Novel Tools for Genetic Manipulation of Follicle Stem Cells in the *Drosophila* Ovary Reveal an Integrin-Dependent Transition from Quiescence to Proliferation

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ABSTRACT In many tissues, the presence of stem cells is inferred by the capacity of the tissue to maintain homeostasis and undergo repair after injury. Isolation of self-renewing cells with the ability to generate the full array of cells within a given tissue strongly supports this idea, but the identification and genetic manipulation of individual stem cells within their niche remain a challenge. Here we present novel methods for marking and genetically altering epithelial follicle stem cells (FSCs) within the *Drosophila* ovary. Using these new tools, we define a sequential multistep process that comprises transitioning of FSCs from quiescence to proliferation. We further demonstrate that integrins are cell-autonomously required within FSCs to provide directional signals that are necessary at each step of this process. These methods may be used to define precise roles for specific genes in the sequential events that occur during FSC division after a period of quiescence.

KEYWORDS follicle stem cell; germarium; integrin; adhesion; quiescence

MANY regenerative tissues possess populations of self-renewing stem cells that are maintained throughout the lifetime of the organism to produce the differentiated daughter cells required for tissue function. Recent work has resulted in the identification of signals from the immediate microenvironment or niche that promote stem cell positioning and self-renewal. Less well understood are the stem cell regulatory signals produced by cells located outside the stem cell niche. For example, a relay of signals involving multiple tissues located far from the ovary results in production of insulin that directly influences proliferation of germ-line stem cells (GSCs)

in the fly (Lafever and Drummond-Barbosa 2005; Geminard *et al.* 2009). In another case, nutritional changes control the production of growth factors by terminal filament and cap cells located at the apical tip of the *Drosophila* ovary (apical cells) that regulate proliferation of follicle stem cells (FSCs) located six to eight cell diameters to the posterior (Hartman *et al.* 2013).

Despite these advances, identification of critical stem cell control mechanisms is limited by our ability to precisely identify stem cell populations and their support cells *in situ* and to manipulate gene function in individual cell types that contribute to stem cell regulation. In flies, the *Gal4-UAS* system has been an extremely powerful tool for analysis of gene function in subsets of cells within a given tissue. Combined with extensive RNA interference (RNAi) libraries and classical genetic mutations, the *Gal4-UAS* system has made it possible to directly assess the effects of altering the activity

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of a single gene on cellular function in many developing tissues.

To enhance our ability to dissect molecular mechanisms of FSC regulation, we conducted a screen designed to identify *Gal4* lines that are expressed in subpopulations of somatic cells within the stem cell compartment of the fly ovary, called the *germarium*. Several populations of somatic cells in the *germarium* have been shown previously to be critical for stem cell regulation. Post-mitotic cap cells constitute the cellular niche for GSCs and provide the signals necessary for self-renewal, prevention of differentiation, and controlled orientation of cell division (Xie and Spradling 1998, 2000; Song and Xie 2002; Song *et al.* 2002; Kai and Spradling 2003; Losick *et al.* 2011). *Terminal filament cells* neighbor cap cells to the anterior and produce growth factors that influence GSC function (Forbes *et al.* 1996b; Xie and Spradling 1998; King and Lin 1999; King *et al.* 2001). Terminal filament and cap cells (collectively referred to as *apical cells*) also generate factors required for FSC proliferation (Forbes *et al.* 1996a, b; Zhang and Kalderon 2001; Song and Xie 2003; Kirilly *et al.* 2005; Hartman *et al.* 2013; Sahai-Hernandez and Nystul 2013). However, FSCs are separated from apical cells by six to eight somatic *inner germarial sheath cells* (IGS cells, also called *escort cells*) that provide additional growth factors for FSC control and adhesive signals that maintain FSC positioning (Song and Xie 2002; Sahai-Hernandez and Nystul 2013). Finally, FSC daughter cells, called *follicle cells*, influence FSC behavior from the posterior by creating gradients of signals that intersect with anterior signals produced by apical cells and escort cells (Vied *et al.* 2012).

Our goal was to identify new methods for marking individual somatic cell populations and tools for genetically manipulating gene function within them in order to probe previously unknown aspects of stem cell function. Here we identify new *Gal4* lines that are expressed in apical cells, cap cells alone, IGS cells, follicle cells, and multiple somatic cell types. We further identify two new *Gal4s* that are particularly useful for manipulation of gene expression in FSCs. Using these new tools, we define the primary defect in FSCs that lack integrin function and define the role of integrin-mediated adhesion in FSC positioning, migration, and long-term self-renewal.

Materials and Methods

Fly strains and genetics

The following stocks obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN) were used for the *Gal4* expression screen: *w**; *P{GawB}109-28, y1 w**; *P{GawB}109-30/CyO, y1 w**; *P{GawB}109-39/TM3, Sb1, y1 w**; *P{GawB}109-53/TM3, Sb1, P{GawB}109C1, y1 w**; *P{GawB}185Y, w¹¹¹⁸, w¹¹¹⁸*; *P{GawB}645b, w**; *P{GawB}bab1Pgal4-2/TM6B, Tb1, P{GawB}c323a, w¹¹¹⁸, w**; *P{GawB}c458, w**; *P{GawB}c532, w¹¹¹⁸*; *P{GawB}c784, P{GawB}cb13, w¹¹¹⁸, w¹¹¹⁸*; *P{GMR52D02-GAL4}attP2, w¹¹¹⁸*; *P{GMR53H05-GAL4}attP2, w¹¹¹⁸*; *P{GMR61C11-GAL4}attP2, w**; *P{en2.4-GAL4}e22c P{UAS-FLP1.D}JD1/CyO*; *P{neoFRT}82B ry506, P{GawB}112A,*

*w**, *w¹¹¹⁸*; *P{GMR28E03-GAL4}attP2, w¹¹¹⁸*; *P{GMR28E04-GAL4}attP2, w¹¹¹⁸*; *P{GMR28D09-GAL4}attP2, P{UAS-Dcr-2.D}1, w¹¹¹⁸*; *P{GawB}pnu^{MD237}/TM3, Ser1, w**; *P{GawB}ptc^{559.1}, w¹¹¹⁸*; *P{GMR30H03-GAL4}attP2, w¹¹¹⁸*; *P{GMR16D01-GAL4}attP2 and w¹¹¹⁸*; and *P{GMR17D09-GAL4}attP2*. Additional lines from the *Drosophila* Genetic Resource Center (Kyoto, Japan) that were used for the *Gal4* screen include *y* P{GawB}boi^{NP4065} w*/FM7c, w* P{GawB}NP4124/FM7c, P{w[+mW.hs]=GawB}sgg^{NP0082} w*/FM7c, y* P{w[+mW.hs]=GawB}sgg^{NP2253} w*/FM7c, y* P{w[+mW.hs]=GawB}sgg^{NP4101} w*/FM7c, y* P{w[+mW.hs]=GawB}sgg^{NP7167} w*/FM7c, P{w[+mW.hs]=GawB}sgg^{NP7270} w*/FM7c, y* w**; *P{w[+mW.hs]=GawB}sl^{iNP1625}/CyO, P{w[-]=UAS-lacZ.UW14}UW14, and w**; *P{w[+mW.hs]=GawB}sl^{iNP2755}/CyO*. Other fly strains were generated using standard *Drosophila* genetic methods.

To characterize *Gal4* expression, males from the preceding *Gal4* lines were mated with virgins from either *UAS-GFP-nls/CyO*, *UAS-GFP-CD8/CyO*, or *UAS-GFP-tau/CyO*. Female progeny obtained within 4 days of eclosion were dissected and subjected to immunohistochemical characterization.

Mosaic analysis with repressible cell marker (MARCM) stocks were generated by crossing males that were *Ub-RFP, Gal80 19AFRT Flp122; UAS-CD8-GFP* to females that were *19AFRT; 109-30-Gal4/CyO*. Experiments using transgenes on the third chromosome [*mys*^{S00043RNAi}, *hh-lacZ*^{P90} (Forbes *et al.* 1996a), *ptc-lacZ*^{559.1} (Forbes *et al.* 1996b)] were done by crossing *Ub-RFP, Gal80 19AFRT Flp122; UAS-CD8-GFP; transgene/TM6b* males to *19AFRT; 109-30-Gal4/CyO* females. Integrin-mutant FSCs were generated by crossing *Ub-RFP, Gal80 19AFRT Flp122; UAS-CD8-GFP; transgene/TM6b* males to *mys* 19AFRT; 109-30-Gal4/CyO* females, where *mys** was *mys*^{M2}, *mys*^{P9}, or *mys*^{b13} (Jannuzzi *et al.* 2002, 2004).

Immunohistochemistry and image analysis

Flies were dissected for immunohistochemistry in Grace's insect medium (Sigma-Aldrich, St. Louis, MO) as described previously (Hartman *et al.* 2013). Ovaries were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 10 min at room temperature, washed three times for 10 min in PBS with 0.03% TritonX-100 (Fisher Scientific), and incubated with primary antibody in PBS-T with 0.5% BSA (Fisher Scientific) overnight at 4°. Ovaries then were washed three times for 10 min in PBS-T and incubated with secondary antibody in PBS-T with 0.5% BSA for 2 hr at room temperature. Primary antibodies were chicken anti-GFP (1:1000; Invitrogen, Carlsbad, CA), mouse anti-Fas3 [1:100; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] (Patel *et al.* 1987), rabbit anti-Vasa (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and rat anti-FC-NA (1:2000) (Hartman *et al.* 2013). Secondary antibodies used were FITC, Cy3, and Cy5 conjugated to species-specific secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Samples were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were collected at room temperature (approximately 22°) using 40×, 1.25 NA (Leica, Buffalo Grove, IL) on an upright microscope (DM5000B; Leica) coupled to a confocal

laser scanner (TCS SP5; Leica). LAS AF SP5 software (Leica) was used for data acquisition. Images representing individual channels of single confocal slices from each germarium were exported as TIFF files, and images were converted to figures using Photoshop software (Adobe Systems, San Jose, CA).

Nutrient restriction

Flies were raised on fruit juice plates containing only simple sugars (50% grape juice, 3% Bacto Agar, 1% glacial acetic acid, and 1% methyl paraben) or on molasses plates [12% Grandma's molasses (B&G Foods, Parsippany, NJ), 3% agar (crystalline, molecular biology grade), and 2.5% methyl paraben] for 3 days. For measuring the time course of quiescence, flies were fed yeast and heat shocked for 2 hr at 37°. Subsequently, flies were kept at 25° on yeast for 3 days for recovery. Nutrient restriction was performed for 1, 2, or 3 days. Fly ovaries were prepared as described by Hartman *et al.* (2010). Ovaries were dissected, fixed in 4% paraformaldehyde, and immunostained with mouse anti-Fas3 (1:25; DSHB) (Patel *et al.* 1987) and rabbit anti-phospho-histone H3 (1:1000; Millipore, Bedford, MA). Dividing FSCs were determined by scoring germaria for phospho-histone H3+ FSCs per germarium. FSCs were identified by their location at the border of germarial regions 2A and 2B, low level expression of Fas3 (Fas3^{lo}), a marker for pre-follicle cells, and the presence of a triangular nucleus, a feature that distinguishes FSCs from their daughter cells and neighboring escort cells (Nystul and Spradling 2007). Student's *t*-tests for two samples were used, with significance achieved at $P \leq 0.05$.

