Alternative direct stem cell derivatives defined by stem cell location and graded Wnt signalling

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Adult stem cells provide a renewable source of differentiated cells for a wide variety of tissues and generally give rise to multiple cell types. Basic principles of stem cell organization and regulation underlying this behaviour are emerging. Local niche signals maintain stem cells, while different sets of signals act outside the niche to diversify initially equivalent stem cell progeny. Here we show that Drosophila ovarian follicle stem cells (FSCs) produced two distinct cell types directly. This cell fate choice was determined by the anterior–posterior position of an FSC and by the magnitude of spatially graded Wnt pathway activity. These findings reveal a paradigm of immediate diversification of stem cell derivatives according to stem cell position within a larger population, guided by a graded niche signal. We also found that FSCs strongly resemble mammalian intestinal stem cells in many aspects of their organization, including population asymmetry and dynamic heterogeneity.

RESULTS

Multiple FSCs reside in a narrow A/P domain of radially symmetric rings

To define the number and location of FSCs in a germarium we used multicolour lineage analysis. Here, mitotic recombination eliminates GFP (G—green), β-galactosidase (B—blue) or RFP (R—red) transgenes, resulting in any one of six distinguishable colours (G, B, BG, GR, BR or BGR) (Supplementary Fig. 1a–c). Recombination was initiated by a heat-shock-induced recombinase and the number of distinct FSC lineages was counted 9 days after the last of a series of heat-shocks.

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Transient clones arising in FCs pass through the ovariole within 5 days⁴ (Fig. 1b) and control animals with no heat-shock included only 3 recombinant clones among 161 ovarioles. We can therefore be certain that almost all experimental clones (over 150 in 50 ovarioles) were induced by heat-shock 9 days earlier and derived from recombination in a stem cell.

An FSC clone that survived for 9 days was defined as including at least one coherent group (‘patch’) of FCs and at least one cell in region
2a/b of the gerarium (an FSC) of the same colour (B in Fig. 1b; B, G, GR and BR in Fig. 1c,d and Fig. 2a–d). Many ovarioles included four or more FSC clone colours and two had all six (Fig. 2i). The distribution of FSC clone colour numbers underestimates the number of surviving FSCs because two or more FSCs may have the same colour (Supplementary Fig. 1c); it best matches expectations for an average of five surviving FSC lineages per gerarium 9 days after marking (see Supplementary Note and Supplementary Fig. 2a).

The majority of FSC clones included more than one candidate FSC. To determine where FSCs reside, we considered clones with only a single candidate FSC in region 2a/b. The single candidate FSC was found at similar frequency in each of the 7–10 confocal z-sections spanning the gerarium, not just in the middle sections as previously proposed (Fig. 1e). We infer that FSCs can occupy any position around the gerarial circumference (Fig. 1f).

Along the A/P axis, 24 FSC lineages had a single candidate FSC immediately anterior to Fas3-positive FCs; we call this ‘layer 1’ (B in Fig. 1c and Supplementary Fig. 1i–n). In 19 lineages the only candidate FSC was in the penultimate Fas3-negative ring (‘layer 2’) (BR in Fig. 1g) and in five lineages it was one cell further anterior (‘layer 3’) (BR in Fig. 1h).

We counted the total number of candidate FSCs in layers 1–3 for all lineages in 36 gerariums (Supplementary Fig. 1i–n). We found an average of 7.6 cells in layer 1, 5.6 cells in layer 2 and 2.0 cells in layer 3, in a distribution centred around a total of 15–16 FSCs (Fig. 1i). The ratio of cells present in layers 1–3 (50%/37%/13%) was very similar to the percentage of ovarioles with the only candidate FSC in layer 1, 2 or 3 (50%/40%/10%), suggesting that all cells in these three layers are FSCs. We also used the MARCM method to label FSC clones with GFP and found a very similar distribution of all candidate FSCs among layers 1–3 (46%/38%/16%, n = 273).

FSCs are maintained by population asymmetry

Although occasional FSC replacement is acknowledged, each FSC is thought to be relatively long-lived, mostly undergoing repeated divisions with asymmetric outcomes. We measured the rate of FSC loss by counting surviving FSC clone colours 14, 20 and 30 days after clone induction. Because it takes 4 days for an FC to transit from birth to the latest egg chambers scored (Fig. 1b) we could also infer the number of FSC colours present 5 days after clone induction by counting the number of FC colours in 9-day ovarioles. The number of FSC colours observed (Fig. 2j) and the inferred number of surviving FSC lineages (Fig. 2k) declined rapidly over time and, by extrapolation, indicates the initial presence of about 16 FSCs (Fig. 2k), very similar to the number of candidate FSCs in layers 1–3 (Fig. 1i).

If some FSC lineages are lost, others must amplify for the total number of FSCs to remain constant. When the average number of surviving FSC lineages was five at day 9 the average number of FSCs per lineage was 3.2 among 50 B, G and BR lineages. A similar number of FSCs per lineage (3.3 at day 9) was measured in a MARCM analysis. The number of FSCs per lineage increased over time (Fig. 2c–h,l).

The rapid loss of some FSCs and amplification of others contradicts earlier models of predominantly asymmetric division of FSCs and instead conforms to a model of population asymmetry, where the fates of two daughters of a stem cell are not necessarily different. Stem cell populations maintained by population asymmetry undergo neutral drift, in which the number of stem cell lineages declines stochastically over time, eventually leaving only one randomly selected lineage per developmental unit. We observed exactly this behaviour from 5 to 30 days following clone induction (Fig. 2c–l), resulting in just one or two lineages in most ovarioles by 20–30 days after clone induction. Correspondingly, the number of FSCs per lineage exhibited stochastic variations at any one time, centred on larger clone sizes as time progressed (Supplementary Fig. 2k–m).

We also counted the number of distinct colours present on each egg chamber (Fig. 1j,k) and the average proportion of each egg chamber contributed by a single colour to deduce that each egg chamber is seeded on average by about four founding FCs (see Supplementary Note). Consistent with the considerable excess of FSCs over founding FCs, individual FSC lineages contributed only sporadically to individual egg chambers (83 of 185 egg chambers for B, G and BG clones at 9 days; 179 of 337 egg chambers for MARCM clones at 9 days) and some FSCs had no matching FCs in the entire ovariole (14 of 63 (22%) B, G and BG clones; 20 of 83 (24%) 9-day MARCM clones; Supplementary Fig. 2d–g).

FSCs are radially mobile

We used live imaging to follow FSCs marked by GFP after embedding dissected ovarioles in Matrigel. Throughout the 3–11 h imaging period, labelled cell bodies in FSC locations moved radially along the gerarial circumference (Fig. 3a,b and Supplementary Videos 1–6). We quantified relative movement between pairs of marked cells (Supplementary Fig. 4). Radial movement was observed between all 34 pairs tracked. Cell pairs switched between periods of moving towards each other or further apart at an average rate of 13% of the circumference (about 45 degrees) per hour and FSCs sometimes crossed paths (Supplementary Fig. 4 and Supplementary Videos 2, 3 and 5). These movements support the idea that FSCs can occupy any radial position and the fluid exchange of radial positions suggests functional equivalency in this dimension.

FSCs produce escort cells

It was first suggested that ECs replenished during adulthood derive from an escort stem cell adjacent to the GSCs. Because there is virtually no somatic cell division anterior to region 2a/b, a more recent study concluded that new ECs derive from occasional division of mature ECs, principally in region 2a/b. We have found that the entire region 2a/b proliferative domain appears to be occupied by FSCs and therefore hypothesized that FSCs produce ECs as well as FCs.

MARCM labelling revealed many ovarioles with marked ECs, FSCs and FCs, consistent with a common FSC origin of both ECs and FCs (Fig. 4a). However, some ovarioles contained only marked FSCs and FCs (Fig. 4b), or only marked ECs, with or without FSCs (Fig. 4c). It has been argued from similar data that ECs and FCs have separate origins; coincident marking was invoked to explain the presence of marked FSCs, FCs and ECs in the same ovariole. We clarified the origin of ECs by examining a time course of MARCM clones.

