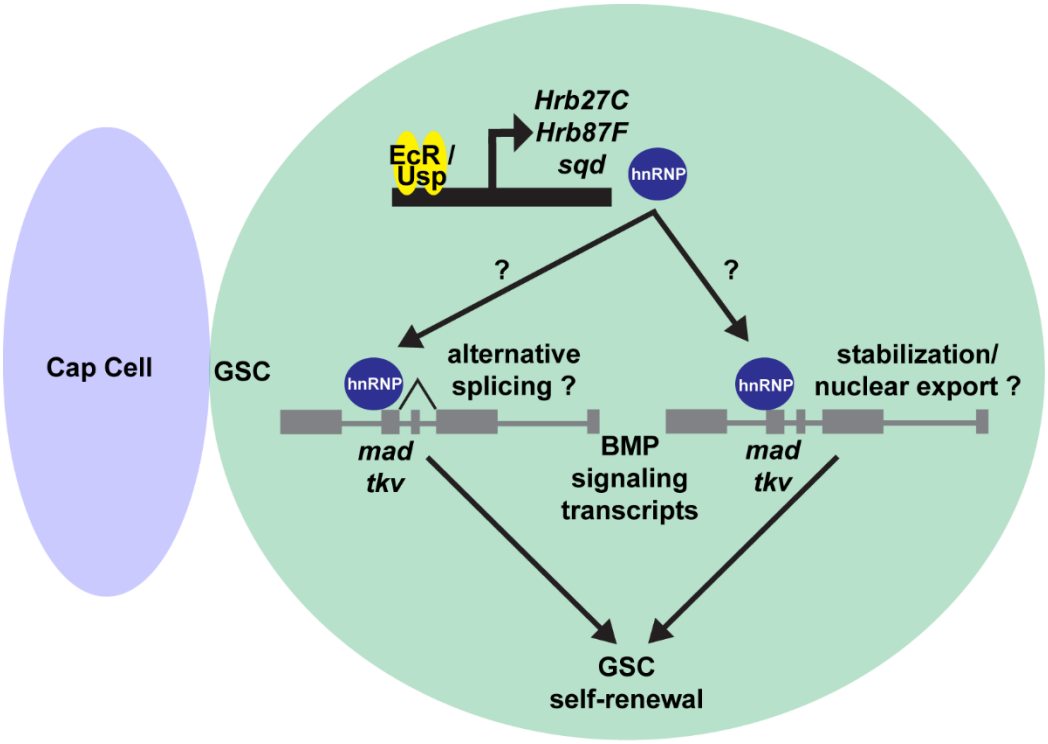

Fig. 7. Model for ecdysone regulation of *Drosophila* ovarian GSC self-renewal. Ecdysone signaling via EcR/Usp transcriptionally regulates expression of specific hnRNPs in GSCs (green). Once translated, these hnRNPs post-transcriptionally regulate splicing, stabilization, or nuclear transport of nascent mRNAs encoding components of the BMP signaling pathway, such as *Mad* or *tkv*, promoting GSC self-renewal.

SUPPLEMENTAL INFORMATION

INVENTORY OF SUPPLEMENTAL INFORMATION:

SUPPLEMENTAL DATA: consists of six figures and one table

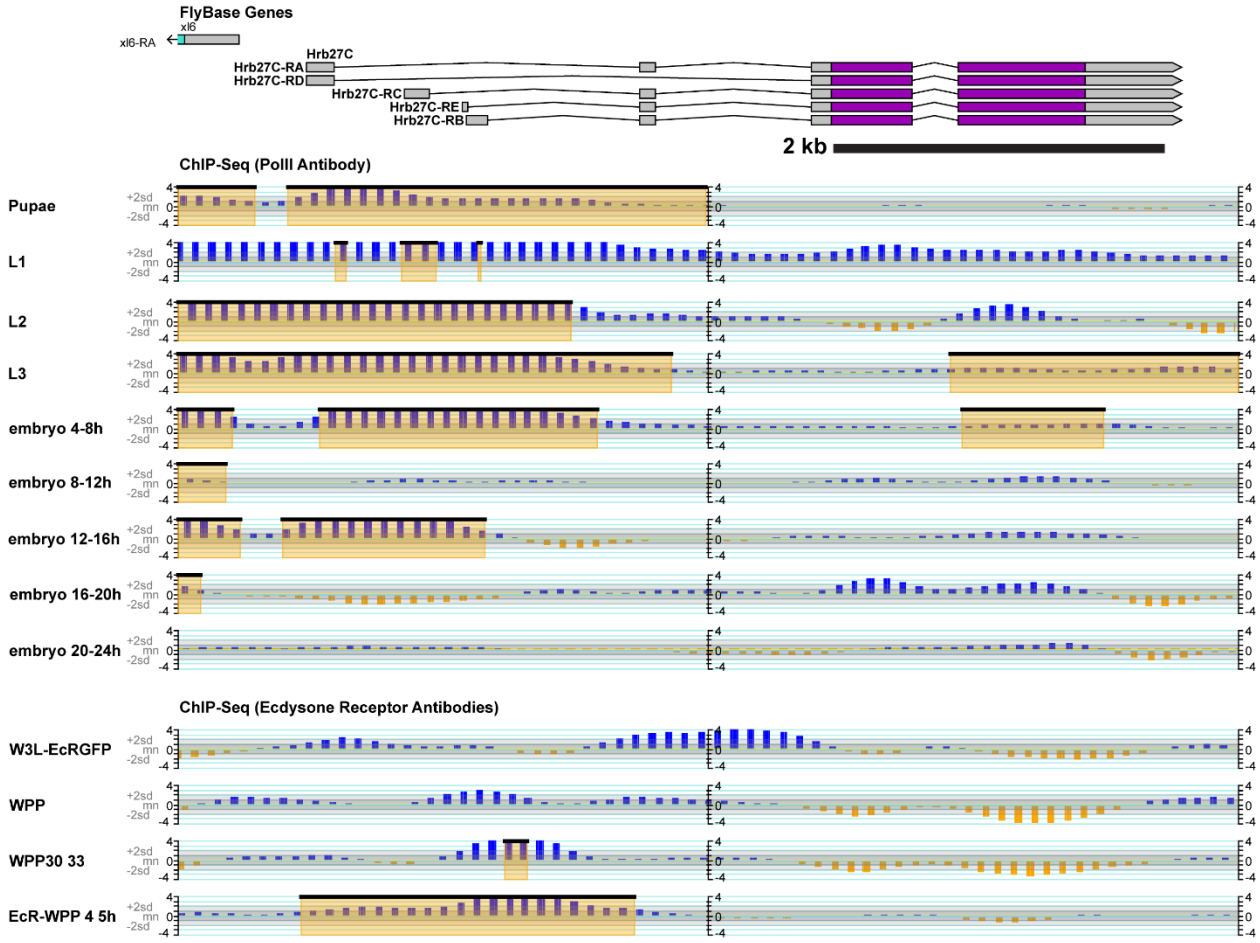

Fig. S1. Screenshot of ModENCODE chromatin immunoprecipitation data for anti-Pol II and anti-EcR at *Hrb27C* gene locus in a variety of developmental stages.

