RESEARCH ARTICLE

Genetic Dissection of a Stem Cell Niche: The Case of the *Drosophila* Ovary

J. Bolívar,[2†] J. Pearson,[1†] L. López-Onieva,[1†] and A. González-Reyes[1*]

In this work, we demonstrate a powerful new tool for the manipulation of the stromal component of a well-established *Drosophila* stem cell niche. We have generated a *bric-a-brac 1* (*bab1*)-Gal4 line that drives UAS expression in many somatic ovary cell types from early larval stages. Using this Gal4 line, we could effectively induce FLP/FRT-mediated recombination in the stromal cells of the ovarian germline stem cell niche. Mutant clones were observed in the developing ovary of larvae and pupae, including in somatic cell types that do not divide in the adult, such as the cap cells and the terminal filament cells. Exploiting the ability of *bab1*-Gal4 to generate large clones, we demonstrate that *bab1*-Gal4 is an effective tool for analyzing stem cell niche morphogenesis and cyst formation in the germarium. We have identified a novel requirement for *engrailed* in the correct organization of the terminal filaments. We also demonstrate an involvement for integrins in cyst formation and follicle cell encapsulation. Finally using *bab1*-Gal4 in conjunction with the Gal80 system, we show that while ectopic *dpp* expression from stromal cells is sufficient to induce hyperplastic stem cell growth, neither activation nor inactivation of the BMP pathway within stromal cells affects germline stem cell maintenance. *Developmental Dynamics 235:2969–2979, 2006.* © 2006 Wiley-Liss, Inc.

Key words: Drosophila oogenesis; ovarian stem cell niche; genetic analysis

Accepted 25 August 2006

INTRODUCTION

The self-renewal of populations of stem cells is critical for animal development, growth, tissue homeostasis, damage repair, and, in the case of germline stem cells (GSCs), reproduction. Both males and females of the fruit fly *Drosophila melanogaster* retain populations of GSCs during most of their adult life that are essential for their fertility. Studies in *Drosophila* have been decisive in the establishment of the conserved "niche model" of stem cell self-renewal, whereby interactions between stem cells and surrounding stromal cells create a unique micro-environment or niche that is permissive for the maintenance of stem cells in a non-differentiated state (Fuchs et al., 2004; Wong et al., 2005).

The ovarian germline stem cell niche is usually occupied by 2–3 GSCs and is located near the anterior tip of each germarium, the region where egg chambers originate (Fig. 1A). GSCs can be readily identified by the presence of an apical spherical spectrin-rich structure known as the spectrosome, and their position in direct contact with somatic cap cells (CpCs; Fig. 1B). CpCs, located between GSCs and the anterior terminal filament (TF), accumulate high levels of the *Drosophila* homologue of β-catenin (Song et al., 2002) (Fig. 1C). The TF is composed of a stack of terminal filament cells (TFCs) that strongly express *engrailed* (Forbes et al., 1996b) (Fig. 1D). Germaria contain several other somatic cell types (Spradling, 1993), including inner germarial sheath (IGS) cells, follicle cells (FCs), and the stem cells from which follicle cells are derived. The latter cell type has previously been referred to as so-

The Supplementary Material referred to in this article can be viewed at www.interscience.wiley.com/jpages/1058-8388/suppmat

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Grant sponsor: Spanish Ministerio de Ciencia y Tecnología; Grant number: BMC2003-01512; Grant sponsor: Junta de Andalucía; Grant number: CVI-280.

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DOI 10.1002/dvdy.20967
Published online 29 September 2006 in Wiley InterScience (www.interscience.wiley.com).

matic stem cells or SSCs. However, the recent identification of novel classes of IGS cells, escort cells (ECs), and escort stem cells (ESCs) (Decotto and Spradling, 2005), means that there are at least two types of somatic stem cells in the germarium. Therefore, in this report we will refer to the cells that give rise to follicle cells as follicle stem cells (FSCs).

The egg chamber is composed of 16 germline cells, 1 posterior oocyte and 15 nurse cells, which are surrounded by somatic follicle cells. The 16-cell cyst forms from a single cystoblast, which divides four times with incomplete cytokinesis in the germarium. CBs result from asymmetrical GSC divisions, which are aligned such that only the daughter cell destined to remain a GSC retains contact with anterior CpCs, while the CB differentiates (Deng and Lin, 1997; de Cuevas and Spradling, 1998). CBs are never in contact with CpCs and their spectrosomes usually lose apical localization. As CBs undergo each of four rounds of mitosis to form mitotic cysts, the CB spectrosome (now called a fusome) becomes branched, interconnecting each cystocyte.

Maintenance of GSCs in the adult ovarian niche is mediated in large part through continued activation of the BMP pathway (reviewed in Wong et al., 2005). BMP ligands are detected via the combined action of a type I receptor, encoded by *thickveins* (*tkv*) or *saxophone*, and a type II receptor, encoded by *punt* (Raftery and Sutherland, 1999). Upon ligand binding, these receptors act to phosphorylate Mad, a Smad transcription factor, which in conjunction with other proteins acts to regulate the expression of target genes. In the adult GSC niche, only the 2–3 anterior germ cells in contact with CpCs possess high levels of BMP pathway activity (phosphorylated Mad protein) (Kai and Spradling, 2003). BMP ligands Dpp (encoded by *decapentaplegic*) and Gbb (encoded by *glass-bottomed boat*) are required for GSC maintenance in both *Drosophila* ovaries and testis (Wong et al., 2005). Mutations in either *dpp* or *gbb* result in a failure to maintain GSCs, whereas the loss of *dpp* from the germline has no effect (Irish and Gelbart, 1987). Germline mutant clones of downstream *dpp*-pathway components, such as *tkv* or *Mad*, result in the rapid loss of GSCs (Wong et al., 2005). The main target of the BMP signaling pathway appears to be the repression of *bag-of-marbles* (*bam*) (Chen and McKearin, 2003, 2005; Casanueva and Ferguson, 2004; Song et al., 2004; Szakmary et al., 2005), whose expression is sufficient to induce GSC differentiation (Ohlstein and McKearin, 1997). Ectopic expression of *dpp* results in the formation of large GSC-like tumors, suggesting that *dpp* is both necessary and sufficient to prevent GSC differentiation (Xie and Spradling, 1998). It is still uncertain which somatic cells in the GSC niche are responsible for the expression of niche signals such as *dpp* and *gbb*, although CpCs are likely candidates given the importance of their association with GSCs for germline maintenance (Song et al., 2002), their expression of *dpp* mRNA (Xie and Spradling, 2000), and the close correlation between CpC and GSC numbers as germaria age (Xie and Spradling, 2000). TFCs express several genes required for stem cell maintenance including *piwi* and *Yb* (Wong et al., 2005). ESCs are closely associated with both GSCs and CpCs (Decotto and Spradling, 2005), and are important for niche function. Thus, the GSC niche appears to be composed of several types of anterior somatic cells, which interact via multiple signals with the GSCs to form a self-renewal permitting environment.