The average number of labelled ECs in EC-containing ovarioles increased over time (Fig. 4e), indicating repeated divisions of a cell labelled at time zero and hence a stem cell origin. The percentage of ovarioles with at least one labelled EC also increased (Fig. 4f),
Figure 2  FSC population asymmetry. (a,b) Diagram of a gerarium (a) and anterior portion of an ovariole illustrating the clones observed in c,d (b). There are G (green), BR (purple), GR (orange) and BGR (white in (b)) FSC clones containing at least one candidate FSC in region 2a/b (BGR FSC is in a different z-section) and one FC patch. (c–h) Examples of ovarioles (single z-sections) (d,f,h) and their germaria (3–5 z-sections combined) at higher magnification (c,e,g) from multicolour lineage experiments 9, 20 and 30 days AHS. The number of distinct FSC lineages declines from four at 9 days (c,d) to two (BG, BR) at 20 days (e,f) and just one (GR) at 30 days (g,h) (single-channel images show all cells have GFP and RFP). In c,e,g, the number of FSCs of one colour (white arrows in c, G; e, BR; and g, GR) is higher when fewer FSC lineages are present (Fas3 staining enhanced by white dotted line). Scale bars, 10 μm (c,e,g) and 50 μm (d,f,h). In d,f, the vertical white lines separate different z-sections (indicated top right) for different regions of the ovariole (full original images for each z-section in Supplementary Fig. 9). In f, the diagonal white line indicates an edge of the original image (shown in Supplementary Fig. 9). (i) Number of ovarioles (among 50) with the indicated number of distinct FSC clone colours 9 days AHS. (j,k) Mean number of distinct multicolour FSC lineages observed (j) and deduced number of FSC lineages surviving (k) for the indicated number of days AHS. In j, the error bars show s.e.m. for n = 50 (5 days), 50 (9 days), 53 (14 days), 47 (20 days) and 60 (30 days) biologically independent ovarioles. The mean number of distinct FSC lineages differed significantly by Pearson’s chi-squared test between day 5 and day 9 (P < 0.0005), day 9 and day 14 (P = 1 × 10⁻⁵), day 20 and day 30 (P < 0.01). The observation of several distinct FSC lineages was not contingent on using multiple heat-shocks for clone induction. An analogous experiment 12 days after a single heat-shock produced ovarioles with one (5/45), two (18/45), three (15/45), four (5/45) and five (2/45) FSC clone colours with FSCs in the same locations as described earlier (Supplementary Fig. 3). (l) Mean number of FSCs labelled per MARCM clone at different times AHS.
Heterogeneity among FSCs

To investigate possible heterogeneity in the FSC population (Fig. 5a) we examined proliferation rates, morphology, gene expression markers and cell fate. We measured proliferation rates by labelling MARCM FSC clones with EdU immediately after ovary removal. EdU incorporation into a greater proportion of layer 1 FSCs (29%) than layer 2 FSCs (16%), while layer 3 FSC labelling (3%) was barely higher than quiescent ECs (1%) (Fig. 5c).

Surface labelling of single cells showed that layer 1 FSCs extended processes over the posterior surface of stage 2b cysts, while layer 2 and 3 FSC processes generally contacted the anterior surface of 2b cysts (Fig. 5d–f and Supplementary Fig. 6a–c). By contrast, region 1 ECs had short processes and region 2a EC processes wrapped around stage 2a cysts (Supplementary Fig. 6d–f), as noted previously.

Some markers showed graded expression among FSCs but all were additionally expressed in FCs, ECs or both. Castor and 109-30-GAL4 were expressed in all FSC layers and FCs but were largely absent from ECs (Fig. 6a–c and Supplementary Fig. 6g–i). Conversely, PZ1444 (ref. 4) and C587-GAL4 (ref. 17) were expressed in ECs, declined over FSC layers, and were largely absent from FCs (Fig. 6d and Supplementary Fig. 6j–l). Fox-GFP was strongly expressed in all FSCs and a limited number of nearby cells (Fig. 6e,f).

To examine functional diversity among FSCs we scored how often germaria with labelled FSCs in only one layer were associated with labelled FCs in the most anterior Fas3-positive cells, indicating very recent FC production. Recently produced FCs were very frequently associated with layer 1 FSCs (85%), but much less frequently with FSCs in layer 2 (17%) or 3 (8%) in 9-day multicolour clones and in MARCM clones (Fig. 5b,g–i). We conclude that layer 1 FSCs produce FCs much more frequently than FSCs in other layers. We propose that FCs derive directly only from layer 1 FSCs.

Conversely, we suggest that ECs derive directly from their immediate neighbours in layer 3 or 2, as might be expected simply on a mechanical basis. Consistent with this proposal, marked FSCs that moved into EC positions during live imaging were always anterior to other FSCs in the same germarium (Fig. 4i and Supplementary Fig. 5). We then asked whether FSCs can move between layers. Approximately two-thirds of RFP-negative multicolour clones (23/34) and MARCM clones (39/57) with two or more marked FSCs included FSCs in more than one layer, even though the overall frequency of those clones suggests that the majority originated from a single marked cell. There must therefore be movement of FSCs between layers. We also saw several examples of FSCs exchanging A/P positions during live imaging (Supplementary Videos 1 and 2). On the other hand, a relatively high proportion of 9-day MARCM FSC clones contained FCs but no ECs or vice versa (Fig. 4b–d), suggesting that FSCs can spend several days in a single layer, producing only one type of derivative. Thus, the FSC population exists in a relatively slow equilibrium

especially when considering only ovarioles that included an FSC (Fig. 4g). Labelled ECs in germaria previously lacking any labelled ECs must derive from a cell type other than an EC. The tight correlation of new ECs with ovarioles containing labelled FSCs strongly suggests that FSCs are the source of new ECs.

The diversity of MARCM clone types can also be readily explained. First, some FSC lineages lack FCs or ECs because an individual FSC produces these cells only sporadically. Accordingly, the proportion of FSC/FC clones lacking ECs declines over time to almost zero, as each surviving FSC clone eventually produces a relatively stable EC (Fig. 4h). Second, individual FSC lineages are also lost frequently (Fig. 2j,k), leaving a residue of any marked ECs produced and hence an increasing proportion of ‘EC-only’ clones over time (Fig. 4d).

We also saw evidence of EC production from FSCs in live imaging studies. In at least four cases we observed extensive migration of a GFP-labelled cell from FSC territory to an anterior position characteristic of ECs (Fig. 4i and Supplementary Fig. 5).

Figure 3 Radial movement of FSCs visualized by live imaging. (a,b) Time-stamped frames up to 3 h 15 min (a) and 3 h 00 min (b) from live imaging of MARCM-labelled FSCs showing frequent radial and independent movement of FSCs (each marked by a coloured arrow). Each image has several z-sections combined, so circumferential movement appears like movement across the interior of the germarium. The frames in a are from Supplementary Video 1, and those in b are from Supplementary Video 2. Supplementary Fig. 4 shows FSC movements tracked in two dimensions around the germarial circumference (from Supplementary Video 6). Scale bars, 20 µm.
along the A/P axis, with individual FSCs occasionally moving from one layer to another and consequently switching between EC and FC production.

**Short-term twin-spot studies confirm FSC origin of ECs and FSC exchange between layers**

We generated multicolour clones using a minimal heat-shock and stained ovarioles 72h later to capture all cells derived from recombination in a single FSC. After recombination the two daughters have known ‘twin-spot’ colour pairings (G–BR, B–GR, GR–BR, BG–GBR; Supplementary Fig. 7). Both daughters of an FC necessarily populate the same egg chamber. Unpaired FC patches must therefore derive from recombination in an FSC and were found up to the fourth egg chamber from the germarium. We concluded that marked FSC derivatives populated the germarium (generally two FC-associated cysts), the first three egg chambers and sometimes the fourth, consistent with budding of new egg chambers every 12h (ref. 4).

The average contribution of each FSC lineage colour to the entire FC population spanning egg chambers 1 to 3 or 4 (as appropriate for each ovariole) was 9.2% (n = 90), approaching expectations for just one of the 14–16 FSCs present. Moreover, we could identify and exclude several exceptional lineages founded by two FSCs on the basis of the presence of overlapping twin-spot pairs (for example, GR lineages in ovarioles with B–GR and BR–GR pairs) to ensure that almost all lineages studied derived from a single cell.

Among the sixteen lineages with at least one EC, nine had matching FCs; in six cases the twin-spot lineage also included FCs (Fig. 7a–d). Of the remaining seven lineages, the twin-spot sister clone included FSCs in five cases, showing that the parent FSC produced both EC and FC; in six cases the twin-spot lineage also included FCs (Fig. 7a–d). Of the remaining seven lineages, the twin-spot sister clone included FSCs in five cases, showing that the parent FSC produced both EC and FC; in six cases the twin-spot lineage also included FCs (Fig. 7a–d). Fas3 (red) and Vasa (blue) stain along the A/P axis, with individual FSCs occasionally moving from one layer to another and consequently switching between EC and FC production.

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In these twenty lineages, the progeny of a single FSC must have moved between layers to produce an FC from layer 1 and an EC from layer 2 or 3. On the other hand, over 40% of EC-containing lineages...
To study how FSC fate is regulated we examined extracellular signals that act on FSCs. Both Hedgehog (Hh) and JAK–STAT signalling promote FSC maintenance. \( ^7, ^19 \) Hh signalling was strong in ECs, FSCs and early FCs, with lower levels in more posterior FCs. \( ^2, ^19, ^21 \) (Supplementary Fig. 6m–o). A JAK–STAT pathway reporter \( ^19, ^22 \) was expressed in FCs, FSCs and region 2a ECs but barely at all in region 1 ECs (Fig. 6a–c). Fz3-RFP reports Wnt pathway activity in the gerarium based on a robust signal that is greatly altered by genetic changes in Wnt pathway activity. \( ^23 \) Fz3-RFP was expressed most strongly in ECs and anterior FSCs, with clearly lower levels in layer 1 FSCs and low or undetectable levels in FCs (Fig. 6g–i). Quantitation showed a consistent decline across the FSC domain (Fig. 8a). FSC clones are poorly maintained in adults if Wnt pathway activity is eliminated or increased \( ^19, ^24 \) but it is not known how Wnt signalling affects FSC maintenance.

