Division-independent differentiation mandates proliferative competition among stem cells

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Cancer-initiating gatekeeper mutations that arise in stem cells would be especially potent if they stabilize and expand an affected stem cell lineage. It is therefore important to understand how different stem cell organization strategies promote or prevent variant stem cell amplification in response to different types of mutation, including those that activate proliferation. Stem cell numbers can be maintained constant while producing differentiated products through individually asymmetrical division outcomes or by population asymmetry strategies in which individual stem cell lineages necessarily compete for niche space. We considered alternative mechanisms underlying population asymmetry and used quantitative modeling to predict starkly different consequences of altering proliferation rate: A variant, faster proliferating mutant stem cell should compete better only when stem cell division and differentiation are independent processes. For most types of stem cells, it has not been possible to ascertain experimentally whether division and differentiation are coupled. However, *Drosophila* follicle stem cells (FSCs) provided a favorable system with which to investigate population asymmetry mechanisms and also for measuring the impact of altered proliferation on competition. We found from detailed cell lineage studies that division and differentiation of an individual FSC are not coupled. We also found that FSC representation, reflecting maintenance and amplification, was highly responsive to genetic changes that altered only the rate of FSC proliferation. The FSC paradigm therefore provides definitive experimental evidence for the general principle that relative proliferation rate will always be a major determinant of competition among stem cells specifically when stem cell division and differentiation are independent.

stem cell | competition | proliferation | population asymmetry | *Drosophila*

Large-scale sequencing of tumor samples, including single cells, provides information about the number and identity of mutations that drive cancer ontogeny, key initiating gatekeeper mutations, and clonal histories (1–3). Understanding how each driver mutation promotes clonal selection throughout this long developmental sequence of changing cellular phenotypes and environments is very challenging, but it is most approachable for the earliest mutations because they occur in the context of normal morphology and physiology. The longevity and proliferative potential of stem cells make it inevitable that the first driver mutations sometimes arise in stem cells, especially for tissues with very active stem cells and short-lived derivatives (1, 4–6). Those first driver mutations (gatekeepers) may act throughout cancer evolution, but they will be especially potent if they provide a selective advantage at the earliest possible stage to stabilize a mutant stem cell lineage and amplify it to provide multiple substrate cells for sampling a variety of potential secondary mutations (6, 7). It is therefore very important to understand what types of mutations favor maintenance and amplification of an affected stem cell, and hence why some gatekeeper mutations may be more potent in one tissue than another.

It might, at first thought, be expected that an increased rate of cell division would inevitably favor the amplification of any cell type. However, stem cells are generally maintained at roughly constant numbers. This constraint, generally imposed by limited space within a supportive niche environment, renders the impact of increased proliferation dependent on the strategies used for stem cell maintenance (8–10) (Fig. 1*A* and Fig. S1*A*). For example, if a stem cell always divides to produce one stem cell and one differentiated cell (single-cell asymmetry; model A, Fig. 1*A*), an increased rate of division of one stem cell will not alter the longevity or representation of that stem cell. Germline stem cells (GSCs) in the *Drosophila* ovary mostly undergo repeated divisions with asymmetrical outcomes, and mutations that alter the rate of GSC divisions do not generally affect GSC maintenance (11–14).

Several types of stem cell, including *Drosophila* follicle stem cells (FSCs), which reside in the same ovaries as GSCs, and mammalian gut stem cells, are instead maintained by population asymmetry (Fig. 1*A* and Fig. S1*A*). The term "population asymmetry" is generally understood to mean that the fates of two daughters of a stem cell are independent. Population asymmetry inevitably creates competition among stem cells for survival and amplification, leading to stochastic expansion of some stem cell lineages, while others are lost ("neutral competition") (15, 16) (Fig. S1*B*). The factors that regulate competition can be uncovered experimentally by identifying hypo- or hypercompetitive genetic variants and the molecular mechanisms they affect. FSC survival can be compromised by reduced activity of adhesion molecules or altered signaling that promotes differentiation (17–19), but both an unbiased genetic screen and analysis of a key niche signal pointed to stem cell division rate as a major determinant of FSC competition (12, 13, 20). By contrast, niche

Significance

Adult stem cells support tissue maintenance throughout life, but they also can be cells of origin for cancer, allowing clonal expansion and long-term maintenance of the first oncogenic mutations. We considered how a mutation that increases the proliferation rate of a stem cell would affect the probability of its competitive survival and amplification for different potential organizations of stem cells. Quantitative modeling showed that the key characteristic predicting the impact of relative proliferation rate on competition is whether differentiation of a stem cell is coupled to its division. We then used *Drosophila* follicle stem cells to provide definitive experimental evidence for the general prediction that relative proliferation rates dictate stem cell competition specifically for stem cells that exhibit division-independent differentiation.

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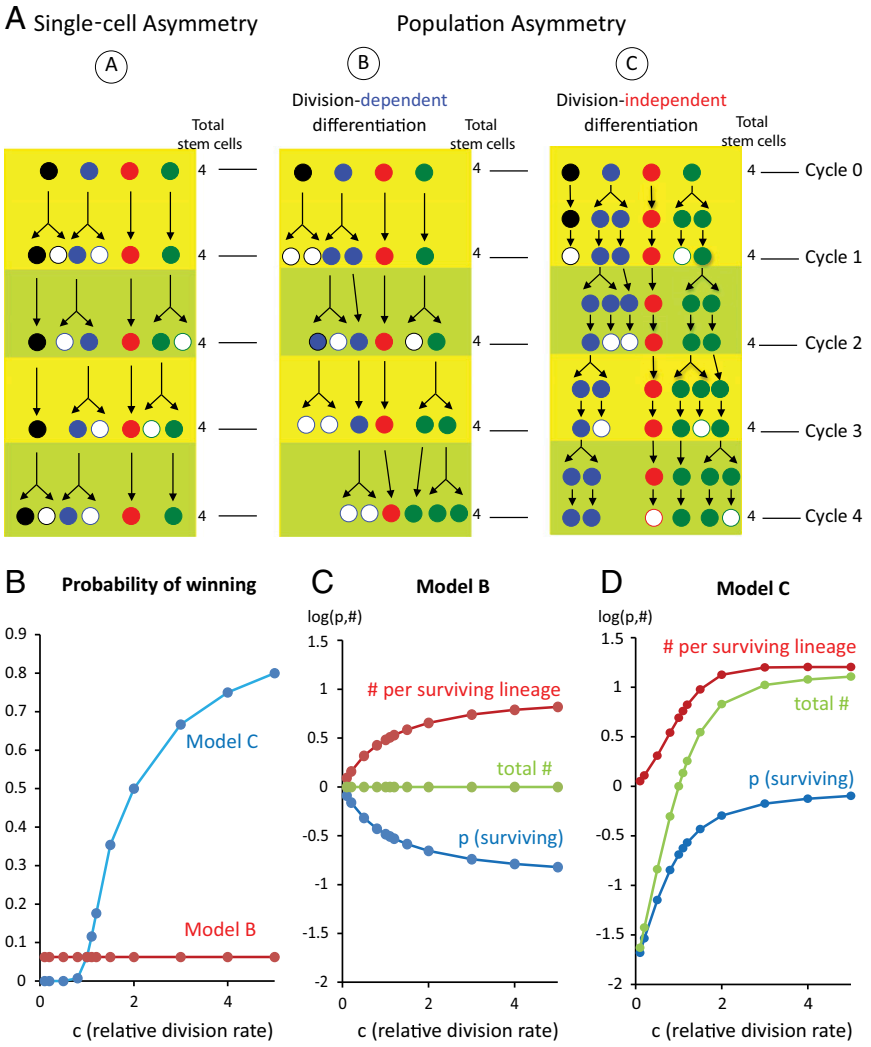