In this work, we have generated a Gal4 line, based on an insertion within the *bric-a-brac 1* locus that is expressed in several ovarian somatic cell types such as CpCs and TFCs. Many of these cells do not divide in the adult, making their genetic manipulation more difficult. We demonstrate that *bab1*-Gal4 represents a valuable new tool for dissecting several aspects of somatic-germline interactions in the germarium, including niche morphogenesis, cyst formation, and niche signaling.

RESULTS

bab1-Gal4 as a Tool for the Genetic Manipulation of Somatic Cell Types Present in the Germarium

Our ability to genetically manipulate the ovarian stem cell niche would be greatly enhanced by the possession of a Gal4 driver that could direct gene expression specifically within niche stromal cells during ovary morphogenesis. The *bric-a-brac 1* gene is essential for the organization of the terminal filament and correct ovary formation (Godt and Laski, 1995; Sahut-Barnola et al., 1995). Using P-element replacement (reviewed in Gloor, 2004), we were able to convert the $bab1^{P}$ enhancer trap line to another encoding the Gal4 transcription factor under the control of a minimal promoter, using a similar strategy as described by Laski and co-workers (Cabrera et al., 2002). The effectiveness of *bab1*-Gal4 for the genetic manipulation of the niche depends greatly upon the timing and specificity of its expression during development. Using a UAS-nod:GFP reporter, we detected *bab1*-Gal4 driven GFP expression in most somatic cells of the ovaries of 3rd instar larvae (Fig. 2A). Examination of pupal ovaries found that very strong expression persisted in TFCs but it could also be detected in the somatic basal stalk and the associated interstitial cells (Fig. 2B). In adult *bab1*-Gal4 ovaries, we observed strong UAS-nod:GFP expression in TFCs and CpCs (Fig. 2C). We also detected GFP expression in anterior IGS cells, which on the basis of their localization and morphology, represent ESCs and ECs (Fig. 2C; see Supplemental Figure 1, which can be viewed at www.interscience.wiley.com/jpages/1058-8388/suppmat). Thus, *bab1*-Gal4 is strongly expressed in most, if not all, somatic cells during ovary morphogenesis and continues to be expressed in the somatic cell types believed to form the GSC niche in the adult.

Many of the signaling pathways so far reported to be involved in the maintenance of stem cells have multiple, essential roles in early development (Wong et al., 2005). Thus, in order to study their contribution to the niche, it is necessary to generate loss-of function and gain-of-function mosaic clones through mitotic recombination. Recombination is often mediated by the ectopic expression of the enzyme Flp recombinase, which catalyses recombination between FRT elements arranged in *cis* or *trans* chromosomal configurations (Blair, 2003).

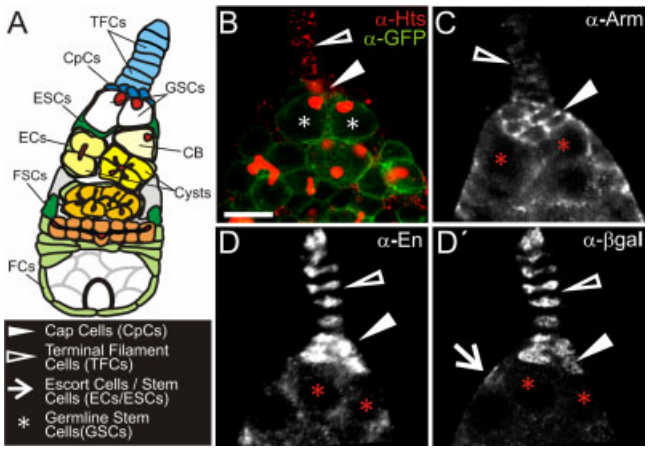

Fig. 1. Cell types present in the germarium and markers for the niche components. **A:** Schematic diagram of a *Drosophila* germarium. TFCs, CpCs, ESCs, ECs, Follicle Stem Cells (FSCs), and Follicle Cells (FCs) are of somatic origin. GSCs can be characterized by the presence of a spectrosome (red dot) and for being in close contact with the CpCs. The cystoblast (CB) undergoes four incomplete rounds of division giving rise to cysts of two, four, eight, and sixteen cells interconnected by branched fusomes (red). Sixteen-cell cysts stretch perpendicular to the anterior-posterior axis of the germarium. **B:** *nanos*-Gal4, UAS-*Src*:GFP germarium double stained with anti-Hts (red) and anti-GFP (green) to visualize the spectrosome and fusomes and germline cells, respectively. **C:** Wild type germarium stained with anti-Armadillo to label CpCs. **D, D':** *bab1*^{P} germarium double stained to visualize Engrailed (D) and βgal (D'). Scale bar = 10 μm. Anterior is up in all figures. Open arrowheads, TFCs; arrowheads, CpCs; arrow, ECs; asterisks, GSCs.

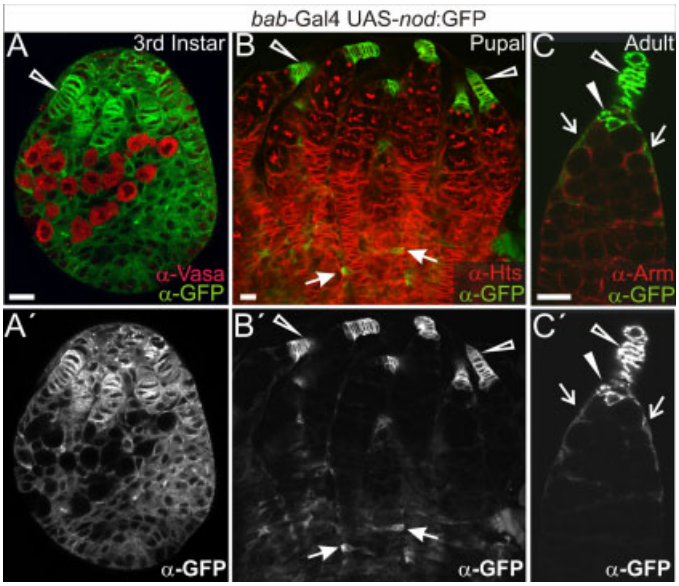

Fig. 2. *bab1*-Gal4 expression in larval and pupal gonads, and in adult ovaries. *bab1*-Gal4 UAS-*nod*:GFP gonads and ovaries were dissected to visualize *bab1*-Gal4-driven GFP. **A:** Third instar larval gonad stained with anti-Vasa and anti-GFP to visualize germline cells (red) and the expression of *nod*:GFP in most somatic cells (green). **B:** Pupal gonad stained with anti-Hts and anti-GFP. Nod:GFP was expressed predominantly in TFCs and CpCs. Weaker staining was also observed in basal stalk and interstitial cells (white arrows). **C:** Adult germarium stained with anti-Armadillo (red) and anti–GFP (green). *bab1*-Gal4 directs GFP expression in TFCs, CpCs, ESCs, and ECs. **A'–C':** Green channel in A, B, and C, respectively. Scale bar = 10 μm. Open arrowheads: TFCs; arrowheads: CpCs; arrow: ECs; asterisks: GSCs.