Increased Wnt pathway activity induced by loss of Axin (Axn) or Adenomatous polyposis coli (APC) activity led to a decline in the frequency of FC-producing FSC clones over time (Fig. 8d,e and Supplementary Fig. 8a,d), as noted previously. \( ^19, ^24 \). There were also significantly fewer FSCs per ovariole than in controls by 12 days (Fig. 8g and Supplementary Fig. 8b). Strikingly, all ovarioles contained

**Figure 5** FSCs are heterogeneous along the A/P axis. (a) Two surfaces of a 3D reconstruction traced from a germarium with multicolour FSC clones, showing germline cells (grey/white), cap cells (pale orange), ECs (yellow) FCs (orange with red Fas3 outlines) and FSCs in layer 1 (blue), layer 2 (pink) and layer 3 (green). (b) Percentage of all clones with FSC(s) in only the indicated layer that are represented in the first layer of Fas3-positive FCs for multicolour clones and MARCM clones. The proportions of layer 1 FSC clones and layer 2/3 FSC clones (combined) with recent FCs were both significantly different to the proportion of all single layer FSC clones with recent FCs (multicolour, \( n = 48 \) (24 layer 1, 19 layer 2, 5 layer 3) biologically independent clones; MARCM, \( n = 30 \) (13 layer 1, 13 layer 2, 4 layer 3) biologically independent clones) by Fisher’s exact two-tailed test \( (P < 0.05) \). s.e.m. indicated by error bars. (c) Fraction of wild-type MARCM-labelled FSCs in each layer labelled by EdU (error bars show s.e.m. from five experiments with \( n = 375 \), 191 and 70 total FSCs scored for layers 1, 2 and 3, respectively). EdU indices for layer 1 versus 2 and layer 2 versus 3 are significantly different \( (P < 0.005) \) by Fisher’s exact two-tailed test. (d–f) Single mid-section layer 1, 2 or 3 FSCs labelled with surface CD8-GFP in Fas3 (red)-stained gerarium. (d) Layer 1 FSC processes contact the posterior surface of a stage 2b cyst. (e,f) Layer 2 (e) and layer 3 (f) FSCs contact the anterior surface of stage 2b cysts. All FSC processes span the gerarium. (g–i) Examples of MARCM clones where the FSCs labelled (green) are in layer 1 (white arrows) (g), layer 2 (cyan arrow) (h) or layer 3 (yellow arrows) (i). Only layer 1 FSCs (g) are associated with (green) labelled FCs (pink arrows) in the first layer of cells expressing Fas3 (red). g and h have six z-sections combined. In g, the diagonal white line indicates an edge of the original image (shown in Supplementary Fig. 9). Scale bars, 20 \( \mu m \).

had no matching FCs (7/16) and almost 80% of FC-containing lineages included no ECs (33/42), suggesting that FSCs also often dwell in an EC-producing or FC-producing layer. When there were two or more surviving FSCs of a single colour, they were confined to the same layer in eight cases and occupied more than one layer in sixteen cases, again indicating a moderate rate of movement between layers (Fig. 7b–f).

Altogether, 43 ECs and 166 FC patches were produced from a total of 152 marked FSCs, suggesting that ECs are produced at a frequency about fourfold lower than FSCs, or roughly one per cycle of egg chamber budding (Fig. 7g). On the basis of the number of FSCs in different layers and their relative EdU indices (Fig. 5c), we estimate that FSC division in layer 1 may not quite suffice to supply FCs without net FSC loss, and hence that there may be a slow net flow from layer 2 to layer 1 (Fig. 7g).

**Wnt signalling controls A/P position and conversion of FSCs to ECs**

To study how FSC fate is regulated we examined extracellular signals that act on FSCs. Both Hedgehog (Hh) and JAK–STAT signalling...
Figure 6 Heterogeneous gene expression among FSCs. (a–i) Expression of markers along the A/P axis; arrows indicate the border of Fas3 expression (green), layer 1 (white), 2 (cyan) and 3 (yellow) FSCs. (a–c) Castor (Cas, red in a,b, grey in c) stains FCs and all FSCs, while the stat-GFP (green) JAK–STAT pathway reporter also extends into region 2a ECs. (d,g–i) PZ1444-lacZ (red) (d) and the Fz3-RFP Wnt pathway reporter (red) (g–i) show strong expression in ECs and weaker expression in FSCs, with reduced or undetectable expression in layer 1 FSCs (white arrows) and FCs (pink arrows); Fas3 (green). h shows Fz3-RFP (grey) from the same gerarium as in g. (e,f) Fax-GFP (green in e, grey in f) is expressed more strongly in ECs than in FCs or ECs. In i, the diagonal white line indicates an edge of the original image (shown in Supplementary Fig. 9). Scale bars, 20 µm.

ECs by 12 days (Supplementary Fig. 8d) with a huge increase in the average number of ECs per ovariole (Fig. 8d,e,g), suggesting that FSCs were lost principally because they became ECs at an unusually high rate. The large number of axn or apc mutant cells in the location of ECs did not incorporate EdU (0/499 at 6 days; 0/1093 at 12 days). Hence, these cells did not amplify after becoming ECs; they exhibit a key characteristic of normal EC cells—quiescence.

The loss of FSCs was slower for axnS0 than for axnF77 and apc1 apc2, but axnS0 clones eventually produced the most ECs per ovariole (Fig. 8g and Supplementary Fig. 8b). This inverse correlation is consistent with greater initial survival and amplification of axnS0 FSCs (relative to axnF77 and apc1 apc2 FSCs) before conversion of the majority of FSCs to ECs, providing further evidence that ECs normally derive from FSCs.

axn and apc mutant FSCs accumulated significantly in layer 3 compared with controls by day 6 (Supplementary Fig. 8c) and by day 12 only 15–20% of surviving FSCs were in layer 1 compared with almost 60% for controls (Fig. 8i). Thus, FSCs with elevated Wnt pathway activity moved to increasingly anterior positions.

EdU incorporation was significantly lower in axn and apc mutant FSCs than in controls, even when considering FSCs only in layer 1 (Supplementary Fig. 8h). We have examined many other mutations that reduce FSC proliferation by similar or greater amounts25–27 but they did not significantly alter FSC location or increase the production of ECs.

When Wnt pathway activity was eliminated by a null arrow mutation FSC/FC clones were lost at a modestly enhanced rate (Fig. 8c,f and Supplementary Fig. 8e), as noted previously19,24. Strikingly, we found that almost no arr mutant ECs were produced (Fig. 8c,f,h and Supplementary Fig. 8e,f). Reduced EC production cannot be attributed simply to loss of FSCs because the ratio of ECs to FSCs was much lower for arr than for controls (0.06 versus 0.19 at 6 days; 0.11 versus 0.37 at 12 days). FSC proliferation was not significantly altered by loss of arr (Supplementary Fig. 8i).

The disposition of arr FSCs was skewed substantially towards layer 1, especially by 12 days (Fig. 8j and Supplementary Fig. 8g). Most clones containing only layer 1 FSCs were associated with marked ECs in the first Fas3-positive cells for arr (86%), as in controls (82%),
Figure 7 Twin-spot clone evidence for a common FSC origin of FCs and ECs. (a-f) Analysis of multicolour FSC twin-spot clones 72 h after induction. Known twin-spot pairings (Supplementary Fig. 7) allow identification of the two sister lineages derived from recombination in an FSC. (a) Illustration of the germarium and egg chambers populated by derivatives of GR and BR daughters of an FSC. (b,c) Two z-sections of the germarium illustrated in a. The BR EC has no corresponding FSC or FC patch, while the GR lineage includes ECs, FSCs and FCs. The twin-spot parent FSC (and the GR daughter) therefore produced both ECs and FCs. (d) A G EC has no corresponding FSC or FC (the only G cell is indicated by a green arrow; all other cells with a similar colour in this image are GR cells) but the twin-spot BR lineage includes FCs and FSCs (as well as an EC in a different z-section). The twin-spot parent FSC therefore produced both ECs and FCs. (e,f) Two z-sections of the same germarium show one G FSC in layer 1 (white arrow) and two G FSCs in layer 2 (cyan arrows), showing that FSCs can move between layers. In b,c the two GR FSCs are both in layer 1, and in d there are BR FSCs in both layer 1 and 2. Dotted white line in b-f indicates anterior limit of Fas3 staining. (g) Summary of FSC dynamics through one cycle (left to right), inferred from the rates of FC production, EC production and FSC division, as well as graded rates of FSC proliferation in layers 1–3. On average, 3–4 of 7–8 FSCs in layer 1 (blue) and 1–2 of 5–6 FSCs in layer 2 (green) divide, while at least 4 layer 1 FSCs become FCs and 1 layer 2 or 3 FSC becomes an EC. Consequently, in some cycles there must also be net movement of a FSC in layer 2 to replenish layer 1 or 3. Scale bars, 20 µm.

indicating recent FC production. The concentration of arr mutant FSC in layer 1 can therefore explain why almost all ovarioles with marked arr mutant FSCs also included marked FCs (Fig. 8f and Supplementary Fig. 8e) and further substantiates the hypothesis that FCs derive directly only from layer 1 FSCs.

