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Mitotic cell cycle progression and differentiation of germline stem cells in Caenorhabditis elegans

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MITOTIC CELL CYCLE PROGRESSION AND DIFFERENTIATION OF GERMLINE STEM CELLS IN *CAENORHABDITIS ELEGANS*

by

Paul Michael Fox

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ABSTRACT OF THE DISSERTATION

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In the *C. elegans* germline, stem cells make a decision to retain the germline proliferative cell fate or differentiate by entering meiosis. Importantly, this decision must be coordinated with progression through the mitotic cell cycle. Previous work has shown that the conserved GLP-1/Notch signaling pathway promotes the proliferative fate while two downstream and redundant pathways are repressed by GLP-1 and promote entry into meiosis: the GLD-1 and GLD-2 pathways. To better understand how the switch to enter meiosis is coordinated with progression through the mitotic cell cycle, I investigated mitotic cell cycle progression among germline proliferative cells. Proliferative cells cycle continuously and have an atypical cell cycle structure in which the G1 phase is omitted. These features of mitotic cell cycle progression are likely explained by continuous CDK-2-CYE-1 activity throughout the cell cycle. In addition to driving cell cycle progression, *cdk-2* and *cye-1* are also important for promoting the proliferative fate. This suggests that CDK-2-CYE-1 may act to coordinate mitotic cell cycle progression with the proliferative

cell fate. While GLP-1 promotes the proliferative fate, GLP-1 does not appear to influence cell cycle rate, suggesting that GLP-1 only regulates proliferative fate, and not mitotic cell division. Whereas CDK-2-CYE-1 may coordinate cell fate and mitotic cell cycle progression, other signaling pathways, such as the GLP-1 pathway, may only regulate cell fate.

To further investigate how the switch to meiosis is coordinated with cell cycle progression, I analyzed the spatial and temporal pattern of meiotic entry among proliferative zone cells following induced loss of *glp-1*. This analysis provided two important conclusions. First, the response of mitotically dividing proliferative zone cells appears to depend on their position in the cell cycle, and proliferative cells likely make the switch to meiosis prior to the initiation of S-phase. Second, actively cycling proliferative zone cells did not appear to display a differential response to loss of *glp-1* other than the meiotic entry timing variation due to cell cycle position. This supports the hypothesis that mitotically cycling proliferative zone cells are developmentally equivalent and that preprogrammed transit amplifying divisions do not occur following loss of GLP-1 activity.

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Table of Contents

List of Figures

List of Tables

#

Chapter 1

Introduction: The *C. elegans* germline as a model for stem cell biology

Multicellular organisms must create new cells for ontogeny, homeostasis and regeneration. However, as cells within an organism mature, they often permanently exit the cell cycle and cannot contribute to this demand. To meet these needs, many tissues set aside stem cells for their source of cell production, particularly for the demands of homeostasis and regeneration. Stem cells have two essential abilities: 1) self renewal by cell division and 2) generation of multiple cell types by differentiation. Central questions in the field of stem cell biology concern how these processes are regulated. Indeed, the field has begun to uncover a complex web of factors. While common mechanisms of regulation apply on occasion, important differences exist among the wide variety of stem cells.

In this thesis, I will describe my work using the *C. elegans* germline as a model for understanding stem cell biology. My work on the *C. elegans germline* addresses four main topics: 1) the characteristics of mitotic cell cycle progression among germline stem cells, 2) the role of CDK-2-CYE-1 in regulating germline stem cell fate and cell cycle progression 3) coordination between cell cycle progression and meiotic entry in germline stem cells and 4) the organization of the germline proliferative zone. Topics 1 and 2 are covered in Chapter 2 which has been submitted as a manuscript to Development. Topics 3 and 4 are covered in Chapter 3 and will be submitted as a second manuscript. Chapter 4 provides general conclusions of the thesis as well as directions for future research. In this chapter, I review the relevant background information.

Regulation of stem cell fate and proliferation

In vertebrate model organisms, stem cells are thought to be relatively rare and have been quite difficult to identify. A number of stem cell locations have been identified, though the individual stem cells within thee locations often remain ambiguous. Important examples that are currently being studied include: epithelial stem cells in the hair bulge, germline stem cells in the basal layer of the seminiferous tubules, neural stem cells in the lateral ventricle subventricular zone, muscle stem cells under the basal lamina of muscle fibres and hematopoietic stem cells in bone marrow (Morrison and Spradling 2008). It is thought that the cells and structures surrounding each of these stem cell types provide an important microenvironment, referred to as the niche, that helps regulate stem cell fate and proliferation (Fuchs et al. 2004; Morrison and Spradling 2008). However, most of these niche microenvironments remain poorly characterized at the molecular level.

Cell-cell interactions between the niche cells and the stem cells are thought to be important for the regulation of stem cell fate and proliferation. A variety of signaling pathways participate in these cell-cell interactions, including the Notch signaling pathway, the Wnt pathway, the FGF pathway, the BMP pathway and the SHH pathway (Morrison and Spradling 2008). Therefore, stem cell regulation needs to be considered in an individual context, as different signaling pathways and mechanisms regulate different stem cell types. Furthermore, gene expression profiling has revealed that stem cells in different tissue types do not share an overall expression profile (Fuchs et al. 2004; Morrison and Spradling 2008). In general, the expression profile of stem cells in a given

tissue more closely resembles that of their surrounding differentiated daughters than that of stem cells from another tissue.

The *C. elegans* germline

The *C. elegans* germline provides an important model system for studying stem cell biology. Adult hermaphrodites contain two independent gonads that share a common uterus (Fig. 1). Each gonad contains ~1000 germ cells that display a distal to proximal polarity, with mitotically dividing stem cells in the distal end of the germline and mature gametes (both oocytes and sperm) at the proximal end. The relatively simple organization of the germline provides a powerful substrate for identifying factors that regulate a wide variety of processes including stem cell self renewal and differentiation. How are the stem cells regulated to provide a proper balance of stem cell renewal and differentiation by entry into meiosis? This is a basic question in stem cell biology and numerous factors have been identified and described that participate in this regulation within the *C. elegans* germline.

GLP-1/Notch signaling provides an important signal for the stem cell fate

Notch signaling pathway is major pathway that regulates development of many tissues throughout the animal kingdom. GLP-1, a *C. elegans* homolog of the Notch receptor, acts cell autonomously to provide an essential signal for stem cell fate in the germline (Austin and Kimble 1987; Berry et al. 1997). The source of GLP-1 ligand comes from a somatic cell, the distal tip cell (DTC), which caps the germline and makes contact with proliferative cells at the very distal part of the germline (Fig. 2) (Kimble

and White 1981). The DTC expresses the GLP-1 ligands, LAG-2 and APX-1 and provides the stem cell niche (Henderson et al. 1994; Nadarajan et al. 2009). Expression of LAG-1 and APX-1 in the DTC mediates their interaction with GLP-1 in the extracellular space of the distal germline, leading to cleavage of the GLP-1 receptor and activation of the GLP-1 signaling pathway. The interaction of GLP-1 with LAG-2 and APX-1 is probably facilitated by the unique structure of the DTC, which makes extensive contact with cells in the distal-most 4 cell diameters but also extends cytoplasmic processes as far as 12-18 cell diameters into the proliferative zone (Hall et al. 1999; Crittenden et al. 2006). However, the importance of the DTC structure remains unclear, as does the distribution of activated GLP-1 throughout the proliferative zone. The lack of information in this regard and the observation that clearly not all cells in the proliferative zone directly contact the DTC has lead to a variety of models that explain the maintenance (at least temporarily) of the proliferative fate in cells displaced from the DTC (see below)(Hansen and Schedl 2006).

Upon binding ligand, consecutive cleavage events free the intracellular portion of GLP-1 (referred to as GLP-1(INTRA)) from the extracellular domain (Greenwald 2005). Three cleavage events are thought to occur during processing of the full length GLP-1 receptor to GLP-1(INTRA). “Site 1” is an extracellular site that is thought to be cleaved during transport to the plasma membrane independent of ligand binding (Greenwald 2005). Interaction of GLP-1 with ligand leads to cleavage at the extracellular “site 2” by an ADAM family metalloprotease. “Site 2” cleavage leads to subsequent cleavage at transmembrane “site 3” by the γ-secretase complex (Goutte et al. 2002).

After release, GLP-1(INTRA) is thought to transfer to the nucleus where it regulates target gene expression (Schroeter et al. 1998; Greenwald 2005). LAG-1 and LAG-3/SEL-8 are two critical cofactors that bind GLP-1(INTRA)(Christensen et al. 1996; Doyle et al. 2000; Petcherski and Kimble 2000). LAG-1 is a member of the CSL family of DNA binding proteins and is thought to play a role in recognizing transcriptional targets of the GLP-1 pathway (Greenwald 2005). LAG-3/SEL-8 is thought to function similar to Mastermind, an essential component of the ternary complex with GLP-1(INTRA) and LAG-1 (Greenwald 2005). Removal of any of these factors from the germline phenocopies the *glp-1* mutant in which germline stem cell maintenance is lost and germ cells prematurely enter meiosis (see below).

Extensive research has recovered a variety of mutations in *glp-1*. The reference null allele *q175*, has an early premature meiotic entry phenotype in which the initial germ cells immediately enter meiosis to form as few as 16 sperm in an otherwise empty germline (Austin and Kimble 1987). Gain-of-function mutants in *glp-1* show an opposite phenotype where germ cells continue to proliferate ectopically at the expense of differentiation and gamete production (Kerins et al. ; Berry et al. 1997; Pepper et al. 2003). In addition, temperature sensitive mutants, both gain- and loss-of-function, have been important tools for sensitized for genetic screens but also provide a method of manipulating GLP-1 activity (Austin and Kimble 1987; Kodoyianni et al. 1992; Qiao et al. 1995; Pepper et al. 2003; Kerins et al. 2010). Temperature sensitive loss of function *glp-1* mutants can lead to an additional type of premature meiotic entry phenotype. Whereas *glp-1* null mutants display premature meiotic entry during the L1 phase of larval

development, loss of *glp-1* by temperature shift in adult animal also causes a premature meiotic entry phenotype in which all proliferative cells enter meiosis.

The Redundant GLD-1 and GLD-2 pathways act downstream of GLP-1 signaling to promote entry into meiosis

Two redundant pathways act downstream of GLP-1 to promote entry into meiosis (Fig. 2). These pathways are referred to as the GLD-1 and GLD-2 pathways. The GLD-1 pathway consists of GLD-1 and NOS-3 while the GLD-2 pathway consists of GLD-2 and GLD-3. Elimination of any of these genes individually does not significantly impair initial entry into meiosis. However, removal of one gene from both the GLD-1 and GLD-2 pathways causes a defect in entry into meiosis and results in ectopic proliferative cells throughout the germline leading to a tumorous phenotype (Kadyk and Kimble 1998; Eckmann et al. 2004; Hansen et al. 2004a). Genetic epistasis has demonstrated that the GLD-1 and GLD-2 pathways act downstream of GLP-1. Triple mutants that eliminate the GLD-1, GLD-2 and GLP-1 pathways phenocopy the germline tumor phenotype of GLD-1 GLD-2 pathway double mutants rather than the premature meiotic entry phenotype of a *glp-1* single mutant (Kadyk and Kimble 1998; Hansen et al. 2004a). This suggests that the primary mechanism by which GLP-1 signaling promotes the proliferative fate is by repressing the activity of the GLD-1 and GLD-2 pathways.

Molecularly, the GLD-1 and GLD-2 pathways support the general idea that post-transcriptional regulation is a critical control mechanism in the germline (Merritt et al. 2008). GLD-1 is a cytosolic RNA binding protein that directly binds mRNA transcripts. A variety of direct mRNA targets of GLD-1 have been identified and shown to be

repressed by GLD-1 (Lee and Schedl 2001; Lee and Schedl 2004; Lakiza et al. 2005; Schumacher et al. 2005; Biedermann et al. 2009). Although these identified targets of GLD-1 are related to other germline functions of GLD-1 (such as sex determination and meiotic prophase progression), a major hypothesis is that GLD-1 also promotes entry into meiosis by repressing key mRNA targets (Hansen and Schedl 2006). This hypothesis predicts that such mRNA targets would encode factors critical for either repressing entry into meiosis or promoting the proliferative fate. NOS-3 is currently the only other known member of the GLD-1 pathway but also encodes a putative translation regulator with similarity to Drosophila nanos (Kraemer et al. 1999; Subramaniam and Seydoux 1999; Hansen et al. 2004b). In the GLD-2 pathway, GLD-2 encodes a poly(A) polymerase that is thought to promote mRNA translation (Wang et al. 2002). GLD-2 directly binds the other member of the GLD-2 pathway, GLD-3, which encodes an RNA binding protein that may direct the catalytic activity of GLD-2 to specific target mRNAs (Eckmann et al. 2002; Eckmann et al. 2004). The GLD-2 pathway regulates GLD-1 accumulation and appears to promote meiotic entry in part through direct regulation of gld-1 mRNA (Suh et al. 2006). Still, the key mRNA targets of the GLD-1 and GLD-2 pathways that promote meiotic entry remain unknown and identifying these targets remains an important goal for future research.

FBF mediates the negative regulation of GLP-1 towards the GLD-1 and GLD-2 pathways

FBF-1 and FBF-2 are nearly identical and are members of the PUF family of RNA binding proteins (Zhang et al. 1997). Collectively referred to as FBF, loss of both

fbf-1 and *fbf-2* results in a complete loss of stem cells and germline self renewal, indicating that FBF has an important role in stem cell maintenance (Crittenden et al. 2002). FBF appears to directly bind and and repress *gld-1* and *gld-3* mRNA transcripts, repressing their accumulation and making FBF a negative regulator of both the GLD-1 and GLD-2 pathways (Crittenden et al. 2002; Eckmann et al. 2004). Furthermore, the presence of LAG-1 binding sites and genetics evidence with *glp-1* suggests that FBF-2 is a direct target of GLP-1 signaling (Lamont et al. 2004). However, the extent of GLD-1 and GLD-3 regulation by FBF remains unclear and additional factors, some yet to be identified, contribute additional regulatory activity (Hansen and Schedl 2006). In certain developmental contexts, FOG-1, FOG-3 and FEM-3, factors with roles in sex determination, appear to cooperate with FBF to promote germline stem cell maintenance (Thompson et al. 2005).

Germline regulation by FBF is complex. In addition to promoting stem cell fate, FBF also has a nonessential role in promoting meiotic entry revealed by the combination of FBF double mutants with a *gld-1* mutation. In these triple mutants, germ cells show a defect in entry into meiosis similar to double mutants that target the GLD-1 and GLD-2 pathways (Crittenden et al. 2002). Recent findings propose an explanation for this double role at the molecular level. FBF directly binds and represses *gld-1* mRNA in the proliferative zone. However, FBF also interacts with GLD-2 and GLD-3 and positively regulates GLD-2 poly(A) polymerase activity in vitro (Suh et al. 2009). This has led to a model in which FBF represses *gld-1* mRNA in stem cells, but switches to positively regulate *gld-1* mRNA upon interaction with GLD-2 (Suh et al. 2009).

Other regulators of proliferation versus meiotic entry

Genetic screens have uncovered a variety of genes and processes that regulate the decision to proliferate or enter meiosis. ATX-2 is another putative translational regulator that acts to promote the proliferative fate (Ciosk et al. 2004; Maine et al. 2004). Based on genetic epistasis, ATX-2 acts downstream or in parallel to GLP-1 in promoting the proliferative fate (Maine et al. 2004). EGO-1, an RNA directed RNA polymerase, also promotes germline proliferation downstream or in parallel with GLP-1 (Vought et al. 2005). A variety of additional genes that promote entry into meiosis have been identified. METT-10, a putative methyl transferase, acts upstream or in parallel to GLP-1 to inhibit the proliferative fate (Dorsett et al. 2009). METT-10 mutations also cause defects in cell cycle progression, indicating that regulation of cell cycle progression can be separated from regulation of the proliferative fate (Dorsett et al. 2009). Analysis of panel of splicing factors including PRP-17 and TEG-4 indicate that pre-mRNA splicing pathways also promote entry into meiosis (Kerins et al. ; Mantina et al. 2009). These examples demonstrate that a variety of inputs balance the proliferative fate with entry into meiosis.

Developmental organization of the proliferative zone and stem cell niche

Despite the extensive characterization of factors that regulate proliferation versus entry into meiosis, few studies have addressed the actual organization of the proliferative zone itself. The adult hermaphrodite germline contain approximately 230 proliferative zone cells that lie within the distal most region of the germline (Fig. 1). These proliferative cells occupy the cell rows from 1 to ~20-25 within the germline (position 1 is the distal-most position). Numerous strategies have been developed for identifying

proliferative cells versus cells that have entered meiosis. The simplest strategy involves staining chromosomes and determining their organization within the nucleus. Upon entry into meiotic prophase, chromosomes cluster to one side of the nucleus, giving them an overall crescent morphology that contrasts with their relatively even distribution in proliferative cells (MacQueen and Villeneuve 2001). While this provides a convenient initial assessment of germ cell status, it does not allow unambiguous identification of entry into meiosis. A more rigorous method of proliferative cell/meiotic cell identification involves staining with antibody markers REC-8 and HIM-3 (Fig. 1)(Hansen et al. 2004a). REC-8 is a meiotic specific cohesin subunit that under mild fixation conditions is observed in the nucleoplasm of proliferative zone cells but absent in cells in meiotic prophase. It serves as a proliferative zone cell marker (Pasierbek et al. 2001; Hansen et al. 2004a). HIM-3 is a chromosomal axes element that loads onto chromosomes at the beginning of meiotic prophase (Zetka et al. 1999). Thus, HIM-3 antibody labels cells in meiotic prophase but not proliferative cells. These antibody markers are nearly entirely mutually exclusive in the germline and serve as important tools for assessing the developmental status of germ cells (Hansen et al. 2004a).

As documented in the *Drosophila* germline, asymmetric division can provide an effective means of balancing stem cell renewal with differentiation and organizing these processes within the context of the niche (Morrison and Spradling 2008). Does the *C. elegans* germline use a similar strategy for balancing self renewal and differentiation? Crittenden et al analyzed a large number of cell divisions within the proliferative zone but failed to detect any pattern in their orientation relative to the DTC, suggesting that this phenomenon does not occur in the *C. elegans* germline (Crittenden et al. 2006).

Since HIM-3 and REC-8 staining provides our effective means of distinguishing proliferative cells versus meiotic cells, we must frame our definition of proliferative zone cells and meiotic cells in terms of their molecular properties. Importantly, HIM-3 and REC-8 change their staining pattern upon entry into meiotic prophase (Fig. 1)(Zetka et al. 1999; Hansen et al. 2004a). Therefore, proliferative zone cells are defined as cells that have not entered meiotic prophase. This definition does not include any reference to self renewal ability or differentiation status because these markers do not specifically relate any information about these properties. For example, meiotic S-phase immediately precedes meiotic prophase, and current markers cannot distinguish meiotic from mitotic S-phase in the proliferative zone. As these cells are likely committed to meiosis, they represent an important group of proliferative zone cells that are not stem cells.

As the example of meiotic S-phase illustrates, a proliferative cell defined by REC-8 immuno-staining is not necessarily a stem cell. However, the proliferative zone must possess stem cells; as a population, the proliferative zone is capable of long term self renewal and the production of differentiated daughter cells. Since individual cells within the proliferative zone cannot be unambiguously identified as stem cells with current techniques, we must consider two important models for the makeup of the proliferative zone (Fig. 3). One possibility is that aside from cells in meiotic S-phase (or that have otherwise initiated a step of meiosis prior to meiotic prophase) the remaining cells in the proliferative zone are all stem cells and have equivalent developmental potential (Hansen and Schedl 2006). A second possibility is that the proliferative zone consists of stem cells with theoretically limitless self renewal ability and transit amplifying cells which undergo

a limited set of mitotic cell divisions before differentiation (meiotic entry)(Hansen and Schedl 2006; Cinquin et al. 2010).

Since the GLP-1/Notch signaling pathway is the major pathway that regulates stem cell fate in the germline (loss of *glp-1* leads to loss of all proliferative cells, stem cells or otherwise), these two hypotheses are typically considered within the framework of GLP-1 signaling within the germline. The most important observation in this regard is that the source of GLP-1 ligand, the DTC, does not make contact with all cells in the proliferative zone (Crittenden et al. 2006). How do cells displaced from the DTC maintain the proliferative fate? One possibility is that these cells do in fact lack active GLP-1 signal (in the form of GPL-1(INTRA)), and some other developmental program instructs them to temporarily proliferate (Hansen and Schedl 2006). The premature meiotic entry phenotype implies that such cells are incapable of long term self renewal, which requires GLP-1 signaling. This hypothesis therefore relates to the idea that some cells within the proliferative zone have lost stem cell fate and are transit amplifying cells, similar to transit amplifying cells in the mammalian testis, or *Drosophila* ovary and testis. Another possibility is that cells displaced from the DTC retain activated GLP-1, likely in the form of GLP-1(INTRA)(Hansen and Schedl 2006). Eventually, this GLP-1(INTRA) disappears and cells then enter meiosis. This possibility suggests that all mitotically cycling proliferative cells are molecularly and developmentally equivalent.

Assuming that all mitotically cycling cells within the proliferative zone are molecularly equivalent, how does geographic displacement from the DTC relate to their stem cell status? Since contact with the DTC is necessary for stem cell maintenance, does this mean that cells displaced from the DTC have effectively become transit-amplifying

cells, regardless of their signaling inputs and molecular makeup? In theory, relocation of displaced germ cells to the DTC niche should restore their stem cell status given that they are molecularly and developmentally equivalent. Work in the *Drosophila* germline as well as the mammalian testis has challenged the idea that some transit amplifying cells in these systems are irreversibly committed to differentiation. In both examples, relocating transit amplifying cells to a depleted stem cell niche restored their stem cell status, suggesting that these are potential stem cells (Brawley and Matunis 2004; Kai and Spradling 2004; Nakagawa et al. 2007). Distinguishing whether the C. elegans proliferative zone contains transit-amplifying cells and whether the nature of their transit amplifying status is due to a molecular signature or geographic location remains an important topic in the field.

Few genes show expression variation among cells in the proliferative zone. GLD-1 is possibly to best characterized example of protein that is not equivalent throughout the proliferative zone. GLD-1 is low in the very distal proliferative zone, but gradually increases as cells move proximally, reaching a high level as they enter meiosis (Jones et al. 1996; Hansen et al. 2004b). GLD-1 also promotes entry into meiosis, and GLD-1 levels are important for the decision to proliferate or enter meiosis (Hansen et al. 2004b). However, it remains unclear whether the differences in GLD-1 levels among proliferative cells reflects an underlying difference in cell fate.

The Mitotic Cell Cycle

Stem cell self renewal relies on progression through the mitotic cell cycle. A vast body of work has described important principles of cell cycle progression and its

regulation (Orford and Scadden 2008). The process includes four phases known as G1, S, G2 and M which coordinate genome replication and division with cell growth in a proper sequence of events. The first GAP phase (G1) includes the start or restriction point, during which cells decide whether or not to initiate a new cell cycle. S phase encompasses the period during which the genome is replicated. During the second GAP phase (G2), cells assess completion of genome replication. Additionally, the GAP phases are also important for cell growth. These steps culminate in mitotic cell division during M-phase. Cyclin dependent kinases (CDKs) partner with co-activators known as cyclins to form master regulators that drive sequential progression through each of these steps. A number of positive and negative regulators act upstream to regulate the activity of CDKs.

Early G1 phase is a critical window during which the decision to progress through the cell cycle is made. Numerous extrinsic signals stemming from nutrient availability, cell density, growth factors, cell-cell contacts, and contact with extracellular matrix converge to determine whether a cell will progress through this initial period and pass the restriction point (Blomen and Boonstra 2007). During the restriction point, active CDK4/cyclinD phosphorylates pRb, a pocket protein that binds and represses E2F transcription factors in its hypophosphorylated state (Dyson 1998). Passage through the R point is defined biochemically by hyperphosphorylation of pRb and is thought to set in motion cell intrinsic signaling events that drive cell cycle progression (Dyson 1998). Relieving the repression of E2F family transcription factors allows them to activate a panel of genes important for progression into S-phase and DNA replication, including cyclin E and components of the DNA replication machinery (DeGregori et al. 1995).

CDK2-cyclin E regulates entry into S-phase

Cyclin-dependent kinases regulate cell cycle progression through phosphorylation of key target genes. Typically, the activity of a given CDK is limited to a specific part of the cell cycle. Canonically, CDK2/cyclin E has highest activity during the G1/S-phase transition (Hwang and Clurman 2005). An important determinant of CDK2 activity is the regulation of cyclin E levels (Hwang and Clurman 2005). Numerous mechanisms serve to regulate cyclin E abundance during the cell cycle. As part of the pRb signaling pathway, cyclin E is transcriptionally regulated by E2F transcription factors (Dyson 1998). Meanwhile, decay of cyclin E protein after enty into S-phase occurs due to targeted protein degradation (Clurman et al. 1996). In *Drosophila* and in mice, the factors involved in targeting cyclin E for degradation include the ubiquitin ligases Cul3 and SCF-Fbw7 (Koepp et al. 2001; Moberg et al. 2001; Strohmaier et al. 2001). This degradation relies in part on autophosphorylation through CDK2 and also phosphorylation by GSK-3β (Clurman et al. 1996; Welcker et al. 2003).

Initial work on CDK2/cyclin E led to a model in which CDK2/cyclin E activity is rate limiting for progression into S-phase (Resnitzky et al. 1994; Duronio and O'Farrell 1995). This predicted that cyclin E and CDK2 would be absolutely required for development. However, mouse knockouts of both cyclin E and CDK2 are viable and lack overt phenotype (Berthet et al. 2003; Geng et al. 2003). Is CDK2/cyclin E an important part of cell cycle progression? Genetic redundancy might explain the lack of phenotypes among various cell cycle gene knockouts. In “lower” metazoans, including *Drosophila* and *C. elegans*, CDK2 and cyclin E have essential developmental functions (Knoblich et al. 1994; Fay and Han 2000).

Cell cycle properties vary among stem cell types

Stem cells exhibit vastly different patterns of cell cycle progression, and variation in cell cycle progression can be observered between tissues and organisms but also within among stem cells in the same niche (Orford and Scadden 2008; Fuchs 2009). Mouse embryonic stem cells have short generation times, appropriate given the behavior of their in vivo counterparts. Between day 4.5 to day 7.0, the mouse embryo expands from 20-25 cells to over 4000, which requires an average cell cycle time of less than 10 hours (White and Dalton 2005). In comparison, various adult stem cells typically exhibit slower cell cycle progression and do not enter the cell cycle frequently. In the mouse hematopoetic stem cell population, which contains both long term hematopoietic stem cells (LT-HSC) and short term hematopoietic stem cells (ST-HSC), LT-HSCs appear to divide only once overy 4-5 months, spending most of their time in G0 (Fuchs 2009).

Mouse embryonic stem cells (mES) display cell cycle features that correlate well with their fast kinetics. mES cells grown in culture have a similar cell cycle structure to epiblast cells harvested from a 6.25dpc embryo, making them a good model for their in vivo counterparts (Stead et al. 2002; White and Dalton 2005). Based on DNA content analysis by flow cytometry, mES cells spend ~50-60% of their time in S-phase, 20-25% in G1 and 20-30% in G2/M with total cell cycle lasting about 11 hours in culture (Stead et al 2002). Whereas G1 typically accounts for the majority of the cell cycle, mES cells achieve a short generation time by significantly decreasing the length of G1 (Orford and Scadden 2008). This cell cycle structure, in which G1 is shortened, is also observed during embryonic cleavage division in a variety of model organisms (including *C.*

elegans: (Edgar and McGhee 1988), *Drosophila*: (Edgar and Lehner 1996), *Xenopus*: (Murray and Kirschner 1989) and Zebrafish: (Yarden and Geiger 1996)). While human embryonic stem cells show a significantly slower cell cycle than mES cells, they too have a shortened G1 phase (White and Dalton 2005).

Regulation of cell progression without the G1 phase

Important variations to canonical cell cycle regulation likely explain how these cell bypass or shorten the G1 phase. Typically, initiation of the cell cycle is though to involve CDK4/cyclin D activation and pRb phosphorylation, leading to cyclin E induction by E2F transcription factors. Could cells bypass this sequence of events by keeping CDK2/cyclin E constitutively active? Indeed, mES cells and other similarly structured cell cycles progress with CDK2/cyclin E active throughout the cell cycle (Orford and Scadden 2008). Furthermore, CDK4/cyclin D activity is low in mES and probably plays little role in the cell cycle progression of these cells (Faast et al. 2004). As mES cells differentiate, G1 lengthens and CDK2/cyclin E activity becomes periodic and dependent on pRb-E2F signaling (Savatier et al. 1994; White et al. 2005). These observations have led to a model in which high CDK2/cyclin E activity throughout the cell cycle renders the CDK4 activation and pRb phosphorylation irrelevant and eliminates the delay during G1 (White and Dalton 2005; Orford and Scadden 2008).

Cell cycle structure and regulation of stem cell fate

Differentiation of mES cells correlates with a lengthening of G1 (White et al. 2005). Is cell cycle structure alteration a cause or consequence of this cell fate change? A growing body of work supports the notion that cell cycle regulation affects cell fate (White and Dalton 2005; Orford and Scadden 2008). This hypothesis stems from the idea that cell fate decisions, such as differentiation or initiation of the cell cycle, are typically made during the G1 phase of the cell cycle (Blomen and Boonstra 2007). By limiting time spent in G1, stem cells might effectively limit their exposure to signals that would cause them to differentiate and lose the stem cell fate (Orford and Scadden 2008; Lange and Calegari 2010). Therefore, the embryonic cell cycle structure may serve as a cell intrinsic mechanism of promoting the self renewal. Hematopoietic stem cells perhaps take an alternate approach to maintaining stem cell fate. In their case, by entering the cell cycle infrequently and remaining quiescent for significant periods of time, they might also effectively avoid differentiation signals by spending time in G0 versus G1 (Orford and Scadden 2008). Another explanation for why hematopoietic stem cells, and perhaps many other adult stem cells, do not enter the cell cycle frequently is to limit the potentially detrimental effects of excess proliferation (Orford and Scadden 2008; Fuchs 2009).

Recent analysis has begun to provide direct support for the hypothesis that cell cycle regulators can influence stem cell fate. Consistent with the hypothesis above, the factors implicated in influencing cell fate are those that act early in the cell cycle during G1 or during progression from G1 into S-phase. Cyclin D contributes to cell fate decisions during development of the mammalian central nervous system (Lange et al. 2009). Meanwhile, cyclin E is required for cell fate decisions in the Drosophila central

nervous system as loss of CycE causes lineage transformation of certain neuroblasts (Berger et al. 2005). Loss of cyclin E or CDK2 also causes differentiation of certain somatic blast cells in C. elegans (Fujita et al. 2007). Recent work in mES cells suggest that CDK2 is necessary for stem cell maintenance as loss of CDK2 causes differentiation of mES cells in vitro (Koledova et al.). This cumulative evidence provides support for the notion that cell cycle factors also regulate cell fate.

Cell cycle regulation is conserved in *C. elegans*

Many of the regulators of cell cycle transition in vertebrates have direct counterparts in *C. elegans* (van den Heuvel 2005). Whereas vertebrate genomes contain multiple copies of many of the cyclins and CDKs implicated in cell cycle progression, the *C. elegans* genome often has one representative. The CDK4/CDK6 homolog, CDK-4, and cyclin D (CYD-1) are both required for cell cycle progression through G1 in larval cells (Park and Krause 1999). In addition, the *C. elegans* genome contains a single copy of cyclin E (*cye-1*) and CDK2 (*cdk-2*) (Fay and Han 2000; Fujita et al. 2007). Loss of these factors also causes severe defects in larval development associated with cell cycle progression, in particular progression into S-phase. These cell cycle arrest phenotypes contrast with the relative lack of phenotypes in knockout mice, indicating that analysis of cell cycle progression in *C. elegans* may avoid the complications of redundancy in the mouse genome.

Cell cycle progression in the *C. elegans* germline

As discussed above, specific stem cells have been associated with reduced cell cycle activity relative to surrounding transit amplifying cells (Fuchs 2009). A lower proliferation rate has been proposed as a mechanism to protect stem cells from exhaustion and might predict that true stem cells in the *C. elegans* germline would also display a difference in cell cycle characteristics relative to surrounding transit amplifying cells (Orford and Scadden 2008; Fuchs 2009). Analysis of cell division frequency among cells in different distal-proximal positions has observed differences within the proliferative zone. As expected, proliferative cells in the proximal-most positions divide less frequently due to enrichment of cells in meiotic versus mitotic S-phase (Hansen et al. 2004a; Crittenden et al. 2006; Maciejowski et al. 2006). The distal-most cells in the proliferative zone appear to divide less frequently (Maciejowski et al. 2006). However, based on S-phase frequency, all proliferative cells have very similar cell cycle characteristics (Hansen et al. 2004a; Crittenden et al. 2006; Maciejowski et al. 2006; Jaramillo-Lambert et al. 2007). In additional, pulse-chase experiments with bromodeoxyuridine (BrdU) failed to identify label-retaining cells in the proliferative zone, arguing that cell cycle differences among proliferative cells are relatively small (Crittenden et al. 2006). Taken together, proliferative cells do not show significant differences in their mitotic cell cycle that would indicate obvious differences in developmental status.

Entry into meiosis requires coordination with mitotic cell cycle progression

For germline stem cells, entry into meiosis is the first step in the differentiation pathway to produce gametes. Meiosis represents a distinct form of chromosome

segregation with significant differences between it and mitosis. However, mitosis and meiosis do share common processes, machinery and regulation. Studies in yeast have provided a foundation for exploring the regulation of meiosis in multicellular organisms. While much of the upstream signaling that regulates meiosis is likely not conserved, the principles of cell cycle regulation and coordination with the mitotic cell cycle may provide important insight to compare and contrast.

Since the initiation of meiosis must be coordinated with mitotic cell cycle progression, and important question in the study of meiosis is: When during the mitotic cell cycle do cells initiate entry into meiosis? Early work in yeast provided evidence that the decision to enter meiosis occurs early in the mitotic cell cycle prior to the initiation of DNA replication. Hirschberg and Simchen used a collection of temperature sensitive, cell cycle arresting cdc mutants to ask when during the cell cycle do cells become committed to completing mitosis (Hirschberg and Simchen 1977). In this analysis, only cells arrested at very early stages of the mitotic cell cycle (prior to initiation of S-phase) were uncommitted to mitosis, suggesting that the decision to enter meiosis must occur prior to S-phase. More recent work has begun to describe molecular changes during the G1-S phase transition as yeast cell switch from mitosis to meiosis, confirming that preparation for meiosis begins early during the cell cycle (Marston and Amon 2004). In budding yeast and fission yeast, distinct regulatory factors are involved in this early preparation for meiosis. In budding yeast, the transcription factor Ime1p induces a panel of genes involved in the switch to meiosis (Honigberg and Purnapatre 2003). In order for induction of Ime1p to occur, the cell must also coordinate repression of specific G1 cyclins that act to repress expression of Ime1p (Colomina et al. 1999). An important

target of Ime1p is Ime2p, a kinase with homology to CDKs that appears to substitute for their activity in promoting initiation of DNA replication (Honigberg and Purnapatre 2003). These molecular events indicate that preparation for entry into meiosis begins early in the cell cycle and involves the repression of key mitosis specific factors as well as activation of meiosis specific factors.

Currently, less is known about how cell cycle factors are regulated during this switch in fission yeast. However, a distinct set of molecular events promotes the switch from mitosis to meiosis during the early part of the cell cycle (Marston and Amon 2004). Therefore, the mechanisms by which initiation of meiosis occurs are not conserved between these distantly related yeast species and may also be divergent in the metazoan lineage. In mice, the decision to enter meiosis appears to occur prior to S-phase, however few details concerning meiosis and mitosis specific factors are known (Baltus et al. 2006). The evidence for the timing of a switch to meiosis comes from analysis of the Stra8 mutant. In Stra8-/- mice, germ cells of the female ovary proliferate normally, however they arrest and fail to enter meiosis during the developmental stage in which meiotic entry normally occurs (Baltus et al. 2006). These arrested cells contained 2n DNA content, indicating that the defect in meiotic entry occurred prior to DNA replication (Baltus et al. 2006).

Why do cells decide to enter meiosis prior to S-phase? Studies have begun to show that a number of meiosis specific chromosomal events initiate during S-phase (Forsburg 2002). An important example is the loading of cohesin proteins during meiotic S-phase, which is necessary for linking both homologous chromosomes and sister chromatids. Loading of cohesin proteins occurs during both mitosis and meiosis.

However, meiosis modifies the cohesion complex by exchanging the mitosis specific Scc1/Rad21 with the meiosis specific subunit Rec8 (Forsburg 2002). Substitution of Rec8 is important for the altered chromosome segregation during meiosis. In a study performed on fission yeast, ectopic induction of entry into meiosis during G2 caused cells to enter meiosis without passing through meiotic S-phase (Watanabe et al 2001). These cells completed equational rather than reductional segregation of chromosomes during the first meiotic division (Watanabe et al 2001). The authors show that reductional division during the first meiotic division requires expression of Rec8 during meiotic S-phase (Watanabe et al 2001). Incorporation of Rec8 during meiotic S-phase provides one explanation of why cells initiate entry into meiosis prior to S-phase.

Homologous chromosome segregation during meiosis I also requires homolog pairing and recombination. A variety of studies have also shown that recombination appears to rely on progression through meiotic S-phase, however the molecular details of this dependency remain unclear (Forsburg 2002). Recombination of homologous chromosomes requires the formation of double strand breaks (DSB). In one study it was observed that yeast mutants that fail to complete DNA replication during meiotic S-phase have a decrease in DSB formation proportional to the amount of the genome that was not replicated (Stuart and Wittenberg 1998). Spo11, the enzyme responsible for generating DSBs, is present during meiotic DNA replication and modulates the duration of S-phase (Cha et al 2000). This has led to the hypothesis that Spo11 may form a pre-recombination complex during S-phase, analogous to the preRC used for DNA replication (Forsburg 2002). These observations support the notion that meiotic S-phase also plays an important role in preparing chromosomes for recombination.