The Flp recombinase is often delivered via heat-shock responsive Flp transgenes. This method has been used extensively in the germarium, especially for cells that continue to divide in the adult female, such as GSCs and follicle cells. In contrast, the generation of clones by mitotic recombination has rarely been used for the study of genes expressed in the germarial niche. Although it is possible to generate somatic clones of terminal filament cells and other somatic cells using heat shock delivery (Godt and Laski, 1995), it is technically problematic. Heat shock must be applied during ovary morphogenesis in the late larval or early pupal stages. The frequency, cell type, and location of clones within the ovary vary greatly depending upon the exact timing of the heat shock (Godt and Laski, 1995). Moreover, because the somatic cells that make up each ovariole are not clonally derived (Godt and Laski, 1995), it is difficult to obtain large, contiguous clones. Also, the ubiquitous nature of heat-shock induction means that recombination can occur in both somatic and germline tissues. As *bab1*-Gal4 is expressed in the somatic cells of larval and pupal ovaries (Fig. 2A,B), we thought it might provide an easier and more effective method for generating loss-of-function and gain-of-function clones of ovarian stromal cells.

First, we examined the effectiveness of *bab1*-Gal4 for driving *cis*-recombination. Recombination between FRT elements arranged in a *cis*-configuration on a single chromosome allows the expression of gain-of-function or dominant-negative transgenes by generating so-called "Flp-out" clones (Blair, 2003). To this end, we used *bab1*-Gal4 to drive Flp expression in females that also possess a transgenic "cassette" containing two FRTs flanking a stop cassette upstream of a lacZ reporter gene. Females containing *bab1*-Gal4, UASt-Flp and "*actin*-FRT>stop>FRT-nls-lacZ" transgenes showed widespread formation of lacZ-expressing clones in the somatic cells of germaria, but not in the germline (Fig. 3A; Table 1). We found that the vast majority of germaria contained large follicle cell clones (95.5%, n = 45). Examination of larval ovaries revealed large numbers of lacZ-expressing clones in the somatic cells of mid 3rd instar larval ovaries (Fig. 3B), consistent with the expression pattern of reporter gene insertions in the *bab1* locus (Sahut-Barnola et al., 1995; Cabrera et al., 2002; this work). Our observations suggest that *bab1*-Gal4 can effectively induce *cis*-recombination throughout the somatic complement of the germarium.

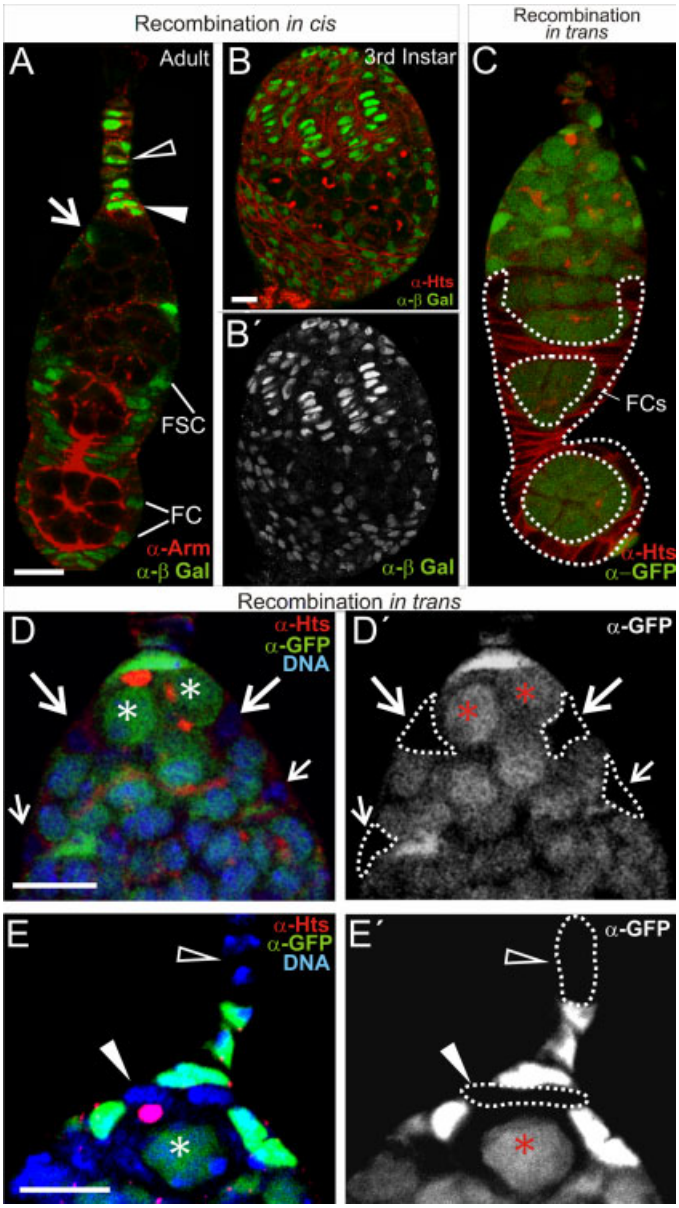

Fig. 3. *bab1*-Gal4-driven Flipase induces recombination in all somatic cell types of the germarium. **A,B:** FLP-mediated recombination in *cis*. Recombinant cells express βgal. Adult germaria (A) and larval gonads (B) were stained with anti-βGal to label recombinant cells (green). Germline cells were visualized with anti-Armadillo (A) or anti-Hts (B) (red). *bab1*-Gal4-induced *cis*-recombination was highly effective as most somatic cells expressed βGal. **C–E:** FLP-mediated recombination in *trans*. In this case, recombinant cells are labeled by the absence of βgal. **C:** Germarium stained with anti-GFP (geen) and anti-Hts (red) to show *trans*-recombination in FSCs and FCs. **D,E:** Germaria stained with anti-GFP (green), anti-Hts (red), and the DNA dye TO-PRO-3 (blue) to document *trans*-recombination in CpCs (arrowheads), TFCs (open arrowheads), and IGSs, which probably represent ESCs (larger arrows) and ECs (smaller arrows). **A',D',E':** Green channel in A, D, and E, respectively.

