The Drosophila ovary produces two waves of adult follicles and a novel pupal wave that turns over

Wayne Yunpeng Fu[1,2,3] and Allan C Spradling[1,2,3,4]

[1]Carnegie Institution for Science

[2]Howard Hughes Medical Institute

[3]Department of Biology, Johns Hopkins University

3520 San Martin Dr.

Baltimore, MD 21218

[4]correspondence: spradling@carnegiescience.edu

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ABSTRACT

Follicles in Drosophila and many other species are thought to develop using a single pathway. In contrast, we show by lineage tracing ~3,000 single-cell clones, that three follicle "waves" arise in pupae that follow distinct programs, suggesting they evolved for distinct functions. Wave 1.5 follicles develop directly from primordial germ cells (PGCs) and provide the first ~100 eggs, enough for some fertility in low nutritional environments. With adequate food, germline stem cell (GSC) divisions produce wave 2 follicles without limit that support high fecundity. Wave 1.5 follicle stem cells (FSCs) are specifically replaced in their niches with new FSCs to coat wave 2 germ cells with unique follicle cells. Previously unknown wave 1 follicles amplify rapidly in number, but soon undergo programmed degeneration and exit the ovary along with some somatic cells as they release lipophilic material. A subpopulation of testis germ cells behave in a similar manner. We propose that wave 1 germ cell breakdown contributes to the concomitant but unexplained pharate adult ecdysone pulse that mediates adult tissue developing, including the rewiring of reproductive behavior.

Keywords

germ cell, ovarian follicle wave, germline stem cell, follicle stem cell, Drosophila melanogaster , pupal ovary

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INTRODUCTION

Some species are known to generate multiple sets of gametes, in spatially or temporally separated groups known as waves (DeFalco and Capel, 2009; Eppig and Handel, 2012; Pazdernik and Schedl, 2013). For example, mice generate three waves of follicles in their ovaries. Wave 1 follicles begin to grow without arrest in the central medullar region, while most follicles, wave 2, reside in the cortex and initially become quiescent as the ovarian reserve (Mork et al. 2012; Rotgers et al. 2018). A recently described third wave of primordial follicles, termed "wave 1.5," develops at the medulla/cortex boundary, arrests for only about 2 weeks, and produces most initially ovulated oocytes rather than wave 1 (Meinsohn et al. 2021; Yin and Spradling, 2025). Most wave 1 follicles undergo "atresia" (Byskov, 1974), in which the oocyte and all but a few granulosa cells turn over, while thecal cells proliferate and interstitial gland cells are recruited. These hormone-producing cells reorganize into a gland-like region that synthesizes mainly androgenic steroids during peri-puberty (Yin and Spradling, 2025), a hormone-sensitive period of juvenile development. Drosophila ovaries may contain similar follicle waves, whose identification requires studying in detail how the ovary develops under control of the steroid hormone ecdysone during larval and pupal stages (Figure 1A; King et al. 1968, King, 1970; Sahut-Barnola et al. 1995; Godt and Laski, 1995; Forbes et al. 1996; Couderc et al. 2002; Camara et al. 2019).

Follicles develop in ordered strings known as ovarioles that begin with the germarium region containing stem cells to produce their constituent germline and somatic cells (Figure 1B). 2-3 GSCs reside in a niche near the germarium's terminal filament (TF) where they give rise to daughters termed cystoblasts (CB) that initiate 16-cell germline cyst formation consisting of one ooycte and 15 nurse cells (Figure 1C, 1E). Two follicle stem cells (FSCs) reside midway through the germarium on opposite sides at the junction between regions 2a and 2b where cysts switch from a temporary

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covering of squamous escort cells (ECs) to a monolayer of epithelial follicle cells (Margolis and Spradling, 1995). Both FSCs are maintained at these sites in niches involving local escort cells and intercellular signals (Nystul and Spradling, 2007; Sahai-Hernandez and Nystul, 2013).

The embryonic ovary acquires primordial germ cells (PGCs) which proliferate throughout larval development in association with somatic intermingled cells (Wieschaus and Szabad, 1979; Zhu and Xie, 2003; Li et al. 2003; Gilboa and Lehmann, 2006; Ganz et al. 2011; Banish et al. 2021; Rosales-Nieves et al. 2025). A pulse of the steroid hormone ecdysone induces the formation of TF structure in third instar larval ovaries that guide ovariole development (Godt and Laski, 1995; Sahut-Barnola et al, 1996; Forbes et al. 1996). Before TFs are completed, a large population of somatic swarm cells migrate from the anterior to the posterior region of the ovary (Couderc et al. 2002). Another key event is GSC niche formation (Lin and Spradling, 1993; Xie and Spradling, 2000; Zhu and Xie, 2003; Gilboa and Lehmann, 2006; Song et al. 2002; Song et al. 2007; Panchal et al. 2017). A TF constitutes the distal part of the niche, whose Notch-dependent development continues during early pupal stages by adding multiple cap cells and specialized basement membranes (Zhu and Xie, 2003; Song et al. 2002; Song et al. 2007; Panchal et al. 2017; Yatsenko and Shcherbata, 2018; Diaz-Torres et al. 2021). 30-40 PGCs enter stem cell niches and become GSCs, but the schedule of GSC activation remains uncertain. About 60 other PGCs directly undergo oogenesis to generate the first ovulated eggs (Zhu and Xie, 2003; Asaoka and Lin, 2004), but their development has not been fully characterized.

Many additional pupal ovarian somatic cell types are known associated with different developmental stages, including peritoneal and epithelial sheath cells, interstitial cells, basal stalk cells and FSC progenitors (King et al. 1968; Slaidina et al. 2020; Slaidina et al. 2021; Reilein et al. 2021). The adult FSC niche utilizes nearby escort cells descended from intermingled cells, as well as both

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short and long-range intercellular signaling pathways (Sahai-Hernandez and Nystul, 2013). Specific ECs in the germarium have been associated with GSC and FSC niches, and with function as a germ cell "differentiation niche" (Kirilly et al. 2007), but when particular ECs differentiate and begin to function is not known. Another major developmental mystery during pupal stage is the source of the largest pupal ecdysone pulse. The prothoracic gland produces larval ecdysone pulses (Figure 1A) but turns over during early pupal development, leaving the origin of the major pharate adult peak (PAP) at 24h APF unexplained (Dai and Gilbert, 1991; Scanlan et al. 2023). Thus, detailed study of pupal ovarian cell lineages may help to identify Drosophila follicle waves, and address other important questions regarding pupal ovary development.

Here we use lineage tracing to identify and characterize three Drosophila follicular waves, which we named based on the three waves observed in the mouse ovary. Drosophila GSCs and FSCs become more active coincident with the 10h APF ecdysone pulse. Directly developing PGCs give rise to "wave 1.5" follicles that produce the first ~100 eggs, after which follicles are exclusively derived from GSC divisions and covered with follicle cells from distinct FSCs to make up "wave 2" follicles. Unexpectedly, a previously undescribed wave of follicles we term "wave 1" develops even earlier but turns over between 22h and 26h APF via an atresia-like process. Wave 1 germ cells break open and release lipophilic cytoplasmic material, before they exit the ovary posterior along with somatic cells. Similar germ cell turnover in the pupal testis takes place at this time, but both ovaries and testes appear fully repaired within a few hours. We propose that wave 1 atretic gametes contribute in a currently unknown manner to the unexplained pharate adult ecdysone peak.

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RESULTS

Analyzing ovarian follicle waves by lineage tracing

To obtain a clearer picture of follicle waves, we used a background-free lineage labeling system based on heat shock-induced FLP-mediated recombination between chromosome 2L homologs (Harrison and Perrimon, 1994; Margolis and Spradling, 1995; Figure S1A). After a heat shock, any mitotically cycling cell has a chance to recombine; but only a small, random fraction of candidate cells actually undergoes recombination, an important feature that allows the progeny of individual cells to be mapped. Moreover, recombination causes only one of the two daughter cells at the next mitosis to become heritably marked (Figure 1D), a property that further enhances information yield in connection with germline cyst and GSC development (Figure 1E, F).

Clone induction was accurately timed ($\pm$2h) by using the short period when a larva stops moving, and begins to tan its puparium cuticle as a benchmark (see Methods). We induced clones at 4-hour intervals between -2h and 14h APF and analyzed them at two periods. The greatest cellular resolution came from scoring lineage-labeled ovaries shortly after eclosion (Figure 1A-C), but lineage-marked pupal ovaries were also scored after clone induction. All of the ovarioles were analyzed for somatic cell clones in both germaria and follicles, whereas we found it sufficient to analyze germ cells in only one replicate of each group due to low variability. Altogether, we quantified 2,368 germ cell clones and all 569 somatic cell clones from a total of 2,270 individual ovarioles (Table S1-S3).

Characterization of wave 2 follicles

First, we analyzed the GSCs that support wave 2 follicle production throughout adulthood. GSC lineage clones can be recognized in newly eclosed adult ovaries by a marked GSC at the tip of the germarium followed by adjacent marked progeny cysts resulting from pupal GSC asymmetric

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divisions (Figure 1C, Figure 2A). The number of GSC clones with these properties increased at 10h APF to reach a labeling frequency similar to that of the adult GSCs (Figure 2C, blue line). Since lineage marker recombination only requires an active, post-G1 cell cycle state, the labeling rate of a cell population positively correlates with cell number, but not cell cycle length. By 10h APF GSCs labeled at rates similar to adult GSCs (Margolis and Spradling, 1995). Marked GSCs produce an average of 4 marked progeny cysts, and since their first division product will usually be unmarked (Figure 1F), it suggests that pupal GSCs undergo an average of 5 total divisions in the ~100h before analysis (Figure 2C, orange line), similar to adult GSC division every 20-24h. Around 4% of ovarioles with GSC clones have all GSCs labeled (Figure 2D, D'); analysis of this subset of ovarioles confirmed that wave 2 GSC progeny cysts do not exit the germarium by eclosion.

A second GSC-related but distinctive class of clones was produced by individual labeled cystoblasts (Figure 2B). CB clones were recognized as single 16-cell labeled cysts located ~5 divisions downstream from unlabeled GSCs, usually in an otherwise unlabeled ovariole. They are predicted to result from the first division of recombined GSCs. Half the time the lineage marker will stay with the GSC daughter yielding a GSC clone, and half the time it will segregate to the CB and develop as a single cyst (Figure 1F). As expected from their origin, across all five time points studied, we observed similar CB and GSC clone numbers: 199 CB clones and 221 GSC clones. Also as expected, CB clones were located an average about one cyst farther from the most GSC-distal labeled cyst in a GSC clone.

Characterization of direct-developing wave 1.5 follicles providing early fertility

The most common type of germ cell labeling pattern were produced by germ cells directly developing from PGCs (wave 1.5). Whether recombination takes place in the PGC, or in a partially developed cyst cell, these unambiguous clones reside within single cysts and label 8, 4, 2 or 1 germ

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cells (Figure 1E; Table S3). Cysts with these patterns were observed farther along the ovariole than GSC and CB clones ensuring they are laid when mature before wave 2-derived eggs. In newly eclosed flies, wave 1.5 cysts constituted all of the 4-5 developing follicles (Figure 2E), while another 1-2 remained in the posterior germarium (Figure 2F, boxed region).

Comparing the labeling pattern of wave 1.5 follicles from each time point revealed several insights. The youngest wave 1.5 follicles increased their labeling frequency coincident with the 10h ecdysone pulse, whereas the two most mature follicles had already undergone more cyst divisions and responded less (Figure 2G). Averaged over all developing wave 1.5 follicles, a steady decline in the percentage of PGC labeling (8-cell clones) was seen with later clone induction times, but a steady increase occurred in the percentage of cysts labeled during successively later rounds of cyst division (Figure 2H). These data quantitate the extent of larval cyst formation and show that cyst divisions during -2h to 14h take place only about every 18h on average, much slower than in older pupae (see below) or in adults. Rarely, more mature follicles scattered among the wave 1.5 follicles contained a 16-cell clone (Figure 2F; Table S3). They result from symmetric division of recombined PGCs before they begin cyst formation and reveal the extent of ongoing PGC division (Figure S2B).

Since all germline cysts and follicles in the ovarioles at eclosion are derived from either GSCs (wave 2) or direct-developing PGCs (wave 1.5), we quantitated the population size of these waves. On average, each ovariole contains 12.5 wave 2 cysts (5 cysts produced per GSC and 2.5 GSCs per ovariole, n=165). Each ovariole therefore contains 6.4 total wave 1.5 follicles, by subtracting wave 2 from 18.9 average total cysts and follicles per ovariole (Figure 2D). This shows that ~100 wave 1.5 follicles are produced per ovary, a much larger population than previously estimated.

