Integrins control the positioning and proliferation of follicle stem cells in the *Drosophila* ovary

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Adult stem cells are maintained in specialized microenvironments called niches, which promote self-renewal and prevent differentiation. In this study, we show that follicle stem cells (FSCs) in the *Drosophila melanogaster* ovary rely on cues that are distinct from those of other ovarian stem cells to establish and maintain their unique niche. We demonstrate that integrins anchor FSCs to the basal lamina, enabling FSCs to maintain their characteristic morphology and position. Integrin-mediated FSC anchoring is also essential for proper development of differentiating prefollicle cells that arise from asymmetrical FSC divisions. Our results support a model in which FSCs contribute to the formation and maintenance of their own niche by producing the integrin ligand, laminin A (LanA). Together, LanA and integrins control FSC proliferation rates, a role that is separable from their function in FSC anchoring. Importantly, LanA-integrin function is not required to maintain other ovarian stem cell populations, demonstrating that distinct pathways regulate niche–stem cell communication within the same organ.

Introduction

Adult stem cells are characterized by the ability to self-renew and to generate the differentiated daughter cells needed for tissue maintenance and repair. The local environment, or niche, in which stem cells reside is critical for their maintenance and function. Positioning of the stem cell within the niche exposes it to survival and self-renewal signals, protects it from differentiation, and guides the orientation of asymmetrical cell divisions that displace nonrenewing daughter cells from the niche. These mechanisms precisely control the balance between quiescence, self-renewal, and differentiation that is required for the long-term survival of the adult stem cell and for tissue homeostasis (Fuchs et al., 2004; Ohlstein et al., 2004; Scadden, 2006).

The *Drosophila melanogaster* ovary contains three stem cell populations, germline stem cells (GSCs), escort stem cells (ESCs), and follicle stem cells (FSCs), that reside at the anterior end of the ovary in a structure called a germarium (Harrison and Harrison, 2006). GSCs divide asymmetrically such that the GSC contacts the niche and the daughter cell that is displaced from the niche initiates differentiation (Xie and Spradling, 2000). The differentiating daughter develops into a 16-cell germline cyst containing an oocyte and 15 germline support cells. In addition to their niche association, GSCs directly contact ESCs (Decotto and Spradling, 2005). Like GSCs, ESCs depend on niche-generated signals for their maintenance. ESCs divide coordinately with GSCs, producing escort cells, which accompany the developing germline cyst as it travels posteriorly through the germarium. Halfway through the germarium, the posterior-most escort cells contact a third population of stem cells, the FSCs (Margolis and Spradling, 1995; Song and Xie, 2002). FSCs generate daughter cells, called prefollicle cells, that form the follicular epithelium surrounding each germline cyst and interfollicular stalk cells, which connect adjacent follicles (King, 1970; Horne-Badovinac and Bilder, 2005). Follicles called egg chambers proceed through 14 well-characterized stages, resulting in mature oocyte production (King, 1970; Spradling, 1993). Defects in any ovarian stem cell population disrupt normal oogenesis, often resulting in female sterility.

Stem cell regulatory signals have been identified for each ovarian stem cell population (Harrison and Harrison, 2006). In general, factors secreted by the niche activate receptor-mediated

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Abbreviations used in this paper: ACI, after clone induction; ESC, escort stem cell; FSC, follicle stem cell; GSC, germline stem cell; LanA, laminin A; WT, wild type.

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signaling cascades in the stem cells that prevent stem cell differentiation and promote self-renewal. As a consequence of this signaling, cells that maintain direct contact with the niche retain stem cell identity, whereas cells that lose contact with the niche differentiate. For GSCs, E-cadherin/Armadillo junctions accumulate at the stem cell–niche cell interface and mediate cell–cell adhesion between the stem cell and cellular components of the niche (Song and Xie, 2002; Song et al., 2002). Similarly, cadherin complexes are localized at the stem cell–niche interface in *Drosophila* male GSCs (Yamashita and Fuller, 2005) and in mammalian hematopoeitic stem cells (Zhang et al., 2003), suggesting that cadherin-mediated cell–cell adhesion may be a general mechanism for maintaining stem cell positioning within the niche.

E-Cadherin complexes provide stem cell daughters with a competitive advantage for self-renewal. In mosaic germaria that contain a GSC with high E-cadherin levels (E-Cad^{hi}) and a GSC with lower levels of E-cadherin (E-Cad^{lo}), the surface of the E-Cad^{hi} GSC can expand its contact with the niche, whereas the E-Cad^{lo} GSC is displaced (Song et al., 2002; Jin et al., 2008). However, when all GSCs in a single germarium lack E-cadherin function, they are positioned correctly and are maintained at rates similar to wild-type (WT) GSCs (Song et al., 2002). These studies demonstrate that higher levels of E-cadherin expression enhance the ability of GSC daughter cells to maintain contact with niche cells (Jin et al., 2008). However, when this competition is removed, other adhesion mechanisms anchor GSCs within the niche.

Mounting evidence supports roles for integrins in stem cell anchoring within niches in other systems. Integrins are heterodimeric receptors consisting of one β subunit and one α subunit that act as cellular anchors by linking ECM molecules to the actin cytoskeleton. High expression levels of particular integrin subunits are found in some stem cell populations relative to their differentiating progeny (Jones and Watt, 1993; Shinohara et al., 1999; Shackleton et al., 2006; Stingl et al., 2006). Integrins are also thought to contribute to stem cell anchoring to the basal lamina, an extracellular structure that is a prominent feature of most stem cell niches (Watt, 2002; Fuchs et al., 2004). Integrin activation through basal lamina association has been implicated in orienting stem cell divisions, proliferation control, regulating stem cell migration and homing, and suppressing differentiation (Hirsch et al., 1996; Andressen et al., 1998; Watt, 2002; Frye et al., 2003; Benitah et al., 2005; Brakebusch and Fassler, 2005; Lechler and Fuchs, 2005). In the *Drosophila* intestine, it has been suggested that the basal lamina is the main, and possibly only, extracellular component of the niche (Ohlstein and Spradling, 2006). Together, these studies strongly support the idea that integrins regulate multiple stem cell properties. Despite substantial evidence supporting stem cell regulatory roles for integrins, loss of expression of specific integrins within stem cells has little or no effect on their positioning or function (Hirsch et al., 1996; Potocnik et al., 2000; Watt, 2002; Lopez-Rovira et al., 2005; Bungartz et al., 2006). Most likely, functional redundancy between different integrin receptors masks the importance of integrin function within these stem cell populations.

To directly assess the contribution of integrins to stem cell regulation, we have analyzed the roles of *Drosophila* integrins and their ligands in stem cell maintenance and regulation in the ovary. Our results demonstrate that one population of ovarian stem cells, the FSCs, depends on adhesive interactions between integrins and the surrounding ECM for their maintenance, anchoring, and proliferation control. We identify individual integrins that regulate these events and examine their function in the progeny produced by asymmetrical FSC division.

Results

Integrins localize to the basal surface of the germarium

We began our investigation of the role of integrins in stem cell regulation by asking whether integrins localize to the surface of stem cells or cells located adjacent to stem cells. We identified three integrin subunits that exhibited prominent cell surface localization within the germarium (Fig. 1 B). The β-integrin subunit βPS and two α-integrin subunits, αPS1 and αPS2 (Brown, 2000), colocalized on the outer, or basal, surface of the germarium. Levels of αPS1 localization were similar all along the surface of the germarium. In contrast, only low levels of localization of βPS and αPS1 were observed in the anterior half of the germarium where escort cells reside. Strikingly, high levels of localization of βPS and αPS1 were observed starting halfway through the germarium, in the approximate location of the FSCs. This localization pattern suggests that integrins may participate in FSC anchoring or regulation. Basal integrin localization remained a prominent feature in prefollicle cells and throughout the follicular epithelium and stalk cells, as previously described (Goode et al., 1996; Bateman et al., 2001; Fernandez-Minan et al., 2007).

Integrins are required for FSC maintenance

To determine whether integrins participate in stem cell regulation, we assessed the effects of loss of integrin function within individual stem cell populations. Marked WT or integrin mutant clones of cells were generated in adult female flies that initially expressed GFP ubiquitously. After a short pulse of expression of the FLP recombinase (Xu and Rubin, 1993), mitotic recombination occurs in some individual cells, resulting in the generation of two daughter cells: one that is marked by the loss of GFP expression and one that continues to express GFP. In our experiments, marked WT or integrin mutant cells lacked expression of GFP. As expected, localization of βPS, αPS1, or αPS2 was eliminated in cells bearing homozygous mutations of the individual genes that encode them (βPS *myospheroid* [*mys*], αPS1 *multiple edematous wings* [*mew*], or αPS2 *inflated* [*if*]; Fig. 1 C; Brown, 2000). In addition, surface localization of both αPS1(*mew*) and αPS2(*if*) was lost in the absence of βPS(*mys*) (Fig. 1 C), demonstrating that βPS(*mys*) is required for their localization. Similarly, localization of βPS(*mys*) was lost in cells lacking both αPS1(*mew*) and αPS2(*if*). In contrast, localization of βPS was not affected significantly in the absence of only one α subunit (Fig. 1 C), suggesting that βPS(*mys*)