Conclusions

Germline stem cells must coordinate mitotic cell cycle progression with entry into meiosis. A variety of studies have indicated that the switch to meiosis is limited to a particular cell cycle phase (prior to S-phase) and may involve regulation of canonical cell cycle factors. In addition, canonical cell cycle factors perhaps also participate in stem cell renewal more generally by contributing to the regulation of stem cell fate. These parallel lines of evidence suggest that mitotic cell cycle progression and the factors that regulate it are central players in regulation of stem cell differentiation to produce the develop to form gametes. However, the connection between mitotic cell cycle progression and germline stem cell regulation has remained unclear. Here, we use the *C. elegans* germline as a model to explore this topic. Chapter two discusses two important contributions towards this end. First, a general characterization of mitotic cell cycle progression among germline proliferative cells is presented. This analysis demonstrates that proliferative cells have a shorter generation time than previously appreciated, are cycling continuous and progression through a cell cycle that lacks a significant G1 phase. Chapter two also characterizes the role that two important cell cycle factors, CYE-1 and CDK-2, play in regulating stem cell versus meiotic cell fate. Chapter three explores the response of proliferative cells to a differentiation signal. The results from this study initially suggest that the switch to meiosis must occur early in the mitotic cell cycle, similar to findings in yeast and mice. In contrast to findings from yeast, mitotic cell cycle arrest in proliferative cells does not block their ability to enter meiosis.

Taken together these results suggest that a variety of complex signals converge on regulation of germline stem cell differentiation into meiosis. While CDK-2/CYE-1 regulation provides and example of a link between mitotic cell cycle progression and meiotic entry regulation, unexpected results from cell cycle arrest studies suggest that mitotic cell cycle progression and the decision to enter meiosis can also be separated.

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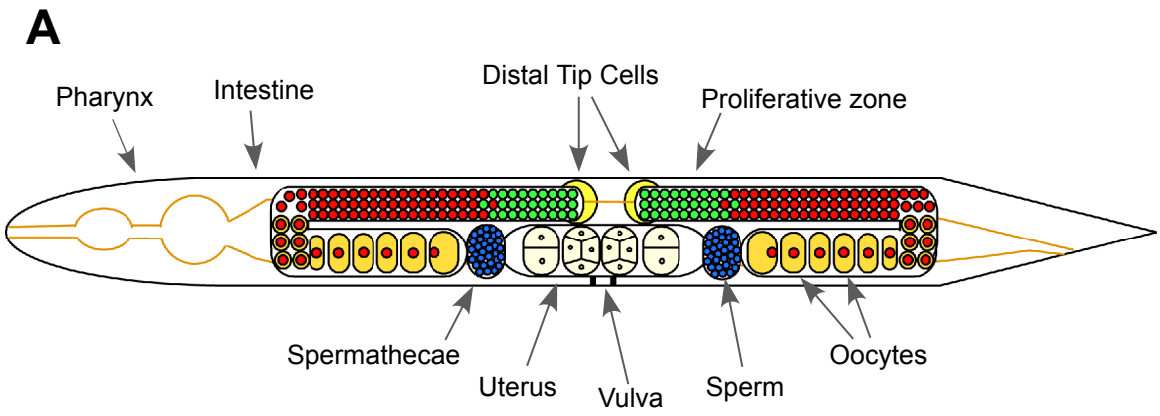
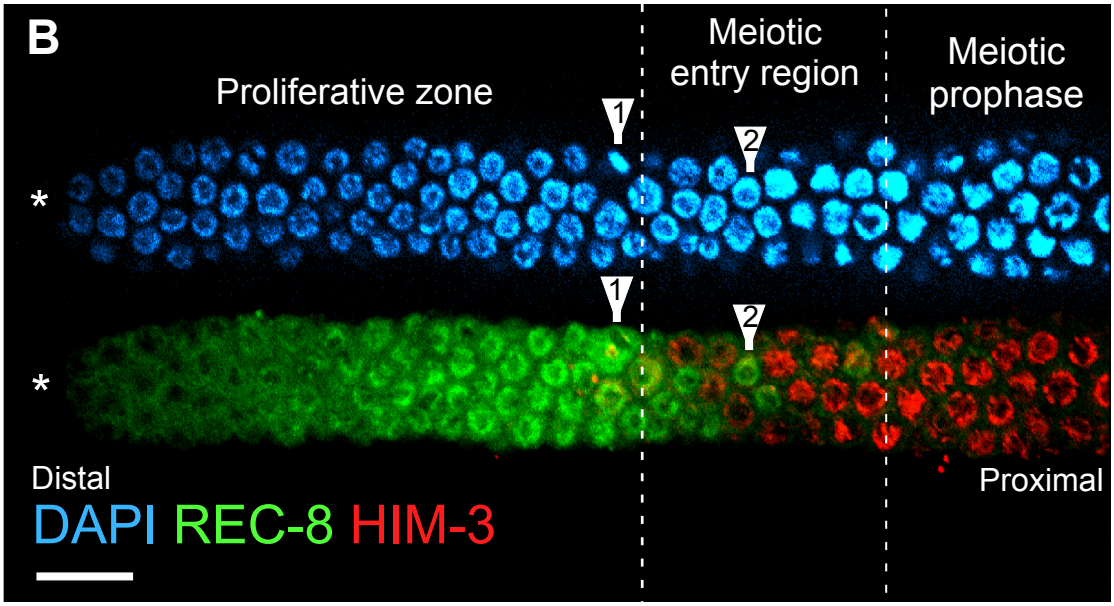
Figure 1


A
Pharynx
Intestine
Distal Tip Cells
Proliferative zone
Spermathecae
Uterus
Vulva
Sperm
Oocytes


B
Proliferative zone
Meiotic entry region
Meiotic prophase
1
2
1
2
*
*
Distal
Proximal
DAPI REC-8 HIM-3

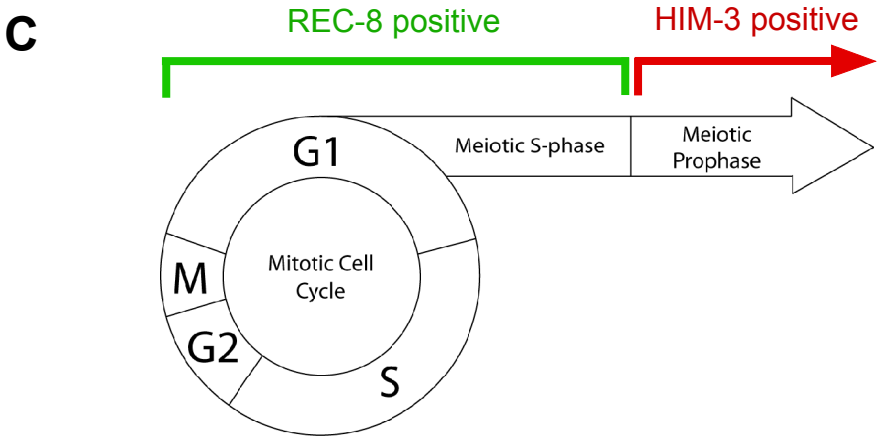

C
REC-8 positive
HIM-3 positive
G1
Meiotic S-phase
Meiotic Prophase
Mitotic Cell Cycle
M
G2
S

Figure 1. Anatomy of the *C .elegans* germline. (A) A schematic drawing of the adult hermaphrodite shows the two gonad arms in color. The gonads display a distal to proximal polarity with respect to their common uterus. The proliferative zone (green) occupies the very distal end of the gonad. Somatic distal tip cells (yellow), cap the gonad arms and provide an signal to promote the proliferative fate. (B) REC-8 (green) and HIM-3 (red) antibodies can be used to distinguish between proliferative zone cells and cells that have entered meiosis (leptotene/zygotene) (Hansen et al. 2004b). Proliferative zone cells are cells that have not initiated meiotic prophase and mitotic M-phase cells are observed throughout the proliferative zone region, although with decreasing frequency at the proximal end (Hansen et al 2004b; Crittenden et al. 2006; Maciejowski et al. 2006). The transition from proliferative zone cell to meiotic prophase occurs within the meiotic entry region. The meiotic entry region begins at the distal-most HIM-3 positive nuclei and ends at the proximal-most REC-8 positive nuclei. Within this region proliferative zone cells and meiotic prophase cells are both present. Among the proliferative zone cells in the meiotic entry region, many are likely in a premeiotic phase such as meiotic S-phase, however mitotic cell divisions can also be detected at a low frequency. These observations illustrate that the transition from proliferative cell occurs over several cell diameters. Arrowhead "1" points to a REC-8 positive proliferative zone cell in M-phase that neighbors the meiotic entry region. Arrowhead "2" points to a REC-8 positive proliferative zone cell that lies within the meiotic entry region. (C) The proliferative zone cells include cells in all stages of the mitotic cell cycle as well as cells that have begun meiotic S-phase. The switch from being a REC-8 positive HIM-3 negative cell to a REC-

8 negative HIM-3 positive occurs between meiotic S-phase and meiotic prophase (Chapter 2).

Figure 2

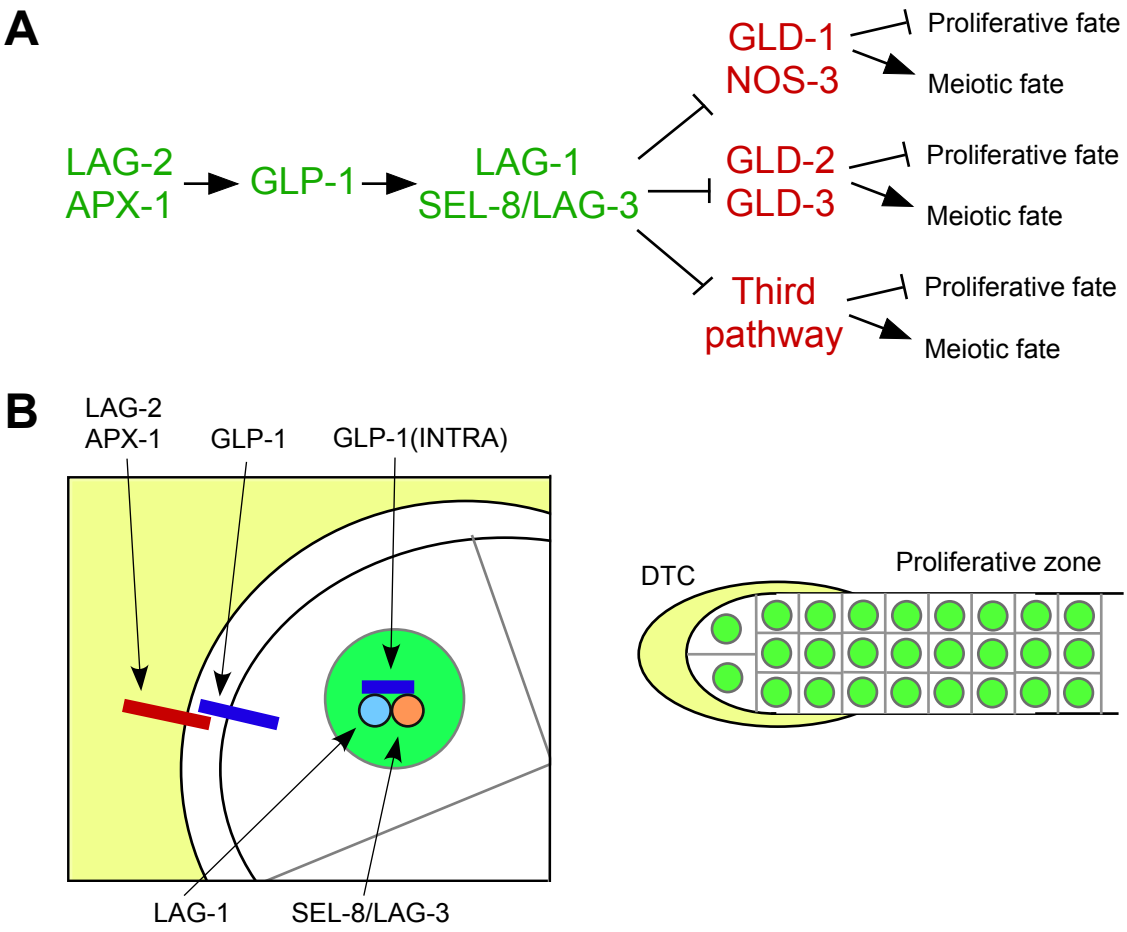

A
LAG-2
APX-1
GLP-1
LAG-1
SEL-8/LAG-3
GLD-1
NOS-3
Proliferative fate
Meiotic fate
GLD-2
GLD-3
Proliferative fate
Meiotic fate
Third
pathway
Proliferative fate
Meiotic fate
B
LAG-2
APX-1
GLP-1
GLP-1(INTRA)
LAG-1
SEL-8/LAG-3
DTC
Proliferative zone

Figure 2. Regulation of the proliferative versus meiotic cell fate decision. (A) The conserved GLP-1/Notch signaling pathway (green) promotes the proliferative fate (Austin and Kimble 1987). GLP-1 signaling acts upstream of and represses at least three redundant pathways that promote entry into meiosis (red) (Kadyk and Kimble 1998; Hansen et al. 2004b). Two of these pathways have been identified, which are the GLD-1 pathway and the GLD-2 pathway. Meanwhile, a third unidentified pathway has been predicted by experimental analysis (Hansen et al. 2004b). (B) The GLP-1 signaling pathway mediates a cell-cell interaction between the DTC and proliferative zone cells. The LAG-2 and APX-1 ligands are expressed in the DTC and interact with GLP-1 receptor expressed in proliferative zone cells (Henderson et al. 1994; Nadarajan et al. 2009). GLP-1 activation results in receptor cleavage and GLP-1(INTRA) translocation to the nucleus, where it is thought to interact with LAG-1 and SEL-8/LAG-3 cofactors to regulate the transcription of target genes (Christensen et al. 1996; Doyle et al. 2000; Petcherski et al. 2000).

Figure 3

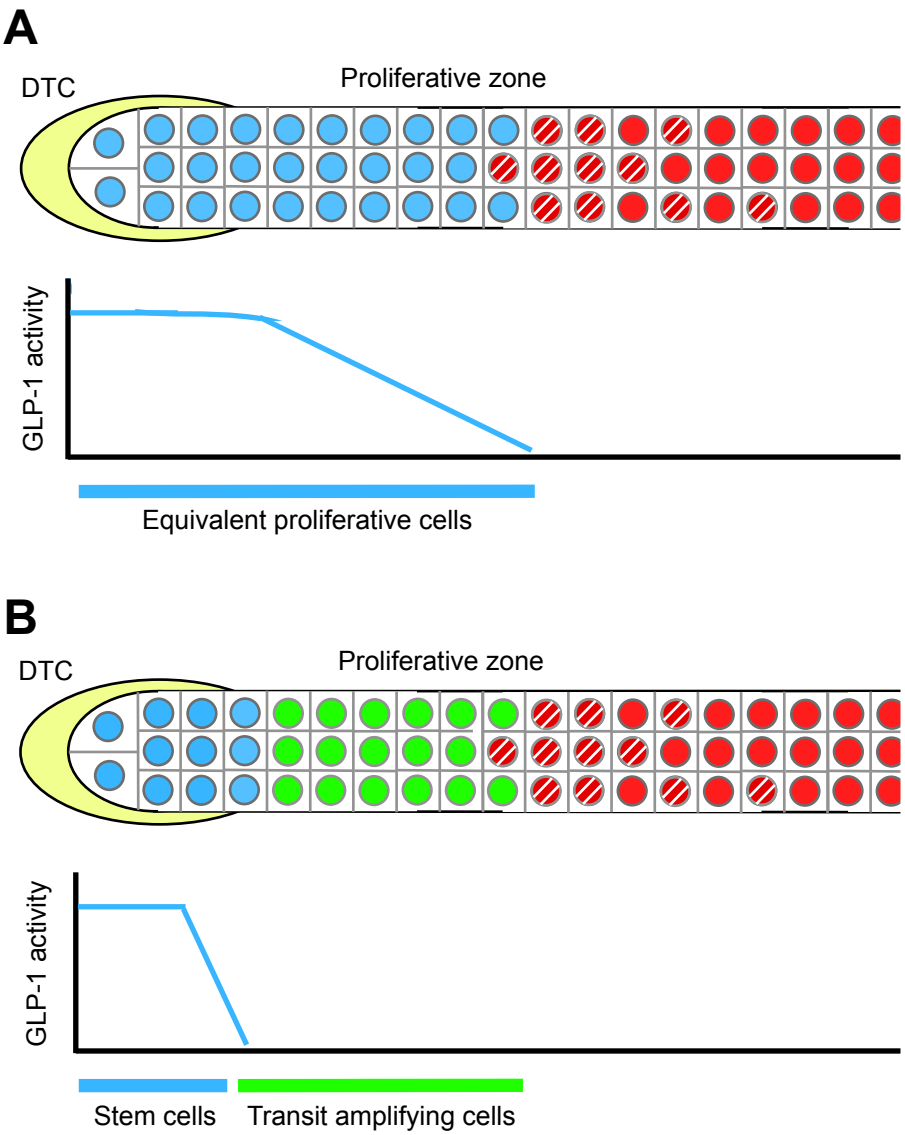

A
DTC
Proliferative zone
GLP-1 activity
Equivalent proliferative cells
B
DTC
Proliferative zone
GLP-1 activity
Stem cells
Transit amplifying cells

Figure 3. Two models for GLP-1 activity and the organization of the proliferative zone. The DTC expresses the GLP-1 ligand and makes contact with proliferative zone cells only in the distal-most region of the proliferative zone. However, proliferative zone cells displaced from this position temporarily retain the proliferative fate and undergo mitotic division. Two models have been proposed to explain how these displaced cells maintain the proliferative fate. (A) GLP-1 activity gradually declines but persists for a period of time after displacement from the DTC niche. This GLP-1 activity is directly responsible for continuation of the proliferative fate and suggests that all proliferative cells are developmentally equivalent. (B) GLP-1 activity turns off in proliferative zone cells immediately after displacement from the DTC niche. These displaced proliferative cells become transit amplifying cells which undergo a programmed set of mitotic division before entering meiosis. This model suggests that the transit amplifying proliferative cells and developmentally distinct from their stem cell counterparts. Legend: stem cells (blue), transit amplifying cells (green), premeiotic S-phase cells (striped red), meiotic prophase (solid red), distal tip cell (yellow).

Chapter 2

Cyclin E/CDK-2 regulates proliferative cell fate and cell cycle progression in the *C. elegans* germline

Summary

The *C. elegans* germline provides an excellent model system for analyzing the regulation of stem cell activity. Proliferative fate of germline stem cells is promoted by the GLP-1/Notch signaling pathway while entry into meiosis is promoted by the redundant GLD-1 and GLD-2 pathways. Here we describe cell cycle kinetics as well as the role of specific cell cycle factors in both mitotic cell cycle progression and the decision between the proliferative and meiotic cell fate. Mitotic cell cycle progression among proliferative cells occurs rapidly, continuously and with little or no time spent in G1 similar to embryonic cell cycles, with cyclin E (CYE-1) levels high throughout the cell cycle. In addition to driving mitotic cell cycle progression, *cye-1* and *cdk-2* also play an important role in promoting the proliferative fate. Depletion of either *cye-1* or *cdk-2* causes proliferative cells to enter meiosis in sensitized mutant backgrounds as well as in specific tumorous mutants. These genetic interactions indicate that CDK-2/CYE-1 act downstream or in parallel to the GLD-1 and GLD-2 pathways and that a third unidentified pathway acts downstream of GLP-1 and in parallel to CDK-2/CYE-1 to promote the proliferative fate. Our results suggest that CDK-2/CYE-1 promotes stem cell self-renewal by promoting both cell cycle progression and stem cell fate.

Introduction

Stem cells achieve self-renewal through the execution of both mitotic cell division and maintenance of stem cell fate. Different stem cell types display distinct patterns in their self-renewal and differentiation. For example, hematopoetic stem cells divide infrequently in comparison to embryonic stem cells (Orford and Scadden 2008). Different modes of stem cell proliferation may necessitate different mechanisms that regulate not only cell cycle progression but also the developmental fate of stem cells. An important goal in stem cell biology is to describe the developmental and cellular processes of stem cells and to identify the molecular mechanisms by which these processes are regulated.

The adult *C. elegans* hermaphrodite germline provides an important model for studying stem cell biology. In adults, all stages of germ cells from mitotic proliferation through meiotic prophase and gametogenesis are present in a linear array (Hansen and Schedl 2006; Kimble and Crittenden 2007). Germ cells divide mitotically in the distal-most part of the germline termed the proliferative or mitotic zone (Fig. 1A). Proliferative cells, defined by the absence of meiotic prophase markers, include stem cells as well as cells that presumably have initiated steps toward differentiation (meiotic-S phase and possibly transit amplifying cells) (Cinquin et al. ; Hansen et al. 2004a; Crittenden et al. 2006; Maciejowski et al. 2006). The transition from a proliferative state to meiotic fate occurs across several cell diameters termed the meiotic entry region, which is delineated by the distal/proximal positions where the distal most cell shows entry into meiotic prophase and the proximal most proliferative cell has not yet enter meiosis (Hansen et al. 2004a). Within this region various cellular processes including mitotic cell division and both mitotic and meiotic S-phase occur in close proximity.

The Notch homolog GLP-1 functions cell-autonomously to promote the proliferative fate (Austin and Kimble 1987). The ligands for GLP-1, APX-1 and LAG-2, are expressed in the somatic distal tip cell (DTC) that makes contact with germ cells present in the distal-most proliferative zone (Henderson et al. 1994; Nadarajan et al. 2009). In distal germ cells with high GLP-1 signaling, downstream cofactors LAG-1 and SEL-8/LAG-3 are thought to co-operate with GLP-1 INTRA to induce transcription of genes that promote the proliferative fate (Christensen et al. 1996; Doyle et al. 2000; Petcherski and Kimble 2000). A major factor regulating the proliferative versus meiotic entry decision is GLD-1 levels (Crittenden et al. 2002; Hansen et al. 2004b). High GLD-1 promotes entry into meiosis while low GLD-1 is important for the proliferative fate (Hansen et al. 2004b). GLD-1, a cytoplasmic translational repressor, defines one of two major pathways that promote entry into meiosis (Francis et al. 1995; Jones and Schedl 1995; Hansen et al. 2004b). These pathways, referred to as the GLD-1 and GLD-2 pathways, act genetically downstream of the GLP-1 signaling pathway (Kadyk and Kimble 1998; Hansen et al. 2004a). Another component of the GLD-1 pathway is NOS-3 (Hansen et al. 2004a). Known components of the GLD-2 pathway include the GLD-2 cytoplasmic poly-(A) polymerase (Wang et al. 2002) and GLD-3, an RNA binding protein (Eckmann et al. 2004). It remains unclear how the regulatory activities of these pathways specifically execute their respective cell fates.

Germline stem cells proliferate by progressing through the mitotic cell cycle; however, daughters that initiate meiosis must leave the mitotic cell cycle. Exit from the mitotic cell cycle to enter meiosis may involve repression of specific cell cycle factors. In yeast, the decision to enter meiosis can only be executed during G1 of the cell cycle, and

regulation of specific cell cycle factors has been shown to play a key role in determining the timing of meiotic entry (Honigberg and Purnapatre 2003; Wittenberg and La Valle 2003). As a mechanism to exclude meiosis during mitosis, G1-specific mitosis-promoting cyclins serve to repress transcription of the key meiosis-inducing transcription factor Ime1p in budding yeast (Colomina et al. 1999). While the underlying mechanisms may differ significantly during animal germline development, regulation of cell cycle factors may also play an important role in regulation of stem cells and determining the timing of meiotic entry.

Further dissection of cell cycle behavior among cells in the proliferative zone of the *C. elegans* germline may shed light on how these cells are regulated to achieve a proper balance between self-renewal and differentiation. We have investigated kinetic and regulatory features of mitotic cell cycle progression of proliferative cells in the adult hermaphrodite germline. Our results describe a previously unappreciated cell cycle structure in which proliferative germ cells progress through the cell cycle without a noticeable G1 phase. We find that this rapid form of cell cycle progression is likely supported by constitutive CDK-2/CYE-1 activity, bypassing the need for upstream CDK-4/CYD-1 activity during G1. In addition, CYE-1 and CDK-2 promote the proliferative fate since RNAi depletion of *cye-1* or *cdk-2* in a *glp-1* partial loss-of-function mutant causes the loss of cells with the proliferative fate due to entry into meiosis (premature meiotic entry). Our results suggest that stem cells coordinate cell cycle progression with maintenance of the stem cell fate through the common positive regulator CDK-2/CYE-1.

Materials and Methods

Nematode maintenance and strains

Animals were propagated under standard procedures at 20°C unless noted otherwise. All strains used in this study are listed in Table S1.

EdU time-course experiments

Plates seeded with MG1693 (*E. coli* stock center) bacteria that had incorporated 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) were prepared similar to the BrdU-labeled bacteria plates (Ito and McGhee 1987) except EdU was substituted for BrdU to a final concentration of 20μM. Animals were raised at 20°C, although certain experiments were repeated at 15°C and 25°C (see Fig. S1), and synchronized by picking L4 animals 24 hours prior to initiation of the time-course experiment. Animals were transferred by a pick from regular OP50-plates to plates containing the EdU-labeled bacteria. Animals were then directly transferred to PBS for dissection (Jones et al. 1996). For pulse chase experiments, animals were transferred from EdU-plates to OP50-plates. After crawling away from the residual EdU-labeled bacteria on the OP50-plates, animals were then picked and transferred to a fresh OP50-plate to minimize the amount of EdU-labeled bacteria that is carried over. Fixed germlines were first incubated with primary and secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) followed by the EdU detection reaction using a EdU-labeling kit (Invitrogen). All samples were analyzed using a PerkinElmer spinning disk confocal microscope and Volocity imaging software. EdU-positive nuclei (Fig. S4) were scored throughout the proliferative zone by assaying multiple focal planes.

Immunohistochemistry

Germlines were extruded, fixed and stained essentially as described previously (Jones et al. 1996). Antibodies: rat anti-REC-8 (1:100), from Joseph Loidl; rabbit anti-HIM-3 (1:100) from Monique Zetka; rabbit anti-phospho-Histone 3 (pH3)(1:400), from Upstate, guinea pig anti-SUN-1 S8-Pi (1:1000), from Verena Jantsch; mouse anti-CYE-1 (1:10) and rabbit anti-pCDC-6 (1:50) from Edward Kipreos. Germlines were imaged using either a PerkinElmer spinning disk confocal microscope or a Zeiss compound microscope. Images were analyzed using Volocity software for confocal images and Axiovision for the Zeiss images. Whole germline images were compiled using Adobe Photoshop and placed on a black background.

DNA Quantification

Animals were staged to 24 hours past L4 and fed EdU-labeled bacteria for 30 minutes prior to dissection/fixation. Extruded germlines were stained for pH3 and EdU as above and stained with DAPI at a concentration of 100 ng/ml. 3D images of the germline were recorded using a PerkinElmer spinning disk confocal microscope with z-stacks spaced every 0.3 microns. Volocity software was used to image nuclei in individual z-stacks and manually draw a region of interest (ROI) encompassing a single nucleus to obtain a fluorescence value corresponding to the DAPI signal within that ROI. The fluorescence values from all z-stacks spanning a given nucleus were summed to obtain a total fluorescence value. Background fluorescence for each image was determined by obtaining the fluorescence value of empty space within the field of view and subtracted from the total fluorescence value. In order to compile data from multiple germlines,

values were normalized to an average value for internal 4n controls (prophase, metaphase and meiotic nuclei) from their respective germline.

RNAi experiments

For RNAi, clones were obtained from Open Biosystems (Kamath et al. 2003), sequence verified and seeded on NGM plates as described elsewhere (Lee et al. 2007). For all analysis of *cye-1* and the additional cell cycle factors, unless noted otherwise, L4 animals were placed on the RNAi plates for 48 hours, dissected and analyzed.

Results

Cell proliferation occurs throughout the proliferative zone

To determine the length, ratio and frequency of the different phases of the cell cycle in the germline, we used various cell cycle markers: EdU for S-phase, phospho-histone 3 (pH3) for M-phase, nucleoplasmic REC-8 under mild fixation conditions as a pan proliferative zone marker (Hansen et al. 2004a) and chromosomal HIM-3 as a marker for germ cells in meiotic prophase (Zetka et al. 1999).

We began our analysis of cell cycle progression in adult hermaphrodites by feeding animals a short pulse (<30 minutes) of EdU (Methods, Fig. 1B). Nearly all cells that labeled with EdU also stain for REC-8, but not HIM-3, and approximately 57% of proliferative zone cells (REC-8 positive) were in S-phase. To assess the frequency of cell proliferation across the distal-proximal axis, we scored the percent of EdU-positive cells per cell diameter. Consistent with previous analysis, cells throughout the proliferative

zone appear to be cycling at equivalent frequencies (Fig. 1C) (Crittenden et al. 2006; Jaramillo-Lambert et al. 2007).

C. elegans germ cells progress through the cell cycle rapidly and continuously

We used pulse-chase analysis as a first step to investigate mitotic cell cycle length. We fed animals on EdU-labeled bacteria for 30 minutes and then transferred them to label free bacteria. By moving animals to unlabeled bacteria, EdU is effectively chased out of the germline (Fig. S2). The ability to perform an effective pulse-chase experiment allows marking a cohort of EdU-positive cells that are in S-phase at the time of the pulse and obtain an estimate of the total cell cycle length by following their progress through subsequent phases of the cell cycle. Specifically, we monitored EdU-positive cells as they passed through M-phase (Fig. 2A). During an 8-hour time course, we observed two waves of EdU-positive cells that go through M-phase, one from hours 2-5 and a second starting at hour 7. These waves indicate two successive cell divisions. Therefore, the total length of the cell cycle could be as short as 5 hours.

This experiment suggests that in 5-6 hours all cells that had been labeled with EdU in mitotic S-phase should have divided at least once, causing a corresponding increase in the number of EdU-positive nuclei per germline. This prediction can be examined by counting the total number of EdU-positive nuclei per germline throughout the time course. However, the proliferative zone consists of both mitotic S-phase cells, which will divide within the boundary of the proliferative zone, and meiotic S-phase cells, which enter an extended meiotic prophase. Since current markers cannot distinguish mitotic from meiotic S-phase, we followed the outcomes of the EdU-labeled

nuclei. We simultaneously counted the number of EdU-labeled nuclei that entered meiotic prophase (REC-8 negative, to estimate meiotic S-phase) while also counting the total increase in EdU-positive nuclei (to estimate mitotic S-phase) throughout the pulse-chase timecourse experiment (Figs 2B-C). From 140 nuclei that are initially labeled as EdU-positive at hour 1 (±24, n=9), we observed an increase of 48 nuclei to 188 total by 5 hours (±40, n=9) and 91 nuclei to 231 total by 6 hours (±39, n=9). Among these, 69 EdU-positive nuclei were in the meiotic prophase by 5 hours (±21, n=9) and 93 were in the meiotic prophase by 6 hours (±26, n=9). Therefore by 5-6 hours, 69-93 EdU-positive nuclei entered meiosis while 48-91 underwent cell division indicating they were in mitotic S-phase at the time of the pulse. In addition, these data suggest that a nearly equal number of cells were going through either meiotic or mitotic S-phase at a given time period. This measurement is not surprising since one cell must enter meiosis for every cell that divides mitotically to maintain a constant number of proliferative zone cells. Taken together, these results are consistent with the idea that the cell cycle may be as short as 5 hours.

Germline mitotic cell cycle lacks a significant G1

We next examined the cell cycle structure by assessing the absolute or relative lengths of each phase of the cell cycle. We began by measuring the length of G2 using a second time course experiment. Animals were continuously labeled with EdU starting at t=0, dissected at 30 minute intervals and stained for pHIS-3 and EdU, allowing us to determine the percentage of M-phase cells that were EdU-positive (Fig. 2D). This experiment reveals the time required for a cell labeled with EdU in S-phase to pass

through G2 and enter M-phase becoming pH3-positive, providing an estimate of the length of G2. According to our data, G2 ranged from 1.5 hours, when EdU-positive M-phase cells were first detected, to 3.5 hours, when all M-phase cells are EdU-positive. By 2.5 hours, approximately 50% of M-phase cells were EdU-positive (Fig. 2D). Thus, the mitotic cell cycle in the germline has a median G2 length of ~2.5 hours with 1.5 and 3.5 hours as the minimum and maximum values respectively.

Next, we measured the length of G2+M+G1 by determining the shortest time of continuous labeling required to mark all proliferative cells with EdU (Crittenden and Kimble 2008). Since the length of M-phase is relatively short, this value allows us to infer the length of G1 through comparison with the length of G2. Figure 2E shows that more than 99% of proliferative cells are EdU-positive after 3.5 hours of continuous EdU feeding. This value of 3.5 hours for the maximum length of G2+M+G1 equals our value for the maximum length of G2 (Figure 2D). This unexpected finding suggests that the length of G1, in addition to M, is very short relative to the length of G2. In support of this we noticed that cells that remained EdU-negative until hour 3.0 were highly enriched for M-phase (Fig. S3), suggesting that soon after completion of M-phase cells return to S-phase. Finally, the fact that all cells in the proliferative zone incorporate EdU during a 3.5 hour pulse confirms previous reports that cells are entering the cell cycle continuously and are not entering significant periods of quiescence (Crittenden et al. 2006).

As an alternate approach to describe mitotic cell cycle structure, we estimated the proportion of proliferative cells in G1 by measuring the DNA content of DAPI stained nuclei (Fig. 3). Since histogram plots of DNA content among cells did not reveal obvious G1, S and G2 populations (Michaelson et al. ; Feng et al. 1999)(Fig. 3B, grey bars), we

used EdU and pH3 markers to identify cells in S-phase and M-phase, respectively. Phospho-H3 positive prophase and metaphase nuclei have 4n DNA content and as expected fall into the right side of the histogram, while individual daughters from anaphase and telophase have 2n DNA content and fall into the left side of the histogram (Fig. 3B, green bars). These internal controls for 2n and 4n DNA content allow us to assign haploid DNA equivalent content to corresponding DAPI fluorescence values for the remaining nuclei. As expected, EdU-positive S-phase cells fall mostly within a relatively even distribution between assigned 2n and 4n DNA content (Fig. 3B, pink bars). The remaining cells in gap-phase correspond to one of two distinct populations: a small population of cells has 2n G1 DNA content while a much larger population has 4n G2 DNA (Fig. 3B, blue bars). The frequency of G2 outnumbers the frequency of G1 roughly 20 to 1, confirming that G1 is a very short compared to G2.

Taken together, the above results allow us to model the mitotic cell cycle structure (Table 1). S-phase, as determined by the labeling index of a short EdU-pulse (<30 minutes), occupies ~57% of the total cell cycle (similar to ~50%, Crittenden et al. 2006 and 40-50%, Jaramillo-Lambert et al. 2007) while M-phase, as determined by the pH3 staining index, occupies 2% of the total cell cycle, consistent with previous results (Hansen et al. 2004a; Crittenden et al. 2006; Maciejowski et al. 2006). G1 and G2 occupy the remaining 41% of the cell cycle during which cells are both pH3 and EdU-negative. Our results above analyzing DNA content indicate that 95% of these cells are in G2. Therefore, G2 represents about 39% (95% of 41%) and G1 represents about 2% (5% of 41%) of the total cell cycle.

When combined with our kinetic measurements, this cell cycle model can be used to extrapolate a total cell cycle length estimate. Using the method in Crittenden et al 2006, whereby the absolute and relative length of G2+M+G1 (3.5 hours and 43%, respectively) are combined to extrapolate a total length of the cell cycle, we obtain 8 hours. However, the assay for measuring the absolute length of G2+M+G1 only provides a maximum value (as opposed to median or average). Using 2.5 hours for the median length of G2 as 39% of the total cell cycle provides an estimate of 6.5 hours for the total cell cycle length, in a similar range as the 5-hour estimate obtained from the pulse-chase experiment in Figure 2. However, these estimates deviate significantly from 16-24 hours reported in Crittenden et al. (2006) where the cell cycle estimate was derived from the measurement of G2+M+G1 (8-12 hours, 50% of the cell cycle). The discrepancy in estimates largely arises from a different measurement of the absolute length of G2+M+G1 (3.5 hours versus 8-12 hours); however this difference cannot be explained by differences between the wild-type strains employed (see Fig. S4). Experimentally, it is unclear how different results were obtained for this estimate.

As an additional assessment of cell cycle activity, we analyzed the output of the proliferative zone (number of cells entering meiotic prophase per unit time) by counting the flux of EdU-labeled cells out of the proliferative zone (Fig. S5). We assume that the output of a proliferative zone of constant size is determined by the number of cells actively dividing and their average cell division rate. Bearing in mind some proliferative cells are in meiotic S-phase, an average germline contains fewer than 230 actively cycling cells (Fig. 1). Experimentally, we observed an output of ~20 cells per hour (Fig. S5). An average cell cycle length of 6.5 hours requires 130 actively cycling cells to

achieve an output of 20 cells per hour while an 8 hour cell cycle requires ~160 cells (in contrast, a 16 hour cell cycle requires >350). These data suggest that ~60-70% of proliferative cells are actively cycling while ~30-40% are premeiotic.

Constant CYE-1/CDK-2 activity may drive rapid and continuous cell cycle progression

What regulatory features underlie this continuous and rapid cell cycle progression? In various cell types that lack G1, high cyclin E/CDK2 activity throughout the cell cycle is thought to drive entry into S-phase independent of the G1 factors cyclin D and CDK4 (Orford and Scadden 2008). Consistent with this, genetic mosaic analysis indicated that *cdk-4* is not required for cell cycle progression in the germline (Fig. S6). Given both a lack of G1 and lack of requirement for CDK-4, we asked whether CYE-1 level is also high throughout the germline mitotic cell cycle. As previously reported, CYE-1 is found in nuclei throughout the proliferative zone (Brodigan et al. 2003; Biedermann et al. 2009)(see below). To investigate whether CYE-1 levels fluctuate according to cell cycle stage, we compared the level of CYE-1 in S-phase cells with cells not in S-phase (predominantly G2 with some M) (Fig. 4A-B) and found that CYE-1 appears constant throughout the cell cycle. In addition, we analyzed the abundance of phospho-CDC-6, a potential CDK-2/CYA-1 substrate that may at least in part be dependent on CDK-2/CYE-1 activity (Kim et al. 2007). Nucleolar localized phospho-CDC-6 is present in all CYE-1 positive cells and is CYE-1 dependent, consistent with constant CDK-2/CYE-1 activity throughout the cell cycle (Fig. S7).

Although CDK-4 is not required for germline cell cycle progression, depletion of CYE-1 or CDK-2 causes cell cycle arrest (Fay and Han 2000). Consistent with these factors specifically functioning to promote progression into or through S-phase, most of the arrested proliferative cells in *cye-1 (RNAi)* treated germlines contained ~2n DNA content (Fig. 4C). Therefore, high CDK-2/CYE-1 activity may allow proliferative germ cells to bypass G1 by promoting progression into S-phase.