<u>A large, novel population of wave 1 germ cells turns over between 22h-26h APF</u>

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Direct examination of pupal ovaries from -2h to 30h APF allowed us to further study developing wave 2 and wave 1.5 follicles, and revealed an unexpected third follicle wave (Figure 3). Germ cells were labeled with a bam-GFP reporter and using Hts/1B1 antibody to visualize fusomes, whose branching is diagnostic of cyst stage. Complete z-series greatly aided stage identification. Cyst development as indicated by Bam reporter expression was observed in late larvae and early pupae and increased at 10h APF (Figure 3A-E). We quantitated the number of future gametes (PGCs plus cysts) as posteriorly migrating apical sheath cells enclosed them in ovarioles by peritoneal sheath deposition (Figure 3K). Sheath formation is complete by 10h, but the number of developing gametes per ovariole continues to increase up to 18h due to ongoing PGC symmetric divisions (Figure S2B).

Beginning as early as 6h APF (Figure 3D), a subpopulation of germ cells was observed to migrate toward the posterior region of the ovary that mostly consists of somatic swarm cells that moved there late in L3 stage (Corderic et al. 2002). Posterior somatic cells form a conical structure that protrudes at this time (Figure 3E-F). Germ cell posterior movement continued, producing a distinct group (Figure 3F-G, L, arrows). The germ cells at the posterior further increase in number as they form cysts and by 22h APF they aggregate to form a mass of 100 to 200 cells (Figure 3G-H, L; Figure 4D, orange line). Between 22-26h, all the posterior germ cells and many adjacent somatic cells break from the ovary posterior and enter the hemolymph (Figure 3I). We term these germ cells "wave 1" because they develop the earliest and undergo an atresia-like turnover process, akin to wave 1 follicles in the mouse (Zheng et al., 2014). All three waves are visible along the A-P axis in Figure 3H. By 30h APF, the ovary has completely lost wave 1 germ cells and its protruding posterior, appearing spherical in shape and to have been repaired (Figure 3J).

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In 10h APF ovaries, vacuoles appear in gaps around germ cells (Figure 3E, M, N), coincident with the small prepupal ecdysone pulse (Figure 1A). Smaller vacuoles were observed inside germ cells, confirming germ cells to be the origin for such vacuoles (Figure 3N-N'). These cells include likely GSCs directly adjacent to TFs (Figure S2C). Such vacuoles disappeared from the ovary within 4 hours without causing apparent morphological changes to the tissue (Figure 3F-G).

Rapid multiplication and clearance of wave 1 germ cells

We analyzed lineage-labeled germ cells directly in young pupal ovaries 24 hours after clone induction to gain further insight into wave 1 multiplication. Analyzing clones induced at -2h and 2h APF in pupal ovaries 24 hours later showed that individual wave 1 clones typically contained multiple nearby cysts that were fully labeled (Figure 4A-C), indicating wave 1 precursor PGCs expanded rapidly first by symmetric division and then by forming germline cysts with typical fusomes. The view facing the ovary posterior shown in Figure 4B-C reveals branched 8-16 cell fusomes in >10 remaining wave 1 cysts. We estimated the cumulative total number of wave 1 germ cells released by first determining that at 18h APF when PGC symmetric division had ceased, there were 22 wave 1 cysts of various sizes indicated by fusome structure (Figure S2D, S2E). At this time before cells began to be extruded, only 15% of cysts contain 16 cells (Figure 4D, blue line). However, at 22h and 26h APF the percentage of 16-cell cysts continued to rise and reached 75%, indicating continuous cyst division even though the total number of wave 1 cells started to fall due to ongoing cell extrusion (Figure 4D). Taking into account the simultaneous proliferation and extrusion, we estimate that in total between 250-350 wave 1 germ cells are expelled. Clusters of wave 1 germ cells and associated somatic cells break off in groups at the posterior (Figure 4E-G).

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Immediately before and as wave 1 germ cells exit the ovary, large spherical vacuoles with significant lipid droplet enrichment appear around them, likely derived from these germ cells (Figure 4H, H'). We examined 22-26h APF testes as well, and found a similar sequence of regional germ cell appearance changes (Figure 4I-I') followed by expulsion from the gonad (Figure 4J-L) with concurrent lipid droplet accumulation (Figure 4K', L'). In the testis, however, cells were lost from the sides of the gonad rather than the posterior, where germ cells are absent.

Two pupal FSCs produce adult-like follicle cell clones to cover wave 1.5 follicles

In uninjured adult ovarioles, two long-lived FSCs ($t_{1/2}$ ~20 days) located at the 2a/2b junction alternately divide to each supply one follicle founder cell to passing germline cysts as they lose their escort cell coverage (Rust and Nystul, 2020). The FSC daughter cells continue dividing to make a continuous monolayer as the maturing cyst moves through the germarium. This process produces a follicular monolayer containing 60-80 cells that is sufficient for follicle budding within about 48 hours. In contrast, how wave 1.5 pupal follicles acquire follicle cells is poorly known. Electron microscopy (King et al. 1968) and our observations (Figure S1C) show that pupal ovarioles even by 40h lack a two-by-two progression of region 2a cysts or a 2a/2b junction. Consequently, it is unclear whether a canonical FSC niche was present during pupal stage when wave 1.5 follicles acquire follicular coverage, which might be derived in a very different manner than in adults.

Lineage analysis scored at eclosion revealed very few FSC or follicle cell clones when induced prior to 10h APF (Figure 5A). Adult-like FSC clones began to be observed frequently starting at 10h APF, we termed them FSC-P (pupal FSC) clones since they provide follicle coverage during pupal stage and strongly resemble adult FSC proliferation pattern (Figure 5B). These clones initiated at precisely the same position at the 2a/2b border described for the location of adult FSCs. However, some FSC-P clones start at variable positions downstream from the 2a/2b junction and lack a marked

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active FSC (Figure 5C-C'). A labeled pupal FSC gave rise to these clones, but it was subsequently displaced from the niche by an unmarked FSC. The location of the last follicle cell in these FSC-P clones is indicative of the time when the labeled FSC was displaced. Almost in a complementary pattern, very short FSC clones that contain an active FSC at 2a/2b junction were also observed, which rarely extend beyond the germarium to cover budded follicles (Figure 5D). These were termed FSC-A (adult FSC) clones since they closely match the extent of wave 2 adult cysts and are newly activated around the time of eclosion (Figure 2B). Short FSC-P clones that only cover 1-3 more downstream wave 1.5 follicles with early FSC loss point were also observed (figure 5D). These clones have a high occurrence rate in the oldest wave 1.5 follicles (Figure 5F), indicating a high frequency of FSC replacement when the first follicles bud off which may result from an immature niche environment. Moreover, same as in adults, when we measured the entire surface of the wave 1.5 follicles covered by FSC-P clones, about 50% of the follicle surface by area was covered by the clone, leaving about 50% unmarked (Figure 5G, G', H; Movie S3-S4). This indicates that two FSCs with equal activity, as in adults, generate most wave 1.5 follicle cells.

Clones of other somatic cell types were also recorded, such as cap cell (CC) and escort cell (EC) clones (Figure S4B-G, K). Their labeling frequency showed a similar amplitude of increase at 10h APF (Figure 5A), but the elevated level was not maintained as in the case of FSC clones and declined significantly by the next 14h APF time point. Since labeling rate is indicative of mitotic activity level, it suggests that ECs and CCs underwent a transient surge of proliferation at 10h APF. This surge temporally overlaps with the 10h prepual ecdysone pulse, which is likely the signal inducing such proliferation. Indeed, observed EC clone sizes confirm the low proliferation level of ECs after 10h APF, with 99% of EC clones containing 4 or less marked ECs and 52% containing only 1 marked EC (Figure S4I). This indicates half of all ECs did not divide throughout the entire pupal stage, and most

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of the rest divided 1-2 times. 84% of CC clones are also single-cell clones, with the rest containing 2 cells (Table S2).

The first wave 2 follicle activates a new set of FSCs

The two FSC clone types are named based on their close spatial correspondence to the two respective follicle waves, whose transition was characterized previously. Since CB clones are the oldest wave 2 cysts in each ovariole, their location can be used to indicate the extent of wave 2 cysts. It was common for CB clones to locate in late region 2b or stage 1 (Figure 2B), meaning 2-3 wave 2 cysts have passed the 2a/2b junction and started to acquire follicle cells from the FSCs. Notably, such extent of wave 2 cysts overlaps with the extent of FSC-A clones (Figure 5D, S3B-S3C). To investigate whether the two types of clones have spatial correlation, we quantified CB clones and FSC-A clones based on their end point cyst position (Figure 5I). Indeed, the distributions of these clones show a matching pattern, both having the highest number of clones that end at the second cyst position in region 2b. In rare ovarioles with coexisting CB clone and FSC-A clone, they end at the same cyst position (Figure 5L). Therefore, FSC-A is likely a distinct group of adult FSCs that are newly activated by the passage of wave 2 cysts across 2a/2b junction.

The long FSC-P clones that lost their active FSC recently all have their last marked cell within the germarium region (Figure 5C, C', K), and this is also the only region where FSC-A clones end. This complimentary pattern indicates that FSC-A is likely what displaced the FSC-P from the niche at 2a/2b junction, and since FSC-A cover wave 2 follicles, FSC-P would selectively cover wave 1.5 follicles. Indeed, when we compared FSC-P clone loss points and CB clone locations, their distributions follow a similar pattern (Figure 5I). FSC-P clones are also observed to end at the advent of a CB clone when these two types of clones are labeled in the same ovariole (Figure 5K), confirming their correlation. Moreover, when a CB clone has not yet reached the 2a/2b junction, the FSC-P that

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makes wave 1.5 follicle cells is not yet lost (Figure 5I). Altogether, these complementarities indicate that FSC-A are wave 2-specific FSCs and FSC-P are wave 1.5-specific FSCs. The transition point between the two follicle waves corresponds to where FSC-P coverage terminates and FSC-A coverage starts. When the first wave 2 cyst that triggers FSC replacement has not reached 2a/2b junction, FSC-P are not yet lost. We also recorded almost exactly the same number of FSC-A clones (n=80) as the total of long FSC-P clones that either retained an active FSC (n=37) or not (n=40), further confirming the replacement model.

The first wave 1.5 follicles have unusual properties

Follicular coverage of the first wave 1.5 follicles in each ovariole included contributions from a different source in addition to FSC-Ps. Terminal wave 1.5 follicle cell clones frequently contain labeled follicle cells in a posterior patch and less often in other positions (Figure S3G-G'). Basal stalk cells were previously reported to contribute to pupal follicle cells (Reilein et al. 2021), and movement of basal stalk cells onto the posterior wave 1.5 follicle layer provides an attractive explanation for the bias terminal follicle clones show toward the posterior region where the stalk attaches. Similar behavior is also documented in the adult ovarioles, where inter-follicular stalk cells are known to sometimes offload extra cells onto the adjoining follicle (Nystul and Spradling, 2010).

The oldest 1-2 wave 1.5 follicles also manifested small developmental changes compared to normal adult follicles. They contain oversized polar cells, suggesting increased polyploidization (Figure S2F). The number and size of border cell clusters, which include the anterior polar cells were sometimes enlarged and delayed in migration (Figure S2F-G). These wave 1.5 follicles also have a high rate of germline cyst developmental abnormality where they contain 8 or 32 cells per follicle(Figure S2H), indicating they underwent one less or one more round of cyst division.

Cap and escort cell differentiation correlates with niche development

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Clones of other somatic cell types were also recorded, such as cap cell (CC) and escort cell (EC) clones (Figure S4B-G, K). Their labeling frequency showed a similar amplitude of increase at 10h APF (Figure 5A), but the elevated level was not maintained as in the case of FSC clones and declined significantly by the next 14h APF time point. Since labeling rate is indicative of mitotic activity level, it suggests that ECs and CCs underwent a transient surge of proliferation at 10h APF. This surge temporally overlaps with the 10h prepual ecdysone pulse, which is likely the signal inducing such proliferation. Indeed, observed EC clone sizes confirm the low proliferation level of ECs after 10h APF, with 99% of EC clones containing 4 or less marked ECs and 52% containing only 1 marked EC (Figure S4I). This indicates half of all ECs did not divide throughout the entire pupal stage, and most of the rest divided 1-2 times. 84% of CC clones are also single-cell clones, with the rest containing 2 cells (Table S2).