In summary, eliminating Wnt signalling in FSCs led to a shift to layer 1, increased FC production by surviving FSCs and an almost complete failure to produce ECs, while elevation of Wnt pathway activity led to FSC movement out of layer 1, reduced FC production by surviving FSCs and a very large increase in EC production. These
correlations provide further evidence that ECs derive directly from anterior FSCs while FCs derive directly from posterior FSCs. The results also show that FSC location and FSC fates are guided by the magnitude of graded Wnt signalling.

**DISCUSSION**

We have uncovered several interesting facets of somatic stem cell organization in the *Drosophila* ovary. First, a relatively large population of FSCs is maintained by population asymmetry. Second,
FSCs produce not only proliferating FCs but also quiescent ECs. Third, FSCs have heterogeneous properties correlated with their position along the A/P axis; most importantly, FCs derive from posterior FSCs while ECs derive from anterior FSCs. Fourth, FSCs are radially mobile and can exchange A/P positions. Fifth, the level of Wnt signalling is graded over the FSC domain, dictating the A/P position of FSCs and whether FSCs produce FCs or ECs.

In this study we identified and counted clones originating in FSCs by the presence of marked FCs at least 9 days after marking, several days beyond the lifetime of an FC, which inevitably passes through the ovariole without delay in association with a germline cyst. We also identified single marked cells of these lineages that persisted in the germainium to define the precise location of FSCs. These criteria meet the fundamental defining characteristics of stem cells.

Our measurement of 14–16 FSCs may not be precise because we could count only six distinct surviving lineages directly and we had to estimate how many lineages were lost before our earliest sampling time (Fig. 2k). Nevertheless, both the total number of FSCs and their distribution among layers deduced from lineage analyses matched the total somatic cell content of layers 1–3 almost perfectly. Several factors contributed to finding more FSCs than previously appreciated. More lineage colours, a high frequency of clone induction and comprehensive cell-by-cell analyses increased our opportunity to count surviving FSCs, while analyses at multiple time points allowed us to deduce that FSCs are maintained by population asymmetry, with many lineages lost very quickly. Earlier estimates of far fewer FSCs were based, in essence, on counting only those stem cells that survived for relatively long periods of time. A-P positions over time and therefore constitute a single stem cell population. Retrospective analysis shows that some FSCs were short-lived and some produced only FCs, while others produced ECs, but these behaviours cannot be forecast for individual FSCs. At any one instant, all FSCs in the population have the potential to produce FCs and ECs, and have an unpredictable lifespan.

Many aspects of FSC organization reported here are remarkably similar to the organization of mammalian intestinal stem cells. FSCs and Lgr5-positive murine intestinal stem cells exist in communities of similar size. Both are maintained by population asymmetry, engendering neutral competition among individual stem cells, but there is also spatial heterogeneity coupled to dynamic exchange (dynamic stem cell heterogeneity) along the major developmental axis. A key difference is that dynamic heterogeneity guides alternative differentiation outcomes only for FSCs. In the gut, diverse secretory cells and transit-amplifying cells are defined, shortly after their derivation from stem cells, by different levels of Wnt and Notch pathway activities. However, it is currently thought that each cell type is initially produced at the same location immediately above the stem cell population, with stochastic expression of Notch ligands determining which cells adopt secretory fates through lateral inhibition.

The choice of an FSC to move anteriorly and become one cell type, to move posteriorly and become a different cell type, or to remain within the stem cell niche presents a potentially general stem cell paradigm. Here, these choices are guided by the magnitude of Wnt signalling at multiple locations within the stem cell domain. Increased Wnt signalling caused layer 1 FSCs to move anteriorly and accelerated the conversion of layer 2/3 FSCs to ECs. Conversely, loss of Wnt signalling caused posterior movement and reduced EC production in layer 2/3 FSCs. Dose-dependent responses at different positions and the observed gradient of Wnt signalling declining from anterior to posterior over the FSC domain suggest that normal FSC behaviour is guided by graded Wnt signalling.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.R. and D.K.; methodology, A.R., E.C., N.T., G.V.-N. and D.K.; formal analysis, A.R., D.M. and D.K.; investigation, A.R., D.M., K.S.P., A.B. and S.F.; writing original draft, D.K.; writing-review and editing, A.R., D.M. and D.K.; visualization, A.R. and D.K.; funding acquisition, D.K.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Multicolour lineage analysis. Two-day old adult Drosophila melanogaster females of genotype yw hs-flp; yw−/−; ubi-GFP FRT40A FRT42B ubi-RFP tub-lacZ. FRT40A FRT42B were given either a single 1 h heat-shock at 37 °C or four 1 h 37 °C heat-shocks spread over 3 days to allow for extensive recombination. The first heat-shock generated a large variety of stem cell lineages. In all experiments flies were maintained at room temperature by frequent passage on normal rich food supplemented with fresh wet yeast. Flies were dissected at 9, 14, 20 and 30 days after the last heat-shock and stained for Fasciclin III (Fas3) and β-galactosidase. Germline cells were imaged in z-interfaces of 3 μm and ovarioles in intervals of 4 μm. MSC lineages were counted at 9 (n = 50); 14 (n = 53) 20 (n = 47) and 31 days (n = 60). Statistical analysis was performed by assessing the number of surviving FSCs per germline deduced from the number of FSC clone colours observed, and the number of founder FCS per egg chamber deduced from FC contributions of a single colour are described in a Supplementary Note. For twin-spot experiments following the fate of all FSC derivatives, two-day-old adult flies of the same genotype as above were heat-shocked at 32 °C for 30 min to induce a low frequency of recombination in FSCs and ovarioles were dissected 3 days later. Two ovarioles contained two non-overlapping pairs of twin-spots (G-GR and BR-GR), twelve ovarioles had overlapping pairs (G-GR and GR-BR), twelve ovarioles had a single pair of colours but most likely derived from two FSCs (from the pattern of FC production or total cells produced) and fifteen ovarioles had a single pair of twin-spots most likely deriving from a single FSC (so that 39 of the 63 lineages seen most likely derived from a single FSC and could be identified as such).

Lineage analysis by MARCM. Two-day-old adult flies of genotype hs-FLP UAS-GFP tub-GAL4; tub-GAL80 FRT40A/NM FRT40A; act > CD2 > GALA were heat-shocked for 15 min at 37 °C. Flies were dissected 9, 14 and 22 days after heat-shock, and stained for GFP and Fas3. FSCs, ECs, and FCs were scored in all z-sections of ovarioles at 9 (n = 148 ovarioles), 14 (n = 77) and 22 days (n = 112).

Immunohistochemistry. Ovaries were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, rinsed three times in PBS with 0.1% Triton and 0.05% Tween-20 (PBST), and blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories) in PBST. Monoclonal antibodies against Fasciclin III and Vasa were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, Iowa 52242. 7G10 anti-Fasciclin III was deposited to the DSHB by C. Goodman, and was used at 1:300 for multicolour lineage experiments and at 1:250 in all other stainings. Anti-Vasa (used at 1:10) was deposited to the DSHB by hand and the nuclei of cells were color-coded by layer using Adobe Photoshop.

Image acquisition and processing. Ovarioles were imaged with a 63 × 1.4 N.A lens on a Zeiss LSM 700 confocal microscope (Carl Zeiss). Zeiss Zen 2012 was used to acquire microscope images of 512 × 512 pixels at 12-bit depth with linear averaging 2 and pixel dwell 3.15 μs at room temperature. Germline and initial egg chambers were acquired with xyz scaling of 0.198 μm, 0.198 μm, 3 μm and larger egg chambers were acquired at 0.5 zoom, xyz scaling of 0.397 μm, 0.397 μm, 4 μm. The range indicator was used to set the appropriate laser intensity for each fluorophore such that the signal was in the linear range. Zen was used to linearly adjust channel levels when a colour was dim rather than setting the laser intensity higher to avoid photobleaching the sample. The presence of a fluorescent signal in each colour was determined by examining each channel individually in the collected image (see Supplementary Fig. 1). Images were digitally zoomed in Zen using digital interpolation and exported as tifs using ‘Contents of Image Window’. Images were rotated in Adobe Photoshop CS to position the anterior of the gerarium and egg chambers to the left. Multicolour ovarioles in Fig. 2 and Supplementary Figs 2 and 3h are composites of gerarium and ovariole images scaled to the same dimensions; different z slices were also chosen to show appropriate clones; borders of individual panels are indicated with a white line.

Measurement of Flizzled3-RFP levels. Levels of Flizzled3-RFP were quantified using Fiji software13. Individual escort cells, FSCs and immediate FCs were outlined using DAPI and Fasciclin III to locate the cells. Each outlined region was transferred onto a greyscale image of F23-RFP using the ROI manager tool and the mean greyscale fluorescence intensity of F23-RFP was measured. For each gerarium, the two brightest region 1 ECs were used as internal controls. The intensities of the two brightest ECs, FSCs and no germ cell generated FCs were measured and their values normalized to the mean region 1 EC intensity.