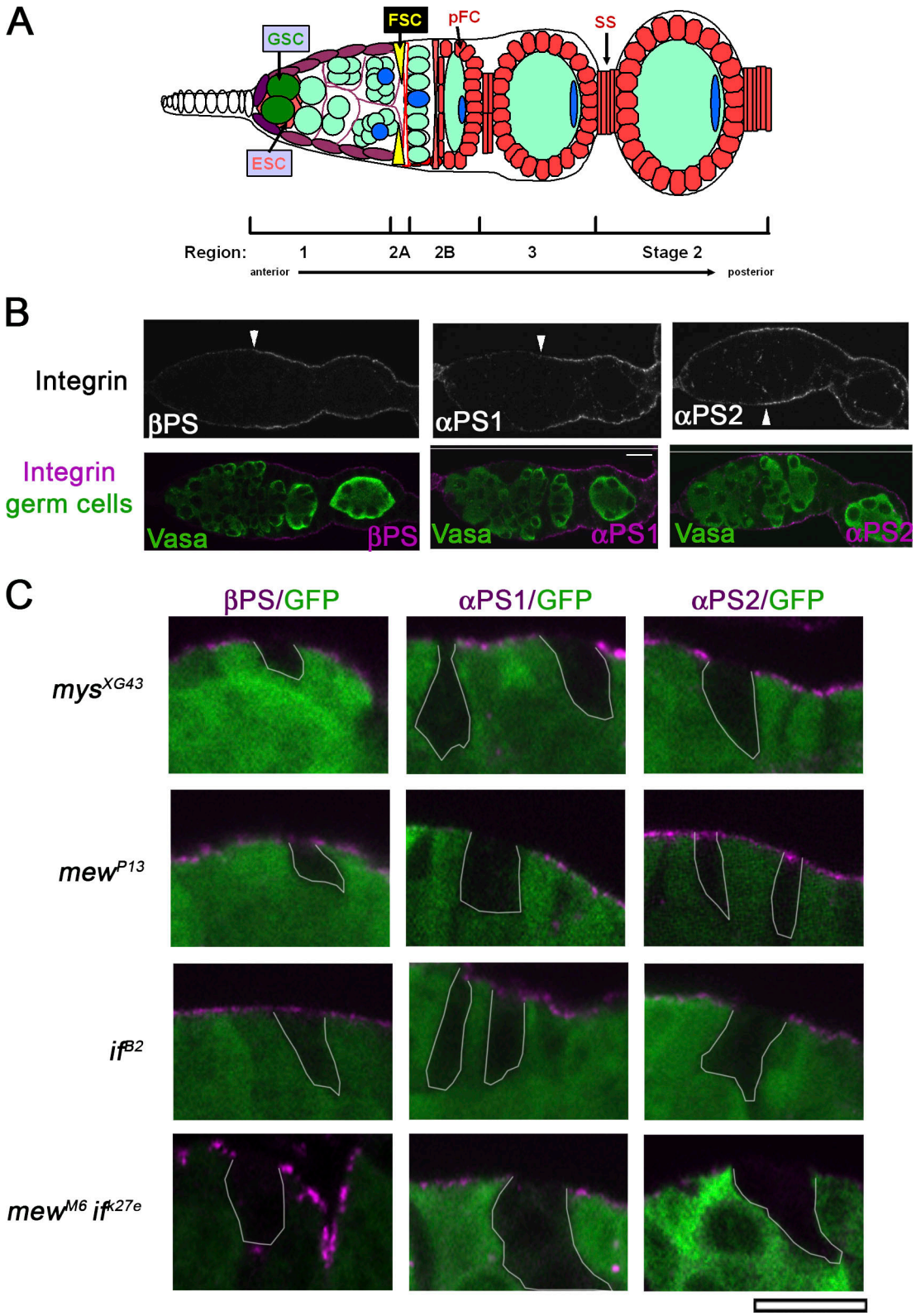


Figure 1. **Integrins localize to the basal surface of the germarium.** (A) Oogenesis schematic. Germline stem cells (GSCs; green) and escort stem cells (ESCs; light pink) reside in a niche consisting of terminal filament (black) and cap cells (purple) in germarium region 1. GSCs generate nonstem cell daughters that produce 16-cell cysts (teal) containing one oocyte (blue). ESCs generate escort cells (dark pink) that accompany cysts through region 1. Follicle stem cells (FSCs; yellow) associate with escort cells in region 2A. FSC daughters, prefollicle cells (pFC; red) that contact germ cells, form an epithelium around the cyst. Prefollicle cells that fail to contact germ cells form stalk cells (SS). Germ cell–follicle cell units bud from the germarium as egg chambers that subsequently develop through 14 stages to form mature eggs. (B) Germaria stained with anti-integrin antibodies (top) or both anti-Vasa, a germ cell marker (bottom, green), and anti-integrin (purple). Three integrin subunits, βPS, αPS1, and αPS2, colocalize to the germarial basal surface with high levels starting at the region 2A/2B border (arrowheads). (C) Prefollicle cell clones (no GFP, green; outlined in white) in germaria 6 d ACI stained with anti-integrin antibodies (purple) as indicated. Localization of βPS, αPS1, and αPS2 was analyzed in prefollicle cell cells bearing mys^{XG43}, mew^{P13}, and if^{B2} or mew^{M6} if^{k27e} mutations, respectively. Bar, 10 μm.

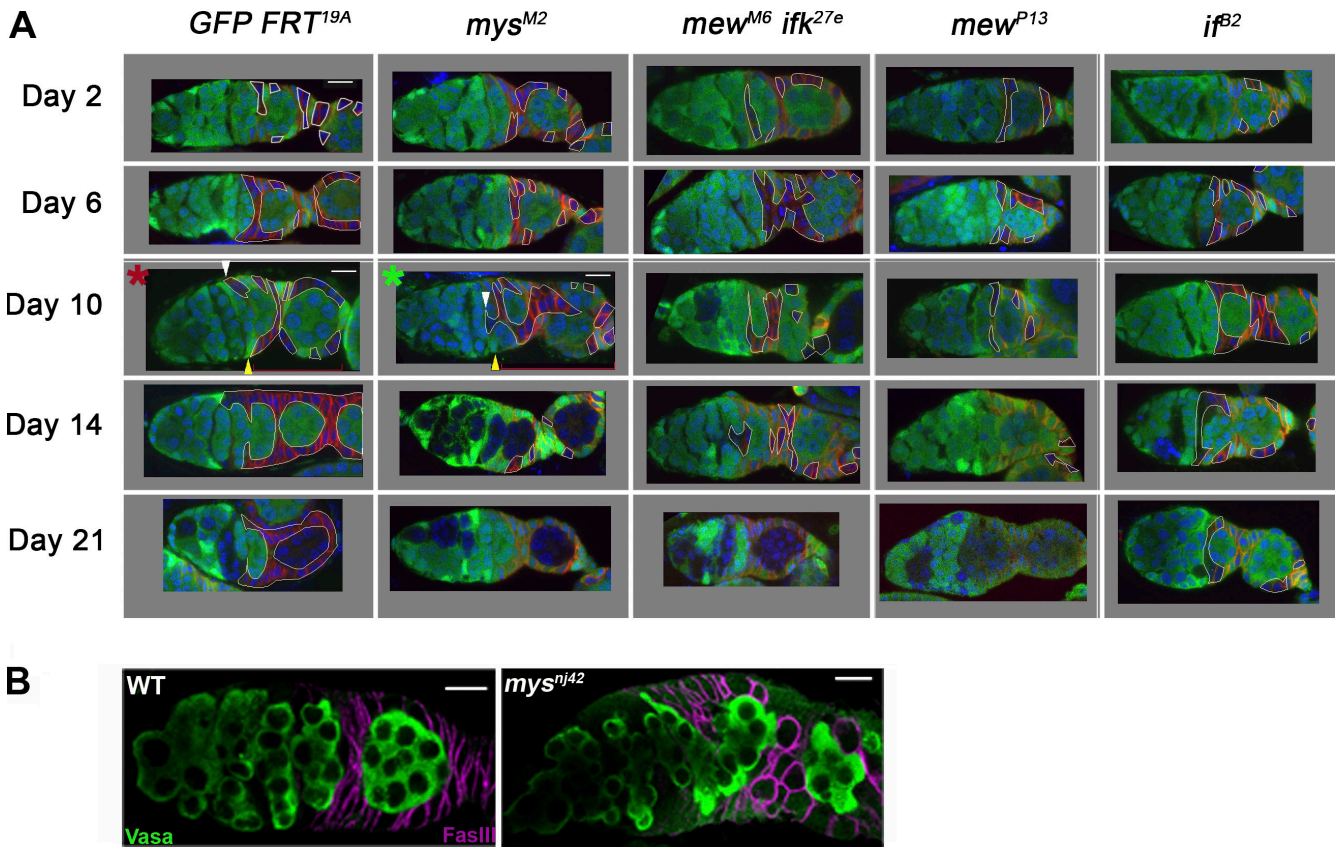


Figure 2. **Integrin mutant FSCs are lost over time.** (A) 3-wk time course of FSC maintenance. Examples of WT (red asterisk) and *mys* mutant (green asterisk) FSCs that lack GFP (white arrowheads; outlined in white) and a GFP-expressing FSC (yellow arrowheads) are indicated. GFP$^-$ progeny (outlined in white) are further to the posterior (red bracket). Days 2, 6, 10, 14, and 21 ACI are shown for germaria bearing clones of the following genotypes: WT (*GFP FRT*^{19A}), *mys*^{M2}(βPS), *mew*^{M6} *if*^{k27e}(αPS1αPS2), *mew*^{P13}(αPS1), and *if*^{B2}(αPS2). Nuclei are stained with propidium iodide (blue). Prefollicle cells are stained with anti-Fas3 antibody (red). (B) WT and temperature-sensitive *mys*^{nj42}(βPS) mutants stained with anti-Fas3 antibody (purple) and anti-Vasa antibody (green) are shown. A single confocal section from the exact center of each germarium is shown. Bars, 10 μm.

can heterodimerize with either α integrin to form an adhesion complex at the basal surface.

To determine whether integrins participate in ovarian stem cell maintenance, we took advantage of established assays for monitoring the retention of marked stem cells over time (Margolis and Spradling, 1995). Mitotic recombination is induced in a subset of ovarian cells, which are marked by the absence of GFP expression. Clones that are initially generated in differentiating progeny cells become incorporated into egg chambers and exit the germarium by 5 d after clone induction (ACI). In contrast, marked WT stem cell clones are maintained within the germarium for long time periods and continue to produce marked progeny cells throughout their lifetime. Based on these established timelines, marked cells present in the germarium at time points later than 5 d ACI must be derived from a marked stem cell. Using this information, the retention rates of WT and mutant stem cells can be compared at multiple time points ACI.

To assess potential roles for integrins in ovarian stem cell maintenance, we compared retention rates of marked WT versus integrin mutant stem cells over time. We found no significant differences in retention rates of WT, *mys*(βPS) mutant, or *mew if*(αPS1αPS2) double mutant GSCs (Figs. 2 and 3 and Table I), indicating that integrins are not required for GSC maintenance. Similarly, we saw no difference in ESC retention between WT clones and integrin mutants (unpublished data).