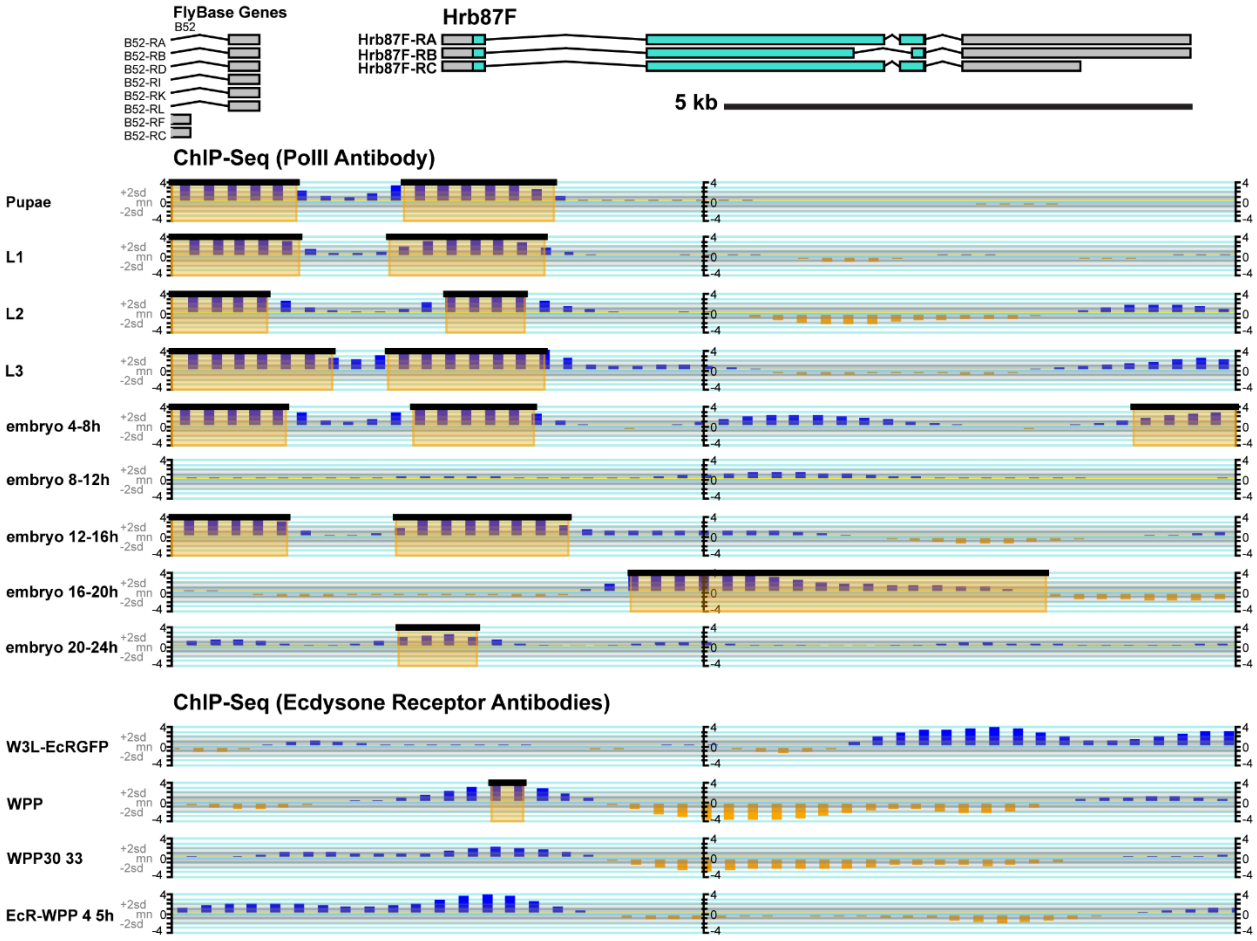

Fig. S2. Screenshot of ModENCODE chromatin immunoprecipitation data for anti-Pol II and anti-EcR at *Hrb87F* gene locus in a variety of developmental stages.

