Cellular Analyses of the Mitotic Region in the *Caenorhabditis elegans* Adult Germ Line

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The *Caenorhabditis elegans* germ line provides a model for understanding how signaling from a stem cell niche promotes continued mitotic divisions at the expense of differentiation. Here we report cellular analyses designed to identify germline stem cells within the germline mitotic region of adult hermaphrodites. Our results support several conclusions. First, all germ cells within the mitotic region are actively cycling, as visualized by bromodeoxyuridine (BrdU) labeling. No quiescent cells were found. Second, germ cells in the mitotic region lose BrdU label uniformly, either by movement of labeled cells into the meiotic region or by dilution, probably due to replication. No label-retaining cells were found in the mitotic region. Third, the distal tip cell niche extends processes that nearly encircle adjacent germ cells, a phenomenon that is likely to anchor the distal-most germ cells within the niche. Fourth, germline mitoses are not oriented reproducibly, even within the immediate confines of the niche. We propose that germ cells in the distal-most rows of the mitotic region serve as stem cells and more proximal germ cells embark on the path to differentiation. We also propose that *C. elegans* adult germline stem cells are maintained by proximity to the niche rather than by programmed asymmetric divisions.

INTRODUCTION

Stem cells are responsible for generating tissues during development and maintaining them during adulthood. To accomplish these tasks, stem cells must produce additional stem cells (self-renewal) as well as differentiated cells. Over the past few years, considerable progress has been made in the analysis of individual stem cells in several organisms, including hematopoietic stem cells (HSC) in mammals (Kiel *et al.*, 2005; Shizuru *et al.*, 2005) and germline stem cells (GSC) in *Drosophila* (Wong *et al.*, 2005). In this article, we investigate the germline mitotic region in the *Caenorhabditis elegans* adult hermaphrodite, which contains GSC. Parallels between *C. elegans* GSC and other stem cell systems include use of Notch signaling to control both HSC and *C. elegans* GSC (Calvi *et al.*, 2003; Kimble and Crittenden, 2005) and use of Puf proteins to control both *Drosophila* and *C. elegans* GSC (Wickens *et al.*, 2002).

C. elegans adult GSC are found at the distal end of the gonadal arm within the “mitotic region,” which is defined by the presence of mitotically dividing germ cells (see Figure 1, A and B). In adults, the single somatic distal tip cell (DTC) is located at the tip of the mitotic region and forms a stem cell niche (Kimble and White, 1981). The distal sheath cells are important for larval germline proliferation (Killian and Hubbard, 2005), but they have little or no contact with the mitotic region in adults (Hall *et al.*, 1999; Killian and Hubbard, 2005). The DTC and the mitotic germline cells are encapsulated by a thin extracellular matrix, which sepa-

rates them from neighboring organs (e.g., intestine; Hall *et al.*, 1999; Lints and Hall, 2004). Proximal to the mitotic region, the “transition zone” contains germ cells in early phases of meiotic prophase (e.g., leptotene and zygotene) and the proximal arm contains maturing gametes (see Figure 1A).

Germline mitotic divisions have been characterized in embryos and young larvae. A single germline precursor cell arises in the early embryo by a series of invariant asymmetric divisions, and that precursor then divides once during embryogenesis (Sulston *et al.*, 1983). The number of germ cells expands during larval development from 2 to ~2000 germ cells in adult hermaphrodites (Hirsh *et al.*, 1976; see Figure 1C). Detailed analyses of early larval divisions revealed variable cleavage planes and daughter cell positions, with individual cells having equivalent size, morphology, and developmental potential (Kimble and White, 1981). Therefore, at least during early larval development, germline stem cells do not rely on programmed asymmetric divisions.

Considerable progress has been made in teasing apart the molecular network of regulators that permit the somatic DTC to maintain the mitotic region and prevent differentiation (reviewed in Kimble and Crittenden, 2005). The DTC uses Notch signaling to maintain mitotic divisions in the germ line and to prevent differentiation. Indeed, Notch signaling is crucial for maintaining the constant overall size of the germ line in adults (Austin and Kimble, 1987). RNA regulators, including FBF and GLD-1, act downstream of Notch signaling to control the balance between proliferation and differentiation as sperm or oocyte. Therefore, the molecular regulation of the *C. elegans* germline mitotic region has become increasingly well defined, but less had been known about its cell biology. Recently, an analysis of the rate and position of adult germline mitoses showed that germ

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cells adjacent to the DTC have a lower mitotic index than more proximal germ cells (Maciejowski *et al.*, 2006). Furthermore, divisions tended to cluster both spatially and temporally (Maciejowski *et al.*, 2006).

In this article, we report the use of bromodeoxyuridine (BrdU) labeling to demonstrate that all germ cells within the mitotic region are actively cycling. We detect no quiescent cells, label-retaining cells, or invariantly oriented cell divisions. We find that a series of short processes of the DTC niche embrace the distal-most germ cells and suggest that these germ cells are anchored within the niche. Finally, we propose that *C. elegans* germline stem cells are maintained by proximity to the niche rather than by programmed asymmetric divisions.

MATERIALS AND METHODS

Nematode Strains

All strains were maintained at 20°C as described (Brenner, 1974). We used the wild-type Bristol strain N2 as well as the following mutants: *LG I: gls-1(q485)* (Jones and Schedl, 1995); *LG II: fbf-1(ok91)* (Crittenden *et al.*, 2002), *fbf-2(q738)* (Lamont *et al.*, 2004), *gld-2(q497)* (Kadyk and Kimble, 1998), *gld-3(q730)* (Eckmann *et al.*, 2002). Transgenes included the *lag-2::GFP* (*gls56*) transcriptional reporter (Bleloch *et al.*, 1999), the *lag-2::LAG-2::MYC* (*qEx318*) translational reporter (this work), and *pie-1::TUBULIN::GFP* (AZ244, Vida Praitis, personal communication). *lag-2::LAG-2::MYC* fuses one MYC tag to the LAG-2 C-terminus and uses the same promoter as *lag-2::GFP*. Animals were staged as mid-to-late L4s and grown for a given number of days before scoring, transferring to new plates if necessary to prevent starvation.