We next assessed the effects of integrin mutation on FSC retention using established criteria. Specifically, we identified marked FSCs by (1) the absence of GFP expression, (2) location at the border of germarial regions 2A and 2B, (3) low level expression of Fas3 (Fas3^{lo}), a marker for prefollicle cells, and (4) the presence of marked progeny within the same ovariole (Margolis and Spradling, 1995; Zhang and Kalderon, 2001; Song and Xie, 2002; Kirilly et al., 2005). Strikingly, *mys*(βPS) or *mew if*(αPS1αPS2) mutant FSCs were lost rapidly relative to marked WT FSCs (Figs. 2 A and 3 and Table I). Consistent with previously published work (Margolis and Spradling, 1995), WT clones were retained over the 3-wk time course at high rates (45–60%). In contrast, only 7–10% of *mys*(βPS) or *mew if*(αPS1αPS2) mutant clones remained after 3 wk. FSCs lacking the individual α-integrin *mew*(αPS1) were also lost rapidly, and very few germaria retained mutant cells after 3 wk (2%). These results demonstrate that the αPS1βPS (*mew/mys*) integrin is a critical regulator of FSC maintenance. FSC loss was more gradual for *if*(αPS2) mutants, and 24% of marked *if* FSCs remained

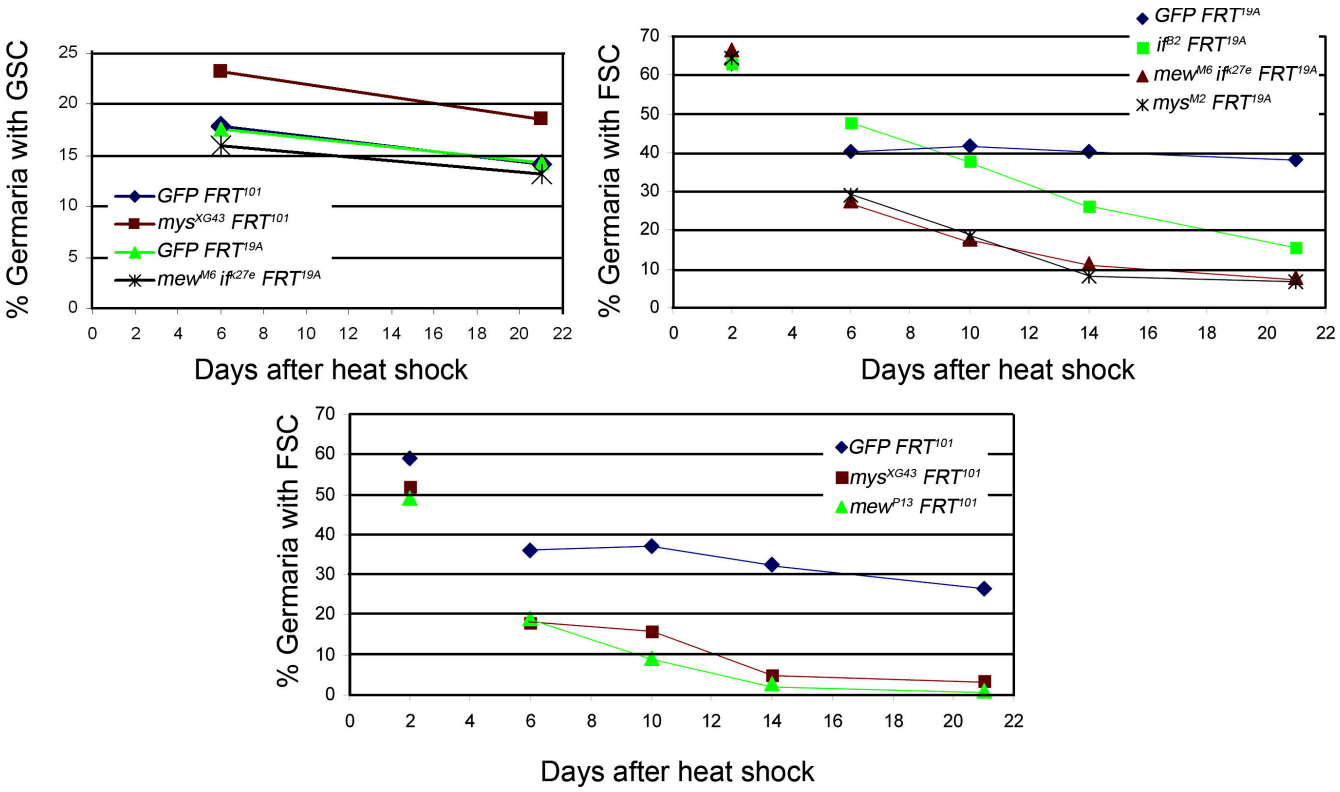


Figure 3. **Integrin mutant FSCs are lost over time.** Quantitation of stem cell maintenance over time. Data are presented as the percentage of germaria containing marked stem cells for each time point. (top) GSC time course. (middle and bottom) FSC time course. The day 2 ACI time point is the initial rate of clone induction and includes both FSCs and prefollicle cells.

after 3 wk. Thus, the αPS2βPS (*if/mys*) heterodimer contributes to FSC maintenance, but αPS1βPS (*mew/mys*) is the primary functional integrin in this process.

Although the number of germaria bearing marked clones was equivalent at 2 d ACI, differences in the numbers of marked WT versus integrin mutant FSCs were obvious by as early as 6 d ACI (Fig. 3 and Table I). By days 10–14 ACI, integrin mutant clones could be seen in germaria that lacked a marked FSC (Fig. 2 A). This indicates that a marked mutant FSC had left the niche and was undergoing differentiation.

Germaria in which both FSCs lacked *mys*(βPS) or *mew if*(αPS1αPS2) were never observed, suggesting that integrin function in FSCs is required for egg chamber formation. Consistent with this idea, removal of *mys*(βPS) function from all cells using a temperature-sensitive *mys* mutant severely disrupted prefollicle cell production (Fig. 2 B), leading to arrested oogenesis.

lanA is required cell autonomously within FSCs

Ligands for both αPS1βPS (*mew/mys*) and αPS2βPS (*if/mys*) integrins have been identified. Whereas αPS2βPS (*if/mys*) binds to ligands containing RGD peptide sequences, the primary ligand for αPS1βPS (*mew/mys*) integrin is laminin A (LanA; also known as α3,5 laminin; Bunch and Brower, 1992; Gotwals et al., 1994). The dramatic loss of FSCs lacking the αPS1βPS (*mew/mys*) integrin suggests that its ligand LanA also may be required for FSC maintenance. To address this question, we examined LanA localization within the germarium. LanA was strongly localized to the basal surface starting about halfway through the germarium, a pattern that overlaps with that of βPS and αPS1 (Fig. 4 A). High levels of LanA and βPS(*mys*) extended to the edge of the marked stem cells, but only low levels of localization were found further to the anterior (Fig. 4 B), supporting the idea that LanA association with αPS1βPS (*mew/mys*) might contribute to FSC retention in the niche. In other systems, integrins are required for production of ECM molecules that accumulate on the epithelial basal surface (Brakebusch and Fassler, 2005), suggesting that LanA production might depend on αPS1βPS (*mew/mys*). However, germaria containing *mys*(βPS) clones exhibited no alterations in LanA levels or localization (Fig. 4 C).

We next asked whether *lanA* mutation affected FSC maintenance. The number of germaria containing WT or *lanA* mutant clones at 2 d ACI was equivalent (Fig. 4 E). However, *lanA* mutant FSCs were lost rapidly relative to WT FSCs (Fig. 4, E and F; and Table I). After 3 wk, very few germaria containing *lanA* mutant FSCs were observed.

One explanation for this result is that production of LanA by the FSC itself might be important for FSC maintenance. Alternatively, reduced production of LanA by all of the mutant cells in a large clone, including the FSC and prefollicle cell progeny cells, might affect basal lamina organization, resulting in FSC loss. If defects in the production of LanA within prefollicle cells affected FSC maintenance, we would expect to see (1) defects in LanA accumulation within the basal lamina in germaria containing mutant cells and (2) greater rates of FSC

Table I. **Quantitation of stem cell retention over a 3-wk time course**

Genotype	Germaria with GSC		Initial clonal rate[b]	Germaria with FSC			
	Day 6[a]	Day 21	Day 2	Day 6	Day 10	Day 14	Day 21
	%	%	%	%	%	%	%
GFP FRT^{101}	17.8 (397)	14.2 (212)	59.0 (256)	35.9 (273)	37.1 (210)	32.4 (216)	26.4 (212)
mys^{XG43} *FRT*^{101}	23.2 (181)	18.5 (248)	52.1 (192)	18.0 (316)	15.8 (221)	5.0 (258)	3.6 (248)
mew^{P13} *FRT*^{101}	ND	ND	49.3 (152)	19.2 (182)	9.3 (183)	2.9 (136)	0.9 (233)
GFP FRT^{19A}	17.6 (159)	14.3 (168)	63.1 (182)	40.2 (159)	41.7 (163)	40.4 (193)	38.3 (154)
mew^{M6}*if*^{k27e}*FRT*^{19A}	15.9 (170)	13.2 (166)	66.4 (164)	27.6 (170)	17.6 (216)	11.3 (186)	7.8 (166)
mys^{M2} *FRT*^{19A}	ND	ND	64.4 (163)	28.9 (180)	18.4 (196)	8.0 (190)	6.8 (192)
if^{B2} *FRT*^{19A}	ND	ND	62.8 (105)	47.7 (132)	37.7 (154)	26.2 (141)	15.6 (179)
GFP FRT^{2A}	ND	ND	47.7 (149)	31 (174)	23.8 (256)	26.8 (190)	14.5 (193)
lanA$^{6-36}$ *FRT*^{2A}	ND	ND	48.3 (248)	20.4 (211)	16.8 (267)	2.8 (245)	0 (213)
lanA$^{9-32}$ *FRT*^{2A}	ND	ND	50.4 (117)	17.2 (58)	8.4 (71)	3.6 (82)	3.3 (61)
lanA^{12641} *FRT*^{2A}	ND	ND	46.1 (65)	22.7 (75)	16.7 (54)	8.0 (100)	5.0 (80)

Data are presented as the percentage of total germaria containing a marked stem cell. Numbers in parentheses are the total numbers of germaria scored for each time point.