Another attractive opportunity provided by clonal analysis was to learn when particular somatic cell types of the germarium differentiate (Table S2). The terminal division of a precursor cell at differentiation may leave a single cell labeled, or if the penultimate precursor recombines, the clone may mark stereotyped final lineage patterns of differentiation. ECs that play important roles in the GSC and FSC niches (Kirilly et al. 2011; Gao et al. 2019; Rust et al; 2020) were of particular interest. We categorized ECs based on their relative location in the germarium, which we named EC1 through EC6 (Figure S4A). EC1-3 locate around the GSC niche, and EC4-6 are close to FSC niches. An EC1 wraps the surface of each GSC, while behind it lies EC2 (Figure S4B-C). A median EC3 is also present between the GSCs (Figure S4D). Three ECs similarly surround both FSC niches (Gao et al. 2019; Rust et al. 2020). EC4 is on the surface of germarium and directly contacts the FSC (Figure S4E), more anterior to it is EC5 (Figure S4F). Another median EC type, EC6, is found

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between the two FSCs located internal in the germarium (Figure S4G). At least in some cases, EC6 cell membranes extend to an adjacent FSC (Sahai-Hernandez and Nystul, 2013).

All EC types had low labeling rate which significantly increased by 10h APF (Figure S4H). The increase was greater for EC1 and EC2, the most anterior ECs next to the GSC niche, than that of other ECs. Since CC clones also showed a similar increase (Figure 5A), GSC niche may be preferentially constructed at this time relative to the FSC niche, and it may take longer than has often been assumed for GSC niches to fully mature during early pupal development, whose completion appears to depend on the 10h APF ecdysone pulse. We also found multiple examples of double and quadruple EC clones (Figure S4I, S4J-J'; Table S2). Given the low frequency of individual EC clones, these likely correspond to the labeling of a single precursor instead of a double labeling event in the same ovariole.

DISCUSSION

Drosophila females produce three waves of follicles

Our experiments demonstrated that the well-studied GSC-based system of follicle production in Drosophila (wave 2) is preceded by two earlier follicle waves. Wave 1.5 and wave 1 follicles directly derive from PGCs that arise in L3 larval ovaries and expand further to a total about 160 PGCs by 18h APF (Figure S2A). Previously, it was thought that larval PGCs not established as GSCs only produced the first 30-60 eggs with identical properties as later eggs (Zhu and Xie, 2003; Asaoka and Lin, 2004). In addition to a larger number of pupal PGCs, we found that ongoing PGC proliferation as late as 16h APF account for the higher number of PGCs that support wave 1 and wave 1.5 development (Figure 3K).

Exactly how many PGCs are needed to produce the 40 GSCs observed (2.7 per ovariole) is not yet precisely known. Our extensive characterization of labeled GSC clones did not enable us to fully

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test the prediction that the first GSC to enter a niche expands symmetrically (Zhu and Xie, 2003), allowing as few as 16 PGCs to produce all GSCs. The clonal marking system generates only one labeled daughter following the first division of a recombined GSC even if that division is symmetric. We found that after an initial GSC division, instead of both daughters remaining in the niche to fill empty sites, the labeled daughter left the niche entirely nearly half the time to produce a CB clone. This indicates that initial niches lack room to support symmetric division, because like adult niches, any space is rapidly filled with a germ cell. Consistent with that conclusion, we observed continued production of the niche component cap, EC1 and EC2 cells, likely allowing the GSC niches to keep growing in size and admitting new stem cells until at least 10h APF. Estimates of when GSCs begin asymmetric divisions have varied (Zhu and Xie, 2003; Song et al. 2007; Banish et al. 2021). We found that the 10h ecdysone pulse correlates with increased numbers of bam-expressing germ cells and GSC division, but most of bam-positive germ cells still reside at the end of the ovarioles, due to PGC direct differentiation into early wave 1.5 cysts. GSCs go on to produce about 5 daughter cysts each by the time of eclosion which remain within the germarium.

The ovary prioritizes rapid production of more than 100 wave 1.5 follicles

Our studies argue that wave 1.5 follicles are intrinsically important, rather than just products of excess PGCs needed to ensure that GSC niches are filled. The PGCs posterior to GSC niches expand by symmetric division (Figure S2B) and directly develop to produce this wave, enough to ensure that all follicles and ~2 posterior cysts per ovariole at eclosion are of wave 1.5 origin. 33% of wave 1.5 PGCs begin cyst formation in the larval ovary and move toward its posterior. The growing number of developing wave 1.5 follicles is responsible for most germ cell bam expression in the middle of the ovary, especially after 10h APF (Figure 3B-E). During the 96 hours of pupal development, wave 1.5 PGCs produce nearly mature follicles at the end of each ovariole. These wave 1.5 follicles will

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have developed from PGC to mature egg in a little more than 5 days. In contrast, adult CBs require 7-8 days to complete oogenesis (King, 1970; Lin and Spradling, 1993). Despite their rapid production, the defects we observed in a minority of only the most rapidly produced terminal wave 1.5 follicles probably are too rare to have much impact on reproduction.

Exceptionally rapid development is one of several indications that wave 1.5 follicles are functionally important. Their follicular covering is produced by a different set of stem cells, FSC-P, than wave 2 follicles, which use FSC-A. Wave 1.5 follicles are less dependent on adult nutrition than wave 2 follicles, because they can acquire sufficient yolk from larval nutrients stored in the fat body. In contrast, wave 2 follicles have not yet left the germarium at eclosion, and their maturation depends almost entirely on finding environmental nutrient sources. Thus, under the limited nutrient conditions most females confront in the wild, at least some wave 1.5 eggs but often no wave 2 eggs will be completed and ovulated. Therefore, wave 1.5 eggs may be specially adapted to limited environmental resources, and wave 2 eggs to nutrient-rich conditions. Our precise characterization of these two follicle waves will now facilitate a search for the molecular differences between wave 1.5 and wave 2 eggs, and between their distinct populations of follicle cells. Differentially expressed genes in FSC-P compared to FSC-A daughter cells might indicate what genetic, molecular and physiological differences facilitate adaptation to different nutrient conditions.

The variation of egg quality

A longstanding question in reproductive biology concerns the degree to which eggs vary in quality. The resolution in cellular behavior made possible by our lineage studies identified increased variation in the first eggs produced in both wave 1.5 and wave 2. The initial wave 1.5 egg produced in each ovariole differed from those produced later in the wave by beginning cyst divisions in the larva, and in acquiring a follicular layer in part from the basal stalk. They also acquire follicle cells

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from recently arrived FSC-Ps operating in an unstable niche. The degree to which these or other differences affect quality is unknown, but we did observe an apparently elevated frequency of morphological deviations in initial wave 1.5 follicles (Figure SF-H). In contrast, wave 2 cysts usually acquire founder follicle cells from two newly installed "FSC-A" stem cells. Our lineage studies showed that at least some initial follicles instead acquire a mosaic layer of FSC-P and FSC-A cells. We observed at least one double clone that suggested the FSC switch had not occurred by the time the first wave 2 cyst arrived and it instead received an FSC-P follicle cell layer (Figure S4J). Thus, follicles arising during a switch between waves have an elevated chance of producing abnormal eggs.

Drosophila wave 1 follicles may contribute to pharate adult ecdysone pulse

Our finding that an early Drosophila germ cell wave produces 250-350 germ cells in germline cysts that turn over between 22h and 26h APF strongly suggests these wave 1 follicles have a function other than gametogenesis. Wave 1 germline cysts are extruded from the ovary together with a group of somatic cells that forms a conical structure at the posterior (Figure 3L), potentially acting synergistically to enable such process to take place. Swarm cells, a population that migrates to the posterior ovary region during L3 associate with wave 1 cysts and may constitute the somatic cells extruded along with them. It is possible that swarm cell migration evolved to support wave 1 function, and swarm cells that are not extruded are not detected in the adult ovary.

Our study shows that wave 1 cells do not represent an obsolete larval structure to be turned over during metamorphosis, but instead are a new cell population produced in a highly programmed manner with rapid proliferation shortly before collective turnover. Wave one turnover occurs at the onset of the pharate adult ecdysone pulse that is critically important for pupal metamorphosis, the source of such pulse was previously proposed to be an unidentified tissue releasing ecdysone

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recycled from earlier times in development and stored in an inactive form (Scanlan et al. 2021). Wave 1 germ cells in the ovary, and a similar group of germ cells in the testis at the same time both turn over as lipid-enriched vacuoles appear around them, suggesting this is not a female-specific process and may have a general purpose during development. We propose that wave 1 follicles may contribute to ecdysone production, either by storing and releasing ecdysone, or indirectly by signaling to other tissues responsible for the release. Determining the true function of transient wave 1 follicles will ultimately require a separate study that is now possible based on their properties reported here.

How similar are the three follicle waves in Drosophila to those in mice?

The general similarity of the three Drosophila follicle waves described here to those found in the mouse ovary was surprising. It is well established that wave 2 follicles in both species provide the major mechanism to produce large numbers of eggs to provide sustained fertility through adulthood. Embryonic development in placental mammals is still dependent on nutritional resources, even though they must be obtained by the mother rather than stored in the egg as yolk. Finally, wave 1 in both species undergoes programmed turnover that temporally overlap with critical hormone pulses mediating adult development, but further experiments are needed to confirm the participation of wave 1 in Drosophila in hormone release.

There are many differences between the waves in Drosophila and mouse that suggest they arose independently in evolution. Wave 2 uses stem cells in Drosophila but follicle quiescence in the mouse to sustain fertility. Wave 1.5 produces about 110 follicles in Drosophila but more than 400 in mouse to generate the first progeny. The "atresia" program of wave 1 differs between these species. Drosophila wave 1 undergoes atresia at an earlier stage of follicle development, possibly associating with escort rather than follicle cells, or with a novel somatic cell type. While both Drosophila and

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mouse wave 1 germ cells turn over, in the mouse steroid hormone is produced by somatic thecal cells and interstitial gland cells (Qi and Spradling, 2025). Further study and comparison of the genetic pathways used by wave 1 germline and somatic cells in both species will likely provide insight into both the mechanisms and similarities between Drosophila and mouse waves.

Drosophila pupal ovarian FSCs offer opportunities to understand stem cell biology

The behavior of stem cell niches represent an important aspect of tissue biology, including homeostatic tissue maintenance and aging (Morrison and Spradling, 2008). Our study of pupal FSCs and their niches documented novel changes in their properties in a normal tissue with the potential to increase our understanding of how stem cells are regulated during development. The FSC-P and FSC-A reside in similar niches supported by ECs and carry out similar functions in follicle production. But the cellular anatomy surrounding their niches and the movement of cysts as they acquire FSC daughters differ between the two.

The replacement of two FSC-P stem cells in each ovariole by two new FSC-A stem cells associated with the switch from wave 1.5 cysts to wave 2 cysts provides one of the clearest examples of developmentally programmed stem cell changes. Previously, stem cells were shown to be capable of replacement when their adhesion to a niche was greater than the adhesivity of the current resident stem cell. It is less clear, however, how such stem cell replacement would take place. The most plausible mechanism would be if early wave 2 cysts carried somatic cells still competent to become FSCs and with higher adhesivity than FSC-Ps. As the first cyst passed the 2a/2b junction, two such cells might contact the niches and induce replacement.

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The dramatic 5-fold increase in FSC-P stability in its niche during early wave 1.5 development (Figure 5F) also presents an opportunity to better understand the initial formation of stem cell niches. Each FSC niche houses only a single stem cell and is consisted of only a few niche cells, making them attractive models. Adult FSCs are subject to regulation by multiple common long-range intercellular signals (Sahai-Hernandez and Nystul, 2013). Thus, it should be possible to determine which developmental parameters are responsible for this initial dramatic change in FSC niche stability.

SUPPLEMENTAL MATERIALS AND METHODS

Fly Husbandry and Developmental Staging

Flies were cultured in 25ºC incubators on standard cornmeal/molasses/agar media, therefore all APF hours mentioned are measured at 25ºC. To collect pupa with accurate APF timing, immobile wandering third instar larva are collected, and 4 hours later only the larva that underwent pupariation are retained while those that are still larva are discarded. This method ascertains that all the remaining pupa to be examined have pupariated at the same time within a ±2 hour window. The X-15-29 and X-15-33 fly lines used for lineage tracing were from Dr. Norbert Perrimon, and the bamP-GFP fly line was from Dr. Mark Van Doren. We used FlyBase (FB2025_05) to find information on phenotypes/function/stocks/gene expression/orthologs etc. Genetic nomenclature also follows Flybase (Öztürk-Çolak et al. 2024).

Lineage Tracing Design

Lineage clones from mitotic recombination were generated using heat shock by the method of Harrison and Perrimon (1993). Females of genotype X-15-33/X-15-29; MKRS, hs-FLP/+ were generated by standard crosses. To induce flippase expression, larva/pupa at various developmental

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times as specified in the results were heat shocked at 37ºC by immersion in a circulating water bath for 1 hour. The recombination enabled by flippase constructs a functional copy of α84Btubulin-LacZ, leading to LacZ reporter being constitutively expressed in the cell where recombination happened. The LacZ-marked clone produced by proliferation of the labeled individual cells in the ovary were subsequently imaged either 24 hours (in pupae) or 5 days (in newly eclosed adult) after heat shock. Quantification of cells in lineage clones was done in Imaris by examining across the entire Z-stack of tissue.