Determination of the MR/TZ Boundary

The MR/TZ boundary was defined as the distal-most row of cells containing multiple nuclei with crescent-shaped DAPI morphology, which is typical of leptotene/zygotene of meiotic prophase I (Francis *et al.*, 1995; Dernburg *et al.*, 1998). A curved metaphase plate is morphologically similar to a nucleus with crescent-shaped DAPI morphology, making the use of a single such nucleus difficult to use for defining the MR/TZ boundary (see also Hansen *et al.*, 2004a).

Cell Number Counts

Cell numbers within specific regions were obtained by first marking the MR/TZ or TZ/pachytene boundaries with microscope cross hairs, and then counting nuclei focal plane by focal plane through the width of the germ line. For some germ lines 3–6 d after L4, germ cell number in the pachytene region was estimated by counting rows and multiplying by typical number of cells/row for that region.

BrdU Labeling

To label *Escherichia coli* with BrdU, a thymidine-deficient *E. coli* strain, MG1693 (from *E. coli* stock center), was grown overnight in M9 with 0.4% glucose, 1 mM MgSO₄, 1.25 µg/ml vitamin B1, 0.5 µM thymidine, and 10 µM BrdU (Ito and McGhee, 1987). To label *C. elegans* with BrdU, hermaphrodites were placed on M9-agar plates seeded with labeled *E. coli* and containing 100 µg/ml ampicillin for varying amounts of time depending on the specific experiment. For chase experiments, worms were transferred to plates containing unlabeled *E. coli* (OP50). Germ lines were dissected, fixed, and stained with anti-BrdU antibodies (B44, Becton-Dickinson, San Jose, CA) and DNA dye TO-PRO-3 (Molecular Probes, Eugene, OR). Z-series of double-labeled germ lines were obtained with a Bio-Rad MRC 1024 confocal microscope (Hercules, CA) and imported into ImageJ v. 1.33 (<http://rsb.info.nih.gov/ij/>). In each z-section, both BrdU-positive and total TO-PRO-3-positive nuclei were counted within a series of outlined areas of two cell diameter widths along the distal-proximal axis, extending to the most proximal BrdU-positive nucleus. Nuclei possessing BrdU staining that colocalized with DNA were scored positive. Such colocalized areas were larger than random specks of staining seen in negative controls (compare Figure 4C inset and Figure 5F to negative control in Figure 5H). Labeling index may also include a small contribution from DNA repair (Pang *et al.*, 2003; Menu dit Huart *et al.*, 2004). Confidence intervals at 95% were determined, and data were graphed using Microsoft Excel (Redmond, WA).

Our BrdU treatments do not appear to have dramatic effects on germline development. We did not see aberrant mitotic arrest, and we did see mitotic figures and PH3-positive nuclei even after long BrdU treatments. In addition, BrdU-labeled nuclei were seen in mature oocytes and embryos, indicating that BrdU did not interfere with oogenesis (Figure 5G and unpublished data).

Mitotic Index

To determine the mitotic index, the number and position of PH3-positive nuclei were scored. The number of PH3-positive nuclei at each position was divided by the average number of germ cells (obtained in separate experiments) at each position along the distal-proximal axis. Confidence intervals at 95% were determined, and data were graphed using Microsoft Excel.

Antibodies and Immunocytochemistry

For anti-GFP (Clontech, Palo Alto, CA), anti-MYC (Roche, Indianapolis, IN), and anti-PH3 (Upstate Biotechnology, Lake Placid, NY) antibodies, germ lines were extruded and fixed in 1% paraformaldehyde for 10 min at room temperature (~22°C) followed by incubation with 0.1% Triton X-100 for 5 min at room temperature. For anti- α -tubulin (Sigma, St. Louis, MO), germ lines were extruded and fixed either in MeOH for 5 min followed by 1% paraformaldehyde for 25 min at room temperature or in -20°C MeOH followed by -20°C acetone for 5 min each (Crittenden and Kimble, 2006). After blocking, fixed germ lines were incubated with antibodies overnight at 4°C.

For double-labeling with anti-BrdU and anti-PH3, germ lines were fixed in -20°C MeOH 2 h to overnight, blocked, and incubated with anti-PH3 overnight at 4°C, followed by postfixation with 1% paraformaldehyde for 15 min at room temperature. After washing, germ lines were treated with 2 N HCl for 15 min at room temperature to denature DNA and expose the BrdU epitope. Germ lines were then neutralized with 0.1 M borate buffer for 15 min at room temperature followed by blocking in phosphate-buffered saline containing 0.5% BSA (modified from Newmark and Sanchez Alvarado, 2000). Anti-BrdU antibodies were used 1:2.5. Fixed germ lines were also stained with DAPI and TO-PRO-3 to visualize DNA. Images were acquired on a Bio-Rad MRC 1024 confocal microscope and processed in Image J and Adobe Photoshop (San Jose, CA).

Scoring DTC Processes

DTC processes were scored in fixed germ lines extruded from worms carrying either *gls56* (*lag-2::GFP*) or *qEx318* (*lag-2::LAG-2::MYC*) and stained with either anti-GFP or anti-MYC. The “extent of cap” was measured as the most proximal point where the germ line had extensive contact with the DTC or its processes. The “extent of longest process” refers to the most proximal point at which a continuous process reached along the germ line. Images are projected confocal z-series taken on a Bio-Rad 1024 confocal microscope. We also examined individual focal planes to look more closely at DTC morphology and interaction with germ cells.