[a]The initial clonal rate for GSCs is the percentage of germaria containing a GSC at day 6 ACI.

[b]The initial clonal rate for FSCs (indicated) was calculated as the percentage of germaria containing marked cells (FSC plus prefollicle cells) on day 2 ACI.

loss when large numbers of mutant progeny cells were present in the germarium. However, significant changes in LanA localization or levels were not observed (Fig. 4 D). Most likely, the WT cells present in the mosaic germaria secreted sufficient levels of LanA. Additionally, the numbers of *lanA* mutant prefollicle cells present in the germarium diminished at time points (e.g., 14 d ACI) when FSC loss was dramatic (Fig. 4 E; and see Fig. 7 C), indicating that *lanA* mutant FSCs were lost most effectively in an environment that was composed largely of WT cells. These results are most consistent with a model in which WT FSCs compete more effectively for occupation of the niche (Nystul and Spradling, 2007) than FSCs lacking *lanA*. This evidence supports the idea that *lanA* is required cell autonomously within the FSC, suggesting that FSCs themselves generate the LanA that is essential for their maintenance.

Integrins anchor FSCs to the niche

Based on our results, we propose a two-step model for FSC anchoring. First, FSCs produce LanA, which becomes incorporated into the basal lamina. Next, LanA activates αPS1βPS (*mew/mys*) on the FSC surface, thus anchoring the FSC to the underlying actin cytoskeleton. The observation that labeled integrin or *lanA* mutant FSCs departed the niche may be explained by a reduction in the attachment strength of the FSC to the basal lamina. To further characterize the role of integrins and LanA in FSC anchoring, we examined the positioning of WT and mutant FSCs. Marked WT FSCs were always found at the germarial surface (Fig. 5 A; King, 1970; Margolis and Spradling, 1995). In contrast, the positioning of many FSCs lacking *mys*(βPS) or both α integrins, *mew if*(αPS1αPS2), was drastically different (Fig. 5 B). *mys*(βPS) or *mew if*(αPS1αPS2) cells frequently were found in the center of the germarium, a location that precludes the possibility of contact with the basal lamina. About half of *mys*(βPS) or *mew if*(αPS1αPS2) mutant cells were misplaced as early as 6 d ACI (53%; $n = 82$), and aberrantly positioned *mys*(βPS) or *mew if*(αPS1αPS2) mutant cells were predominant by 14 d ACI (85.9%; $n = 39$). The changes in positioning of *mys*(βPS) or *mew if*(αPS1αPS2) mutant cells were not the result of changes in the levels or localization of E-cadherin (Fig. 5, D and E), which has been implicated in FSC anchoring to neighboring escort cells (Song and Xie, 2002). Thus, integrins act as FSC anchors independently of previously identified adhesion mechanisms.

Displaced integrin mutant FSCs may function as stem cells, generating new mutant progeny cells concomitantly with WT FSCs in the same germarium. Consistent with this idea, displaced mutant FSCs retained many characteristics of normal FSCs, including a low level expression of Fas3, direct association with escort cells, and the capacity to divide (Fig. 5, B and C; and see Fig. 7 G). In some cases, mutant progeny cells were adjacent to centrally localized mutant FSCs (Fig. 5, B and D), suggesting that they may have been generated by division of the displaced FSC. However, some mutant FSCs could be found at the germarial surface, even at late time points (14.1% of mutant

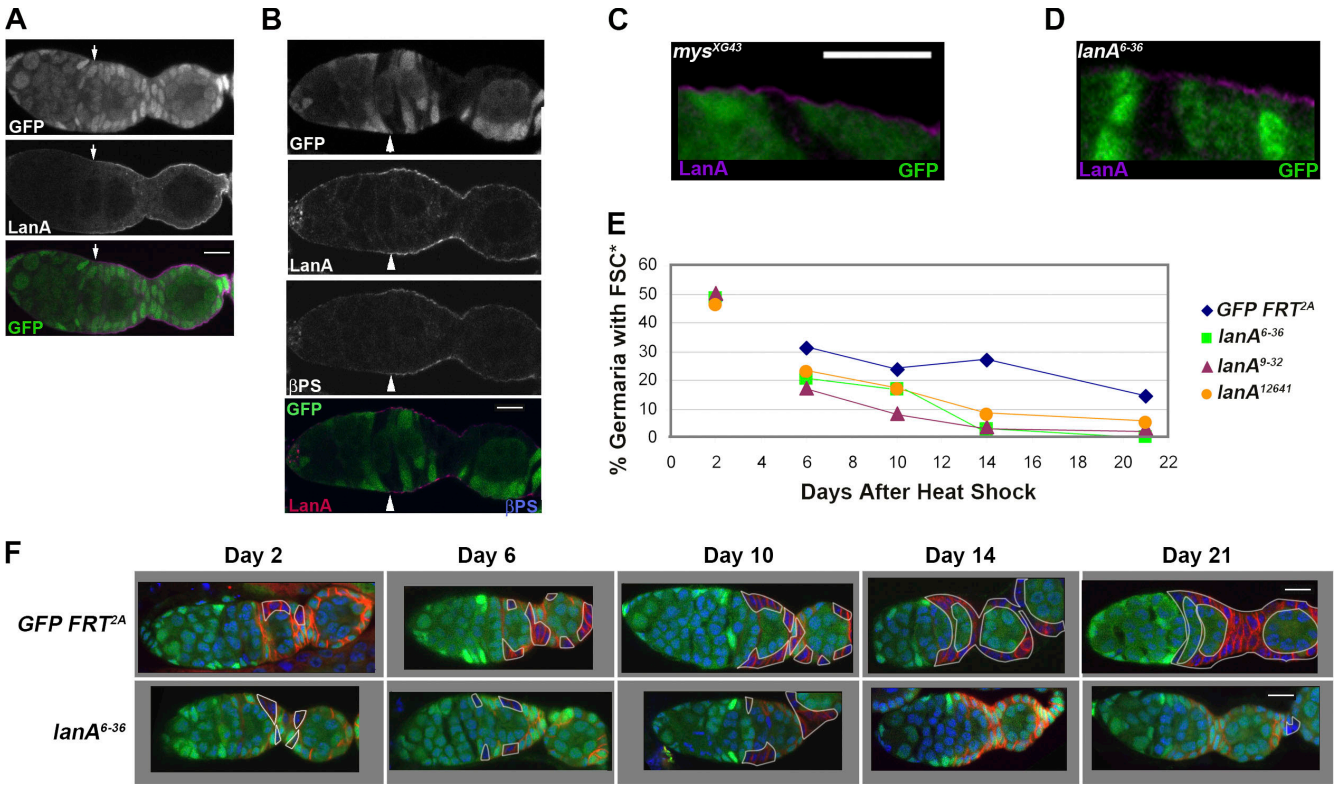


Figure 4. **FSCs lacking *lanA* are lost from the niche.** (A) High levels of LanA (purple) localize to the basal lamina starting near the region 2A/2B border (arrows). Nuclei are labeled with GFP. (B) LanA (red) and βPS(*mys*) (blue) localization is highest beginning near FSCs (no GFP; arrowheads). (C and D) LanA (purple) localizes normally in *mys*(βPS) cells (C; no GFP) or $lanA^{6\text{-}36}$ cells (D; no GFP). (E) Quantitation of $lanA^{6\text{-}36}$, $lanA^{9\text{-}32}$, and $lanA^{12641}$ mutant FSC loss over time. The percentage of germaria containing marked FSCs for each time point is shown. The day 2 ACI time point is the initial rate of clone induction and includes both FSCs and prefollicle cells. (F) 3-wk time course of FSC maintenance. Days 2, 6, 10, 14, and 21 ACI are shown for germaria bearing $GFP\ FRT^{2A}$ clones or $lanA^{6\text{-}36}$ mutant clones (no GFP; outlined in white). Nuclei are stained with propidium iodide (blue), and prefollicle cells are stained with anti-Fas3 antibody (red). A single confocal section from the exact center of each germarium is shown. Bars, 10 μm.

FSCs was surface localized on day 14, and 10.2% was surface localized on day 21). These properly localized mutant FSCs may have generated all of the mutant progeny observed before losing their positioning and moving toward the interior. The rates of loss of mutant FSCs and the numbers of displaced mutant FSCs are consistent with this possibility. Most likely, displaced mutant FSCs could function only briefly as stem cells, and loss of normal positioning within the niche predestined them for loss.

In addition to altered positioning, the morphology of *mys*(βPS) or *mew if*(αPS1αPS2) mutant FSCs was defective. WT FSCs exhibited a triangular shape, with the basal surface abutting the basal lamina and lateral surfaces joining in the center of the germarium (Fig. 5 A; Tanentzapf et al., 2000; Nystul and Spradling, 2007). *mys*(βPS) or *mew if*(αPS1αPS2) mutant FSCs lost their triangular appearance, instead adopting a rounded morphology that occasionally included a flattened surface, where they attached to an escort cell (Fig. 5 B). FSCs lacking *mys*(βPS) that localized to the center of the germarium lacked the high levels of basal cortical actin and the planar polarized basal actin fibers seen in WT FSCs (Fig. 5, F–I; Bateman et al., 2001; Frydman and Spradling, 2001; Deng et al., 2003). In contrast, *mys*(βPS) mutant FSCs residing at the germarial surface exhibited normal shape and actin organization. Most likely, integrins contribute to the basal lamina anchoring that enables WT FSCs to adopt their characteristic appearance. In the absence of integrins, this attachment is weakened, leading to dramatically altered FSC morphology.

We next examined the effects of mutation of individual α integrins or *lanA* on FSC anchoring and morphology. Surprisingly, FSCs lacking *mew*(αPS1), *if*(αPS2), or *lanA* were positioned correctly, exhibited a normal triangular shape, and had normal actin organization (Fig. 6). This result suggests that either αPS1βPS (*mew/mys*) binding to LanA or αPS2βPS (*if/mys*) binding to its extracellular ligand is sufficient to anchor FSCs to the basal lamina. However, FSCs that are apparently anchored normally, such as those lacking *mew*(αPS1) or *lanA*, still are lost from the niche over time (Figs. 2 and 3 and Table I). This observation suggests that anchoring the FSC in itself is not sufficient for FSC maintenance.