Immunostaining and Light Microscopy

Drosophila pupal and adult ovaries were dissected in phosphate-buffered saline (PBS) followed by immediate fixation in 4% paraformaldehyde in PBS (made from stock 16% Paraformaldehyde Aqueous Solution EM Grade, Electron Microscopy Sciences, SKU#15710) for 30 minutes at room temperature on rocking platform. Samples were then washed and permeabilized twice with 1% PBT (Triton X-100 in PBS) at 10 minutes each, blocked in 1% PBTB (0.6% BSA and 5% NGS in 1%PBT) overnight at 4ºC, and incubated with primary antibodies in 0.3% PBTB (0.6% BSA and 5% NGS in 0.3%PBT) overnight at 4ºC. Next, samples were washed 2 times with 0.3% PBT and 1 time with 0.3%PBTB at 30 minutes each. Secondary antibodies were then added in 0.3% PBTB and incubation was done overnight at 4ºC, then samples were washed 3 times with 0.3% PBT, the second time including 1:10,000 DAPI if nuclear stain was desired. Lastly, the sample was briefly washed in PBS before mounting on slide in Vectashield Antifade mounting medium. All overnight incubations were performed on a platform rocker and other shorter incubations on a nutator.

Primary antibodies used were GFP (1:200, chicken, Abcam Ab13970), LacZ (1:200, chicken, Abcam Ab134435), vasa (1:50,000, rabbit, from Dr. Ruth Lehmann) and 1B1/hts (1:50, mouse, from Developmental Studies Hybridoma Bank). Secondary antibodies used were Invitrogen AF-488, AF-

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568 and AF-680, all at 1:250. Images were acquired on Leica TCS SP8 confocal microscope, using 10X/NA0.25 objective for ovarioles and 63X/NA1.4 objective for germaria and pupal ovaries.

Image Analysis and Cell Quantification

Z-stack images acquired were re-constructed into 3D object using Imaris software (v10.0.0). Statistical analysis of cells was conducted using the spot function with manual spot assignment and automated quantification. Lineage tracing clone size was determined based on the number of nls.GFP+ nuclei, and PGC/cyst number in the pupal ovaries was determined based on the number of 1B1+ spectrosomes/fusomes (sample shown in Supplemental Movie S2, a 6h APF pupal ovary with white circles showing spots assigned to each spectrosome/fusome).

DATA AVAILABILITY

Complete results from the lineage studies are included in the paper and Supplemental material. Original Z-stack images are available upon request.

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FIGURE LEGENDS

Figure 1. Strategy for analyzing ovarian follicle waves.

A. Timeline of Drosophila development and ecdysone expression during the third larval instar and pupal stage (modified from Scanlan et al. 2024). Puparium formation occurs coincidental with a major peak of ecdysone at 0h APF. A small peak at 10-12h APF corresponds to pupariation and a broader pharate-adult peak reaches its maximum about 24-28h APF. **B-C**: samples of lineage analysis after ovary development to eclosion. **B.** Example of an ovariole from a newly eclosed fly heat shocked at 2h APF. One wave 1.5 follicle clone labels 4 of the 16-cells in the germline cyst within a single developing Stage 8 follicle of one ovariole isolated from a newly eclosed fly ovary. S1: a stage 1 follicle, S2-3: a stage 2 or 3 follicle, etc. **C.** The germarium from a newly eclosed fly heat shocked at 2h APF. One GSC was lineage labeled (GSC, green) along with its 4 progeny cysts. One GSC remains unlabeled that made unlabeled progeny cysts. Somatic cell types: TF--terminal filament, CC--cap cells, EC--escort cells, FSC--follicle stem cell, FC--follicle cells, spectrosome and fusome shown in magenta are stained by 1B1. R1, R2a, R2b are germarium regions, S1 is the first follicle budding from the germarium. Scale bars are 50μm in B and 20μm in C. **D.** Brief schematic of the lineage labeling system. Heat shock (ΔT) activates a constitutive marker gene (green) in one daughter cell of the next round of mitosis. **E.** Diagram shows the cyst formation trajectory from symmetrically dividing GSC/PGC, to cyst-forming cystoblast (CB) which undergoes 4 rounds of mitotic division to eventually construct a 16-cell cyst (CC). The number of labeled cyst cells expected if activation takes place in only 1 germ cell at the indicated stages is shown below the arrows. **F.** The two possible clone patterns resulting from a labeled GSC. In the GSC clone where the lineage marker is segregated into the GSC instead of the CB, The GSC clone will contain 1 less total labeled cyst (N-1) compared to the number of total divisions it underwent (N).

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Figure 2. Germline lineage clones of wave 2 and wave 1.5 follicles.

A. Germarium from a newly eclosed fly heat shocked at -2h APF. One of its two GSCs is labeled (green) and has generated a lineage of 3 progeny cysts (arrowheads) while daughters of the unlabeled GSC are unlabeled. **B.** An ovariole showing a cystoblast (CB) clone identified by all 16 of its germ cells being labeled. Its position in region 2b (R2b) corresponds to the first unlabeled cyst downstream of the GSC clones in 2A as expected (Figure 1F). **C.** Summary diagram of wave 2 GSC clones induced at the 5 time points studied, labeling rate is calculated by dividing total number of clone-containing ovarioles over all ovarioles. The frequency of clone labeling (blue) rapidly increased at 6h APF and remains at around 30% of ovarioles. The number of divisions the GSCs have undergone (orange) stayed uniform around 4 cysts/GSC across the 16hr duration. n=346, 303, 129, 224, 164 for the 5 time points. The adult labeling frequency data point is from Margolis and Spradling, 1995. Error bars represent SD across animals. **D-D'.** An ovariole with all GSCs and their progenies labeled, where all unlabeled cysts and follicles minus the first 2 unlabeled wave 2 CB cysts are wave 1.5. D' is the magnified view of the germarium region, arrowheads point to GSC spectrosomes. **E.** Ovarioles with unlabeled germaria showing wave 1.5 follicles with 8/16 labeled cyst cells (5 visible), or 4/16 (2 visible) labeled. **F.** An ovariole showing one 16/16 fully labeled wave 1.5 follicle at stage 4-5, while the less mature wave 1.5 follicle at stage 1 in the same ovariole is only 8/16 labeled. **G.** Percentage comparison of wave 1.5 PGCs that have initiated cyst division at HS time points before 10h (blue) vs after 10h (yellow), that make wave 1.5 follicles at different positions (Stage 2-5 vs Stage 6-9). Sample population is the same as in 2C. Error bars represent SD across time points in each group. **H.** Percentages of wave 1.5 follicles at different stages of cyst development (PGC/single cell, 2CC, 4CC, 8CC), vs time of clone induction showing progressive growth. Sample population is the same as in 2C. Scale bars are 20μm in A, B, D', 50μm in D, F, G.

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Figure 3. Time course of pupal germ cell development in three follicle waves.

A. Diagram of a 10h APF pupal ovary oriented as in panel images, anterior to the left and posterior to the right. The wave 2 arrow points to PGCs that become GSCs, wave 1.5 arrow points to PGCs that directly undergo cyst division inside ovarioles, and wave 2 arrow points to PGCs that localize towards the posterior. **B-J.** A time sequence of pupal ovaries from -2h APF to 30h APF stained for germ cells (Vasa, red), forming cysts (bam-GFP, green), fusomes and somatic cell membranes (Hts/1B1, magenta) and nuclei (DAPI, blue). Arrowheads in D indicate newly formed ovarioles where ovariolar germ cells are separated by the sheath, a feature not observed in earlier time points (B, C). Yellow arrows in F, G show the posterior movement of wave 1 germ cell precursors, which develop into cysts, aggregate (H) and leave the ovary along with a large number of posterior somatic cells (I). White arrows in E, H, I point to vacuoles devoid of antibody labeling that appear around germ cells. The distinction of 3 waves is outlined in H. **K.** Quantification of total single germ cells and cysts per ovariole from -2h to 30h APF. Error bars represent standard deviation of average across ovaries. Ovary age at analysis is indicated in hours APF. n=10 ovaries for each time point. **L.** A stacked image of 10 Z-series planes to show the large aggregation of all wave 1 germ cells into a disk-shaped mass in the posterior (arrow) of a 22h APF ovary. **M-N.** 10h APF germ cells magnified to show vacuoles and gaps between cells. Scale bars are 50μm in B-J and L, 10μm in M-N, N'.

Figure 4. Rapid proliferation of ovarian wave 1 germ cells and cysts.

A. Pupal ovary lineage labeled at -2h APF and analyzed at 22h APF. Wave 1 germ cells at the posterior (p) include lineage labeled cysts with fusomes (A', Hts/1B1 channel). A few smaller cysts were labeled within the ovarioles. **B.** A pupal ovary labeled as in A, but viewed from the posterior to

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reveal 14 wave 1 cysts, all with fusome (B'); 4 cysts are labeled in all cells. Yellow arrow indicates site where wave 1 germ and somatic cells have broken off the ovary. **C.** Pupal ovary labeled at 2h APF and analyzed at 26h APF. A large segment of ovarian somatic cells has separated (upper right). C' shows that all 4 cysts have 16-cell fusomes, 3 are lineage marked in all cells. **D.** Quantification of wave 1 cysts and germ cells (orange) from the time they start to become spatially separated from the other 2 more anterior waves (14h APF) until the time they have all extruded from the ovary (30h APF). Points on dashed lines are cumulative total wave 1 germ cells ever produced discounting loss from extrusion and considering the continuous cyst amplification, as shown by the constant increase of 16 cell-cyst percentage out of all cysts in wave 1 (blue). n=6,8,8,8 ovaries, respectively. Error bars represent SD across ovaries. **E.** Pupal ovary labeled at 2h APF and analyzed at 26h APF showing significant germ cell extrusion from the posterior, among which two large clusters are lineage positive. **F-G.** 22h APF bam-GFP fly line pupal ovaries showing germ cell extrusion similar to that seen in lineage tracing fly line ovaries. **H.** 22h APF ovary with lipid-enriched vacuoles stained with BODIPY embedded in the ovary next to wave 1 germ cells. H' is the brightfield image of the same ovary showing morphology of the vacuoles, indicated by arrows. **I.** A 22h APF testis with a large cluster of dying germ cells. I' shows a magnified view of the dying germ cell cluster under brightfield, revealing the morphological changes of those cells that makes them distinct from surrounding testical tissue. **J.** A 22h APF testis showing significant amount of cell loss on the upper left corner. Areas in squares are magnified in K and L. **K-L.** Magnified images of 4J showing dying germ cells that still have some remnant vasa expression. K' and L' show lipid enrichment similar to that of 4H. Scale bars are 50μm in all images.

Figure 5. Progenies from a temporary FSC population cover wave 1.5 follicles.

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A. Somatic cell clone labeling rate as a function of induction times in hours APF. All cell types exhibit a marked increase in labeling rate at 10h. While FSC clones maintained the elevated rate, that of cap cell (CC) and escort cell (EC) clones dropped subsequently at 14h. n=621, 517, 449, 224, 459 total scored ovarioles. **B.** A lineage-labeled wave 1.5 FSC clone marked at 10h APF and analyzed 5 days later at eclosion. A clone encompassing about 50% of the follicle cells is seen extending over two region 2B cysts and all budded follicles. Arrowhead points to the marked active FSC at 2a/2b junction. **C.** Example of wave 1.5 FSC clone without an active FSC labeled, where the labeled wave 1.5 FSC was replaced by an unlabeled FSC after its progeny has covered parts of 4 sequential wave 1.5 follicles. **C'.** A magnified image of a wave 1.5 FSC clone that lost its active FSC, showing the absence of active FSC at 2a/2b junction location marked by arrowheads. **D.** A short wave 2 FSC clone marked at 14h APF that by eclosion has only covered two R2b cysts, corresponding to the extent of wave 2 cyst progression in the germarium. **E.** A wave 1.5 FSC clone that lost its FSC early, only making enough FC to cover 2 follicles. **F.** Quantification of wave 1.5 FSC clones based on the follicle position where their FSC was lost. n=273 FSC-P clones scored. **G-G'.** Example showing how wave 1.5 FSC clone coverage was calculated in Imaris. G shows the raw stacked image and G' shows the wave 1.5 FSC clone coverage in blue and total FC layer on follicles containing the clone in magenta. **H.** FSC clone coverage percentage calculated directly by surface area of the 3D objects created in Imaris, or by surface area converted from volume of such objects. n=25 wave 1.5 follicles measured. **I.** Quantification of CB clone, wave 2 FSC clone and wave 1.5 FSC clone based on the location of the labeled CB, the first labeled wave 2 FC and the last labeled wave 1.5 FC (lost FSC), respectively. R2b+1 stands for the first R2b cyst position, and so on. Only clones that involve a position in R2b and S1 are recorded in the graph. n=130,54,40 clones for each clone type. **J.** An ovariole with a short wave 2 FSC clone surrounding the CB clone. Scale bars are 20μm in all images.