Scoring Orientation of Divisions

Division orientations were scored using either tubulin staining or DAPI in wild-type or *pie-1::TUBULIN::GFP* germ lines extruded from adults 24 h after L4. Images were obtained on a Zeiss Axioskop (Thornwood, NY) with a Hamamatsu Orca digital camera (Bridgewater, NJ) using Improvision Open-labs software (Lexington, MA). Images were processed in Adobe Photoshop.

RESULTS

Germ Cell Numbers Maintained in Adults

To ask whether the number of germ cells is stable during adulthood, we counted germline nuclei in DAPI-stained animals on 6 consecutive days of adulthood, beginning with young adults 1 d past the fourth larval stage (L4). Although germ cells are continuously lost during this interval to gametogenesis and cell death (Gumienny *et al.*, 1999), the total number of germ cells was essentially constant: day 1, 919 $\pm$ 96 (n = 8); day 2, 869 $\pm$ 72 (n = 11); day 3, 1140 $\pm$ 127 (n = 5); day 4, 892 $\pm$ 91 (n = 6); day 5, 1013 $\pm$ 102 (n = 4); and day 6, 1038 $\pm$ 113 (n = 4). Therefore, the germ line must be continuously replenished by stem cells. The number of germ cells within the mitotic region was also essentially constant during the same interval: day 1, 243 $\pm$ 25 (n = 8); day 2, 232 $\pm$ 22 (n = 11); day 3, 227 $\pm$ 16 (n = 5); day 4, 222 $\pm$ 35 (n = 15); day 5, 214 $\pm$ 34 (n = 9); and day 6, 238 $\pm$ 12 (n = 4). Cells within the mitotic region must self-renew as they are not depleted during this period of germline maintenance. Together, these results confirm the existence of stem cells in the adult germ line.

S-Phase and M-Phase Indices in the Mitotic Region

In certain vertebrate tissues and in plant meristems, stem cells appear to be slow cycling or quiescent (Zhang *et al.*,

2003; Passegue *et al.*, 2005; Stahl and Simon, 2005). To begin to explore the idea that similar cells might exist within the *C. elegans* germline mitotic region, we first used BrdU labeling to detect nuclei in S-phase, a common marker of progression through the cell cycle. Our first BrdU experiment assessed S-phase index (also called labeling index), which refers to the percentage of nuclei in S-phase. Specifically, we exposed young adult hermaphrodites (24 h past the L4 stage) to BrdU for 15 min, prepared their germ lines without any appreciable chase, and then scored the number of S-phase nuclei using BrdU-specific antibodies and total number of nuclei with the TO-PRO-3 DNA dye. Each nucleus was assigned a position according to its distance from the DTC, measured in cell diameters along the distal-proximal axis (Figure 1B, bottom). For example, all germ cells directly adjacent to the DTC were assigned to row 1 and so forth (Figure 1B). We counted BrdU-positive and TO-PRO-3-positive nuclei in 2-row intervals and then graphed the percentage of nuclei in S-phase with respect to position (Figure 2A). In rows 1–16, ~50% of nuclei were labeled, demonstrating that about half of the nuclei were in S-phase at any given time (Figures 2A and 4A). Because nuclei in row 1 were of particular interest, being located immediately adjacent to the DTC, we also scored row 1 on its own and found it to have a BrdU-labeling index similar to that of rows 1 and 2 combined (40 ± 16 vs. $43 \pm 15\%$, $p = 0.72$). The average labeling index in rows 1 and 2 was not significantly different from the average of rows 3–10 (43 ± 15 vs. $55 \pm 6\%$, $p = 0.13$); however, the labeling index was more variable in rows 1 and 2 (8–71%, $n = 12$) than in rows 3–10 (38–76%, $n = 12$). More proximally (rows 11–20), the labeling index decreased until no BrdU-labeled nuclei were seen after row 20 (Figure 2A).

As an alternate measure of mitotic cycling, we examined the percentage of nuclei in M-phase (mitotic index). To this end, we stained M-phase nuclei with anti-PH3 antibodies (Hendzel *et al.*, 1997) and all nuclei with DAPI or TO-PRO-3 DNA dyes. The mitotic index was $\sim 3.5 \pm 1\%$ in rows 1–16, $\sim 0.4 \pm 0.3\%$ in rows 17–23 and 0% in more proximal rows ($n = 102$; Figure 2B). The mitotic index of rows 3–10 appeared somewhat higher than rows 1–2 or rows 10–16 (4.3% in rows 3–10 vs. 2.9% in rows 1–2, $p = 0.04$; and 2.7% in rows 11–16; $p = 4 \times 10^{-5}$). These experiments can be interpreted to indicate that either the cell cycle is ~1.5 times longer or M-phase ~1.5 times shorter in rows 1 and 2 (see below for additional data). A lower mitotic index in the distal-most germ cells has also been reported with a larger data set (Maciejowski *et al.*, 2006).

The S- and M-phase indices in Figure 2 were graphed with respect to distance from the DTC. We next graphed the same data sets relative to the boundary between mitotic region and transition zone (MR/TZ). These alternative graphs were done because the position of the MR/TZ boundary varies from germline to germline (Figure 3A). We assigned negative numbers to rows distal to the boundary and positive numbers to rows proximal to the boundary (Figure 3, B and C). Labeling index was ~50% in the six rows of germ cells distal to the MR/TZ boundary, dropped to ~25% in the first two rows of the transition zone, and continued to decrease in the next 4 rows (Figure 3B). By contrast, the mitotic index dropped in the 3 to 4 rows just distal of the transition zone and remained low 3 rows into the transition zone (Figure 3C). We conclude that the S-phase index is equivalent throughout the mitotic region (about half the nuclei are in S-phase at any position) but that the M-phase index drops dramatically in the proximal-most rows of the mitotic region. The most likely explanation is that many of the S-phase