Integrin and *lanA* FSCs exhibit reduced proliferation rates

In addition to FSC anchoring, integrins may contribute to FSC maintenance by regulating FSC survival or proliferation. Reduced rates of FSC proliferation or survival are predicted to create a competitive disadvantage between mutant and WT FSCs for maintaining niche association (Nystul and Spradling, 2007). Thus, defects in FSC proliferation or survival might contribute to FSC loss over time. We examined the effects of integrin or *lanA* mutation on FSC survival using TUNEL and vital dye

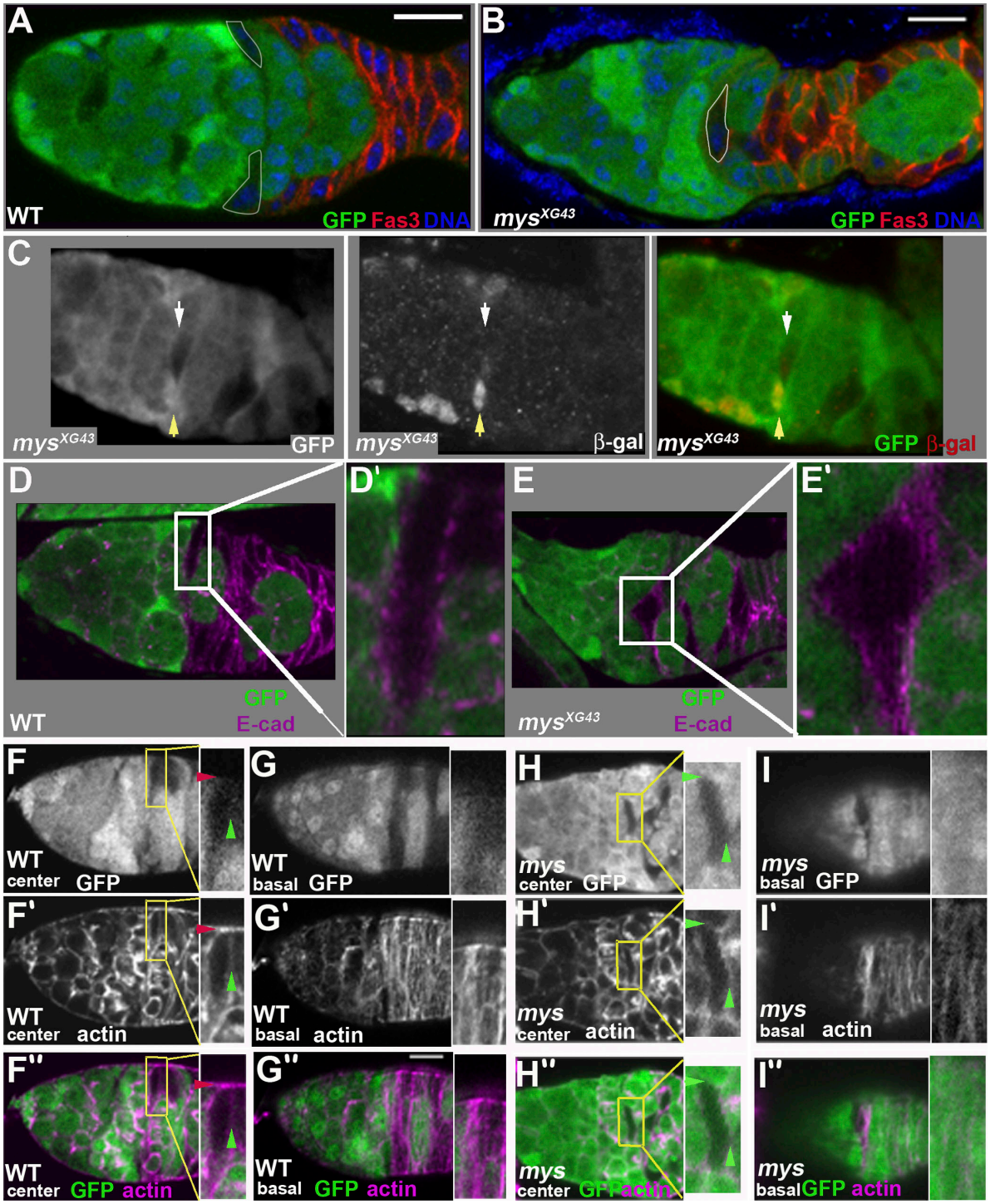


Figure 5. **Aberrant positioning and morphology of *mys*^{XG43} mutant FSCs.** (A and B) Wild type (WT; A) or *mys*^{XG43}(βPS) mutant (B) FSCs (no GFP; outlined in white). (C) *mys*^{XG43} mutant FSCs lack GFP (white arrows). The *P* element PZ1444 leads to expression of β-gal in escort cells (yellow arrows; red). (D and E) WT (D) or *mys*^{XG43} mutant (E) FSCs (no GFP; white boxes) stained with anti–E-cadherin antibodies (purple). Insets are magnified views of boxed areas. (F–I) WT or *mys*^{XG43} mutant cells (no GFP) labeled for actin (F′–I′) or both GFP and actin (purple; F′′–I′′). (F and H) Single confocal section at the center of the germarium. Boxed FSCs are magnified at the right. (G and I) Basal surface of the germaria in F and H. FSCs are magnified at the right. (F and F″) WT cells (no GFP) at the germarial center. Basal actin (red arrowheads) and internal cortical membranes (green arrowheads) are indicated. (G–G′′) Basal surface of the germarium in F. (H–H′′) *mys*^{XG43} mutant cells (no GFP) at the germarial center. Actin on all cortical membranes (green arrowheads) is equal. (I–I′′) Basal surface of the germarium in H. Areas of nonmutant cells are magnified at the right. Bars, 10 μm.

exclusion assays. Although we observed dying germline cysts (4–9% of germaria) and escort cells (6–18% of germaria), we did not observe dying WT ($n = 48$) or *mys*(βPS) ($n = 90$) mutant FSCs. This result is consistent with previous data suggesting that FSCs are lost primarily through differentiation (Zhang and Kalderon, 2001; Song and Xie, 2002; Nystul and Spradling, 2007) and

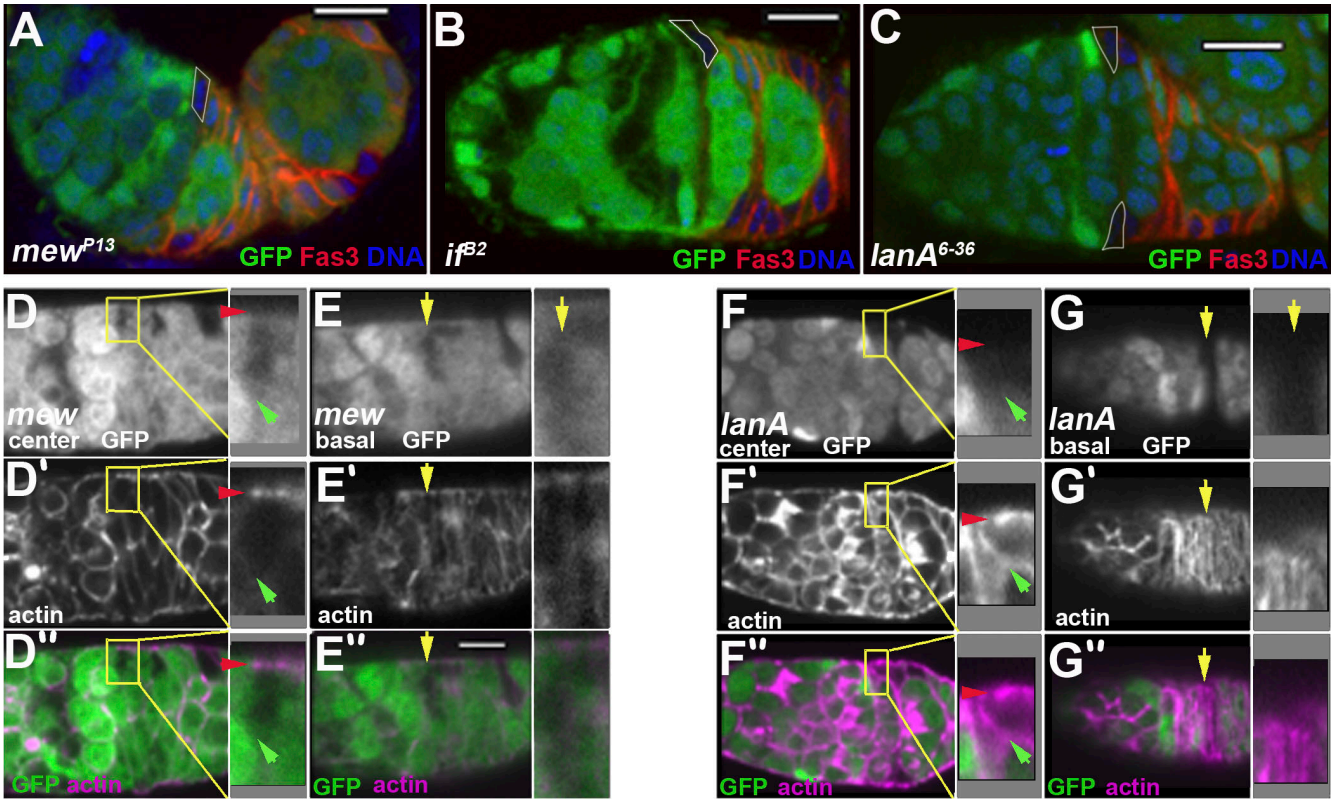


Figure 6. ***mew* and *lanA* mutant FSCs exhibit normal positioning, shape, and actin organization.** (A–C) Labeled *mew*^{P13}(αPS1) (A), *if*^{B2}(αPS2) (B), or *lanA*$^{6\text{-}36}$ (C) mutant FSCs (no GFP; outlined in white). In B, GFP$^-$ germline clones also are visible. (D–G) GFP$^-$ cells labeled for actin (D′–G′) or both GFP (green) and actin (purple; D′′–G′′). (D and F) Single confocal section at the center of the germarium. FSCs are magnified at the right. Basal actin (red arrowheads) and internal cortical membranes (green arrowheads) are indicated. (D) *mew*^{P13} mutant cells (no GFP). (F) *lanA*$^{6\text{-}36}$ mutant cells (no GFP). (E and G) Basal surface of the germaria in D and F, respectively. FSCs are magnified at the right. Yellow arrows indicated GFP$^-$ clones. Bars, 10 µm.