Fig. 1. Stem cell organization dictates the impact of proliferation rate on stem cell competition. (*A*) Possible set of trajectories for a population of four stem cell lineages (black, blue, red, and green) through four cycles for three different types of organization. By the end of each cycle, two of the four stem cells have divided, the total number of stem cells (filled circles) remains constant (at four), and two nonstem cells (open circles) have been produced. In model A, each stem cell division always produces one stem cell and one nonstem cell ("single-cell asymmetry"). Each lineage is maintained in equal proportion no matter what its relative rate of division (here, the blue stem cells divided four times and the red stem cells did not divide at all). Models B and C represent different mechanisms of population asymmetry. In model B, nonstem cells are only produced when a stem cell divides ("division-dependent differentiation"), but each division can produce two stem cells or two nonstem cells (with equal frequency) or one of each (also Fig. S1*A*). The relative proliferation rate of a variant stem cell does not affect its predicted competitive success (from quantitative modeling). In this example, the red stem cell survives despite failing to divide, while the blue stem cells are extinguished despite dividing more times per cycle (four of six) than even the green stem cells (three of five). In model C, nonstem cells are produced at any time, independent of division history (division-independent differentiation), and the total number of nonstem cells produced equals the total number of stem cell divisions over the whole population to maintain constant stem cell numbers ("population asymmetry") (also Fig. S1*A*). The cartoon shows an intermediate stage in each cycle to illustrate that division and differentiation are separate processes. Division is shown first, but these processes would not be rigidly ordered (and are not ordered in mathematical modeling). For model C, the relative proliferation rate of a variant stem cell has a large impact on its competitive success. In this example, the red stem cell is (by chance) relatively resistant to differentiation, remaining a stem cell for three of four cycles, but that lineage is nevertheless extinguished eventually because the red stem cell did not divide (contrast with A and B). Conversely, even though the blue stem cells became nonstem cells at almost half of the possible opportunities (three of eight), this lineage amplified because of frequent divisions (contrast with A and B, where divisions were at least as frequent). (*B–D*) Graphical representation of results from quantitative modeling (Dataset S1) of stem cell models B and C, considering in each case a population of 16 stem cells that initially includes one variant with division frequency altered by a factor of c. (*B*) Probability that the variant stem cell lineage is ultimately the sole surviving ("winning") lineage (constant at 1/16 for model B in red). The probability of survival of the variant lineage (p; blue); the expected number of stem cells in a surviving variant lineage (#; red); and, hence, (the product of p and #) the expected total number of variant stem cells present (green) for model B (*C*) and model C (*D*) are shown, on a $\log_{10}$ scale, after a fixed time interval (this would correspond to roughly 12 cycles of egg chamber budding, or 6 d, for FSCs, where roughly six FSCs divide per budding cycle; $t = 12 \times 6/16 = 4.5$) (also Fig. S1*B*).

adhesion, resistance to differentiation, and quiescence are more commonly cited as key parameters favoring longevity of various other stem cells, including *Drosophila* GSCs (9, 21). We wished to understand whether a fundamental principle of stem cell organization might explain a causal connection between proliferation and competition by using FSCs as a model stem cell.

GSCs and FSCs are housed in the germarium, which lies at the anterior of each egg-producing ovariole (Fig. 2*A*). In the anterior half of the germarium, escort cells (ECs) support the differentiation

of GSC derivatives into 16-cell cysts (22). Follicle cell precursors (FCs) then associate with germline cysts midway through the germarium and proliferate to form an expanding monolayer epithelium (23). A subset of FCs differentiates early to form polar cells and stalk cells, which allow budding of fully enveloped cysts from the posterior of the germarium to produce new egg chambers roughly every 12 h throughout the life of well-fed adult females. Until recently, it was thought that each germarium contained only two, or perhaps three, FSCs; that FSCs produced only FCs; and that the majority of FSC divisions produced one FSC and one FC (23–25). However, we recently reported that each germarium contains many more FSCs (about 14–16), that FSCs produce quiescent ECs as well as transit-amplifying FCs, and that FSCs are maintained by population asymmetry (18).

We first considered two potential mechanisms for population asymmetry (models B and C in Fig. 1*A* and Fig. S1*A*) from a theoretical perspective and used quantitative modeling to conclude that the impact of proliferation rate on stem cell competition should depend critically on whether "differentiation" (the production of a transit-amplifying cell or differentiated cell) is independent of stem cell division. We then examined FSC organization in more detail, specifically to discover whether FC production was temporally coupled to FSC division and to test rigorously the impact of mutations that altered FSC proliferation rate on FSC representation. The experimental results provide