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K. An ovariole with a CB clone past the 2a/2b junction also contains a wave 1.5 FSC clone that does not contain an active FSC, and the last wave 1.5 FCs in the clone are at the same position as the CB clone. It is the magnified germarium region of the ovariole shown in panel C. **L.** An ovariole where a CB clone has not yet passed the 2a/2b junction also contains a wave 1.5 FSC clone that has not lost its active FSC.

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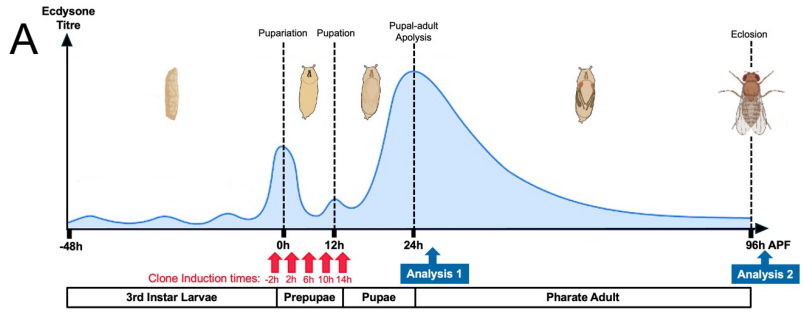
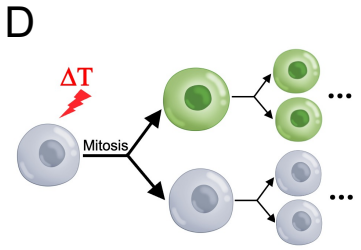
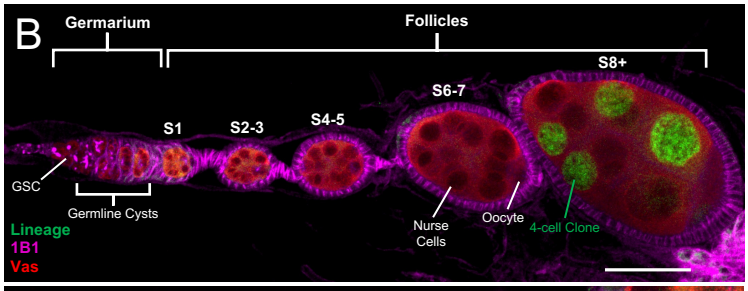
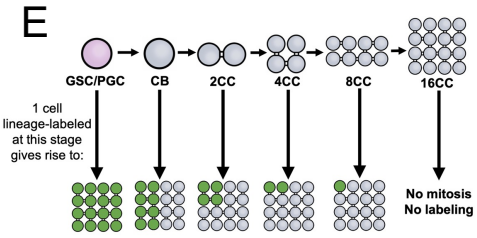
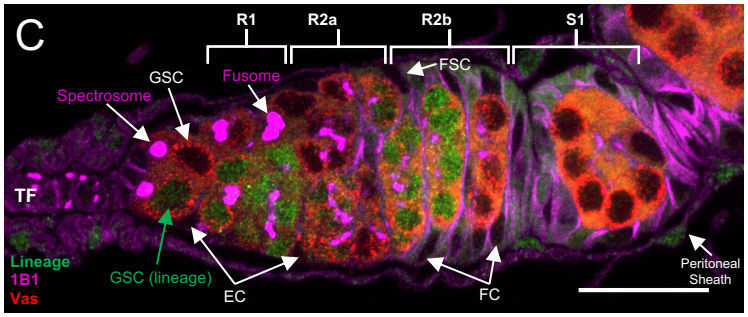
Fig.1


A
Ecdysone Titre
Pupariation
Pupation
Pupal-adult Apolysis
Eclosion
-48h
0h
12h
24h
96h APF
Clone Induction times: -2h 2h 6h 10h 14h
Analysis 1
Analysis 2
3rd Instar Larvae
Prepupae
Pupae
Pharate Adult


D
ΔT
Mitosis


B
Germarium
Follicles
S8+
S6-7
S1
S2-3
S4-5
GSC
Germline Cysts
Nurse Cells
Oocyte
4-cell Clone
Lineage
1B1
Vas


E
GSC/PGC
CB
2CC
4CC
8CC
16CC
1 cell lineage-labeled at this stage gives rise to:
No mitosis
No labeling


C
R1
R2a
R2b
S1
GSC
Fusome
FSC
Spectrosome
TF
Lineage
1B1
Vas
GSC (lineage)
EC
FC
Peritoneal Sheath

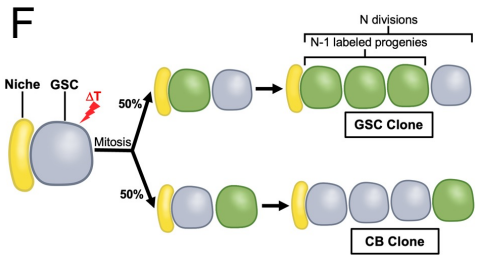

F
N divisions
N-1 labeled progenies
Niche
GSC
ΔT
Mitosis
50%
50%
GSC Clone
CB Clone

Fig.2

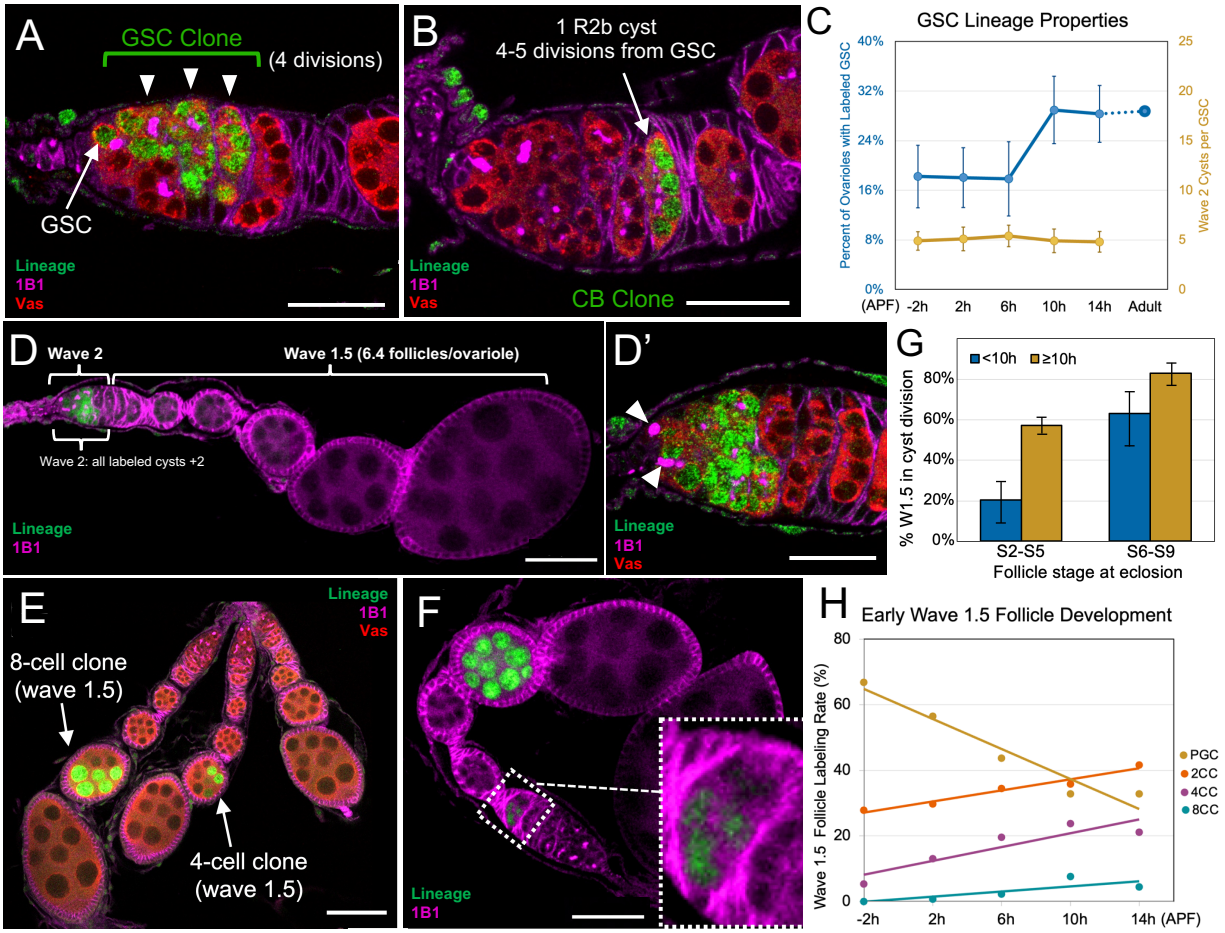

A
GSC Clone
(4 divisions)
GSC
Lineage
1B1
Vas
B
1 R2b cyst
4-5 divisions from GSC
Lineage
1B1
Vas
CB Clone
C
GSC Lineage Properties
Percent of Ovarioles with Labeled GSC
Wave 2 Cysts per GSC
40%
32%
24%
16%
8%
0%
25
20
15
10
5
0
(APF) -2h
2h
6h
10h
14h
Adult
D
Wave 2
Wave 1.5 (6.4 follicles/ovariole)
Wave 2: all labeled cysts +2
Lineage
1B1
D'
Lineage
1B1
Vas
G
<10h
≥10h
% W1.5 in cyst division
80%
60%
40%
20%
0%
S2-S5
S6-S9
Follicle stage at eclosion
E
Lineage
1B1
Vas
8-cell clone
(wave 1.5)
4-cell clone
(wave 1.5)
F
Lineage
1B1
H
Early Wave 1.5 Follicle Development
Wave 1.5 Follicle Labeling Rate (%)
80
60
40
20
0
-2h
2h
6h
10h
14h (APF)
PGC
2CC
4CC
8CC