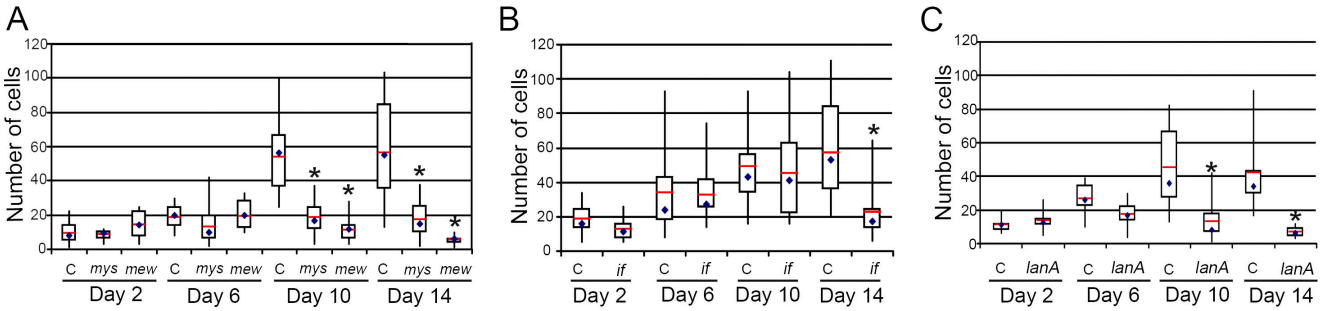
indicates (1) that loss of integrin or *lanA* mutant FSCs is not caused by cell death and (2) that cell survival regulation is not a primary integrin function in FSCs.

We next assessed the effects of integrin and *lanA* mutation on FSC proliferation. First, we determined the number of progeny within the germarium that were derived from marked mutant or WT FSCs (Fig. 7, A–C). FSCs with reduced proliferation rates are expected to produce fewer progeny than WT FSCs. Consistent with this hypothesis, by 10–14 d ACI, *mys*(βPS) clones had an average of threefold fewer cells than WT clones (Fig. 7 A). Similar effects were seen in *mew*(αPS1) and *lanA* clones (Fig. 7, A and C). *if*(αPS2) clones were smaller than WT clones at very late time points (Fig. 7 B), but the effects were far less dramatic than those observed for *mys*(βPS), *mew*(αPS1), or *lanA* mutant clones. These results may be explained by a requirement for integrins in controlling proliferation of FSCs, their progeny, or both. Additionally, these results support the idea that αPS1βPS (*mew/mys*) is the primary integrin in FSC regulation and that αPS2βPS (*if/mys*) contributes to a lesser extent.

Next, we directly measured the numbers of FSCs or prefollicle cell progeny undergoing mitosis by immunostaining with anti–phosphohistone H3 antibodies. We observed clear differences in the proliferation rates of WT versus integrin or *lanA* FSCs using this method (Fig. 7, D and E). FSCs lacking *mys*(βPS) divided 2.5-fold less frequently than WT FSCs, which is consistent with the reduced number of prefollicle cells derived from *mys*(βPS) mutant FSCs. Similar differences between proliferation rates of WT and *mys*(βPS) mutant FSCs was observed when BrdU incorporation was used to mark cells in S phase (Fig. 7 F). In contrast, *mys*(βPS) mutant and WT prefollicle cells divided at equal rates (Fig. 7 G; Fernandez-Minan et al., 2007), suggesting that the reduced numbers of differentiating progeny cells was exclusively caused by diminished FSC proliferation. Despite the reduced proliferation rates of *mys*(βPS) mutant FSCs, *mys*(βPS) mutant FSCs located in the center of the germarium were able to divide occasionally, indicating that displaced FSCs can respond to proliferation signals to some degree (Fig. 7 H). Although mutation of *mew*(αPS1) or *lanA* did not detectably affect FSC positioning, FSCs lacking either gene divided less frequently than WT FSCs (*mew* = approximately twofold, *lanA* > 1.9-fold; Fig. 7, D and E). These results suggest that LanA engagement of the αPS1βPS (*mew/mys*) receptor controls FSC proliferation, even when FSC anchoring and position are not affected. In contrast to clear requirements for *mys*(βPS), *mew*(αPS1), and *lanA*, mutation of *if*(αPS2) had minimal effects on FSC proliferation rates (Fig. 7 D).

FSCs lacking *mys*(βPS) produce defective progeny

During our examination of integrin function in FSCs, we also observed morphological abnormalities in the prefollicle cells



D

Mitotic rate	*GFP FRT*^{101} (172)	*mys*^{XG43} *FRT*^{101} (127)	Fold Reduction	*mew*^{P13} *FRT*^{101} (114)	Fold Reduction	*GFP FRT*^{19A} (47)	*if*^{B2} *FRT*^{19A} (116)	Fold Reduction	*GFP FRT*^{2A} (56)	*lanA*$^{6-36}$ *FRT*^{2A} (133)	Fold Reduction
PH3+FSC / total (+/- 2(SEM))	0.047 (+/- 0.01)	0.019 (+/- 0.02)	2.5	0.026 (+/- 0.02)	1.8	0.021	0.024 (+/- 0.02)	0.9	0.019 (+/- 0.02)	0.000 (+/- 0)	>1.9

E

Mitotic rate	Control	*mys*^{XG43}	Fold Reduction (N)
BrdU+FSC / total (+/- 2(SEM))	0.34 (+/- 0.06)	0.07 (+/- 0.07)	4.8 (33)

F

Mitotic rate	*GFP FRT*^{101} (N=227)	*mys*^{XG43} (N=269)
PH3+pFC / total (+/- 2(SEM))	0.48 (+/- 0.14)	0.60 (+/- 0.12)

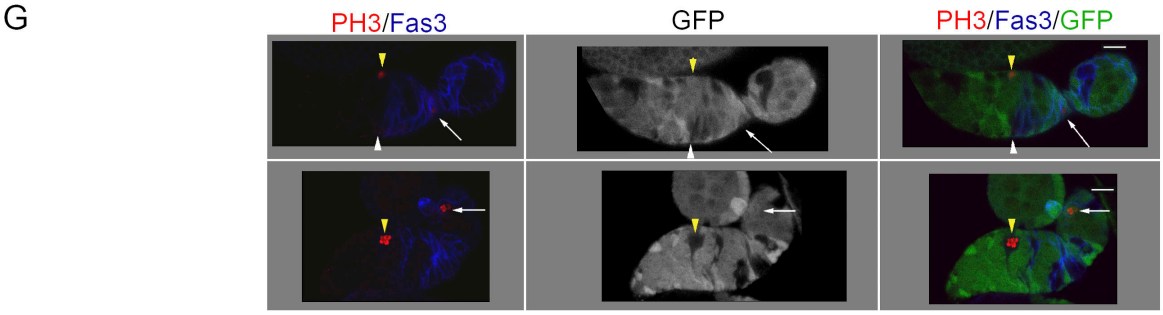


Figure 7. **Reduced proliferation rates in integrin mutant FSCs.** (A–C) Box and whisker plots represent the distribution of marked control or integrin cells in individual germaria containing a GFP$^-$ FSC. The top of the box is the 75th percentile, and the bottom of the box is the 25th percentile. Whiskers extend above the box to the maximum cell number and below the box to the minimum cell number. Red lines represent the mean, and blue diamonds represent the median. At least 10 germaria were scored per genotype. Statistically significant differences calculated using a *t* test in the numbers of WT versus mutant cell numbers are indicated with asterisks (*, $P < 0.002$). The appropriate *GFP FRT* clones are WT controls for integrin mutants on the same FRT chromosome. (D and E) Rates of FSC proliferation. Phosphohistone H3 (PH3)–positive WT or mutant FSCs (no GFP) were divided by the total number of germaria. Error = twice the SEM (± 2[SEM]). Fold reduction = WT proliferation divided by the mutant rate. N = total number of germaria scored/genotype. (E) FSC proliferation rates calculated by BrdU labeling. The percentage of BrdU WT FSCs from mosaic germaria containing mutant FSCs is the control and is based on the presence of two FSCs/germarium (Nystul and Spradling, 2007). (F) Rates of prefollicle cell proliferation. The rate equals the number of GFP$^-$ phosphohistone H3$^+$ cells in regions 2B and 3 of germaria containing a marked FSC divided by the total number of germaria scored. Error = ±2(SEM). Arrows indicate dividing prefollicle cells. (G) Mosaic germaria containing phosphohistone H3$^+$ (red) WT (GFP positive) or *mys*(βPS) mutant (GFP$^-$) FSCs. FSCs (arrowheads) express low levels of Fas3 (blue). Bars, 10 μm.

generated by integrin mutant FSCs. Consistent with previous studies, we saw no significant effect on cell polarization, clone size, or migration patterns of prefollicle cells when *mys*(βPS) mutant clones were generated in cells that had already initiated differentiation (Figs. 7 A and 8 A; Devenport and Brown, 2004; Fernandez-Minan et al., 2007). In contrast, loss of *mys*(βPS) in FSCs led to dramatic defects in their progeny. Prefollicle cells derived from *mys*(βPS) mutant FSCs were often round, exhibiting inappropriate invasion, splitting of germline cysts, loss of contact inhibition, and failure to maintain stalk cell polarization (Fig. 8 B). These prefollicle cells lacked basal domains, instead exhibiting the distribution of lateral markers such as Fas3 on all nonapical membranes (Fig. 8 B). Collectively, these results suggest that the establishment of basal and lateral domains within prefollicle cells depends on basal polarization of the FSC from which they are derived.