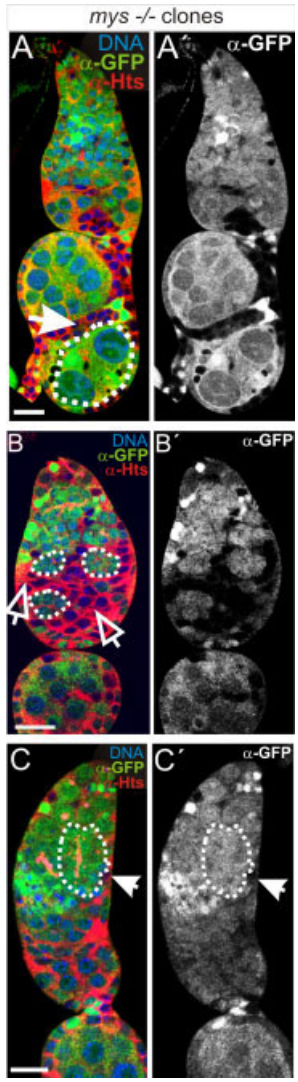

Fig. 4. *myospheriod* is required for egg chamber formation and niche integrity. **A–C:** Germaria were stained to detect GFP (green), Hts (red), and DNA (blue). Lack of *mys* function in somatic cells in the germarium led to an absence of interfollicular stalks between egg chambers (large arrow), cysts containing less than 16 cells (dotted lines in A, B), failed follicle cell migration (empty arrow), and led to the abnormal orientation of cysts. C: The dotted line delimits a wild type 16-cell cyst associated with a mutant somatic cells (small arrow) migrating parallel to the anterior-posterior axis of the germarium.

Next, we examined the effectiveness of *bab1*-Gal4 driven Flp expression to mediate *trans*-recombination between FRT chromosomes. *Trans*-recombination is usually tracked by the loss of a marker, typically GFP, present only on the wildtype chromosome (Blair, 2003). First, we examined recombination between FRT insertions on the first chromosome. We found GFP-negative clones in the somatic cells of over half of the germaria analyzed (56.3%, n = 53) (Fig. 3C–E). As with *cis*-recombination, we observed clones in all the somatic cell types of the germaria. Importantly, unlike *cis*-recombination, *bab1*-induced *trans*-recombination requires cell division to form mutant clones (data not shown). As TF and CpC structures are fully formed by pre-pupal stages and do not undergo cell division in the adult (King, 1970; Godt and Laski, 1995), it is likely that *bab1*-Gal4-induced clone formation in these cell types principally occurred during larval stages. We found that the frequency of clone formation was high, albeit signifi-

TABLE 1. Induction of *cis*-Recombination Using the *bab1*-Gal4 UAS-Flp System in Germaria[a]

Germaria analyzed	Germaria with visible somatic cell clones	Follicle cell clones[b]	Cap cell clones[b]	Terminal filament cell clones[b]	Inner germarial sheath cell clones[b]
45 (100%)	43 (96.5%)	43 (95.5%)[c]	18 (40%) 2.1 cells/clone	31 (68.9%) 3.9 cells/clone	24 (53.3%) 13.2 cells/clone

[a]Summary of the frequency of *bab1*-Gal4-directed recombination within the same chromosome (*cis*-recombination) in females containing *bab1*-Gal4, UAS-Flp, and *actin*-FRT>stop>FRT-nls-LacZ transgenes. [Removal of the termination sequence by recombination between FRT sites] should result in clones that constitutively express lacZ (see text for details). Prior to dissection, adult females of the above phenotype were raised at 25°C and aged for 4–5 days after eclosion. Follicle cell clones were scored in germarial cysts. LacZ-expressing clones were identified by staining the dissected ovaries with anti-β Gal and anti-Hts antibodies. The average number of cells per clone is indicated for several of the cell types.
[b]Frequency of germaria containing at least one visible GFP-positive clone.
[c]Most follicle cell clones had at least 10 cells in each clone.

TABLE 2. Induction of *trans*-Recombination Using the *bab1*-Gal4 UAS-Flp System in Germaria[a]

Adult female genotype	Germaria analyzed	Germaria with visible somatic cell clones	Follicle cell clones[b]	Cup cell clones[b]	Terminal filament cell clones[b]	Inner germarial sheath cell clones[b]
mys^{XG43} FRT101/*Ubi*-nlsGFP FRT101; *bab1*-Gal4 UAS-Flp/+	53 (100%)	30 (56.6%)	19 (35.8%)[c]	15 (28.3%) 2.1 cells/clone	8 (15.1%) n.d. n.d.	23 (43.4%) 9.6 cells/clone
FRT42D *Df(2R) en*^{E}/ FRT42D *Ubi*-nlsGFP; *bab1*-Gal4 UAS-Flp /+	69 (100%)	69 (100%)	67 (97.1%)[c]	41 (59.4%) 1.7 cells/clone	46 (66.7%) 3.4 cells/clone	41 (59.42%) 5.1 cells/clone

[a]Two experiments tested the frequency of *bab1*-Gal4 directed recombination between homologous chromosomes (*trans*-recombination; see text for details). Prior to dissection, adult females of the above phenotype were raised at 25°C and aged for 4–5 days after eclosion. Follicle cell clones were scored in region 2a, 2b, and 3 germarial cysts, as well as in stage 2 and 3 egg chambers. Mutant follicle cells, cap cells, and terminal filament cells were identified by staining the dissected ovaries with a-GFP and a-Hts antibodies. n.d. = not determined.
[b]Frequency that scored germaria contained at least one visible GFP negative clone.
[c]Most follicle cell clones had at least 10 cells in each clone.

cantly lower than that for *cis*-recombination (Table 2). To examine whether the frequency of somatic *trans*-recombination might be affected by FRT position or genetic background, we also studied the frequency of recombination between FRT insertions on the second chromosome (Table 2). In this case, we found that 100% of germaria (n = 69) contained somatic clones resulting from recombination between the FRT sites on the second chromosome. Clones in stromal CpCs, TFCs, or IGS cells were found in over half of all germaria analyzed. In addition, virtually all germaria observed carried follicle cell clones. As in the example shown in Figure 3C, we find cases in which all FSCs have lost the GFP expression, giving rise to egg chambers containing only GFP-negative follicle cells. Thus, *bab1*-Gal4 appears an effective tool for driving *trans*-recombination in the stromal cells due to its specificity and timing of expression in the somatic component of the ovary during niche formation.