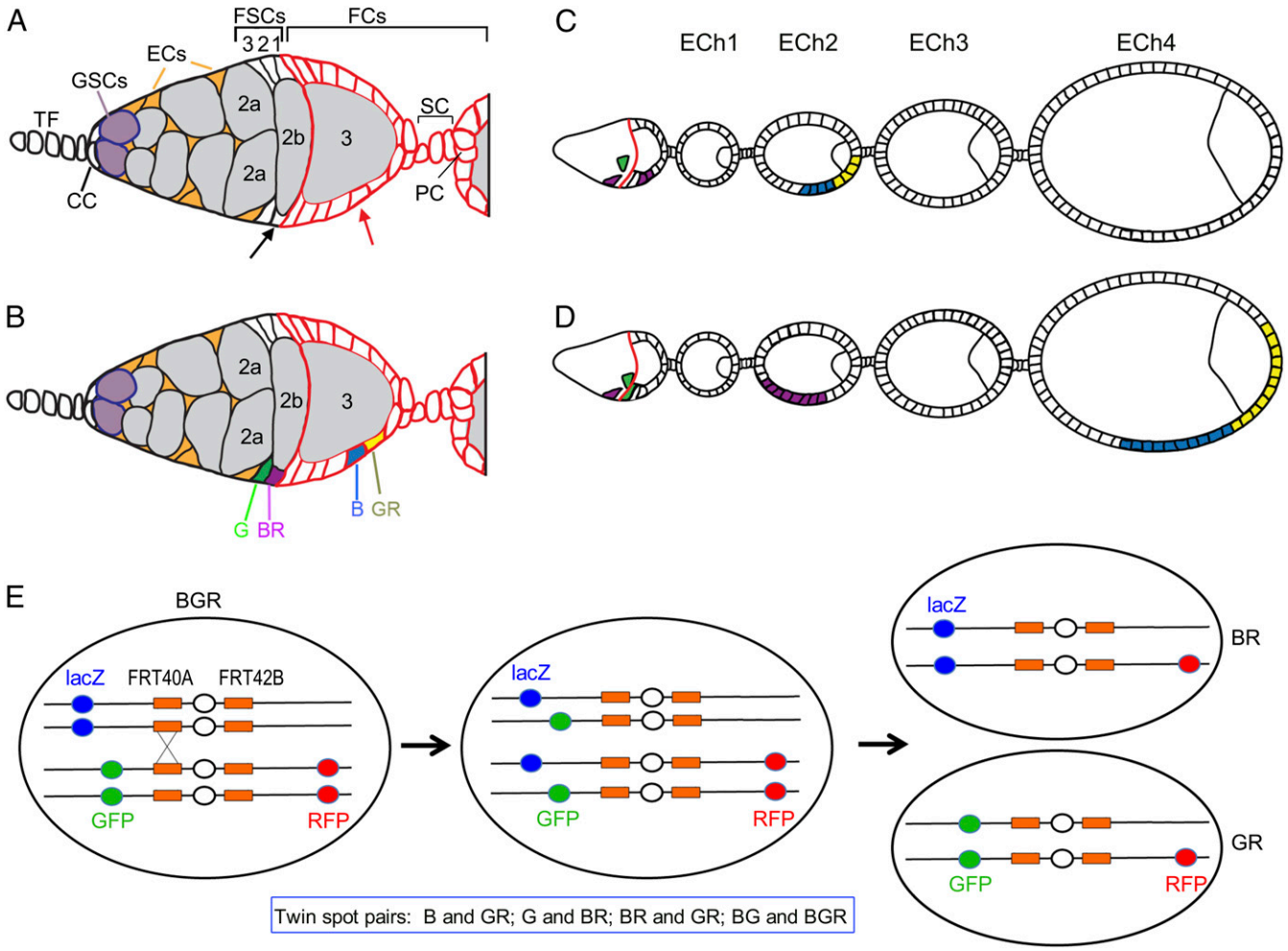


Fig. 2. *Drosophila* oogenesis and twin-spot analysis of FSC daughter fates. (*A–D*) Illustration of FSC and FC twin-spot clones. (*A*) Germarium diagram showing terminal filament (TF) cells, cap cells (CCs), GSCs, GSC daughters developing into 16-cell germline cysts (light gray), ECs (orange), FSCs, and FCs, including stalk cells (SCs) and polar cells (PCs) from anterior (left) to the newest egg chamber. Fas3 expression on FC surfaces is shown in red. The anterior limit of Fas3 staining, running along the posterior surface of a stage 2b germline cyst, provides a key landmark. FSCs lie in three layers ("3-1") immediately anterior to Fas3 but posterior to stage 2a cysts. The progression over time of the products of mitotic recombination in an FSC (black arrow) and an FC (red arrow) is illustrated. (*B*) Germarium showing twin-spot daughters immediately after recombination in an FSC (green, G; purple, BR) and in an FC (blue, B; yellow, GR). Letters indicate the presence of a given transgene (B, blue *lacZ*; G, green GFP; R, red, RFP). (*Right*) B and GR FC daughters proliferate to form patches, which are always on the same egg chamber, as it grows and moves to the posterior (right) along the ovariole two cycles (*C*, 24 h) and four cycles (*D*, 48 h) after initial marking. Egg chambers bud from the germarium roughly every 12 h. A BR FC produced in the previous cycle has divided once (*C*), leading to an FC patch on the second egg chamber (ECh2) two cycles later (*D*). Unpaired FC patches, as shown here for BR, must derive from recombination in an FSC and were never observed beyond the fourth egg chamber 72 h after heat shock. (*E*, *Left*) Starting genotype at the time of mitotic recombination is shown for the second chromosome of flies used for twin-spot lineage marking. The *tub-lacZ* (lacZ), *ubi-GFP* (GFP), and *ubi-RFP* (RFP) transgenes, as well as *FRT 40A* and *FRT 42B* recombination targets (orange) on either side of the centromere (white oval), are indicated. Heat-shock induction of an *hs-flp* transgene on the X-chromosome can induce recombination at either or both pairs of homologous FRTs (*E*, *Center*), followed by segregation to yield two daughter cells with recombinant genotypes in predictable twin-spot pairings (*E*, *Right*) (here, BR and GR daughters are produced; other possible pairings are B:GR, G:BR, and BG:BGR).

definitive evidence for the general principle that stem cell competition depends on relative proliferation rates specifically when stem cell division and differentiation are independent processes.

Results

Contrasting Impacts of Altered Proliferation for Different Population Asymmetry Mechanisms. We considered three idealized strategies for stem cell maintenance to evaluate from a theoretical standpoint how stem cell organization controls the impact of cell proliferation rates on stem cell competition. If each stem cell division produces an asymmetrical outcome (model A in Fig. 1*A*), there will be no competitive advantage or disadvantage for a stem cell that divides at a different rate. *Drosophila* ovarian GSCs appear to show this organization and indifference to stem cell division rates (12, 13).

For stem cells governed by population asymmetry, two contrasting mechanisms have not generally been explicitly distinguished experimentally or conceptually. The predicted consequences of altered proliferation are widely different for the two models. If stem cell division and differentiation are rigidly coupled (model B in Fig. 1*A*), then an individual stem cell that proliferates faster than others (blue and green stem cells in Fig. 1*A*) will have a higher chance of amplification during a fixed time interval, but it will also have a proportionally higher chance of being lost. Hence, a qualitative appraisal suggests there will be little or no net consequence on stem cell competition. In principle, the organization depicted in model B might apply to stem cells that must maintain contact with a limited niche surface to be maintained by short-range signals or by adhesion (9, 26) because cell division characteristically reduces cell contacts with neighbors and the extracellular matrix (27–29) and generates two daughters potentially competing for a space that previously supported only one stem cell (Fig. S1*A*). Such mechanisms are commonly associated with oriented cell divisions and single-cell asymmetry; however, in the absence of rigidly oriented divisions, the resulting intermediate levels of short-range signaling or adhesion for both daughters could plausibly result in the retention of zero, one, or two stem cells (Fig. S1*A*). In practice, model B has often been assumed in fitting mathematical models to the results of lineage studies, as exemplified by several studies of mammalian epidermal stem cells (30–33), and some studies suggest it applies to *Drosophila* intestinal stem cells (34–37), but there is, as yet, no definitively proven example of model B.

If stem cell division and differentiation are independent processes that are not linked mechanistically or temporally for an individual stem cell (model C in Fig. 1*A* and Fig. S1*A*), then each stem cell division initially produces two stem cells and a stem cell can differentiate at any time. Here, an increase in the proliferation rate of one stem cell relative to others will inevitably lead to a higher likelihood of amplification and a reduced likelihood of losing the variant lineage (blue stem cells in Fig. 1*A*). Conversely, stem cells that rarely divide (red lineage in Fig. 1*A*) can survive for long periods if differentiation is coupled to cell division (models A and B) but are very likely to be lost within a few cycles if differentiation is independent of stem cell division (model C) because there is a chance to differentiate at every cycle (time period). In summary, there is a strong likelihood that slower proliferating stem cells will be lost and faster proliferating stem cells will amplify only when there is division-independent differentiation (model C).

The different organizations described above for population asymmetry were translated into a quantitative model to evaluate whether an altered proliferation rate has any effect on stem cell competition in model B and to predict the magnitude of such effects in model C (Fig. S1*B* and Dataset S1). In each case, the model was constrained to maintain a constant total number of stem cells. Thus, if an extra stem cell is produced at any time (by a division producing two stem cells), this was immediately followed by stem cell loss (by a division producing two nonstem cell daughters in model B or by differentiation of one stem cell in model C), and, conversely, stem cell loss was followed by stem cell duplication. Additionally, the probabilities of a division yielding two stem cells or two nonstem cells were considered to be equal in model B. These models can be treated as classical Markov chains (details are provided in Dataset S1).

For model B, a variant stem cell with an altered division rate (by a factor c) has an unchanged probability of being the sole lineage remaining after all others have been lost (Fig. 1*B*, Fig. S1*B*, and Dataset S1). By contrast, in model C, the probability of being the winning (sole remaining) lineage increases greatly for a variant stem cell that divides faster than its competitors (Fig. 1*B* and Dataset S1). For a population of 16 stem cells, a 50% increase in proliferation rate raises the probability of indefinite survival from 1/16 to about 1/3 (more than fivefold) and reduces the expected average time to achieve that state twofold (Dataset S1).