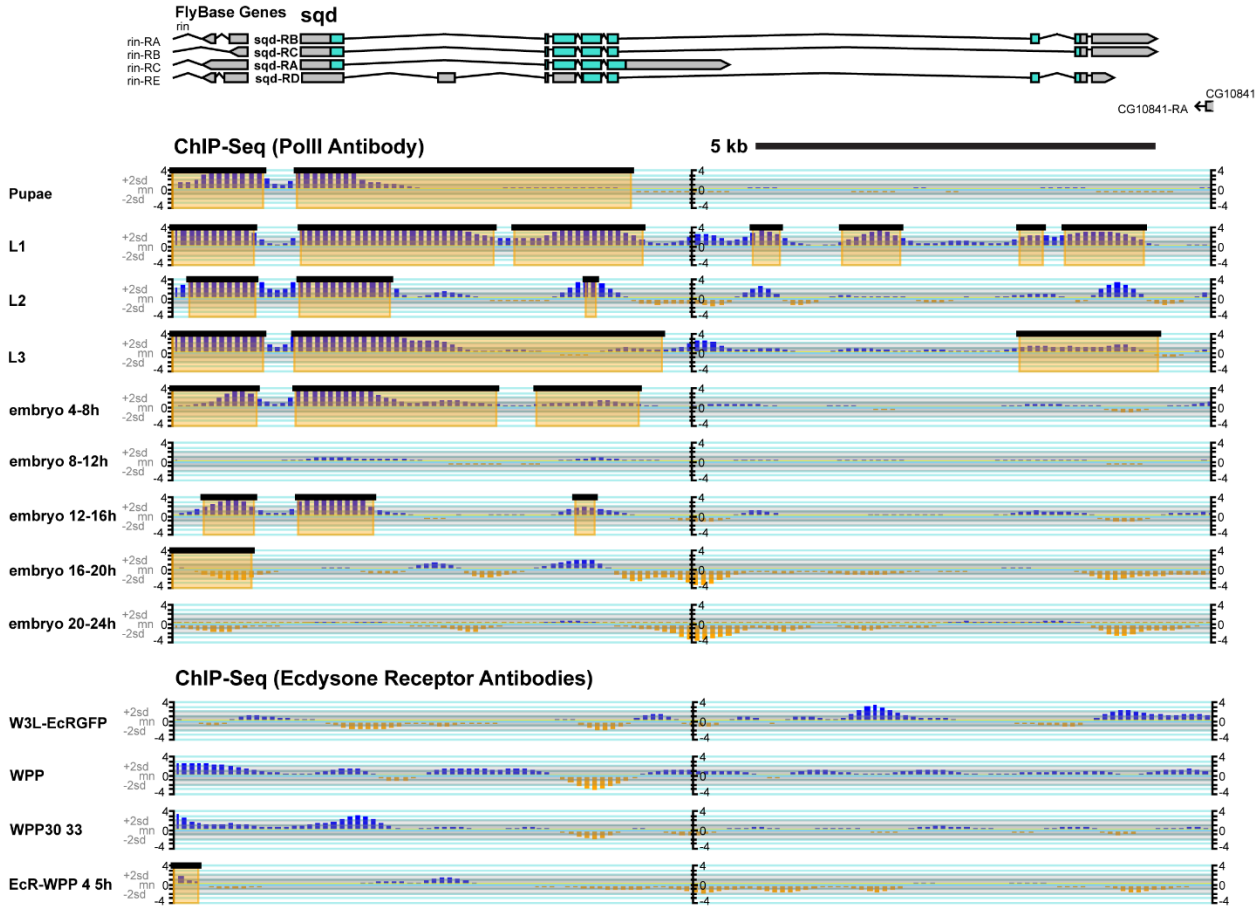

Fig. S3. Screenshot of ModENCODE chromatin immunoprecipitation data for anti-Pol II and anti-EcR at *sqd* gene locus in a variety of developmental stages.