A Genetic Tool to Study Cyst Formation in the Germarium: A New Role for Integrins

Proper egg development requires a complex series of interactions between somatic and germline tissues (Dobens and Raftery, 2000; Huynh and St Johnston, 2004a). FSCs, located in the middle region of the germaria, divide to produce the follicle cells that encapsulate 16-cell germline cysts. Following encapsulation, a subpopulation of follicle cells differentiates to form the interfollicular stalks that separate each egg chamber.

We decided to test the effectiveness of *bab1*-Gal4 as a tool for studying the role of the soma in cyst encapsulation. The proper encapsulation of germline cysts by follicle cells depends on extensive cell–cell interactions and cell–cell adhesion (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998). Integrins are known to be required in the soma for aspects of follicle cell organization (Bateman et al., 2001; Devenport and Brown, 2004);

however, no role for integrins has been described during cyst formation in the germarium. *myospheriod* (*mys*) encodes the *beta* PS-integrin subunit, which is required to form most integrin complexes in *Drosophila* (Brown et al., 2000). Using the *bab1*-Gal4 tool, we found that the loss of *mys* function in follicle cells caused a frequent and dramatic disruption in egg chamber organization (Fig. 4A–C). Mutant egg chambers were often separated by two follicle layers without an interfollicular stalk and frequently contained less than 16 cells (Fig. 4A,C). This suggests the loss of integrin activity may affect the initial follicle cell migration that encapsulates each cyst. In support of this hypothesis, we observed cases where presumed germ cell clusters in regions 2b and 3 of the germarium with less than 16 cells were surrounded by mutant follicle cells (Fig. 4B). Taken together, our observations suggest that the lack of integrin function severely disrupts the ability of follicle cells to migrate normally during cyst encapsulation in region 2b of the germarium.

In wild type germaria, 16-cell cysts in region 2b become flattened into a lens shape, which is aligned perpendicular to the AP axis (Huynh and St Johnston, 2004b). We found that when such cysts were in contact with mutant somatic cells, they were often abnormally aligned along the AP axis (Fig. 4C). This suggests that somatic cells may play a role in the arrangement of germline cysts prior to region 2b, a role most likely played by Escort Cells (ECs), which migrate with germline cysts through regions 1 and 2 of the germarium (Decotto and Spradling, 2005). Alternatively, it is possible that *mys* mutant follicle cells migrate abnormally towards the anterior of the germarium and there affect cyst orientation. To test this, we generated *mys* mutant clones using the *e22c*-Gal4 line (Duffy et al., 1998), which is expressed in follicle stem cells and not in ECs. Mosaic germaria harbouring follicle cell mutant clones show encapsulation phenotypes indistinguishable from those present in *bab1*-Gal4 induced clones (our unpublished observations). In contrast, we never see 16-cell cysts aligned parallel to the AP axis of the germarium in *e22c*-Gal4 experimental ovaries. These results argue that ECs are involved in integrin-mediated cyst migration in the germarium.

Genetic Study of Terminal Filament Morphogenesis Using bab1-Gal4

The ability to efficiently generate relatively large mutant clones in the stromal niche cells opens the way to address the function of genes known to be expressed within somatic cells of the GSC niche but whose function is unknown. One such example is *engrailed* (*en*), a transcription factor that plays multiple roles during development (Hidalgo, 1996), which is strongly expressed in TFCs and CpCs (Forbes et al., 1996b) (Figs. 1D, 5A).

Using *bab1*-Gal4-mediated recombination with a strong *engrailed* deletion allele (en^E) (Gustavson et al., 1996), we were able to generate clones of cells within terminal filaments that lack *engrailed* expression. Large en^E clones in late 3rd instar larval gonads were often associated with a disorganized TF, in contrast to the precisely organized arrangement of adjacent wildtype TFC stacks (Fig. 5B,C). Terminal filaments are formed from a layer of intercalated somatic cells that separate through a process of cell margin bundling into groups of 5–6 TFCs, followed by cell flattening, alignment, and the recruitment of additional TFCs to the posterior of each stack (Godt and Laski, 1995). As the organization of adjacent TFs does not appear to be affected, and the en^E mutant cells have adopted the flattened shape typical of TFCs, it suggests that *engrailed* may be involved specifically in the alignment of TF stacks. Alternatively, the presence of adjacent wildtype TFCs may be sufficient for the mutant cells to adopt these TFC characteristics. We found that large *en* TF clones in the pupal ovaries were also associated with a failure to properly organize the TF stack (Fig. 5D). However, smaller posterior TFC clones of cells, which were recruited after the initial partitioning of terminal filament precursors (Godt and Laski, 1995), had no detectable effect on pupal TF stack organization (Fig. 5E). Taken together, our observations suggest that *engrailed* expression, at least in the 5–6 initial TF precursors, is necessary for proper organization and alignment of TF cells into their characteristic stack structure.

Genetic Dissection of Signaling Mechanisms Responsible for GSC Maintenance

One of the most important pathways known to be involved in the maintenance of GSCs is the BMP pathway. Most evidence points to a model in which the source(s) of Dpp and Gbb ligands are the anteriorly-located stromal cells, which are in close proximity with the GSCs such as CpCs, and/or ESCs (Xie and Spradling, 2000; Zhu and Xie, 2003). However, partly due to the difficulty of visualizing *dpp* expression in the somatic cells (Xie and Spradling, 2000), the above model has been difficult to prove definitively.

On the basis of the germarial expression pattern observed for reporter genes driven by *bab1*-Gal4 (see Fig. 2C), we thought that *bab1*-Gal4 might be useful for the ectopic expression of ligands in the stromal CpCs, TFCs, and IGS cells. To test this, we wanted to determine if *bab1*-Gal4-mediated expression of *dpp* could induce hyperplastic GSC growth, characterized by the presence of hundreds of spherical spectrosome containing GSC-like cells, which has previously been observed when *dpp* was expressed in FCs and IGS cells (Kai and Spradling, 2003; Zhu and Xie, 2003; Song et al., 2004; Xi et al., 2005). However, we found that ectopic expression of *dpp* when driven by *bab1*-Gal4 was lethal (data not shown). This is most likely the result of embryonic and larval Dpp expression in the appendages and the abdomen, where the *bab1* genes and *bab1* enhancer traps are also expressed (Godt et al., 1993; Kopp et al., 2000; Cabrera et al., 2002; Chu et al., 2002). We were able to overcome this lethality by blocking *bab1*-Gal4 expression during embryonic, larval, and pupal development using the temperature-sensitive Gal4 repressor $Gal80^{ts}$ (Lee and Luo, 1999). As we cannot visualize directly Dpp, we first examined control germaria containing a UAS-lacZ reporter. When adult flies were shifted to the $Gal80^{ts}$ restrictive temperature of 31°C, we found that we could readily detect strong expres-