Figure 1. Background on *C. elegans* hermaphrodite germ line. (A) Schematic of adult hermaphrodite germ line. Somatic DTC, red; mitotic region, yellow; pachytene region, green; oocytes, pink; sperm, blue. Somatic tissues other than DTC are omitted for simplicity. (B) The adult mitotic region (MR). Above, extruded gonad stained with actin antibodies to outline “cell” boundaries. Below, diagram of actin-stained gonad, color-coded as in A. Transition zone, TZ. The germline MR extends from the distal end (arrowhead) to the distal edge of the transition zone (dashed line). Most germ “cells” are located peripherally and are connected by a cytoplasmic bridge to a cytoplasmic core running the length of the germline tissue (asterisk; Hirsh *et al.*, 1976); the germ cells are technically part of a syncytium, but are referred to as “cells” because they are partially enclosed by membranes and because mitoses are not synchronized. Germ cell position is determined by counting cell diameters along the distal-proximal axis (D/P axis, numbers shown above lower diagram). The transition zone is dominated by meiotic prophase nuclei (Crittenden *et al.*, 1994; Dernburg *et al.*, 1998; Hansen *et al.*, 2004a). We define the boundary between the MR and TZ by the point at which multiple germ cells acquire the crescent-shaped DAPI staining within their nuclei that is typical of early meiotic prophase (Eckmann *et al.*, 2004). (C) Postembryonic germline development. Animals are outlined in gray; color coding as in A. L1–L4, first to fourth larval stage. Adult (24 or 96 h), adult 24 or 96 h past mid-L4; #GC, total number of germ cells. Approximate germ cell number for each developmental stage is listed at right.

germ cells in the few rows distal to the MR/TZ boundary, as well as those in the transition zone, are in premeiotic S-phase. Thus the transition from the mitotic cell cycle to the meiotic cell cycle occurs over multiple cell diameters (see also Hansen *et al.*, 2004a).

Figure 2. S- and M-phase indices scored in hermaphrodites 24 h past L4. Solid line indicates region of mitotic cell cycle in all germ lines. Dotted line spans positions of MR/TZ boundaries in all germ lines scored. Dashed line indicates region of meiotic prophase in all germ lines. (A) Labeling-index. % BrdU-positive cells at positions along the distal-proximal axis. Animals were fed BrdU for 15 min with no chase. Bars, 95% confidence limit. Data from 12 germ lines. (B) Mitotic index. Percent PH3+ cells at positions along the distal-proximal axis. Data from 102 germ lines.

No Quiescent Germline Nuclei in the Mitotic Region

To ask whether *all* germline nuclei within the mitotic region could be labeled with sufficiently long exposure, we exposed adults to BrdU for increasing time. We began BrdU treatment at 24 h past the L4 stage, as done in the previous experiment, but extended the exposure by 2-h intervals. Because the 4 rows preceding the MR/TZ boundary had a lower mitotic index and are likely to be in premeiotic S-phase (see above), we focused on the distal 16 rows for this experiment, which we refer to as the high mitotic index region. Within that high mitotic index region, the percentage of BrdU-labeled nuclei increased progressively from ~50% after 15 min (Figure 4A), to ~75% after 4 h (Figure 4B), to 100% after 8–12 h (Figure 4C) of BrdU treatment (Figure 4D). After 8 h, three of eight germ lines possessed 100% labeled germline nuclei, although some nuclei were only partially labeled (Figure 4C, inset), which we interpret as having spent less time in S-phase. After 12 h, all germ lines had 100% BrdU-positive nuclei ($n = 4$). Therefore, within an 8–12-h period, all cells within the high mitotic index region had entered or progressed through S-phase.

To ask whether any difference in complete labeling time could be discerned between germ cells at distinct locations within the high mitotic index region, we graphed the increase in S-phase index with increased BrdU exposure times for each of three positions along the proximal-distal axis (Figure 4E). All had 100% BrdU-positive nuclei within an

8–12 h period (Figure 4E). Indeed, the time course for rows 1 and 2 was similar to that for rows 3–16. We conclude that no quiescent nuclei are present in the mitotic region and that cell cycles are similar throughout the region.

Estimate of Cell Cycle Length in the Mitotic Region

The data in Figures 2A and 4D permit an estimate of the average cell cycle length for germ cells within the mitotic region. S-phase appears to take roughly half of the cell cycle, because at any one time, about half the germ cells take up BrdU. Furthermore, the interval spanning G1, G2, and M can be estimated from the 8–12 h necessary to obtain >99% BrdU incorporation (Aherne *et al.*, 1977). Taking these data together, we estimate the length of the mitotic cell cycle to be 16–24 h for germ cells in the high mitotic index region of the adult hermaphrodite germ line. This timing contrasts with cell cycle length during the proliferative phase of germline development, which averages ~4 h (Kipreos *et al.*, 1996).

No Label-retaining Cells in the Germline Mitotic Region

In some systems, stem cells are defined by their ability to retain BrdU label after a long chase (Braun and Watt, 2004; Fuchs *et al.*, 2004; Potten, 2004). To learn whether the *C. elegans* adult germ line might contain such “label-retaining” cells, we exposed animals at varying stages to extensive BrdU pulses (e.g., 24–48 h), which were followed with increasingly long BrdU-free chases (Figure 5A). For each ex-

Figure 3. Labeling and mitotic indices normalized to MR/TZ boundary from data sets in Figure 2. (A) Percent germ lines with MR/TZ boundary at a given position. (B and C) Labeling index and mitotic index were calculated with respect to the first row of the transition zone instead of distance from the DTC. Rows in transition zone and proximal end are labeled 1, 2, etc., where the first row of the transition zone is 1. Rows distal to transition zone are labeled -1, -2 etc. (B) Labeling index normalized to MR/TZ boundary. (C) Mitotic index normalized to MR/TZ boundary. Data from 66 germ lines.