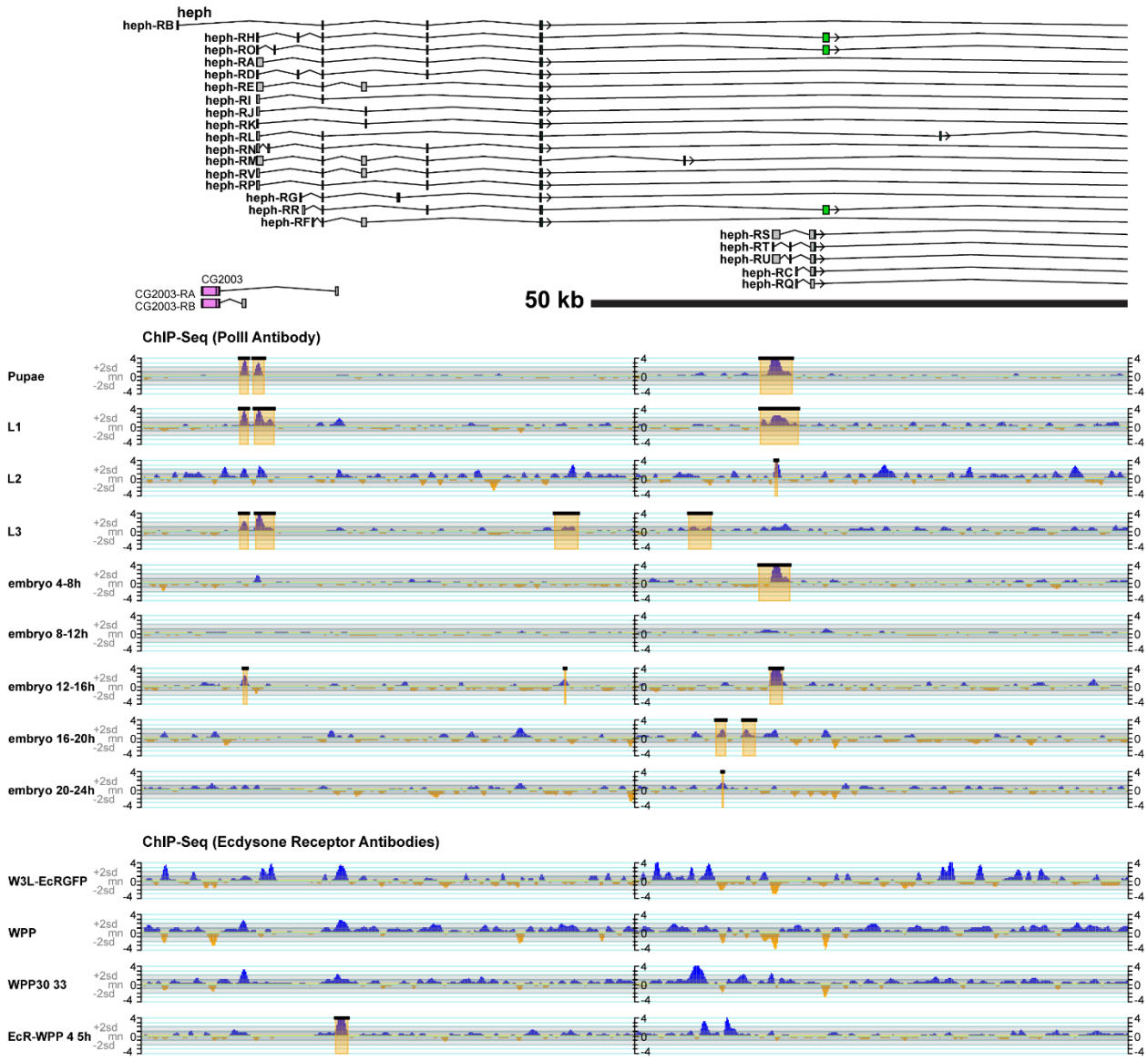

Fig. S4. Screenshot of ModENCODE chromatin immunoprecipitation data for anti-Pol II and anti-EcR at 5' end of *heph* gene locus in a variety of developmental stages.