The average number of stem cells in a lineage initiated from a single variant stem cell at any time before completion of clonal evolution (Fig. S1*B*) is predicted to be the same for all values of c in model B (green line in Fig. 1*C*). That is because changes in the average number of stem cells per surviving variant lineage are exactly offset by inverse changes in the probability that the variant lineage survives (Fig. 1*C*). By contrast, both of these parameters increase for greater c values in model C, and therefore sum to give an even larger response for the total number of variant stem cells present [1.0 (c = 1.0), 1.8 (c = 1.2), 3.5 (c = 1.5), and 6.7 (c = 2.0) expected variant stem cells; Fig. 1*D*]. In summary, quantitative modeling shows that for model B, an altered rate of proliferation has no impact on cell competition measured by the eventual winner of clonal competition or stem cell representation at earlier times, whereas altered division rates have a large effect on both measures of competition for stem cells organized as in model C.

Twin-Spot Lineage Analysis to Follow FSC Behavior over 72 h. To test the theoretical predictions connecting stem cell organization to the impact of altered proliferation on competition, which should apply to all types of stem cell, we turned to *Drosophila* FSCs. To determine whether FSC differentiation into FCs occurs only at the time of FSC division (model B) or independent of FSC division (model C), we tried to reconstruct the precise behavior of FSCs over a 72-h period through a detailed lineage analysis. Marked clones were created at a fixed time by using a heat shock-induced *flp* recombinase to promote mitotic recombination at *FRT* sites located at the base of chromosome arms harboring *GFP* ("G"), *β-galactosidase* ("B"), and *RFP* ("R") transgenes (Fig. 2*E*). We showed previously that in these flies, fewer than one in 100 ovarioles produced recombinant FSC genotypes in the absence of heat shock (18). Hence, we can be sure that virtually all recombination events occur shortly after heat shock.

Our first objective was to define the first egg chambers populated by FC derivatives of recombination in an FSC. Both FSCs and their proliferative FC progeny can undergo mitotic recombination to produce twin-spot daughters with predictable pairs of color combinations (B:GR, G:BR, BR:GR, and BG:BGR daughter pairs) (Fig. 2*E*). However, the earliest FCs are distinguished from FSCs by their association with a developing germline cyst, leading to the inevitable passage of an FC and all of its progeny through the ovariole. Recombination in an FC therefore always produces two daughters associated with the same germline cyst; those daughters will then proliferate to form paired twin-spot FC patches on the same egg chamber (illustrated for B:GR FC daughters in Fig. 2 *A–D*). By contrast, an FC patch that has no paired twin spot on the same egg chamber must have derived from recombination in an FSC (illustrated for G:BR FSC daughters in Fig. 2 *A–D*).

At 72 h after heat shock, unpaired FSC-derived FC patches were found in the fourth youngest egg chamber for more than a quarter of all ovarioles; older egg chambers always contained only paired twin-spot FC patches. We therefore deduced that the first opportunity for a marked FSC daughter to become an FC in any of the experimental ovarioles was the passage through the FSC region of a germline cyst that will become the fourth youngest egg chamber 72 h later (as seen for a BR FSC daughter in Fig. 3*D*). This deduction is consistent with the expectation that egg chambers bud from the germarium roughly every 12 h (23) and that the germarium generally contains two cysts posterior to the FSCs, leading to a maximum of six cycles of FC recruitment over 72 h (Fig. 3*D*). Egg chamber production in all ovarioles of the experimental flies was likely synchronized to within 12 h. We therefore made the conservative assumption that marked FSC daughters had the opportunity to contribute to all egg chambers