CYE-1 and CDK-2 promote the proliferative fate

CYE-1 could act to promote the stem cell fate in addition to promoting cell cycle progression, with the prediction that depletion of CYE-1 or CDK-2 could cause proliferative cells to prematurely enter meiosis. Since depletion of CYE-1 or CDK-2 leads to cell cycle arrest (Fig 4C) which may mask meiotic entry, we asked whether RNAi depletion could promote meiotic entry of proliferative cells in a sensitized genetic background containing the *glp-1(bn18)* mutation (Qiao et al. 1995). We depleted CYE-1 by RNAi from *glp-1(bn18)* L4 animals, at the permissive temperature, in parallel with control *gfp(RNAi)* (Fig. 5A-C). After 48 hours of RNAi treatment, no cells with the proliferative fate were evident in *glp-1(bn18); cye-1(RNAi)* germlines as meiotic cells extend to the distal end, a phenotype indicating premature meiotic entry. This loss of proliferative cells due to increased meiotic entry indicates that CYE-1 plays an important role in regulating the decision to proliferate versus enter meiosis.

To address whether this premature meiotic entry phenotype is specific to *cye-1* or whether general loss of cell cycle function can also promote premature meiotic entry, we performed an RNAi screen for enhancement of *glp-1(bn18)* with a panel of cell cycle

factors, as well as a set of GLD-1 mRNA targets that are enriched for cell cycle genes (Fig. 5D; Fig. S8). Strikingly, while multiple factors produced significant cell cycle defects, only *cye-1* and *cdk-2* enhanced *glp-1(bn18)* to produce premature meiotic entry. Thus, the ability of *cye-1* and *cdk-2* to promote the proliferative fate is not likely a general cell cycle property but rather a function specific to these factors.

***cye-1*/*cdk-2* is epistatic to known meiotic entry regulatory pathways**

We next asked where CYE-1/CDK-2 act relative to the currently described genetic pathway for regulation of the proliferative versus meiotic cell fate decision. The conserved GLP-1/Notch signaling pathway promotes the proliferative fate. Acting downstream of GLP-1 are two redundant pathways that promote meiotic entry, the GLD-1 and GLD-2 pathways. While loss of GLP-1 function causes proliferative cells to enter meiosis prematurely, simultaneous loss of both the GLD-1 and GLD-2 pathways causes a defect in entry into meiosis; this leads to germline overproliferation and prevents gamete production (Austin and Kimble 1987; Kadyk and Kimble 1998; Hansen et al. 2004a). We asked whether CYE-1 or CDK-2 depletion could promote meiotic entry independent of the activity of the GLD-1 and GLD-2 pathways. Thus we performed *cye-1(RNAi)* or *cdk-2(RNAi)* in a series of double mutants containing putative null alleles of one gene in GLD-1 pathway and another gene in the GLD-2 pathway (Table 2) and asked whether loss of CYE-1 or CDK-2 would cause ectopic proliferative cells to enter meiosis. As previously shown, when germlines were mutated in both the GLD-1 and GLD-2 pathways, germlines display ectopic proliferative cells with some or no evidence of meiotic entry depending on the genes used, according to HIM-3 and pSUN-1 (an

additional marker for initiation of meiotic prophase (Penkner et al. 2009)) staining (Fig. 6A, Fig. S9). However, upon depletion of CYE-1 or CDK-2, we observed large numbers of the germ cells entering meiosis following 48 hours of RNAi treatment. As a control, when we depleted CDK-1 by RNAi from germlines lacking the GLD-1 and GLD-2 pathways, we observed cell cycle arrest but did not observe increased entry into meiosis as determined by REC-8, HIM-3 and pSUN-1 staining (Table 2, data not shown). Therefore, loss of CYE-1 or CDK-2 is able to promote meiotic entry independent of the activity of either the GLD-1 or GLD-2 pathway. Based on this, we conclude that CYE-1/CDK-2 promotes the proliferative fate downstream of or in parallel to the GLD-1 and GLD-2 pathways.

Although meiotic entry occurred throughout much of the germline in the above experiments, we never observed meiotic entry in the distal-most cells in the germline (Fig. 6, Fig. S9). This distal region corresponds to the proliferative zone in wild-type where LAG-2 and APX-1 expression in the DTC mediates activation of the GLP-1 signaling. We hypothesized that GLP-1 activity, restricted to these distal proliferative cells, acts independent of CDK-2/CYE-1 to promote the proliferative fate. Since the GLD-1 and GLD-2 pathways are genetically downstream of GLP-1 signaling, *gld-1* pathway *gld-2* pathway; *glp-1* null triple mutants are tumorous (Kadyk and Kimble 1998, Hansen et al 2004a). Therefore, we asked whether *cye-1(RNAi)* could cause distal germ cells to enter meiosis in a series of *gld-1 gld-2* pathway; *glp-1* triple mutants. While *gld-1 gld-2* pathway; *glp-1* mutants remain tumorous when treated with *gfp(RNAi)*, we observed widespread meiotic entry in the adult germlines after 48 hours of *cye-1(RNAi)* (Table 2, Fig. 6B, Fig. S9). Importantly, we observed meiotic entry occurring in the

distal-most cells, in contrast to the absence of meiotic entry in the distal-most cells in the *gld-2 gld-1* pathway*; cye-1(RNAi)* germlines. We interpret these results to indicate that *glp-1* can act independent of *cye-1*/*cdk-2* to promote the proliferative fate. Furthermore, these results indicate that GLP-1 signaling must have an additional activity besides regulating the GLD-1 and GLD-2 pathways since, as assessed by *cye-1(RNAi)*, GLP-1 can promote the proliferative fate/inhibit meiotic entry even in the absence of their activity. Furthermore, this result sheds light on our initial analysis of CYE-1/CDK-2 depletion in the germline. *cye-1(RNAi)/cdk-2(RNAi)* in wild-type causes proliferative cells to arrest in the cell cycle but does not cause premature meiotic entry. In contrast, we observed premature meiotic entry in a sensitized background with decreased GLP-1 activity. In conclusion, loss of CYE-1/CDK-2 only causes meiotic entry when GLP-1 signaling is reduced.

CYE-1 is targeted for degradation upon entry into meiosis

CYE-1 remains high throughout the mitotic cell cycle, but its expression sharply decreases as germ cells enter meiosis. GLP-1 signaling, however, is not necessary for CYE-1 accumulation as CYE-1 accumulates in proliferative cells of *gld-1 gld-2* pathway; *glp-1* null triple mutants (Fig. S10). Previously, it has been reported that GLD-1 binds and represses *cye-1* mRNA during the pachytene stage of meiotic prophase, suggesting that GLD-1 could be responsible for the repression of CYE-1 upon entry into meiosis (Biedermann et al. 2009). However, we found that the initial down regulation of CYE-1 remains intact in *gld-1* null mutants, indicating that a redundant mechanism may play a role in mediating CYE-1 repression in this region (Fig. 7A-B). To investigate whether

CYE-1 is targeted for degradation upon meiotic entry, we analyzed CYE-1 in germlines depleted of candidate ubiquitin ligase factors (data not shown). This analysis implicated three components of an SCF ubiquitin ligase that are required for CYE-1 repression: CUL-1 (Kipreos et al. 1996), SKR-1/2 (Nayak et al. 2002) and PROM-1 F-box-like (Jantsch et al. 2007). In *prom-1* mutants for example, CYE-1 decreases gradually after entry into meiosis, in contrast to the immediate repression in wild-type germlines (Figure 7A, C). This suggests that a PROM-1 dependent pathway may act together with GLD-1 to repress CYE-1 expression. To examine this, we analyzed *gld-1prom-1* double mutants and found that CYE-1 remains high throughout the germline (Fig. 7D). These data suggest that GLD-1 acts in parallel with an SCF^{prom-1} ubiquitin ligase to repress and maintain low CYE-1 upon meiotic entry. Importantly, even in the presence of ectopic CYE-1, germ cells still enter meiosis in both *prom-1* single mutants as well as *gld-1prom-1* double mutants. Therefore, while CYE-1 is necessary to maintain the proliferative fate in certain instances, CYE-1 alone is not sufficient to promote the proliferative fate. Additionally, the combined activities of the GLD-1 and GLD-2 pathways are not sufficient for down regulation of CYE-1 as germ cells enter meiosis; in the few germ cells of *gld-1gld-2* pathway double mutants that enter meiosis, CYE-1 levels fall (Fig. S10, data not shown).

Discussion

Cell cycle progression in the *C. elegans* germline

Proliferating germ cells in the adult hermaphrodite display three important kinetic characteristics: 1) rapid cell cycle progression, 2) continuous cell cycle progression and

3) a cell cycle structure in which G1-phase is highly abbreviated or absent. Our results indicate that the average length of the cell cycle at 20°C under standard laboratory conditions is ~6.5-8 hours. Our results also describe the length of individual phases of the cell cycle: S-phase comprises ~57% of the total cell cycle; G2 comprises ~39%; M and G1 comprise a small fraction of the cell cycle (Fig. 8A). Our data provide a model that ties together the rate of cell division, meiotic cell production and the number of actively proliferating cells (Fig. 8B).

Additional observations on regulatory features of the germline cell cycle correlate well with the cell cycle kinetics. CDK-4, a cyclin-dependent kinase required for cell cycle progression in larval somatic cells with a significant G1 phase (Park and Krause 1999), is not required for cell cycle progression in the adult germline. In addition, CYE-1 is present throughout the germline mitotic cell cycle in contrast to its canonical periodic expression in somatic cells. High levels of CYE-1 may be responsible for driving germ cells through the cell cycle without an appreciable delay in G1 and requirement for G1-CDK, CDK-4. High levels of CYE-1 are also observed in other rapidly dividing cells that lack a significant G1 phase, including mouse embryonic stem cells (Stead et al. 2002) and the *Drosophila* and *Xenopus* embryos (Richardson et al. 1993; Rempel et al. 1995). Thus high CYE-1 levels may contribute both to the brief G1-phase and continuous cell cycle progression in the adult germlines.

Role of CDK-2/CYE-1 in promoting the proliferative fate

We have shown that CDK-2/CYE-1 regulates cell fate, adding to a growing body of evidence supporting this role, including previous examples in *Drosophila* (Berger et al.

2005), *C. elegans* (Fujita et al. 2007) and mouse embryonic stem cells (Neganova et al. 2009). Cyclin dependent kinases, including CDK2, are well-documented cell cycle progression regulators through phosphorylation of key targets, and CDK-2/CYE-1 may promote the proliferative fate by a similar mechanism. Alternatively, CDK-2/CYE-1 may promote the proliferative fate by regulating cell cycle structure rather than via phosphorylation of specific regulatory factors *per se*. For example, in mouse ES cells, one model for maintenance of pluripotency is through limiting the time spent in G1-phase, when differentiation-inducing factors are thought to act upon stem cells. This mechanism allows cells to maintain their pluripotent and self-renewal potential by avoiding the differentiation permissive G1-phase (Orford and Scadden 2008). The mechanism by which CDK-2/CYE-1 regulates cell fate awaits further investigation and may be distinct in different cell types.

In *C. elegans*, CDK-2/CYE-1 promotes the germline proliferative fate and endogenous repression of CYE-1 as cells enter meiosis may be critical for signaling meiotic entry. In both yeast cells and the mouse germline, the decision to enter meiosis is thought to occur prior to meiotic S-phase (Baltus et al. 2006). Paradoxically, CYE-1 is repressed at the time of morphological entry into meiotic prophase, after meiotic S-phase, raising the question of how CDK-2/CYE-1 influences the timing of meiotic entry. Perhaps CYE-1/CDK-2 pathway activity is not primarily regulated by CYE-1 levels, but rather by regulation of another pathway component or a separate posttranslational modification. Alternatively, the decision to enter meiosis in *C. elegans* could occur after meiotic S-phase. Finally, repression of CYE-1 could simply serve to reinforce meiotic entry following the initiation of another signal. Consistent with these models, we find that

ectopic CYE-1 in germlines depleted of an SCF ubiquitin ligase is not sufficient to promote the proliferative fate. Regardless, CYE-1/CDK-2 activity is necessary for maintaining the proliferative fate in the presence of reduced GLP-1 activity, and high CYE-1 throughout the cell cycle may be a necessity of this role. Periodic or otherwise unstable CYE-1 levels could lead to unstable maintenance of the proliferative fate or proliferative zone size.

Analysis of genetic interactions among CYE-1, GLP-1 and the GLD-1 and GLD-2 pathways reveals two important findings in terms of where CYE-1/CDK-2 act relative to these factors in the decision between proliferation versus entry into meiosis (Fig. 8C-D). First, depletion of CYE-1 or CDK-2 promotes meiotic entry independent of the activity of the GLD-1 and GLD-2 pathways. Second, GLP-1 can act independent of CYE-1 or CDK-2 to promote the proliferative fate. This first finding indicates that CYE-1/CDK-2 acts downstream of or in parallel to the GLD-1 and GLD-2 pathways. Intriguingly, *cye-1* mRNA is a known target of GLD-1, suggesting CYE-1 may be a true downstream factor in this pathway (Biedermann et al. 2009). While this in turn may place CYE-1 downstream of GLP-1, our second finding adds an important point to this genetic model: GLP-1 retains the ability to promote the proliferative fate despite RNAi knockdown of CYE-1/CDK-2. While we cannot exclude the possibility of residual CDK-2/CYE-1 kinase activity, this result suggests that GLP-1 provides some activity in parallel to CDK-2/CYE-1 as well as the GLD-1 and GLD-2 pathways. Indeed, previous work suggested that there may be a third pathway that acts downstream of GLP-1 to promote meiotic entry (Hansen et al. 2004a).

In the *Drosophila* ovary, similar to what we observe in the *C. elegans* germline, decreased cyclin E causes premature meiotic entry during cystocyte transit amplification (Lilly and Spradling 1996), and cyclin E levels drop as germ cells enter meiotic prophase, at least in part due to SCF mediated protein degradation (Doronkin et al. 2003; Narbonne-Reveau and Lilly 2009). However, the precipitous fall in cyclin E levels as germ cells enter meiotic prophase, in both *C. elegans* and *Drosophila*, is not likely a trigger for entry into meiosis because it occurs after meiotic S-phase. The *Drosophila* ovary also displays similarities in cell cycle structure with *C. elegans* among the germline stem cells (Hsu et al. 2008). However, a few differences exist between *C. elegans* and *Drosophila* germlines: In stem cells and transit-amplifying cells of *Drosophila* ovary, cyclin E level decreases during S-phase through SCF mediated degradation (Lilly et al. 2000; Hsu et al. 2008; Narbonne-Reveau and Lilly 2009). Whereas in *C. elegans*, CYE-1 levels are remain high throughout the cell cycle, suggesting the SCF mediated CYE-1 degradation is inactive in proliferating cells. Additionally, while it is currently unclear if *C. elegans* has transit-amplifying cells, it does not have proliferative cells analogous to cystocytes, which undergo a stereotypical pattern of cell divisions that are synchronous and uncoupled from cell growth.

Conclusion

CDK-2/CYE-1 acts not only in cell cycle progression but also in stem cell maintenance. This dual activity allows CDK-2/CYE-1 to coordinate self-renewal of stem cells. CDK-2/CYE-1 may also perform this role in other stem cells, with most likely candidates including embryonic stem cells. However, our results also indicate that other

pathways, in this case the GLP-1/Notch pathway, may also act independently to regulate stem cell fate.

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Figure 1

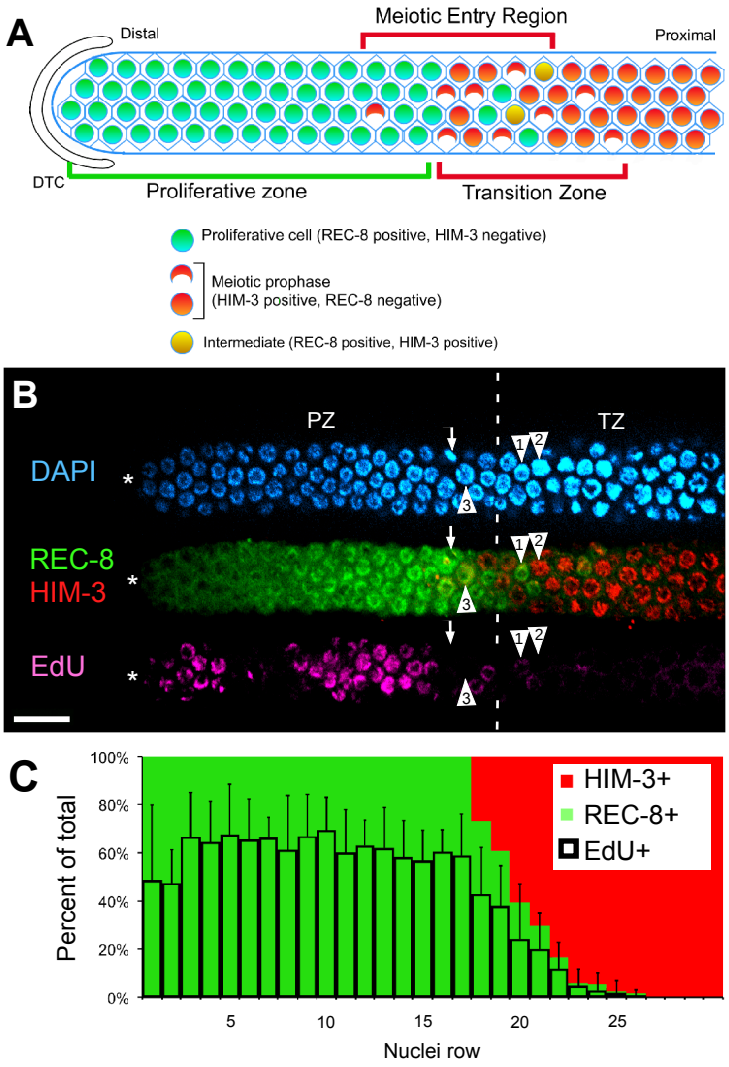

A
Distal
Meiotic Entry Region
Proximal
DTC
Proliferative zone
Transition Zone
Proliferative cell (REC-8 positive, HIM-3 negative)
Meiotic prophase
(HIM-3 positive, REC-8 negative)
Intermediate (REC-8 positive, HIM-3 positive)
B
PZ
TZ
DAPI
REC-8
HIM-3
EdU
C
Percent of total
100%
80%
60%
40%
20%
0%
5
10
15
20
25
Nuclei row
HIM-3+
REC-8+
EdU+

Figure 1. S-phase occurs equivalently throughout the proliferative zone. (A) The adult proliferative zone in the distal end of the germline contains ~230 cells that are defined by the presence of proliferative zone markers and the absence of meiotic prophase markers (REC-8 positive and HIM-3/pSUN-1 negative). The meiotic entry region contains proliferative cells as well as cells that entered meiotic prophase (REC-8 negative and HIM-3/pSUN-1 positive). The transition zone (TZ) marks the distal-proximal boundaries where crescent-shaped leptotene/zygotene meiotic prophase nuclei are observed. The distal boundary of the TZ is determined by the first row with multiple crescent shaped nuclei. (B) Confocal section of the surface layer of the distal portion of a dissected adult hermaphrodite germline. Proliferative cells are identified using anti-REC-8 staining (green) while cells in meiotic prophase are identified using anti- HIM-3 staining (red). Cells in S-phase (pink) are labeled with a <30-minute EdU-pulse. HIM-3 positive nuclei are EdU-negative. Arrow points to an M-phase cell near the TZ. Arrowheads point to adjacent nuclei within the TZ that are in distinct stages of development: (1) an EdU-and REC-8-positive nucleus (EdU-signal is dim) that is likely in meiotic S-phase, (2) an EdU-negative HIM-3-positive nucleus that is in meiotic prophase and (3) an EdU-negative HIM-3-negative REC-8-positive nucleus (scale bar=20μm). (C) Cells in rows 1-30 were analyzed for three characteristics on a per row basis: REC-8, HIM-3 and S-phase (EdU incorporation after short pulse). Graph shows percentage of cells in each row that are positive for each marker. For all figures, error bars show standard deviation.

Figure 2

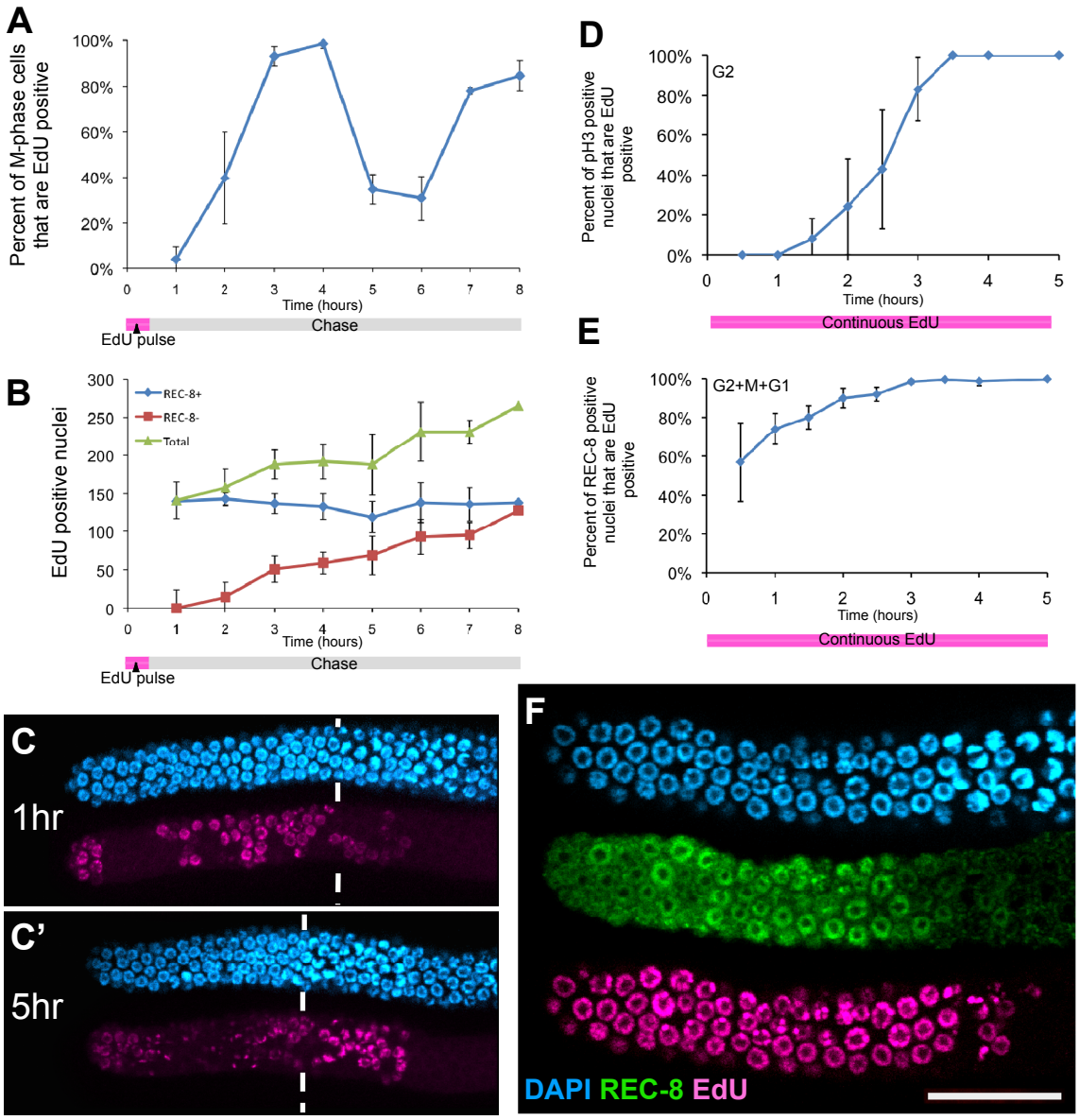

A
Percent of M-phase cells that are EdU positive
100%
80%
60%
40%
20%
0%
0 1 2 3 4 5 6 7 8
Time (hours)
Chase
EdU pulse
B
EdU positive nuclei
300
250
200
150
100
50
0
REC-8+
REC-8-
Total
0 1 2 3 4 5 6 7 8
Time (hours)
Chase
EdU pulse
D
Percent of pH3 positive nuclei that are EdU positive
G2
100%
80%
60%
40%
20%
0%
0 1 2 3 4 5
Time (hours)
Continuous EdU
E
Percent of REC-8 positive nuclei that are EdU positive
G2+M+G1
100%
80%
60%
40%
20%
0%
0 1 2 3 4 5
Time (hours)
Continuous EdU
C
1hr
C'
5hr
F
DAPI REC-8 EdU

Figure 2. Kinetic analysis of mitotic cell cycle progression in the germline. (A) Mitotic cell cycle length was estimated by a pulse-chase experiment. Cohorts of EdU-pulsed (S-phase labeled) cells were examined as they traversed the cell cycle and passed M-phase. Animals from the pulse-chase experiment were dissected at one-hour intervals and stained for EdU incorporation, pH3 and REC-8. Graph plots the percent of cells in M-phase (pH3-positive) that contain EdU. (B) Plot of total number of EdU-positive nuclei from pulse-chase experiment scored as REC-8 positive or negative. Averages are from two separate experiments. (C-C') Representative images of germlines from 1 and 5 hours of the pulse-chase experiment shows that by 5 hours there is a decrease in EdU signal among distal nuclei due to cell division while proximal nuclei, that retain high EdU intensity, have entered meiosis. (D-F) Animals were fed EdU continuously starting at t=0 and dissected at 30 minute intervals to obtain estimates of phases of the cell cycle. (D) Length of G2 estimated by analyzing the percent of cells in M-phase (pH3-positive) that are EdU-positive during the time course. (E) Length of G2+M+G1 was estimated by analyzing the percent of all REC-8 positive nuclei that are EdU-positive. Averages are compiled from three experiments with at least 10 germlines analyzed per time-point in each experiment. (F) Representative germline after 3.5 hours of continuous EdU labeling showing all proliferative cells have incorporated EdU (scale bars=20μm).

Figure 3

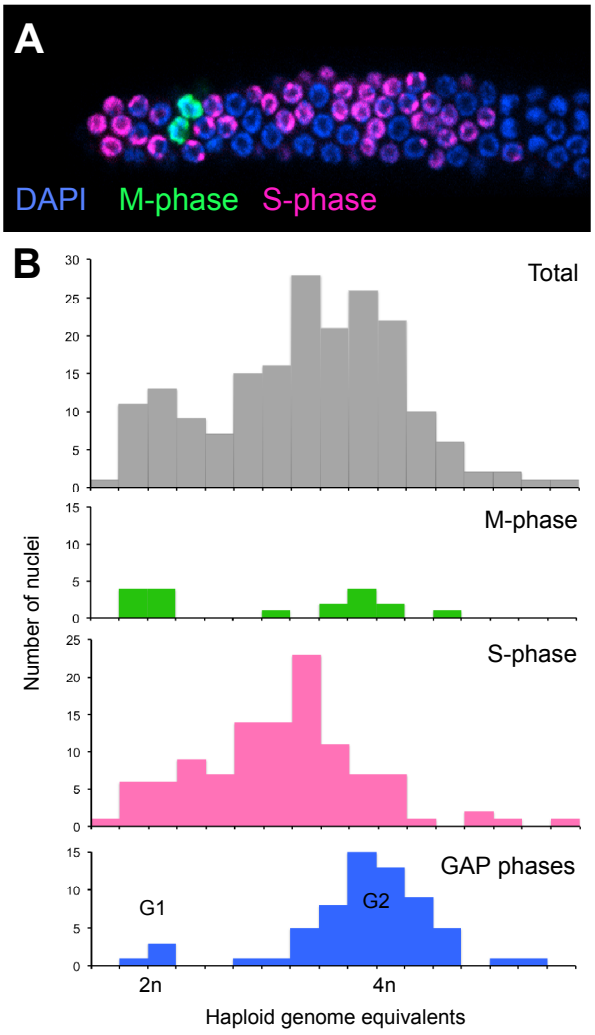

A
DAPI
M-phase
S-phase
B
Total
M-phase
S-phase
GAP phases
G1
G2
2n
4n
Number of nuclei
Haploid genome equivalents

Figure 3. G1 is largely absent from the germline mitotic cell cycle. (A) Animals were fed a 30-minute pulse of EdU, immediately dissected and stained for EdU incorporation (S-phase) and pH3 (M-phase). (B) DNA content of proliferative cells was assessed by confocal microscopy, and cells were scored for M-phase, S-phase and unlabeled GAP phase. The fluorescent intensity values corresponding to 2n and 4n DNA content, x-axis, were assessed by analyzing prophase and metaphase nuclei (4n) and individual daughters from anaphase and telophase (2n) as internal controls.

Figure 4

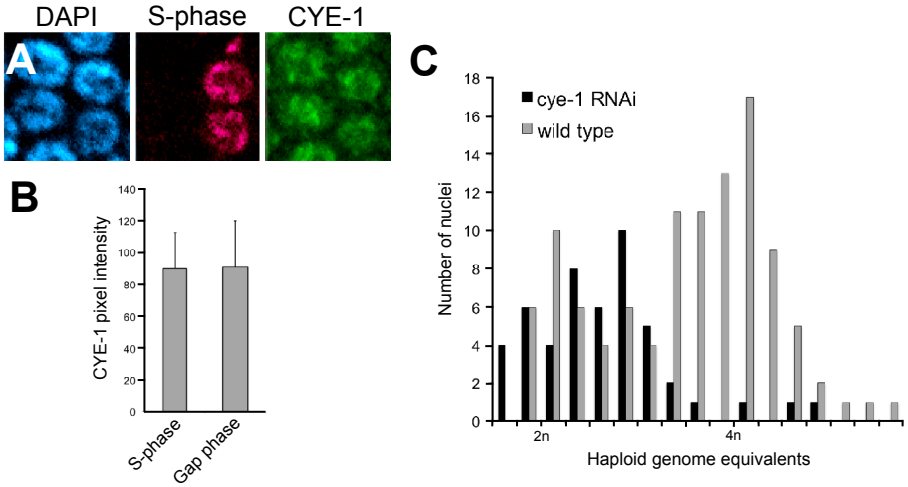

DAPI
S-phase
CYE-1
A
B
CYE-1 pixel intensity
140
120
100
80
60
40
20
0
S-phase
Gap phase
C
cye-1 RNAi
wild type
Number of nuclei
18
16
14
12
10
8
6
4
2
0
2n
4n
Haploid genome equivalents

Figure 4. CYE-1 remains high throughout mitotic cell cycle progression and is required for passage through S-phase. (A) CYE-1 is present at equal levels in S-phase and GAP phase nuclei. S-phase cells were identified by EdU incorporation (pink) after a short pulse and CYE-1 protein (green) was visualized by anti-CYE-1 staining. (B) Average fluorescence values were determined by assaying the pixel intensity of nuclear CYE-1 from confocal images. (C) DNA content of *cye-1(RNAi)* cell cycle arrested or wild-type control proliferative zone nuclei was determined by measuring DAPI fluorescent intensities from confocal image stacks.

Figure 5

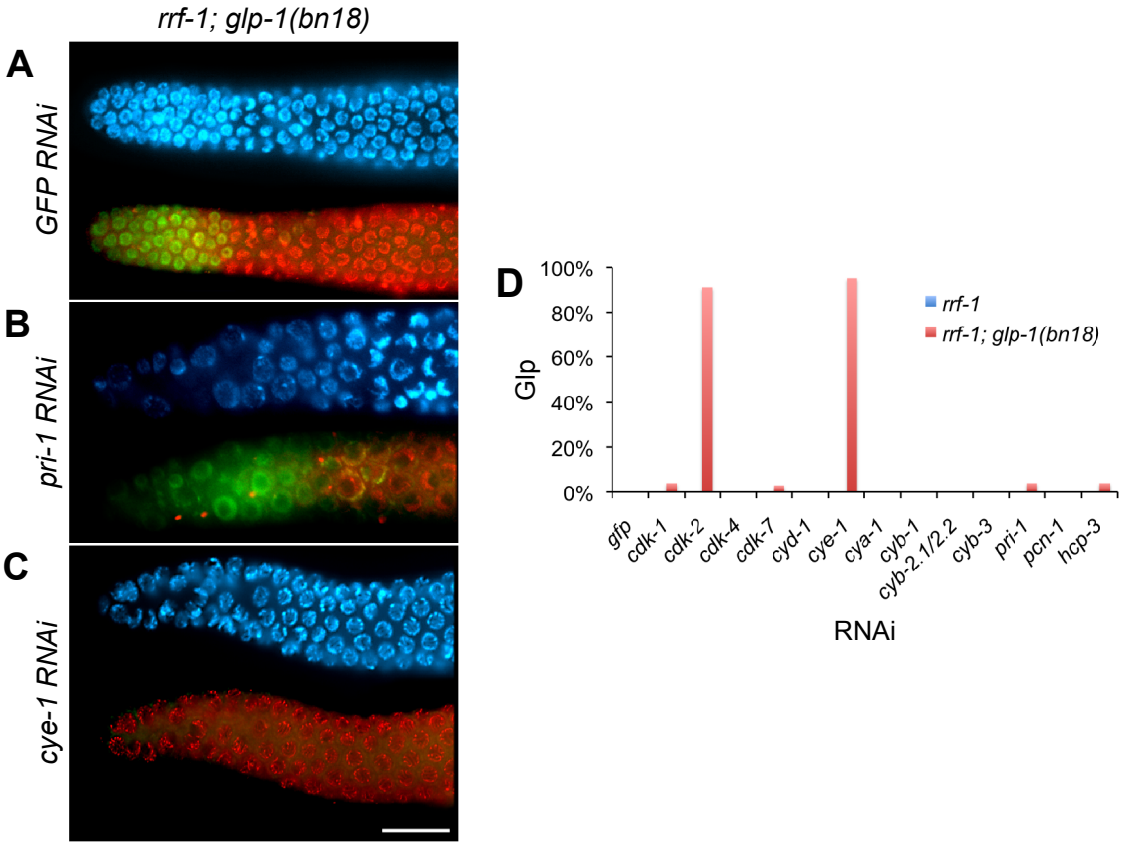

rrf-1; glp-1(bn18)
A
GFP RNAi
B
pri-1 RNAi
C
cye-1 RNAi
D
100%
80%
60%
40%
20%
0%
Glp
rrf-1
rrf-1; glp-1(bn18)
gfp
cdk-1
cdk-2
cdk-4
cdk-7
cyd-1
cye-1
cya-1
cyb-1
cyb-2.1/2.2
cyb-3
pri-1
pcn-1
hcp-3
RNAi

Figure 5. CDK-2 and CYE-1 promote the proliferative fate of germ cells. *glp-1(bn18)* was used as a sensitized genetic background for monitoring premature meiotic entry. To examine premature meiotic entry in *glp-1(bn18)* adult hermaphrodites, L4s were fed RNAi bacteria for 48 hours. Proliferative cells were identified via anti-REC-8 staining (green) and cells in meiotic prophase identified via anti-HIM-3 staining (red). *pri-1(RNAi)* serves to represent the enlarged nuclei cell cycle arrest phenotype when most cell cycle factors were depleted by RNAi (B and D). In contrast, depletion of *cye-1* or *cdk-2* in *glp-1(bn18)* mutants resulted in a complete loss of the proliferative zone due to premature meiotic entry (C and D)(scale bar=20μm).

Figure 6

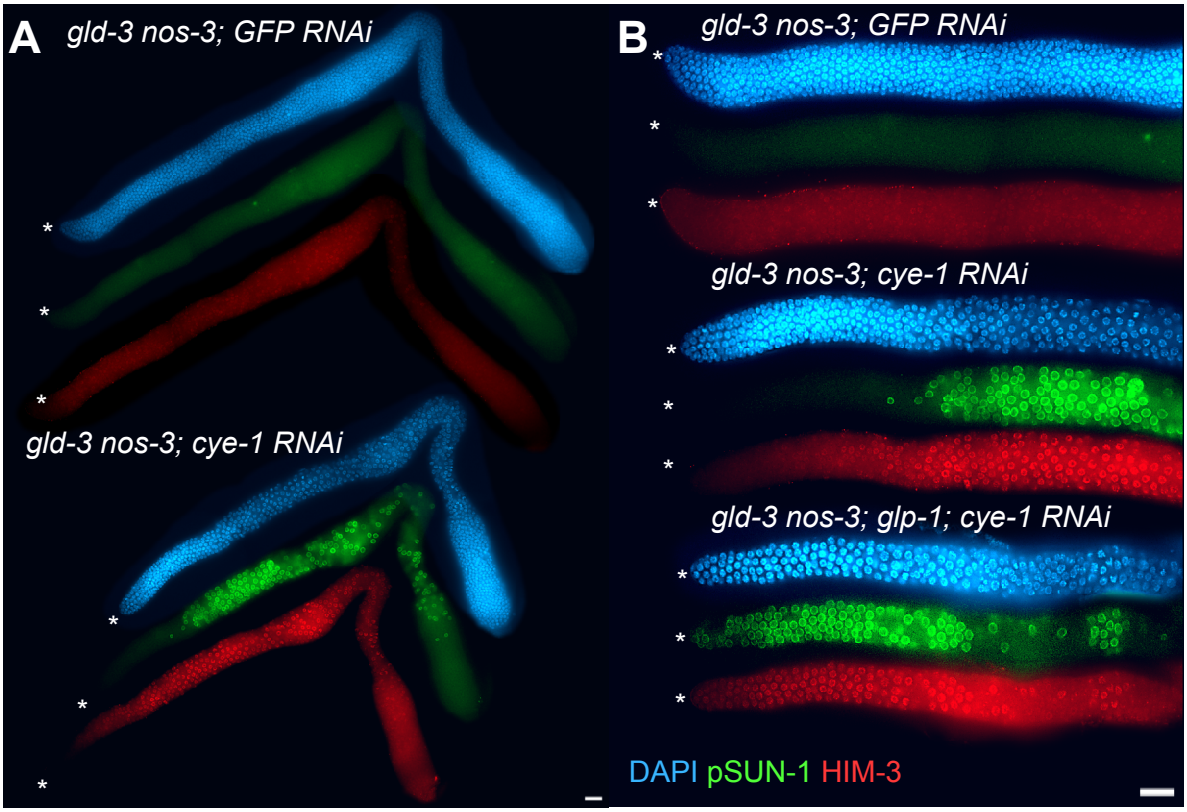

A
gld-3 nos-3; GFP RNAi
gld-3 nos-3; cye-1 RNAi
B
gld-3 nos-3; GFP RNAi
gld-3 nos-3; cye-1 RNAi
gld-3 nos-3; glp-1; cye-1 RNAi
DAPI pSUN-1 HIM-3

Figure 6. CYE-1 is epistatic to the GLD-1 and GLD-2 pathways and acts in parrallel to GLP-1. (A) *gld-3(q730)nos-3(oz231)* null double mutants eliminate the function of both the GLD-1 and GLD-2 pathways, displaying a complete germline tumorous phenotype. Knockdown of CYE-1 by RNAi suppresses the tumorous phenotype by causing proliferative cells to initiate meiotic development, shown by positive HIM-3 (red) and pSUN-1 (green) nuclear staining. Meiotic entry is observed throughout the germline except in the distal region that corresponds to the proliferative zone in wild-type. (B) *gld-3(q730)nos-3(oz231); glp-1(q175)* null triple mutants have a tumorous germline phenotype since loss of the GLD-1 and GLD-2 pathways is epistatic to loss of GLP-1. Here, *cye-1(RNAi)* in a *gld-3(q730)nos-3(oz231); glp-1(q175)* triple mutant causes germ cells at all position, including distal, to enter meiosis. *Marks the distal end (scale bar=20μm).