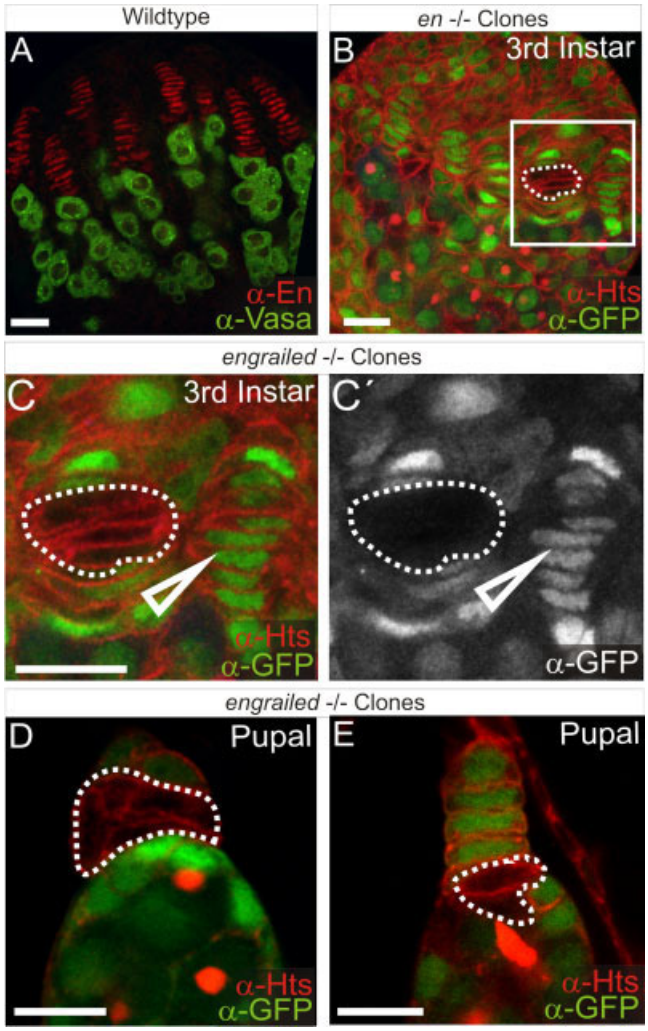

Fig. 5.

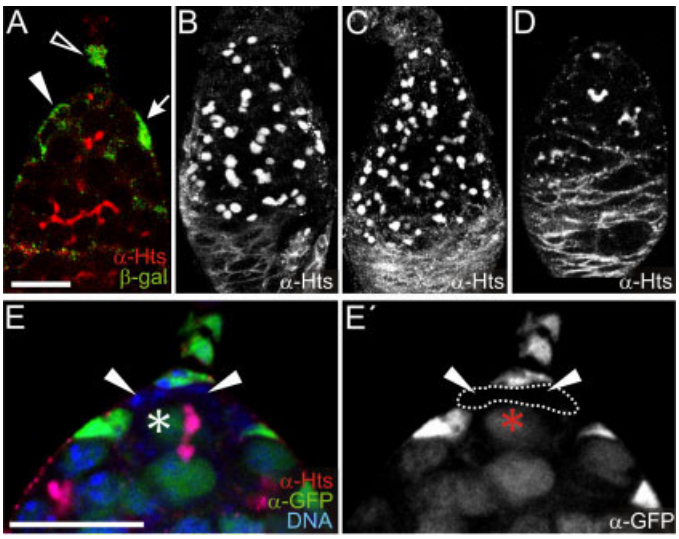

Fig. 6.

sion of lacZ in anterior somatic niche cells (Fig. 6A). Next, we examined the effect of expressing *dpp* from the somatic niche cells in the adult ovary. After 3 days in conditions permissive for UAS-*dpp* expression, we found a strong GSC tumor phenotype (Fig. 6B), similar to what has previously been found by the transient expression of *dpp* by heat-shock (Xie and Spradling, 1998) or by expression of an activated *dpp* pathway receptor (Tkv*) in the germline (Fig. 6C). This demonstrates that the expression of the Dpp ligand from the somatic CpCs, TFCs, and anterior IGS cells, is capable of inducing hyperplastic stem cell growth.

Evidence from an enhancer trap reporter of a target of the BMP pathway, *dad*-lacZ, suggests that the BMP pathway may be activated in some anterior somatic IGS cells (Casanueva and Ferguson, 2004; Song et al., 2004;

Fig. 5. *engrailed* is required for normal TF formation. **A:** Wildtype pupal gonad stained with anti-Engrailed (red) and anti-Vasa (green). En is present in TFCs and CpCs. **B,C:** Gonad from a third instar female larva stained with anti-GFP (green) and anti-Hts (red) to label *engrailed* (*en*) mutant cells. **C:** Magnified view of the white square in B. The cluster of *en* mutant TFCs was not organized properly such as the wild type filament to its right (open arrowhead). **D,E:** Pupal germaria stained with anti-GFP (green) and anti-Hts (red). D: Loss of *en* activity in large clones gives rise to aberrant terminal filaments. E: Small *en* clones can incorporate into wild-type looking terminal filaments. Dotted lines delineate clusters of *en* mutant cells. Absence of GFP indicates a homozygous mutant condition.

Fig. 6. A functional BMP pathway is not essential within CpCs for GSC maintenance. **A:** Germarium from a *tub*- Gal80^{ts}/UAS-lacZ; *bab1*-Gal4/+ female stained with anti-βGal (green) and anti-Hts (red) to show *bab1*-Gal4-driven expression of UAS-lacZ. **B:** Germarium from a *tub*-Gal80^{ts}/+;*bab1*-Gal4/UAS-*dpp* female stained with anti-Hts to show the tumor of spectrosome-containing cells produced after *bab1*-Gal4-driven overexpression of Dpp in adult germaria. **C:** Overexpression of UAS-*tkv**, driven in the germline by *nanos*-Gal4, induces overproliferation of GSC-like cells, as shown by anti-Hts staining. **D:** UAS-*tkv**/+;*tub*-Gal80^{ts}/+; *bab1*-Gal4/+ germarium stained with anti-Hts. Overexpression of *tkv** in adult somatic niche cells does not have significant effects on the germline. **E:** Germarium stained with anti-GFP (green), anti-Hts (red), and TO-PRO-3 (blue) to show a wild type GSC in contact solely with two *Mad* mutant CpCs (dotted line). Open arrowheads, TFCs; arrowheads, CpCs; arrow, ECs; asterisks, GSCs.