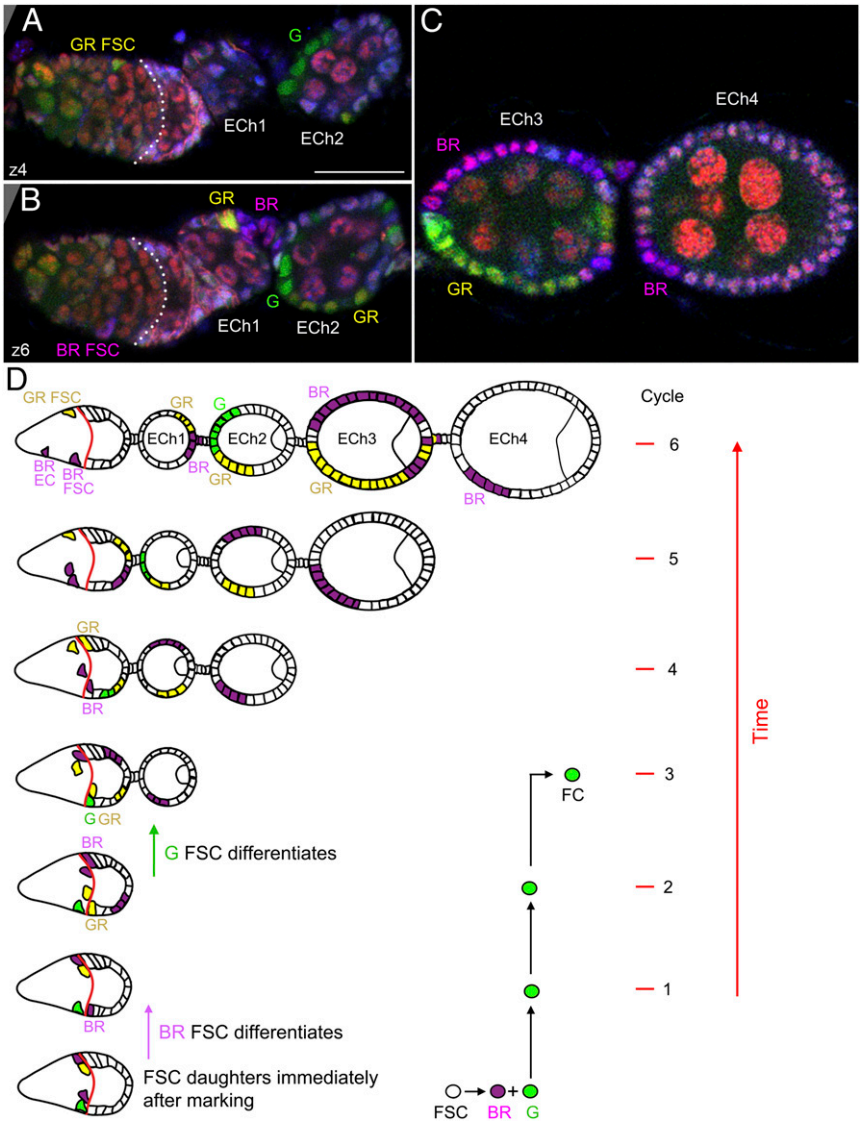


Fig. 3. Twin-spot lineage analysis to determine when an FSC daughter becomes an FC. (*A*–*D*) Analysis of FSC twin-spot clones 72 h after induction. Two z-sections of the germarium and the first two egg chambers (ECh1 and ECh2) (*A* and *B*) and ECh3 and ECh4 (*C*) of an ovariole are illustrated in the top cartoon (*D*). All z-sections were examined in each fluorescence channel to assign all cell colors definitively (Fig. S2). The anterior (left) limit of surface Fas3 staining (pink), which labels FCs, is outlined with white dotted lines. [Scale bar: 20 μm (same for *A*–*C*).] The BR (purple) FC patch in ECh4 had no matching twin-spot FCs (would be G or GR) in the same egg chamber, and must therefore derive from recombination marking in an FSC. Hence, all marked cells up to and including ECh4 derive from daughters of recombination in an FSC. Colors present up to ECh4 are G, BR, and GR, showing that one FSC produced a G:BR twin-spot pair (illustrated at bottom right of *D*) and a second FSC produced a GR:BR twin-spot daughter pair. There is only one G FC patch, no G FSC, and no G EC, implying that there were no divisions of the G daughter of the FSC after it was born (shortly after the time of heat shock). The G FC patch is in ECh2, and was therefore produced two cycles (about 24 h) after the first opportunity to become an FC (in ECh4, as for the BR FC). Thus, the G daughter of an FSC became an FC long after it was born (about 24 h, two cycles of egg chamber budding). (*D*) Inferred histories of the G:BR and BR:GR twin-spot pairs are illustrated from immediately after recombination marking (bottom) through each 12-h cycle of egg chamber budding up to the final stained ovariole at 72 h after heat shock. All FCs produced from cycles 1–5 are labeled on the cartoons at the cycle produced and in the final ovariole. To produce an FC at cycles 2, 3, and 4, the GR FSC must also divide in these cycles. One BR FSC becomes an FC in cycle 1 (the same cycle that produced the BR cell). The other BR FSC daughter contributes an FC in cycle 2, and must therefore also divide in that cycle. Thereafter, it contributes an FC in cycle 4 and an EC at an unknown time, while leaving one FSC. The BR FSC must therefore divide at least once during cycles 3 and 4, and twice in total from cycles 3–6 (shown as cycles 4 and 5 in the schematic). A second illustration of inferred histories is presented in Fig. S3.

up to the third youngest (five cycles of egg chamber budding in total) for ovarioles with no unpaired FC patches in the fourth youngest egg chamber (as in Fig. S3).

Division-Independent Differentiation of FSCs to Become FCs. For each lineage derived from mitotic recombination in an FSC, we could see how many FSCs remained after 72 h, whether any ECs had been produced, and exactly when any FCs had been produced because the order of egg chambers displays the time at which a founder FC associated with a passing germline cyst (Fig. 3*D*). We looked for examples of an FSC daughter lineage that included only a single patch of FCs, no ECs, and no FSCs. That pattern reports a daughter of mitotic recombination in an FSC that became a founder FC without any intervening divisions; it is exemplified by the G lineage in Fig. 3 (single channels shown in Fig. S2) and by the B lineage in Fig. S3 (also Dataset S2 and Fig. S4). We found 17 such examples. In six cases, the solitary FC patch was in the fourth youngest egg chamber (egg chamber 4), implying that the marked cell became an FC immediately or shortly after the FSC division where mitotic recombination occurred. In three cases, the FC patch was in the third youngest egg chamber. In these three examples, we cannot be certain if FC production was immediate or delayed by one cycle because the germline cyst that became the fourth youngest egg chamber may also have been available for population after the marked FSC daughters were born. Importantly, in eight cases (including the G lineage in Fig. 3 and the B lineage in Fig. S3), the FC patch was in egg chamber 2 or younger; moreover, an older egg chamber contained FCs from a different marked FSC derivative. In these eight cases, we can deduce that a marked FSC daughter was born shortly after heat shock, did not divide subsequently, and then became an FC only after one or more cysts had passed through the FSC region. In other words, those FSC daughters only became FCs 12–60 h after birth (Fig. 3*D* and Fig. S3*D*). We repeated the experiment, examining a new set of ovarioles 72 h after heat shock, and found a similar distribution of locations for solitary marked FC patches (22 of 35 before egg chamber 3). These observations provide direct evidence that an FSC can become an FC at any time, not just immediately after cell division. Thus, FSCs exhibit "division-independent differentiation" and conform to model C (Fig. 1*A* and Fig. S1*A*).

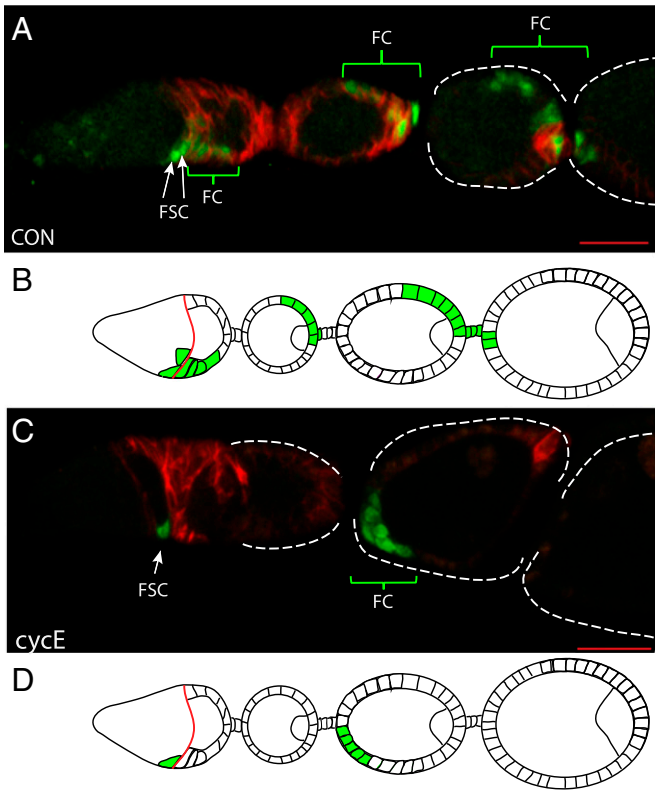
We also examined all ovarioles harboring only one pair of twin spots that likely derived from a single stem cell (Dataset S3) to determine the immediate behavior of FSC daughters. If a marked daughter produced two or more cells (ECs, FC patches, or FSCs) by 72 h, we deduced that it must have divided as an FSC before any subsequent differentiation event (GR FSC in Fig. 3 and BR FSC in Fig. S3). In nine cases, both FSC daughters (18 cells in total) divided as a stem cell before any further events; in two instances, both daughters became FCs without any intervening divisions; and in two cases, one daughter divided as a stem cell, while the other became an FC (one example) or an EC (one example) without an intervening division. Altogether, 20 FSC daughters subsequently divided as a stem cell, while five became FCs and one became an EC without dividing again as an FSC. These outcomes are consistent with a key aspect of division-independent differentiation (model C in Fig. 1*A* and Fig. S1*A*), namely, the initial production of two stem cells from all FSC divisions.

Genetic Evidence for Division-Independent Differentiation. As a further test of whether or not production of FCs is contingent on concurrent FSC division, we examined genetic conditions that reduced the rate of FSC division substantially. We previously identified loss-of-function mutations affecting the cell cycle regulator, Cyclin E (CycE), and a DNA replication component, Cutlet, as reducing FSC maintenance (12, 13). We also showed that loss of Yorkie (Yki) activity reduced FSC proliferation and FSC maintenance (20). Here, we found that the rate of FSC division, measured by 5-ethynyl-2′-deoxyuridine (EdU) incorporation 6 d after clone induction, was indeed greatly reduced relative to controls for *cycE* (17%), *cutlet* (34%), and *yki* (11%) mutant FSCs (Fig. 4*E*) marked as GFP-positive by the mosaic analysis with a repressible cell marker (MARCM) technique (38). To measure FC production, we examined all ovarioles with a marked FSC and counted the proportion of germarial cysts and egg chambers that included a marked FC patch (Fig. 4). All three proliferation-defective mutant FSC genotypes produced substantial numbers of FC patches (Fig. 4). Indeed, the proportion of cysts and egg chambers with marked FCs per marked *cycE*, *cutlet*, or *yki* FSC was 11.5% (combining all three genotypes), only marginally lower than for controls (average of 14.5%). These measures of FC production are not precise because we do not know the number of marked FSCs present throughout the measured time course of FC production. Consequently, the results show that FC production cannot be rigidly coupled to FSC division because FC production is clearly not reduced in proportion to the reduced FSC division rate (three- to ninefold), but they do not prove that FSC differentiation is entirely unaffected by *cycE*, *cutlet*, or *yki* mutations.