Figure 7

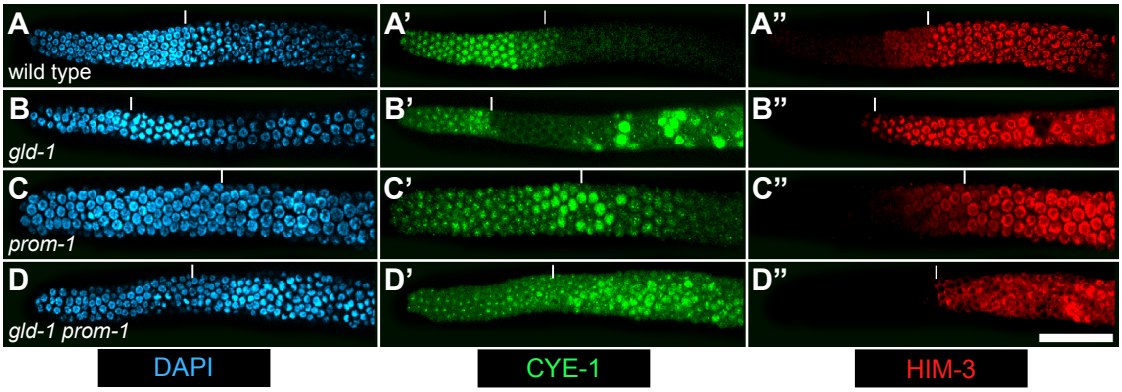

A
wild type
A'
A"
B
gld-1
B'
B"
C
prom-1
C'
C"
D
gld-1 prom-1
D'
D"
DAPI
CYE-1
HIM-3

Figure 7. PROM-1 and GLD-1 act to repress CYE-1 in the germline. (A-D) Animals of the indicated genotype were staged at 24 hours past L4, dissected and germlines examined by anti-CYE-1 (green) and anti-HIM-3 (red) staining. A) Wild-type germlines show immediate repression of CYE-1 upon entry into meiosis. (B) *gld-1(q485)* null mutants fail to maintain low CYE-1 during meiotic prophase; however, initial repression of CYE-1 upon meiotic entry is intact. (C) *prom-1(ok1140)* null mutants display a significant delay in repressing CYE-1 relative to the onset of meiotic entry. (Previous work showed that pairing is defective in prom-1 null (Jantsch et al. 2007); however, the pairing defect can not be solely due to ectopic CYE-1 as *cye-1(RNAi)* fails to suppress this defect (data not shown). (D) *gld-1(q485)prom-1(ok1140)* null double mutants fail to show any signs of CYE-1 repression.

Figure 8

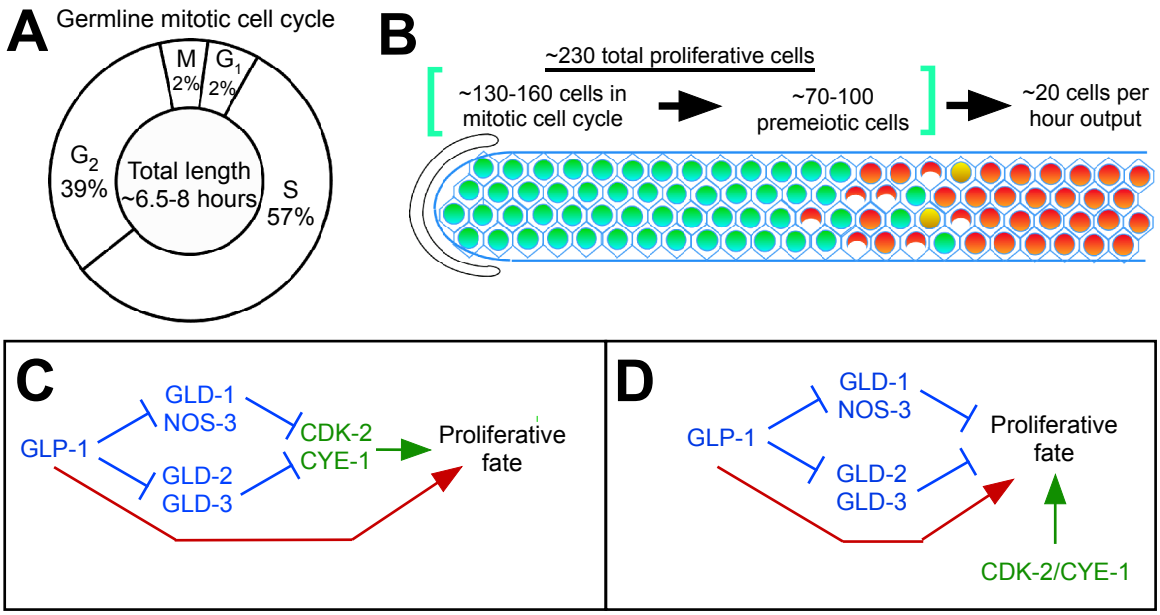

A
Germline mitotic cell cycle
M
2%
G1
2%
G2
39%
Total length
~6.5-8 hours
S
57%
B
~230 total proliferative cells
~130-160 cells in
mitotic cell cycle
~70-100
premeiotic cells
~20 cells per
hour output
C
GLP-1
GLD-1
NOS-3
GLD-2
GLD-3
CDK-2
CYE-1
Proliferative
fate
D
GLP-1
GLD-1
NOS-3
GLD-2
GLD-3
Proliferative
fate
CDK-2/CYE-1

Figure 8. Summary and Model. (A) The mitotic cell cycle in the adult hermaphrodite germline lacks a significant G1-phase. S-phase, as demonstrated here and by others, occupies the largest part of the cell cycle. We found that G2 also comprises a significant part of the cell cycle. This model is consistent with regulatory characteristics and marker accumulation. (B) Our data combine to describe how proliferation in the germline is balanced by production of cells that enter meiosis. Proliferating germ cells undergo cell division on average once every 6.5-8 hours, which is balanced by ~20 cells entering meiosis per hour. This rate of cell production predicts that ~130-160 cells or ~60-70% of the proliferative zone is undergoing mitotic cell cycle progression. This leaves an additional ~70-100 premeiotic cells that are not engaged in mitotic cell cycle progression (see Fig. S5). (C-D) Interactions among CYE-1/CDK-2, GLP-1 and the GLD-1 and GLD-2 pathways are consistent with at least two genetic models. Previous work indicates that the GLD-1 and GLD-2 pathways lie downstream of GLP-1. (C-D) CDK-2/CYE-1 (green) may lie downstream of the GLD-1 and GLD-2 pathways (C) or act in parrallel (D). In addition and relevant to both models, our data indicate that GLP-1 activity (red) also promotes the proliferative fate/inhibits meiotic development by a mechanism independent of CDK-2/CYE-1 and the GLD-1 and GLD-2 pathways.

Table 1. Cell cycle summary

Proliferative zone cells[1]	231±23 (n=18)
S-phase cells[2]	133±20 (n=18)
S-phase index[3]	57±5%
S-phase length[4]	3.7 hours
M-phase cells[5]	5.2±2.3 (n=37)
M-phase index[6]	2%
G1 index[7]	2%
G2 index[7]	39%
G2 length[8]	2.5 hours
Cell cycle length (Based on G2 mean)[9]	6.5 hours
Cell cycle length (Based on G2+M+G1 maximum value)[10]	8 hours

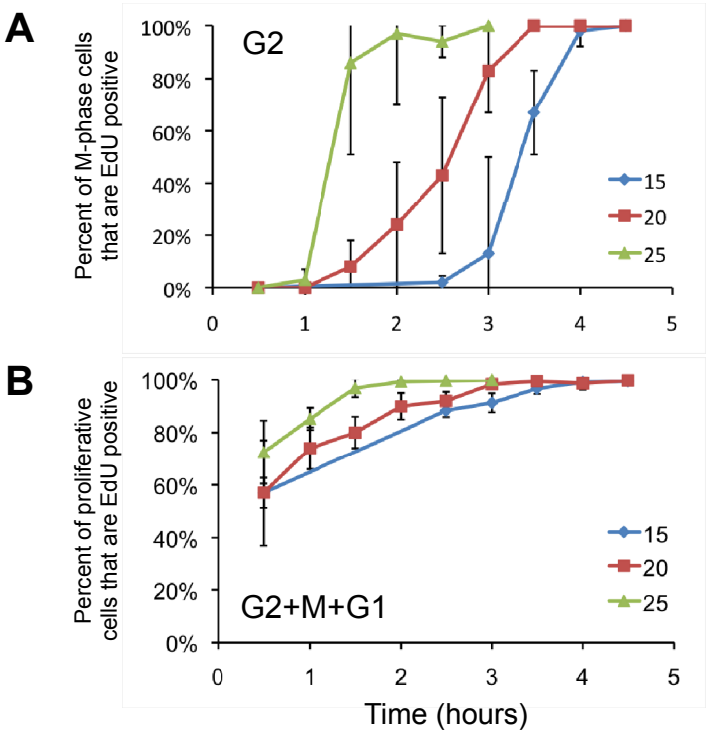
All measurements performed on N2 hermaphrodites 24 hours past L4 at 20°C. [1]Average proliferative zone cells determined by counting REC-8 positive cells (see Fig. 1).
[2]S-phase determined by pulsing animals with EdU for 30 minutes and counting total cells positive for EdU (see Fig. 1).
[3]S-phase index determined by dividing number of S-phase positive cells by number of REC-8 positive cells.
[4]Length of S-phase determined as 57% of 6.5 hours.
[5]M-phase determined by counting pH3 positive nuclei.
[6]M-phase index determined by dividing number of M-phase positive cells by number of REC-8 positive cells.
[7]G1 and G2 index determined from DNA content analysis (see Fig. 3).
[8]Mean G2 length obtained from G2 timecourse experiment. (see Fig. 2C).
[9]Cell cycle length extrapolated from the mean length of G2 and the G2 index. [10]Cell cycle length extrapolated from 3.5 hours for G2+M+G1 (see Fig. 2D) which represents 43% of the total cell cycle.

Table 2. Loss of cye-1 suppresses germline tumors by promoting meiotic entry

Genotype	RNAi	Percent of germlines with meiotic entry	Extent of meiotic entry[1]	Percent meiotic entry within distal 10 cell diameters	Average distance to first meiotic cell[3]	n
wild type		100%	NA	0%	20	31
glp-1(ar202gf) 25C	*GFP*	100%	**	0%	NA	21
glp-1(ar202gf) 25C	*cye-1*	100%	**	0%	NA	27
gld-2 gld-1	*GFP*	100%	***	0%	15	24
gld-2 gld-1[2]	*cye-1*	100%	***	3%	13	28
gld-2 gld-1[2]	*cdk-2*	100%	***	0%	15	28
gld-2 gld-1; glp-1	*GFP*	85%	**	12%	NA	41
gld-2 gld-1; glp-1	*cye-1*	100%	***	91%	2	22
gld-2 gld-1; glp-1	*cdk-2*	100%	***	32%	NA	34
gld-2 gld-1; glp-1	*cdk-1*	100%	**	0%	NA	19
gld-3 nos-3	*GFP*	0%	*	0%	NA	32
gld-3 nos-3	*cye-1*	100%	***	0%	21	25
gld-3 nos-3	*cdk-2*	88%	***	5%	14	18
gld-3 nos-3	*cdk-1*	0%	*	0%	NA	24
gld-3 nos-3; glp-1	*GFP*	0%	*	0%	NA	23
gld-3 nos-3; glp-1	*cye-1*	100%	***	75%	6	20
gld-3 nos-3; glp-1	*cdk-2*	81%	***	55%	NA	22
gld-2; nos-3; glp-1	*GFP*	100%	**	4%	17	23
gld-2; nos-3; glp-1	*cye-1*	100%	***	90%	2	20
gld-1; gld-3	*GFP*	93%	**	0%	NA	30
gld-1; gld-3	*cye-1*	100%	***	10%	13	20
gld-1; gld-3	*cdk-2*	100%	***	0%	NA	27

[1]Extent of meiotic entry is scored by counting the number of nuclei rows that contain cells in meiotic prophase as assayed by HIM-3 and pSUN-1 staining: * corresponds to <5 cell diameters of meiotic entry, ** corresponds to 5-30 cell diameters, and *** corresponds to >30 cell diameters.
[2]Baseline meiotic entry in *gld-2 gld-1* at 48 hours past L4 is high (***), precluding straightforward quantification; however, following *cye-1(RNAi)* there is a clear qualitative increase in meiotic entry (see Fig. S9).
[3]In mutant strains assigned "NA", the first meiotic cell did not occur in a reproducible position near the distal end of the gonad.

Supplemental Figure 1



C

Temperature (°C)	15	20	25
Proliferative Zone Cells[1]	239±27 (n=18)	231±23 (n=18)	206±19 (n=11)
S-phase nuclei[2]	136.6±13.2 (n=12)	133±20 (n=18)	149.3±26.5 (n=9)
M-phase nuclei[3]	7.0±3.3 (n=36)	5.2±2.3 (n=37)	3.8±2.0 (n=38)
M-phase index[4]	3%	2%	2%
S-phase index[5]	57%	57%	72%
G2 length (mean)[6]	3.5 hrs	2.5 hrs	1.5 hrs
G2+M+G1 length (maximum)[7]	4 hrs	3.5 hrs	2 hrs
Cell cycle length (based on G2+M+G1 max length)[8]	9.3 hrs	8.1 hrs	7.1 hrs

All measurements performed on N2 hermaphrodites 24 hours past L4. [1]Proliferative cells determined by counting REC-8 positive cells. [2]S-phase cells determined by pulsing animals with EdU for 30 minutes. [3]M-phase cells determined by pH3 antibody labeling. [4]M-phase index determined by dividing the number of M-phase cells by the total number of proliferative cells. [5]S-phase index determined by dividing the number of S-phase cells by the total number of proliferative cells. [6]G2 length obtained by EdU timecourse experiment in (A). [7]G2+M+G1 length determined by EdU timecourse experiment in (B). [8]Cell cycle length extrapolated from G2+M+G1 length in combination with its percentage of the total cell cycle.

Figure S1. Cell cycle length varies inversely with temperature. Animals maintained at 15°C, 20°C or 25°C were fed EdU continuously starting at t=0 and dissected at 30 minute intervals to obtain estimates of individual phases of the cell cycle. (A) As in Fig. 3, G2 was estimated by analyzing the percent of cells in M-phase (phospho-H3 positive) that are EdU-positive during the time-course. In brief, this time course measures the time required for a germ cell to incorporate EdU tracer in S-phase and pass G2 to enter M-phase. (B) Also as in Fig. 3, G2+M+G1 was estimated by analyzing the percent of all REC-8 positive nuclei that are EdU-positive. At least ten germlines were analyzed for each time point. (C) Summary of the adult hermaphrodite proliferative zone parameters at 15°C, 20°C and 25°C. Consistent with our expectations, the cell cycle length varies inversely with temperature.

Supplemental Figure 2

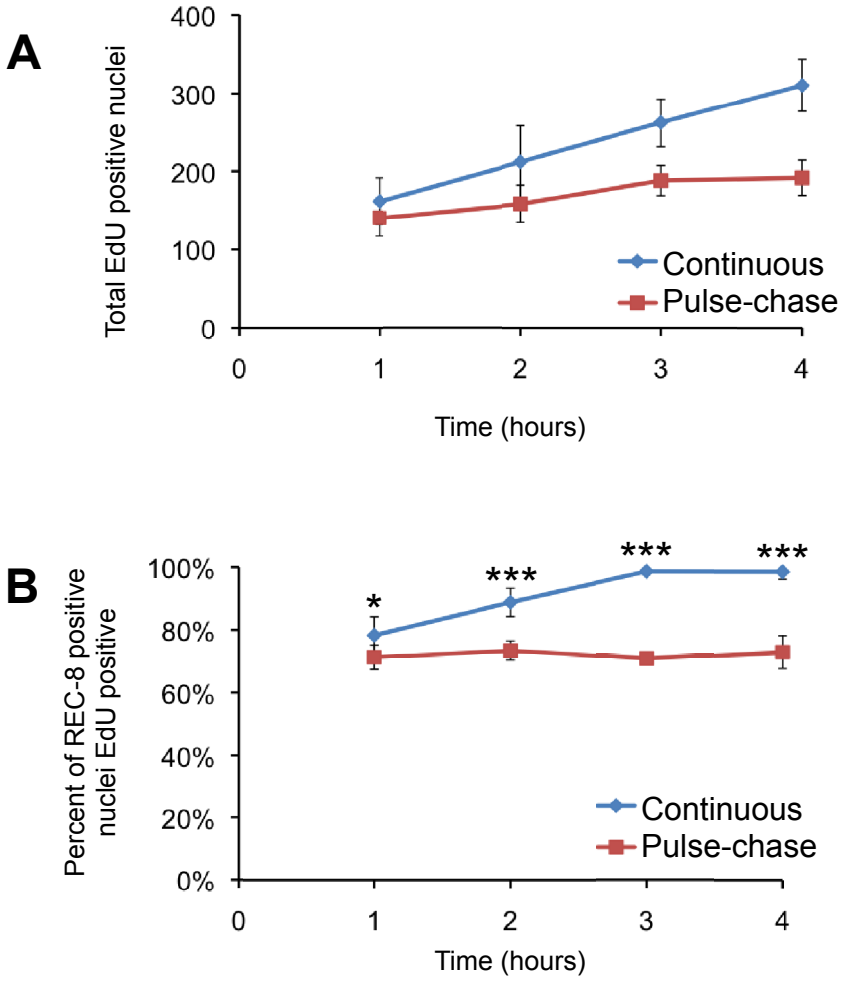


Figure S2. EdU can be effectively chased out of germline. Animals were fed EdU bacteria either continuously (continuous) or given a single 20 minute pulse and then chased by moving to label free bacteria (pulse-chase). Germlines were harvested at 1 hour intervals starting 1 hour after being initially placed on EdU bacteria. The total number of EdU-positive nuclei in each germline was counted (A) and the percent of proliferative nuclei (REC-8 positive) (B) was determined for each germline and then averaged. The difference between the "pulse-chase" and "continuous" feeding regimens indicates that the EdU is effectively chased out of the germline by transfering the animals to label free bacteria. Student t-tests were performed to compare the "continuous" versus "pulse-chase" values for each timepoint (two tailed distribution, equal variance, *$p<0.05$, **$p<0.005$, ***$p<0.0005$). Between seven and twelve germlines were analyzed for each timepoint. Error bars show standard deviation.

Supplemental Figure 3

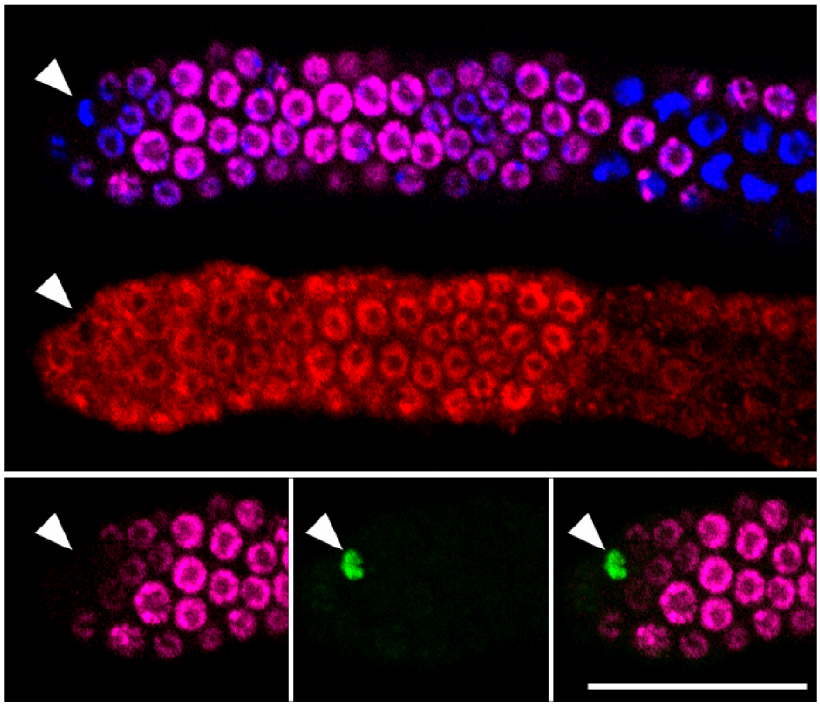


Figure S3. Final proliferative cells to label in EdU time-course are enriched for cells in M-phase. Representative image of the proliferative zone taken from the 3 hour time-point from the continuous EdU labeling time-course from Fig. 3. Surface plane of this germline contains a single proliferative zone cell (arrowhead) which is EdU-negative and in M-phase (pH3 positive). (DAPI, blue; EdU, pink; REC-8, red; pH3, green)

Supplemental Figure 4

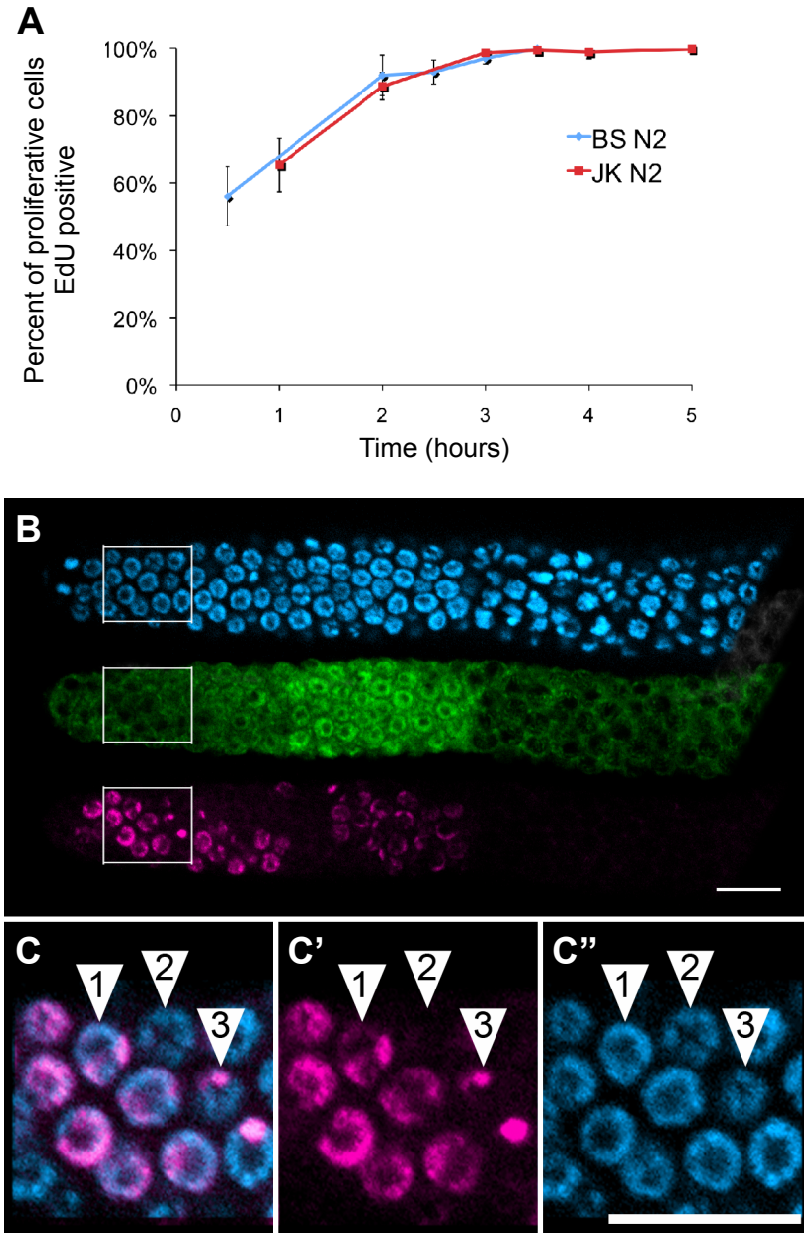
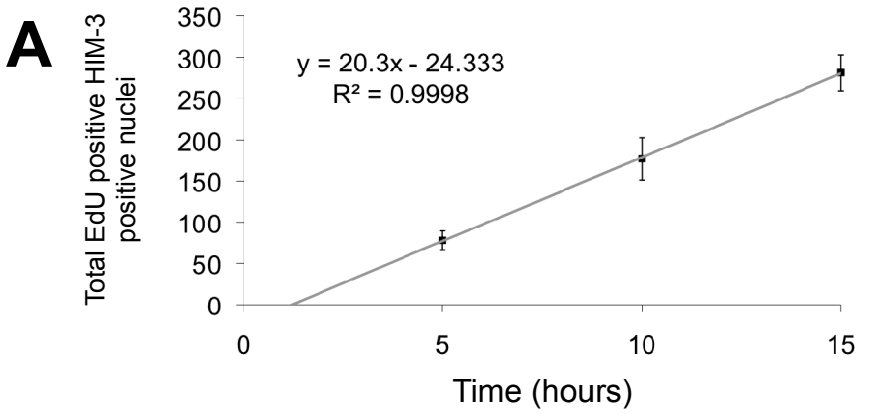


Figure S4. Comparison of cell cycle in different Bristol N2 wild-type strains. (A) The length of G2+M+G1 in wild-type adult hermaphrodites from the Bristol N2 strain provided by Sarah Crittenden (JK, from the laboratory of Judith Kimble, the University of Wisconsin, Madison, WI) were compared with the Bristol N2 strain (BS) used in this study as described in Fig. 3E. Both strains give an estimate of G2+M+G1 of 3.5 hours. The same samples were also scored by analyzing EdU incorporation among nuclei in the ten distal-most cell rows of the proliferative zone (in contrast to scoring all REC-8-positive nuclei) and essentially identical results were obtained (data not show). (B-C) Germlines after 30 minutes of feeding on EdU labeled bacteria examined for EdU incorporation (pink) and REC-8 staining (green). Nuclei that incorporated EdU often display varying levels of EdU signal, which presumably reflects the time spent in S-phase during the labeling period. Any amount of signal above background, which is generally very low, that co-localized with DAPI signal was scored as an EdU-positive nucleus. Arrowheads in (C) point to EdU-positive and negative nuclei: (1) label is present throughout nucleus, (2) label is absent and (3) nucleus is partially labeled. Similar results were obtained using BrdU as a tracer in place of EdU. After 3 hours of BrdU feeding, 223 germ cells (±24 n=8) are BrdU positive versus 227 (±21 n=13) for 3 hours of EdU feeding. Consistent with previous results (Critteden et al 2006), we did not observe abnormal nuclear morphology or a decrease in cell division frequency after 24 hours of BrdU or EdU feeding (data not shown), suggesting that neither BrdU nor EdU have dramatic effects on the germline.

Supplemental Figure 5



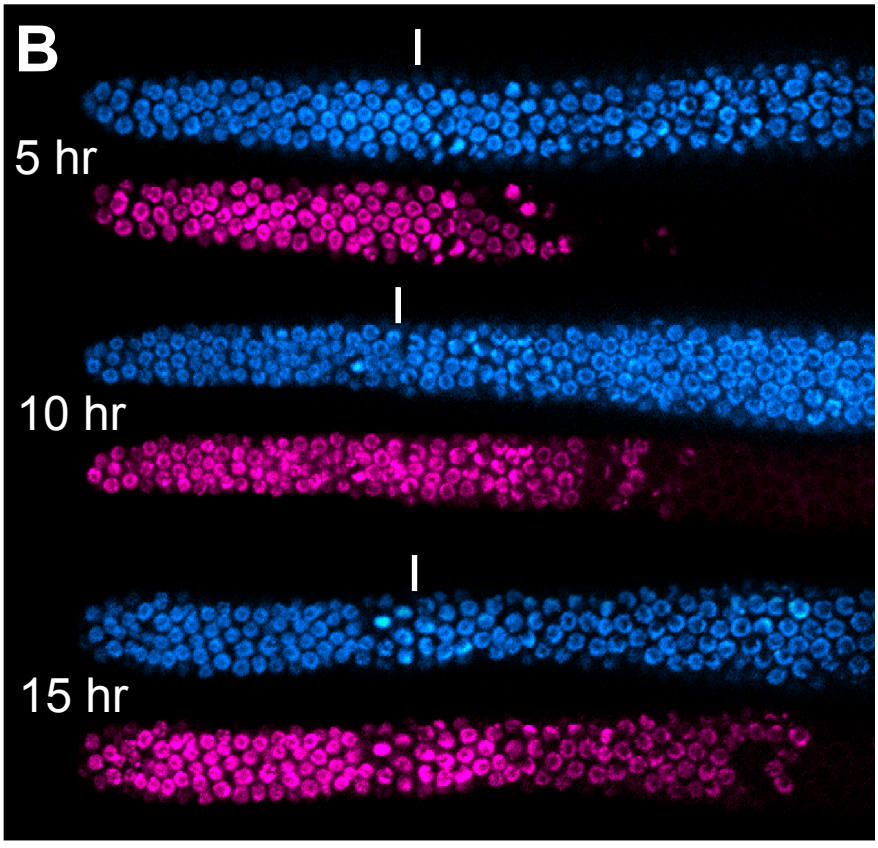
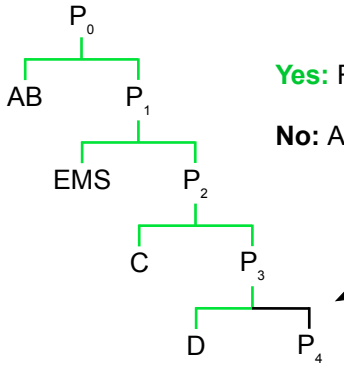


Figure S5. The proliferative zone produces approximately 20 new cells per hour. (A-B) Wild type animals were fed EdU bacteria continuously and dissected at 5-hour intervals after the start of the EdU feeding. Dissected germlines were stained for EdU along with REC-8 and HIM-3 to detect cells that have entered meiosis. (A) The number of EdU-positive nuclei that have entered meiosis at each time-point is graphed. The line, with a slope of ~20 (EdU-labeled HIM-3 positive cells per hour), is a measure of the proliferative zone output per hour. This output value suggests that ~130-160 cells are actively cycling within the proliferative zone based on our estimates for average cell cycle length (6.5 hour cell cycle estimate translates to ~130 cells while an 8 hour cell cycle estimate translates to ~160 cells). Out of ~230 cells observed in the proliferative zone on average, this leaves ~70-100 cells that are not mitotically cycling, with the majority in meiotic S-phase. Interestingly, the x-intercept of the line is non-zero/positive; our interpretation is that there is an interval of up to ~1 hour separating incorporation of EdU into cells in meiotic S-phase and the entry of those cells into meiotic prophase as defined by HIM-3 staining. This indicates that not all of the ~70-100 non-mitotically cycling cells are in meiotic S-phase. An example of a REC-8 positive, EdU-negative and HIM-3 negative nucleus is shown in Fig. 1B, arrowhead 3, and may represent a nucleus that has finished meiotic S-phase but has not loaded sufficient HIM-3 to be categorized as having entered meiosis. (However, other than its proximal position, we cannot rule out that it may be in G2 of the mitotic cycle.) (B) Representative images of the three time points show the increase in EdU positive nuclei that have entered meiosis. The vertical white bar indicates the position where cells have started to enter meiosis. DAPI (blue) EdU (pink).

Supplemental Figure 6

ozEx76[sur-5::dsRed; CDK-4::GFP]



Array present in germline?

Yes: Rescued progeny, positive for sur-5::dsRed

No: All progeny negative for sur-5::dsRed, larval arrest

16/1187 germline mosaics (1.3%)

Genotype	Array present in germline?	Number of Progeny
cdk-4(gv4); Ex[sur-5::dsRed; CDK-4::GFP]	YES	177±45 (n=8)
cdk-4(gv4); Ex[sur-5::dsRed; CDK-4::GFP]	NO	197±14 (n=5)

Figure S6. CDK-4 is not required for germline cell cycle progression. In order to test whether CDK-4 is required for germline development, *cdk-4(gv4)* null mutants (Park and Krause, 1999), which arrest at the L1-L2 larval stage, were rescued with an extrachromosomal array (*ozEx76*) that contains wild-type *cdk-4* and *sur-5::dsRed* that marks nuclei of all somatic cells. L4 hermaphrodites were cloned and screened to identify individuals in which all F1 progeny lack the extrachromosomal array as determined by both 100% penetrance of the larval arrest phenotype and complete lack of the *sur-5::dsRed* visible marker. 16 out of 1187 animals were identified as germline mosaics. Based on visualization of the germline with DIC optics and quantification of brood size of the *cdk-4* germline mosaics, *cdk-4* is not required for germline proliferation. Similar mosaic analysis results were obtained with an independently derived extrachromosomal array from Park and Krause (1999).

Supplemental Figure 7

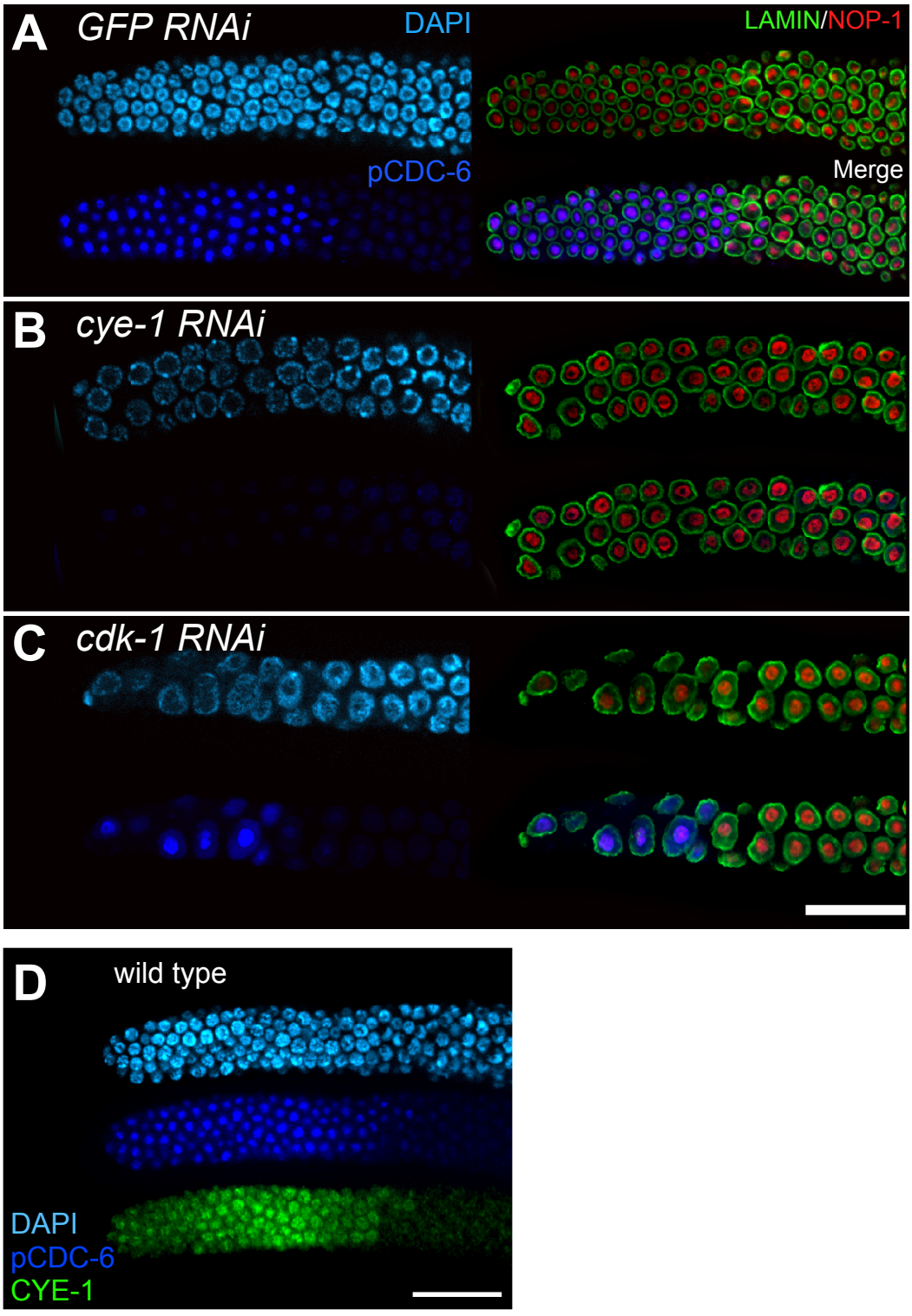


Figure S7. Nucleolar phospho-CDC-6 is present throughout the proliferative zone and is dependent on *cye-1*. Wild-type hermaphrodites were treated with either *gfp* (A), *cye-1* (B) or *cdk-1* (C) RNAi for 48 hours starting at L4. Germlines were dissected and stained with antibodies against NOP-1, LMN-1 and pCDC-6. In controls, pCDC-6 colocalized with NOP-1 in the nucleoli of proliferative cells but diminished in cells in meiotic prophase. pCDC-6 levels appear equivalent in nucleoli throughout the proliferative zone. After *cye-1* depletion, pCDC-6 levels in the nucleoli of proliferative zone cells that were cell cycle arrested (enlarged) were diminished to a comparable level of cells in meiotic prophase. In contrast, *cdk-1* depletion did not reduce nucleolar pCDC-6 levels in arrested proliferative cells.