Fig.3

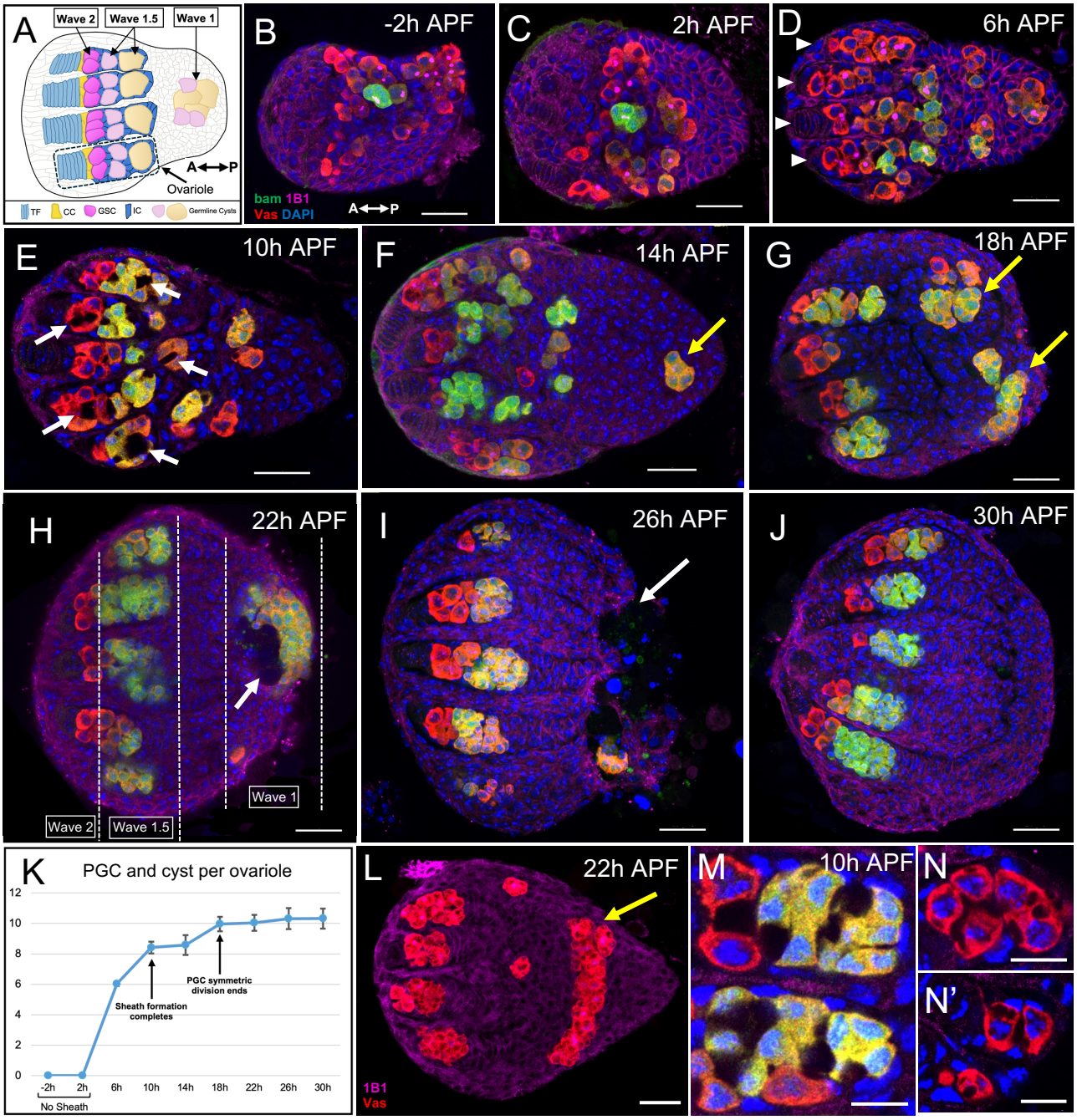

A
Wave 2
Wave 1.5
Wave 1
A↔P
Ovariole
TF
CC
GSC
IC
Germline Cysts
B
-2h APF
bam 1B1
Vas DAPI
A↔P
C
2h APF
D
6h APF
E
10h APF
F
14h APF
G
18h APF
H
22h APF
Wave 2
Wave 1.5
Wave 1
I
26h APF
J
30h APF
K
PGC and cyst per ovariole
12
10
8
6
4
2
0
-2h
2h
6h
10h
14h
18h
22h
26h
30h
No Sheath
Sheath formation completes
PGC symmetric division ends
L
22h APF
1B1
Vas
M
10h APF
N
N'

Fig.4

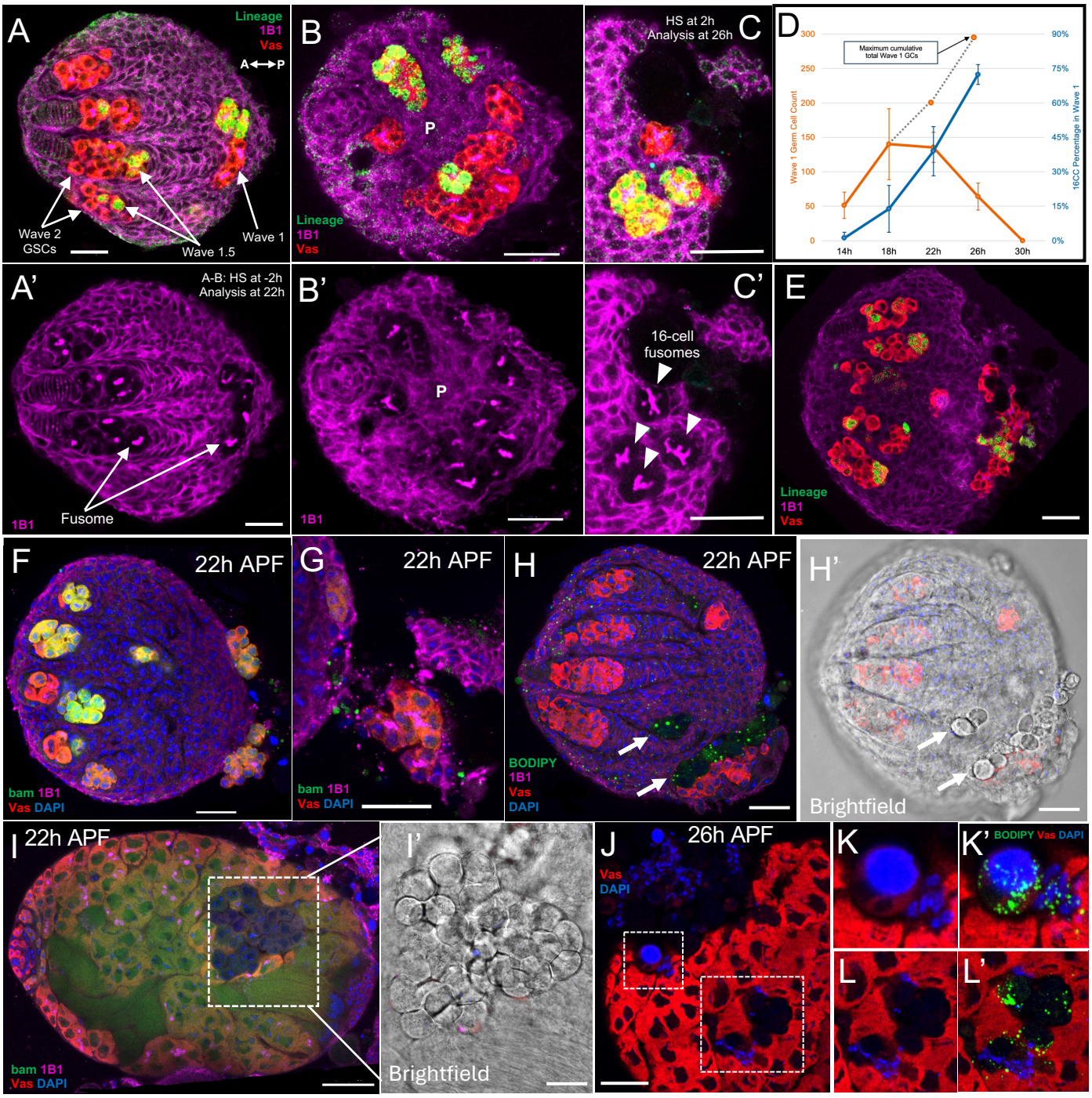
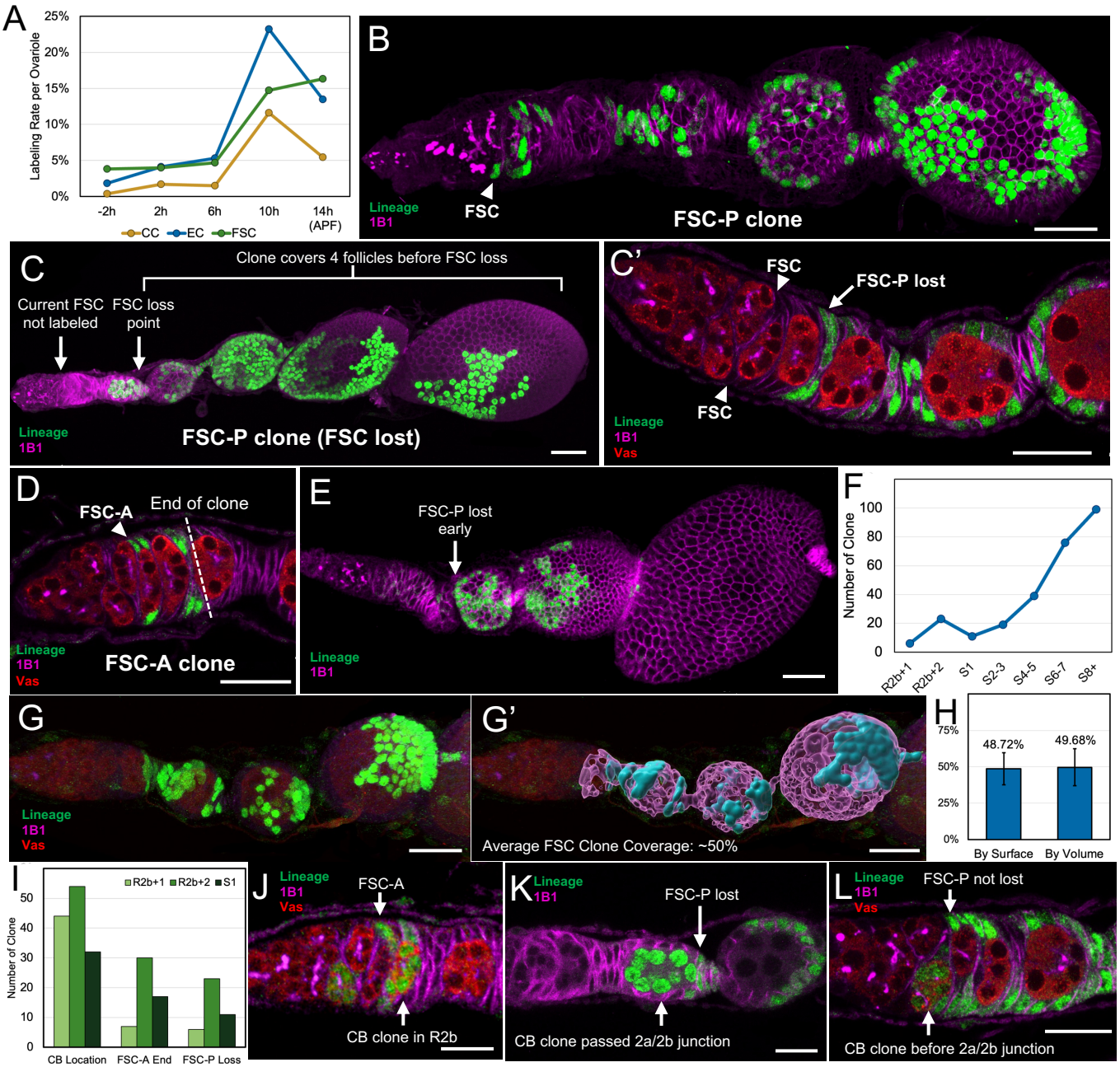

A
Lineage
1B1
Vas
A↔P
Wave 2 GSCs
Wave 1.5
Wave 1
B
P
Lineage
1B1
Vas
HS at 2h
Analysis at 26h
C
D
Maximum cumulative total Wave 1 GCs
Wave 1 Germ Cell Count
16CC Percentage in Wave 1
300
250
200
150
100
50
0
90%
75%
60%
45%
30%
15%
0%
14h
18h
22h
26h
30h
A'
A-B: HS at -2h
Analysis at 22h
Fusome
1B1
B'
P
1B1
C'
16-cell fusomes
E
Lineage
1B1
Vas
F
22h APF
bam 1B1
Vas DAPI
G
22h APF
bam 1B1
Vas DAPI
H
22h APF
BODIPY
1B1
Vas
DAPI
H'
Brightfield
I
22h APF
bam 1B1
Vas DAPI
I'
Brightfield
J
26h APF
Vas
DAPI
K
K'
BODIPY Vas DAPI
L
L'

Fig.5


A
Labeling Rate per Ovariole
25%
20%
15%
10%
5%
0%
-2h
2h
6h
10h
14h (APF)
CC
EC
FSC
B
Lineage
1B1
FSC
FSC-P clone
C
Clone covers 4 follicles before FSC loss
Current FSC not labeled
FSC loss point
Lineage
1B1
FSC-P clone (FSC lost)
C'
FSC
FSC-P lost
FSC
Lineage
1B1
Vas
D
FSC-A
End of clone
Lineage
1B1
Vas
FSC-A clone
E
FSC-P lost early
Lineage
1B1
F
Number of Clone
100
80
60
40
20
0
R2b+1
R2b+2
S1
S2-3
S4-5
S6-7
S8+
G
Lineage
1B1
Vas
G'
Average FSC Clone Coverage: ~50%
H
75%
50%
25%
0%
48.72%
49.68%
By Surface
By Volume
I
R2b+1
R2b+2
S1
Number of Clone
50
40
30
20
10
0
CB Location
FSC-A End
FSC-P Loss
J
Lineage
1B1
Vas
FSC-A
CB clone in R2b
K
Lineage
1B1
FSC-P lost
CB clone passed 2a/2b junction
L
Lineage
1B1
Vas
FSC-P not lost
CB clone before 2a/2b junction

Supporting information for
The Drosophila ovary produces three follicle waves similar to those in mice

Wayne Yunpeng Fu and Allan C Spradling

Corresponding author: Allan C Spradling
Email: spradling@carnegiescience.edu

The supporting information includes:

Supplemental Table S1 to S2
Supplemental Figures S1 to S4
Legends for Supplemental Figures S1 to S4
Legends for Movies S1 to S4

Other supporting materials for this manuscript include the following:

Supplemental Table S3.xlsx

Movies S1 toS4

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Supplemental Tables

Table S1

	GSC	R2a	R2b		Stage 1		Stage 2-3		Stage 4-5		Stage 6-7		Stage 8-9		Stage 10A	
	16	16	4-8	16	4-8	16	2-8	16	2-8	16	1-8	16	1-8	16	1-8	16
-2h	18.2%	3.8%	1.7%	9.5%	6.9%	2.6%	10.7%	1.7%	9.8%	1.4%	17.1%	1.2%	17.3%	0.3%	0.6%	0%
2h	18.0%	11.2%	1.0%	16.8%	8.3%	2.6%	16.8%	3.6%	21.5%	2.3%	21.5%	1.0%	26.7%	0.0%	22.1%	0%
6h	17.8%	2.3%	1.6%	11.6%	8.5%	1.6%	11.6%	1.6%	21.7%	0.8%	16.3%	0.8%	37.2%	0%	38.0%	0%
10h	28.9%	6.3%	8.0%	14.7%	16.5%	1.8%	22.8%	2.2%	25.0%	1.8%	39.3%	0%	51.8%	0.4%	10.7%	0%
14h	28.3%	7.9%	10.4%	13.4%	16.5%	2.4%	23.8%	3.0%	34.1%	1.8%	51.8%	0.6%	65.2%	0%	0.6%	0%

Table S1. Germ cell clone quantification. Lineage labeling frequency (number of clones/number of total ovarioles) of germ cell clones at different sizes, stages and HS time points. The first header row indicates the location of recorded germline clones, and the second header row indicates the size of such clones (e.g. 4-8 represents tally of 4-cell and 8-cell germline clones, etc.) For germ cells, only one replicate group is fully quantified due to low level of variation (n=346, 303, 129, 224, 164 ovarioles for each time point, respectively).