periment, the level of labeling decreased uniformly from all cells within the germline mitotic region (Figure 5B). Figure 5, C–G, shows a representative set of germ lines, taken from animals whose labeling regimen began when they were between the L2 and L3 stages and ended 36–48 h later, when they became adults 24 h past the L4 stage. BrdU was therefore incorporated during the larval proliferative phase of germline development, when the cell cycle averages ~4 h in length (Kipreos *et al.*, 1996). Immediately after the pulse (0-h chase), all germline nuclei in both mitotic and meiotic regions were fully labeled, including oocytes (unpublished data). After a 12-h chase, BrdU staining had decreased within the mitotic region, although all nuclei remained labeled (Figure 5D). After 24-, 36-, and 48-h chases, BrdU staining decreased more and more, but speckles remained associated with nuclei in the mitotic region (Figure 5, E and F, unpublished data). As a control, we examined germ lines from animals that had not been treated with BrdU and found small background speckles that did not colocalize with nuclei (Figure 5H).

Figure 4. Active cycling in the mitotic region. (A–C) Single confocal sections of germ lines extruded after BrdU pulses of increasing times. Pink, anti-BrdU; blue, TO-PRO-3 DNA dye; dashed line, mitotic region/transition zone boundary, arrowheads, distal end. (A) 15-min BrdU pulse. BrdU labels nuclei in both mitotic region left of dashed line and transition zone to its right. Nuclei with crescent-shaped DAPI staining are BrdU-negative and are likely to be in meiotic prophase; nuclei whose DAPI staining appears round are BrdU-positive and are likely to be in premeiotic S-phase (white arrowheads). (B) 4-h BrdU pulse. (C) 8-h BrdU pulse. All mitotic germ cells contain some BrdU label. Inset, boxed region. Lightly labeled germ cell in box (white arrowhead) is shown in a different focal plane in inset (white arrowhead). (D) Percent BrdU-positive nuclei in the high mitotic index region at increasing times of BrdU treatment. Each circle represents one germ line; red filled circles represent germ lines in which all germ cells show some BrdU labeling. Red lines indicate mean. This data represents all except the proximal 4 rows of the mitotic region; more proximal rows frequently contained several unlabeled nuclei even after 12 h, but most (>95%) were labeled. (E) Percent BrdU-positive nuclei at different positions along the distal-proximal axis. Each line represents data from all nuclei in a 2-row interval along the distal-proximal axis. Time course for complete incorporation is similar at these positions.

The progressive reduction of BrdU staining in the mitotic region appeared essentially uniform along the distal-proximal axis, but BrdU staining remained high in meiotic nuclei. We suggest that BrdU loss from the mitotic germ line results from at least two factors: dilution by replication in the absence of BrdU and movement of germ cells from the mitotic region into meiotic zones (Figure 5, E–G; see below). The speckles that remained associated with nuclei in the mitotic region even after a 48-h chase are likely to represent chro-

Figure 5. No label-retaining cells in germline mitotic region. (A) Summary of BrdU pulse-chase experiments. Left, developmental stage indicated, including either hours after hatching (top) or hours after L4 (bottom). Red bar, interval feeding BrdU-labeled bacteria; blue bar, interval feeding unlabeled bacteria; *, experiments in C–G are examples from these time points. (B) Percent BrdU-positive nuclei in mitotic region. (n), number germ lines scored. (C–G) Projected confocal z-series of representative germ lines after BrdU pulse and chase. Arrowhead, distal end; pink, BrdU; blue, TO-PRO-3 DNA staining; dashed line, MR/TZ boundary. (C) 0-h chase. All germ cells in mitotic region are BrdU labeled. (D) 12-h chase. BrdU staining has decreased in mitotic region. (E) 24-h chase. BrdU staining has further decreased in mitotic region. (F) 48-h chase. Little or no BrdU staining in mitotic region. No complete nuclei are stained; flecks of staining are likely to label bits of chromosomes. (G) Lower magnification image of entire germ line shown in part in F. BrdU has moved into mature oocytes. (H) Negative control. Germ line extruded from an adult that has not been exposed to BrdU, but is stained with anti-BrdU antibodies.

mosomal segments that were retained by chance. We did not notice a reproducible pattern from germ line to germ line; in particular we did not see more BrdU labeling in the distal-most 1 or 2 rows of mitotic germ cells relative to the more proximal mitotic germ cells. In other systems, label-retaining nuclei are easy to detect based on their high levels of BrdU compared with nuclei in surrounding cells (Braun and Watt, 2004; Potten, 2004); such nuclei were not observed. We conclude that the *C. elegans* germ line does not contain label-retaining nuclei and that BrdU turnover is essentially uniform in all cells within the mitotic region.

Germ Cells in the Mitotic Region Move Proximally into the Meiotic Region

We next focused on movement of BrdU-labeled nuclei from the mitotic region into meiotic zones. To this end, adults (24 h past L4) were fed BrdU for 30 min, and the position of BrdU label was determined during the course of a 24-h chase. Immediately after the 30-min BrdU pulse, BrdU-positive nuclei were scattered throughout the mitotic region in a variable pattern similar to that observed after a 15-min pulse (Figures 4A and 6A). After a 12-h chase, intensely labeled BrdU-positive nuclei had just moved into the pachytene region, ~12 rows past the MR/TZ boundary (Figure 6B), and after a 24-h chase they had moved yet more proximally into the pachytene region, ~27 rows past the MR/TZ boundary (Figure 6C). To estimate the rate of movement, we scored the proximal border of intensely labeled nuclei (inverted triangle in Figure 6, A–C), which moved proximally during the 24-h chase (Figure 6, A–D). On average, the border moved at approximately 1 row per hour (Figure 6D). In these same germ lines, we also saw uniform loss of BrdU label from germ cells within the mitotic region (Figure 6, A–C; unpublished data). We conclude that germ cells move from the mitotic region into the meiotic zones and that germ cells move through the meiotic region at a rate of ~1 row per hour.