Reconstructing FSC Histories to Detail FSC Dynamics. We also used the detailed record of FSC behavior manifest by twin-spot clones to confirm and extend previous conclusions about FSC numbers, FSC dynamics, and FC production. In the two twin-spot experiments, the average percentage contribution of a single color to the FCs of an egg chamber for B, G, and BG clones was 18.9% (18.9% and 18.8% in the two sets of experiments), and for solitary FC patch clones (where single FC founders are almost certain), it was 17.8% (19.0% and 16.5%). Hence, our best estimate of the average number of founder FCs per egg chamber is between five and six (1/0.189 = 5.3, 1/0.178 = 5.6). We also measured the early rates of FSC loss and FSC amplification. We found that 25 of the 49 marked daughters likely arising from a single stem cell were lost (becoming FCs or ECs) over the next 3 d. This high rate of loss supports a model of population asymmetry, where individual stem cells are frequently lost or amplified in a stochastic process of neutral competition.

Finally, we derived explicit histories of FSC behavior for all marked FSC daughter lineages to calculate the average frequency of FSC divisions and the average frequency of differentiation to FCs and ECs. To facilitate modeling, and in keeping with our deduction of division-independent differentiation, we artificially split each cycle of egg chamber budding into an opportunity for all FSCs to divide, followed by an opportunity for all FSCs to become an FC or EC (as in model C of Fig. 1*A*). The stained ovarioles at 72 h showed the total number of FSCs, FC patches, and ECs produced by each lineage, as well as the cycle at which founder FCs were produced (Fig. 3 and Fig. S3). The cycles at which marked FSCs divided were either definitively compelled or highly constrained by the sequence of FC production, together with the total number of FSCs and ECs produced (legends for Fig. 3*D* and Fig. S3). Wherever FSC divisions could equally likely have occurred at either of two different cycles, assignments were made so that FSC divisions were spaced as evenly as possible.

By combining the cycle-by-cycle inferred histories of 79 lineages (illustrated and tabulated for one ovariole in Fig. S3), we found that marked FSCs divide at 44% (221 of 501) of available opportunities (each cycle represents an opportunity for each FSC) and that marked FSCs become FCs at 21% (159 of 747) of available opportunities, while producing 43 ECs over the same period (1 per 3.7 FCs) (Table 1). If each egg chamber is seeded by five founder FCs, then 1.4 (5/3.7) ECs are produced at each cycle, on average, and a total of 6.4 FSC divisions would therefore maintain homeostasis. For 6.4 divisions at an average frequency of



E	# Germaria with FSCs	Total # FSCs	Average # FSCs per germarium	Average FSC EdU index	# Cysts Examined	% Cysts with marked FC	% Cysts with marked FC per marked FSC
Control	39	205	5.3	0.28	143	57	11
cycE	30	61	2.0	0.049	105	34	16
Control	44	158	3.6	0.28	158	58	16
yki	41	64	1.9	0.031	128	18	9
Control	42	169	4.0	0.27	163	52	13
cutlet	24	75	3.1	0.093	91	29	9
Average Control	--	--	*4.2*	*0.28*	--	*56*	*13*
Average Mutant	--	--	*2.1*	*0.06*	--	*27*	*11*

Fig. 4. Proliferation-deficient FSCs still produce FCs. (*A–D*) Ovarioles with MARCM clones for control (CON) genotype (*A*) and $cycE^{WX}$ (cycE) genotype (*C*) labeled with Fas3 (red) 6 d after clone induction. Green MARCM-labeled FSCs (white arrows) are within three cell diameters to the left (anterior) of the Fas3 staining border. FC patches in the germarium or egg chambers (outlined with dashed white lines where the Fas3 staining outline is weak) are indicated by brackets. (Scale bars: 20 μm.) (*B* and *D*) Schematics of ovariole images above, showing the locations of marked FSCs and FCs. (*E*) Summary of FSC division rates (EdU index), FSC numbers, FC patches, and FC patches per FSC for mutants with reduced FSC proliferation, together with their controls.

0.44 per FSC, there must be, on average, 14.5 (6.4/0.44) FSCs at the start of a cycle. Similarly, to produce five FCs per cycle at the frequency observed (0.21 per FSC), there should be 23.8 FSCs (5/0.21) in the middle of a cycle (Table 1). At that stage, the number of FSCs is artificially inflated by 6.4 in our model because FSCs have divided, but none has become an FC or EC. So, the true estimate of the average number of FSCs based on FC production rate is 17.4 (23.8–6.4). These two estimates [14.5 and 17.4, based on FSC division and FC production frequencies, respectively (Table 1)] are in good agreement with the earlier estimate of 14–16 FSCs based on counting the number of surviving FSC lineages over time and counting the total number of cells within the FSC domain (18). Thus, our analysis of twin-spot FSC lineages has confirmed our recent conclusions about FSC numbers and FSC maintenance by population asymmetry, it has revised our best estimate of the number of FC founders per egg chamber, and it has demonstrated that differentiation of an FSC to an FC is not dependent on FSC division.

FSC Competition Is Dictated by Relative Rates of Proliferation: Experimental Evidence. Division-independent differentiation of FSCs predicts that competition among FSCs will be highly responsive to their relative rates of proliferation (Fig. 1). There is already substantial evidence that FSC proliferation rate strongly influences FSC competition (12, 13, 20). However, previous analyses of competition between FSCs was limited to measuring the loss of a marked variant FSC lineage over time and, for the rare changes that enhanced competitive success, to counting the proportion of ovarioles containing "all-marked" clones, where a single lineage contributes all FCs to several successive egg chambers (12, 20). The recent findings, confirmed here, that each germarium contains many FSCs (14–16) and that the number of FSCs in each marked lineage changes over time as a result of competition (18) allow a better measure of stem cell competition as the average number of FSCs present at a fixed time after FSC clone induction (Fig. S5).

Here, we measured FSC proliferation rates according to EdU incorporation over 1 h of in vitro incubation immediately after ovary dissection (Fig. 5 *B–D*). We measured FSC competition (how well a variant stem cell survives and amplifies within a niche containing a constant total number of stem cells) by counting the average number of marked FSCs per ovariole at 6 d and 12 d after clone induction for a variety of FSC clone genotypes expected to affect proliferation (Fig. S5). FSC clones with a homozygous mutation or expressing a *GAL4/UAS*-driven transgene were generated and marked as GFP-positive by the MARCM technique (38). These clones were compared with control FSC clones generated in the same way and strictly in parallel in flies lacking the mutation or transgene under investigation.

We found that *yki*, *cycE*, and *cutlet* mutations reduced FSC proliferation and also substantially reduced the average number of marked FSCs per germarium at 6 d and 12 d (Fig. 5 *A–C* and Fig. S6). Moreover, expression of excess CycE restored both the proliferation rate and the average number of marked *cutlet* mutant FSCs to levels above those of controls (Fig. 5*A* and Fig. S6). Conversely, excess CycE alone, loss of *hpo* (which increases Yki activity), or increased PI3-kinase pathway activity due to mutation of *PTEN* (12) increased both the proliferation rate of marked FSCs and the average number of marked FSCs per germarium (Fig. 5 *A* and *D* and Fig. S6).