Supplemental Figure 8

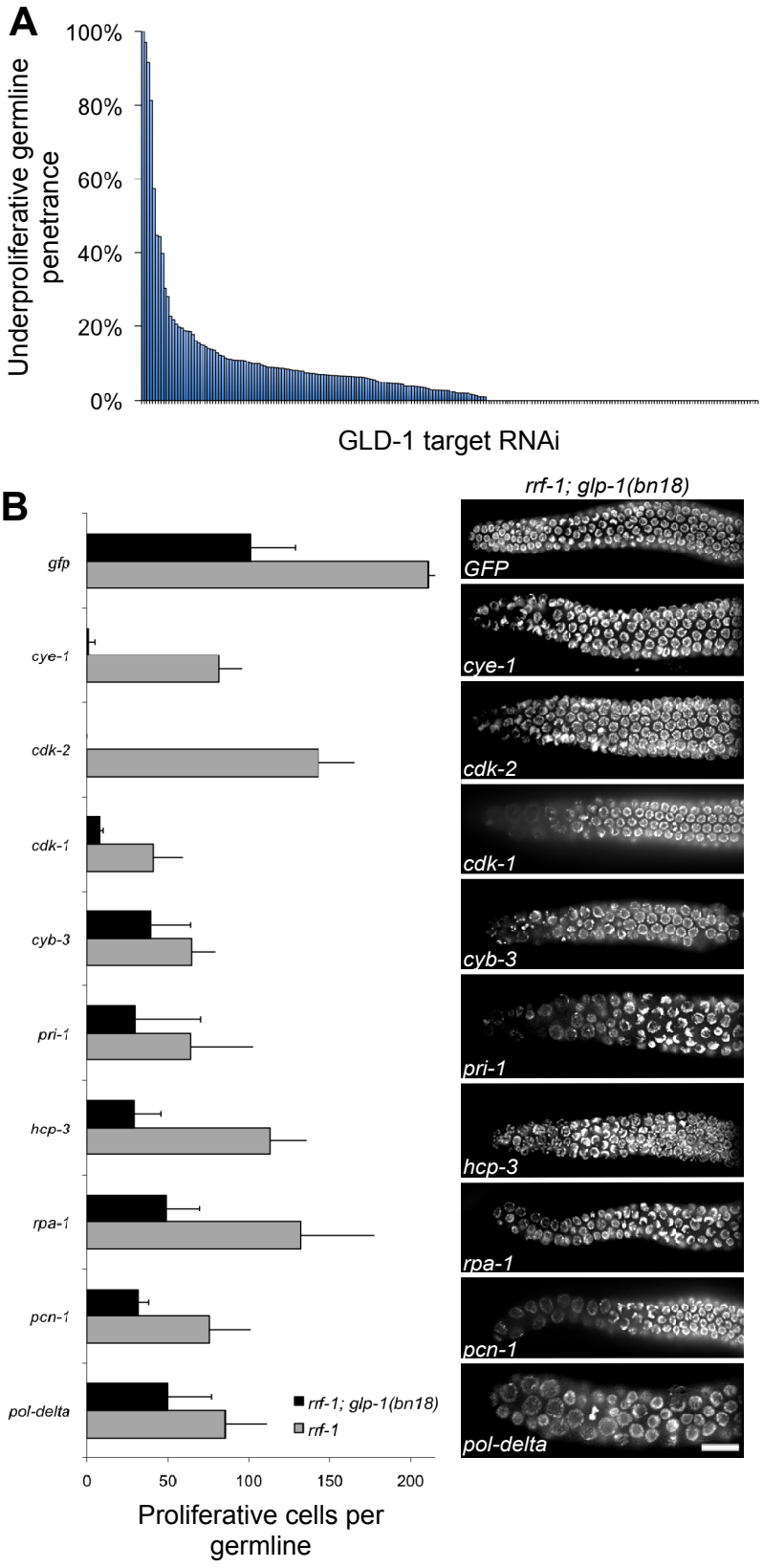


Figure S8. General cell cycle arrest does not lead to premature meiotic entry. (A) A panel of ~200 putative GLD-1 targets (to be described elsewhere, M-H. Lee, unpublished) were screened by RNAi in *rrf-1;glp-1(bn18)* to identify new factors that promote the proliferative fate. RNAi was performed by placing P0 adult hermaphrodites on RNAi feeding plates. F1 progeny were staged at 24 hours past L4 and stained with DAPI. Germlines were analyzed for a defect in proliferation or maintaining proliferative cells by scoring whether germlines were normal or underproliferative (contain fewer than ~100 cells by gross inspection). Chart graphs the the penetrance of the underproliferative phenotype with individual genes listed in rank order along the x-axis. GFP RNAi was performed as a control in parallel during each experiment. The background penetrance of the underproliferative phenotype in this control varied between 0-20%. Therefore, we interpreted genes with >20% penetrance of the underproliferative phenotype as positives. These genes were subjected to a secondary screen (B) to determine whether RNAi depletion leads to a defect in proliferation (i.e. cell cycle arrest) or a failure to maintain the proliferative fate. (B) *rrf-1* and *rrf-1;glp-1(bn18)* animals were fed RNAi bacteria targeting the indicated gene for 48 hours starting at L4. Dissected germlines were stained with REC-8 and HIM-3 antibodies to assay premature meiotic entry. The total number of REC-8 positive nuclei was counted for each genotype. Representative images of DAPI stained germlines illustrate the enlarged nuclei phenotype that occurs with most cell cycle arrest. For most cell cycle factors accept *cye-1* and *cdk-2*, RNAi leads to a decrease in the size of the proliferative zone but does not cause complete premature meiotic entry.

Supplemental Figure 9

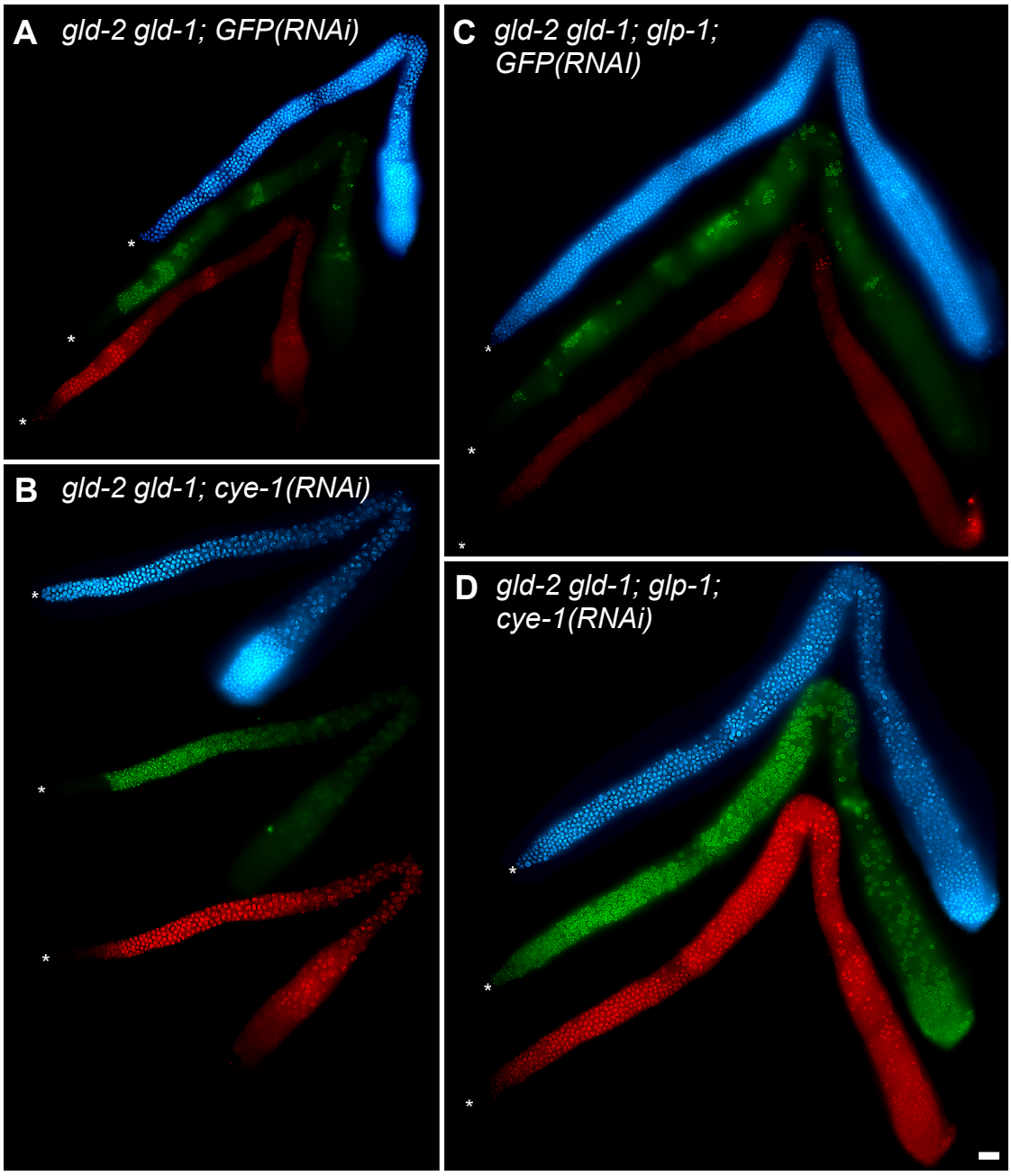


Figure S9. CYE-1 depletion suppresses a *gld-2 gld-1* germline tumor by promoting meiotic entry. L4 hermaphrodites were fed GFP or *cye-1* RNAi bacteria for 48 hours starting at L4. (A, C) *gld-2(q497)gld-1(q485)* and *gld-2(q497)gld-1(q485); glp-1(q175)* triple null mutant animals fed GFP bacteria show tumorous germlines as revealed by a low number of nuclei entering meiosis and labeling positively for HIM-3 (red) and pSUN-1 (green) staining. (B,D) *gld-2(q497)gld-1(q485)* and *gld-2(q497)gld-1(q485); glp-1(q175)* triple null mutant animals fed *cye-1* RNAi bacteria display a significant increase in entry into meiosis. In *glp-1(+)* animals (B), germ cells in the distal germline fail to enter meiosis in response to *cye-1* depletion (asterix indicates the distal tip of the germline). In *glp-1(-)* animals (D), cells throughout the germline (including the distal most cells) enter meiosis in response to *cye-1 (RNAi)*.

Supplemental Figure 10

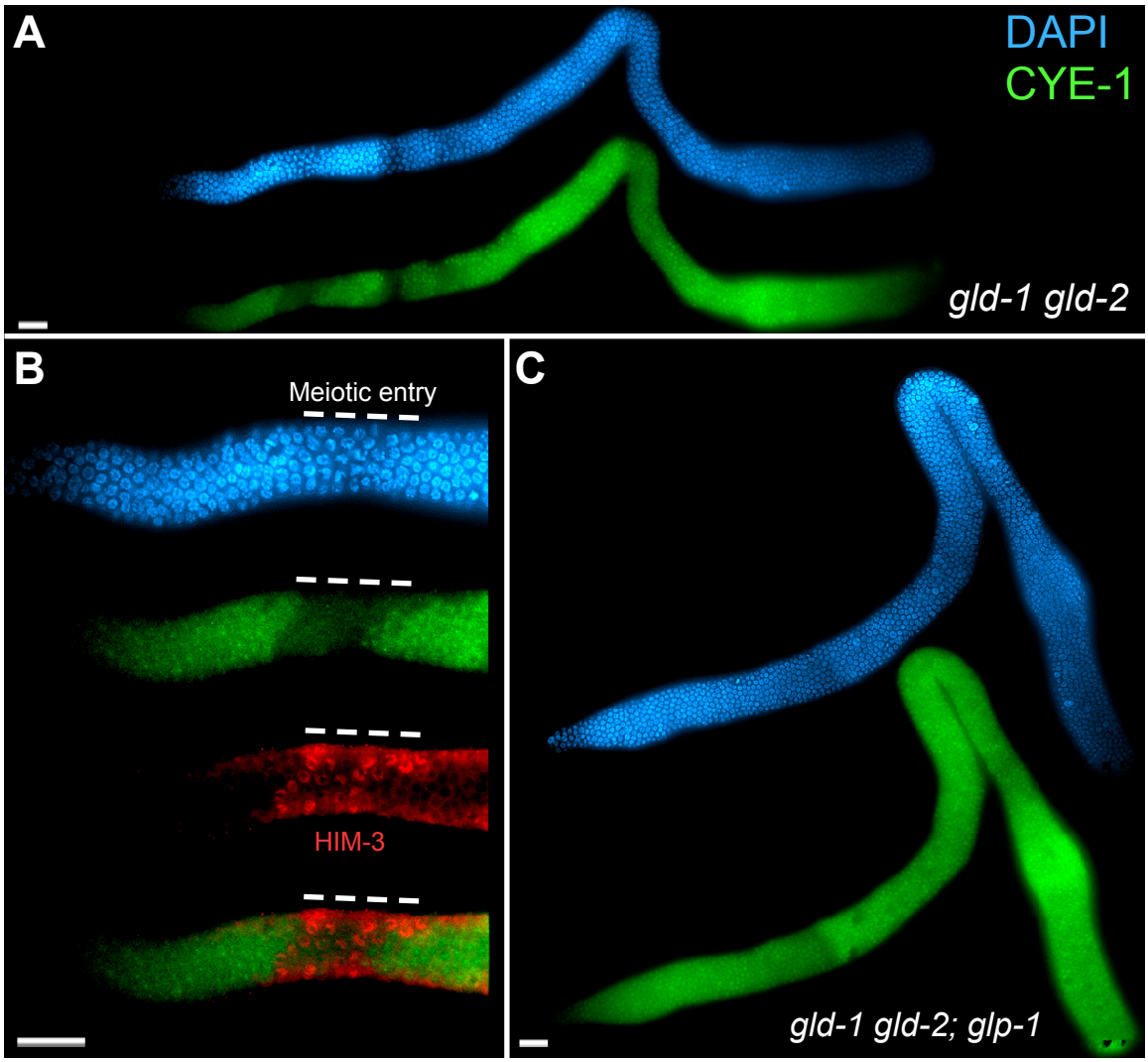


Figure S10. CYE-1 expression in *gld-2gld-1* and *gld-2gld-1; glp-1* mutants. Adult hermaphrodites 24 hours past L4 were dissected and analyzed for CYE-1 expression with CYE-1 antibody (green). (A-B) *gld-2(q497)gld-1(q485)* null double mutants show CYE-1 throughout the germline. Scattered nuclei which enter meiosis (HIM-3-positive, red) display significant CYE-1 repression. (C) *gld-2(q497)gld-1(q485); glp-1(q175)* triple null mutants show CYE-1 throughout the germline, indicating that GLP-1 is not necessary for CYE-1 expression.

Table S1. List of strains used

Stain name	Genotype
N2 Bristol (BS)	wild type
N2 Bristol (JK)	wild type
PD8488	*rrf-1(pk1417)*
BS3679	*rrf-1(pk1417); glp-1(bn18)*
BS3538	*rrf-1(pk1417); glp-1(ar202)*
BS4019	*prom-1(ok1140) unc-55(e405)*
BS673	*gld-1(q485)/hT2gfp*
BS3369	*gld-2(q497) gld-1(q485)/hT2gfp*
BS3392	*gld-2(q497) gld-1(q485)/hT2gfp; unc-32(e189) glp-1(q175)/hT2gfp*
BS3792	*gld-3(q730) nos-3(oz231)/mIn1::GFP*
BS5444	*gld-3(q730) nos-3(oz231)/mIn1::GFP; unc-32(e189) glp-1(q175)/hT2*
BS3855	*gld-1(q485)/ccIs4251 unc-13(e51); gld-3(q730)/mIn1::GFP*
BS5264	*gld-2(q497)/hT2; nos-3(oz231)*
BS5268	*gld-2(q497)/hT2; nos-3(oz231); unc-32(e189) glp-1(q175)/hT2*
BS4026	*gld-1(q485) prom-1(ok1140)/hT2gfp*
KM48	*cdk-4(gv3)/szT1*
KM123	*cdk-4(gv3)/szT1; Ex[PpPD95.67, CDK-4::GFP]*
BS1175	*cdk-4(gv3)/szT1; ozEx76[sur-5::dsred, CDK-4::GFP]*

Appendix to Chapter 2

How do proliferative zone cells in the adult hermaphrodite respond to a decrease in nutrition?

Summary

We used the feeding defective mutant *eat-2* to investigate how proliferative cells respond to decreased nutrition. The proliferative zone in *eat-2* mutants has a significantly reduced output (number of daughter cells that enter meiosis per unit time) indicating that nutrition is important for the rate of germ cell production. *eat-2* mutants show a striking decrease in proliferative cell number but do not show a significant alteration in cell cycle kinetics relative to well fed wild type animals under standard laboratory conditions. These results suggest that proliferative zone output can be regulated by varying the number of proliferating cells.

Introduction

The regulation of cell cycle progression usually occurs during the G1 phase of the cell cycle (Blomen and Boonstra 2007). At this time a variety of extrinsic inputs regulate progression past the restriction point of the cell cycle. Passage through the restriction point involves activation of CDK4-cyclin D and phosphorylation of pRb (Blomen and Boonstra 2007). Downstream of the restriction point, CDK2-cyclin E activation drives the transition to S-phase (Hwang and Clurman 2005). An important extrinsic determinant for restriction point passage is nutrient availability. The absence of sufficient metabolites or the absence systemic growth signals such as insulin often inhibit passage through the restriction point (Blomen and Boonstra 2007). However, delays in other stages of the cell cycle can also occur. For example, germline stem cells in the *Drosophila* ovary delay

progression through G2, as well as G1, and normal progression rates in G2 depend on insulin signaling (Hsu et al. 2008). Similarly, insulin signaling is required for normal germline proliferation rates during larval development in *C. elegans*, and the absence of insulin signaling causes an apparent delay in G2 (Michaelson et al. 2010).

We have shown that the mitotic cell cycle of proliferative cells in *C. elegans* lacks a significant G1 phase (Chapter 2). Consistent with this cell cycle structure, CYE-1 levels are continuous throughout the cell cycle, suggesting that CDK-2-CYE-1 activity is uncoupled from the typical signaling events associated with the restriction point. Most notably, CDK-4 is not required for cell cycle progression in the germline (Chapter 2). How do these cells regulate cell proliferation?

Previous work on the germline has shown that insulin signaling is important for germline proliferation in larval development (Michaelson et al. 2010). Defects in the insulin signaling pathway lead to decreased expansion of the proliferative cell pool during larval development, resulting in an adult germline with fewer proliferative zone cells (Michaelson et al. 2010). While insulin signaling is a likely mediator of low nutrition feedback, the direct effect of lower nutrition on germline proliferation has not been rigorously investigated. To investigate this topic, we utilized *eat-2* mutant in which food intake is decreased due to a defect in the rate of pharyngeal pumping (Avery 1993). *eat-2* mutants have been used in a variety of studies that analyze the relationship between caloric intake and aging (Lakowski and Hekimi 1998). However, germline proliferation in *eat-2* mutants has not been characterized. Here we present an analysis of cell cycle progression of proliferative cells in adult *eat-2* mutants. Consistent with a decrease in nutrient intake, we show that the adult *eat-2* proliferative zone has a lower output than

wild type animals. However, we do not observe a significant difference in the kinetics of cell cycle progression among proliferative cells. Rather, we observe that the size of the adult proliferative zone is significantly smaller than the wild type proliferative zone. Therefore, these results suggest that the adult proliferative zone can alter its output by modulating the number of mitotically dividing cells as opposed to altering their generation time.

Results and Discussion

Proliferative zone output is decreased in *eat-2* mutants

We first asked whether the presumptive nutrient decrease in *eat-2* mutants causes a decrease in the rate of germ cell production. In adult animals, the proliferative zone is maintained at steady state by balancing mitotic cell division with differentiation of germ cells into meiosis. Therefore, the output of the proliferative zone is essentially the number of cells that enter meiosis. We used EdU to label germ cells in the proliferative zone and quantified the rate with which these labeled cells enter meiosis. We compared the total number of EdU positive nuclei that entered meiosis after continuous EdU feeding in wild type versus *eat-2* germlines. After 10 hours of EdU feeding, fewer EdU labeled nuclei entered meiosis in *eat-2* (63±27) than in wild type (177±55) germlines (Fig. 1). Therefore, the decrease in nutrient intake causes an overall decrease in germ cell production by the proliferative zone.

Cell cycle progression is not significantly affected in *eat-2* mutants

The decreased cell output from the proliferative zone indicates that either 1) fewer cells are actively cycling or 2) cell cycle kinetics are slower (or a combination of these). These changes are not mutually exclusive and could both contribute to a decreased output. To investigate whether the decrease in germ cell production (proliferative cell output) is due to a change in cell cycle kinetics, we analyzed several markers for mitotic proliferation. First, we analyzed the frequency of cell division using pH3 antibody to label cells in M-phase, and we analyzed the frequency of S-phase by giving animals a short pulse of EdU (<30 minutes) (Fig. 2). Both M-phase index and S-phase index were indistinguishable in *eat-2* versus wild type animals, suggesting that the cell cycle structure, and likely overall cell cycle kinetics, is not significantly altered. To more specifically analyze cell cycle kinetics, we measured two additional parameters: the length of G2 and the length of G2+M+G1. We observe that the maximum values for both the length of G2 and the length of G2+M+G1 are equivalent in *eat-2* and wild type (~3.5 hours)(Fig. 2). Therefore, a decrease in cell cycle kinetics does not explain the significant decrease in germ cell production in *eat-2* mutants. Furthermore, comparison of the length of G2 with G2+M+G1 suggests that *eat-2* proliferative zone cells also lack a G1 phase, similar to wild type germline (Chapter 2). Consistent with these cell cycle observations, CYE-1 is also expressed continuously throughout the proliferative zone, similar to wild type (data not shown, Chapter 2). Therefore, cell cycle progression in *eat-2* proliferative zone cells appears be very similar to wild type. However, we note that *eat-2* proliferative zone cells may display a larger variation in G2 length and G2+M+G1 length (Fig. 2). These parameters may suggest that the average cell cycle length is somewhat altered in *eat-2* mutants.

eat-2 germlines contain fewer proliferative cells

The similar cell cycle kinetics in *eat-2* versus wild type animals suggests that the decrease in *eat-2* proliferative zone output is due to fewer actively cycling cells. In the germline, mitotic division occurs in the proliferative zone, which can be labeled by REC-8 antibody. To determine whether *eat-2* mutants have a smaller proliferative zone, we dissected eat-2 and wild type germlines and counted the number of REC-8 positive nuclei. Indeed, *eat-2* mutants contained a significantly smaller proliferative zone than wild type animals (Fig. 3).

Taken together, the above results suggest that the adult proliferative zone decreases cell output by regulating the number of actively cycling cells. This mechanism of output regulation may be a consequence of the cell cycle structure of proliferative cells. Since they lack a significant G1 phase, proliferative cells may lack a stable arrest point in the cell cycle, causing them to rely on modulation of the number of actively cycling cells. However, since the eat-2 decreases nutrition throughout development, the decrease in proliferative zone cells may stem from a decrease in expansion of the proliferative zone population during larval development. Therefore, it remains unclear whether dynamic changes in proliferative cell number act as an endogenous mechanism for responding to sudden decreases in nutrition (see Chapter 4).

Materials and Methods

Nematode strains and growth conditions: The following strains were used: N2 Bristol (wild type) and *eat-2(ad465)*(Avery 1993). Animals were grown under standard growth

conditions at 20°C (Brenner 1974). For all experiments, animals were maintained in the presence of food at all times. Animals were staged to 24 hours past L4.

Proliferative zone output: Animals were fed EdU-labeled bacteria continuously for 10 hours. Dissected germlines were labeled with REC-8 and HIM-3 antibody and for EdU incoporation. Proliferative zone output was compared by counting the total number of EdU positive nuclei that had entered meiosis (REC-8 negative and HIM-3 positive) by the 10 hour timepoint.

Cell cycle analysis: M-phase index, S-phase index, G2 length and G2+M+G1 length were determined as described in Chapter 2. Importantly, eat-2 mutants did not show a delay in EdU incorporation due to a failure to ingest the bacteria. This is indicated by the relatively equivalent S-phase index after a <30 minute pulse of EdU-bacteria.

Proliferative zone size: The proliferative zone size was determined by counting the total number of proliferative cells (labeled positive with REC-8 antibody).

References

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Hsu, H.J., LaFever, L., and Drummond-Barbosa, D. 2008. Diet controls normal and tumorous germline stem cells via insulin-dependent and -independent mechanisms in Drosophila. *Dev Biol* **313**(2): 700-712.

Hwang, H.C. and Clurman, B.E. 2005. Cyclin E in normal and neoplastic cell cycles. *Oncogene* **24**(17): 2776-2786.

Lakowski, B. and Hekimi, S. 1998. The genetics of caloric restriction in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **95**(22): 13091-13096.

Michaelson, D., Korta, D.Z., Capua, Y., and Hubbard, E.J. 2010. Insulin signaling promotes germline proliferation in C. elegans. *Development* **137**(4): 671-680.

Figure 1

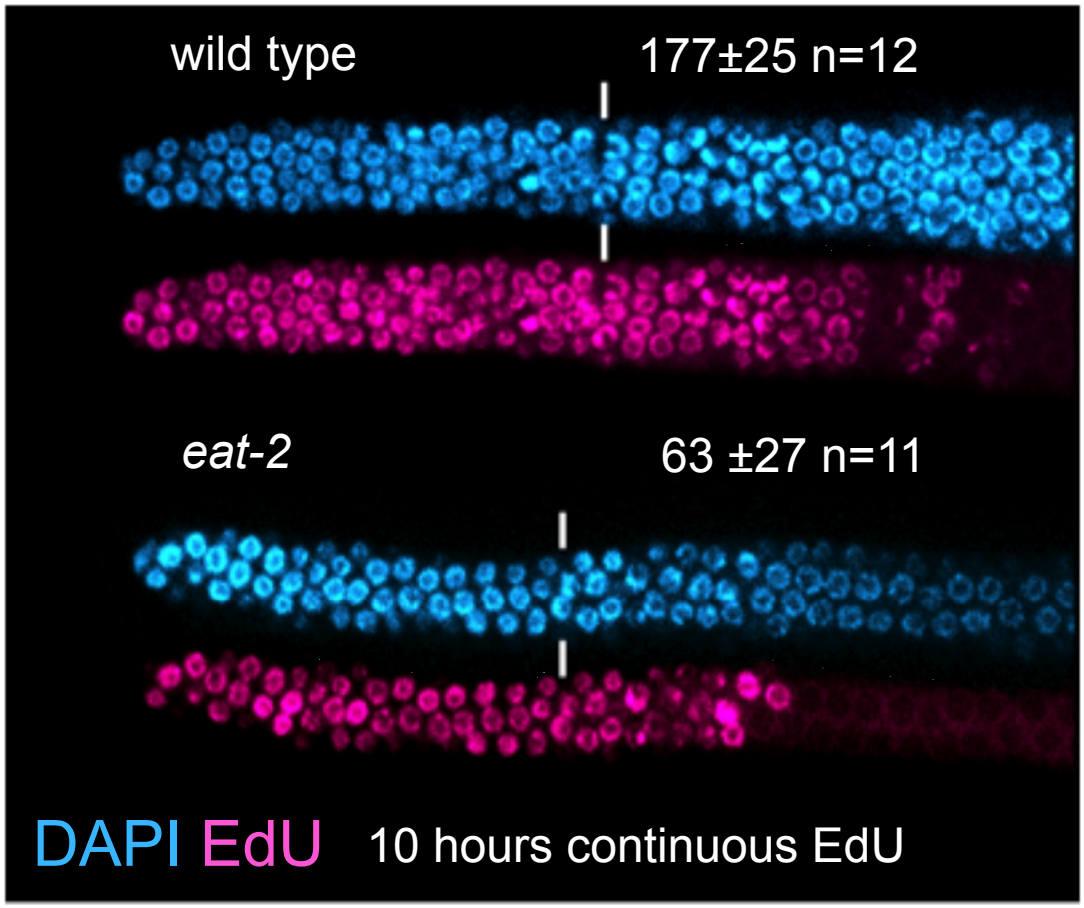
wild type
177±25 n=12
eat-2
63 ±27 n=11
DAPI EdU
10 hours continuous EdU

Figure 1. The proliferative zone in *eat-2* mutants has a lower cell output. Wild type and *eat-2* adults were fed EdU-bacteria for 10 hours at 20°C. Dissected germlines were stained with DAPI, REC-8 antibody, HIM-3 antibody and for EdU incorporation (pink). REC-8 and HIM-3 are not shown. The horizontal bar indicates the start of the transition zone. The total number of EdU positive meiotic prophase cells (REC-8 negative, HIM-3 positive) were counted as a measure of proliferative zone output.

Figure 2

A

	M-phase index	S-phase index
n2	2.8% n=36	57±8% n=14
eat-2	2.8% n=23	54±13% n=15

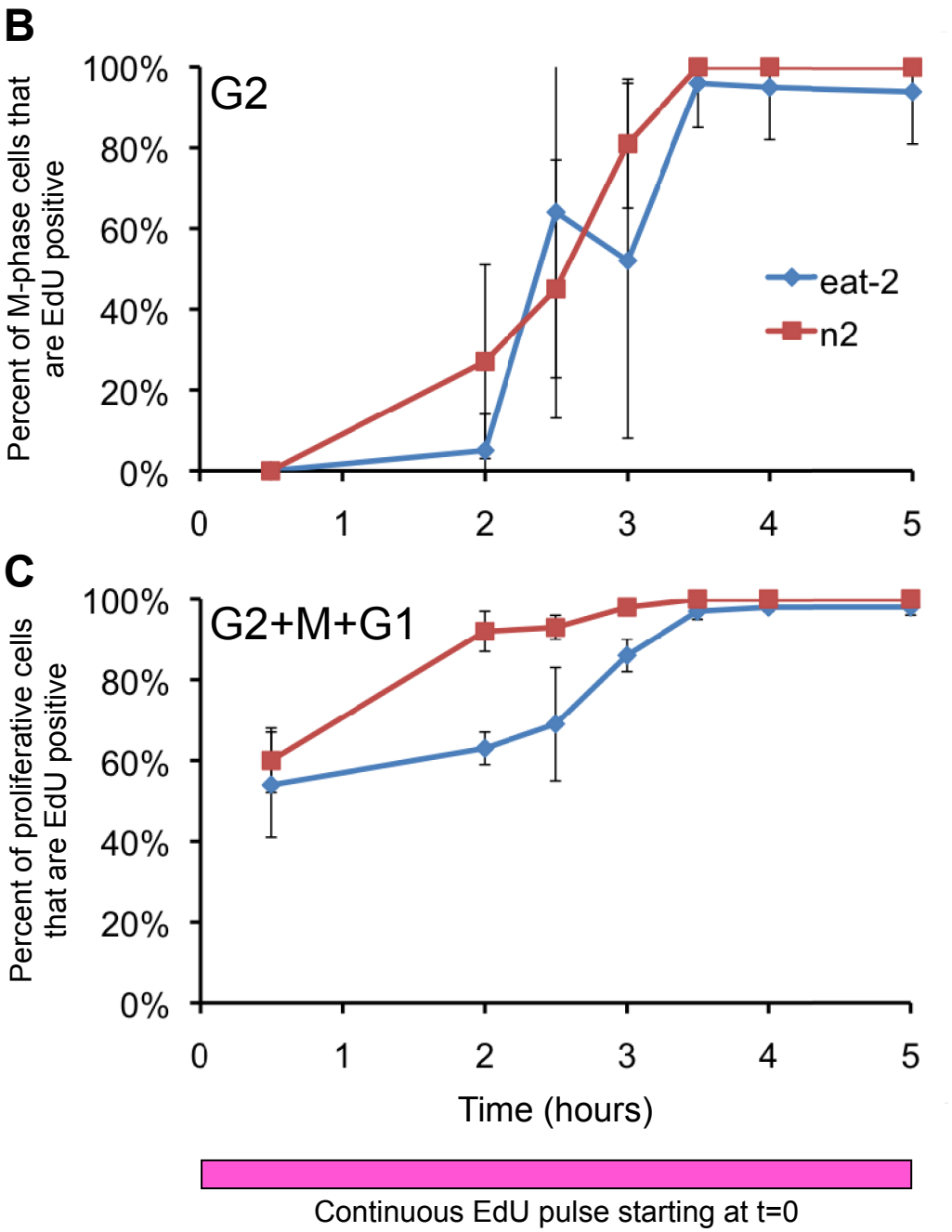

Figure 2. Cell cycle kinetics are not affected in *eat-2* proliferative cells. (A) M-phase index (number of M-phase cells/number of proliferative zone cells) and S-phase index (number of S-phase cells/number of proliferative zone cells) are equivalent in *eat-2* and wild type adults. (B) The length of G2 was estimated by measuring the length of time for EdU labeled cells in S-phase to reach M-phase. Animals were fed EdU continuously and dissected over a time-course. Germlines were labeled for EdU incorporation and stained with pH3 antibody (to label M-phase). By 3.5 hours, nearly all M-phase cells in both wild type and *eat-2* germlines are EdU positive, indicating that this is maximum length of G2. (C) The total length of G2+M+G1 was measured by determining the shortest continuous pulse required to label all proliferative cells positive for EdU. In both wild type and *eat-2* germlines, nearly all proliferative cells are EdU positive after 3.5 hours of continuous EdU feeding, indicating that the maximum length of G2+M+G1 is 3.5 hours. Error bars show standard deviation.

Figure 3

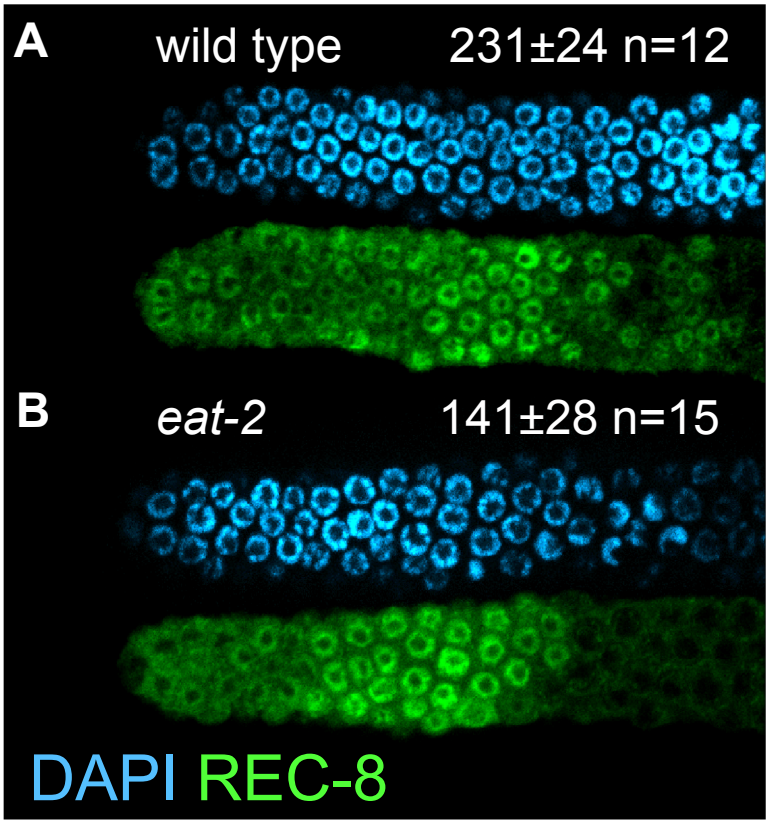

Figure 3. *eat-2* mutants have a smaller proliferative zone. (A-B) wild type (A) and *eat-2* (B) adults were dissected 24 hours past L4. Germlines were labeled with REC-8 antibody (green) and the total number of proliferative zone cells (REC-8 positive) was counted. Wild type animals have a significantly larger proliferative zone than *eat-2* mutants.

Chapter 3

Response of proliferative zone cells in the adult hermaphrodite germline to loss of *glp-1* activity

Introduction

Germline stem cells give rise to daughter cells that enter meiosis. As these cells divide mitotically to achieve self renewal, entry into meiosis must be coordinated with progression through the mitotic cell cycle. Extensive research in yeast has demonstrated that the decision to enter meiosis occurs early in the mitotic cell cycle, prior to the start of S-phase and a recent study in mice suggests that this requirement may be conserved in metazoa (Baltus et al. 2006). However, many of the details of meiotic entry in multicellular organisms remain unclear.

C. elegans is an important model organism for studying reproductive development. The adult hermaphrodite germline consists of approximately 1000 germ cells, with all stages of germ cell development, from germline stem cell to mature oocytes and sperm, displayed in a linear array. The production of new germ cells begins in the distal part of the germline in the proliferative zone. Here, germline stem cells divide mitotically to produce differentiated daughters as well as maintain a steady state proliferative zone population. Cells in this region are referred to as proliferative zone cells, although the actual developmental identity of individual cells within the proliferative zone remains ambiguous. Cells in the proliferative zone stain positive with an antibody against REC-8, whereas cells that have entered meiosis are positive for HIM-

3 (Zetka et al. 1999; Pasierbek et al. 2001; Hansen et al. 2004a). These mutually exclusive markers are useful for identifying proliferative zone cells and cells that have entered meiosis (Hansen et al. 2004a). However, within the proliferative zone, a number of cells have initiated meiotic S-phase and do not contribute to mitotic proliferation (see Chapter 2). In addition, it remains unclear whether some cells are transit amplifying cells with restricted developmental potential as opposed to all mitotically cycling cells having equivalent developmental potential (Hansen and Schedl 2006; Cinquin et al. 2010). An important goal is to better characterize the organization of the proliferative zone with respect to these different developmental stages.

The major signaling pathway that promotes the proliferative fate is the conserved GLP-1/Notch signaling pathway (Hansen and Schedl 2006; Kimble and Crittenden 2007). The somatic distal tip cell (DTC) expresses the ligands LAG-2 and APX-1 that activate GLP-1 receptor expressed in the proliferative zone cells (Austin and Kimble 1987; Crittenden et al. 1994; Henderson et al. 1994; Nadarajan et al. 2009). Active GLP-1 is important for the proliferative fate as demonstrated by *glp-1* mutant analysis. Loss of function *glp-1* mutants fail to maintain proliferative cells while gain of function *glp-1* mutants have ectopic proliferative cells that lead to tumor formation (Austin and Kimble 1987; Berry et al. 1997; Pepper et al. 2003). While the DTC appears to increase its contact with the proliferative zone through cytoplasmic extensions, many proliferative cells do not appear to receive direct contact with the DTC and presumably fail to receive GLP-1 activating ligand (Crittenden et al. 2006). This observation has led to two hypotheses regarding the developmental status of proliferative cells with regard to GLP-1 activity (Hansen and Schedl 2006). 1) Loss GLP-1 signal activity during displacement

from the DTC initiates a series of programmed transit amplifying divisions. 2) Cells displaced from the DTC temporarily retain GLP-1 signal activity and remain developmentally equivalent stem cells until GLP-1 activity is lost and then germ cells enter meiosis.

In addition to GLP-1 regulating the proliferative fate, two downstream and redundant pathways promote entry into meiosis. These are the GLD-1 and GLD-2 pathways which likely perform their regulatory function by regulating mRNA translation (Hansen and Schedl 2006; Kimble and Crittenden 2007). Neither of these pathways are individually essential for meiotic entry to occur, as loss of a single gene from either pathway does not completely inhibit entry into meiosis (Kadyk and Kimble 1998; Hansen et al. 2004a). However, loss of one gene from each pathway results in a near complete meiotic entry failure. GLD-1 is one of these genes, and regulation of its abundance is an important determinant in the decision to either remain a proliferative cell or enter meiosis (Hansen et al. 2004b). Furthermore, GLD-1 abundance increases from the distal end of the proliferative zone to proximal end of the proliferative (Jones et al. 1996; Hansen et al. 2004b). This increase in GLD-1 is consistent with the hypothesis that cells in the proximal region of the proliferative zone may have a difference developmental potential than cells in the distal region of the proliferative zone and that high levels of GLD-1 promote meiotic entry.