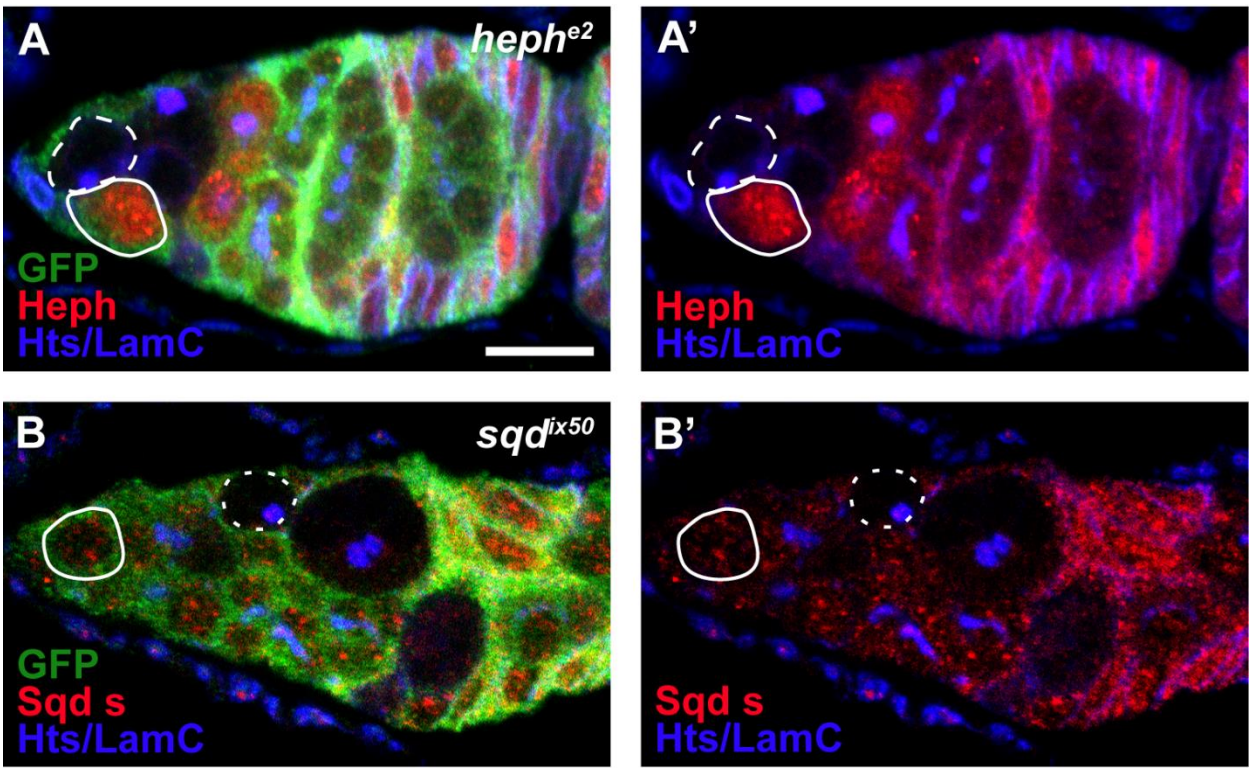

Fig. S5. Anti-Heph and anti-Sqd antibodies are specific. (A) *heph*^{e1} mosaic germaria stained with anti-GFP (green; wild-type cells), anti-Heph (red), anti-Hts (blue; fusomes, and follicle cell membranes), anti-LamC (blue; nuclear envelope of cap cells). (B) *sqd*^{ix50} mosaic germaria stained with anti-GFP (green), anti-Sqd (red), anti-Hts (blue), anti-LamC (blue). (Green channel removed in A' and B' for clarity.) GSCs are outlined in white (wild-type = solid line; mutant = dotted line). Scale bar = 5 μm.