Specificity of FSC clone induction

Flies of the genotype *Ub-RFP, Gal80 19AFRT Flp122/19AFRT; UAS-CD8-GFP/109-30-Gal4* were heat shocked as (1) third-instar larvae or (2) 5- to 7-day-old post-eclosion adults. Isolated ovaries were fixed in 4% paraformaldehyde in PBS and immunostained as described earlier using antibodies against GFP, Vasa, and Fas3. Total numbers of germaria bearing clones in IGS cells (defined as somatic cells anterior to the region 2A–2B border), FSCs (cells at the region 2A–2B border just anterior to the first flattened germ-line cysts that produced labeled follicle cells), or follicle cells were scored. Germaria with clones in more than one cell type were scored more than once. The difference between the percentage of germaria with IGS cells in larval heat shock vs. adult heat shock is statistically significant ($P < 0.00001$).

To mark IGS cells in a MARCM background, flies of the genotype *Ub-RFP, Gal80 19AFRT Flp122; UAS-CD8-GFP/109-30-Gal4/hh-lacZ/+* or *Ub-RFP, Gal80 19AFRT Flp122; UAS-CD8-GFP/109-30-Gal4/ptc-lacZ/+* were heat shocked as 5- to 7-day-old posteclosion adults. Isolated ovaries were fixed in 4% paraformaldehyde and immunostained with antibodies against GFP, β -Gal, and Fas3.

Integrin analysis

To evaluate integrin function on FSC maintenance, *19A FRT; 109-30/CyO, mys^{P9} 19A FRT; 109-30/CyO, mys^{m2} 19A FRT;*

109-30/CyO, mys^{b13} 19A FRT; 109-30/CyO virgins were mated with *Ub-RFP, Gal80 19AFRT Flp122; UAS-GFP-nls* or *UAS-GFP-CD8/CyO* males. Appropriate female and male progeny were collected 3–5 days after eclosion and incubated overnight on nutrient-poor food to arrest FSC division. The following day, proliferation was activated with yeast for 4 hr at room temperature. Flies then were heat shocked for 3 hr at 37°. Following heat shock, flies were incubated at room temperature for 4 days to expel transiently GFP-labeled cells from the germarium, after which they were transferred to fruit juice plates containing only simple sugars (50% grape juice, 3% Bacto Agar, 1% glacial acetic acid, and 1% methyl paraben) for 3 days. Flies were dissected and ovaries were isolated at this point for starvation evaluation, as described earlier. To examine FSC proliferation and migration, after flies were starved for 3 days, they were transferred to new vials containing yeast. To examine the initial division, ovaries were harvested 6 hr after stimulation to maximize potential FSC division (Hartman *et al.* 2013). To examine long-term FSC maintenance and proliferation, flies were maintained at steady state for 1, 2, and 3 weeks, after which they were dissected and imaged as described earlier.

To evaluate how integrin function determines FSC maintenance and behaviors, 385–692 flies per genotype per time point were scored [wild type (WT): week 1 $n = 429$, week 2 $n = 576$, week 3 $n = 504$; *mys^{P9}*: week 1 $n = 437$, week 2 $n = 537$, week 3 $n = 448$; *mys^{m2}*: week 1 $n = 692$, week 2 $n = 536$, week 3 $n = 437$; *mys^{b13}*: week 1 $n = 462$, week 2 $n = 385$, week 3 $n = 554$]. FSC maintenance was determined by identification of a GFP-labeled cell with low Fas3 expression within the germarium. Concurrently, the same germaria were used to determine FSC mislocalization as those cells within the germarium that retain GFP expression but lose their position at the region 2A–2B plane. Proliferative potential was measured as the percentage of ovarioles containing greater than four GFP-labeled daughter cells. Lastly, projection targeting was evaluated by determining whether the GFP-labeled projection extended toward the opposite FSC pole or acquired an abnormal phenotype. Statistical significance was determined using unpaired Student's *t*-tests for two samples, with significance achieved at $P \leq 0.05$.

Analyzing 3D renderings of germaria using IMARIS software

LIF files obtained using LAS AF SP5 software also were uploaded into Imaris 3D software (Bitplane, Zurich, Switzerland), and three-dimensional (3D) renderings of the series were analyzed. Using the 3D editing option, we were able to isolate the region 2A–2B divisionary plane containing a GFP-labeled cell and lacking Fas3 staining. Using the Surpass View, we quantitatively measured both average projection length (WT: $n = 21$; *mys^{P9}*: $n = 26$) and average FSC distance from the midline (WT: $n = 15$; *mys^{P9}*: $n = 38$; Fend *mys^{S00043RNAi}*: $n = 34$) using the Measurement Points option and measuring from the center of the cell body to either the projection tip or the center of the midline. Total average clonal volume was quantitated using the

Surface option (WT: week 1 $n = 11$, week 2 $n = 10$, week 3 $n = 11$; *mys*^{P9}: week 1 $n = 9$, week 2 $n = 14$, week 3 $n = 10$) within objects with a GFP intensity of 15–256 pixels to differentiate cells and projections from background staining. Statistical significance was determined using unpaired Student's *t*-tests for two samples, with significance achieved at $P \leq 0.05$.

Results

Controlled expression of UAS-dependent genes in terminal filament and cap cells

Large-scale genetic screens have led to the generation of hundreds of transgenic fly lines bearing random insertions of *Gal4* throughout the genome. Patterns of *Gal4* expression are determined by promoters located adjacent to the insertion site. In most cases, expression patterns have been mapped in developing embryos and selected tissues, but patterns of expression within subsets of cells in the germarium have not been clearly defined. To identify *Gal4* lines that are expressed in somatic cells of the germarium, including (1) terminal filament and cap cells, (2) IGS cells, (3) FSCs, and (4) follicle cells (Figure 1A), we used a candidate approach in which lines that contain *Gal4* inserted into known genes that function in a particular somatic cell population within the ovary [e.g., *bric-à-brac 1* (*bab1*) and *hedgehog* (*hh*) function in terminal filament and cap cells (Cabrera *et al.* 2002; Hartman *et al.* 2010, 2013; Sahai-Hernandez and Nystul 2013)] were screened as well as lines in which previous studies had indicated general ovarian expression (Table 1).

bab-Gal4 exhibited robust expression in terminal filament and cap cells, as well as stalk cells and polar cells that separate adjacent egg chambers later in development, as reported previously (Cabrera *et al.* 2002). In addition, weak expression in developing follicle cells was observed (Figure 1B) (Cabrera *et al.* 2002; Hartman *et al.* 2010). *109-53 Gal4* exhibited a more specific expression pattern in terminal filament and cap cells, as well as stalk cells, with no detectable expression in follicle cells (Figure 1B) (Hartman *et al.* 2013). Three independent *Gal4* insertions into the *hh* locus (*hh*^{GMR28E03}, *hh*^{GMR29D09}, and *hh*^{GMR28B04}) gave very specific expression in terminal filament and cap cells (Eliazar *et al.* 2011) but no detectable expression in IGS cells, FSCs, or follicle cells in the germarium (Figure 1B). This is consistent with previous data demonstrating that Hh protein is present and functional in these cells (Hartman *et al.* 2010, 2013; Sahai-Hernandez and Nystul 2013) and distinguishes these lines from a *hh-lacZ* reporter gene that also is expressed in escort cells (Forbes *et al.* 1996a). As expected, insertions in other components of the Hh signaling pathway such as *boi-Gal4*^{NP4065} gave similar patterns of expression (Figure 1B).

In addition to *hh*, *wingless* (*wg*) is expressed in terminal filament and cap cells (Forbes *et al.* 1996b; Song and Xie 2003; Sahai-Hernandez and Nystul 2013). Surprisingly, we found that *Gal4* insertions in *shaggy* (*sgg*), a negative regulator of the Wg signaling pathway that normally functions in Wg-receiving cells, promoted GFP expression in cap cells, as

well as reduced or mosaic expression in the terminal filament. GFP was expressed in both terminal filament and cap cells in the *sgg-Gal4*^{NP7270} and *sgg-Gal4*^{NP2253} lines and very weakly expressed in IGS cells (Figure 1C). Some *Gal4* insertions in the *sgg* locus exhibited higher expression in cap cells, mosaic expression in the terminal filament, and weak or undetectable expression in IGS cells, including *sgg-Gal4*^{NP7167}, *sgg-Gal4*^{NP4101}, and *sgg-Gal4*^{NP7142} (Figure 1C). Other *sgg-Gal4* lines were more broadly expressed in all anterior somatic cells of the germarium, including the terminal filament, cap cells, and IGS cells (*sgg-Gal4*^{NP0082}) (Figure 3B). Additional *Gal4* insertions *cb13-Gal4*, *185Y-Gal4*, and *109C1-Gal4* exhibited GFP expression in terminal filament and cap cells (Figure 1D). *slit* (*slit*)-*Gal4*^{NP2755}, *c458-Gal4*, and *109-28-Gal4* were specific to cap cells (Figure 1D). GFP expression levels among the different *Gal4* lines ranged from very weak [e.g., *hh-Gal4*^{GMR28B04}, *hh-Gal4*^{GMR29D09}, *brother of ihog* (*boi*)-*Gal4*, and *cb13-Gal4*] to strong (e.g., *bab-Gal4*, *109-53-Gal4*, *slit-Gal4*^{NP2755}, and *sgg-Gal4*^{NP7167}). In some cases, expression could only be detected using UAS-Tau-GFP, a much stronger reporter of transcriptional activation than UAS-GFP-nls (e.g., *boi-Gal4*^{NP4065}, *sgg-Gal4*^{NP4101}, *sgg-Gal4*^{NP0082}, and *sgg-Gal4*^{NP7270}) (Figure 2A). This supports findings from many studies that the location of the *Gal4* insertion within the promoter determines the resulting expression level and pattern.

The range of expression of different *Gal4* lines is useful for manipulating protein expression in terminal filament and cap cells. For example, expression of genes required within terminal filament and cap cells for Hh signaling, including *boi* and *DHR96*, was reduced effectively by expressing targeting RNAs under the control of strong drivers such as *bab-Gal4* and *109-53-Gal4* (Hartman *et al.* 2013). However, the expression levels of these drivers are too strong for some experiments. Attempts to express active Hh ligand using *bab-Gal4* or *sgg-Gal4*^{NP7270} were unsuccessful because of early developmental defects (data not shown). Weakly expressing drivers such as *sgg-Gal4*^{NP4101}, in contrast, enabled isolation of phenotypically normal adult flies expressing Hh-GFP in terminal filament and cap cells (Figure 2B). As expected, increased expression of Hh-GFP led to excessive proliferation of FSCs and their progeny, resulting in longer stalks between egg chambers that contained far more cells than WT stalks (Figure 2B) (Forbes *et al.* 1996a, b). Thus gene expression levels in terminal filament and cap cells can be controlled using these newly identified *Gal4* lines, adding simplified tools to previously existing approaches such as the use of temperature-sensitive alleles of *Gal80* for controlling gene expression levels in these cells.