3D gerarium reconstruction. For each optical z-section, Fas3 staining was traced by hand and the nuclei of cells were colour-coded by layer using Adobe Photoshop. Velocity 3D image analysis software (Perkin Elmer) was used to render the 3D reconstruction in Fig. 5a.

Live imaging. Imaging chambers were fabricated with poly(dimethylsiloxane) (PDMS, Sylgard 184 distributed by Dow Corning). A degassed 10:1 w/v solution of base and curing agent was poured into an apt container and allowed to polymerize at 65 °C for 3 h. Three 8-mm-diameter wells were coated by coring the elastic PDMS with biopsy punches. Perpendicular channels with a smaller diameter (1.5 mm) were then created to connect the main wells. The central chamber was dedicated to the ovarioles, while the lateral chambers acted as medium reservoirs, feeding the middle chamber via connecting channels. Two 8-mm-thick PDMS slabs were stacked in two layers (the top one having only the 3 chambers and no channels) to increase the volume of media in the reservoirs. To ensure hydraulic sealing, the slabs were irreversibly bonded via air plasma treatment; another plasma treatment sealed the assembled chambers to a 75 × 25 mm glass coverslip. Thin PDMS membranes were used as lids to prevent medium evaporation from the chambers.

GFP-marked FSC clones were induced in flies of genotype hs-flp UAS-GFP tub-GAL4; tub-GAL80 FRT40A/NM FRT40A; act > CD2 > GALA or hs-flp; UAS-GFP act > CD2 > GALA; FRT282 tubGalo80/282BNM. Ovaries were dissected into imaging medium formulated in ref. 36 (20% FBS in Schneider's insect medium, 0.2 mg ml−1 insulin, penicillin and streptomycin). After separating ovaries into individual ovarioles, 135 μl ovarioles were mixed with 15 μl Matrigel (Corning), added to the imaging chamber and left covered for 15 min to gel. Wells were then filled to the top with imaging medium. Germline cells were imaged every 15 min on a Zeiss LSM 700 confocal microscope. Cells appeared healthy throughout, with many cell divisions observed (for example, see Supplementary Video 1).

EdU labelling. Ovaries were dissected directly into 15 μm EdU in Schneider's insect medium, incubated for 1 h at room temperature and then fixed for 10 min at room temperature in 4% paraformaldehyde. EdU incorporation was detected using the Click-iT Plus EdU Imaging Kit C1063B (Life Technologies).

Markers and clonal analysis of mutant genotypes. Flies with alleles on an FRT42D chromosome; + (control) or arr+, were crossed to hs-flp, UAS-GFP, tub-GAL4; FRT42D tub-GAL80/Gy; act > CD2 > GALA flies to produce MARCM clones (abbreviated as FRT). Flies with arr+ on FRT42D chromosome NM (Notch-Myc, control), axnFRT42D (abbreviated as axnFRT), axn+77 or apcFRT were crossed to hs-flp; UAS-GFP act > CD2 > GALA; FRT82B tubGalo80/TM2 flies. Marker expression analysis were performed with PZ1444, F3-RFP, CS7-Gal4, 109-Gal4, stat-GFP (10 Stat-binding sites in GFP promoter) and fagx-GFP. Flies of genotype yw/yw; hs-flp; FRT40A UAS-cd2-RFP UAS-GFP-mRNA/FT40A UAS-dE-GFP UAS-cd2-mRNA; act > CD2 > Gal4/4 were heat-shocked to produce surface-labelled clones by the twin-spot generator technique19 to examine FSC and EC shapes.

Statistics and reproducibility. All images shown are representative of at least ten examples. In most cases the number is much higher and is given explicitly where relevant for statistical analysis of outcomes. No statistical method was used to predetermine sample size but we used prior experience to establish minimal sample sizes. No samples were excluded from analysis, provided staining was of high quality. The experiments were not randomized; all samples presented as groups in the results were part of the same experiment and treated in exactly analogous ways without regard to the identity of the sample. Investigators were not blinded during outcome assessment, but had no pre-conception of what the outcomes might be. Standard statistical tests are described in each figure legend; sample sizes were appropriately large with appropriate distributions; relevant means, standard errors of the mean and P values are cited together with explicit sample sizes, which always represent biological replicates.

Data availability. Source data for Figs 2, 4, 8 and Supplementary Fig. 8 have been provided as Supplementary Table 1. All data supporting the findings from this study are available from the corresponding author on reasonable request.