Movement appeared to stall at a position of ~30 cell diameters from the DTC; that position corresponds roughly

Figure 6. Germ cells move proximally from mitotic region into meiotic zone. Animals were pulsed for 30 min with BrdU and then chased for increasing times. (A–C) Projected z-series of extruded germlines stained with anti-BrdU (pink) and TO-PRO-3 (blue). Arrowhead, distal end; dashed line, MR/TZ boundary. The white arrowhead marks the proximal edge of BrdU staining. (A) 0-h chase. Most BrdU-labeled nuclei are in mitotic region with some in transition zone. (B) 12-h chase. BrdU-labeled nuclei have just moved into the pachytene region (~12 rows past MR/TZ boundary). (C) 24-h chase. BrdU-labeled nuclei have moved further proximally into the pachytene region (~27 rows past the MR/TZ boundary). (D) Position of proximal boundary of BrdU-positive nuclei (x-axis) graphed with respect to length of chase (y-axis).

Figure 7. Configuration and extent of DTC processes. (A) Adult hermaphrodite germ line, 24 h past mid-L4. Each part of the panel is a projection of two confocal sections in adjacent focal planes from the same germ line. Green, GFP; red, anti-GLP-1; blue, TO-PRO-3. The DTC extends short processes that intercalate between and nearly surround germ cells in the first 2 rows along the distal-proximal axis. (B–F) Images are projections of confocal z-series. Genotype and reporter GFP indicated in figure. Green, GFP; pink, anti-PH3; blue, TO-PRO-3. Green triangle, proximal extent of extensive DTC contact; yellow circle, proximal extent of longest DTC process; diamond with dashed line, MR/TZ boundary. (B) +; *qls56* L4 germ line. (C) +; *qls56* adult germ line, 24 h after L4. (D) +; *qls56* adult germ line, 72 h after L4. (E–G) DTC process length does not change in mutants that affect the position of meiotic entry. (E) *fbf-1(ok91); qls56*. (F) *fbf-2(q738); qls56*. DTC maintains extensive contact with distal-most 4 rows of germ cells and extends processes up to 12 cell diameters in a wild-type background as well as in mutants that shorten (*fbf-1*) and lengthen (*fbf-2*) the mitotic region. (G) +; *qEx319*. Germline from animal carrying the *lag-2::LAG-2::MYC* construct. Green is anti-MYC, pink is anti-PH3, blue is TO-PRO-3. *LAG-2::MYC* fusion protein is found throughout the DTC and its processes. (H) Graph comparing position of MR/TZ boundary to extent of DTC cap and length of longest continuous process. DTC maintains extensive contact with distal-most 3 or 4 rows of germ cells (green square) and has no long processes in L4, extends processes ~12 and 18 cell diameters proximal from the DTC body 24 and 72 h after L4, respectively. Although the DTC processes lengthen, the beginning of meiotic prophase occurs closer to the DTC body with age. Note that mitoses, as indicated by PH3 staining, can occur in germ cells that do not contact the DTC or its processes (L4 and 24 h). This is also apparent in older animals when individual z-slices are examined. Although the MR shortens, cell number does not decrease dramatically with age (see first section of *Results*). (I and J) Charts with data for wild-type at different stages (I) and mutants at 24 h after L4 (J).

to the border between transition zone and pachytene region. The germ nuclei become organized at the periphery of the germline tube when they enter pachytene; perhaps the slowed movement reflects the time it takes for this organization.

We next estimated the number of germ cells in premeiotic S-phase. That estimate is based on the idea that germ cells in premeiotic S-phase will move proximally into the meiotic region and remain intensely BrdU labeled. By contrast, germ cells in mitotic S-phase will divide and label will be diluted during the subsequent S-phase. We found ~60 strongly stained nuclei in the meiotic region after a 30-min pulse and a 16-h chase ($n = 11$; average 59, range 42–78). With no chase, ~10 nuclei in the transition zone incorporate BrdU, which leaves ~50 nuclei that are likely to have been in premeiotic S-phase within the mitotic region, probably in the 5 to 6 rows just distal to the MR/TZ boundary.

Extent of DTC and Its Processes

The DTC expresses the LAG-2/Delta ligand to control germline mitotic divisions (Kimble and White, 1981; Henderson *et al.*, 1994). We examined the extent of the DTC and its processes using two reporters: a *lag-2::GFP* transcriptional reporter highlights DTC cytoplasm and its processes (Blelloch *et al.*, 1999), and a *lag-2::LAG-2::MYC* translational reporter reveals functional LAG-2 protein within the DTC

(this work). The main body of the DTC caps the distal end of each gonadal arm during larval development and remains there during adulthood (Kimble and Hirsh, 1979; Figure 7; unpublished data). In this work, we focus on DTC processes, extending previous work (Fitzgerald and Greenwald, 1995; Hall *et al.*, 1999; Finger *et al.*, 2003).

We examined individual confocal sections and followed GFP-positive DTC processes that partially surround or extend between the distal-most germ cells (Figure 7A). Such processes embraced germ cells in row 1 (17/17 germ lines) as well as cells in rows 2–4 (15/17 germ lines; Figure 7A). We estimate that ~5 germ cells occupy row 1, ~7 reside in row 2, and ~10 reside in each of rows 3 and 4. Therefore, the DTC processes partially enclose ~30 germ cells in rows 1–4. We suggest that the germline stem cells reside in these distal-most rows of the mitotic region (see *Discussion*).