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Table S2

	1CC	2CC	1EC	2EC	2+EC	FSC-A	FSC-P (without FSC)	FSC-L (with FSC)
-2h	0.36%	0%	0.65%	0.83%	0.33%	1.05%	1.09%	1.09%
2h	1.36%	0.33%	1.76%	2.34%	0%	2.79%	0.47%	0.56%
6h	1.17%	0.31%	3.43%	1.25%	0.63%	3.03%	0.94%	0.70%
10h	8.48%	3.13%	10.71%	4.91%	7.59%	7.59%	2.68%	4.46%
14h	4.86%	0.58%	8.30%	4.44%	0.72%	6.39%	5.01%	3.27%

Table S2. Somatic cell clone quantification. The percentage are labeling rates of each type of somatic cell clone (number of ovarioles with such clone over the total number of ovarioles). 1CC: clone of 1 cap cell; 2CC: clone of 2 cap cells; 1EC: clone of 1 EC; 2EC: clone of 2 ECs; 2+EC: clone with more than 2 ECs (87% are 4-EC clones); FSC-A: number of short adult FSC clones that correspond to wave 2 follicles (see text); FSC-P (without FSC): number of pupal FSC clones covering wave 1.5 follicles that are missing an active FSC (see text); FSC-P (with FSC): number of long FSC clones that contains an active pupal FSC (see text). n=621, 517, 449, 224, 459 total scored ovarioles for each time point, respectively.

External Supplemental Tables

Table S3.xlsx. Raw data recording the location and size of each individual observed clone. For interpretation please view the first legends tab inside the excel file.

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Supplemental Figures S1 to S4

Figure S1. Additional Information on Experimental Design and PGC biology
A. Schematic showing the detailed recombination process that gives rise to a lineage-labeled cell. **B.** An asymmetry in ovariole formation and PGC distribution exists in early pupal ovary, most prominent at 2h-6h APF and progressively becomes less evident over time. No significant asymmetry is observed by 18h APF. Ovarioles on the top of the image include more germ cells than the ovarioles on the bottom, as indicated by the brackets. PGCs posterior to the ovarioles are also unevenly distributed, more enriched on one side. 3D view shown in Movie S1. **C.** Example ovariole at 30h APF and 40h APF, both lacking the adult 2a/2b junction and R2b cyst morphology. **D-E.** Summary diagrams showing the labeling status of germ cells in wave 1.5 follicles following induction at -2h (F), or 14h (G).

Figure S2. Additional Quantifications of Wave 1 germ cells
A. Quantification of PGCs and cysts in the entire pupal ovary from -2h to 30h APF, showing the extent of PGC symmetric division and the dip caused by wave 1 loss starting at 22h APF. Quantification is based on counting the number of spectrosomes/fusomes, example counting process shown in Movie S2. **B.** Images showing the last rounds of PGC division take place among PGCs that give rise to wave 1.5 follicles at 14h APF. These are the PGCs that become included in the forming ovarioles but are too posterior to become GSCs. No more such divisions are observed at any time point later than 14h APF. **C.** A zoomed-out image of Figure 3N', showing that the single germ cells with vacuole inclusion are indeed GSCs next to the terminal filament, appearing as stacks of DAPI+ flat cells to the left side of the image. **D.** Quantification of wave 1 germ cells (red) and cysts (blue) from 14h to 22h APF. Germ cell count stays the same while cyst number drops from 18h to 22h APF, due to the loss of wave 1 cysts initiating at 22h while the overall number being compensated by continuous cyst division. **E.** Zoomed-in display of the blue "Total Cysts" bars in S2A, detailing the relative proportion of cysts at different sizes in the total population. **F.** A final wave 1.5 follicle now at stage 9 that was lineage labeled at 14h and contains a follicle cell clone which extends to the previous follicle. The border cell cluster at the anterior is mosaic (magnified in the box below) with 3 marked cells, and migrates slower than in adults toward the posterior. Other examples show the large size of the polar cells in these clusters and the presence of 3-4 labeled cells. **G-G'.** More examples showing polar cells migrate posteriorly at different speed than main body follicle cells, while in adults they migrate together. **H-H'''.** Ovarioles of newly eclosed flies showing abnormal cyst size of 8 cells and 32 cells in the last two wave 1.5 follicles, and the disproportionate polyploidization of several nurse cells.

Figure S3. Additional examples of FSC clones
A-A'. Ovarioles with long FSC-P clones that retained an active pupal FSC. **B-C.** Examples of FSC-A clones. C' is the magnified germarium region of the ovariole in C. **D-D'.** Ovarioles with long FSC-P clones that do not contain an active pupal FSC, replaced by an unlabeled FSC. **E-E'.** Short FSC-P clones on the older wave 1.5 follicles that had an early FSC loss. **F-F'.** The shortest FSC-P clones that only cover the anterior of oldest wave 1.5 follicle, complementing the basal stalk follicle cell clone shown in G-G'. **G-G'.** Small patches of follicle cell clone that only cover

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the most mature wave 1.5 follicles, most likely originating from the basal stalk cells as previously reported (Reilein et al. 2021).

Figure S4. Lineage labeled escort cell (EC) clones.
A. A germarium stained to mark the ECs using the enhancer trap line PZ1444. Two GSCs (unlabeled) are seen at the front associated with cap cells (arrowhead). In addition, 8 specific ECs are labeled and indicated with arrowheads and assigned corresponding names. B-L show germaria following clonal marking to identify EC-specific clones. **B-B''.** Example germaria each with one labeled EC1 (arrows). **C-C'.** Germaria with single EC2 clone. **D.** Germarium with an EC3 clone. **E-E'.** Germaria with single EC5 clone. **F-F'.** Examples of EC6 clone, immediately next to FSCs. **G.** An EC6 clone in the middle of the germarium at 2a/2b junction. **H.** Labeling frequency of EC clones based on the number of ECs each clone contains. **I.** Frequency of ovarioles with clones of ECs by type (EC1-6). **J.** Two adjacent labeled ECs, E5 and E4 are located directly adjacent to an unlabeled FSC. **J'.** An ovariole containing 4 ECs that are close to the 2a/2b junction. **K-K''.** Examples of cap cell clones. K' contains 2 labeled cap cells. **L.** An ovariole containing both a CB clone and a FSC-P clone that has lost its active pupal FSC, showing FSC-P loss happened slightly after the CB clone passed by 2a/2b junction.

Legends for Movies S1 to S2

Movie S1: Rotation of a 6h APF ovary showing the asymmetry of PGC distribution along a dorsal-ventral axis.

Movie S2: Example process of PGC counting in the pupal ovary. Spectrosomes/fusomes shown as magenta dots stained by Hts/1B1 are circled, as they are counted in Imaris using the Spot function.

Movie S3-S4: Examples showing how the quantification of follicle cell coverage in Figure 5K-L was done in Imaris in 3D reconstruction. Movie S3 shows an FSC-L clone and Movie S4 shows an FSC-M clone.

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Figure S1

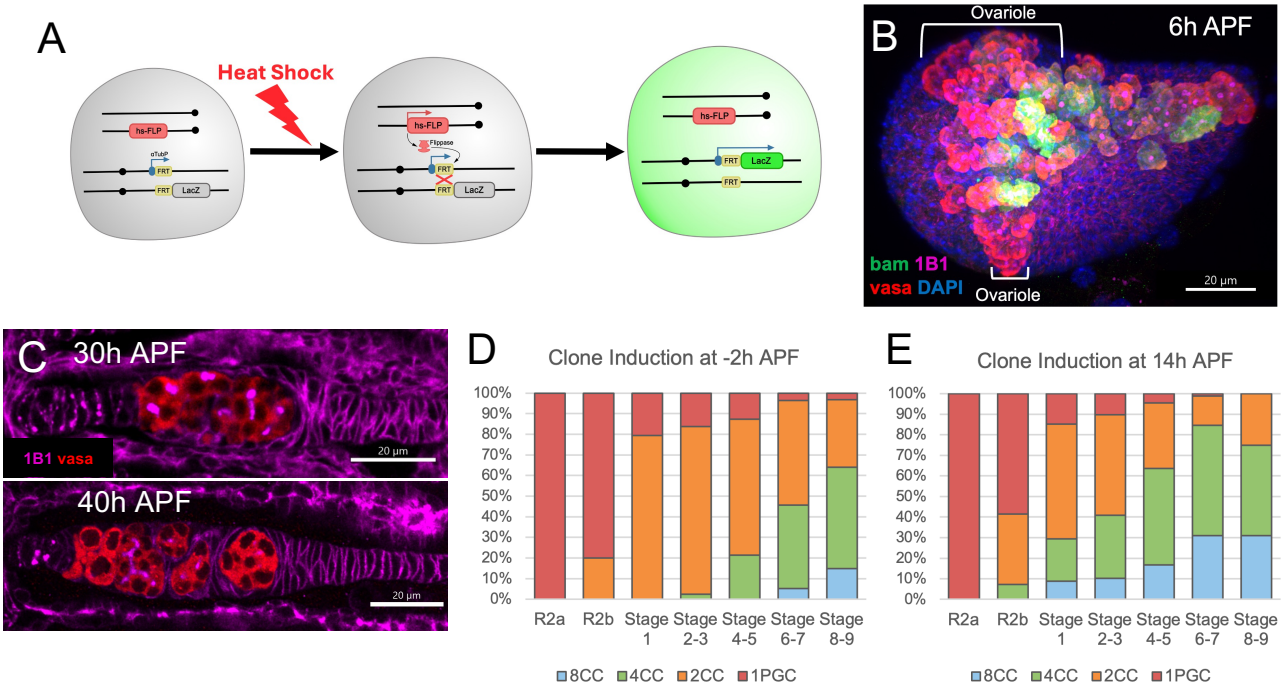

A
Heat Shock
hs-FLP
αTubP
FRT
LacZ
Flippase
B
Ovariole
6h APF
bam 1B1
vasa DAPI
Ovariole
20 µm
C
30h APF
1B1 vasa
20 µm
40h APF
20 µm
D
Clone Induction at -2h APF
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
R2a
R2b
Stage 1
Stage 2-3
Stage 4-5
Stage 6-7
Stage 8-9
8CC
4CC
2CC
1PGC
E
Clone Induction at 14h APF
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
R2a
R2b
Stage 1
Stage 2-3
Stage 4-5
Stage 6-7
Stage 8-9
8CC
4CC
2CC
1PGC