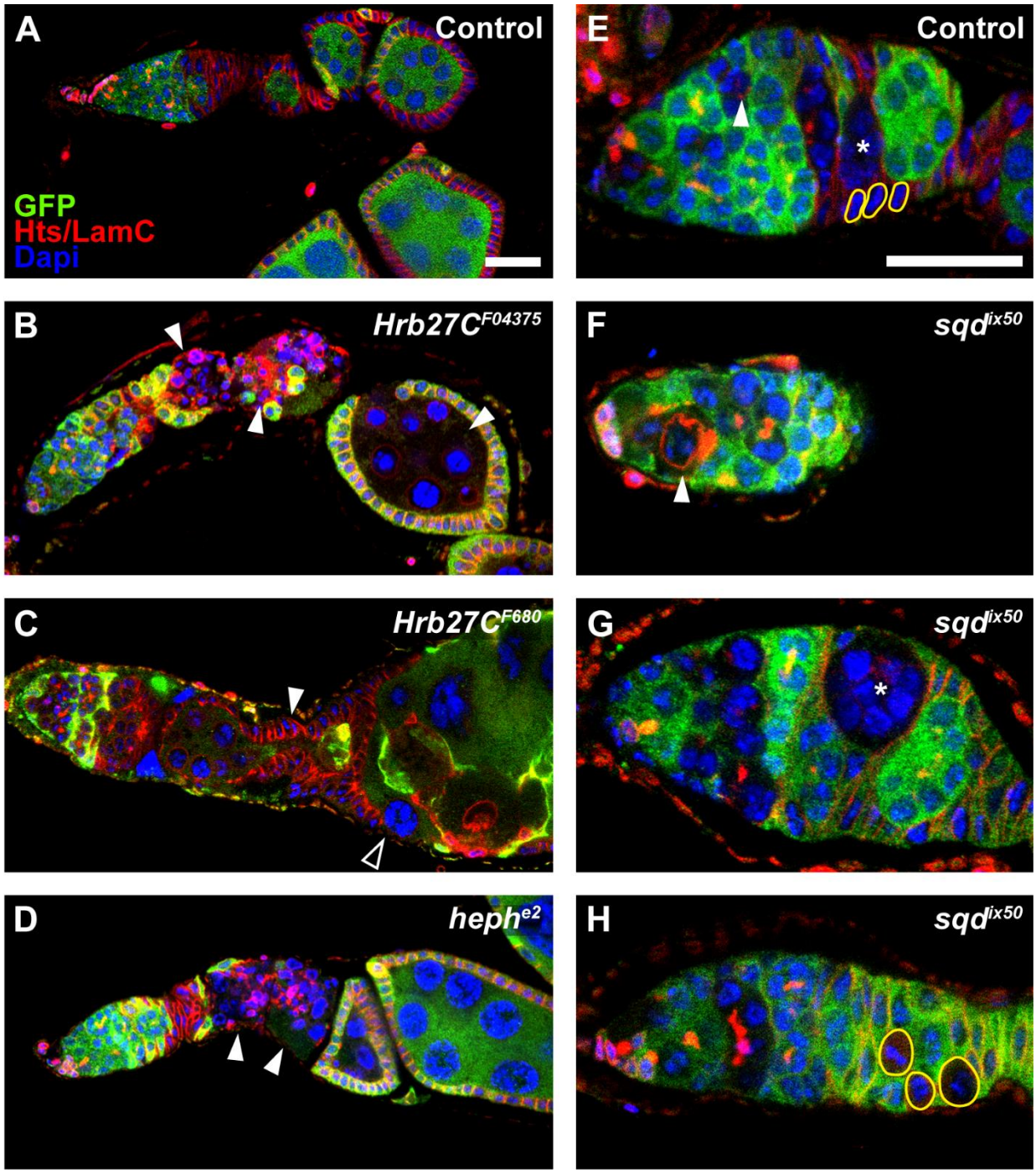

Fig. S6. *Hrb27C, heph*, and *sqd* mutants have additional somatic or early germline defects. (A-D) Ovarioles labeled with anti-GFP (green; wild type cell), anti-Hts (red; fusomes, and follicle cell membranes), and anti-LamC (red; nuclear envelope of cap cells). (A) Control ovariole. (B) *Hrb27C*^{F04375} mutant follicle cells lead to cyst death (arrowheads). (C) *Hrb27C*^{rF680} mutants have enlarged germaria. Mutant follicle cells fail to pinch off cyst (filled in arrowhead) and do not form a complete monolayer around cyst (outline arrowhead). (D) *heph*^{e2} mutant follicle cells lead to cyst death (arrowheads). (E) Control germarium (arrowhead indicates

nuclear envelope; asterisk indicates lens-shaped 16-cell cyst; follicle cells demarcated by yellow circle). (F-H) *sqd*^{ix50} mutant germline and somatic cell defects include: irregular, enlarged nuclear membranes (arrowhead), circular cyst that lack a branching fusome (asterisk) and enlarged, circular follicle cells (yellow line). Scale bars = 20 μm.

Table S1. Primer pairs used for quantitative RT-PCR.

hnRNP	Forward	Reverse
Hrb27C	ACATGCCACCTAACTCTGCC	TTGAGCACGCGAGTACATGT
Glo	AACGCAGACGTGCAATTTAAC	CGTTGTACTGGTCATCCTCATC
heph	ACCGCCCATAGCGACTACA	TTGAGCTGTTTGCATTGTTGC
Hrb57A	ATGAACTTTGACCGCGTATATGC	CTCCGTTACGATTGTTTCCCC
Hrb98de	CCCCACAAGATCGACGGTC	GGCATTCGGGGAATCAATGT
NonA	GCCCAGAATCAAAACCAGAACC	CGAACCCACCCTTGTTGTTTC
Pep	GCAACCGCAATTTTGGAGGT	TTCCCAAGGCGACATGTTCA
rump	GGACGCTAGTAACTCGGTGG	CTTCAGATCCTGCCAACGGT
sm	TGGTGCAAATGGGAGACGC	AAGCGATCTGTATCTTGCCAC
syp	CTCTCTAGCCAAACCCCC	ACGAGCACGCAGAATCTCC
sqd	CGCAAAGGATTCTGCTTCATCA	CACGCTTAACATCGACCTCC
Hrb87f	CACGTACTCCCAGTCGTACAT	GCAGGCACTCTTCATCGTGA
RP49	TGTGCCAAATTGTACCCG TG	GCTTGTTCGATCCGTAACCG