As described in Chapter 2, we and others have studied mitotic cell cycle proliferation among cells in the proliferative zone and observed important characteristics of the germline mitotic cell cycle: 1) the cell cycle lacks a significant G1 phase, 2) proliferative zone cells do not enter quiescence (Crittenden et al. 2006) and 3) cell cycle

progression is relatively rapid (average generation time at 20°C is ~6-8 hours). The observed continuous CYE-1 throughout the cell cycle likely contributes to these cell cycle features and may render early G1 regulatory events irrelevant (Orford and Scadden 2008). Consistent with this notion, CDK-4, a cyclin dependent kinase generally implicated in G1 regulation and required for larval cell cycle progression in *C. elegans* (Park and Krause 1999), is not required for cell cycle progression in the germline. Given the nature of cell cycle progression in the *C. elegans* germline, how is entry into meiosis coordinated with mitotic cell cycle progression? Typically, entry into meiosis as well as developmental decisions regarding differentiation are thought to be made during the G1 phase of the cell cycle (Marston and Amon 2004).

To further characterize how the proliferative zone is organized and how cells coordinate mitotic cell cycle progression with entry into meiosis, we have studied the process by which cells enter meiosis. We have used the *glp-1(bn18)* temperature sensitive loss of function mutant which allows us to manipulate meiotic entry among proliferative zone cells. *glp-1(bn18)* has a A1034T change in the fourth ankyrin repeat in the intracellular domain of GLP-1 protein (Kodoyianni et al. 1992). According to crystal structure, this residue is predicted to interact with the SEL-8/LAG-3 cofactor (Kodoyianni et al. 1992; Wilson and Kovall 2006). At the permissive temperature, *glp-1(bn18)* mutants are relatively wild type (Kodoyianni et al. 1992). However, shifting these mutants to the restrictive temperature causes a loss of GLP-1 activity resulting in loss of proliferative zone cells due to meiotic entry (Kodoyianni et al. 1992). Similarly, loss of GLP-1 signaling appears to be an important endogenous mechanism by which germ cells decide to enter meiosis. As proliferative zone cells move proximal in the

germline, they move away from the source of GLP-1 activating ligand, which likely plays an important role in promoting the switch to meiosis.

We have used a panel of cell cycle and cell fate markers to analyze the spatial and temporal pattern in which proliferative zone cells enter meiosis in a *glp-1(bn18)* up-shift experiment. In our analysis, we confirm that the proliferative zone is not uniform since cells in the proximal part of the proliferative zone enter meiosis first. These proximal proliferative zone cells are likely cells that have already initiated the process of meiotic entry (in meiotic S-phase) allowing them to complete entry into meiosis prior to more distal cells. The remaining cells appear to respond equivalently to a loss of *glp-1*, however the kinetics with which cells enter meiosis appears to depend on their position in the cell cycle at the time of the up-shift. The equivalent behavior of these cells suggests there are not developmentally distinct transit amplifying cells, or if there are transit amplifying cells, they are equivalent to stem cells in their requirement for GLP-1 activity.

By combining this up-shift experiment with in depth cell cycle analysis, we observe that cells in early stages of the mitotic cell cycle are committed to completing the mitotic cell cycle before they can switch to meiosis. Surprisingly, arresting cells in the mitotic cell cycle by hydroxyurea treatment or RNAi of critical cell cycle factors does not inhibit their ability to enter meiosis following loss of *glp-1*activity. This suggests that the decision to enter meiosis can be uncoupled from mitotic cell cycle progression.

Materials and Methods

Nematode maintenance and strains

Animals were cultured using standard techniques (Brenner 1974). The following strains were used in this analysis: N2 Bristol wild type, *glp-1(bn18)*, *gld-1(q485)/ccIs4251 unc-13; glp-1(bn18)*.

Immunofluorescence imaging of dissected germlines

Germlines were dissected as described elsewhere (Jones et al. 1996). Samples were fixed in 3% Formaldehyde/0.1 M K_2HPO_4 (pH7.2) for ten minutes at room temperature followed by 5 minutes in methanol at –20°C. Samples were then incubated with primary antibody overnight at room temperature. Primary antibody was removed and washed 3x prior to incubation with secondary antibody for 4 hours at room temperature. Samples were then washed 3x and stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 100 ng/ml. For EdU experiments, fixation, antibody and DAPI staining were performed as described aboved. Samples were then stained for EdU incorporation using an EdU detection kit (Invitrogen). Samples were imaged using a PerkinElmer spinning disk confocal microscope. Images were processed using Adobe Photoshop.

Cell cycle analysis

M-phase Index: Dissected germlines were labeled with REC-8 and phospho-Histone 3 (pH3) antibodies to identify proliferative cells and cells in M-phase, respectively. REC-8 positive nuclei and pH3 positive nuclei were counted per germline and M-phase index was determined by dividing the total number of pH3 positive nuclei by the total number of REC-8 positive nuclei.

S-phase Index: To identify cells in S-phase, animals were given a 25-30 minute pulse of EdU and dissected immediately without an appreciable chase. Dissected germlines were labeled for EdU incorporation with REC-8 antibody to identify proliferative cells. S-phase index was determined by dividing the total number of EdU positive nuclei per germline by the number of REC-8 positive nuclei.

Length of G2+M+G1: The length of G2+M+G1 was measured by determining the minimum continuous EdU pulse required to label all proliferative cells positive for EdU incorporation.

Temperature shift experiments

For all temperature shift experiments, animals were raised at 15°C and staged by picking L4 animals 24 hours prior to the start of the temperature shift. Animals were shifted to 25°C by picking animals to plates pre-warmed at 25°C. For temperature shift experiments involving EdU incoporation, HU treatment or both, plates were pre-incubated to desired temperature.

EdU experiments

MG1693 strain bacteria were grown in the presence of EdU and used to seed EdU-plates as described in chapter 2. Animals were picked to EdU-plates (pre-incubated to desired temperature) for 25 minutes (unless stated otherwise). For pulse-chase experiments, animals were then transferred to OP50 plates (Brenner 1974) and allowed to crawl away from the cross contaminating EdU-bacteria. Animals were then transferred to a second fresh OP50 plate.

HU treatment

For HU treatment, NGM plates pre-seeded with bacteria (or EdU-plates for EdU experiments) were supplemented with hydroxyurea to a final concentration of 250uM. Animals were then picked to HU-containing plates using standard techniques.

Results

Cell cycle progression in the proliferative zone

Our previous work on adult germline proliferative zone cells highlighted several important characteristics of their proliferation under standard conditions: 1) roughly 60% of cells within the proliferative zone are actively mitotically cycling while the remaining cells are in meiotic S-phase; 2) the mitotic cell cycle lacks a significant G1 phase; and 3) all mitotically cycling cells are actively cycling, there are no quiescent or slowly cycling cells (See Chapter 2). How are these parameters affected by changes in the proliferative zone size and regulation? GLP-1/Notch is part of a central signaling pathway that instructs proliferative cells to maintain their proliferative fate, however previous work suggests that it is not essential for actual mitotic cell cycle progression (for review see Hansen and Schedl 2006). To determine the affect of GLP-1 on germline proliferation, we analyzed the temperature sensitive loss of function mutant *glp-1(bn18)* (Maine and Kimble 1989; Kodoyianni et al. 1992; Qiao et al. 1995). First, we addressed whether *glp-1(bn18)* mutants at the permissive temperature (15°C) have a reduced proliferative zone size which would indicate a decrease in GLP-1 signaling. Using REC-8 antibody as a marker for proliferative zone cells we observed that *glp-1(bn18)* mutants have a smaller

proliferative zone than wild type animals (166±19 n=20 versus 240±27 n=18). The smaller proliferative zone size confirms that *glp-1(bn18)* mutants have reduced GLP-1 activity in the germline at the permissive temperature.

Next, we asked whether reduced GLP-1 activity causes a change in mitotic cell cycle rate among the remaining proliferative cells. As an initial test, we determined both the S-phase and M-phase index in the *glp-1(bn18)* adult proliferative zone at the permissive temperature (Fig. 1). Despite the decrease in proliferative zone size, the remaining proliferative zone cells showed the same S-phase index and M-phase index as wild type. We extended this cell cycle analysis by determining the length of G2+M+G1, which also allows us to extrapolate a total cell cycle length. The length of G2+M+G1 was determined by measuring the minimum length of continuous EdU feeding required to label all nuclei in the proliferative zone positive for EdU (Crittenden and Kimble 2008). In *glp-1(bn18)* mutants at 15°C, 5 hours of continuous EdU feeding was required for all proliferative cells to incorporate EdU, similar to 4.5 hours for wild type germlines. These values can be used to extrapolate that the maximum length of the cell cycle. The S-phase index for wild type and *glp-1(bn18)* suggests that S-phase occupies ~60% of the total cell cycle, indicating that G2+M+G1 occupies ~40% of the total cell cycle. Therefore, we can extrapolate that the total length of the cell cycle in wild type versus *glp-1(bn18)* mutants animals is ~11 hours versus ~12 hours. An important note for this estimate is that our assay for the length of G2+M+G1 provides a maximum value due to the nature of the assay. The cell cycle time in the proliferative zone almost certainly has a range, suggesting that the cell cycle may often be shorter than the values estimated by this method. Furthermore, the 1 hour difference is within the error of the method and

therefore we do not think that the difference between 11 and 12 hours is significant. Therefore, the decrease in GLP-1 activity due to the *glp-1(bn18)* mutation causes a decrease in proliferative zone size but does not affect the cell cycle kinetics of the proliferative cells present.

We also performed our analysis of G2+M+G1 length on *glp-1(bn18)* and wildtype animals shifted from 15°C to 25°C (25°C is the restrictive temperature for the *glp-1(bn18)* mutant). In both *glp-1(bn18)* and wild type germlines shifted to the restrictive temperature, all proliferative zone cells had incorporated EdU after 3 hours of continuous feeding (Fig. 1). Using this value for G2+M+G1, we can extrapolate that the total cell cycle may be as long as 7 hours for both wild type and *glp-1(bn18)* mutant germlines. However, this again likely represents a maximum estimate and experiments below indicate that cell cycle progression at 25°C can occur significantly faster.

Since *glp-1(bn18)* mutants at the permissive temperature display similar cell cycle kinetics but have a smaller proliferative zone, the proliferative zone should have decreased cell output (cells enter meiosis per unit time) in comparison with wild type germlines. To compare the output, we fed *glp-1(bn18)* and wild type adults EdU for 10 hours at 15°C and analyzed how many EdU labeled nuclei had entered meiosis by co-staining the dissected germlines with REC-8 and HIM-3 antibodies. The EdU positive HIM-3 positive, REC-8 negative germ cells are cells which incorporated EdU and entered meiosis during the 10 hour feeding period. In *glp-1(bn18)* mutants, we counted 107±19 n=11 EdU positive HIM-3 positive REC-8 negative germ cells while in wild type animals, we counted 154±31 n=9. These values represent the approximate output of the proliferative zone over a 10 hour period. Since cell cycle kinetics are similar in glp-

1(bn18) and wild type animals, the output should be proportional to the number of proliferative zone cells. Indeed, the ratio of 10 hour output to the number of proliferative zone cells is ~0.65 for each, indicating that the output for these two strains is proportional to the number of proliferative cells. This observation further supports the idea that cell cycle kinetics in *glp-1(bn18)* germlines are equivalent to wild type germlines. In addition, the proportional proliferative zone output values suggest that an equivalent ratio of the proliferative zone is actively mitotically cycling.

Proliferative cells enter meiosis in *glp-1(bn18)* mutants at the restrictive temperature

We considered two alternative hypotheses to explain the organization of the proliferative zone: 1) proliferative cells have different levels of developmental maturity in progressing from a stem cell to a differentiated early meiotic prophase cell (see Figure 3)(Hansen and Schedl 2006; Cinquin et al. 2010) and 2) proliferative cells are developmentally equivalent. In order for these hypotheses to be instructive, we must define different levels of developmental maturity. An important hypothesis in the field suggests that germ cells that leave the stem cell niche in the distal end of the germline will complete one or more cell divisions (transit amplifying divisions) before entering meiosis (Hansen and Schedl 2006; Cinquin et al. 2010), analogous to germ cells in the *Drosophila* testis and ovary (Fuller and Spradling 2007). Therefore, we define this hypothetical developmental maturation process as progression through a set (>1) of cell divisions.

By shifting *glp-1(bn18)* temperature sensitive mutants from the permissive temperature (15°C) to the restrictive temperature (25°C), we can force all proliferative zone cells to enter meiosis (Austin and Kimble 1987; Kodoyianni et al. 1992; Cinquin et al. 2010). These alternative hypotheses make experimental predictions in regards to the process of differentiation to meiotic prophase. Our first hypothesis is that proximal cells will be more mature and the first to enter meiosis upon loss of GLP-1 activity, initiating a wave a meiotic entry that would proceed from the proximal boundary of the proliferative zone to the distal end (Fig. 3). Previous work by Cinquin et al has independently considered this hypothesis and suggested that indeed proliferative cells undergo a process of maturing as they switch from a stem cell to meiotic prophase since they observed a proximal to distal wave of meiotic entry following loss of *glp-1*(Cinquin et al. 2010). However, analysis of meiotic entry in this experiment was performed primarily by scoring GLD-1 protein levels as a proxy for differentiation, which does not unambiguously distinguish proliferative zone cells from cells in meiotic prophase. The second hypothesis predicts that proliferative zone cells will enter meiosis simultaneously since they are developmentally equivalent.

We analyzed the spatial and temporal pattern with which the proliferative zone cells enter meiosis in *glp-1(bn18)* mutants shifted to the restrictive temperature by employing a battery of markers. We began our characterization by performing a time course analysis of when exactly proliferative zone cells (positive for REC-8 and negative for HIM-3) enter meiosis (become negative for REC-8 and positive for HIM-3). Figure 2A shows the number of proliferative zone cells per germline over time after animals were shifted to the restrictive temperature, a trend that was highly reproducible in repeat

experiments. This loss of proliferative zone cells due to entry into meiosis was a direct cause of the *glp-1(bn18)* mutation as proliferative cells do not prematurely enter meiosis in wild type germlines shifted to the restrictive temperature (however a slight decrease in the proliferative zone cell number may occur due to temperature shock).

This initial analysis provides a framework for dissecting the response of proliferative cells to loss of GLP-1. First, the spatial and temporal patterns in which proliferative cells enter meiosis are reproducible and rapid (statistically significant differences from the 15°C steady state can be detected after 2 hours at the restrictive temperature). This suggests that inactivation of *glp-1(bn18)* at the restrictive temperature is rapid and robust. In addition, cells in the proliferative zone do not enter meiosis simultaneously but rather over an ~8 hour time window. What accounts for the spatial and temporal pattern in which the proliferative zone enters meiosis?

To test whether a difference in developmental maturation may distinguish proximal proliferative zone cells from distal proliferative zone cells in their ability to enter meiosis, we analyzed the position, on a cell by cell basis, of proliferative zone cells (REC-8 positive, HIM-3 negative) and meiotic cells (REC-8 negative, HIM-3 positive) in our temperature shift experiment. Figure 4 shows the distribution of REC-8 positive cells by cell diameter row at time points throughout the *glp-1(bn18)* upshift time course. REC-8 positive nuclei are initially present throughout the 20 distal most cell diameters of the germline. The 12 distal most cell diameters only contain REC-8 positive cells and the next 8 cell diameters contain a mixture of REC-8 positive cells and REC-8 negative cells with a decreasing percentage of REC-8 positive cells from distal to proximal. Between hours 0-4, the distribution of REC-8 positive cells does not change significantly among

the 12 distal most cell diameters. In the next 8 cell diameters, there is an overall decrease in REC-8 positive cells that occurs at each position. From hour 4 to hour 6, the largest decrease in REC-8 positive cells occurs. This change is not restricted to any position as both distal and proximal throughout the germline show a significant decrease in REC-8 positive cell frequency. The REC-8 positive cells that remain are located within the 12 distal most cell diameters. The decrease in proliferative cell frequency continues throughout these 12 cell diameters through hour 8 until all proliferative cells have entered meiosis by hour 10.

Do we observe a pattern of meiotic entry that suggests a distal to proximal gradient of developmental maturity is present in the steady state proliferative zone? First, as depicted in Figure 3, we believe that the initial cells that enter meiosis (between hour 0 and hour 4) were cells that had already initiated meiosis or would have done so regardless of a loss of *glp-1*. This notion is based on the observation that these cells are located in the meiotic entry region of the germline, where the majority of cells are thought to have exited the mitotic cell cycle and be in meiotic S-phase (or another pre-meioitc phase such as pre-meiotic G1 or G2). The remainder of our analysis will focus on the cells that enter meiosis after the 4 hour time point, when we suspect that cells that were mitotically dividing at the time of the temperature up shift enter meiosis. Importantly, after the hour 4 time point, meiotic entry is not limited to one region of the proliferative zone; a large percentage of cells in the very distal rows have entered meiosis by hour 6. Therefore, the majority of cells throughout the proliferative zone have similar kinetics in which they enter meiosis. This result suggests that proximal versus distal cells are not distinguished by developmental maturation through a series of transit amplifying divisions. However,

some cells do not enter meiosis until after hour 6. Therefore, while we did not observe a proximal to distal wave as predicted by the "Non-equivalent" model in Figure 3, proliferative zone cells did not entirely adhere to the "Equivalent" model either. We hypothesize that the asynchrony in meiotic entry among proliferative cells is likely a result of their position in the mitotic cell cycle at the time of the temperature shift (see below).

The results presented here do not rule out the possibility of a proximal to distal wave of meiotic entry occurring between hour 4 and hour 6 among the cells which enter meiosis by hour 6. However, our cell cycle analysis indicates that the length of the mitotic cell cycle in temperature shifted germlines is ~7 hours (although this is a maximum estimate). Therefore, progression through an extra cell division would not be able to explain a difference in meiotic entry kinetics less than 2 hours. Accordingly, if a proximal to distal wave of meiotic entry occurs between hour 4 and hour 6, it likely does not indicate a difference in distal-proximal maturation based on progression through a set of cell divisions.

Mitotic cell cycle progression after loss of GLP-1

In yeast, entry into meiosis begins during the G1 phase of the cell cycle and cells in other phases of the cell cycle are committed to completing the remaining mitotic cell cycle before they can enter meiosis (Hirschberg and Simchen 1977; Honigberg and Purnapatre 2003; Simchen 2009). We hypothesize that the position of a cell in the mitotic cell cycle at the time of the temperature shift affects the kinetics with which that cell enters meiosis. To begin to address this hypothesis, we analyzed cell division and DNA

replication among the proliferative zone cells remaining at each time point during our *glp-1(bn18)* upshift time course (Fig. 2). As shown in Figure 2, cell division continues until hour 6, with a significant number of cell divisions occuring at hour 4. This confirms that proliferative cells complete at least one mitotic cell cycle prior to meiotic entry. In addition, we observe cells in S-phase at hour 6 and hour 8 with a decrease in S-phase index among remaining proliferative cells at hour 8. The fact that S-phase can be observed later in the timecourse than M-phase is likely due to cells undergoing meiotic S-phase as the final step before entry into meiosis. These results suggest that loss of *glp-1* does not interfere with intervening cell cycle progression prior to meiotic entry. In addition, the order in which cell cycle events are completed corresponds to the expected sequence of events in which cells enter meiosis (after completing cell division, cells undergo meiotic S-phase).

Our previous work on the wild type proliferative zone (see chapter 2) estimates that 55-70% of the proliferative zone is actively mitotically cycling. Extrapolated to the reduced size of the *glp-1(bn18)* proliferative zone, this suggests that 85-115 cells are actively dividing (a reasonable extrapolation since cell cycle kinetics are similar and proliferative zone output is proportional). Do all of the cycling cells complete their cell division and do some of these cells divide more than once? To address this question we determined how many cell divisions occurred in our mutant proliferative zones after the temperature shift by counting an increase in EdU labeled nuclei. After 5 hours of EdU feeding at 15°C, over 99% of all proliferative cells in both wild type and *glp-1(bn18)* mutant germlines are EdU positive (Fig.1; Fig. 5). As labeled nuclei continue to divide, the number of EdU labeled nuclei within the germline will increase. Once all proliferative

cells become labeled, any additional increase in the number of EdU positive nuclei per germline must be solely due to cell division. We fed wild type and mutant animals with EdU labeled bacteria for 5 hours at 15°C and then shifted animals to 25°C while continuing to feed them EdU labeled bacteria. Wild type germlines show a steady increase in the total number of EdU labeled nuclei as labeled proliferative cells continue to divide at 25°C (Fig. 5). In *glp-1(bn18)* mutants, the total number of EdU labeled nuclei increases initially by reaches a plateau at 6 hours, the point at which cell division is no longer observed among mutant germlines (Fig. 5). From the time at which animals are shifted to the restrictive temperature until cell division ceases at hour 6, we observed an increase of ~110 EdU labeled nuclei, indicating that ~110 cell divisions occur (Fig. 5). Since we predicted that adult hermaphrodite *glp-1(bn18)* germlines at 15°C contain ~85-115 actively cycling cells, this suggests that these cells all divide approximately once prior to entry into meiosis.

Proliferative cells in Mitotic S-phase and G2 are likely committed to mitosis

When during cell cycle progression do proliferative cells switch from the mitotic cell cycle and initiate meiotic development? The above results suggest that all actively cycling cells complete a single cell division prior to entering meiosis. This observation suggests that cells are committed to completing mitosis early during the cell cycle. However, the total number of cell divisions could be due to a subset of cells dividing more than once, thus complicating the interpretation of this result. Therefore, we analyzed more specifically whether cells in S-phase and G2 are committed to completing mitosis.

First, we asked whether cells in G2 are committed to mitosis or if these cells could switch directly to meiotic prophase. If cells in G2 are committed to mitosis, they must also proceed through meiotic S-phase prior to meiotic prophase whereas if cells in G2 can proceed directly into meiotic prophase without an intervening division, they do not execute DNA replication (Fig. 6A). Therefore, we can test whether these cells can enter meiotic prophase without an intervening S-phase by assaying for EdU incorporation. Experimentally, we placed *glp-1(bn18)* mutants on EdU plates while simultaneously shifting the mutants to the restrictive temperature and asked whether cells within the distal 15 cell diameters (location of the proliferative zone at the time of the shift) are able to enter meiosis without incorporating EdU (Fig. 6B). As shown by a representative image in Figure 6, we never observed EdU negative cells within this region after entry into meiosis was completed at the restrictive temperature (Fig. 6C). The absence of proliferative zone cells entering meiosis without incorporating EdU indicates that these cells were unable to switch from mitotic G2 directly into meiotic prophase.

To determine whether cells in mitotic S-phase can switch to meiosis without an intervening cell division, we analyzed the order in which cells in S-phase versus cells in G2 enter meiosis after shifting *glp-1(bn18)* mutants to the restrictive temperature. We traced cells that were initially in S-phase at the time of the temperature shift by giving animals a short pulse of EdU for the first 20 minutes at the restrictive temperature of a typical *glp-1(bn18)* up-shift experiment. We then analyzed the percent of REC-8 positive nuclei that were EdU positive or negative during the temperature shift time course (Fig. 7). Our analysis indicates that in the hour 6 and hour 8 timepoints, immediately prior to all cells having entered meiosis, the remaining REC-8 positive cells are mostly EdU

negative. This suggests that the final cells to enter meiosis were mostly in G2 (non-S-phase) at the time of the temperature shift and may indicate that cells in S-phase entered faster than cells in G2, possibly because they were not committed to mitosis or because they completed fewer intervening mitotic cell divisions.

At the hour 6 and hour 8 time points of the *glp-1(bn18)* upshift timecourse, very few REC-8 positive cells remain. Perhaps, these cells took longer to complete entry into meiosis because they executed a second mitotic cell division while the surrounding proliferative cells only divided once. To test this possibility, we analyzed when these EdU positive and EdU negative cells divide during the meiotic entry time course. Performing essentially the same pulse-chase-temperature shift experiment, we assayed whether cells in M-phase were EdU positive or negative over the first 6 hours of the *glp-1(bn18)* upshift time-course (Fig. 8A). As shown in Figure 8, EdU negative cells divide first and are observed M-phase at hour 1 and hour 2. By hour 3, nearly all cells in M-phase are EdU positive. At hour 4 and hour 5, we observed a second, albeit decreased, round of EdU negative cells in M-phase. We interpret these results to indicate that a portion of EdU negative cells divide twice before entering meiosis, once during hours 1-2 and again during hours 4-5. Based on this timing of the second division, we observe that cells shifted to 25C can complete the cell cycle within ~4 hours, significantly faster than the maximum cell cycle length of 7 hours that we estimated above (see Fig. 1). We note that the cell cycle length has a range of values, which was also observed with wild type germline using similar assays (see Chapter 2). The result that a fraction of cells that were EdU negative (in G2) at the time of the temperature shift divide twice before entering meiosis likely explains why the final cells to enter meiosis were EdU negative (in G2) at

the time of the temperature shift. Some of these EdU negative cells completed two rounds of cell division before entering meiosis whereas EdU positive nuclei divide only once.

Why do a subset of proliferative cells divide twice while the majority appear to divide only once before entering meiosis? One hypothesis is that these cells may occupy a specific developmental position within the proliferative zone in which they are programmed to divide twice after loss of GLP-1 signal. However, based on the position of the remaining REC-8 positive nuclei at hour 6 and hour 8, the final cells to enter meiosis do not appear to occupy a specific distal-proximal position within the proliferative zone (Fig. 4). Consistent with this, we did not observe the final cell divisions restricted to a specific distal-proximal position (data not shown). Rather than a consequence of progression through programmed transit amplifying divisions, we suspect that the variation in intervening cell divisions depends on cell cycle position at the time of the temperature shift. If cells become committed to completing the mitotic cell cycle early during cell cycle progression, cells in late G2 may have completed their first division and passed the window during which they could switch to meiosis before the drop in GLP- activity directed them to enter meiosis. Thus, the second division may be the direct result of a delay in GLP-1 inactivation in temperature shift experiment.

Commitment to meiosis

In yeast, commitment to meiosis does not occur until after completion of meiotic S-phase (Simchen 2009, also see Discussion). We investigated commitment to meiosis in the *glp-1(bn18)* upshift experiment by returning animals to the permissive temperature at various time points following the upshift and analyzing their long term ability to sustain

mitotic proliferation (Fig. 9A). If proliferative cells were committed to meiosis by the time that they were returned to the permissive temperature, long term mitotic proliferation would not occur. We observed that *glp-1(bn18)* mutants returned to the permissive temperature after 3 hours can successfully maintain mitotic proliferation (Fig. 9B). However, when animals were returned to the permissive temperature after spending longer than 3 hours at the restrictive temperature, all proliferative zone cells entered meiosis (Fig. 9B). How does this commitment relate to the cell cycle events occurring at this timepoint. During the *glp-1(bn18)* upshift timecourse, mitotic cell division persists until 4 and 5 hours, albeit at a reduced frequency (Fig. 2). Therefore, although cells remain in the mitotic cell cycle at these timepoints, they are not capable of maintain long term mitotic proliferation. While this suggests that cells may become committed to meiosis even before mitotic cell cycle exit, there is an important caveat to this analysis. Unfortunately, it is unclear what are the dynamics of *glp-1* reactivation upon returning *glp-1(bn18)* mutants to the permissive temperature. If refolding and/or new synthesis of GLP-1(bn18) upon return to the permissive temperature is significantly delayed, then this reactivation delay could be the limiting factor. Alternatively or in addition, GLD-1 has been shown to bind and repress glp-1 mRNA (Marin and Evans 2003). GLD-1 accumulates as germ cells enter meiosis (see below)(Jones et al. 1996; Hansen et al. 2004b) and may reach a high enough level to block new GLP-1(bn18) synthesis. Therefore, it remains unclear whether endogenous or experimental factors have led to commitment to meiosis after hour 3 of the *glp-1(bn18)* upshift experiment.

Meiotic entry during cell cycle arrest in *glp-1(bn18)* temperature upshift

How does arrest in the mitotic cell cycle affect the ability of proliferative cells to enter meiosis? In fission yeast, blocking DNA replication activates a meiotic DNA replication checkpoint similar to that observed in mitosis and prevents the onset of meiosis (Murakami and Nurse 1999). To extend these studies in *C. elegans*, we asked whether arresting cells with hydroxyurea (HU) inhibits entry into meiosis. We incubated *glp-1(bn18)* mutants on HU-plates for five hours at the permissive temperature (see Materials and Methods, Fig. 10), which completely inhibits any detectable DNA replication (data not shown), and then shifted animals to the restrictive temperature for ten hours (Fig. 10). Importantly, HU treatment alone does not cause premature meiotic entry (Fig. 10). To our surprise, in HU-treated mutants shifted to the restrictive temperature, proliferative cells display premature meiotic entry, based on loss of nucleoplasmic REC-8 and HIM-3 loading on the chromosomes, indicating that HU treatment did not block meiotic entry (Fig. 10). In addition, we confirmed the DNA replication inhibition was successful by assaying for EdU incorporation in HU treated mutants at the restrictive temperature. Indeed, proliferative cells with a block in DNA replication still appear to enter meiotic prophase after loss of GLP-1 signaling and did not show any detectable EdU incorporation (Fig. 10C).

This result is surprising given the precise coordination of meiotic entry with the mitotic cell cycle in wild type germlines. Is the appearance of meiotic entry an artifact of our marker selection? To investigate this, we analyzed other signs of differentiation and meiotic entry in these HU treated mutants. We labeled germlines with a panel of differentiation and meiotic entry markers for the germline including: phospho-SUN-1 (Penkner et al. 2009) (Fig. 11) and chromosomal reorganization using NOP-1/LMN-1

antibodies (MacQueen and Villeneuve 2001) (Fig. 12) and GLD-1 (Jones et al. 1996) (Fig. 13). In addition, we asked whether CYE-1 repressed upon entry into meiosis (Fig. 11). This additional characterization confirmed that DNA replication blocked proliferative cells still appear to enter meiosis upon loss of GLP-1.

Multiple forms of cell cycle arrest fail to block meiotic entry

HU causes arrest in S-phase by blocking DNA replication. How does the induction of meiotic entry respond to other forms of cell cycle arrest? CDK-1 is required for M-phase but not S-phase in mitotic cell division in *C. elegans* (Boxem et al. 1999). We used *cdk-1 RNAi* to cause cell cycle arrest in the germline and asked whether proliferative cells in *glp-1(bn18); cdk-1 RNAi* mutants can still enter meiosis. After 24 hours of *cdk-1 RNAi* in the *glp-1(bn18)* mutant at 15°C, little or no proliferation was observed among the proliferative cells (data not shown). However, after shifting these animals to the restrictive temperature for 10 hours, all cells appeared to enter meiotic prophase (data not show). Therefore, the ability of arrested cell to enter meiosis following *glp-1(bn18)* upshift is not specific to the HU form of arrest and can occur during multiple types of cell cycle arrest.

Kinetics of meiotic entry in cell cycle arrested proliferative cells mimic those of non-arrested cells

Our previous results suggest that the kinetics with which cells enter meiosis depends on their position in the cell cycle (Fig.4; Fig.7). How are these kinetics affected by cell cycle arrest? To investigate this, we analyzed the total number of proliferative

cells in HU treated *glp-1(bn18)* mutants over a time course at the restrictive temperature (Fig. 10B). A gradual decrease in proliferative cells begins soon after mutants are shifted to the restrictive temperature, and the major drop in proliferative cells occurs between hour 6 and 8. This drop in proliferative cells occurs at a similar time to when non-arrested proliferative cells enter meiosis (Fig. 2 and Fig. 10B). Based on these results, it is tempting to speculate that a timing mechanism acts to coordinate entry into meiosis with cell cycle progression.

GLD-1 promotes entry into meiosis independent of mitotic cell cycle progression

GLD-1 plays an important role in promoting entry into meiosis (Francis et al. 1995; Kadyk and Kimble 1998; Crittenden et al. 2002; Hansen et al. 2004a; Hansen et al. 2004b). Previous work indicates that control of GLD-1 protein levels is a key factor in the decision to either remain a proliferative cell or enter meiosis (Hansen et al. 2004b). In the germline, GLD-1 expression is low in the distal part of the proliferative zone (Jones et al. 1996; Hansen et al. 2004b). Its expression gradually increases moving proximal and reaches high expression just prior to the start of the meiotic entry region (Jones et al. 1996; Hansen et al. 2004b). However, mitotic cell divisions still occur among proliferative zone cells that have accumulated high levels of GLD-1 (Hansen et al. 2004b). Therefore, while GLD-1 accumulation plays an important role in promoting entry into meiosis, its accumulation is likely regulated independent of mitotic cell cycle progression. In order to coordinate the switch to meiosis with the proper timing during the mitotic cell cycle, proliferative zone cells may temporarily override GLD-1 activity. To further investigate the role of GLD-1 in meiotic entry, we analyzed GLD-1 expression

after shifting *glp-1(bn18)* mutants to the restrictive temperature (Fig. 14A). In the distal proliferative zone, GLD-1 showed a gradual increase in expression during hour 2 and hour 4 relative to the baseline expression in unshifted controls. Between hour 4 and hour 6, we observed the greatest increase in GLD-1 expression, consistent with previously reported observations with another *glp-1(ts)* mutant (Cinquin et al 2010). This increase in GLD-1 occurs at the same time during which we observe the majority of proliferative cells entering meiosis (Fig. 2), confirming that GLD-1 accumulation plays an important role in the meiotic entry process.

While meiotic entry does occur in *gld-1* mutants, we decided to further investigate whether *gld-1* alters the timing or process by which cells enter meiosis. We constructed double mutants in which we introduced a *gld-1(null)* mutation into the *glp-1(bn18)* strain. Similar to *gld-1(null)* single mutants, *gld-1(null); glp-1(bn18)* show characteristic germline phenotypes (Francis et al. 1995). There is a recognizable proliferative zone in the distal part of the germline followed by a meiotic entry region. However, as in *gld-1(null)* single mutants, germ cells in the *gld-1; glp-1(bn18)* double mutants have a defect in meiotic prophase progression and eventually re-enter the mitotic cell cycle, forming a proximal tumor (Francis et al. 1995). While these germlines have an obvious tumor phenotype, germ cells still enter meiosis in the normal location, indicating that GLD-1 is not absolutely required for entry into meiosis. To determine whether the *gld-1* mutation causes a defect in the timing of meiotic entry, we shifted *gld-1(null); glp-1(bn18)* mutants to the restrictive temperature and monitored proliferative zone cells and meiotic cells using REC-8 and HIM-3 antibody. The initial size of *gld-1(null); glp-1(bn18)* proliferative zone is smaller than that of *glp-1(bn18)* single mutants. However, whereas

all REC-8 positive proliferative zone cells in *glp-1(bn18)* single mutants have entered meiosis by 10 hours and become REC-8 negative HIM-3 positive, *gld-1(null); glp-1(bn18)* double mutants still retain REC-8 positive cells in the distal proliferative zone after 24 hours at the restrictive temperature (Fig. 14C). Therefore, although *gld-1* mutants still induce some degree of meiotic entry upon loss of *glp-1*, the absence of *gld-1* appears to cause a defect in this meiotic entry process, either by delaying meiotic entry or preventing a number of cells from initiating meiosis altogether. These phenotypes are consistent with the known function of GLD-1 in promoting meiotic entry.

Our above results with *glp-1(bn18)* single mutants suggest that cell cycle position is an important determinant in meiotic entry timing. Therefore, a defect in cell cycle progression or a increase in cell cycle length might explain the defect in meiotic entry among proliferative zone cells in the *gld-1(null); glp-1(bn18)* double mutant. To investigate this possibility we analyzed cell cycle progression in *gld-1(null); glp-1(bn18)* mutants using pH3 antibody to analyze M-phase and EdU incorporation to analyze S-phase. However, *gld-1(null); glp-1(bn18)* double mutants did not show any significant difference in cell cycle progression in comparison with *glp-1(bn18)* single mutants (data not shown). Therefore, the defect in meiotic entry in *gld-1(null); glp-1(bn18)* mutants is not likely due to a defect in cell cycle progression.

The fact that cell cycle arrested cells can still be forced to enter meiosis in *glp-1(bn18)* mutants at the restrictive temperature suggests that a major regulatory mechanism is promoting entry into meiosis independent of mitotic cell cycle progression. We hypothesized that GLD-1 may contribute to this since GLD-1 accumulation in wild type germlines appears to occur regardless of cell cycle position. In support of this idea

GLD-1 accumulates in proliferative zone cells of *glp-1(bn18)* mutants shifted to the restrictive temperature, even when these proliferative cells are arrested with HU (Fig. 13). Does GLD-1 also function in these cell cycle arrested cells to promote meiotic entry? To investigate this possibility we treated *gld-1(null); glp-1(bn18)* mutants with hydroxuyrea, shifted them to the restrictive temperature and monitored the status of proliferative zone cells with REC-8 and HIM-3 antibody. Whereas *gld-1(null); glp-1(bn18)* mutants display a significant decrease in proliferative zone size, there is no decrease in REC-8 positive proliferative zone cell in HU-treated *gld-1(null); glp-1(bn18)* double mutants compared to unshifted controls. Therefore, *gld-1* activity is important for promoting the meiotic entry we observe in cell cycle arrested proliferative cells. This suggests that the function of GLD-1 in promoting meiotic entry acts independent of mitotic cell cycle progression.

Discussion

GLP-1 does not directly affect progression through the mitotic cell cycle

The GLP-1 signaling pathway plays a major role in promoting the proliferative fate (Hansen and Schedl 2006; Kimble and Crittenden 2007). Recent studies (see Chapter 2) have demonstrated that the proliferative fate may, in part, be linked to active mitotic cell cycle progression. Proliferative cells appear to be constantly cycling under normal conditions and an important cell cycle regulator, CDK-2-CYE-1, may contribute to this link between the proliferative cell fate and active cell cycle progression by acting as a positive regulator to both process. This raises the possibility that GLP-1 signaling may also regulate cell cycle progression, aside from simply providing a permissive

environment by promoting the proliferative cell fate. A direct connection between GLP-1 signaling and mitotic cell cycle progression predicts that loss of function *glp-1* mutants might show defective or abnormal cell cycle progression. However, our results here indicate that mitotic cell cycle progression in *glp-1(bn18)* mutants is normal, both at the permissive temperature (15°C) and at restrictive temperature while proliferative cell fate persists (25°C). This observation suggests that GLP-1 does not directly regulate cell cycle progression. In this study, we employ the *glp-1(bn18)* mutant to study the process of meiotic entry and many of our observations are correlated with mitotic cell cycle events. The fact that *glp-1(bn18)* does not alter mitotic cell cycle events allows us to refer our observations back to the biology of the wild type germline.