Despite the absence of basal domains in *mys*(βPS) mutant cells, contact with germ cells enabled prefollicle cells to form apical domains. The apical marker Bazooka (Fig. 8 C) accumulated where *mys*(βPS) mutant prefollicle cells contacted germ cells but was not observed in *mys*(βPS) mutant cells that failed to contact germ cells. This result supports previous data indicating that basal domain specification is dispensable for prefollicle cells to receive germline-derived polarization signals (Tanentzapf et al., 2000). However, these prefollicle cells failed to incorporate into the normal follicular epithelium and were displaced by WT cells before stage 10 (Devenport and Brown, 2004; Fernandez-Minan et al., 2007; and unpublished data). These results suggest

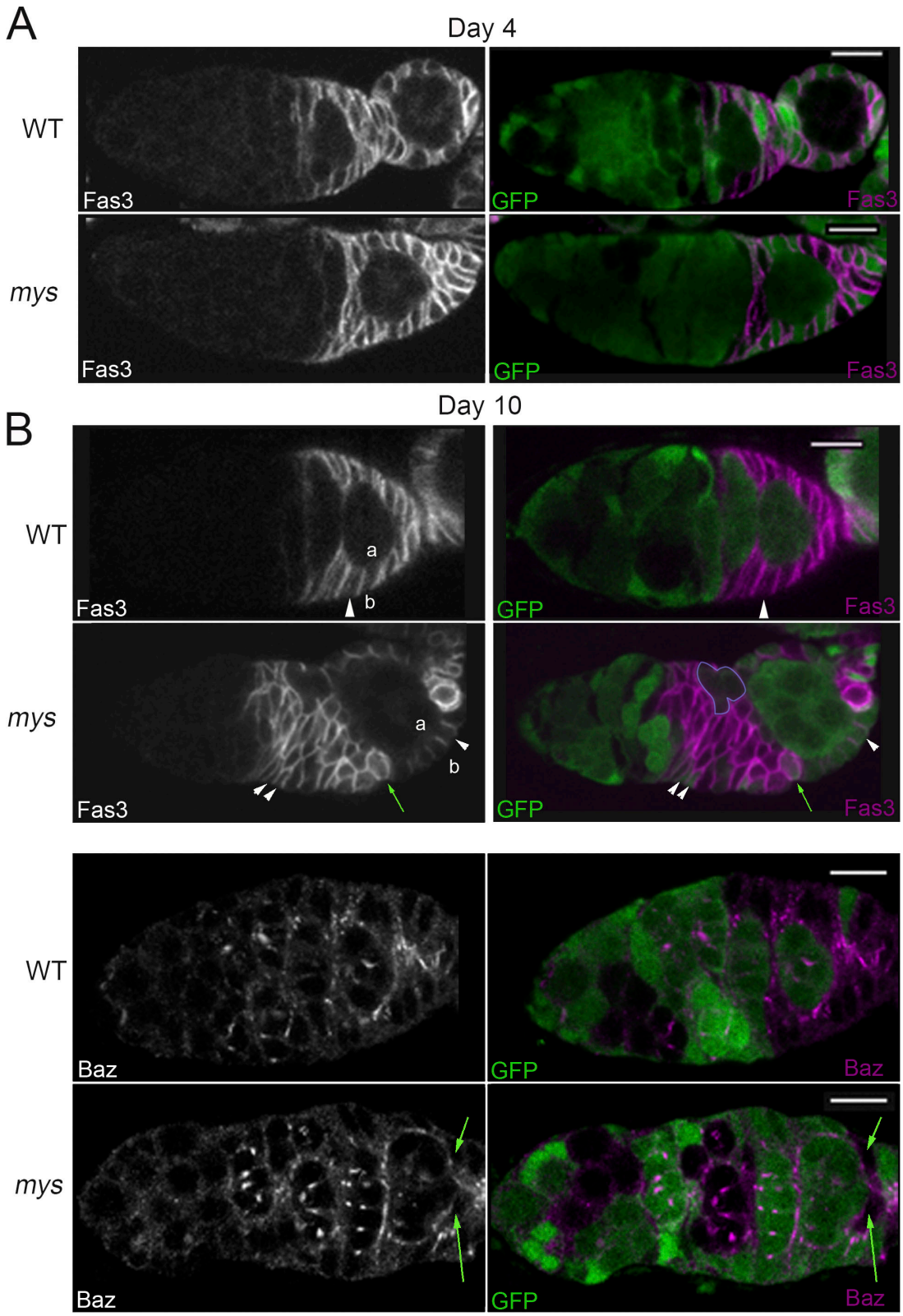


Figure 8. ***mys*(βPS) mutant FSCs produce defective progeny.** (A) Germaria 4 d ACI bearing WT or *mys* mutant cells (no GFP) labeled with Fas3 antibodies (purple). (B) Germaria 10 d ACI bearing WT or *mys* mutant cells labeled as in A. White arrowheads indicate WT cells at the germarial surface. Fas3 localizes to lateral membranes but is excluded from apical (a) or basal (b) membranes. Round *mys* mutant cells (green arrows) pile up, and Fas3 localizes to all nonapical membranes. Invasive *mys* mutant cells separate three germ cells from the cyst (outlined in blue). (C) Germaria 10 d ACI bearing WT or *mys* mutant cells (no GFP) are labeled with anti-Bazooka antibodies (purple). Apical domains form normally (green arrows). Bars, 10 μm.

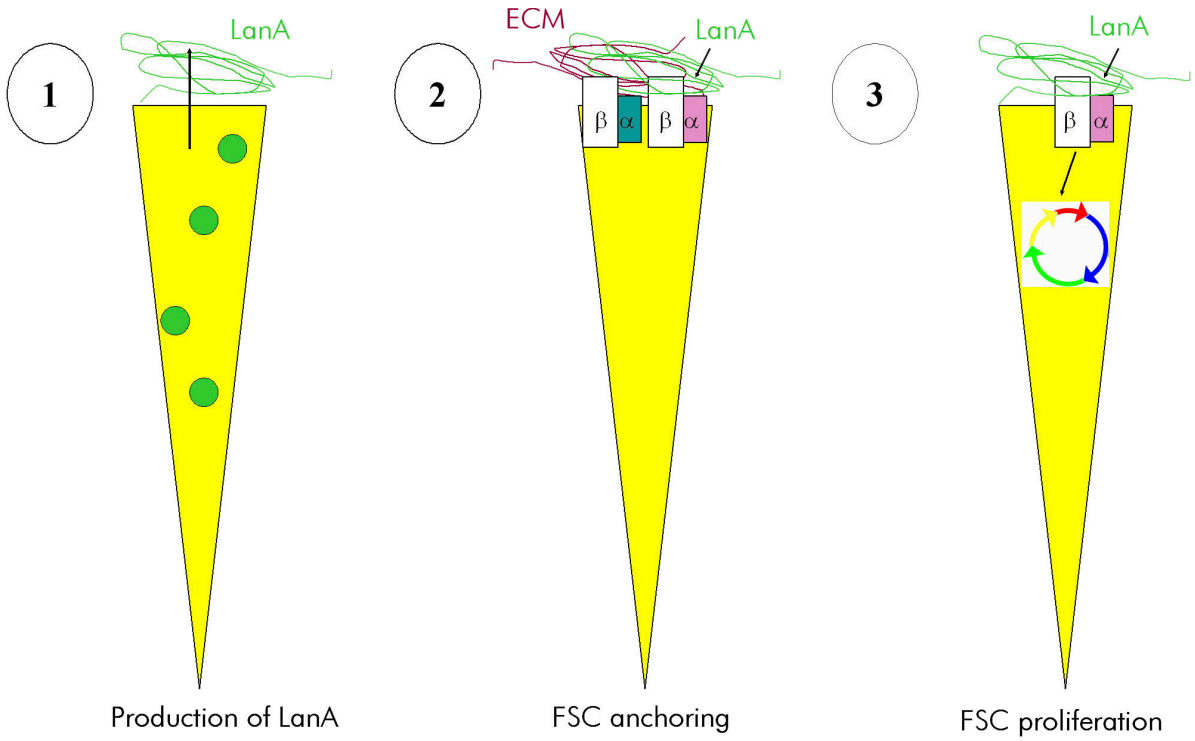


Figure 9. **Model for integrin function in FSCs.** (1) FSCs (yellow triangles) secrete LanA (green) into the ECM to generate a critical niche component. (2) Engagement of αPS1βPS (*mew/mys*, white/pink) by LanA and of αPS2βPS (*if/mys*, white/blue) by an RGD peptide–containing extracellular ligand (ECM, red) anchors the FSC to the basal lamina, maintaining its position within the niche. (3) LanA binding to αPS1βPS (*mew/mys*, white/pink) receptors also controls FSC proliferation rates. Together, these three mechanisms contribute to FSC maintenance regulation.

that other adhesion mechanisms and polarizing signals can partially compensate for the absence of integrins in prefollicle cells but that integrin mutation within FSCs has severe consequences on subsequent development.

Discussion

Stem cells depend on the surrounding microenvironment to provide signals that control their identity, self-renewal, and position and to prevent differentiation. The identification of cellular and molecular stem cell niche components has broadened our understanding of the complex interplay between stem cells and niches. In this study, we investigate the roles of integrins and their ligands in stem cell regulation in the *Drosophila* ovary. Our results support the model that one population of ovarian stem cells, the FSCs, produce the integrin ligand LanA, generating a critical component of their own niche (Fig. 9). Activation of integrin receptors expressed on the FSC surface maintains FSC position and enables FSCs to receive proliferative cues. This mechanism is not required to maintain other ovarian stem cell populations. Thus, distinct adhesion pathways regulate communication between stem cells and their niche within the same organ. The differentiating daughters of FSC divisions, prefollicle cells, also rely on integrins within the FSC to regulate their subsequent polarization and function. Therefore, integrin function within FSCs is critical for the maintenance and development of both FSCs and their progeny.

Emerging evidence suggests that there are two general categories of stem cell niche. Stable niches, such as the mammalian hematopoeitic stem cell niche and GSC niches in flies, can exist in the presence or absence of stem cells and provide sufficient information for cells to acquire or retain stem cell fate (Xie and Spradling, 2000; Kai and Spradling, 2003, 2004; Wilson and Trumpp, 2006). In these cases, differentiated support cells perform most niche functions, including anchoring, maintenance of asymmetrical cell divisions, and the generation of factors that control proliferation and survival and prevent differentiation (Harrison and Harrison, 2006; Wilson and Trumpp, 2006). The architecture and position of other niches is less clearly defined, perhaps enabling the movement of stem cells across the tissue rather than fixing them in a defined location. In such "flexible" niches, there is no obvious differentiated support cell to provide anchoring function. Instead, stem cells may attach to a prominent basal lamina that surrounds the entire tissue. It is possible that stem cells in these tissues can change location in response to local signals, thus functioning where they are needed at a given point in time (Ohlstein and Spradling, 2006).