We also examined projections of confocal z-series to determine the length of DTC processes along the distal-proximal axis (Figure 7, B–G). These processes lengthened with age (Figure 7, H and I). Similar results were found using either transcriptional or translational reporters (Figure 7, G and J; unpublished data). In contrast to DTC process lengthening, the boundary between the mitotic region and transition zone shortened with age (Figure 7H). Therefore, DTC process length does not correlate with extent of the mitotic region along the distal-proximal axis (Figure 7H). Note that

Figure 8. Orientation of cell divisions in mitotic region. (A) The orientation of mitotic spindles and/or metaphase plates was scored as along, across or oblique to the distal-proximal axis at each position. The majority of mitotic germ cells are found in the first 16 rows of the germ line; the likelihood of finding a germ cell in M-phase decreases more proximally. (B–E) Germ lines were stained with DAPI (white) or DAPI (white) and anti-tubulin (green). Arrowheads, distal ends. Germ cells in two different germ lines dividing across the distal-proximal axis at position 1, adjacent to the DTC (DAPI in B and anti-tubulin in C), oblique to the distal-proximal axis at position 1 (DAPI in D), or along the distal-proximal axis at position 5 (DAPI and anti-tubulin in E).

although the number of cell diameters between the DTC and TZ decreases with age, the total number of germ cells in the mitotic region remains relatively constant, because of an increased density of nuclei (unpublished data).

We also examined DTC processes in mutants with mitotic regions that are either longer or shorter than normal: DTC process lengths in the mutant were similar to those in wild type, even though mitotic region lengths were different (Figure 7, E, F, and J). We conclude that the DTC retains extensive contact with the distal-most 3 or 4 rows of germ cells throughout germline development and that DTC process length does not control the length of the mitotic region.

Mitotic Spindles Orient Randomly with Respect to the Distal-Proximal Axis

In the *Drosophila* ovary and testis, stem cells reproducibly orient their mitotic spindles so that the self-renewing daughter is born adjacent to the niche, whereas the differentiating daughter is born away from the niche (Xie and Spradling, 2000; Yamashita *et al.*, 2003). To ask whether a similar orientation could be observed in the *C. elegans* germline mitotic region, we examined the orientation of mitotic spindles, metaphase plates, and/or anaphase chromosomes with respect to the distal-proximal axis of extruded germ lines stained with anti-tubulin and/or DAPI. The plane of division was scored as parallel, perpendicular, or oblique to the distal-proximal axis at each position (Figure 8A). We found no dramatic bias in orientation at any position (Figure 8A). In particular, germ cells in the distal-most rows were not reproducibly oriented along the axis (Figure 8, B–E).

DISCUSSION

GSC Are Likely to Reside Adjacent to DTC

The localization of germline stem cells (GSC) within the adult mitotic region has been a mystery. We have found that the GSC are not readily distinguishable by their cell cycle properties or their division orientation. However, several lines of evidence from this work, taken together with previous findings and work on other stem cell systems, indicate

that the GSC reside in row 1 and perhaps rows 2–4 within the niche.

The mitotic region maintains essentially the same number of cells despite active cycling of all germ cells in that region. Therefore, over the course of several days, many and perhaps most germ cells in the mitotic region move proximally and enter meiosis. Consistent with this idea, BrdU-labeled cells move from the mitotic region into meiotic domains (e.g., transition zone, pachytene region; this work), and cell death is not observed in the distal germ line (Gumienny *et al.*, 1999). We suspect that germ cells retain their relative positions during proximal movement, because each germ “cell” is linked by an intercellular bridge to a central core of cytoplasm that runs along the length of the germ line (see Figure 1). Therefore, movement is not likely to rely on active migration of individual cells that move past other cells. Instead, germ cells probably move as a consequence of cell divisions.

Although most germ cells in the mitotic region move proximally and enter meiosis, GSC must remain within the niche. The simplest model is that GSC are located immediately adjacent to the DTC and that germ cells further from the DTC move proximally and embark on the path to differentiation. Consistent with this idea, germ cells in the distal-most 1 to 4 rows are nearly surrounded by short DTC processes, but germ cells more proximally lose that extensive DTC contact (Finger *et al.*, 2003; this work). In *Drosophila*, adherens junctions anchor germ cells in the niche (Song *et al.*, 2002; Yamashita *et al.*, 2005), but in the *C. elegans* germ line, no specialized junctions have been found between the DTC and adjacent germ cells (Hall *et al.*, 1999; Lints and Hall, 2004; Y-J Li and J. Kimble, unpublished results). An attractive idea is that the short DTC processes surrounding the distal-most germ cells anchor those germ cells within the niche. By this model, the DTC is not only responsible for signaling to germ cells, but also for holding GSC in the niche. The GSC may also remain at the distal end because they are not being “pushed” proximally by other dividing cells. Indeed, one characteristic of a stem cell compartment is the lack of input cells (Aherne *et al.*, 1977), and the distal-most germ cells, those in row 1, fit this criterion.

In a variety of vertebrate tissues, the stem cell cycle is distinct from that of cells that have left the stem cell compartment (Braun and Watt, 2004; Fuchs *et al.*, 2004; Walkley *et al.*, 2005). In the *C. elegans* germ line, the distal-most germ cells (those in rows 1 and 2) have a lower M-phase index than more proximal germ cells (this work; Maciejowski *et al.*, 2006; Figure 9A). However, germ cells in rows 1 and 2 do not appear different from more proximal germ cells (rows 3–16) with respect to labeling index or overall cell cycle length. Specifically, the fraction of nuclei that incorporate BrdU is the same throughout this region, and the time required to BrdU-label all nuclei is the same throughout this region. Therefore, one simple explanation for the lower mitotic index in the distal-most germ cells is that they have a shorter M-phase. We conclude that germ cells in rows 1–16 of the mitotic region have similar, albeit not identical, cell cycle properties. Therefore, unlike vertebrate stem cells, *C. elegans* GSC do not cycle more slowly and are not quiescent.