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Supplementary Figure 1 Multicolour labeling method, imaging and scoring. (a) Second chromosome genotype (top) of flies used for lineage marking, showing tub-lacZ (lacZ), ubi-GFP (GFP) and ubi-RFP (RFP) transgenes and FRT 40A and FRT 42B recombination targets either side of the centromere (white oval). Heat-shock (HS) induction of a hs-flp transgene on the X-chromosome can induce recombination independently at either pair of FRTs, making one or both (2L and 2R) chromosome arms homozygous in daughter cells, thereby eliminating one or more of the marker genes (one possible outcome for one daughter cell is shown). The resulting daughter cell is designated “BR” to indicate the presence of just two colours (Blue and Red) compared to the three colours (BGR) present for parental cells after staining (B- Blue lacZ, G-Green GFP, R- Red, RFP). (b) The nine possible genotypes that can be produced by FRT-mediated recombination are indicated by the combination of 2L genotypes (labeled vertically) and 2R genotypes (labeled horizontally; “+” indicates the absence of the RFP transgene). The resulting diploid genotypes are displayed within the nine large boxes; each vertical column of small coloured squares represents one chromosome that may include lacZ (Blue), GFP (Green) or RFP (Red) transgenes or not (white). Two copies of ubi-RFP could not reliably be distinguished phenotypically from one, so there are only six distinguishable phenotypes (indicated by the combination of Blue (B), Green (G) and Red (R) colours present). Only the three RFP-negative phenotypes on the right (BG, B and G) are produced by just a single genotype. Accordingly, these RFP-negative phenotypes are expected to be the least frequent. In the 9d AHS (after heat-shock) experiment RFP-negative FSC lineages were indeed present at a frequency (52/177) roughly half that of RFP-positive FSC lineages (125/177). (c) Illustration of one possible distribution of FSC genotypes in a germarium. Each FSC is represented by a coloured circle within one of the nine boxes representing the nine possible genotypes. Note that two (or more) FSCs may acquire identical genotypes (BGR, top left) or may share the same RFP-positive phenotype despite having different genotypes (for example, GR FSCs in the left and middle columns). Hence, in this germarium, seven FSCs exhibit just four distinct phenotypes. (d-n) Images of the same ovariole shown in Fig. 1c,d to illustrate how single channel images are used to assign transgenes present and how a complete series of z-sections is used to assign FSC positions. Images show all channels, (e) Fas3 antibody staining (white), (f) β-galactosidase antibody staining (blue, “B”), (g) GFP (green, “G”) and (h) RFP (red, “R”) for a central z-section. The red channel includes some bleed-through from Fas3 staining (from Alexa 546) but the RFP signal is distinguished as nuclear, while Fas3 staining is at the plasma membrane (germline nuclei also stain in one or more channels but are easily distinguished by their size and location). Successive z-sections encompassing the germarium (one section between j and k is omitted because it shows only FSCs seen in adjacent sections) with each FSC labeled according to colour (BGR, GR, B, BR). (i) The B FSC seen in (k) and the two lower GR FSCs seen in (m) are also seen in this section but are not labeled, so that each FSC is labeled only once in (i-n). Yellow arrows mark the borders of Fas3 expression (pink in composites). Brackets or arrows marking FSCs are labeled according to layers, with layer 1 (white) adjacent to Fas3, layer 2 (cyan) immediately anterior (left) and layer 3 (yellow) one cell further anterior (left). Fourteen FSCs are shown (1 B, 2 BR, 8 GR, 3 BGR), with one (GR*) in (n) in the process of mitosis. Note that there is a single candidate B FSC (as stated for Fig. 1c) and also note that the BGR FSCs are not seen in central z-sections (the 60° sectors shown in Fig. 1e). Also, there are no G or BG FSCs even though G and BG FCs are present (f-h and Fig. 1d), indicating that G and BG FSCs were lost between day 5 and day 9. Scale bar of 20μm applies to all panels (all are same magnification).
Supplementary Figure 2 Decline in number of FSC lineages over time indicates population asymmetry. (a-c) Percentage of ovarioles (among 50 scored) with the indicated number of distinct clones 9d AHS defined by inclusion of (a) both FSCs and FCs (“FSC clones”), (b) FSCs (with or without matching FCs) and (c) FCs (with or without FSCs), representing all active FSCs present at 5d. To the right of each observed distribution is the expected distribution of distinct colours predicted from a model (in blue) for the number of FSCs (5, 6 and 10, respectively) that best fits the adjacent experimental distribution (see Supplementary Note). (d–j) Examples of (f,g,j) ovarioles and (d,e,h,i) their germaria at higher magnification from multi-colour FSC lineage experiments examined at 9 days AHS. (d–g) Illustration of an FSC-only clone (such clones are included in (b) but not (a)). The same (d,e) germarium and (f,g) ovariole with (d,f) all colours present or (e,g) only the green channel (to clearly distinguish B from BG cells), showing B FSCs with no matching FC. (d,e) have seven z-sections combined to show all FSCs. (f,g) Vertical white lines separate different z-sections (indicated top right) for different regions of the ovariole (full original images for each z-section in Supplementary Fig. 9). (h–j) Illustration of an FC-only clone (such clones are included in (c) but not (a)). (h,i) Two combinations of z-sections (z1–3 and z5–8 to show all FSCs) of one germarium and (j) egg chambers from the same ovariole, showing BG FC patches with no BG FSC. Scale bars are 20μm. (k–n) Percentage of all B, G and BG multicolour or GFP+ MARCM FSC clones that contain a given (k,l,n) number or (m) range of FSCs when analyzed at the stated times after clone induction. The total number of biologically independent clones analyzed was (k) n=64, (l) n=83, (m) n=83 (9d), n=43 (22d) and (n) n=20.
Supplementary Figure 3 FSC locations from multicolor lineages induced by a single heat-shock. (a-h) Examples of (a,c,e,g) germaria (at higher magnification) and (b,d,f,h) their associated ovarioles from multi-colour FSC lineage experiments examined 12 days after a single heat-shock. (a,b) The only candidate FSC of the BR lineage is in layer 1 ("BR", white arrow). (c,d) The only candidate FSC of the BGR lineage is in layer 2 ("BGR", cyan arrow). (e,f) The only candidate FSC of the BR lineage is in layer 3 ("BR", yellow arrow). (g,h) The only candidate FSC of the BG lineage is in layer 1 and is at the bottom surface of the germarium, not in a mid-section ("BG", white arrow). The border of Fas3 staining (pink) is outlined by a white dotted line in (a,c,e,g). (a,c) have two z-sections combined and (e) has four z-sections superimposed. (h) Vertical white line separates different z-sections (indicated top right) for different regions of the ovariole (full original images for each z-section in Supplementary Fig. 9). (b,d,h) Diagonal white lines indicate an edge of the original image (shown in Supplementary Fig. 9). Scale bar 20μm in (a,c,e,g) and 50μm in (b,d,f,h).
Supplementary Figure 4 Quantitation of Radial Cell Movements in the FSC region by Live Imaging. (a) Graphical representation of radial cell movements for the germarium shown in Supplementary video 6. Positions of cell bodies at each time point were placed around a circle representing a germarial cross-section in the FSC region. Each circle was subdivided into 12 sectors and 9 z-sections (red lines) to place the cells accurately based on their xyz coordinates at each imaging time-point, generally at 15 min intervals. Each GFP-marked cell nucleus could be tracked without ambiguity and is artificially coloured. The whole germarium sometimes moved, including rotations. The critical parameter is therefore the movement of one cell nucleus relative to another. Cell pairs continually switched between periods of moving towards one another and moving apart. (b) For each pair of cells (A-B, A-C etc.) we estimated and tabulated relative movement as the fractions of a sector (1/12 of the circumference) by which two cells separated (positive values in blue) or approached (negative values in pink) over the time interval during which they maintained their approach or separation. The minimum resolvable distance was 0.25 of a sector, so distances were estimated as multiples of quarter sectors. When it was too difficult to judge whether the relationship of cell pairs had changed, the movement was scored as 0. The proportion of time intervals during which cell pairs altered their radial separation was summed over the entire movie (only the first 4h of the full 7h movie are displayed as circles and tabulated here) as “% time moving”. The average rate at which each cell pair altered their separation during periods of movement was calculated as the percentage of the circumference moved per hour (“% circ. moved/h”). The overall averages for 34 pairs of cells analyzed in four full movies are given in the two bottom cells. All 34 cell pairs exhibited continual back and forth movements.
Supplementary Figure 5 Anterior movement of FSCs into EC territory. (a-c) Three time-stamped series of live imaging frames up to (a) 6h 45min, (b) 3h 45min and (c) 3h 37min from germaria where one marked cell (white arrow) moves anteriorly (left) from the FSC region to EC territory. In (c) a second FSC (purple arrow) moves a shorter distance anteriorly. All FSCs marked by arrows in (a-c) moved radially relative to each other. Panel a frames are from Supplementary video 4, panel b from Supplementary video 5, panel c from Supplementary video 6. We observed four clear examples of large anterior movements, likely representing FSCs becoming ECs, in a total of about 80 recorded germaria, each containing an average of four labeled FSCs, viewed on average for about half a cycle (6h). We deduced in our twin-spot analyses that approximately one EC is produced per cycle from the whole FSC population. We would therefore expect an EC to be produced from a quarter of the FSC population (4 labeled FSCs) viewed over half a cycle roughly once in every eight videos. Large anterior movements were observed in roughly 1/20 of videos (4/80). The frequency of these movements is consistent with FSCs becoming ECs but suggests that at least as many ECs were also produced as a result of smaller anterior movements of FSCs in the same set of videos. Scale bars are 20μm.
Supplementary Figure 6 FSC heterogeneity along the A/P axis. (a-f) Single cells labeled with surface CD8-GFP in Fas3 (red)-stained germaria. (a-c) FSCs in a section from the top or bottom third of the z-stacks, showing processes extending bilaterally from (a) layer 1, (b) layer 2 or (c) layer 3 FSCs. (d-g) ECs have (d) short processes in anterior regions but (e,f) longer processes contacting stage 2a cysts if in region 2, closer to the location of FSCs. (g-i) 109-30 GAL4 enhancer trap stained for the products of UAS-GFP (green), PZ1444-lacZ (red) and Fas3 (blue), showing 109-30-GAL4 expression in FSCs and FCs (similar to the pattern of Castor expression but less uniform). (g) and (h) are the same image with or without the blue channel. (i) is the projection of four z-sections, spanning 9μm. (j-l) C587-GAL4 enhancer trap expressing UAS-CD8-RFP (red in j, gray-scale in l) and stained for expression of PZ1444-lacZ (green in j, gray-scale in k) and Fas3 (blue) showed strong C587-GAL4 expression in ECs and weaker expression in FSCs (similar to the pattern of PZ1444-lacZ expression but extending weakly also into FCs). (m-o) ptc-lacZ expression in green (m) or gray-scale (n) from the same germarium and (o) in green in another example is high in ECs, FSCs and the earliest FCs. Arrows indicate Fas3 staining border (green), FSCs in layer 1 (white), layer 2 (cyan) and layer 3 (yellow), and early FCs (pink). All scale bars are 20μm.
Supplementary Figure 7  Twin-spot method. Recombination can occur at either FRT40A or FRT42B or both followed by a variety of possible segregation patterns. In the example shown, the daughter pairs are B and GR. All possible daughter pairs are listed but we did not score BG/BGR pairs because the BGR daughter cannot be distinguished from the many cells not undergoing any recombination.
Supplementary Figure 8 Early (6d) effects of altered Wnt pathway activity on FSC behaviour. (a-c) Increased Wnt pathway activity due to axn or apc mutations (a) decreased the proportion of FSC clones associated with FCs, (b) increased the average number of labeled ECs per germarium, and (c) decreased the proportion of FSCs in layer 1 in favor of more anterior positions. These phenotypes at 6d were generally less pronounced than at 12d (Fig. 8). (e-g) Loss of Wnt pathway activity (e) increased the proportion of FSC clones associated with FCs and reduced the percentage of ovarioles with labeled ECs, (f) reduced the average number of labeled ECs per germarium and (g) increased the proportion of FSCs in layer 1 versus layers 2 and 3. (d) At 12d AHS increased Wnt pathway activity due to axn or apc mutations decreased the percentage of FSC clones associated with labeled FCs and increased the percentage of ovarioles with labeled ECs. (h,i) The fraction of MARCM-labeled FSCs labeled by EdU at 6d (h) was decreased significantly for layer 1 FSCs and the total FSC population by increased Wnt pathway activity and (i) was slightly but not significantly reduced in each layer by loss of Wnt pathway activity. In all cases there is still no significant EdU incorporation into ECs (not shown). Error bars show SEM for (a,b) n=68 (WT), n=65 (axn50), n=67 (axnE77) and n=65 (apc1apc2) biologically independent ovarioles or germaria, (c) n=237 (WT), n=311 (axn50), n=239 (axnE77) and n=119 (apc1apc2) FSCs, (d) n=75 (WT), n=67 (axn50), n=64 (axnE77) and n=63 (apc1apc2) biologically independent germaria, (e,f) n=78 (WT) and n=89 (arr) biologically independent ovarioles or germaria, (g) n=228 (WT) and n=189 (arr) FSCs, (h) n=112 (WT), n=132 (axn50), n=104 (axnE77) and n=68 (apc1apc2) layer 1 FSCs, n=197 (WT), n=321 (axn50), n=237 (axnE77) and n=125 (apc1apc2) total FSCs, (i) n=136 (WT) and n=134 (arr) layer 1 FSCs, n=228 (WT) and n=189 (arr) total FSCs. Significant differences from controls (WT) were assessed by Pearson’s chi-squared test (* p<0.005, # p<0.05). (a,c,d,e,g,h,i) Significant differences from controls (WT) were assessed by Fisher’s exact two-tailed test (* p<0.001, # p<0.05). See Supplementary Table 1 for supporting data.
Supplementary Figure 9  Original images for panels with more than one z-section or image edges. (a-c,h,i,j,n,u-x) Original images for Figure panels where a diagonal white line indicates the edge of an original image. (d-i,o-t,w,x) Original images for each z-section for Figure panels where different z-sections are shown divided by vertical white lines. Some panels were derived from different z-sections showing neighbouring segments of an ovariole because different z-sections were optimal for highlighting the key colours present in a given egg chamber. In all cases, original images were rotated, if necessary, to give a conventional orientation in final panels of anterior to the left.
**SUPPLEMENTARY INFORMATION**