It is possible that changes in the activity of Cutlet, the PI3-kinase pathway, Yki, or even CycE may have affected FSC competition by changing a property other than proliferation rate. Alterations to Wnt signaling provide a precedent for changes that affect FSC numbers by altering the likelihood of FSC differentiation. Increased Wnt signaling caused FSC loss due to excessive conversion into ECs, whereas loss of Wnt signaling increased the likelihood of conversion into FCs, which was measured by the accumulation of FSCs in the most posterior, FC-adjacent FSC layer ("layer 1") and by the proportion of FSC clones associated with FCs (18). We therefore measured these parameters for the genetic changes we used to alter FSC proliferation rates.

Genetic changes that reduced marked FSC numbers did not increase the number of marked ECs produced, the proportion of marked FSCs in layer 1, or the proportion of marked FSC clones with marked FCs, ruling out enhanced differentiation to ECs or FCs as responsible for the FSC deficit (Fig. S7). Conversely, these measures of marked EC and FC production were not decreased by alterations that increased marked FSC numbers (Fig. S7 *A* and *B*). Indeed, slower dividing FSC variants were slightly biased toward anterior layers, and the number of marked ECs present generally correlated positively with the number of

Table 1. Summary of number of marked FSC behaviors summed from inferred histories in 79 ovarioles to calculate FSC division frequency, FSC differentiation frequency, and total number of FSCs, given separate deduction of about five FCs (and hence 1.4 ECs) produced per cycle, on average

Parameter	Value	Inferred value
No. of marked FSC divisions	221	
No. of marked FSCs at start of cycle	501	
Proportion of FSCs that divide in each cycle	221/501	44%
No. of FSCs at start of each cycle to generate 6.4 new FSCs	6.4/0.44	14.5
No. of marked FC founders produced	159	
No. of marked ECs produced	43	
No. of marked FSCs at middle of cycle	747	
Proportion of FSCs that become FCs each cycle	159/747	21%
Proportion of FSCs that become ECs each cycle	43/747	
No. of FSCs at middle of each cycle to generate five FCs	5/0.21	23.8
Deduced number of FSCs at start of cycle	23.8–6.4	17.4

marked FSCs, consistent with changes in EC production simply following a primary change in the number of their progenitor FSCs. These observations fully support the conclusion that the drastic decreases (*yki*, *cycE*, and *cutlet*) or increases (*hpo*, *UAS-cycE*, and *pten*) in marked FSC numbers we observed were caused by changes in the rate of proliferation, per se, rather than by any unanticipated secondary effects on FSC location or differentiation (Fig. 5*A* and Table S1). Thus, FSCs provide robust direct evidence for a general model of organization of stem cells, namely, population asymmetry with division-independent differentiation, where

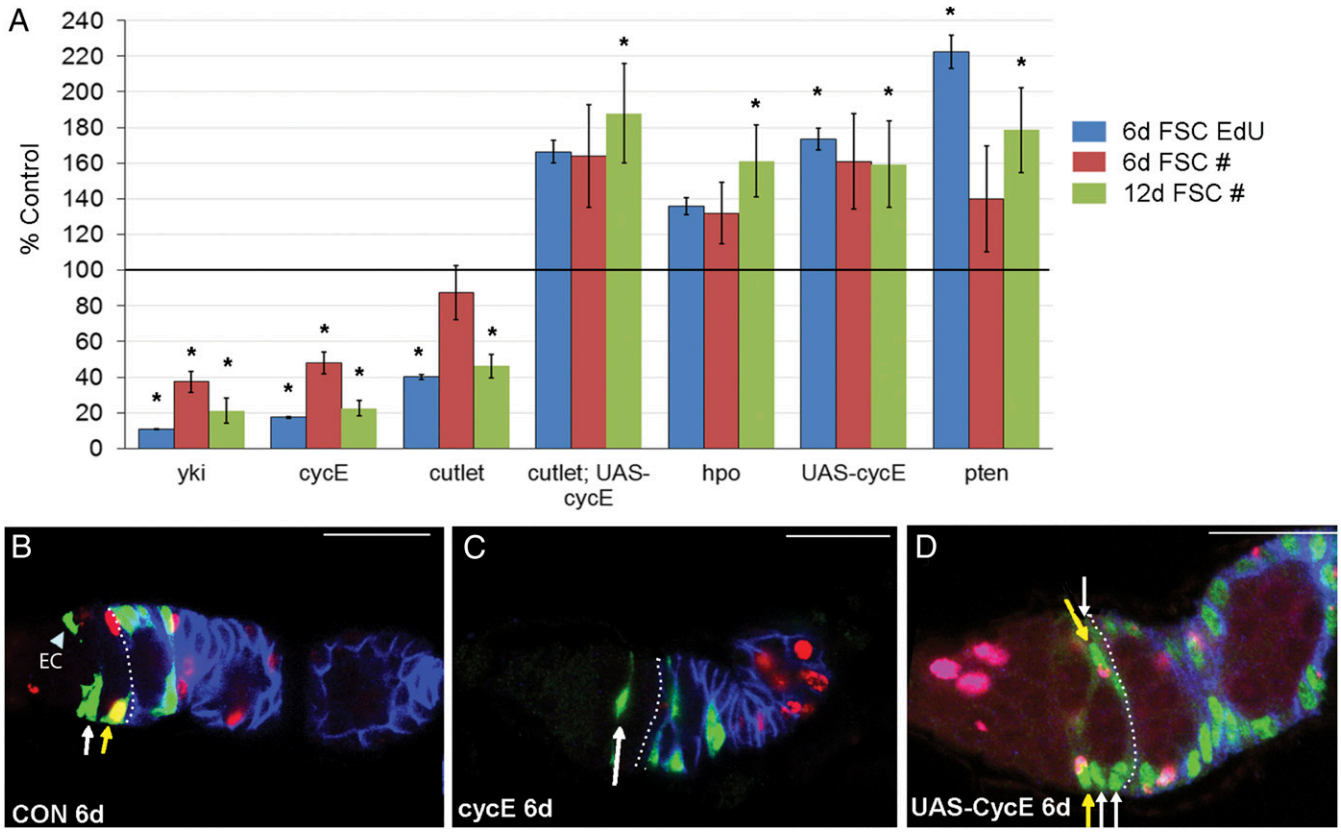


Fig. 5. FSC competition is determined by relative FSC proliferation rates. (*A*) Correlation between proliferation rate (blue, FSC EdU index) at 6 d and average number of marked FSCs per ovariole at 6 d (red) and at 12 d (green), expressed as a percentage of control values for MARCM FSC clones of the listed genotypes. Error bars show SEM [EdU: $n = 64$ (158), $n = 61$ (205), $n = 75$ (159), $n = 173$ (159), $n = 201$ (158), $n = 193$ (159), and $n = 141$ (159) FSCs in the order shown; 6-d-old FSCs: $n = 58$ (54), $n = 38$ (61), $n = 35$ (65), $n = 43$ (65), $n = 52$ (54), $n = 49$ (65), and $n = 41$ (65) ovarioles in the order shown, 12-d-old FSCs: $n = 58$ (69), $n = 53$ (55), $n = 37$ (71), $n = 56$ (71), $n = 63$ (69), $n = 56$ (71), and $n = 54$ (71) ovarioles in the order shown; values in parentheses are for the associated controls]. Significant differences to control EdU index (by Fisher's exact two-tailed test, $^*P < 0.05$) and marked FSC number (by Student's *t* test, $^*P < 0.05$) are indicated. (*B–D*) MARCM clones (marked by GFP, green) of the designated genotypes, labeled to visualize EdU incorporation (pink) and Fas3 (blue) 6 d after clone induction. FSCs are within three cell diameters of the left (anterior) border of Fas3 staining (dotted white line). ECs (arrowheads) are further anterior. All green MARCM-labeled FSCs with EdU (yellow arrows; green and pink often adjacent in the same nucleus rather than overlaid) or without EdU (white arrows) are indicated (also Fig. S6). (Scale bars: 20 μm.)

relative proliferation rate is both predicted and shown experimentally to be a key determinant of which stem cells are the most competitive.