Response of proliferative zone cells to loss of *glp-1*

We have used the *glp-1(bn18)* temperature sensitive mutant to characterize the response of proliferative zone cells to a loss of *glp-1*. In the gonad, GLP-1 ligand is provided by the DTC and GLP-1 receptor is present on germ cells throughout the proliferative zone. Movement of germ cells away from the DTC is thought to cause a drop in GLP-1 activity, which plays a major role in instructing proliferative cells to enter meiosis. However, it is unknown when and where the drop in GLP-1 activity actually occurs. As discussed in Figure 3, two basic models have been proposed for the temporary maintenance of the proliferative fate after cells become displaced from the DTC niche. These models make distinct predictions for the pattern of meiotic entry of proliferative cells following a sudden drop in GLP-1 activity throughout the germline by shifting *glp-1(bn18)* to the restrictive temperature. Whereas equivalent proliferative cells should enter

meiosis nearly simultaneously, developmentally distinct proliferative cells may enter meiosis with varying kinetics as a consequence of their developmental maturation process. While our results did not follow either of these simplified models, they suggest that by and large proliferative zone cells respond equivalently to a loss of *glp-1*. The spatial and temporal pattern of meiotic entry did suggest that a group of cells (~25% of total population) in the proximal region was somewhat distinct from the majority of the proliferative zone cells located more distally and entered meiosis during the 0-4 hour period after the temperature shift. These cells likely represent cells that were in meiotic S-phase at the time of the temperature shift or would have normally entered meiosis during this time period. For the remaining proliferative zone cells, there was no position specific effect on their meiotic entry timing, but rather a temporal difference in meiotic entry timing that appeared to result from their initial asynchrony in the mitotic cell cycle at the time of the temperature shift.

Based on the response of proliferative zone cells to a loss of *glp-1*, we propose that temporary maintenance of the proliferative fate in wild type germ cells after displacement from the DTC relies upon direct GLP-1 activity. This is supported by the observation that loss of *glp-1* by shifting *glp-1(bn18)* to the restrictive temperature causes a rapid meiotic entry response. If another signaling mechanism was responsible for temporary proliferative fate maintenance in the cells displaced from the DTC, a more significant delay (in terms of both time and intervening cell divisions) should have preceded meiotic entry throughout the proliferative zone.

Commitment to meiosis and mitosis

In budding yeast, the commitment to meiosis and mitosis do not occur at analogous stages of the cell cycle (Simchen 2009). Yeast cells become committed to mitosis early during progression through the mitotic cell cycle, specifically prior to the start of S-phase (Hirschberg and Simchen 1977). Once cells begin mitotic S-phase, they cannot switch to meiosis until after completion of mitotic division. This commitment to mitosis likely occurs because cells need to perform a specialized meiotic S-phase (Forsburg 2002). However, cells that undergo meiotic S-phase are not committed to meiosis until just before the first meiotic division is executed (Simchen et al. 1972). If the nutritional cues that initiate meiosis in budding yeast, namely glucose and nitrogen starvation in the presence of acetate, are reversed, cells can revert to the mitotic cell cycle (Simchen et al. 1972). Therefore, commitment to meiosis occurs long after the initial meiotic preparatory events in G1 occur.

In yeast, meiosis occurs in response to environmental cues, but in multicellular organisms, entry into meiosis is less dependent on environment. When the decision to enter meiosis occurs in addition to when commitment to mitosis and meiosis occur has not been analyzed in multicellular organisms. In particular, we were interested in investigating this in the *C. elegans* germline where an apparent lack of the G1 phase of the cell cycle may result in important differences in these processes. Our ability to induce meiotic entry with *glp-1(bn18)* and analyze cell cycle progression allows us to investigate commitment of proliferative cells to both mitosis and meiosis with respect their progression through the cell cycle. We observed that nearly all actively mitotically cycling cells are committed to completing mitotic cell division prior to entering meiosis, suggesting that commitment to mitosis occurs early in the cell cycle. Consistent with this,

neither cells in mitotic G2 nor mitotic S-phase appear to be able to directly switch to meiosis. This is consistent with the idea that meiotic S-phase is a critical step in meiosis.

Meiotic entry occurs in *glp-1(bn18)* despite cell cycle arrest

We observed that proliferative zone cells can be induced to enter meiosis despite being arrested in the cell cycle. In fission yeast, blocking DNA replication by HU treatment prevents entry into meiosis that is induced by nitrogen starvation (Murakami and Nurse 1999). However, entry into meiosis can be induced under abnormal conditions by utilizing variety genetic tricks. In fission yeast, the initiation of meiosis requires a signaling cascade that includes the Mei2 RNA binding protein, the Pat1 protein kinase and Mei3, which binds and represses Pat1 (Watanabe et al. 2001). The initiation of meiosis relies upon Mei3 induction, which causes Mei3 to inhibit Pat1. However, induction of Mei3 is specifically restricted to the G1 phase of the cell cycle (Watanabe et al. 2001). Therefore, the initiation of meiosis normally only occurs during the G1 phase of the cell cycle. However, cells in G2 can be induced to enter meiosis directly (without undergoing mitotic division and meiotic S-phase) by ectopically activating Mei3 or repressing Pat1 (Watanabe et al. 2001). In budding yeast, meiotic entry can be induced in mutants in which DNA replication checkpoints fail (Forsburg 2002). Whereas HU treatment normally blocks the ability to enter meiosis, yeast cells can be induced to enter meiosis when DNA replication checkpoints are inactivated (Stuart and Wittenberg 1998). An example of this occurs in *clb5 clb6* double mutants, where meiotic DNA replication is blocked yet cells still attempt to undergo meiosis (Stuart and Wittenberg 1998). As these

examples demonstrate, abnormal entry into meiosis can occur by manipulating downstream regulatory mechanisms or checkpoint control mechanisms.

An important consideration for our analysis of *glp-1(bn18)* is the degree to which ectopic induction of meiosis by loss of *glp-1* relates to the endogenous process of meiotic entry. Does the temperature shift inducing loss of *glp-1(bn18)* provide a useful framework for exploring the meiotic entry process or does this loss of *glp-1* cause an abnormal meiotic entry response? In the wild type germline, loss of GLP-1 activity appears to be a major signal for inducing meiotic entry. As proliferative zone cells move away from the DTC, they presumably receive a decrease in GLP-1 activity and this decrease likely plays a major role in the endogenous meiotic entry process. However, the decrease in GLP-1 activity may be regulated in the germline in a way that is not reproduced in the *glp-1(bn18)* temperature upshift experiment. First, loss of GLP-1 activity may somehow be coordinated with progression through the cell cycle. It is conceivable that endogenous GLP-1 activity decrease is coupled to cell division, as distribution of GLP-1(INTRA) molecules between daughter cells could cause a sharp decrease in GLP-1 activity. Second, the decision to enter meiosis in wild type proliferative zone cells may occur as a result of secondary signaling cues prior to GLP-1 activity actually reducing to the level achieved in the *glp-1(bn18)* up shift experiment. Testing these hypotheses awaits the development of methods for analyzing GLP-1 activity.

GLD-1 may act independent of the mitotic cell cycle to promote entry into meiosis

The observation that loss of *glp-1(bn18)* causes entry into meiosis regardless of mitotic cell cycle arrest suggests that an important meiotic entry promoting signal is acting independent of mitotic cell cycle progression. The redundant GLD-1 and GLD-2 pathways act to promote entry into meiosis (Kadyk and Kimble 1998; Hansen et al. 2004a). Therefore, one or both of these pathways may play a role in promoting meiotic entry independent of mitotic cell cycle progression. GLD-1 is a critical member of the GLD-1 pathway and its expression level is important for regulating the decision of germ cells to either retain the proliferative fate or enter meiosis (Crittenden et al. 2002; Hansen et al. 2004b). GLD-1 protein gradually accumulates in the wild type proliferative zone as proliferative zone cells move proximal and near the meiotic entry region (Jones et al. 1996; Hansen et al. 2004b). This accumulation of high GLD-1 levels occurs independent of cell cycle progression, since equally high GLD-1 can be observed in adjacent cells despite the fact that they may be in different mitotic cell cycle stages or beginning meiotic prophase (Hansen et al. 2004b). Here, we show that GLD-1 may act independently of cell cycle progression to promote entry into meiosis. When HU arrested proliferative zone cells lack *gld-1*, they do not enter meiosis upon loss of *glp-1*. This observation is supported by the fact that GLD-1 accumulation is cell cycle independent and that GLD-1 accumulation promotes entry into meiosis. However, in a wild type germline, proliferative zone cells properly coordinate the switch to meiosis with mitotic cell cycle progression. Therefore, in proliferative zone cells with high GLD-1, the coordination of meiotic entry with the appropriate stage of the mitotic cell cycle depends on temporarily overriding the effects of GLD-1 accumulation. This coordination is somehow disrupted by cell cycle arrest and/or loss of *glp-1* when HU-arrested

proliferative cells enter meiosis in the *glp-1(bn18)* upshift experiment. Taken together, these results suggest that coordination of the switch to meiosis with mitotic cell cycle progression depends on a complex web of regulatory factors

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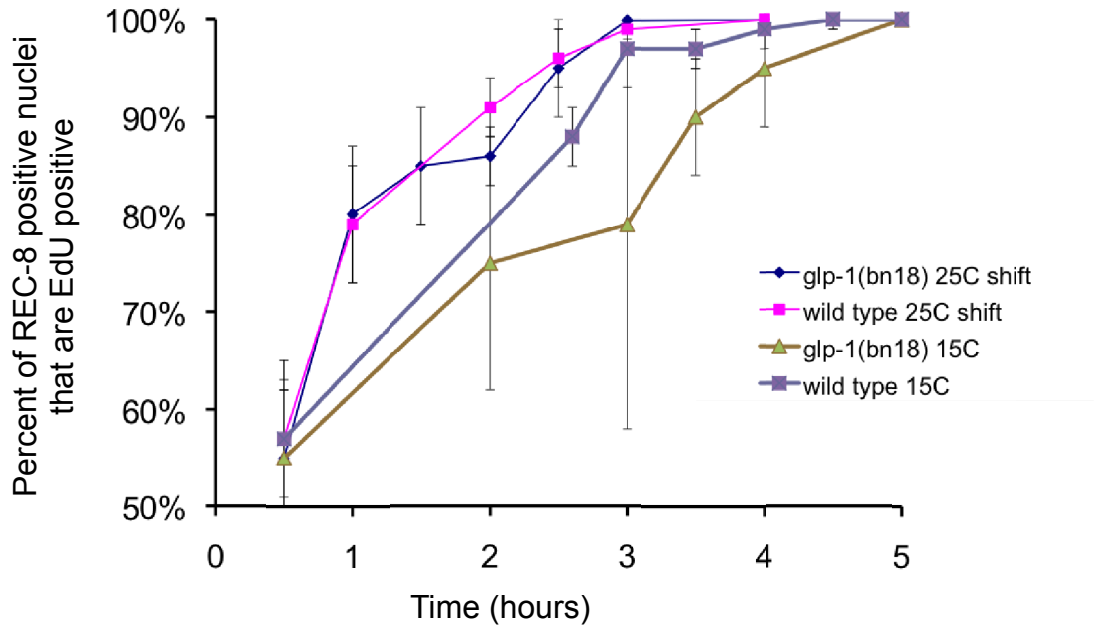
Figure 1

A



B

	Wild type	***glp-1(bn18)***
Proliferative cells (15°C)	240±27 n=18	166±19 n=20
S-phase index (15°C)	63±8% n=18	59±7% n=20
M-phase index (15°C)	3±1% n=49	3±1% n=54
Maximum length of G2+M+G1 (15°C)	4.5 hours	5 hours
Maximum length of G2+M+G1 (15°C to 25°C shift)	3 hours	3 hours
10 hour Output (15°C)	154±31 n=9	107±19 n=11

Figure 1. Cell cycle progression in *glp-1(bn18)* mutants is unaffected. Cell cycle progression was analyzed in *glp-1(bn18)* temperature sensitive mutants at the permissive and restrictive temperature. (A) A continuous EdU feeding timecourse was used to measure the length of G2+M+G1. Animals were raised at the permissive temperature (15°C) and placed on EdU-plates and incubated at 15°C or at 25°C (restrictive temperature). Animals were dissected at various intervals during the time-course and dissected germlines were stained with REC-8 antibody to identify proliferative zone cells and for EdU incorporation. The time required to label all proliferative zone cells positive for EdU provides an estimate of the total length of G2+M+G1. The length of G2+M+G1 is 4.5 hours for N2 and 5 hours for *glp-1(bn18)* 15°C, a difference that is within the error of these experiments. In addition, the length of G2+M+G1 decreases at higher temperature for both wild type an *glp-1(bn18)* strains. (B) Chart shows a summary of cell cycle parameters for *glp-1(bn18)* mutants versus wild type animals at the restrictive temperature (see Materials and Methods). Three important observations are made: 1) cell cycle progression in *glp-1(bn18)* is essentially identical to wild type, 2) the size of the proliferative zone in *glp-1(bn18)* is smaller in comparison to wild type germlines and 3) the output of the proliferative zone in *glp-1(bn18)* mutants is lower but proportional to the decrease in proliferative zone size.

Figure 2

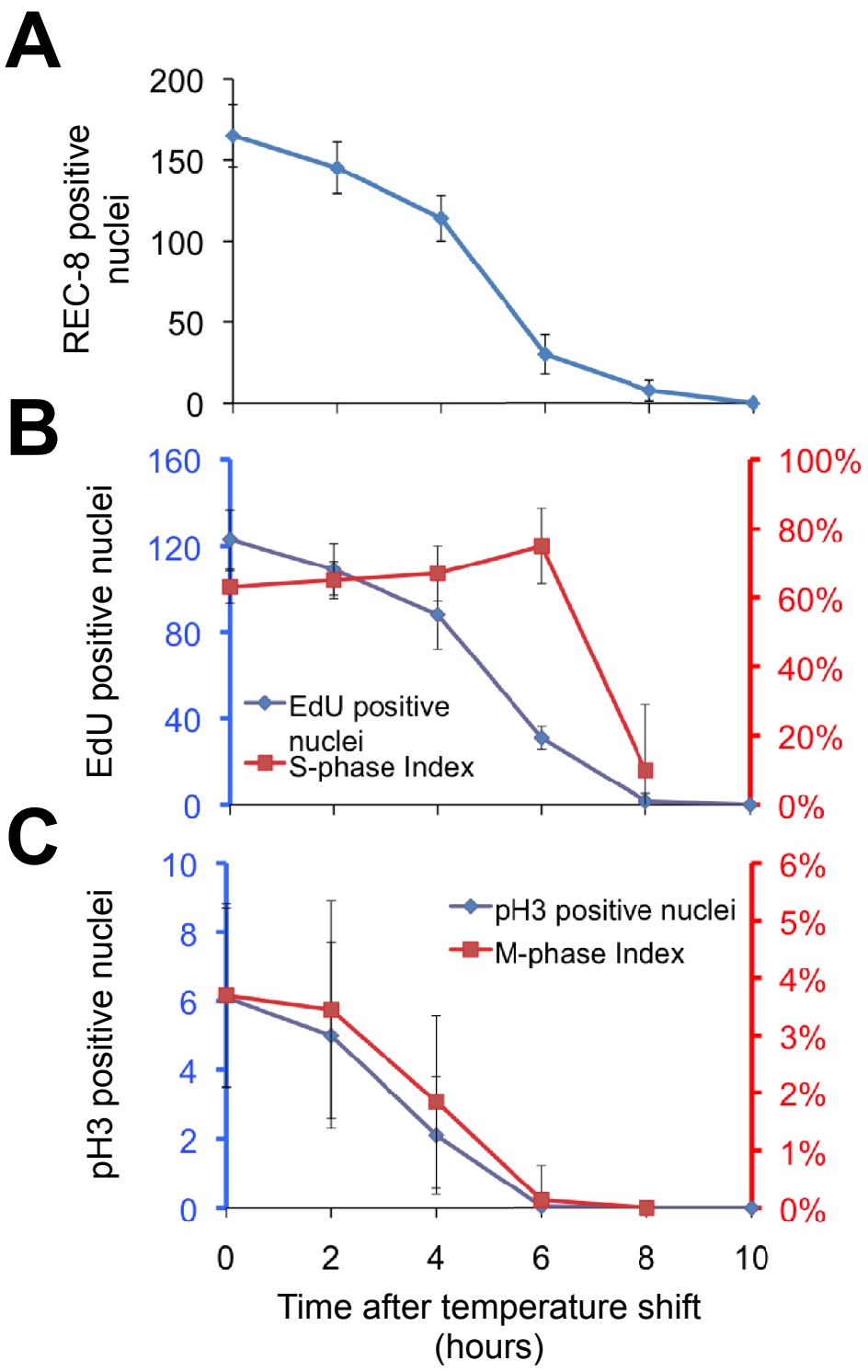

A
REC-8 positive nuclei
200
150
100
50
0
B
EdU positive nuclei
160
120
80
40
0
100%
80%
60%
40%
20%
0%
EdU positive nuclei
S-phase Index
C
pH3 positive nuclei
10
8
6
4
2
0
6%
5%
4%
3%
2%
1%
0%
pH3 positive nuclei
M-phase Index
0
2
4
6
8
10
Time after temperature shift (hours)

Figure 2. Time course of meiotic entry in *glp-1(bn18)* shifted to 25°C. *glp-1(bn18)* mutants were raised at 15°C until 24 hours past L4, shifted to 25°C and animals were dissected every two hours. (A) Dissected germlines were labeled with REC-8 and HIM-3 antibody to identify proliferative zone cells and cell in meiotic prophase. REC-8 positive, HIM-3 negative nuclei were counted to plot the decrease in the proliferative zone as cells enter meiosis. (B) *glp-1(bn18)* mutants were manipulated essentially as described in (A) except animals were given a 30 minute pulse of EdU immediately prior to dissection to identify cells in S-phase. Germlines were then labeled with REC-8 antibody and EdU incorporation. The total number of EdU positive nuclei per germline was counted as well as the total number of proliferative zone cells. S-phase index was determined by dividing the number of EdU positive nuclei by the total number of REC-8 positive nuclei. (C) *glp-1(bn18)* mutants were manipulated as described in (A) and labeled with REC-8 and pH3 antibody. M-phase index was determined by dividing the total number of pH3 positive nuclei by the number of proliferative zone cells. Error bars show standard deviation.

Figure 3

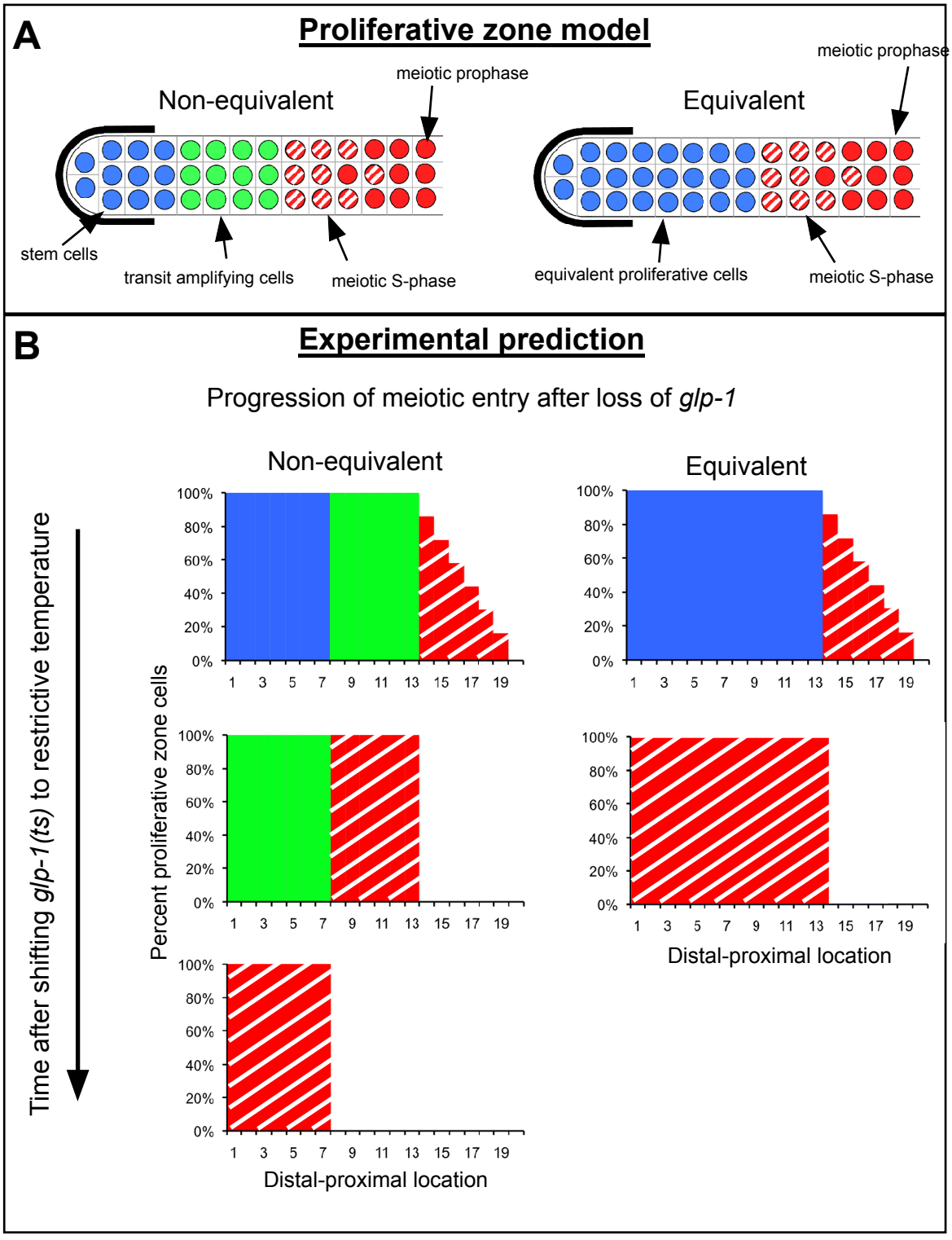

A
Proliferative zone model
Non-equivalent
meiotic prophase
stem cells
transit amplifying cells
meiotic S-phase
Equivalent
meiotic prophase
equivalent proliferative cells
meiotic S-phase
B
Experimental prediction
Progression of meiotic entry after loss of glp-1
Non-equivalent
Equivalent
Time after shifting glp-1(ts) to restrictive temperature
Percent proliferative zone cells
100%
80%
60%
40%
20%
0%
1 3 5 7 9 11 13 15 17 19
Distal-proximal location

Figure 3. Two hypotheses and a test for the organization of the proliferative zone. (A) Two hypotheses are proposed to explain the organization of the proliferative zone with respect to developmental potential of proliferative zone cells. In the non-equivalent model, proliferative zone cells progress through a multiple steps in the transition from stem cell to meiosis. Stem cells are shown in blue. Transit amplifying stages are shown in green. Cells in meiotic S-phase are striped red. Cells in meiosis are solid red. A second model proposes that proliferative zone cells are developmentally equivalent (depicted as being all blue with meiotic S-phase cells still striped red). These models are largely based on the observation that the DTC niche only contacts the distal most cells (shown in blue) in the "non-equivalent" model. (B) These models present distinct experimental predictions regarding the temporal and spatial pattern of meiotic entry following loss of *glp-1*. The non-equivalent model predicts that proliferative zone cells will enter meiosis in a proximal to distal wave. After loss of GLP-1, proliferative cells must complete the intermediate stages of development in the transition from stem cell (blue) to meiosis (red). The fact that subsets of proliferative zone cells have already initiated this process through becoming transit amplifying cells will be reflected in the differential timing with which they complete entry into meiosis. In contrast, the equivalent model predicts that proliferative cells will respond similarly to the loss of *glp-1* regardless of distal-proximal position. It is important to note that this experimental test does not definitively prove either model. Also, superimposed on DTC-GLP-1 signaling is the fact that cells are in different stages of the mitotic cell cycle. If entry into meiosis is restricted to a certain stage in the mitotic cell cycle, then there will be a delay in entry until cell cycle progression proceeds to the appropriate time window. Therefore, simultaneous entry into

meiosis is still not expected since proliferative cells are not synchronized in cell cycle progression. For simplicity, this additional layer of complexity is not considered in this prediction.

Figure 4

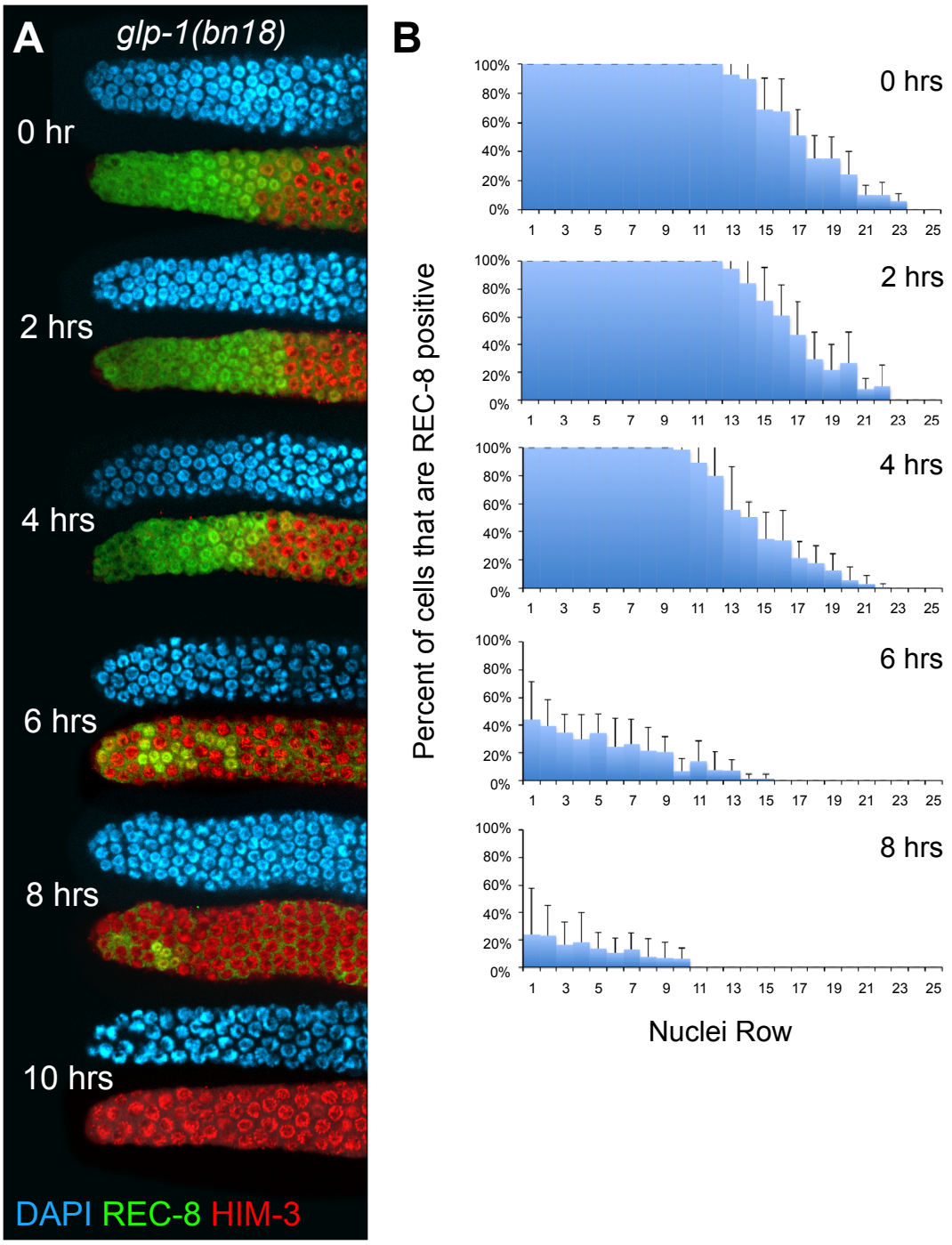
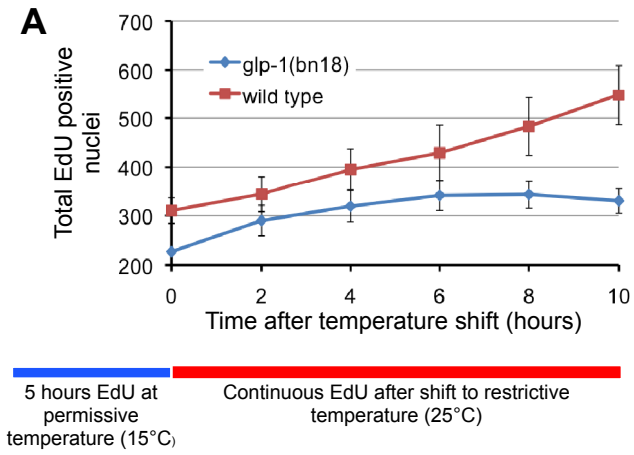

A
glp-1(bn18)
0 hr
2 hrs
4 hrs
6 hrs
8 hrs
10 hrs
DAPI REC-8 HIM-3
B
Percent of cells that are REC-8 positive
0 hrs
2 hrs
4 hrs
6 hrs
8 hrs
100%
80%
60%
40%
20%
0%
1 3 5 7 9 11 13 15 17 19 21 23 25
Nuclei Row

Figure 4. Distribution of proliferative cells in *glp-1(bn18)* at the restrictive temperature. (A) Representative images of *glp-1(bn18)* germlines at the restrictive temperature for the indicated time. REC-8 (green) labels proliferative zone cells while HIM-3 (red) labels cells in meiotic prophase. (B) Graphs show the percentage of cells positive for REC-8 for cell diameter positions in the 25 most distal cell rows of the germline. At least 10 germlines were scored for each time point. Graph time point corresponds to the time point of the adjacent image in panel (A).

Figure 5


A
Total EdU positive nuclei
700
600
500
400
300
200
glp-1(bn18)
wild type
0
2
4
6
8
10
Time after temperature shift (hours)
5 hours EdU at permissive temperature (15°C)
Continuous EdU after shift to restrictive temperature (25°C)

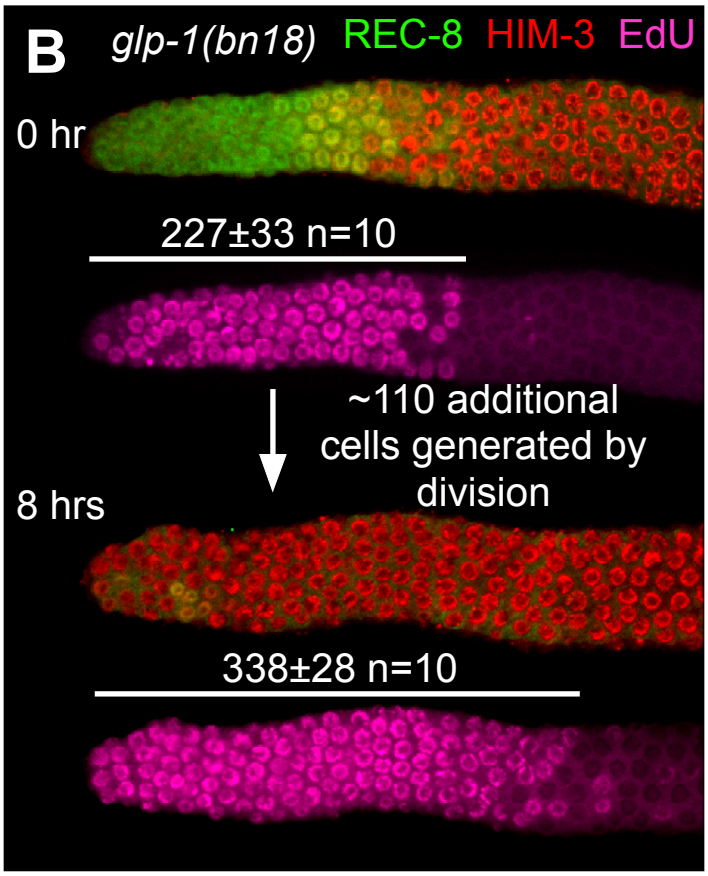
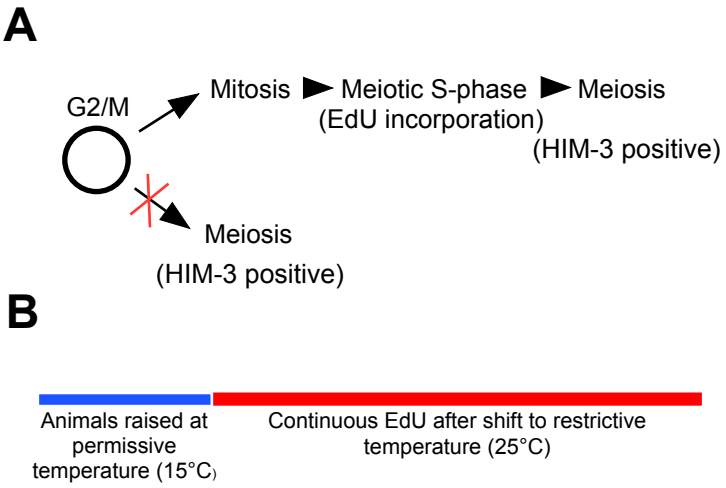

B
glp-1(bn18)
REC-8
HIM-3
EdU
0 hr
227±33 n=10
~110 additional cells generated by division
8 hrs
338±28 n=10

Figure 5. Approximately 110 additional cells generated before all proliferative zone cells enter meiosis in *glp-1(bn18)* upshift experiment. (A-B) Wild type and *glp-1(bn18)* animals were raised at 15°C and staged to 24 hours past L4. Animals were then fed EdU continuously for 5 hours which is sufficient to label all proliferative cells positive for EdU. Animals were then shifted to 25°C without stopping the EdU feeding. t=0 is when animals were shifted to 25°C. Animals were dissected and labeled with REC-8 and HIM-3 antibodies as well as for EdU incorporation. (A) Total number of EdU positive nuclei in wildtype and *glp-1(bn18)* germlines is plotted over time. While the total number of EdU positive nuclei in wild type germlines continues to increase due to continued cell division, the increase in EdU positive nuclei in *glp-1(bn18)* animals reaches a plateau at hour 6 when cell division is no longer observed (see also Figure 2 and Figure 5). (B) Representative images of *glp-1(bn18)* at hour 0 and hour 8.

Figure 6


A
G2/M
Mitosis
Meiotic S-phase
Meiosis
(EdU incorporation)
(HIM-3 positive)
Meiosis
(HIM-3 positive)
B
Animals raised at permissive temperature (15°C)
Continuous EdU after shift to restrictive temperature (25°C)

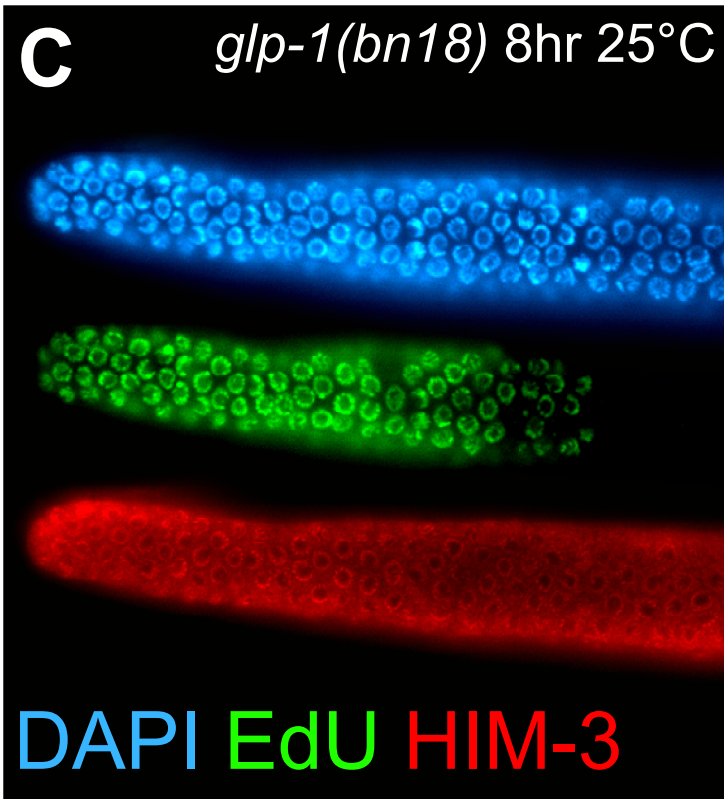
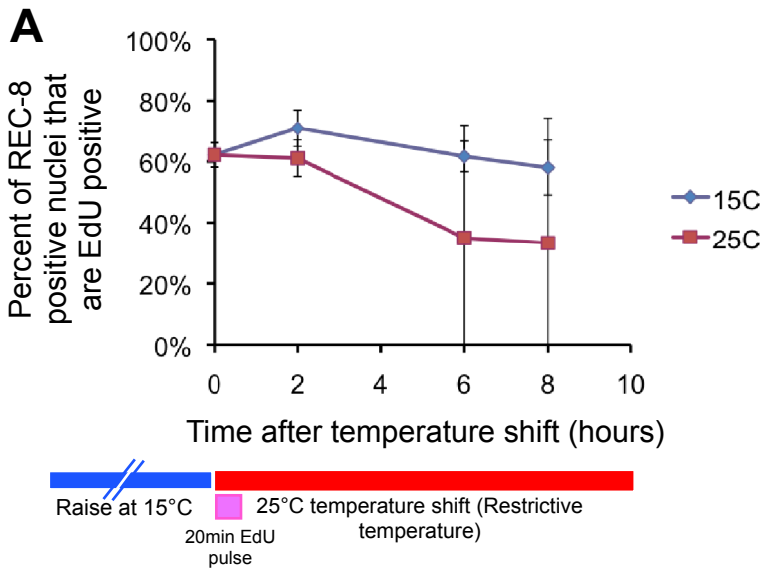

C
glp-1(bn18) 8hr 25°C
DAPI EdU HIM-3

Figure 6. Proliferative cells in G2/M are committed to mitosis. (A) In order to enter meiosis with 4n DNA content, proliferative cells in G2/M with 4n DNA content could either enter meiosis directly (become HIM-3 positive) or divide mitotically and then enter meiosis after completing meiotic S-phase. To determine whether cells in G2/M are obligated to first divide mitotically before entering meiosis, we raised *glp-1(bn18)* mutants at the permissive and shifted them to the restrictive temperature while simultaneously initiating continuous EdU feeding (B). If cells in G2/M are committed to completing mitosis before they can enter meiosis, all proliferative cells should pass through S-phase prior to entering meiosis. (C) A representative image of the distal germline after 10 hours at the restrictive temperature and ten simultaneous hours of EdU feeding shows cells throughout the first 15 cells diameters are all EdU positive, indicating that cells throughout the proliferative zone must pass through an S-phase before entering meiosis. Therefore, G2/M proliferative zone cells could not directly enter meiosis, but rather were committed to completing the mitotic cell cycle.