We propose that FSCs reside in a dynamic niche that retains characteristics of both stable and flexible niches. Normally, the FSC niche is found halfway through the germarium on its outer surface (Margolis and Spradling, 1995; Nystul and Spradling, 2007) and is therefore positionally defined. Additionally, FSCs associate transiently with supporting escort cells via E-cadherin–mediated adhesion (Song and Xie, 2002; Nystul and Spradling, 2007). FSCs lacking E-cadherin are lost, indicating that this mechanism contributes to FSC maintenance. However, in agametic germaria, which lack germ cells and escort cells, FSCs

function normally despite their drastically altered position and the absence of cellular components of the niche (Kai and Spradling, 2003; Kirilly et al., 2005). Thus, the FSC niche may be flexible, enabling FSCs to self-renew and produce progeny in the absence of a structurally and positionally defined niche. Our results demonstrate that integrin interactions with the basal lamina regulate FSC maintenance, positioning, and proliferation in normal germaria. Integrins also are present on the FSC basal surface in agametic germaria (Tanentzapf et al., 2000), suggesting that this regulatory mechanism is functional even when cellular components of the niche are absent. Our results support the idea that at least one component of the niche, the integrin ligand LanA, is generated by FSCs themselves. This unique property may permit FSCs to adjust their local environment, enabling them to function in disparate locations.

Previously identified stem cell regulatory factors often are expressed or localized asymmetrically between the stem cell and its progeny cells. For example, higher levels of E-cadherin are found at the interface between ovarian GSCs and niche cells than are found between germ cells (Song et al., 2002; Jin et al., 2008). In contrast, integrins are expressed at apparently uniform levels in FSCs and their progeny. However, integrins are critical for FSC function and are dispensable in progeny that have already initiated differentiation. This suggests that asymmetry is generated functionally rather than by differential expression or localization.

Functional asymmetry might be achieved in two possible ways. First, FSC positioning at the anterior-most point of the developing epithelium may make it uniquely capable of receiving secreted signals produced by differentiated cells further to the anterior (Kirilly et al., 2005). Activation of downstream pathways within the FSC, in concert with integrin-dependent signals, may promote expression of a unique set of genes that control FSC identity and behavior. Second, FSC positioning may make it uniquely dependent on integrin-mediated adhesion for its maintenance. In addition to integrin-mediated basal positioning information, differentiating prefollicle cells receive lateral and apical signals from neighboring prefollicle cells and germ cells, respectively. No current evidence suggests that FSCs receive positioning information from either germ cells or their differentiating progeny. Additionally, FSCs are only partially polarized, with clear basal domains but intermixed lateral and apical components (Tanentzapf et al., 2000). Thus, FSCs may depend on integrins for their positioning and polarization in the absence of additional signals.

Dynamic interaction between integrins and locally produced ECM ligands is an important mechanism for controlling changes in cell migration, adhesion, and polarization that are required for development (Li et al., 2003; Nelson and Bissell, 2006). Laminin binding to integrin receptors both activates intracellular signaling pathways and promotes biochemical changes in the laminin network that are necessary for basement membrane formation. This process can occur in many cells simultaneously, resulting in the polarization of an entire epithelium or in individual cells to temporally and spatially control adhesion versus migration decisions (Li et al., 2003; Medioni and Noselli, 2005).

We found that FSCs lacking the integrin ligand (*lanA*) or a subunit of its receptor (*mew*(αPS1)) present identical FSC maintenance and proliferation phenotypes (Figs. 2–4). Additionally, the LanA produced by neighboring WT cells apparently is not sufficient to maintain mutant FSCs within the niche. Thus, reciprocal signaling between laminin and integrins may depend on cell-autonomous production of LanA by FSCs after each cell division. Local production of LanA in the immediate vicinity of the FSC may be required for efficient activation of integrin signaling cascades or to maintain a stable structure for FSC anchoring. Alternatively, FSCs may need to produce LanA during the migration or displacement steps that occur during the process of FSC replacement (Nystul and Spradling, 2007). In all cases, WT FSCs expressing LanA would be expected to compete more effectively than *lanA* mutant FSCs for positioning within the niche. These results suggest that FSCs have the capacity to generate critical components of the niche that then directs their stem cell behavior.

Studies on the roles of growth factors in FSC regulation have indicated a link between FSC positioning and proliferation control. Current data suggest that the secreted factor, Hedgehog, controls FSC proliferation rates through regulation of FSC niche size and location (Zhang and Kalderon, 2001). Additionally, FSCs in close proximity to the source of proliferative BMP signals respond robustly. In contrast, the response of FSCs residing in their normal niche, three to five cell diameters away from the signal source, is dampened (Margolis and Spradling, 1995; Kirilly et al., 2005). These results are consistent with a model in which the positioning of the FSC niche at the region 2A/2B border is an important factor in FSC proliferation control.

In this study, we demonstrate critical requirements for integrins in determining FSC positioning. Loss of the β-integrin *mys*(βPS) or both α integrins (*mew if* (αPS1αPS2)) resulted in detachment of FSCs from the basal lamina and displacement to the center of the germarium. The anchoring defects were associated with reduced proliferation rates in mutant FSCs, supporting a link between FSC positioning and proliferation control. However, proliferation rates also were drastically reduced in FSCs lacking *mew*(αPS1) or *lanA*, which remained properly localized. Therefore, changes in FSC positioning are not sufficient to explain the reduced proliferation rates in all integrin mutants.

These observations suggest that integrins independently regulate FSC positioning and proliferation. Whereas our data support the idea that FSC positioning depends on integrin-mediated adhesion to the basal lamina, the integrin-dependent mechanisms that control FSC proliferation are unclear. LanA and/or integrins may participate in niche formation and maintenance, perhaps cooperating with Hh signals (Zhang and Kalderon, 2001). Integrins also may modify the FSC response to growth factor signals, a mechanism that is well documented in mammalian cultured cells (Lee and Juliano, 2004). Finally, activation of integrins by LanA and/or other ligands may initiate signaling cascades that regulate proliferation independently of other signals. Further analysis will be required to determine the precise molecular role of integrins in FSC proliferation control.

Our results demonstrate critical roles for integrins in regulating epithelial stem cells. Integrins may play similar roles in other epithelia, such as the mammalian skin and intestine, where stem cell adhesion to the basal lamina is thought to anchor the stem cell to its niche, enabling it to receive signals that control differentiation and proliferation. In these tissues, like the fly ovary, integrin function also is critical for proper development of stem cell progeny. FSCs lacking integrin function produced severely defective progeny, but mutation within differentiating progeny cells had little or no effect. This suggests that genetic alteration within stem cell populations has more severe defects on tissue development than identical mutations in differentiated cells within the same tissue. These results may have important implications for how mutation of critical genes within stem cell populations affect tissue development and health.

Materials and methods

Drosophila stocks and husbandry

mys^{XG43} *FRT*^{101}, *mew*^{P13} *FRT*^{101}, and *if*^{B2} *FRT*^{19A} are null alleles of *myospheroid*, *multiple edematous wings*, and *inflated*, respectively, recombined on to X chromosomes containing FRT sites as indicated. Additional integrin alleles used were *mys*^{M2} *FRT*^{19A}, a hypomorphic *mys* allele, and the double mutant *mew*^{M6} *if*^{k27e}*FRT*^{19A}, which eliminates the function of both *mew* and *if*. *lanA*$^{6-36}$ *FRT*^{2A} and *lanA*$^{9-32}$ *FRT*^{2A} are null alleles of *lanA* recombined on to *FRT2A*. *lanA*^{12641} *FRT*^{2A} is an ethane methyl sulfonate–induced allele of *lanA* that was isolated in an unrelated screen (Grueber et al., 2007) and fails to complement *lanA*$^{9-32}$. Ovary clones of integrin or *lanA* mutant cells were generated in adult virgin females. Females were subjected to a 60-min heat shock at 37°C to express FLP recombinase under control of the heat shock promoter, inducing FRT-mediated recombination (Xu and Rubin, 1993). After the heat shock, females were placed at 25°C with males in yeasted vials. Flies were placed into fresh vials every day for 2–21 d before the ovaries were isolated for analysis. The β-gal insertion line PZ1444 (Margolis and Spradling, 1995) was used to mark escort cells.

Immunocytochemistry and imaging

Ovary dissections and fixation were described previously (Guarnieri et al., 1998). Live staining for βPS (*mys*), αPS1 (*mew*), and αPS2 (*if*) was performed as described previously (Brower et al., 1984; Goode et al., 1996). BrdU and ApopTag labeling were performed as described previously (Kai and Spradling, 2003). Phalloidin (Invitrogen) or propidium iodide staining was described previously (Guarnieri et al., 1998), as was immunofluorescence analysis with antibodies against βPS, αPS1, αPS2 (1:100; Brower et al., 1984), LanA (1:5,000; Fessler et al., 1987), GFP (1:1,000; Invitrogen), Bazooka (1:500; Wodarz et al., 1999), DE-cadherin (1:25; Developmental Studies Hybridoma Bank [University of Iowa]; Oda et al., 1994), Fas3 (1:1,000; DSHB), phosphohistone H3 (1:2,000; Millipore), and Vasa (1:2,000; Hay et al., 1990). Appropriate secondary antibodies conjugated to FITC, Cy3, or Cy5 were obtained from Jackson ImmunoResearch Laboratories. Samples were mounted in Vectashield mounting medium (Vector Laboratories). Images were collected at room temperature (~22°C) using 100× NA 1.3 or 60× NA 1.4 oil immersion lenses (Nikon) on upright (Eclipse E600; Nikon) or inverted (TE2000-U; Nikon) microscopes coupled to MRC1024 (Bio-Rad Laboratories) or C1 (Nikon) confocal laser scanners, respectively. Lasersharp or NIS-Elements software (Nikon) was used for data acquisition. Image processing was performed using ImageJ (National Institutes of Health) and Photoshop software (Adobe). For most figures, single confocal slices of the exact center of each germarium are shown, as indicated in the figures.

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