Gal4 expression in escort cells overlaps with neighboring cap cells or FSCs

IGS cells were identified originally as support cells for developing germ-line cysts in the most anterior regions of the germarium (King 1970). IGS cells line regions 1 and 2 of the germarium and extend thin cytoplasmic projections to separate adjacent developing germ-line cysts (King 1970; Mahowald and Kambyzellis 1980; Schulz *et al.* 2002; Decotto and Spradling

Figure 1 *Gal4* expression in terminal filament and cap cells. (A) Schematic diagram showing somatic cell populations in the gerarium, including terminal filament cells (blue), cap cells (magenta), germ-line stem cells (GSCs, orange), inner germarial sheath cells (IGS cells, also known as escort cells, turquoise), follicle stem cells (FSCs, green), follicle cells (immunostained with Fas3, blue), and germ cells (immunostained with Vasa, red). (B) Expression of previously reported *Gal4* lines (*bab-Gal4* and 109-53-*Gal4*) and Hh-pathway *Gal4* insertions (*hh-Gal4*, *boi-Gal4*) in terminal filament and cap cells. (C) Five *shaggy* (*sgg*)-*Gal4* lines exhibit expression in terminal filament and/or cap cells. (D) *Gal4* insertions in unidentified genes exhibit expression in terminal filament and/or cap cells. Germaria in B–D are immunostained with Vasa (blue, germ cells) and either Fas3 (red, follicle cells) or FC-NA (red, high in FSCs and follicle cells). Scale bars are indicated.

Table 1 Quantitation of Expression of UAS-GFP-nls by Given *Gal4* Insertions

<i>Gal4</i>	Insertion	Inserted gene	Reference	Total	No exp.	% No exp.	Total TF cells	% TF cells	Total CCs	% CCs	Total IGS cells	% IGS cells	Total FSCs	% FSCs	Total FCs	% FCs
<i>boi</i> ^{NP4065}	<i>P{GawB}NP4065</i>	<i>boi</i>	Hayashi et al. 2002	80	52	65.00	28	35.00	28	35.00	0	0.00	0	0.00	0	0.00
<i>bab</i> ^{P^{Gal4}-2}	<i>P{GawB}bab1</i>	<i>bric-à-brac</i>	Bolivar et al. 2006	230	0	0.00	230	100.00	128	55.65	54	23.48	0	0.00	180	78.26
<i>dlp</i> ^{GMR53H05}	<i>P{GMR53H05-GAL4}attP2</i>	<i>dally-like</i> <i>protein</i>	Pfeiffer et al. 2011	81	38	46.91	0	0.00	27	33.33	32	39.51	0	0.00	4	4.94
<i>dlp</i> ^{GMR52D02}	<i>P{GMR52D02-GAL4}attP2</i>	<i>dally-like</i> <i>protein</i>	Pfeiffer et al. 2011	53	16	30.19	25	47.17	4	7.55	3	5.66	0	0.00	16	30.19
<i>dlp</i> ^{GMR61C11}	<i>P{GMR61C11-GAL4}attP2</i>	<i>dally-like</i> <i>protein</i>	Pfeiffer et al. 2011	76	68	89.47	0	0.00	7	9.21	2	2.63	0	0.00	0	0.00
<i>e22c</i>	<i>P{GawB}e22c</i>	<i>engrailed</i>	Duffy et al. 1998	89	12	13.48	3	3.37	22	24.72	76	85.39	55	61.80	40	44.94
<i>fend</i> ^{NP112A}	<i>P{GawB}112A</i>	<i>forked ends</i>	Larkin et al. 1996	47	7	14.89	0	0.00	0	0.00	45	95.74	33	70.21	3	6.38
<i>fend</i> ^{NP5045}	<i>P{GawB}fend^{NP5045}</i>	<i>forked ends</i>	Hayashi et al. 2002	60	36	60.00	12	20.00	17	28.33	1	1.67	1	1.67	2	3.33
<i>fend</i> ^{NP4124}	<i>P{GawB}fend^{NP4124}</i>	<i>forked ends</i>	Hayashi et al. 2002	112	7	6.25	0	0.00	105	93.75	105	93.75	105	93.75	0	0.00
<i>hlh</i> ^{GMR28E04}	<i>P{GMR28E04-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	99	76	76.77	20	20.20	12	12.12	3	3.03	0	0.00	1	1.01
<i>hlh</i> ^{GMR28E03}	<i>P{GMR28E03-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	82	0	0.00	82	100.00	82	100.00	82	100.00	82	100.00	16	19.51
<i>hlh</i> ^{GMR28D09}	<i>P{GMR28D09-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	83	33	39.76	12	14.46	4	4.82	1	1.20	1	1.20	40	48.19
<i>pnr</i> ^{MD237}	<i>P{GawB}pnrMD237</i>	<i>pannier</i>	Brand and Perrimon 1993	58	42	72.41	6	10.34	1	1.72	10	17.24	1	1.72	0	0.00
<i>ptc</i> ^{559.1}	<i>P{GawB}ptc559.1</i>	<i>patched</i>	König et al. 2011	76	0	0.00	40	52.63	75	98.68	76	100.00	76	100.00	61	80.26
<i>sgg</i> ^{NP0082}	<i>P{GawB}sgg^{NP0082}</i>	<i>shaggy</i>	Hayashi et al. 2002	67	67	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
<i>sgg</i> ^{NP2253}	<i>P{GawB}sgg^{NP2253}</i>	<i>shaggy</i>	Hayashi et al. 2002	58	45	77.59	12	20.69	6	10.34	2	3.45	0	0.00	0	0.00
<i>sgg</i> ^{NP4101}	<i>P{GawB}sgg^{NP4101}</i>	<i>shaggy</i>	Hayashi et al. 2002	64	50	78.13	0	0.00	14	21.88	0	0.00	0	0.00	0	0.00
<i>sgg</i> ^{NP7167}	<i>P{GawB}sgg^{NP7167}</i>	<i>shaggy</i>	Hayashi et al. 2002	96	86	89.58	10	10.42	0	0.00	0	0.00	0	0.00	0	0.00
<i>sgg</i> ^{NP7270}	<i>P{GawB}sgg^{NP7270}</i>	<i>shaggy</i>	Hayashi et al. 2002	230	201	87.39	22	9.57	2	0.87	0	0.00	6	2.61	2	0.87
<i>slf</i> ^{GMR30H09}	<i>P{GMR30H09-GAL4}attP2</i>	<i>slf</i>	Pfeiffer et al. 2011	55	29	52.73	26	47.27	10	18.18	4	7.27	0	0.00	0	0.00
<i>SNF4A</i> ^{y^{GMR31C04}}	<i>P{GMR31C04-GAL4}attP2</i>	<i>SNF4Agamma</i>	Pfeiffer et al. 2011	64	46	71.88	11	17.19	8	12.50	6	9.38	0	0.00	0	0.00
<i>SNF4A</i> ^{y^{GMR30H03}}	<i>P{GMR30H03-GAL4}attP2</i>	<i>SNF4Agamma</i>	Pfeiffer et al. 2011	101	75	74.26	23	22.77	4	3.96	8	7.92	0	0.00	0	0.00
<i>wg</i> ^{GMR16D01}	<i>P{GMR16D01-GAL4}attP2</i>	<i>wingless</i>	Pfeiffer et al. 2011	52	25	48.08	18	34.62	10	19.23	5	9.62	0	0.00	8	15.38
<i>wg</i> ^{GMR17D09}	<i>P{GMR17D09-GAL4}attP2</i>	<i>wingless</i>	Pfeiffer et al. 2011	99	45	45.45	37	37.37	26	26.26	36	36.36	0	0.00	2	2.02
<i>109-28</i>	<i>P{GawB}109-28</i>	<i>Unknown</i>	Younger 2001 ^a	70	36	51.43	23	32.86	24	34.29	1	1.43	0	0.00	0	0.00
<i>109-30</i>	<i>P{GawB}109-30</i>	<i>Unknown</i>	Hartman et al. 2010	92	0	0.00	65	70.65	65	70.65	7	7.61	92	100.00	92	100.00
<i>109-53</i>	<i>P{GawB}109-53</i>	<i>Unknown</i>	Assa-Kunik et al. 2007	85	0	0.00	85	100.00	85	100.00	0	0.00	0	0.00	85	100.00
<i>109C1</i>	<i>P{GawB}109C1</i>	<i>Unknown</i>	Younger 2001 ^a	103	12	11.65	91	88.35	0	0.00	0	0.00	0	0.00	0	0.00
<i>185Y</i>	<i>P{GawB}185Y</i>	<i>Unknown</i>	Manseau et al. 1997	81	5	6.17	46	56.79	0	0.00	0	0.00	0	0.00	76	93.83
<i>645b</i>	<i>P{GawB}645b</i>	<i>Unknown</i>	Manseau et al. 1997	73	5	6.85	21	28.77	20	27.40	64	87.67	28	38.36	25	34.25
<i>c323a</i>	<i>P{GawB}c323a</i>	<i>Unknown</i>	Manseau et al. 1997	73	37	50.68	3	4.11	21	28.77	35	47.95	33	45.21	29	39.73
<i>c458</i>	<i>P{GawB}c458</i>	<i>Unknown</i>	Manseau et al. 1997	56	12	21.43	2	3.57	15	26.79	0	0.00	0	0.00	0	0.00
<i>cb13</i>	<i>P{GawB}cb13</i>	<i>Unknown</i>	Ward et al. 2002	97	67	69.07	29	29.90	5	5.15	1	1.03	0	0.00	0	0.00
<i>GR1</i>	<i>P{GawB}GR1</i>	<i>Unknown</i>	Gupta and Schüpbach 2003	55	29	52.73	18	32.73	7	12.73	2	3.64	0	0.00	14	25.45
<i>55b</i>	<i>P{GawB}Eip63F-1^{55b}</i>	<i>Eip63F-1</i>	Brand and Perrimon 1993	61	24	39.34	31	50.82	13	21.31	9	14.75	0	0.00	4	6.56
<i>c135</i>	<i>P{GawB}c135</i>	<i>Unknown</i>	Manseau et al. 1997	68	47	69.12	20	29.41	3	4.41	3	4.41	0	0.00	0	0.00

^a Personal communication.

Columns titled "Total" represent the number of germaria exhibiting a given expression pattern and percentages of germaria; columns titled "%" exhibit a given expression pattern. No expression ("No exp."), terminal filament cells ("TF cells"), cap cells ("CCs"), IGS/escort cells ("IGS cells"), follicle stem cells ("FSCs"), and follicle cells ("FCs") were assessed. References are given for the first description of patterns of expression during oogenesis or for uncharacterized *Gal4* lines for the generation of the line.

Figure 2 Weak *Gal4* drivers can be used to promote expression of potentially lethal ligands. (A) *boi*^{NP4065} (top), *sgg*^{NP4101}-*Gal4* (middle), and *sgg*^{NP7270} (bottom) weakly promote expression in terminal filament and cap cells. *UAS-GFP-nls* (green, weak, left) and *UAS-Tau-GFP* (green, strong, right) are easily visualized. (B) *Hh-GFP* expression (green) under control of *sgg*^{NP4101}-*Gal4* drives FSC proliferation, resulting in long stalks (arrowheads, Fas3+, red) between developing germ-line cysts (Vasa, blue).

2005; Morris and Spradling 2011). The posterior-most IGS cell directly contacts FSCs (Margolis and Spradling 1995; Nystul and Spradling 2007, 2010; Xie 2013), and cell-cell adhesion between this posterior-most IGS cell and FSCs is thought to be essential for long-term FSC maintenance (Song *et al.* 2002). Previous work suggested that a subset of IGS cells originated from a stem cell population located adjacent to germ-line stem cells (Decotto and Spradling 2005). These escort cells were thought to travel together with developing germ-line cysts and pass them forward to waiting FSCs for envelopment by a layer of follicle cells. More recent work indicates that IGS cells are stationary and are not likely to be derived from a stem cell population (Kirilly *et al.* 2011; Morris and Spradling 2011). Direct imaging of early germ-line cyst development revealed that cysts are passed instead from IGS cell to IGS cell via dynamic cellular projections (Morris and Spradling 2011). Multiple studies have assessed the effects of genetic mutations on IGS cells using drivers such as *c587-Gal4* and *patched (ptc)-Gal4*. Clear conclusions can be drawn for the effects of these changes on IGS cells located anterior to the region 2A–2B border. However, both *Gal4* drivers are also expressed in FSCs and their pre-follicle cell daughters in addition to IGS cells (Song *et al.* 2004; Decotto and Spradling 2005; Eliazar *et al.* 2014), making it challenging to definitively distinguish labeled posterior IGS cells

from FSCs at the region 2A–2B border in order to analyze IGS cell dynamics and identify the mechanisms that control their behavior.