Kirilly et al., 2005). To test the significance of the activation of the BMP pathway in somatic cells, we again used Gal80^{ts} to allow the development of flies containing *bab1*-Gal4 and activated *tkv* receptor (*tkv**) under the control of the UAS promoter. In contrast to the expression of UAS-*dpp*, we did not observe any abnormal expansion of the stem cell niche in response to the somatic expression of the activated *tkv** receptor (Fig. 6D). Thus, the ectopic activation of the BMP pathway in stromal cells is not sufficient to induce hyperplastic stem cell growth.

It has been reported that the BMP pathway–responsive *dad*-LacZ is expressed in CpCs (Casanueva and Ferguson, 2004). However, other reports found *dad*-LacZ expressed in some IGS cells but not in the CpCs themselves (Song et al., 2004; Kirilly et al., 2005). To examine if the BMP pathway is functionally required in CpCs for GSC maintenance, we used *bab1*-Gal4 to drive the formation of mutant clones for the essential Smad effector, *Mad* (Raftery and Sutherland, 1999). We carefully examined mosaic germaria containing GSCs in contact only with *Mad* mutant CpCs and found that the lack of *Mad* expression in these cells 12–14 days after adult eclosion did not have any detectable effect upon GSC renewal (Fig. 6E; n = 7). Although we cannot rule out the possibility that the loss of *Mad* in these cells is compensated for by the presence of other wildtype CpCs in the niche, or that it may take longer than 12–14 days for the removal of Mad to have an effect, taken together our results suggest that the activation of the BMP pathway in the CpCs is not essential for GSC maintenance. These findings exclude a requirement for both *gbb* and *dpp* within the CpCs, as all BMP family ligands are believed to be mediated via *Mad* in *Drosophila* (Raftery and Sutherland, 1999). Thus, the ability of individual CpCs to perceive BMP ligands is not essential for the maintenance of GSCs with which they are in contact. However, further investigation will be required to determine the longer term consequences, if any, of the loss of *Mad* from CpCs to the GSC niche.

DISCUSSION

The genetic and cell biological tools available for the study of stem cell populations in *Drosophila* have played a large part in establishing the niche paradigm (Fuchs et al., 2004; Wong et al., 2005). In this regard, clonal analysis has been instrumental in the study of *Drosophila* oogenesis. However, such clones have usually been limited to cell lineages, which undergo frequent division in the adult, such as germline and follicle cells. One of the main aims of this study was to generate a stromal cell specific Gal4 line and determine its usefulness for dissecting the role of stromal cells in stem cell maintenance. We have shown that *bab1*-Gal4 can strongly express transgenes in all somatic cells of the germarium that form part of the GSC niche, and can be used to induce very high frequencies of recombination in most somatic cell types, including those most likely to be involved in stem cell maintenance. On the basis of the results we have presented in this report, *bab1*-Gal4 makes the generation of stromal cell clones a practical proposition, and thus should allow several key questions about stem cell niches to be addressed. The ability to express transgenes specifically in anterior stromal cells may be useful to investigate the roles of other putative stem cell niche ligands such as Gbb (Song et al., 2004), Hh (King et al., 2001; Zhang and Kalderon, 2001; Zhu and Xie, 2003) or Upd (Decotto and Spradling, 2005). Moreover, we have shown that the combination of *bab1*-Gal4 with Gal80^{ts} affords the flexibility to test the effect of ectopic expression of transgenes without deleterious effects on early development.

Relatively little is known about ovary morphogenesis or how stem cell niches are formed. The TFCs arise from the medial layer of somatic cells during the third instar stage of development at around the time that *bab1* expression is detected (King, 1970; Godt and Laski, 1995; Sahut-Barnola et al., 1995). The expression of conserved developmental regulators *en* and *hh* in TFCs during ovary morphogenesis may provide further insights into this process (Forbes et al., 1996b; Zhu and Xie, 2003). En is a transcription factor, while *hh* encodes a short-range morphogen (Lawrence and Struhl, 1996). Both genes are highly conserved and play fundamental roles in pattern formation during *Drosophila* development (Lawrence and Struhl, 1996; Hidalgo, 1996). In the ovary, expression of *hh* from the soma is essential for the maintenance of FSCs (Zhang and Kalderon, 2001) but apparently dispensable for GSC self-renewal (King et al., 2001). However, the role of these genes in stem cell niche formation is unknown. We have shown that loss of *en* from TFCs within a stack is associated with a disruption of the alignment of TFC within each filament. These data are the first evidence that *en* is functionally required for terminal filament morphogenesis. It has been proposed that *en* and *hh* might mediate their role in the segregation of cell populations of the wing by affecting cell adhesion in distinct populations of cells (Dahmann and Basler, 1999, 2000). It has also been suggested that cell adhesion might play a critical role in ovary morphogenesis (Godt and Laski, 1995). The defects we observed in the alignment of en^{E} mutant TFC clones could be explained by a failure of adhesion between these cells. Interestingly, each stage of the TF reorganization is associated with dynamic changes in the distribution of β-catenin (Godt and Laski, 1995), supporting the idea that the formation of terminal filaments may depend on the regulation of cell–cell adhesion. Moreover, the loss of integrins from follicle cells in the germarium resulted in failure of proper cyst formation, suggesting that integrin-mediated adhesion or signaling may play a crucial role in organizing the envelopment of germline cysts by migrating follicle cells. Our observations demonstrate the value of *bab1*-Gal4 for studying genes involved in stem cell niche morphogenesis. This approach could be applied to other genes such *D*E-cadherin (*shg*) or *armadillo* (*arm*), which have been proposed to play an adhesive role in the morphogenesis of terminal filaments (Godt and Laski, 1995) and the anchoring of GSCs into the niche (Song et al., 2002).

BMP signaling in the germline is essential for GSC maintenance (Wong et al., 2005). Although somatic cells

are thought to be the source of BMP ligands, the requirement of the BMP pathway in the soma has been largely untested. Based on the expression of an enhancer trap line of a downstream target of Dpp signaling (*dad*-lacZ), it has been reported that the BMP pathway might be stimulated in CpCs (Casanueva and Ferguson, 2004). In apparent contradiction, other studies have documented that, while levels of *dad*-lacZ expression were high in some anterior IGS cells and/r ESCs, lacZ expression was undetectable in CpCs (Song et al., 2004; Kirilly et al., 2005). This might reflect experimental differences or could reflect a transient activation of the BMP pathway in CpCs, such as has been described for FSCs (Kirilly et al., 2005). Using *bab1*-Gal4 we were able to test the functional requirement of BMP signaling in CpCs by looking at *Mad* mutant clones, and found that the expression of *Mad* in CpCs was not essential for GSC maintenance. Further analysis of *Mad* mutant clones generated by *bab1*-Gal4 should allow us to determine the requirement for the BMP pathway in other somatic cells, such as the ESCs.