**Supplementary Table Legend**

**Supplementary Table 1** Statistics Source Data (Tab 1) Number of FSC lineages 5-30d AHS (Fig. 2). Number of distinct FSC lineages scored in each ovariole to produce average number of lineage colours reported in Fig. 2j and deduced number of FSC lineages reported in Fig. 2k. Number of FSCs per FSC clone for MARCM analyses 9-22d after clone induction, used to derive Fig. 2l. (Tab 2) MARCM clone types 9-22d after clone induction used for graphical summaries of Fig. 4. The table shows the total number of ovarioles examined (n) and the prevalence of different types of clones containing only the named cell types. Prevalence is expressed as both the absolute number of ovarioles of a given type (n) and the percentage of all ovarioles with this composition (%) for ovarioles examined 9, 14 and 22 days after MARCM clone induction with a single heat-shock. Distribution of FSC among different layers is also shown. (Tab 3) MARCM clone types at 12d AHS (supporting Fig. 8) and (Tab 4) 6d AHS (supporting Supplementary Fig. S8) for genotypes affecting Wnt signaling. Format is exactly as described above for Tab 2 (substituting different genotypes for different time points).

**Supplementary Video Legends**

**Supplementary Video 1** (corresponding to Fig. 3a). Radial movement of FSCs. Maximum projections of the top 3 to 6 z-sections to show relationships of 3 presumed FSCs indicated by arrows. We included 3 to 6 z-sections in a projection in order to capture the highlighted cells throughout the imaging period despite their movement between z-sections. The projections consequently capture only one dimension of the cells’ radial movement. All cells moved regardless of radial position, but since cells in the middle z stacks principally move up and down through z stacks their movement is not captured in this compressed 2-dimensional video. The “white” cell (marked by the white arrow) moved further around the germarium at 4h 15min and was temporarily lost from the movie. At 4h 15min, the “yellow” cell divided; both daughters are subsequently indicated by yellow arrows. Additional presumed FSCs moved into view from the other side of the germarium beginning at 3h 45min but are not indicated by arrows. At 6h the germarium began to disintegrate as cells moved out of the posterior half. We have observed twisting rotations in germaria as they appear to attempt egg chamber budding and during these rotations cells move out of the germaria. Bar, 20 µm.

**Supplementary Video 2** (corresponding to Fig. 3b). Radial movement of FSCs. Maximum projections of the top 2 or 3 z-sections to show the three cells indicated by arrows throughout the movie. The “white” and “yellow” cells crossed one another radially. After 3h of imaging, cells began to move out of the germarium. Bar, 20 µm.

**Supplementary Video 3** (corresponding to Fig. 4i). FSC movement into EC territory. Maximum projections of the top 2-4 z stacks are shown. The “white” and “magenta” cells moved anterior into Escort Cell territory. The “white” and “red” cells crossed paths radially at 2h and again at 4h.

**Supplementary Video 4** (corresponding to Supplementary Fig. 4a). FSC movement into EC territory. Two labeled cells started in the 2a/b region and the “white” cell moved anterior into Escort Cell territory.

**Supplementary Video 5** (corresponding to Supplementary Fig. 4b). FSC movement into EC territory. Maximum projections of the top 4-6 z-sections are shown in order to include the 5 cells indicated by coloured arrows. All cells moved back and forth in radial movements and the “white” cell moved anteriorly into Escort Cell territory. The “red” and “yellow” cells crossed radially.

**Supplementary Video 6** (corresponding to Supplementary Fig. 4c). FSC movement into EC territory. Maximum projections of all z-sections are shown. Initially the “red”, “yellow”, and “magenta” cells were in the top half (5 z-sections), whereas the “white” cell was in the last z-section (on the other side of the germarium). The “magenta” cell travelled around to the other side of the germarium. Both white and magenta cells moved anterior into Escort Cell territory.
SUPPLEMENTARY NOTE

Number of FSCs per germarium and founder FCs per egg chamber: statistical methods

1. Best fit for number of FSCs per germarium based on the distribution of numbers of different FSC colors observed

For statistical modeling we assumed for simplicity that all ovarioles have the same number of surviving FSC lineages at the time of analysis (9d after clone induction for the first analysis). In ovarioles 9 days after clone marking we observed experimentally that each of the three types of RFP-negative FSC clones (B, G and BG) were present at a frequency of roughly 1/9 of the total, while the three types of RFP-positive FSC clones (BR, GR, BGR) were present at a frequency of roughly 2/9. We therefore assumed in our statistical modeling that the different colors of FSC clone were present in those same proportions (B:G:BG:BR:GR:BGR = 1:1:1:2:2:2). Those proportions are exactly what would be expected if each of the nine possible genotype was present at the same frequency. This outcome was very convenient for simple modeling. It represents a balance between too few recombination events (leading to an excess of GFP/lacZ and RFP/+ genotypes) and too many successive recombination events (which lead to an excess of the genotypes stable to further recombination- GFP/GFP, lacZ/lacZ, RFP/RFP and +/+).

Assuming that the nine genotypes in the FSCs are independent and identically distributed (as found experimentally), the probability of observing exactly k different FSC colors when there are n surviving FSC lineages per germarium at the time of analysis is given by the formula below.

\[
\sum_{c_1=(k-3)}^{3k} \sum_{m=c_1}^{n-(k-c_1)} \binom{n}{m} \left( \frac{1}{3} \right)^m \left( \frac{2}{3} \right)^{n-m} f(c_1, m) f(k - c_1, n - m)
\]

where

\[
f(0, 0) = 1 \\
f(\overline{z}, 0) = 0 \quad \text{where } z \text{ is a non-zero integer} \\
f(1, r) = 3(1/3)^r \\
f(2, r) = 3(2/3)^r - 2(1/3)^r \\
f(3, r) = 1 - f(1, r) - f(2, r)
\]
The expected percentages of ovarioles with 1-6 distinct FSC colors calculated from this formula are presented below for different numbers of surviving FSC lineages (n= 4, 5, 6 etc.). The calculated distributions that most closely match observed distributions (from 50 ovarioles expressed as percentages) at 9d are shown below for (a) FSCs with matching FCs, (b) all FSCs (with or without matching FCs) and (c) the number of FC-producing FSC colors present at 5d (equal to the number of distinguishable FC patches at day 9 (Supplementary Fig. S2c)).

(a) Best fit of predicted color distributions to experimental data for FSC clones with matching FCs at 9d.

| k  | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|----|----|----|----|----|----|----|--------------------------|
| FSC+FC 9d observed | 2  | 12 | 44 | 30 | 8  | 4  |                          |
| n=4 prediction   | 1  | 20 | 55 | 24 | 0  | 0  | 302                      |
| n=5 prediction   | 0  | 8  | 42 | 42 | 7  | 0  | 185                      |
| n=6 prediction   | 0  | 3  | 28 | 48 | 19 | 1  | 795                      |

Best match is for n=5 FSCs (lowest value for sum of differences squared).

The theoretical distribution above for 5 FSCs is shown in Supplementary Fig. 2a, next to “FSC clones” data.

(b) Best fit of predicted color distributions to experimental data for FSC clones (with or without matching FCs) at 9d.

| k  | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|----|----|----|----|----|----|----|--------------------------|
| FSC 9d observed | 0  | 6  | 34 | 36 | 14 | 10 |                          |
| n=4 prediction   | 1  | 20 | 55 | 24 | 0  | 0  | 1078                     |
| n=5 prediction   | 0  | 8  | 42 | 42 | 7  | 0  | 253                      |
| n=6 prediction   | 0  | 3  | 28 | 48 | 19 | 1  | 295                      |
| n=7 prediction   | 0  | 1  | 18 | 47 | 30 | 4  | 694                      |

Best match for n= 5-6 FSCs.

The theoretical distribution above for 6 FSCs is shown in Supplementary Fig. 2b, next to “FSCs” data.

(c) Best fit of predicted color distributions to experimental data for FSC clones with matching FCs at 5d.

| k  | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|----|----|----|----|----|----|----|--------------------------|
| FSC+FC 5d observed | 0  | 6  | 18 | 20 | 32 | 24 |                          |
| n=8 prediction   | 0  | 1  | 11 | 41 | 39 | 8  | 820                      |
| n=9 prediction   | 0  | 0  | 7  | 34 | 45 | 14 | 622                      |
| n=10 prediction  | 0  | 0  | 4  | 28 | 48 | 20 | 568                      |
| n=11 prediction  | 0  | 0  | 3  | 22 | 49 | 33 | 635                      |
Best match for n=10 FSCs.