Fifteen *Gal4* lines that drove expression of *UAS-GFP* in IGS cells were identified in our screen. *ptc-Gal4* and *sli-Gal4*^{NP1625} promoted *UAS-GFP* expression in all somatic cells in the anterior half of the germarium, including terminal filament and cap cells, IGS cells, and FSCs (Figure 3A and Table 1) (Casanueva and Ferguson 2004; Gancz *et al.* 2011; König *et al.* 2011; Sahai-Hernandez and Nystul 2013). In some germaria, the domain of expression extended further to the posterior and included FSC progeny, the early follicle cells (Forbes *et al.* 1996b; Casanueva and Ferguson 2004; Gancz *et al.* 2011; König *et al.* 2011; Sahai-Hernandez and Nystul 2013). Like *ptc-Gal4* and *sli-Gal4*^{NP1625}, *dally-like (dlp)*^{GMR53H05}-*Gal4*, *dlp*^{GMR61C11}-*Gal4*, and *sgg-Gal4*^{NP0082} promoted *UAS-GFP* expression in all anterior somatic cells as well as in FSCs and occasionally in FSC progeny (Figure 3B). In contrast, two *Gal4* insertions into the *forked end (fend)* locus (*fend-Gal4*^{112A} and *fend-Gal4*^{NP4124}) and *engrailed (en)-Gal4*^{e22c} exhibited more restricted expression in IGS cells, with occasional cap cells and FSCs also observed (Figure 3, C and D). Three additional *Gal4* drivers, *645b-Gal4*, *323a-Gal4*, and *c532-Gal4*, promoted expression in most cell types in the germarium, including

terminal filament and cap cells, IGS cells, FSCs, and follicle cells (Figure 3E). These new tools will be useful for altering gene expression within IGS cells and FSCs (see below) as well as for delineating commonalities in gene expression between these two cell types.

Utility of new *Gal4* lines during late development

The *UAS-GFP-nls* line used in our expression screen exhibited *Gal4*-independent expression in later stages of oogenesis in anterior follicle cells, posterior follicle cells, and border cells (Figure 4A), but no expression was observed in the germarium (Figure 4F). However, several *Gal4* lines exhibited expression patterns that were distinct from the background *UAS-GFP-nls* expression (Table 2). Strong GFP expression in posterior and lateral follicle cells was observed in *bab-Gal4*, *e22c-Gal4*, *fend*^{NP4124}-*Gal4*, *ptc-559.1-Gal4*, *GR1-Gal4*, *GawB-55b-Gal4*, *c135-Gal4*, *109-30-Gal4*, *109-53-Gal4*, *645b-Gal4*, and *c323-Gal4* (Table 2 and Figure 4, C and D). *bab-Gal4*, *109-30-Gal4*, and *GR1-Gal4* were expressed in polar cells (Figure 4E). Finally, *bab-Gal4*, *109-53-Gal4*, *109-30-Gal4*, *fend*^{NP4124}-*Gal4*, and *185Y-Gal4* promoted expression in stalk cells throughout development (Figure 4F).

109-30-Gal4-labeled FSCs exhibit novel cellular projections

A major goal of our screen was to identify new methods for marking and altering gene expression in FSCs. FSCs are long-lived cells that divide frequently, enabling them to be labeled using standard mitotic recombination marking techniques such as the MARCM system (Nystul and Spradling 2007). FSCs remain in position in the germarium, while their follicle cell daughters wrap around developing germ-line cysts and move out of the germarium within 4–5 days of clone induction. Thus label-retaining cells that are located at the region 2A–2B border exhibit a distinctive triangular shape due to engagement with the basement membrane and express low levels of markers of polarized follicular epithelial cells such as *Fas3* that are defined as FSCs (Margolis and Spradling 1995; Zhang and Kalderon 2000, 2001; Nystul and Spradling 2007; Wang *et al.* 2012). Use of any of these criteria in the absence of others can lead to confusion between FSCs and their pre-follicle cell daughters. For example, low *Fas3* expression is also a characteristic of a population of FSC daughter cells called *pre-follicle cells* that are thought to migrate across the germarium after completion of cell division. While these cells clearly retain sufficient properties of FSCs to actually acquire an FSC fate, they are considered to be distinct from *bona fide* FSCs (Nystul and Spradling 2007, 2010; Sahai-Hernandez and Nystul 2013).

All *Gal4* drivers that have been used to genetically manipulate FSCs also exhibit expression in other somatic cells within the germarium. In situations where a genetic mutation alters the positioning of FSCs, definitive identification and assessment of the effects of the defect on FSCs specifically are challenging. For example, integrin-mutant FSCs lose contact with the basement membrane, a defect that leads to altered cell shape and an inability to maintain positioning at the surface of the

Figure 3 *Gal4* expression in IGS cells. (A) *ptc-Gal4* and *sli-Gal4* promote GFP (green) expression in all somatic cells in the anterior half of the germarium, including terminal filament and cap cells, IGS cells, FSCs, and pre-follicle cells. (B) GFP (green) expression in anterior somatic cells of the germarium under control of *dlp-Gal4* and one *sgg-Gal4* line (*sgg*^{NP0082}). (C and D) *fend-Gal4* and *en-Gal4* exhibit more restricted GFP (green) expression in IGS cells and FSCs. (E) Broad GFP (green) expression in all somatic cells within the germarium, including terminal filament and cap cells, IGS cells, FSCs, pre-follicle cells, and follicle cells, is observed in *645b-Gal4*, *323a-Gal4*, and *c532-Gal4*. Cells in all panels are immunostained with Vasa (blue) to label germ-line cells and *Fas3* (red) to label follicle cells. Scale bars are indicated in each panel.

Figure 4 *Gal4* expression in late-stage somatic cells. (A) *Gal4*-independent expression of UAS-GFP (green) is observed in anterior and posterior follicle cells. No GFP expression is observed in the germarium. (B) Many *Gal4* lines exhibit only nonspecific UAS-GFP expression (e.g., *GMR^{30H03}-Gal4*). (C and D) Specific, strong GFP expression is observed in lateral and posterior follicle cells and border cells in (C) *GR1-Gal4* and (D) *c135-Gal4-Gal4* and *c135-Gal4*. *Gal4*-mediated GFP (green) expression is observed in (E) polar cells (*bab-Gal4*) and (F) stalk cells (*185Y-Gal4*). Germ-line cells (blue) and follicle cells (Fas3, red) are labeled. Scale bars are indicated.

germarium (O'Reilly *et al.* 2008). These mutant cells exhibit all other characteristics of FSCs, including label retention, the ability to generate daughter cells that are incorporated into the follicular epithelium (albeit with significant morphologic defects), and expression of low levels of Fas3 (O'Reilly *et al.* 2008). Moreover, loss of integrin function in follicle cells that have already initiated differentiation has no effect on their morphology and development (O'Reilly *et al.* 2008), supporting the idea that the primary function of integrins is to maintain the location and morphology of FSCs themselves. However, difficulties with definitive FSC identification leave open the possibility that integrin mutation affects the ability of developing follicle cells to incorporate into the follicular epithelium and that the integrin-mutant-label-retaining cells that are observed are displaced follicle cells rather than mutant FSCs.

To address this problem, we developed two new methods for genetically manipulating FSCs without affecting neighboring cells. The *109-30-Gal4* driver is expressed in FSCs and all FSC daughter cells up to stage 3 (Figure 5A) (Hartman *et al.* 2010). After stage 3, *109-30 Gal4* promotes UAS-GFP expression in stalk cells only (Figure 5B) (Hartman *et al.* 2010). To determine whether mitotic FSC clones can be generated using *109-30-Gal4*, 2- to 7-day-old females were heat shocked to induce *109-30-Gal4* expression via the MARCM system (*Ub-RFP, Gal80 19AFRT Flp122/19AFRT; 109-30/UAS-GFP*) and analyzed weekly for GFP expression. One week after clone induction, a single label-retaining WT cell located at the region 2A–2B border that expressed low levels of Fas3 was observed in a small number of germaria (<5%) (Figure 5C). Labeled

cells were able to produce daughter cells that polarized and became incorporated into the follicular epithelium (Figure 5D). By 2 weeks after clone induction, cells located at the anterior end of the follicular epithelium at the region 2A–2B border and many daughter cells were labeled using this approach (Figure 5D). By 3 weeks after clone induction, the follicular epithelium in some germaria was 100% derived from GFP+ FSC clones (Figure 5D), again emphasizing the location and morphology of the FSC as critical criteria for identification.

A previous report suggested that the *109-30-Gal4* driver is expressed in a small number of IGS cells in addition to FSCs and their daughter cells (Sahai-Hernandez and Nystul 2013). Consistent with this, ovaries isolated from flies that were heat shocked as third-instar larvae and incubated for 7 days after heat shock exhibited GFP-labeled MARCM clones in IGS cells, FSCs, and follicle cells, all cell populations that divide frequently at that stage of development (Figure 5, E and G). In contrast, ovaries isolated from heat-shocked adults exhibited a high percentage of GFP-labeled FSC clones and follicle cell clones, with rare cases of labeled cells that might be IGS cells or FSCs (Figure 5, C, D, F, and G), supporting previous observations that escort/IGS cells rarely divide in adult ovaries (Decotto and Spradling 2005; Kirilly *et al.* 2011; Morris and Spradling 2011; Sahai-Hernandez and Nystul 2013). These results suggest that 9 of 10 GFP-labeled cells observed at the region 2A–2B border in ovaries isolated from heat-shocked adults are FSCs rather than IGS cells. The remaining cell might be an IGS cell or an FSC (Figure 5C). To further assess the ability of *109-30-Gal4* to generate GFP-labeled FSCs, the

Table 2 Quantitation of Expression of UAS-GFP-nls by Given *Gal4* Insertions during Late Development