In the wildtype situation, BMP pathway activation in the germline is restricted to only GSCs in contact with CpCs plus occasional "pre-CBs" (Kai and Spradling, 2003; Casanueva and Ferguson, 2004; Song et al., 2004). The formation of GSC tumors in response to *bab1*-Gal4-driven expression of *dpp* in anterior somatic cells, the likely source of BMP ligands in the GSC niche, suggests that the levels of BMP/Dpp ligand are an essential limiting factor in the normal restriction of GSC maintenance to those cells in contact with the CpC cells. In this regard, we note that *dpp* expression primarily from somatic cells within region 2 of the germaria also induces similar GSC tumors (Kai and Spradling, 2003; Zhu and Xie, 2003; Song et al., 2004), suggesting that GSC tumor phenotypes are sensitive to the availability, but not to the pattern, of ligand expression within the germarium.

Another gene that could be involved in spatially restricting BMP pathway activation is *piwi*. A member of the Argonaute family of RNA binding proteins, *piwi* is expressed in both somatic and germline cells, and is required within the soma for the maintenance of GSCs (Cox et al., 1998, 2000). Somatic overexpression of *piwi* causes a reversible increase in the number of anterior GSC-like germ cells (Cox et al., 2000; King et al., 2001). Recent evidence suggests that *piwi* expression in somatic cells is required to maintain the BMP-dependent repression of *bam* in GSCs (Chen and McKearin, 2005; Szakmary et al., 2005). However, somatic *piwi* appears to act on GSCs via a yet-unidentified BMP independent pathway that impinges on a downstream component of the BMP pathway in the GSCs (Szakmary et al., 2005), possibly by preventing the degradation of Mad protein (Chen and McKearin, 2005). The use of the *bab*-Gal4 line opens the possibility to directly address the role of *piwi* in the stromal cells.

In summary, we have demonstrated that the *bab1*-Gal4 is a useful tool for the genetic manipulation of the somatic cells that are critical for the maintenance of stem cells in *Drosophila* and to unravel the complex cell–cell interactions that define a stem cell niche. However, it is clear that the *bab1*-Gal4 line that we have characterized has some limitations. Firstly, the non-clonal mechanism by which individual germaria are formed makes it difficult to generate clones in which all the CpCs or TFCs in a given niche are mutant. Expression of *bab1*-Gal4 in non-ovarial tissues means that for some transgenes (e.g., UAS-*dpp*), Gal4 expression must be repressed during development to prevent lethality. The *bab1* locus is a complex regulatory region with different insertions giving similar but distinct expression patterns (Cabrera, 2002, no. 1). Isolation of the individual genetic elements that make up this region may allow the generation of novel Gal4 lines, which are more specific to the somatic cells within the ovary. Furthermore, it may allow the development of lines that are expressed in specific types of stromal cells, which would hasten the dissection of the complex interactions between stem and stromal cells that form the basis for stem cell maintenance within regulatory microenvironments.

EXPERIMENTAL PROCEDURES

Immunohistochemistry and Microscopy

Immunohistochemistry was performed at room temperature using standard procedures (detailed protocols are available upon request). Primary antibodies were used at the following concentrations: rabbit anti-Vasa (a gift from R. Lehmann) 1/2,000; mouse anti-Hts 1/50, mouse anti-Armadillo 1/100, and mouse anti-Engrailed 1/50 were obtained from the Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA; rabbit anti β-Galactosidase (Cappel™) 1/10,000; rabbit anti-GFP (Molecular Probes™) 1/500. Secondary antibodies Cy2 and Cy3 (Jackson InmunoResearch Laboratories, Inc.) were used at 1/200. DNA staining was performed using the DNA dye TO-PRO-3 (Molecular Probes™) at 1/1,000. Images were captured with a Leica TCS-SP2 confocal microscope and processed with Adobe Photoshop.

Fly Stocks

Targeted transposition of the Gal4 element p{GawB,*w*+} element was used to convert the $bab1^{P}$ enhancer trap line to *bab1*-Gal4 (Godt et al., 1993; Sepp and Auld, 1999). $bab1^{P}$ carries an insertion of the P{A92} element close to the 5′ end of the first intron (Couderc et al., 2002). Dysgenic *w*/Y; p{GawB,*w*+}, CyO 760/+; $bab1^{P}$ (ry^{+})/TMS, Δ2–3 males were crossed to *w*; TM3/TM6B females. Twenty-eight lines with a w^{+} insertion on the IIIrd chromosome were established and tested for GFP expression. Of those, 8 showed GFP expression in the germarium. The tkv^{*} cDNA (a constitutively active form of a *dpp* receptor carrying the Q199D aa substitution) (Das et al., 1998) was cloned in the UASp expression vector (Rorth, 1998) to make UASp-tkv^{*} construct and used to generate *tkv** containing transgenic lines. In order to obtain adult females overexpressing *lacZ, dpp*, or *tkv** under the control of *bab1*-Gal4, we crossed *w*; *tub*-Gal80^{ts}/CyO; *bab1*-Gal4/TM2 with *w*; UASt-lacZ/CyO, *w*; UASt-*dpp*/TM3 or UASp-tkv^{*}, respectively. The offspring were grown at 18°C and adult F1 flies were shifted to 31°C for

3 days. Ectopic expression of Nod:GFP (Bolívar et al., 2001), Src:GFP (González Reyes, 2003) or *tkv** in germline cells was achieved by crossing the respective UASp lines with *nanos*-Gal4 (Van Doreen et al., 1998). The UASt-RedStinger stock was obtained from the Bloomington Stock Centre.

Generation of Somatic and Germline Clones

To generate somatic mutant clones, we used the following chromosomes: *mys*^{11} FRT101; *Df(2R) en*^{E} FRT42D; *Mad*^{12} FRT40. A UASt-Flp insertion on the third chromosome was recombined with the *bab1*-Gal4 no. 48 line. In the case of *Mad* mutant clones, adult females were dissected 12–14 days after eclosion. The *e22c*-Gal4 UASt-FLP chromosome was used to generate *mys* mutant follicle cell clones (Duffy et al., 1998). In the *cis*-recombination experiments, *bab1*Gal4 UASp-Flp/TM3 flies were crossed to *actin*-FRT>stop>FRT-nls-LacZ transgene-containing flies.

ACKNOWLEDGMENTS

We are grateful to Jan Adams for making the UASp-*tkv** construct. We thank L. Cobreros, M. Domínguez, M. González-Gaitán, I. Guerrero, E. Sánchez Herrero, the DSHB, and the Bloomington Stock Centre for fly stocks and reagents.

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Supplemental Fig. 1. *bab1*-Gal4 expression in adult ovaries, as visualized with a nuclear marker. *bab1*-Gal4 UAS-Red-Stinger ovaries were dissected to visualize *bab1*-Gal4-driven nuclear DsRed.