Figure S2

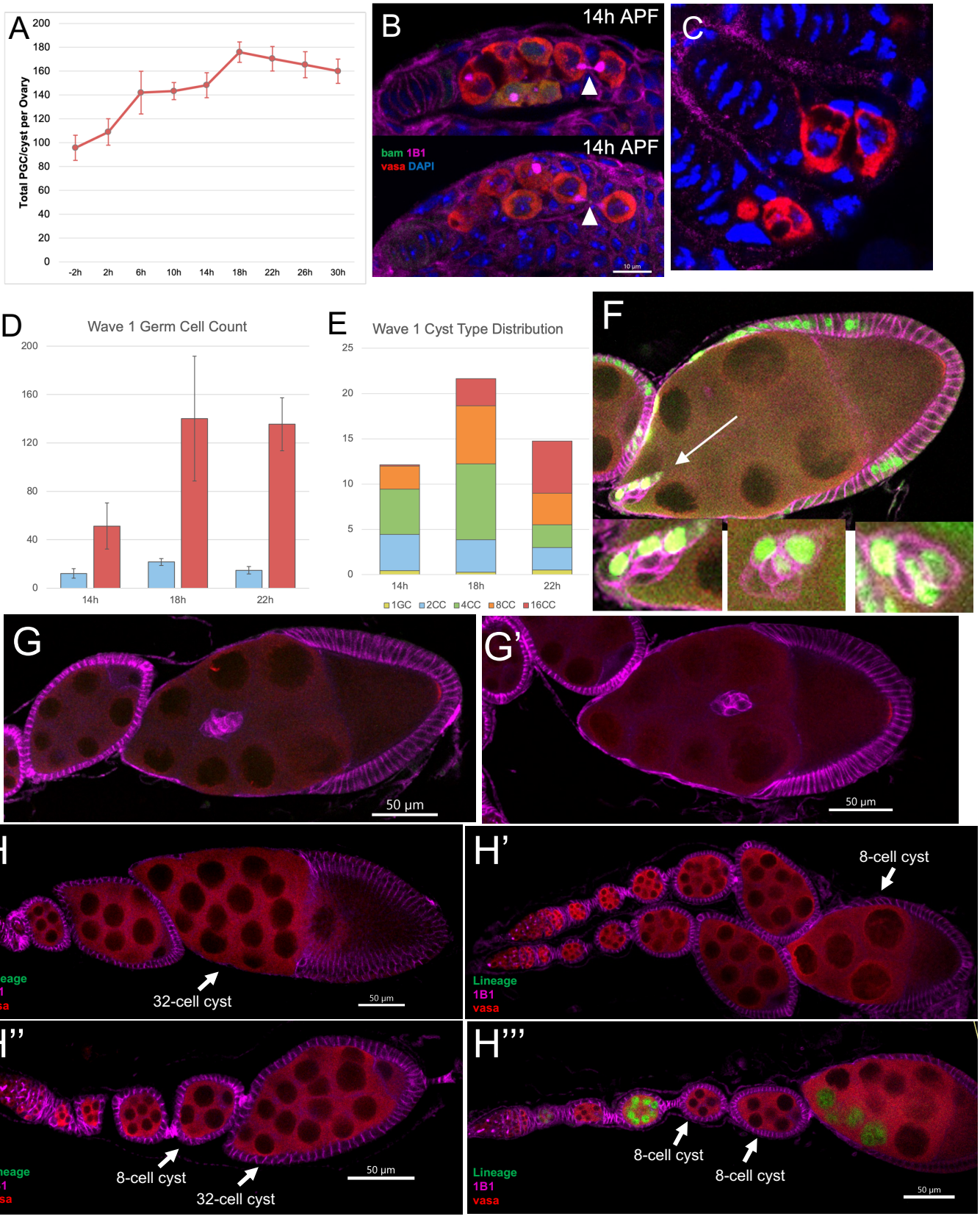

A
Total PGC/cyst per Ovary
200
180
160
140
120
100
80
60
40
20
0
-2h
2h
6h
10h
14h
18h
22h
26h
30h
B
14h APF
bam 1B1
vasa DAPI
14h APF
10 µm
C
D
Wave 1 Germ Cell Count
200
160
120
80
40
0
14h
18h
22h
E
Wave 1 Cyst Type Distribution
25
20
15
10
5
0
14h
18h
22h
1GC
2CC
4CC
8CC
16CC
F
G
50 µm
G'
50 µm
H
Lineage
1B1
vasa
32-cell cyst
50 µm
H'
8-cell cyst
Lineage
1B1
vasa
H''
Lineage
1B1
vasa
8-cell cyst
32-cell cyst
50 µm
H'''
8-cell cyst
8-cell cyst
Lineage
1B1
vasa
50 µm

Figure S3

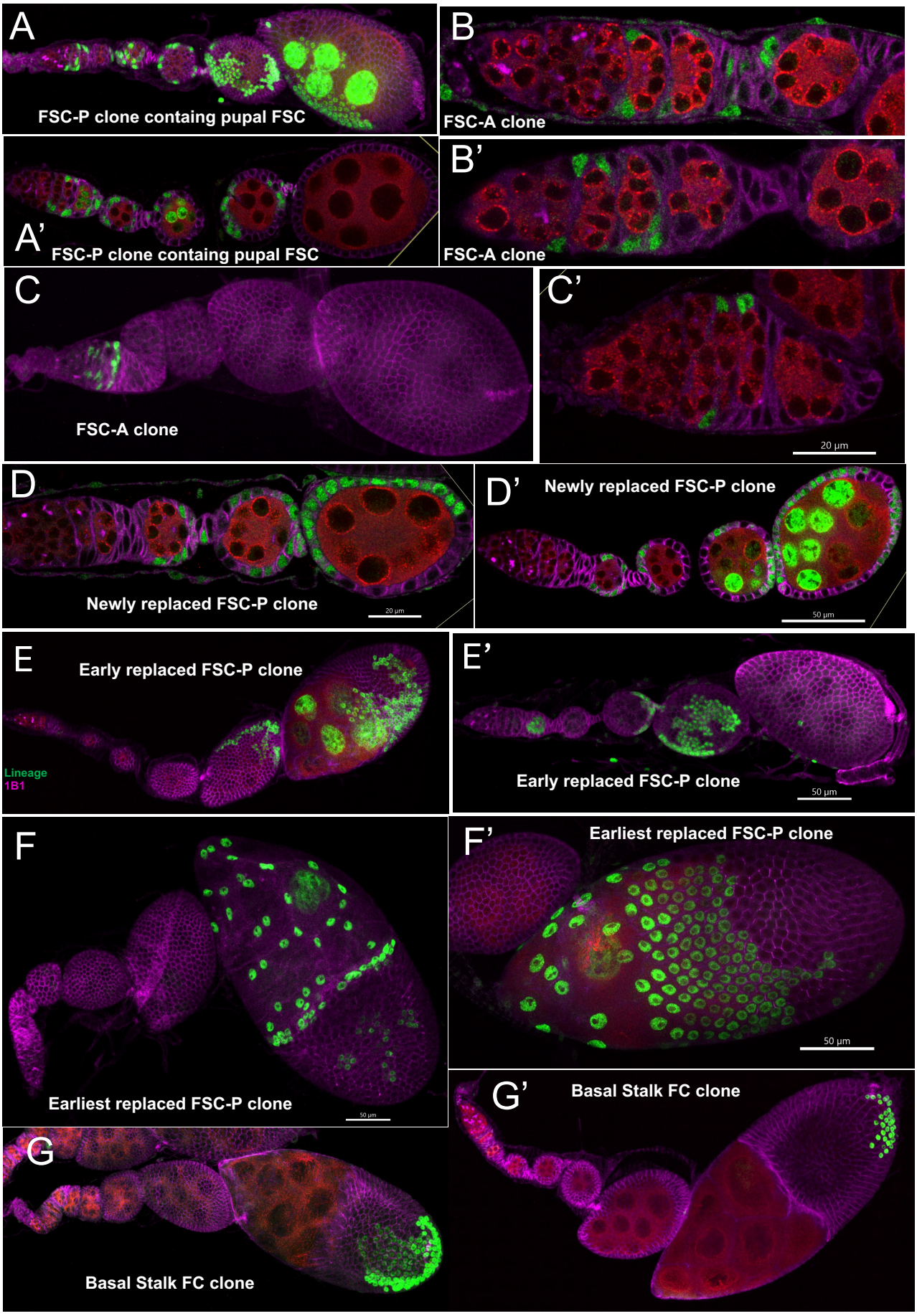
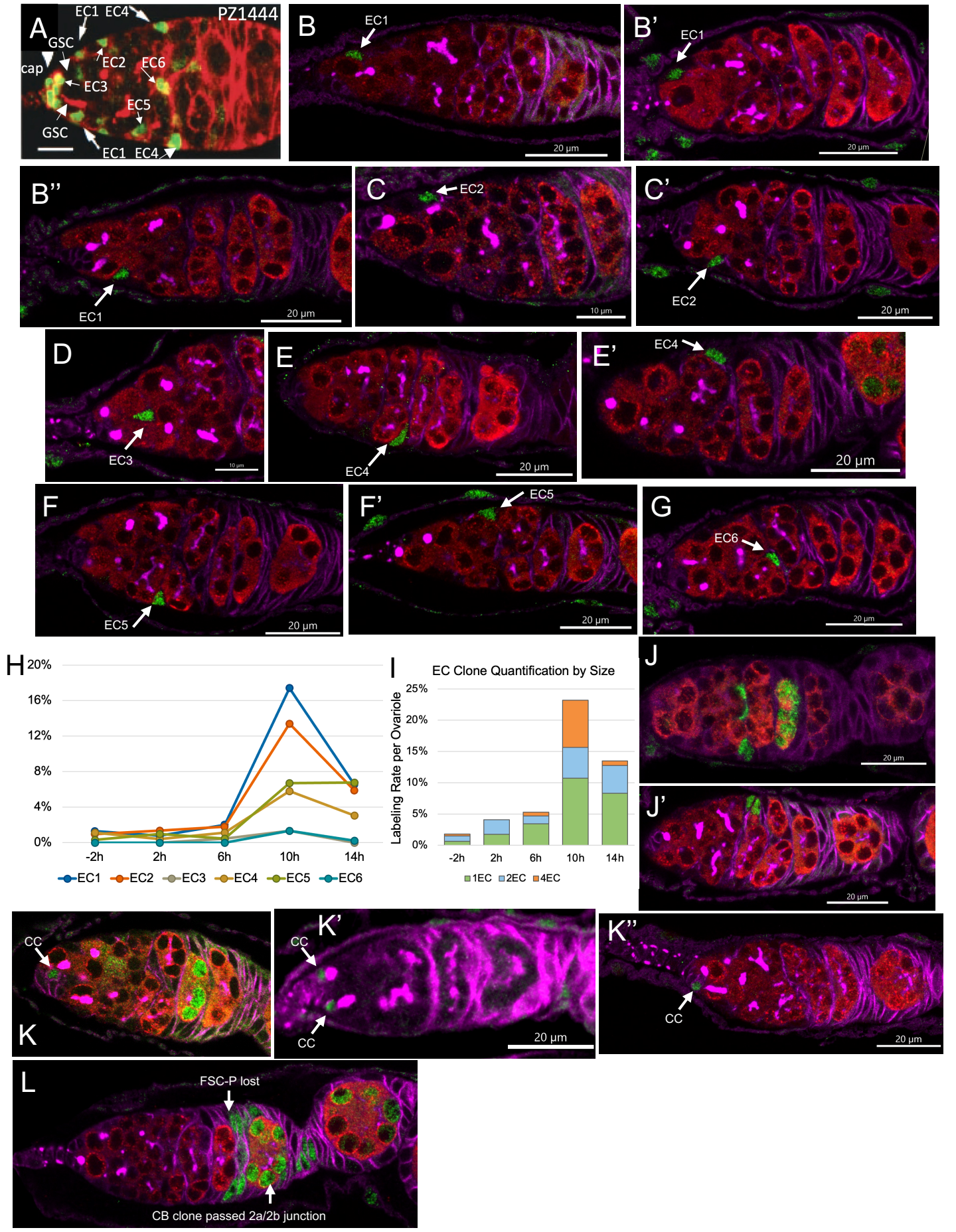

A
FSC-P clone containg pupal FSC
B
FSC-A clone
A'
FSC-P clone containg pupal FSC
B'
FSC-A clone
C
FSC-A clone
C'
20 µm
D
Newly replaced FSC-P clone
20 µm
D'
Newly replaced FSC-P clone
50 µm
E
Early replaced FSC-P clone
Lineage
1B1
E'
Early replaced FSC-P clone
50 µm
F
Earliest replaced FSC-P clone
50 µm
F'
Earliest replaced FSC-P clone
50 µm
G
Basal Stalk FC clone
G'
Basal Stalk FC clone

Figure S4


A
PZ1444
EC1 EC4
GSC
cap
EC2
EC6
EC3
EC5
GSC
EC1 EC4
B
EC1
20 µm
B'
EC1
20 µm
B''
EC1
20 µm
C
EC2
10 µm
C'
EC2
20 µm
D
EC3
10 µm
E
EC4
20 µm
E'
EC4
20 µm
F
EC5
20 µm
F'
EC5
20 µm
G
EC6
20 µm
H
20%
16%
12%
8%
4%
0%
-2h
2h
6h
10h
14h
EC1
EC2
EC3
EC4
EC5
EC6
I
EC Clone Quantification by Size
Labeling Rate per Ovariole
25%
20%
15%
10%
5%
0%
-2h
2h
6h
10h
14h
1EC
2EC
4EC
J
20 µm
J'
20 µm
K
CC
K'
CC
CC
20 µm
K''
CC
20 µm
L
FSC-P lost
CB clone passed 2a/2b junction