<i>Gal4</i>	Insertion	Inserted gene	Reference	Total Exp.	No Exp.	% No exp.	% PF cells	% AF cells	% LF cells	% Polar	% Border	% Stalk
<i>boj</i> ^{NP4065}	<i>P{GawB}NP4065</i>	<i>boi</i>	Hayashi et al. 2002	80	0	0.00	0.00	100.00 I(8–12)	0.00	0.00	100.00 S(8,9)	0.00
<i>bab</i> ^{Gal4-2}	<i>P{GawB}bab1</i>	<i>bric-à-brac</i>	Bolivar et al. 2006	230	0	0.00	100.00 W(≥8)	100.00W(≥8)	100.00 W(2–5)	100.00 S(2–10)	100.00 W(≥7)	100.00 S
<i>dlp</i> ^{GMR53H05}	<i>P{GMR53H05-GAL4}attP2</i>	<i>dally-like protein</i>	Pfeiffer et al. 2011	81	40	49.38	0.00	50.62 W(8)	0.00	0.00	32.10 W(9–11)	0.00
<i>dlp</i> ^{GMR52D02}	<i>P{GMR52D02-GAL4}attP2</i>	<i>dally-like protein</i>	Pfeiffer et al. 2011	53	8	15.10	0.00	84.91 S(≥6)	0.00	0.00	1.89 W(9)	0.00
<i>dlp</i> ^{GMR61C11}	<i>P{GMR61C11-GAL4}attP2</i>	<i>dally-like protein</i>	Pfeiffer et al. 2011	98	27	27.55	0.00	72.45 I(≥5)	0.00	0.00	72.45 W(≥8)	0.00
<i>e22c</i>	<i>P{GawB}e22c</i>	<i>engrailed</i>	Duffy et al. 1998	89	0	0.00	100.00 W(5–9)	100.00 W(5–8)	100.00 W(5)	0.00	100.00 W(8–10)	0.00
<i>fend</i> ^{NP112A}	<i>P{GawB}112A</i>	<i>forked ends</i>	Larkin et al. 1996	53	1	1.89	0.00	98.11 W(≥8)	0.00	0.00	98.11 W(≥8)	0.00
<i>fend</i> ^{NP5045}	<i>P{GawB}fend^{NP5045}</i>	<i>forked ends</i>	Hayashi et al. 2000	62	0	0.00	14.52 I(≥8)	100.00 S(≥6)	0.00	0.00	35.48 S(≥8)	0.00
<i>fend</i> ^{NP4124}	<i>P{GawB}fend^{NP4124}</i>	<i>forked ends</i>	Hayashi et al. 2002	112	0	0.00	100.00 W(≥8)	100.00 S(≥8)	100.00 S(9,10)	0.00	100.00 S(≥8)	100.00 S
<i>hh</i> ^{GMR28E04}	<i>P{GMR28E04-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	99	28	28.28	0.00	71.72 W(≥6)	0.00	0.00	3.03 W(9)	0.00
<i>hh</i> ^{GMR28E03}	<i>P{GMR28E03-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	82	13	15.85	84.15 W(≥8)	84.15 W(≥8)	0.00	0.00	84.15 W(≥8)	0.00
<i>hh</i> ^{GMR28D09}	<i>P{GMR28D09-GAL4}</i>	<i>hedgehog</i>	Pfeiffer et al. 2011	90	48	53.33	0.00	46.67 W(6–8)	0.00	0.00	0.00	0.00
<i>pnr</i> ^{MD237}	<i>P{GawB}pnrMD237</i>	<i>pannier</i>	Brand and Perrimon 1993	61	0	0.00	27.87 W(6–7)	100.00 S(≥6)	0.00	0.00	37.70 W(≥8)	0.00
<i>ptc</i> ^{559.1}	<i>P{GawB}ptc559.1</i>	<i>patched</i>	König et al. 2011	86	11	12.79	86.05 S(≥8)	86.05 S(8–10)	0.00	0.00	87.21 S(≥8)	0.00
<i>sgg</i> ^{NP0082}	<i>P{GawB}sgg^{NP0082}</i>	<i>shaggy</i>	Hayashi et al. 2002	74	12	16.22	40.54 W(≥6)	83.78 W(≥5)	0.00	0.00	14.86 W(≥8)	0.00
<i>sgg</i> ^{NP2253}	<i>P{GawB}sgg^{NP2253}</i>	<i>shaggy</i>	Hayashi et al. 2002	59	28	47.46	0.00	52.54 W(6–8)	0.00	0.00	0.00	0.00
<i>sgg</i> ^{NP4101}	<i>P{GawB}sggNP4101</i>	<i>shaggy</i>	Hayashi et al. 2002	69	16	23.19	46.38 I(≥6)	76.81 I(≥4)	0.00	0.00	40.58 W(≥8)	0.00
<i>sgg</i> ^{NP7167}	<i>P{GawB}sggNP7167</i>	<i>shaggy</i>	Hayashi et al. 2002	96	0	0.00	0.00	0.00	0.00	0.00	100.00 I(≥8)	0.00
<i>sgg</i> ^{NP7270}	<i>P{GawB}sggNP7270</i>	<i>shaggy</i>	Hayashi et al. 2002	114	26	22.81	35.96 W(≥6)	77.19 I(≥6)	0.00	0.00	24.56 W(≥8)	0.00
<i>sl</i> ^{GMR30H09}	<i>P{GMR30H09-GAL4}attP2</i>	<i>sli</i>	Pfeiffer et al. 2011	55	14	25.45	0.00	0.00	0.00	0.00	74.55 W(≥8)	0.00
<i>SNF4A-γ</i> ^{GMR31C04}	<i>P{GMR31C04-GAL4}attP2</i>	<i>SNF4Agamma</i>	Pfeiffer et al. 2011	65	41	63.08	9.23 W(6–8)	36.92 W(6–8)	0.00	0.00	4.62 W(≥9)	0.00
<i>SNF4A-γ</i> ^{GMR30H03}	<i>P{GMR30H03-GAL4}attP2</i>	<i>SNF4Agamma</i>	Pfeiffer et al. 2011	103	44	42.73	13.59 W(9)	57.28 W(6–10)	0.00	0.00	6.80 W(9–10)	0.00

(continued)

Table 2, continued

Gal4	Insertion	Inserted gene	Reference	Total	No Exp.	% No exp.	% PF cells	% AF cells	% LF cells	% Polar	% Border	% Stalk
wg ^{GMR16D01}	P{GMR16D01-GAL4}attP2	wingless	Pfeiffer et al. 2011	57	24	42.11	0.00	57.89 W(≥8)	0.00	0.00	0.00	0.00
wg ^{GMR17D09}	P{GMR17D09-GAL4}attP2	wingless	Pfeiffer et al. 2011	123	23	18.70	0.00	73.98 I(≥8)	0.00	0.00	73.98 I(≥8)	0.00
109-28	P{GawB}109-28	Unknown	Younger 2001 ^a	97	20	20.62	0.00	79.38 W(≥8)	0.00	0.00	79.38 W(≥8)	0.00
109-30	P{GawB}109-30	Unknown	Hartman et al. 2010	92	0	0.00	100.00 I(4)	100.00 S(≥4)	100.00 I(4-8)	100.00 S(≥4)	100.00 S(≥8)	100.00 S
109-53	P{GawB}109-53	Unknown	Assa-Kunik et al. 2007	85	0	0.00	100.00 W(≥4)	0.00	0.00	0.00	100.00 W(≥8)	100.00 S
109C1	P{GawB}109C1	Unknown	Younger 2001 ^a	121	25	20.66	0.00	0.00	0.00	0.00	79.34 I(≥8)	0.00
185Y	P{GawB}185Y	Unknown	Manseau et al. 1997	81	0	0.00	0.00	0.00	0.00	0.00	4.94 W(8)	95.05 S
645b	P{GawB}645b	Unknown	Manseau et al. 1997	80	1	1.25	52.50 S(≥8)	98.75 S(≥8)	100.00 S(≥10)	0.00	100.00 S(≥10)	0.00
c323a	P{GawB}c323a	Unknown	Manseau et al. 1997	73	13	17.81	82.19 I(≥8)	82.19 I(≥8)	82.19 I(≥8)	0.00	82.19 I(≥8)	0.00
c458	P{GawB}c458	Unknown	Manseau et al. 1997	67	7	10.45	23.88 W(≥8)	89.55 S(≥8)	0.00	0.00	0.00	0.00
cb13	P{GawB}cb13	Unknown	Ward et al. 2002	102	24	23.53	76.47 W(6-8)	76.47 S(≥6)	0.00	0.00	1.96 W(9)	0.00
GR1	P{GawB}GR1	unknown	Gupta and Schüpbach 2003	55	0	0.00	100.00 S(≥5)	100.00 S(≥5)	100.00 S(≥5)	100.00 S(≥4)	100.00 S(≥5)	0.00
55b	P{GawB}Eip63F-1 ^{55b}	Eip63F-1	Brand and Perrimon 1993	64	10	15.63	6.25 W(≥7)	84.38 I(≥7)	84.38 I(≥7)	0.00	0.00	0.00
c135	P{GawB}c135	unknown	Manseau et al. 1997	68	0	0.00	100.00 S(≥4)	100.00 S(≥4)	100.00 S(≥4)	0.00	1.47 W(≥9)	0.00

^a Personal communication.

Columns titled "Total" represent the number of late-stage egg chambers exhibiting a given expression pattern, and columns titled "% No exp." exhibit a given expression pattern. No expression ("No exp."), posterior follicle cells ("PF cells"), anterior follicle cells ("AF cells"), lateral follicle cells ("LF cells"), polar cells ("Polar"), border cells ("Border"), and stalk cells ("Stalk") were assessed. References are given for the first description of patterns of expression during oogenesis or for uncharacterized Gal4 lines for the generation of the line. Strength of GFP expression is indicated with S (strong), I (intermediate), or W (weak). Numbers in parenthesis indicate stage of GFP expression observed. Shaded rows indicate Gal4 drivers with expression in addition to the leaky expression of GFP-nls.

location of GFP-labeled *109-30-Gal4*-induced MARCM clones relative to IGS cells expressing *lacZ* under control of the *hh-lacZ* (Forbes *et al.* 1996a) or *ptc-lacZ* (Forbes *et al.* 1996b) promoters was examined. Ovaries isolated from heat-shocked adults exhibited GFP-labeled cells located one cell posterior to *hh-lacZ*-expressing cells (Figure 5H). Overlapping expression of GFP and *hh-lacZ* was never observed ($N = 54$). Because *hh-lacZ* strongly labels terminal filament and cap cells with weaker expression in IGS cells and no expression in FSCs (Forbes *et al.* 1996a; Sahai-Hernandez and Nystul 2013), these data strongly support the idea that *109-30-Gal4* predominantly marks FSCs and not IGS cells under these conditions.

Many germaria exhibited *109-30-Gal4*-stimulated GFP-labeled clones located one cell posterior to *ptc-lacZ*+ cells, a pattern similar to that observed for *hh-lacZ* (Figure 5I). However, *ptc-lacZ* expression also was observed in GFP-labeled cells at the region 2A–2B border and occasionally in cells further to the posterior (Figure 5J). These results support previous reports that the *ptc* promoter drives expression in multiple somatic cell populations throughout the germarium (Forbes *et al.* 1996b; Casanueva and Ferguson 2004; Gancz *et al.* 2011; König *et al.* 2011; Sahai-Hernandez and Nystul 2013) and emphasizes the need for caution in using *ptc* as a marker for distinguishing FSCs from IGS cells and pre-follicle cells.

In addition to previously reported characteristics of FSCs, including location, low *Fas3* expression, and the ability to generate clonally identical daughter cells, MARCM-labeled FSCs extended a thick, axon-like projection from the marked FSC across the germarium, ending at the opposite surface of the germarium on the border of regions 2A and 2B (Figure 5K). This projection was seen when FSCs were marked either with *UAS-CD8-GFP*, which labels the plasma membrane (Figure 5K); *UAS-GFP-nls*, in which GFP localizes primarily to the nucleus but also weakly to the cytoplasm (Figure 5L); and *UAS-Tau-GFP*, which binds to microtubules (Figure 5M). This suggests that FSCs project a prominent microtubule-containing cytoplasmic extension, a previously undescribed feature of FSCs.