An important challenge for the future is to identify which germ cells stay in the niche and which move proximally. Lineage tracing in the *C. elegans* germ line has been problematic—in part because of variable germline divisions (Kimble and Hirsh, 1979; this work) and in part because of transgene silencing (Kelly *et al.*, 1997). However, technical advances have recently accomplished transgene expression in the distal germ line (Praitis *et al.*, 2001; G. Seydoux,

Figure 9. Summary and working model for the germline mitotic region. (A) Diagram of germ cells in the mitotic region. Red, distal tip cell. Undifferentiated germ cells, yellow; germ cells showing some marker of differentiation, green or gradient of yellow to green. MR/TZ, average boundary between mitotic region and transition zone; TZ/PR, average boundary between transition zone and pachytene region. Proposed sites of GSCs (orange dotted line) and the switch (graded yellow to green dotted line) from mitosis to meiosis, based on the estimated position of premeiotic S cells (green dotted line) and GLD-1 expression (green bar). GLD-1 expression was reported elsewhere (Jones *et al.*, 1996; Hansen *et al.*, 2004b). Black bars summarize cell cycle data with respect to position along the distal/proximal axis. The lower mitotic index in the distal-most germ cells also seen by Maciejowski *et al.* (2006). (B) Control by stem cell asymmetric divisions. Above, each stem cell generates one stem cell daughter (self-renewal) and one daughter destined to differentiate. Below, the two daughters occupy distinct positions relative to the niche (red). (C) Control by a stem cell population. Above, stem cells generate stem cell daughters as well as daughters destined to differentiate, either by symmetrical or asymmetrical divisions. Below, daughter cell position relative to the niche (red). Daughters within niche retain stem cell characteristics. We propose that this is how GSCs are controlled in the *C. elegans* adult hermaphrodite germline.

personal communication), and lineage tracing should now be feasible. In the absence of this definitive assay, GSC number can be roughly estimated from the total germ cell number in the distal-most rows: row 1 (~5 cells), row 2 (~7 cells), row 3 (~10 cells), and row 4 (~10 cells). However, the number of germ cells with GSC potential may be much greater, and indeed, may include most of the germ cells within the mitotic region.

Switch from Mitosis to Meiosis within the Mitotic Region

Where do germ cells switch from the mitotic cell cycle to the meiotic cell cycle? Nuclei in early meiotic prophase (e.g., leptotene/zygotene) have clearly made that switch, but less was known about nuclei in earlier stages. This work provides evidence that most BrdU-labeled germ cells in the proximal ~4 rows of the mitotic region are in premeiotic S-phase, and therefore that they have entered the meiotic cell cycle within the "mitotic region" (Results; Figure 9A). Furthermore, BrdU-labeled germ cells in the transition zone must also be in premeiotic S-phase; the mitotic index within the transition zone is low (0.1–0.3%), and mitoses are restricted to the distal 3 rows of the transition zone. Therefore, the switch from the mitotic cell cycle into the meiotic cell cycle does not occur at a sharp boundary, but instead can occur over a relatively broad domain that spans the MR/TZ boundary (Figure 9A; also see Hansen *et al.*, 2004a).

The idea that many germ cells in the proximal rows of the mitotic region have entered premeiotic S-phase is consistent with previous findings. Specifically, GLD-1 protein is first detected at a low level approximately midway through the mitotic region (Jones *et al.*, 1996; Hansen *et al.*, 2004b), and

GLD-1 is a key regulator of the switch from the mitotic to meiotic cell cycle (Jones *et al.*, 1996; Kadyk and Kimble, 1998; Hansen *et al.*, 2004b). Furthermore, germ cells in the proximal rows of the mitotic region begin to express HIM-3, a component of the synaptonemal complex and marker of meiosis (Zetka *et al.*, 1999; MacQueen and Villeneuve, 2001; Hansen *et al.*, 2004a). A simple explanation is that GLD-1 initiates the switch into the meiotic cell cycle midway through the mitotic region, but that germ cells vary in their ability to respond, perhaps because of cell cycle differences in this virtually asynchronous population (Figure 9A; see also Hansen *et al.*, 2004a).

GSC Divisions Can Be Symmetrical

A popular model for stem cell control has been that stem cells divide asymmetrically to generate one stem cell daughter and one daughter destined to differentiate (Spradling *et al.*, 2001; Clevers, 2005; Yamashita *et al.*, 2005). In *Drosophila*, GSC divisions are oriented in both ovary and testis, and daughter cells are asymmetrically positioned relative to the niche (Hardy *et al.*, 1979; Deng and Lin, 1997; Xie and Spradling, 2000; Yamashita *et al.*, 2003; Li and Xie, 2005; Figure 9B). By contrast, in *C. elegans*, we have found no evidence for divisions that are reproducibly oriented, either in the larval germ line (Kimble and Hirsh, 1979) or in adults (this work). Indeed, germ cells in row 1 can divide perpendicular to the long axis of the gonad, so that daughter cells have equivalent positions within the niche (Figure 9C). Similarly, germ cells throughout the mitotic region can divide along virtually any axis. Therefore, although *Drosophila* GSC normally divide asymmetrically, *C. elegans* GSC appear capable of

symmetrical division, at least with respect to daughter cell position. The most likely explanation is that GSC are maintained by proximity to the niche rather than by programmed asymmetric divisions. By this scenario, self-renewal and generation of differentiated progeny can be accomplished at a population level (Figure 9C; Morrison and Kimble, 2006).

A separate question is whether *C. elegans* GSC divisions produce daughters of the same or different developmental potential. During early larval development, GSC divisions produce daughter cells with equivalent potential (Kimble and White, 1981), and in both *Drosophila* larval and adult germ lines, GSC divisions can similarly produce daughter cells with equivalent developmental potential (Brawley and Matunis, 2004; Kai and Spradling, 2004). Indeed, *Drosophila* cystoblasts are now proposed to represent a reservoir with stem cell potential (Kai and Spradling, 2004). We suggest that a similar situation is likely to be the case for the adult *C. elegans* germ line.

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