Discussion

We have followed the behavior of individually marked FSCs in detail to show that FSC differentiation is not coupled to FSC division. This organization represents a subset of population asymmetry models and predicts that stem cell proliferation rate will be a major determinant of stem cell competition. In line with this prediction, we confirmed prior strong evidence of a causative link between proliferation rate and competition among FSCs (12, 13, 20) still more rigorously by measuring the proliferation rates and competitive outcomes for a number of genetic alterations that appear only to affect proliferation. The important general implication of these findings is that an analogous organization of any stem cell population, defined by the key characteristic of division-independent differentiation, will necessarily render those stem cells prone to cancer-promoting gatekeeper mutations that increase the rate of stem cell proliferation.

Stem Cell Dynamics Constrained by Niche Space. Stem cells generally require a specific environment to be maintained. If that requirement limits the space where stem cells can survive, then a specific stem cell lineage can only expand at the expense of others; it cannot expand independently or indefinitely (21, 39). This constraint applies to the normal FSC niche; to our theoretical modeling; and, for example, to mammalian intestinal stem cells in a single crypt. It is a key reason why only one category of stem cell organization (Fig. 1*A* and Fig. S1*A*) permits a causal connection between differential proliferation and competition.

Some mutations that alter stem cell proliferation might additionally relieve or substitute for required niche factors, and therefore allow the entire stem cell domain to expand. Those mutations could be particularly potent primary changes leading to expansion of a stem cell lineage within a single niche, or they could lead to a secondary expansion of a lineage, as in the accelerated colonization of neighboring intestinal crypts (40, 41). Those consequences would not be limited to stem cell populations exhibiting division-independent differentiation, but the effects on stem cell competition would also not be due solely to a change in stem cell proliferation rate. For FSCs, strong hyperactivation of JAK-STAT signaling appears to expand the FSC domain dramatically (42); the genetic changes studied in this work did not show any clear evidence of altering the FSC domain.

FSCs and Mammalian Intestinal Stem Cells as Archetypes of Proliferation-Dependent Competition. It has generally not been possible to follow endogenous stem cell behavior in enough detail to determine whether stem cell differentiation is coupled to cell division. The two notable exceptions before our work were live imaging studies of mammalian epidermal and intestinal stem cells. In both cases, conversion of stem cells to nonstem cells (judged by location) was seen in the absence of recent cell division (43, 44), just as we observed for FSCs. Interestingly, prior reports measured lineage products at fixed time points (rather than continuous observation) to infer a division-dependent differentiation model for epidermal stem cells, in which over 80% of division outcomes were deduced to be asymmetrical (30–32, 44). It remains to be seen whether further studies will confirm or contradict assertions of division-dependent differentiation based on discontinuous sampling of marked lineages for other stem cells, such as *Drosophila* intestinal stem cells (34–37).

Even though the most direct studies to date for *Drosophila* FSCs and mammalian epidermal and intestinal stem cells show that differentiation is largely uncoupled from stem cell division, it remains a challenge to provide definitive evidence that the two processes are entirely independent or, potentially in other cases, that differentiation is always coupled to cell division. Moreover, it is possible that some stem cells may exhibit intermediate behaviors. These uncertainties do not detract from the important concept that division-independent differentiation, in pure or hybrid form, is key for proliferation rate to alter stem cell competition.

The overall organization of FSCs and mammalian intestinal stem cells is remarkably similar. This includes the size of the stem cell population; rapid stem cell divisions; and, now, division-independent differentiation (18, 45, 46). It has also been proposed that activating mutations in the Wnt or Ras pathway that promote mammalian intestinal stem cell survival and amplification might act by promoting stem cell proliferation, although it was not explicitly tested whether other effects of those pleiotropic pathways, such as directly modulating differentiation, might be responsible (47, 48). In fact, Wnt signaling is known to affect intestinal cell locations and the nature of stem cell products, while Ras activation was also shown to increase the rate of crypt fission, effectively expanding the niche for an otherwise spatially constrained stem cell population (7, 47). Despite these reservations about experimental proof of a causal connection, we can confidently predict that intestinal stem cells must indeed exhibit a strong influence of proliferation rate on stem cell competition specifically because they undergo division-independent differentiation. This connection was not previously highlighted as causative or fundamental (43). Our study of FSCs explicitly spells out this important, universally applicable connection and provides robust experimental evidence of causality between stem cell proliferation rates and stem cell competition, as described below.

Previously, the major niche signal, Hedgehog (Hh), was shown to regulate FSC competition principally by transcriptionally inducing the coactivator Yki, and Yki was shown to act by inducing CycE to induce an increased rate of stem cell division (20). Here, we showed that alteration of Yki activity and additional manipulations of factors expected to alter only proliferation (CycE and Cutlet), as well as changes to PI3-kinase activity, produced corresponding changes in FSC proliferation rate and FSC numbers: fewer FSCs in response to reduced proliferation and more FSCs when proliferation rates were higher. Moreover, other potential causes of the observed changes in FSC numbers (FSC location and the rate of conversion of FSCs to ECs or FCs) were ruled out by directly measuring these parameters. Hence, the cumulative experimental evidence linking stem cell proliferation rate to competition is currently stronger for FSCs than for any other stem cell (49). Moreover, the consequences of activating mutations in the Hh or Hpo/Yki pathway in FSCs provide a clear paradigm for how a gatekeeper mutation affecting a signaling pathway that controls stem cell proliferation can lead to precancerous amplification of an affected stem cell (20, 50).

Proliferation-Dependent Competition and Stem Cell Exhaustion: Different Time Scales or Stem Cells? Our studies concern a relatively short time frame that is plausibly relevant for the amplification of a stem cell harboring a primary mutation that could eventually lead to cancer. Some mutations that increase proliferation and lead to stem cell amplification in the short term might also eventually have a deleterious effect on stem cell survival, perhaps because of DNA damage from excessively fast or incessant replication. The latter possibility, sometimes termed "stem cell exhaustion," is often cited for hematopoietic stem cells (HSCs) and provides an attractive general rationale for minimizing the normal replicative duties of at least a subset of stem cells, as observed experimentally for HSCs (51–53). Intestinal crypts also contain relatively quiescent stem cells that can replace the population of actively dividing stem cells in emergency situations (45). We have also observed spatial heterogeneity of proliferation rates among normal FSCs in a germarium, and it is not yet known whether quiescent ECs might become FSCs under normal or stress conditions (18).

For HSCs, many, but not all, genetic changes that increased proliferation rate led to a long-term reduction in HSC potency measured by a transplantation assay, while HSC function over the short term and under physiological conditions was not measured (51, 52, 54). The organization of HSC niches and HSC dynamics are also not sufficiently well understood at present to know whether differentiation depends on stem cell division. Consequently, the relevance of the concepts discussed in this work to normal HSCs and early steps in blood cancers is not excluded by earlier conclusions of proliferative stem cell exhaustion and remains to be explored. Conversely, while further studies are warranted, we are not aware of significant evidence for proliferative exhaustion of FSCs or mammalian intestinal stem cells (15, 16, 45). Instead, over the time scales discussed in this work, we have observed only a robust positive, causal impact of proliferation rate on stem cell competition that can be attributed to a key attribute of organization of those stem cells, namely, division-independent differentiation.

Methods

Multicolor twin-spot lineage analysis, image acquisition and processing, MARCM lineage analysis of mutant genotypes, EdU labeling, and immunohistochemistry are described in *SI Methods*.

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