Stimulation of the FSC quiescence-to-proliferation transition by feeding

Although *109-30-Gal4* has been used previously to mark and genetically manipulate FSCs and their immediate daughter cells (Hartman *et al.* 2010, 2013; Sahai-Hernandez and Nystul 2013), the straightforward MARCM approach does not allow direct analysis of FSC dynamics without complications of label expression in the daughter cells of FSC divisions. To address this, we developed a method for analysis of single, marked FSCs before they have generated any daughter cells. Our goal was to induce a quiescent FSC state and then provide a stimulus to initiate FSC division in a temporally predictable manner. Previous work demonstrated that FSC division slows dramatically when flies are transferred to “poor food,” composed primarily of molasses, a substance that is rich in complex carbohydrates but lacks sufficient protein and lipid to support normal ovarian stem cell proliferation (Drummond-Barbosa

and Spradling 2001). This suggested the possibility that changes in nutrient status might be useful for controlling the quiescence-to-proliferation transition of FSCs. Whereas FSCs continued to divide at a dramatically reduced rate when flies were raised on molasses plates (Drummond-Barbosa and Spradling 2001), FSC division was arrested in flies raised on grape juice plates (Figure 6A), demonstrating induction of a quiescent state. FSC quiescence was achieved after 2 days of culture on grape juice plates, and no FSC division was observed after this time point (Figure 6B). Feeding flies with yeast paste stimulated FSC division within 6 hr of feeding, as expected (Hartman *et al.* 2013). These results suggest that the FSC quiescence-to-proliferation transition can be manipulated by changes in nutrient status.

Displacement of FSC daughters around the circumference of the germarium and establishment of a web of projection at the region 2A–2B border

To assess the morphologic changes in FSCs that occur during cell division, positively marked FSC clones (*Gal80 19AFRT Flp122/19AFRT; 109-30-Gal4/UAS-GFP*) were generated in well-fed adult flies with dividing FSCs by heat shocking on day 0. On day 4 after clone induction, when differentiated cells that were labeled by the initial heat shock were incorporated into follicles and exited the germarium, FSC quiescence was induced by transferring heat-shocked flies to grape juice plates. In flies that were nutrient deprived for 3 days, non-dividing, positively marked FSC clones were easily identified by robust GFP expression, location on the region 2A–2B border, triangular morphology, low *Fas3* expression, and an axon-like projection that extended across the germarium to the opposite surface when viewed in two dimensions (Figure 7A). About 61% of germaria contained a marked FSC ($N = 344$), but fewer than 5% contained a single labeled cell. Six hours after feeding, a small number of germaria with only two–four GFP-labeled cells were observed, indicating that cell division had occurred as expected (Figure 7B) (Hartman *et al.* 2013).

Examination of 3D reconstructions of cross sections of the region 2A–2B border revealed that FSCs in nutrient-restricted flies remained close to the surface of the germarium, with short, thick projections extending from either side along the basement membrane (Figure 7C). FSC projections elongated in germaria isolated 6 hr after feeding that exhibited one–four labeled cells, with daughter cells displaced along the extended projections (Figure 7D). Labeled cells generated from subsequent cell divisions were arrayed side by side around the surface of the germarium but at a constant distance from the central axis of the germarium (Figure 7, E and F). Most germaria exhibited side-by-side labeled cells at 1 week (13 of 14 germaria), 2 weeks (16 of 17 germaria), and 3 weeks (18 of 22 germaria) after feeding. In some cases, labeled cells were observed on opposite sides of the germarium (1 of 14 at 1 week, 1 of 17 at 2 weeks) or located at a distance from other labeled cells but connected by a labeled projection (4 of 22 at 3 weeks). Two or three weeks after feeding, some germaria (7 of 39)

Figure 5 *109-30-Gal4* as a tool for visualizing FSCs. (A) *109-30-Gal4* promotes GFP (green) expression in FSCs and follicle cells up to stage 3. (B) After stage 3, *109-30-Gal4* promotes GFP (green) expression in polar and stalk cells. (C) Single cells at the region 2A–2B border can be labeled using *109-30-Gal4* and the MARCM system. (D) *109-30-Gal4/UAS-GFP*-labeled cells (green) produce clonally identical daughter cells. (E) Larval heat shock results in GFP labeling (green) of IGS cells (yellow arrow), FSCs (white arrow), cap cells (pink arrows), and follicle cells (blue arrow). (F) Adult heat shock results in GFP labeling (green) of cells resembling FSCs (low Fas3, red) and generation of GFP-labeled follicle cells. (G) Quantitation of the percent of germlaria bearing GFP-labeled IGS cells, FSCs, or follicle cells (fc) in heat-shocked larvae ($N = 192$) or adults ($N = 211$). Total numbers of germlaria bearing clones in the indicated cell type were scored. IGS cell numbers in larval heat shock vs. adult heat shock are statistically different ($P < 0.00001$). (H) GFP-labeled FSCs (green, white arrowhead) are located posterior to *hh-lacZ*-expressing IGS cells (β -Gal, red, yellow arrowhead). *hh-lacZ* is also expressed in the terminal filament and cap cells (blue arrow). (I) GFP-labeled FSC (green, white arrowhead) is located posterior to *ptc-lacZ*-expressing IGS cells (β -Gal, red, yellow arrowheads). (J) *ptc-lacZ* (β -Gal, red) is expressed in IGS cells (yellow arrowheads), FSCs (white arrow), and early follicle cells (blue arrowheads). (K) *109-30-Gal4*-labeled cells (CD8-GFP, green) exhibit axon-like projections (arrowhead). (L) GFP-nls-labeled cytoplasm (green) and (M) Tau-GFP (green) are present in projections. Germaria in all panels are labeled with Vasa (blue, germ cells) Fas3 (red, follicle cells), or FC-NA (red, nuclei) and GFP (CD8-GFP, Tau-GFP, or GFP-nls, as indicated, green). White arrowheads indicate the locations of FSCs.

were observed in which all cells were derived from the same original FSC, with labeled cells ringing the germlarium at the region 2A–2B border (Figure 7G). Cellular projections extended from FSCs and their labeled daughter cells, creating a weblike structure that increased in size with the number of labeled cells, eventually spanning the region 2A–2B border (Figure 7, H–J). Fully clonal germlaria generated extensive interconnected projection networks, with

some projections extending anterior to the region 2A–2B border (Figure 7, J–L).

fend-Gal4 predominantly marks FSCs independently of other cell types

Combined with carefully timed clone generation, these results suggest that *109-30-Gal4* will be a powerful tool for measuring the morphology and location of FSCs and their daughter cells

Figure 6 Nutrient deprivation induces a quiescent state in FSCs. (A) FSCs fail to divide in flies raised on fruit juice-only plates ($N = 622$). Flies fed molasses maintain a constant rate of FSC division ($N = 680$). (B) 0.5–1% of germaria isolated from flies fed yeast exhibit proliferating FSCs ($N = 647$). Proliferation rates are 1 day after transfer to fruit juice plates ($N = 132$), but FSC proliferation arrests at 2 days ($N = 168$), and quiescence is maintained at 3 days ($N = 345$), a time point that is used for the experiments described later.

at specific time points after induction of the quiescence-to-proliferation transition. One of our primary goals, however, was to develop tools for genetically manipulating FSCs without affecting other cells within the germarium. We noticed that all *Gal4* drivers that exhibited strong expression in IGS cells also were expressed in FSCs (Figure 3). In contrast, most of these drivers were not expressed in differentiating follicle cells. IGS cells divide very infrequently in adult flies in contrast to FSCs (Margolis and Spradling 1995; Decotto and Spradling 2005; Morris and Spradling 2011; Sahai-Hernandez and Nystul 2013), suggesting that these drivers might be useful for generating marked clones in mitotically active FSCs without affecting the dormant IGS cells that also express *Gal4*. To test this idea, MARCM clones were generated using *fend-Gal4*^{112A} and *fend-Gal4*^{NP4124}, both of which are insertions in the *forked ends* (*fend*) locus.

We were initially interested in *fend-Gal4*^{112A} because of its mosaic expression pattern. In most germaria, UAS-GFP expression was observed in cap cells, IGS cells, and FSCs (Figure 3B). Occasional GFP-expressing somatic cells were observed in region 3 of the germarium and in stalks and older egg chambers (data not shown). In rare cases, expression was limited to FSCs alone (Figure 8A), suggesting that *fend-Gal4*^{112A} might be useful as a FSC-specific tool. To test this idea, MARCM clones were generated using *fend-Gal4*^{112A} together with *UAS-Tau-GFP*. Labeled cells exhibited morphology that was indistinguishable from *109-30-Gal4*-generated FSC clones, including positioning at the region 2A–2B border, association with the

extracellular matrix, characteristic triangular shape, low Fas3 expression, and a prominent projection extending to the opposite surface of the germarium (Figure 5G and Figure 8B). Similar results were observed using *fend-Gal4*^{NP4124} (Figure 8C). GFP-labeled cells generated using *fend-Gal4*^{NP4124} produced marked follicle cells over the course of 3 weeks after clone induction (Figure 8D), indicating that the labeled cells were FSCs. About 12.5% of germaria bearing a *fend-Gal4*^{NP4124}-induced FSC clone also exhibited one or more marked IGS cells (Figure 8, E and F). Unlike marked cells generated using *109-30-Gal4*, which were always located at the region 2A–2B border, *fend-Gal4*^{NP4124} induced marked IGS cell clones located several cell diameters anterior of the region 2A–2B border (Figure 8E). This suggests that this combined approach can be used to label FSCs specifically in >85% of germaria. However, data obtained using this approach should take into account a potential 12.5% error rate, an issue that can be addressed through careful analysis of the positioning of marked clones, colabeling with *hh-lacZ*, measuring the ability of labeled cells to produce marked follicle cells, and assessment of sufficient numbers of germaria for statistical power.

We found that FSCs marked using *fend-Gal4*^{NP4124} and *109-30-Gal4* exhibited the same morphologic features at time points after the feeding-stimulated transition from starvation-induced quiescence to proliferation (Figure 8, G–J). Newly generated daughter cells appeared to track along cellular projections extended by FSCs along the circumference of the germarium, maintaining a constant distance from the central axis of the germarium (Figure 8J). Thus two independent FSC marking techniques enable the analysis of FSC morphology during division after a period of cell cycle arrest and will be useful for assessing the effects of genetic mutations within FSCs on these sequential events.

Integrins provide directional information to FSCs during cell division

Our previous work demonstrated that integrins are necessary for FSC positioning, morphology, and proliferation (O'Reilly *et al.* 2008). However, integrin-mutant FSCs were displaced from the niche and exhibited alterations in FSC location and shape (O'Reilly *et al.* 2008), making it difficult to definitively distinguish *bona fide* mutant FSCs from mislocalized daughter cells. As expected from our previous work, FSCs lacking expression of the β PS-integrin encoded by the *myospheroid* (*mys*) gene that were MARCM labeled with *109-30-Gal4* (*mys*^{M2} FRT 19A/RFP *Gal80* 19A FRT *Flp122*; *109-30-Gal4/UAS-CD8-GFP*) exhibited dramatic differences in morphology relative to WT FSCs (Figure 9). By 1 week after clone induction, integrin-mutant FSCs lost their triangular shape and were mislocalized to the center of the germarium (Figure 9A). Fewer labeled integrin-mutant FSCs relative to WT cells were observed at 1 week after clone induction, consistent with our previous report (Figure 9D) (O'Reilly *et al.* 2008). Labeled FSC clones expressing a β -subunit lacking the cytoplasmic domain (*mys*^{P9} 19AFRT/*Ub-RFP*, *Gal80* 19AFRT *Flp122*; *109-30 Gal4/UAS-GFP*) exhibited the same

dramatic morphologic changes as FSCs lacking β PS expression altogether (Figure 9, B and D). Similar results were observed with additional *mys* mutants that influence adhesion strength to the extracellular matrix (Figure 9, C and D (Jannuzzi *et al.* 2004)).