The theoretical distribution above for 10 FSCs is shown in Supplementary Fig. 2c, next to “FC” (= FSC/FC clones at 5d) clone data.

Similar modeling for the observed distribution of distinguishable FC-producing FSC lineages at days 14 (n= 53 ovarioles), 20 (n=47 ovarioles) and 30 (n=60 ovarioles) is shown below. The average number of distinguishable lineages from day 5 to day 30 is plotted in Fig. 2j and the inferred number of surviving FSC lineages is presented in Fig. 2k.

(d) Best fit of predicted color distributions to experimental data for FSC clones with matching FCs at 14d.

| k   | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|-----|----|----|----|----|----|----|------------------------|
|     | FSC+FC 14d observed | 17 | 66 | 13 | 2  | 2  | 0                      |
| n=1 prediction | 100 | 0  | 0  | 0  | 0  | 0  | 11422                  |
| n=2 prediction | 19  | 81 | 0  | 0  | 0  | 0  | 406                    |
| n=3 prediction | 4   | 44 | 52 | 0  | 0  | 0  | 2182                   |
| n=4 prediction | 1   | 20 | 55 | 24 | 0  | 0  | 4624                   |

Best match for 2 FSCs.

(e) Best fit of predicted color distributions to experimental data for FSC clones with matching FCs at 20d.

| k   | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|-----|----|----|----|----|----|----|------------------------|
|     | FSC+FC 20d observed | 30 | 53 | 15 | 2  | 0  | 0                      |
| n=1 prediction | 100 | 0  | 0  | 0  | 0  | 0  | 7938                   |
| n=2 prediction | 19  | 81 | 0  | 0  | 0  | 0  | 1079                   |
| n=3 prediction | 4   | 44 | 52 | 0  | 0  | 0  | 2126                   |

Best match for 2 FSCs.

(f) Best fit of predicted color distributions to experimental data for FSC clones with matching FCs at 30d.

| k   | 1  | 2  | 3  | 4  | 5  | 6  | Σ(Observed-Predicted)^2 |
|-----|----|----|----|----|----|----|------------------------|
|     | FSC+FC 30d observed | 56 | 39 | 5  | 0  | 0  | 0                      |
| n=1 prediction | 100 | 0  | 0  | 0  | 0  | 0  | 3482                   |
| n=2 prediction | 19  | 81 | 0  | 0  | 0  | 0  | 3158                   |
| n=3 prediction | 4   | 44 | 52 | 0  | 0  | 0  | 4938                   |

Best match for 1-2 FSCs (plotted as 1.5 in Fig. 2k)
2. Number of FSCs per germarium deduced from the percentage contribution from each FSC lineage color.

The number of FC-producing FSCs can also be calculated as the reciprocal of the FC population contributed by a single FSC. We calculated the contribution of a single FSC lineage color to the FC population. This number underestimates the true number of FSCs because each color may include more than one FSC.

To determine the contribution of a single FSC lineage color to the entire FC epithelium of an ovariole from the 9d multicolor data we considered only G, B and BG lineages because these are present at an average frequency of 1/9 rather than 2/9 and are therefore more often initiated by single FSCs (Supplementary Fig. 1b,c). We also considered only ovarioles with at least four egg chambers and lineages that included a surviving FSC and at least one matching FC patch, even if present only in the germarium. We estimated the proportion of each egg chamber occupied by the color under examination, scoring all egg chambers in the ovariole (including those with no matching FCs). The average contribution of a single FSC color was close to 1/8 of all FCs (12.0%; n=30, SEM= 2.6%). An analogous analysis of 9-day FSC clones generated by the MARCM method showed an average contribution close to 1/7 of all FCs (15.3%; n=63, SEM=1.8%).

These data would lead to an estimate of 7-8 FSCs if each lineage color contained only a single FSC during the period of measurement (5-9d). However, even if each colored clone were initiated from a single FSC immediately after recombination marking, several FSCs would later amplify. The average amplification of surviving FSCs would be the reciprocal of the proportion of initially marked FSC lineages that survive. Hence, the number of FSCs estimated to be present during 5-9d by calculating the contributions of individual colors is equivalent to an estimate of the number of surviving FSCs during the period from 5-9d. The value of 7-8 surviving FSC lineages fits well with the time-course of surviving FSC lineages measured by counting the number of distinct colors present at different time points (Fig. 2k).
3. Number of founder FCs per egg chamber deduced from percentage contribution from each FC color.

The number of founder FCs per egg chamber can be calculated as the reciprocal of the average contribution of a single FC color, provided that a single color represents a single founder FC. To minimize the frequency of two or more founder FCs sharing the same color we only considered ovarioles with B, G and BG FSC clones. There were 83 patches containing B, G or BG FCs, with an average contribution of 30.4% per patch to the FC epithelium of a single egg chamber.

To derive the contribution per founding FC we must also estimate how many of the scored FC patches derived from one, two, three or more founder FCs of the same color. To do this we assumed that each founder FC is drawn independently from a group of source cells and hence that the number of founder cells of a given color will show a binomial distribution. To determine the probability, p, that a founder FC of a specific color (B, G or BG) is drawn we counted the number of egg chambers with zero FCs of the specified color (among ovarioles with an FSC clone of the specified color). For B, G and BG lineages combined we found that 102 of 185 egg chambers had no FC contribution from the interrogated genotype. Hence, \((1-p)^k = 102/185\) where \(k\) is the number of founder FCs per egg chamber.

We then performed separate calculations of predicted binomial distributions of the number of founder FCs of the same color per egg chamber for different values of \(k\). We present the results below only for \(k = 3, 4\) and 5 because these emerge as the only plausible candidates. From the p value inferred (from the experimental determination that \((1-p)^k = 102/185\) we calculated the fraction of patches predicted to be founded by \(i\) (= 1, 2, 3, 4 or more) founder FCs per egg chamber as \(f(i) = p^i(1-p)^{k-i}i!/i!(k-i)!\) and hence the inferred average number of founding FCs per patch of a given color as \((f(1)+2f(2)+3f(3)+4f(4))/(f(1)+f(2)+f(3)+f(4))\). Occupancy per founding FC was then calculated as occupancy per patch divided by inferred number of founding FCs per patch. The reciprocal of this value was our best estimate of the number of founding FCs per egg chamber. Even though different values of \(k\) produced small differences in the inferred number of founding FCs per patch the best estimate for the number of founding FCs per egg chamber is 4 whether we initially use values of \(k\) anywhere from 3 to 5.
(a) **Number of founder FCs per patch and hence founder FCs per egg chamber based on binomial distribution: multicolor clones**

|   | Probability that an FC patch is founded by the indicated number of FCs (i) of the same color | Inferred average number of founder FCs per patch | Experimental occupancy per patch | Inferred occupancy per founder FC | Inferred # founder FCs |
|---|----------------------------------|-----------------------------------------------|---------------------------------|---------------------------------|-----------------------|
|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| k=3 | 0.180 | 0.363 | 0.080 | 0.006 | 0 | 0 | 1.20 | 30.4% | 25.2% | 4.0 |
| k=4 | 0.138 | 0.354 | 0.085 | 0.009 | 0 | 0 | 1.23 | 30.4% | 24.8% | 4.0 |
| k=5 | 0.112 | 0.348 | 0.088 | 0.011 | 0.001 | 0 | 1.25 | 30.4% | 24.3% | 4.1 |

A MARCM FSC clonal analysis was scored and analyzed in exactly analogous fashion. Average occupancy was 31.2% over 179 patches and 158/337 egg chambers had zero FC contribution, scoring only ovarioles that included marked FSC clones.

(b) **Number of founder FCs per patch and hence founder FCs per egg chamber based on binomial distribution: MARCM clones**

|   | Probability that an FC patch is founded by the indicated number of FCs (i) of the same color | Inferred average number of founder FCs per patch | Experimental occupancy per patch | Inferred occupancy per founder FC | Inferred # founder FCs |
|---|----------------------------------|-----------------------------------------------|---------------------------------|---------------------------------|-----------------------|
|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| k=3 | 0.223 | 0.404 | 0.116 | 0.011 | 0 | 0 | 1.26 | 31.2% | 24.8% | 4.0 |
| k=4 | 0.173 | 0.391 | 0.123 | 0.017 | 0.001 | 0 | 1.30 | 31.2% | 23.9% | 4.2 |
| k=5 | 0.141 | 0.384 | 0.126 | 0.021 | 0.002 | 0 | 1.33 | 31.2% | 23.4% | 4.3 |

For the MARCM data the percentage occupancy per founding cell is close to 25% (for all values of k, including k=4), giving a best estimate of an average of four founding FCs per egg chamber, just as for the multicolor FC patches. It is clear, however, that there can sometimes be at least five founding FCs (Fig. 1k).

Also, a variety of departures from our simplifying statistical assumptions, such as unequal numbers of FSCs of different colours and heterogeneous potential for immediate FC production (both of which we observe), would lead to under-estimating how frequently a single FC colour is founded by a single FC, and would therefore raise our estimate of the number of founding FCs to a value higher than four.