Surprisingly, however, a significant number of germaria retained MARCM-labeled integrin-mutant FSCs throughout the time course [13–30% of germaria, $N = 448$ (*mys^{P9}*), 437 (*mys^{m2}*), and 554 (*mys^{b13}*)], and the rate of FSC loss was not statistically different from that observed for WT FSCs ($N = 504$) (Figure 9, A–D). By 3 weeks after clone induction, 81.5% of integrin-mutant FSCs were located in the center of the germarium compared with WT FSCs, which exhibited only 2.8% displacement from the basement membrane [$N = 448$ (*mys^{P9}*) and 504 (WT)] (Figure 9, A–C and E). Some labeled integrin-mutant cells were located anterior to the region 2A–2B border in region 2A and occasionally in region 1 [21.7% of displaced FSCs ($N = 60$)] (Figure 9, A and B). It is formally possible that these cells are IGS cells labeled in the initial heat shock. This is unlikely because of the very low induction of IGS cell clones in heat-shocked adults (Figure 5), the complete absence of labeled cells in regions 1 and 2 in matched WT control experiments ($N = 940$), and the aberrant morphology of labeled mutant cells observed that bears no resemblance to normal IGS cells (Figure 9A, 3 weeks). Most likely, these are displaced FSCs that would not have been detected with the negative marking technique used in our original work. Although they were retained over long time periods, morphologically defective integrin-mutant FSCs were not competitive for niche occupancy, as indicated by the lack of labeled cells in the FSC niche or labeled daughter cells at late time points (Figure 9, E and F). Projections in integrin-mutant FSCs were defective, exhibiting random orientation relative to WT FSCs, which always projected toward the opposite pole of the germarium (Figure 9G). In addition to dramatic morphologic defects, germaria bearing integrin-mutant FSCs exhibited very few clones of marked daughter cells containing more than four cells,

during the transition from quiescence to proliferation. (A) Short projections are extended by *109-30-Gal4* MARCM-labeled FSCs (green) in nutrient-restricted flies viewed in two dimensions. (B) Six hours after feeding, labeled FSCs (green) divide, with labeled daughter cells displaced along the germarial surface. (C) Labeled FSC (green) extends projections (arrowheads) around the circumference of the germarium. A cross section of the region 2A–2B border viewed from the anterior in nutrient-restricted flies is shown. (D) Six hours after feeding, FSC (green) projections lengthen (arrowheads), and a daughter cell (green) is displaced along the extended projection. (E) FSCs and daughter cells (green) become positioned around the circumference of the germarium and (F) remain at a constant distance from the central axis of the germarium. Distances between cells A and C from the central axis (B) are shown. (G) Three weeks after feeding, 10–14 labeled cells (green) ring the germarium. *109-30-Gal4*-labeled cells (CD8-GFP, green) at the region 2A–2B border (H) 1 week, (I) 2 weeks, and (J) 3 weeks after feeding viewed in cross section from the anterior. (K and L) CD8-GFP-labeled cells ring the germarium and extend projections viewed in cross section (K) or in three dimensions (L). Germaria are labeled with Vasa (blue, germ-line cells), Fas3 (red, follicle cells), and GFP (green).

Figure 8 *fend-Gal4* as a tool for analysis of the transition of FSCs from quiescence to proliferation. (A) *fend-Gal4*-labeled FSCs extend Tau-GFP (green)-containing projections. FSCs are labeled with (B) *fend*^{112A}-*Gal4* or (C) *fend*^{NP4124}-*Gal4* driving *UAS-Tau-GFP* (green) 1 week after clone induction. (D) FSC clones induced by *fend*^{NP4124}-*Gal4* populate the follicular epithelium after 3 weeks. (E) About 12.5% of germaria bearing *fend*^{NP4124}-*Gal4* FSC clones (white arrowhead) have a marked IGS cell (yellow arrowhead). (F) Quantitation of the numbers of marked IGS cells, FSCs, or follicle cells (fc) in germaria isolated from heat-shocked adults is shown. *fend*^{NP4124}-*Gal4*-labeled FSC (green) in (G) nutrient-restricted flies and (H) 6 hr after feeding. (I) Three weeks after feeding, multiple-labeled cells (green) cover half the germarium. (J) Labeled cells (A and C) maintain a constant distance from the central axis of the germarium (large white dot). Germaria are labeled with FC-NA (blue, nuclei), Vasa (blue, germ cells), Fas3 (red, follicle cells), and GFP (green) as indicated.

indicating reduced proliferation (Figure 9F). By 3 weeks after clone induction, only 38% of germaria bearing a marked integrin-mutant FSC contained four or more GFP-labeled daughter cells compared with 95% of germaria bearing WT FSC clones [$N = 448$ (*mys*^{P9}) and 504 (WT)] (Figure 9F). These results are consistent with our previous observations that the rate of FSC proliferation in integrin mutants is dramatically reduced relative to WT cells (O'Reilly *et al.* 2008) and suggests that long-lived integrin-mutant FSCs are generally quiescent.

Recently, it was shown that differentiated daughter cells influence FSCs via a signaling feedback mechanism (Vied *et al.* 2012). This makes it challenging to pinpoint whether defects observed in integrin mutants are due to loss of function within the FSC itself (O'Reilly *et al.* 2008) or whether defective signals from clonally identical integrin-mutant daughter cells influence FSC function (Vied *et al.* 2012). A third possibility is that the marked cells observed are not FSCs but instead are differentiated daughter cells that cannot effectively incorporate into the developing epithelium and are gradually pushed toward the anterior. To address this, we analyzed the effects of integrin mutation on FSCs before they have generated any

daughter cells. Marked FSC clones were induced in well-fed flies with 109-30-*Gal4*, and then FSC division was arrested by transferring the flies to nutrient-restricted food, as described earlier (Figure 6). A small number of germaria that exhibited one to four labeled cells were assessed. In three dimensions, integrin-mutant FSCs in nutrient-restricted flies exhibited similar morphology to WT FSCs, including location close to the surface of the germarium and extension of projections (Figure 10A). On feeding, however, dramatic differences were observed. By 6 hr after feeding, daughters of integrin-mutant FSC divisions were displaced toward the central axis of the germarium rather than maintaining a constant distance along the circumference (Figure 10, B–D). These daughter cells exhibited numerous short, radially arrayed, thick projections that lacked directionality similar to those seen for marked integrin-mutant cells in steady-state conditions (Figure 9 and Figure 10, C–E). The total length and volume of projections were dramatically reduced in integrin mutants (WT: $N = 21$; *mys*^{P9}: $N = 26$) (Figure 10, E and F). Unlike WT FSCs, which produced daughter cells arrayed around the circumference of the germarium over time, integrin-mutant cells were solitary,

Figure 9 *mysospheroid* (*mys*) mutation leads to morphologic and proliferation defects in FSCs. (A–C) 109-30-*Gal4*-labeled (A) *mys^{M2}*, (B) *mys^{P9}*, or (C) *mys^{b13}* mutant FSCs (*UAS-CD8-GFP*, green) at 1 week (top), 2 weeks (middle), and 3 weeks after clone induction (bottom). *Fas3* (red) marks follicle cells, and *FC-NA* (red) marks nuclei. (D) FSC loss over time for WT (Control, blue) and *mys^{P9}* mutants (*mys^{P9}*, red). (E) The percentage of displaced FSCs is shown for WT (Control, blue) and *mys^{P9}* mutants (*mys^{P9}*, red). (F) Proliferation of WT (Control, blue) vs. *mys^{P9}* FSCs (*mys^{P9}*, red) is indicated by the number of germaria containing clones of more than four daughter cells. Statistical differences ($P < 0.0001$) were observed at 2 and 3 weeks after clone induction. (G) *mys^{P9}* mutant projections lack direction, indicated by the percentage of labeled *mys^{P9}* mutant (*mys^{P9}*, red) vs. WT FSCs (Control, blue) extending a projection toward the opposite pole of the germarium. *N*-values for all panels: WT: week 1 $N = 429$, week 2 $N = 576$, week 3 $N = 504$; *mys^{P9}*: week 1 $N = 437$, week 2 $N = 537$, week 3 $N = 448$; *mys^{M2}*: week 1 $N = 692$, week 2 $N = 536$, week 3 $N = 437$; *mys^{b13}*: week 1 $N = 462$, week 2 $N = 385$, week 3 $N = 554$. *** $P < 0.0001$. Scale bars are indicated.

remaining close to the central axis throughout the 3-week time course (WT: $N = 15$; *mys*^{P9}: $N = 38$; *fend-Gal4*; *mys*^{S00043 RNAi}: $N = 26$) (Figure 10, D and G). As a result, they failed to generate the web of projections observed in WT germaria (Figure 7J vs. Figure 10, I–K). Similar results, including displacement of daughter cells toward the central axis and changes in FSC morphology, were observed when integrin function was reduced specifically in FSCs by generating MARCM clones with *fend-Gal4*^{NP4124}, which simultaneously drives expression of both GFP and an RNAi targeting *mys* (*fend-Gal4*^{NP4124}19AFRT/+; *mys*^{RNAi}^{S00043}) (Figure 10, H and L). These results indicate that cell-autonomous expression of integrins in FSCs is required to provide the directional information required for proper orientation of cell division and for niche occupancy.

Discussion

Controlling the transition of stem cells from quiescence to proliferation is critical for normal tissue maintenance and for potential use of adult stem cells in regenerative medicine. Both intrinsic and extrinsic signals contribute to this transition, but the sources of the signals and their response pathways remain largely undefined. Here we have identified new tools that permit genetic manipulation within subsets of somatic cells in the germarium in order to define the signals that directly influence FSC function, as well as other aspects of early oogenesis. Using these tools, we show that quiescent FSCs in nutrient-restricted flies extend projections from the body of the cell arching around the germarium and along the basement membrane. After division, daughter cells appear side by side along the track laid down by the FSC projections. The new cell appears to stabilize its position and produce its own projections in preparation for the next round of division. In germaria in which all the cells become clonally identical to the originally labeled FSC, daughter cells encircle the germarium, interconnected by a complex network of projections. Consistent with our previous work (O'Reilly *et al.* 2008), loss of integrin function has dramatic effects on the ability of FSCs to retain their positioning along the basement membrane. Integrins also are required for displacement of daughter cells along projections extended by FSCs and for formation of the array of labeled cells around the surface of the germarium. Although integrin-mutant FSCs exhibit projections, they lack direction, often extending into the central axis of the germarium rather than around its circumference. Together these results indicate that integrin-mediated adhesion provides directional information that maintains positioning of FSCs and their pre-follicle cell daughters along the basement membrane. This is particularly important early in the transition of FSCs from quiescence to proliferation because cells lacking directional information become mislocalized and unable to produce a normal follicular epithelium.

The projections extended by WT FSCs may promote communication between FSCs. Previous work suggests that there are exactly two FSCs in each germarium, located opposite each other in a central optical slice of the germarium (Margolis and

Spradling 1995; Nystul and Spradling 2007, 2010). In two dimensions, FSC projections appear to cross the germarium and contact the FSC niche on the opposite surface (Figure 5, E–G), supporting the idea that distant FSCs may communicate via axon-like projections. In three dimensions, however, the projection is more complex, appearing as a weblike structure that appears to contact multiple cells at the region 2A–2B border (Figure 7, H and I). In some germaria, labeled pre-follicle cell daughters become arrayed around the circumference of the germarium over time and extend projections that integrate to form a weblike barrier that spans the Region 2A–2B border (Figure 7, J–L, and Figure 11). One appealing possibility is that germ-line cysts shedding IGS cell projections in region 2A contact this web, simultaneously inducing wrapping of the cyst by the pre-follicle cells generating the net and triggering their proliferation (Figure 11). This mechanism would create a physical switch that promotes transitioning of germ-line cysts from the anterior half of the germarium, where they associate with IGS cell projections, to the posterior, where proliferating follicle cells surround each cyst to form the follicular epithelium (Figure 11). Defects in formation of the web structure are observed when integrin function is reduced in FSCs (Figure 9). This phenotype correlates with disruption of germ-line cyst architecture and malformation of developing follicles in germaria bearing an integrin-mutant FSC (Figure 8 and Figure 9) (O'Reilly *et al.* 2008). Perhaps the failure of mislocalized integrin-mutant FSCs to produce this physical switch contributes to the defects in follicle formation observed.

Our 3D analysis supports previous observations that pre-follicle cells are displaced along the basement membrane around the circumference of the germarium following FSC division (Figure 7, D and E) (Nystul and Spradling 2007, 2010). It appears that labeled FSCs and pre-follicle cells become evenly spaced in a circumferential array around the entire germarium when proliferation is stimulated by feeding after a period of starvation (Figure 7G). This may occur because of active migration of pre-follicle cells along projections extended by the FSC, generating the cross-migrating cells that have been reported to traverse along the anterior surface of the germ-line cyst moving through the region 2A–2B border or posterior migrating cells that traverse the posterior surface (Nystul and Spradling 2007, 2010). Consistent with this idea, labeled cells that lacked an apparent physical connection were observed located on opposite sides of the germarium. Under the conditions used for these experiments, however, most germaria observed 1–3 weeks after feeding exhibited labeled pre-follicle cells located side by side, apparently filling the half of the germarium closest to the labeled FSC and then spreading around the entire circumference over time (Figure 7, C–E). This suggests the possibility that in response to feeding-stimulated proliferation, pre-follicle cells in some germaria passively fill in space on subsequent divisions of FSCs and/or pre-follicle cells. This model resembles that observed in the mammalian intestinal stem cell niche (Snippert *et al.* 2010), where symmetric stem cell division generates identical daughter cells that expand laterally around the circumference

Figure 10 *myospheroid (mys)* is required for the transition from quiescence to proliferation. (A–D) Germaria are labeled with GFP (green), Vasa (blue, germ cells), and Fas3 (red, follicle cells). Scale bars are indicated. (A) In three dimensions, *mys^{P9}* mutant FSCs (green) from nutrient-restricted flies extend short projections (arrowheads) similar to those seen in WT cells. (B) Six hours after feeding, *mys^{P9}* mutant daughter cells (green) are displaced toward the central axis of the germarium rather than around its circumference. (C) Projections extended by *mys^{P9}* mutant cells are short and lack direction. (D) *mys^{P9}* mutant cells (A and B, white dots) are located close to the central axis of the germarium (C, white dot). (E) Average projection length 6 hr after feeding from labeled WT (GFP FRT19A, $N = 21$) or *mys^{P9}* mutant (*mys^{P9}* FRT19A, $N = 26$) FSCs. (F) Average volume of projections calculated for WT FSCs (Control, blue, week 1 $N = 11$, week 2 $N = 10$, week 3 $N = 11$) or *mys^{P9}* mutant FSCs (*mys^{P9}* FRT19A, red, week 1 $N = 9$, week 2 $N = 14$, week 3 $N = 10$). P -values at 2 and 3 weeks after feeding are shown. (G) The distance from the central axis of the germarium of labeled WT (GFP FRT19A, $N = 15$) or *mys^{P9}* mutant (*mys^{P9}* FRT19A, $N = 38$) cells is shown. ** $P < 0.0002$. (H) *fend-Gal4* was used to simultaneously label FSCs and reduce β PS levels by

of the crypt base and compete for occupancy of a disklike niche. Cells displaced from the niche by crowding move up the crypt and undergo differentiation (Snippert *et al.* 2010). This stochastic evolution model eventually results in a clonally identical stem cell pool within one niche. A similar stochastic model applies to germ-line stem cell maintenance in the mouse male germ line (Klein *et al.* 2010).

An appealing possibility in the fly ovary is that newly generated FSC daughter cells take up “empty slots” where new cells are needed to generate the web described earlier. In this case, daughter cells might migrate to distant sites or simply occupy empty space close to the location of the original cell division. Our results support previous observations that FSCs and their daughter cells exhibit many similar properties, including indistinguishable morphology of the cells arrayed around the circumference of the germarium (Figure 7G), contribution of projections to the web at the region 2A–2B border (Figure 7, H–L), and strikingly similar expression patterns of Patched, IMP, Slit, Notum, and G protein-coupled receptor kinase-2 (13C06-*Gal4*) in FSCs, pre-follicle cells, and IGS cells (Nystul and Spradling 2010; Sahai-Hernandez and Nystul 2013). Here we describe *Gal4* insertions into the *fend*, *shaggy*, *engrailed* (e22c), and *wingless* (16D01-*Gal4*) loci and two *Gal4s* inserted into unidentified loci (645b and c323a) as additional genes that exhibit similar expression patterns (Figure 3). Rare, if any, expression of these markers was observed in differentiated follicle cells, suggesting that overlapping expression patterns in FSCs and pre-follicle cells may be functionally relevant. Moreover, FSCs and pre-follicle cells are both competent to function as FSCs (Nystul and Spradling 2007), raising questions about the features that distinguish the two cell populations. Our results are consistent with the presence of a pool of cells that includes FSCs and pre-follicle cells located in a circumferential array at the region 2A–2B border. These cells all are competent to compete for niche occupancy and contribute to the web of projections that spans the region 2A–2B border. The new tools described here, combined with previously used clonal labeling techniques (Nystul and Spradling 2007), will provide opportunities to define precisely the distinguishing features of the two cell populations and the functional role of the projection web in follicle development.

A critical mechanism for maintaining FSC positioning is integrin-mediated adhesion (Figure 9) (O'Reilly *et al.* 2008). FSCs with reduced integrin function are rapidly lost from the niche relative to WT FSCs and undergo differentiation as follicle cells (Figure 9) (O'Reilly *et al.* 2008). In some cases, however, integrin-mutant FSCs are retained close to the region 2A–2B border for several weeks (Figure 9, A–D). These cells are found close to the central axis of the germarium, outside the FSC niche (Figure 10). As a result of this mispositioning, integrin-mutant FSCs are replaced readily by WT FSCs in the

same mosaic germarium and are generally quiescent, only rarely generating aberrant daughter cells that fail to incorporate properly into the follicular epithelium (Figure 9 and Figure 10) (O'Reilly *et al.* 2008). The effects of integrin mutation are cell autonomous because the only cells lacking integrin function in our experiments are FSCs themselves (Figure 9 and Figure 10). These results suggest that adhesion to the basement membrane is required for FSCs to maintain their position within the niche and to compete for niche occupancy with resident FSCs. The orientation of cell division, displacement of daughter cells along FSC projections, positioning of pre-follicle cells, and encapsulation of germ-line cysts all are affected by the presence of integrin-mutant FSCs within the germarium (Figure 9 and Figure 10). These defects can be attributed to the loss of directional information provided by association of integrins with components of the basement membrane at the surface of the germarium. In addition, integrin-mediated cytoplasmic signaling may contribute to receipt of proliferation signals by FSCs. Exclusive expression in FSCs of a form of the β -integrin *mysospheroid* that lacks the cytoplasmic signaling domain leads to defects that are indistinguishable from those seen in FSCs lacking all *mys* expression (Figure 9), supporting the idea that integrins may contribute to FSC regulation via downstream signaling. However, many reports have demonstrated that anchoring of the cytoplasmic domain of integrins to the actin cytoskeleton is essential for full adhesion strength (Fu *et al.* 2012). Further work will be required to identify the relative contributions of integrin-mediated adhesion and signaling in FSC proliferation regulation.

A primary goal of our study was to generate a genetic toolbox to enable analysis of the contributions of specific cell types to FSC regulation. *Gal4* lines that promote expression in individual cell types, including cap cells only and terminal filament cells only, were identified (Figure 1). These lines may be useful for dissecting the relative roles of proteins produced by both terminal filament and cap cells in regulation of germ-line stem cells, IGS cells, and FSCs. Other lines exhibited expression in more than one cell type, with a sufficient number of lines identified that allow differential levels of expression of transgenes (Figure 1, Figure 2, and Figure 3). In terminal filament and cap cells, for example, weak expression of growth factors may be achieved using *boi-Gal4* or *sgg-Gal4* without having dramatic effects on the development of the fly (Figure 2). *bab-Gal4* remains the most robust driver for terminal filament and cap cells, with many examples of its successful use for reducing gene expression with RNAi (Bolívar *et al.* 2006; Hartman *et al.* 2010, 2013; Sahai-Hernandez and Nystul 2013; Xin *et al.* 2013). All the *Gal4* lines we identified that promoted expression in IGS cells also exhibited expression in other cell types, including cap cells, FSCs, and/or pre-follicle cells. These lines may be useful when combined with temporal generation

expression of RNAi targeting the *mys* transcript. Distances from the central axis of labeled WT cells (FRT 19A $N = 15$) or *mys* knockdown (*mys* RNAi^{S00043}, $N = 34$) cells are shown. $**P < 0.05$. (I–K) Germaria are labeled with GFP (green), Vasa (blue, germ cells), and Fas3 (red, follicle cells). Scale bars are indicated. Projections extending from *mys*^{P9}-labeled cells (I) 1 week, (J) 2 weeks, and (K) 3 weeks after feeding. (L) Distance from the central axis of *fend-Gal4*-mediated *mys*^{RNAi}-expressing FSCs (green).

Figure 11 Proposed role for the web of projections in transitioning germ-line cysts from region 2A to region 2B. (A) Prior to contact by a developing germ-line cyst (blue), FSCs and pre-follicle cells ring the germarium (green circles), extending projections that form a weblike barrier (green) at the region 2A–2B border. In a cross section viewed from the anterior (A'), the web appears as a relatively solid surface. (B) Contact between the germ-line cyst in region 2 and the web initiates a series of cellular interactions that promote proliferation of follicle cells and wrapping of the posterior portion of the germ-line cyst. The web is pushed toward the posterior by the germ-line cyst that is now visible in the cross section (B'). (C) As the germ-line cyst exits region 2, proliferation follicle cells wrap the anterior end of the cyst, completing encapsulation. The cyst pushes the web into region 2B and possibly forces the exit of pre-follicle cells at the region 2A–2B border. Arrows indicate the position of FSCs.

of labeled clones in pupal stages, where IGS cells are still mitotic (Sahai-Hernandez and Nystul 2013). However, FSCs also are labeled when clones are generated in pupal stages, raising questions about how to distinguish MARCM-labeled IGS cells from FSCs when a *Gal4* driver that is expressed in both cell types is used. Genetic manipulation of IGS cells thus remains a challenge. The combined approach of timed generation of labeled mutant clones and stimulation of FSC proliferation after a period of starvation-induced quiescence now allows analysis of the roles of individual genes in the early steps of FSC division. Together these new tools will be useful for many scientists interested in the molecular control of early events in oogenesis.

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