Figure 7


A
Percent of REC-8 positive nuclei that are EdU positive
100%
80%
60%
40%
20%
0%
0
2
4
6
8
10
Time after temperature shift (hours)
15C
25C
Raise at 15°C
20min EdU pulse
25°C temperature shift (Restrictive temperature)

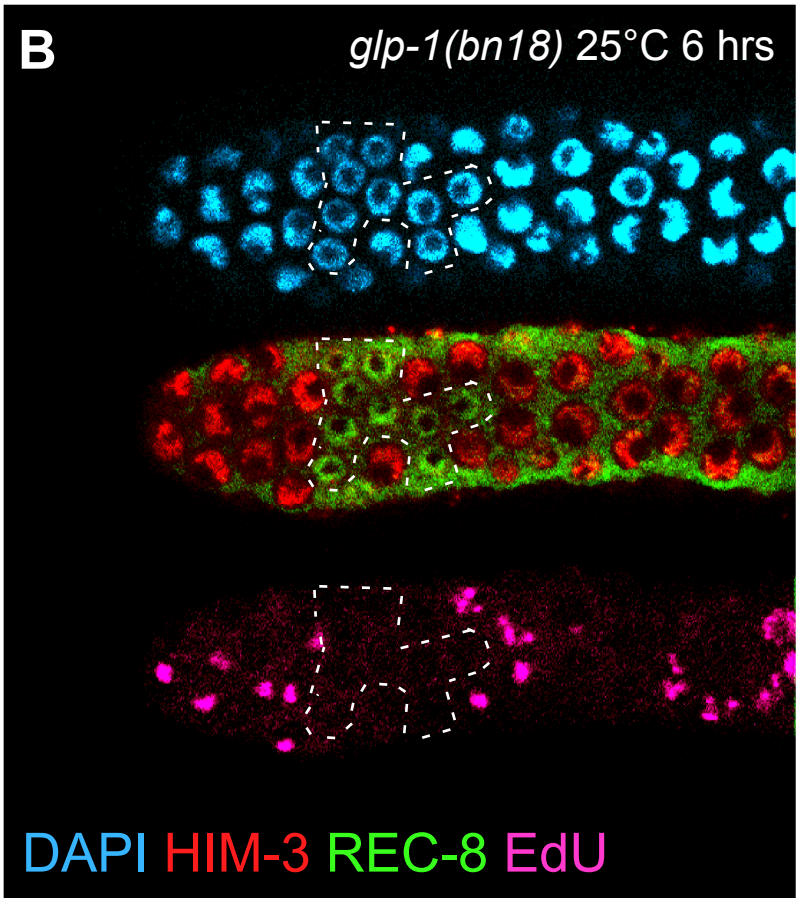

B
glp-1(bn18) 25°C 6 hrs
DAPI HIM-3 REC-8 EdU

Figure 7. The final cells to enter meiosis are enriched for cells that were in G2/M at the time of the temperature shift. *glp-1(bn18)* animals were raised at the permissive temperature and shifted to the restrictive temperature 24 hours past L4. Animals were given a 20 minute pulse of EdU at the time of the temperature shift. Dissected germlines from the indicated time points were labeled with REC-8 and HIM-3 antibody and for EdU detection. (A) The percent of REC-8 positive nuclei that were also EdU positive were scored for the indicated time points. (B) A representative germline from the 6 hour time point shows a cluster of 9 REC-8 positive cells in the distal portion of the germline. All nine of these cells are EdU negative but are surrounded by EdU positive cells that have already entered meiotic prophase (REC-8 negative, HIM-3 positive). This indicates that cells that were in S-phase (EdU) positive at the time of the temperature shift entered meiosis prior to when cells in G2/M/G1 entered meiosis. On average, the number of cells in G2 greatly exceeds the number of cells in M or G1 (Chapter 2). Therefore, the EdU negative cells were likely in G2 at the time of the temperature shift.

Figure 8

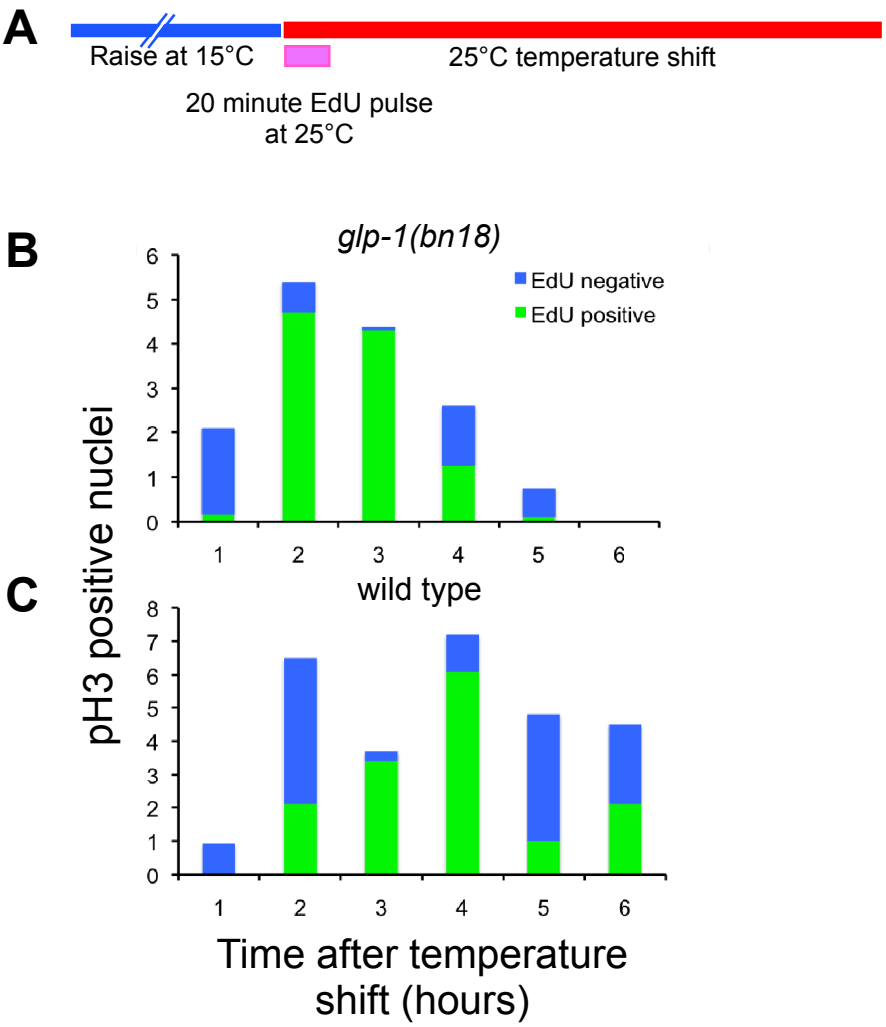

A
Raise at 15°C
25°C temperature shift
20 minute EdU pulse
at 25°C
B
glp-1(bn18)
EdU negative
EdU positive
C
wild type
pH3 positive nuclei
Time after temperature
shift (hours)

Figure 8. A portion of cells that were in G2/M/G1 at the start of *glp-1(bn18)* temperature shift will divide twice before entering meiosis. (A) *glp-1(bn18)* mutants were raised at the permissive temperature and shifted to the restrictive temperature 24 hours past L4. Animals were were shifted to the restrictive temperature and given an EdU pulse for the first 20 minutes at the restrictive temperature. Dissected germlines from the given time points were labeled with pH3 antibody and for EdU detection. (B-C) Phospho-H3 positive nuclei were counted and scored as EdU positive or negative for the indicated time points. In both *glp-1(bn18)* mutants (B) and wild type animals (C), the initial cells to divide are EdU negative, followed by cells that are EdU positive dividing between hours 2-5. In wild type animals were cell division continues indefinitely, EdU negative cells begin to divide a second time starting at hour 4. In *glp-1(bn18)* mutants, a small number of EdU negative cells appear to divide again at hours 4-5. This timing indicates that mitotic cell cycle progression at 25°C can be completed in ~4 hours. In both wild type animals and *glp-1(bn18)* mutants, a noticeable dip in cell division during the first hour is likely due to the temperature shock. Taken together, the results indicate that a small number of EdU negative (cells that were in G2/M at the time of the temperature shift/EdU pulse) divide twice before entering meiosis. The EdU negative cells which divide at the 4-5 hour time points are not the result of outlying cells with a greater than average G2 length because in separate experiments in which animals were continuously fed EdU from the time of the temperature shift, all cells dividing after 3 hours were EdU positive.

Figure 9

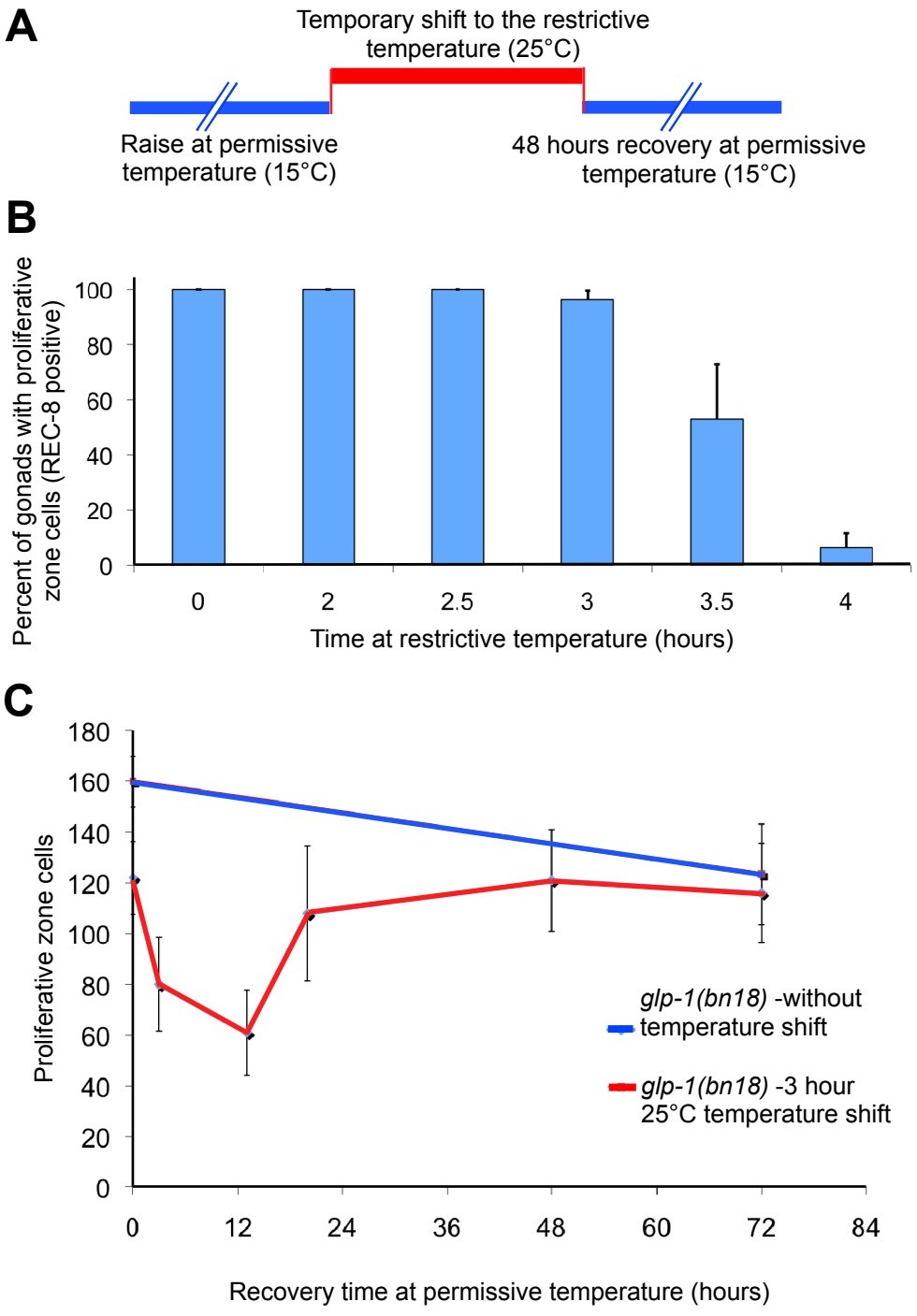

A
Temporary shift to the restrictive temperature (25°C)
Raise at permissive temperature (15°C)
48 hours recovery at permissive temperature (15°C)
B
Percent of gonads with proliferative zone cells (REC-8 positive)
100
80
60
40
20
0
0
2
2.5
3
3.5
4
Time at restrictive temperature (hours)
C
Proliferative zone cells
180
160
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
84
Recovery time at permissive temperature (hours)
glp-1(bn18) -without temperature shift
glp-1(bn18) -3 hour 25°C temperature shift

Figure 9. Analysis of commitment to meiosis in *glp-1(bn18)* mutants shifted temporarily to the restrictive temperature. (A-B) *glp-1(bn18)* mutants were raised at the permissive temperature (15°C) and shifted to the restrictive temperature (25°C) as staged adults 24 hours past L4. After a temporary period at the restrictive temperature, animals were returned to the permissive temperature and allowed to recover for 48 hours. Animals were dissected and germlines stained with antibodies against REC-8 and HIM-3 to identify proliferative zone cells. (B) The dissected germlines were scored as being either positive or negative for proliferative zone cells (REC-8 positive) in animals temporarily shifted to the restrictive temperature for the indicated period of time. After 3 hours at the restrictive temperature followed by 48 hours of recovery, over 95% of gonads still had proliferative zone cells. (C) *glp-1(bn18)* mutants were shifted to the restrictive temperature for 3 hours and then returned to the permissive temperature. After the return to the permissive temperature (time point 0), germlines were dissected at the indicated time points and stained with REC-8 and HIM-8 antibody. The total number of proliferative zone cells (REC-8 positive) were counted.

Figure 10

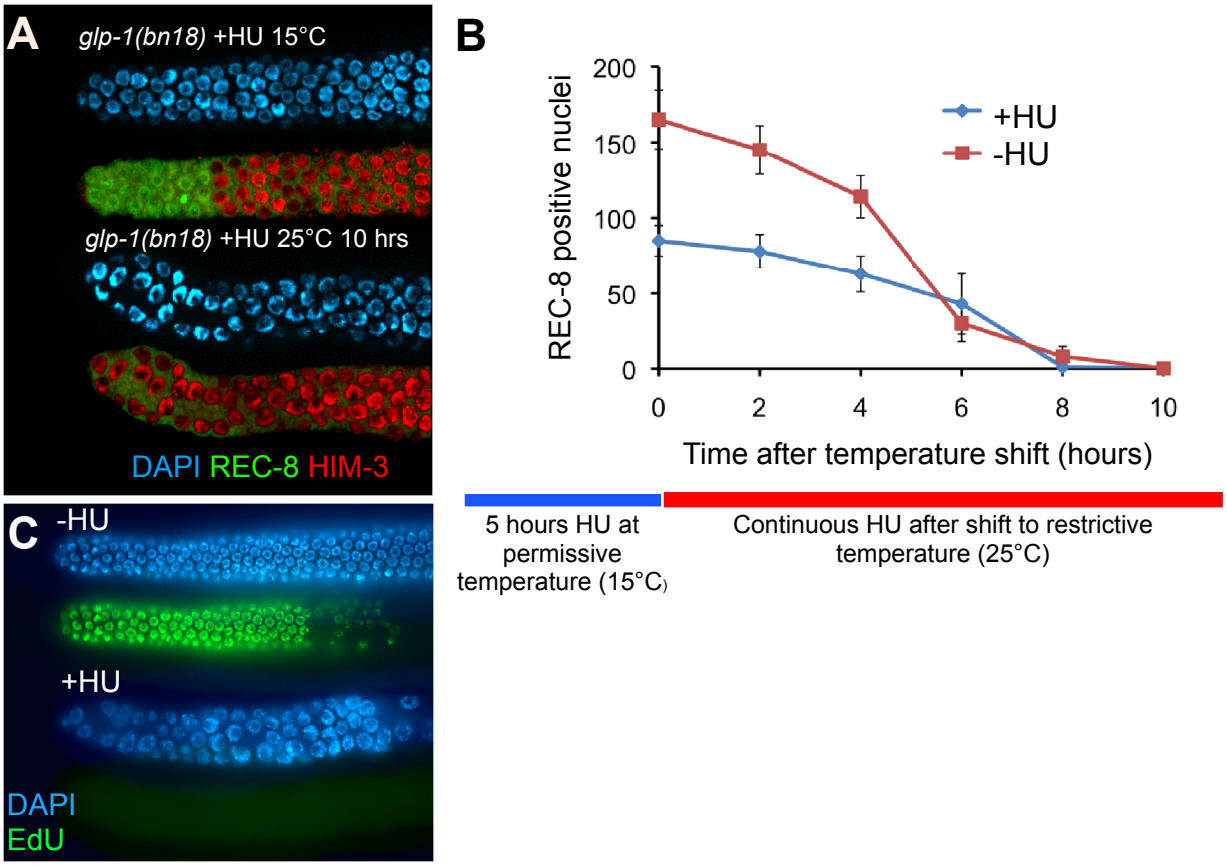

A
glp-1(bn18) +HU 15°C
glp-1(bn18) +HU 25°C 10 hrs
DAPI REC-8 HIM-3
B
REC-8 positive nuclei
200
150
100
50
0
0
2
4
6
8
10
Time after temperature shift (hours)
+HU
-HU
5 hours HU at permissive temperature (15°C)
Continuous HU after shift to restrictive temperature (25°C)
C
-HU
+HU
DAPI
EdU

Figure 10. Hydroxyurea does not block entry into meiosis in *glp-1(bn18).* *glp-1(bn18)* mutants staged to 24 hours past L4 were treated with HU for 5 hours at 15°C, shifted to 25°C and HU treatment was continued at 25°C until dissected at the indicated time point. (A) A representative image shows a HU-treated *glp-1(bn18)* germline maintained at the permissive temperature and one that was shifted to the restrictive temperature for 10 hours (15 hours of HU treatment in both cases). Germlines were stained with REC-8 and HIM-3 antibody. The germline shifted to the restrictive temperature shows a complete loss of REC-8 positive nuclei as all cells become HIM-3 positive and appear to initiate meiotic prophase. (B) The number of REC-8 positive nuclei per germline was counted for each time point. For both HU+ and HU- *glp-1(bn18)* mutants, initiation of entry into meiosis (assayed by loss of REC-8 and gain of HIM-3) is completed at approximately the same time. (C) *glp-1(bn18)* mutants were treated with HU or vehicle as described above but also fed EdU bacteria continuously after the shift to the restrictive temperature. While control germlines show EdU incoporation throughout (similar to the results from Fig. 5 above), HU-treated germlines show no sign of EdU incorporation, confirming that HU successfully blocked DNA replication.

Figure 11

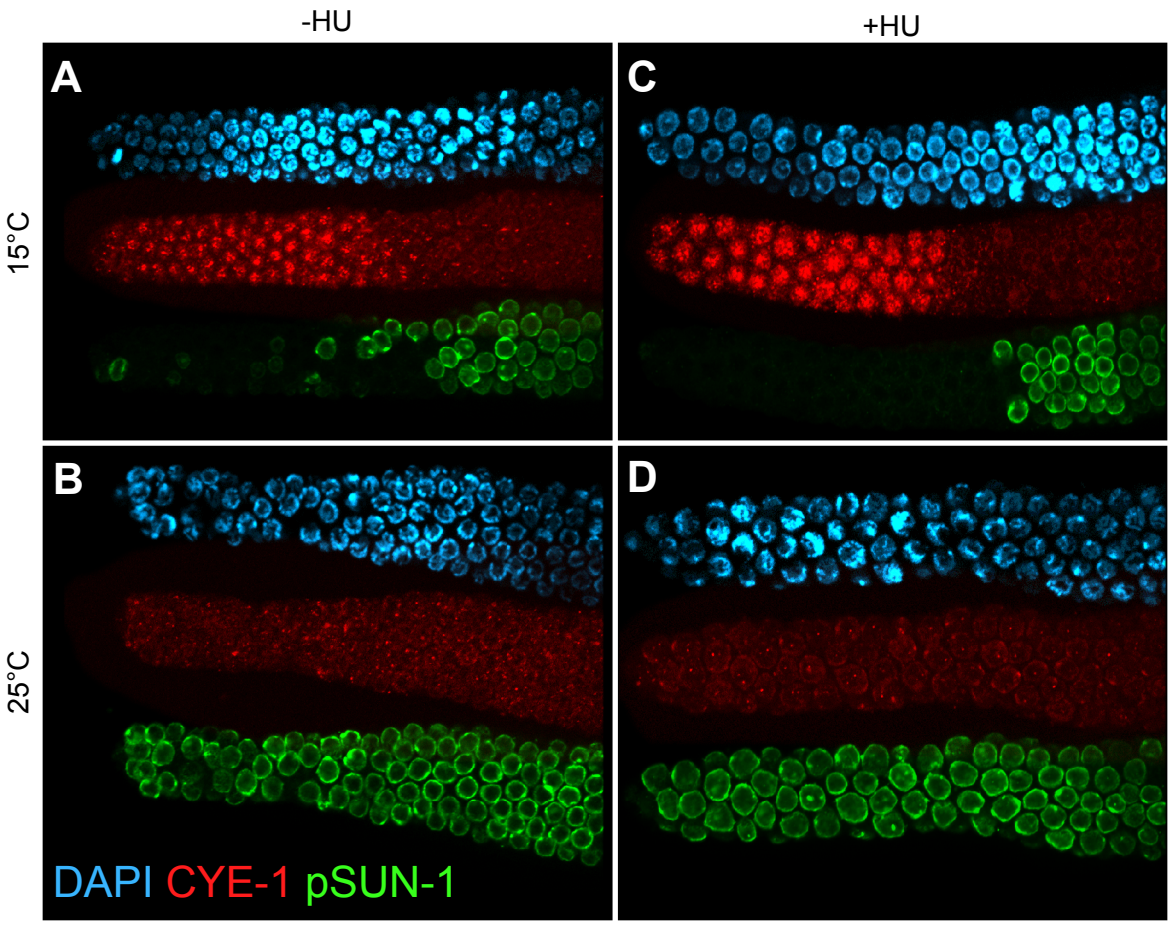


Figure 11. HU arrested cells successfully repress CYE-1 and accumulate phosphorylated SUN-1 upon initiation of meiotic prophase. As described in Fig. 10 above, *glp-1(bn18)* mutants were raised at the permissive temperature and treated continuously with hydroxyurea or PBS control for 5 hours prior to being shifted to the restrictive temperature for ten hours as adults (staged 24 hours past L4). Hydroxyurea treatment continued as animals were maintained at the restrictive temperature. Germlines were dissected after 10 hours at the restrictive temperature (or an additional 10 hours at the permissive temperature for controls) and stained with phospho-SUN-1 antibody and CYE-1 antibody. In both untreated (A) and hyrdoxyurea treated (C) *glp-1(bn18)* germlines at the permissive temperature, CYE-1 is high in proliferative zone cells but low in meiotic cells, similar to wild type (Chapter 2). In contrast, phospho-SUN-1 is low in proliferative zone cells (except cells in M-phase) but high in the early stages of meiotic prophase (Penkner et al. 2009). In both untreated (B) and hydroxyurea treated (D) *glp-1(bn18)* mutants shifted to the restrictive temperature for 10 hours, CYE-1 is completely repressed and phospho-SUN-1 extends to the distal end of the germline.

Figure 12

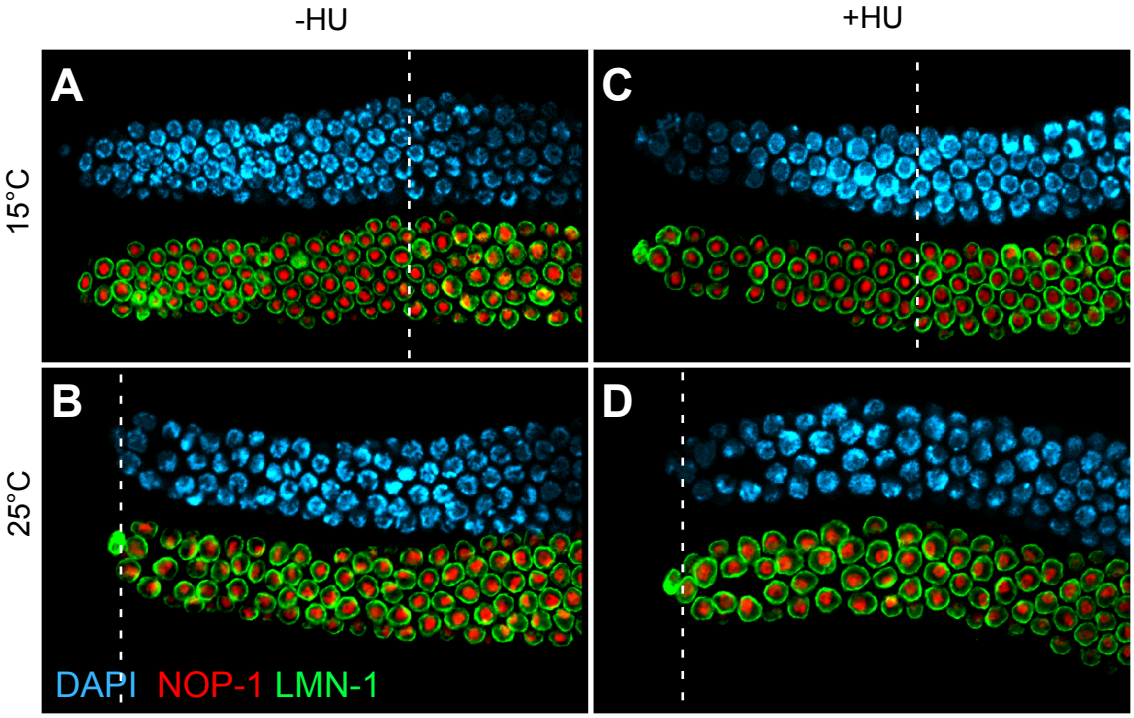


Figure 12. HU arrested cells display normal chromosomal reorganization upon entry into meiosis. During entry into meiotic prophase, chromosomes undergo nuclear reorganization and cluster to one side of the nucleus in a process that is thought to be important for homologous chromosome pairing. This temporary nuclear reorganization can be observed in nuclei with DAPI stained chromosomes and by a shift in the nucleolus (NOP-1 staining) relative to the nuclear envelope (LMN-1 staining) (MacQueen and Villeneuve 2001). As described in Fig. 10 above, *glp-1(bn18)* mutants were raised at the restrictive temperature and treated continuously with HU or vehicle control for 5 hours prior to being shifted to the restrictive temperature for ten hours as adults (staged 24 hours past L4). Hydroxyurea treatment continued as animals were maintained at the restrictive temperature. Germlines were dissected after 10 hours at the restrictive temperature (or an additional 10 hours at the permissive temperature for controls) and stained with NOP-1 antibody and LMN-1 antibody. In both untreated (A) and HU treated (C) *glp-1(bn18)* germlines at the permissive temperature, nuclear reorganization occurs in the transition zone, 10-15 cell diameters proximal to the distal tip. In both untreated (B) and HU treated (D) *glp-1(bn18)* mutants shifted to the restrictive temperature for 10 hours, nuclear reorganization is observed in cells in the very distal positions of the germline, where cells are now in meiotic prophase. Start of the transition zone is indicated by the dashed vertical white line.

Figure 13

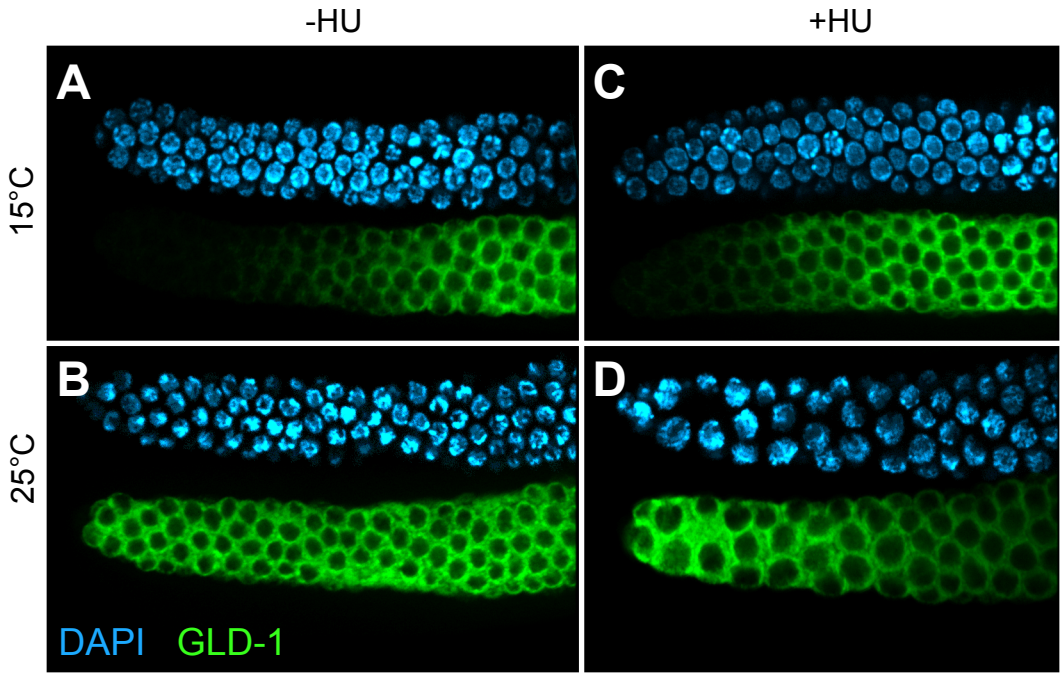


Figure 13. HU arrested cells display a normal increase in GLD-1 levels upon entry into meiosis, a factor itself involved in promoting entry into meiosis. As described in Fig. 10 above, *glp-1(bn18)* mutants were raised at the restrictive temperature and treated continuously with HU or vehicle control for 5 hours prior to being shifted to the restrictive temperature for ten hours as adults (staged 24 hours past L4). HU treatment continued as animals were maintained at the restrictive temperature. Germlines were dissected after 10 hours at the restrictive temperature (or an additional 10 hours at the permissive temperature for controls) and stained with GLD-1 antibody. In both untreated (A) and HU treated (C) *glp-1(bn18)* germlines at the permissive temperature, GLD-1 is low in the distal proliferative zone but high in the proximal proliferative zone and in meiotic prophase. In both untreated (B) and HU treated (D) *glp-1(bn18)* mutants shifted to the restrictive temperature for 10 hours, high GLD-1 extends to the distal end of the germline.

Figure 14

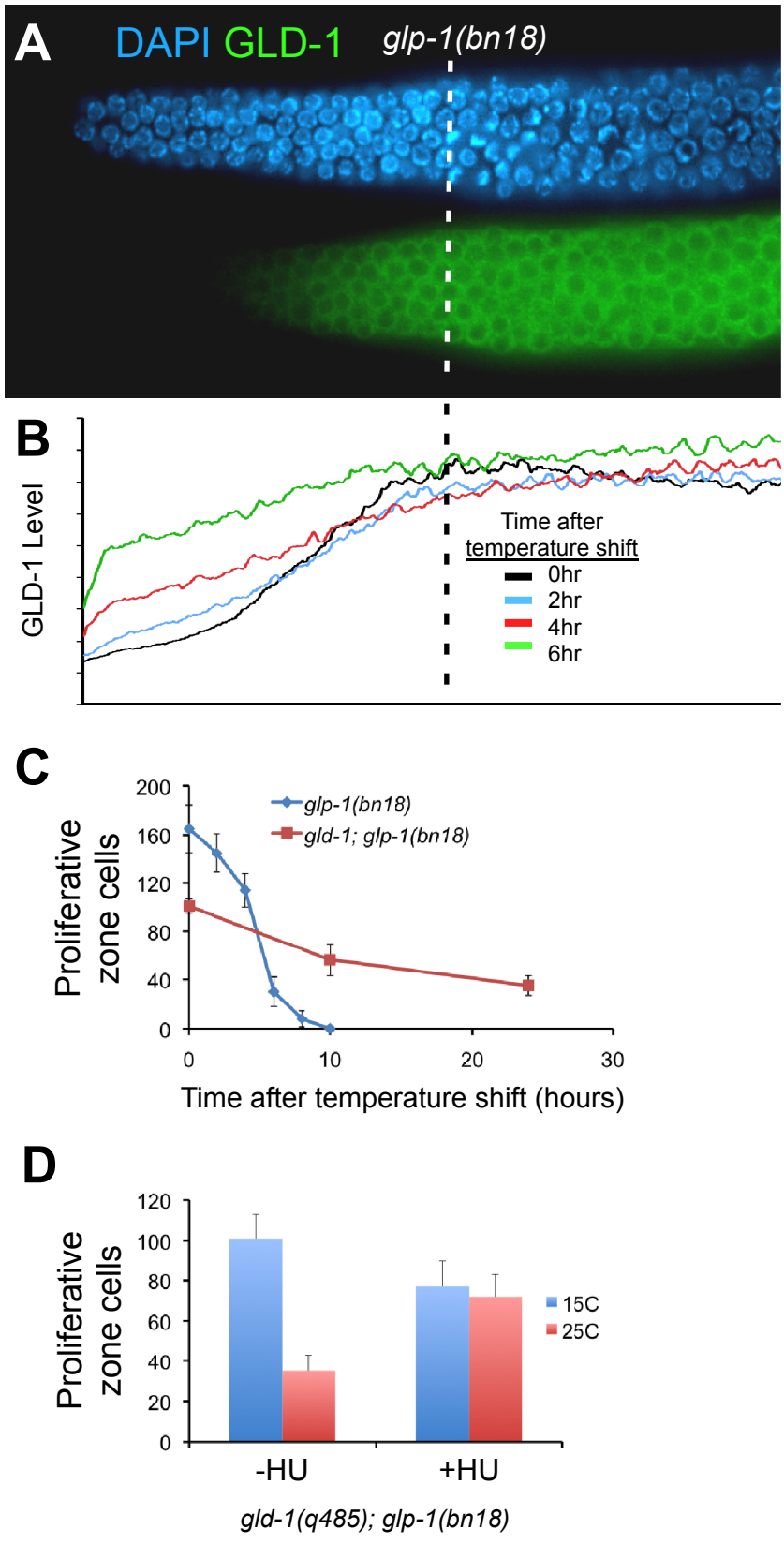

A
DAPI GLD-1
glp-1(bn18)
B
GLD-1 Level
Time after
temperature shift
0hr
2hr
4hr
6hr
C
Proliferative zone cells
glp-1(bn18)
gld-1; glp-1(bn18)
200
160
120
80
40
0
0
10
20
30
Time after temperature shift (hours)
D
Proliferative zone cells
120
100
80
60
40
20
0
15C
25C
-HU
+HU
gld-1(q485); glp-1(bn18)

Figure 14. GLD-1 promotes meiotic entry independent of mitotic cell cycle progression. (A) GLD-1 is expressed in the cytoplasm and its expression increases in a distal proximal gradient, low in the distal end of the proliferative zone and high in the proximal end as cells near meiotic entry. (B) GLD-1 levels were analyzed by ImageJ software in *glp-1(bn18)* mutants after a shift to the restrictive temperature. GLD-1 accumulation in the distal part of the germline begins to increase early and reaches near maximal level by 6 hours, when many cells in the proliferative zone have entered meiosis. (C) *glp-1(bn18)* and *gld-1(null); glp-1(bn18)* mutants were shifted to the restrictive temperature and analyzed for the number of REC-8 positive HIM-3 negative cells in the distal proliferative zone. *The gld-1(0); glp-1(bn18)* proliferative zone retains proliferative zone cells until at least 24 hours after the shift to the restrictive temperature whereas all proliferative zone cells in *glp-1(bn18)* single mutants enter meiosis within 10 hours. (D) *gld-1(0); glp-1(bn18)* mutants were treated with HU or vehicle control for 5 hours at the permissive temperature and then shifted to the restrictive temperature while maintaining HU or control treatment. After 24 hours at the restrictive temperature, control mutants show a significant decrease in proliferative zone size, however HU treated germline show no difference between germlines at the restrictive temperature versus germlines maintained at the permissive temperature. Therefore, GLD-1 is required for HU arrested cells to enter meiosis.

Chapter 4

Conclusions and Future Directions

Conclusions

Coordination of mitotic cell cycle progression and the proliferative fate

Stem cells face two major decisions. In regards to cell fate, stem cells must decide to either remain a stem cell or to differentiate. In addition, they must decide whether to enter the mitotic cell cycle or remain quiescent. For germline stem cells, these decisions must be coordinated. The cell fate decision to differentiate and enter meiosis is not compatible with a decision to progress through the mitotic cell cycle. In the *C. elegans* germline, proliferative cells integrate a variety of signaling inputs to execute these binary decisions, and it is plausible that proliferative cells receive varying degrees of conflicting inputs. How do the cells respond to these inputs with compatible cell fate and proliferation decisions? Our analysis of the proliferative zone suggests that the proliferative cell fate is closely linked to active progression through the mitotic cell cycle. In support of this, mitotic cell cycle progression is continuous among proliferative cells. Furthermore, CDK-2-CYE-1 regulates both mitotic cell cycle progression and the proliferative fate. CDK-2-CYE-1 acts to promote the proliferative fate and/or repress entry into meiosis in addition to driving mitotic cell cycle progression (Chapter 2). It is possible that proliferative cells employ CDK-2-CYE-1 regulation as a mechanism to coordinate mitotic cell cycle progression with the appropriate cell fate.

Cell fate regulation by CDK-2-CYE-1

Among cell cycle factors that function in the germline, CDK-2 and CYE-1 are unique because in addition to driving cell cycle progression, they are also regulate the proliferative fate. In mES cells, CDK2-cyclin E is hypothesized to indirectly promote stem cell maintenance by limiting the length time spent in G1 and thus decreasing their window for differentiation (White and Dalton 2005; Orford and Scadden 2008). If CDK-2-CYE-1 indirectly promotes the proliferative cell fate by limiting G1, differentiation of proliferative cells should correlate with an increase in G1 length. However, we do not observe a significant G1 in any part of the germline proliferative zone under normal conditions, suggesting that differentiation in the germline does not involve a cell cycle structure change (Chapter 2). We propose that CDK-2-CYE-1 directly regulates the proliferative fate, likely through phosphorylation of specific targets. If this hypothesis proves correct, an important goal will be identifying the downstream targets of CDK-2-CYE-1 and determining whether common targets mediate cell cycle progression and stem cell fate.

Although cell cycle structure may not regulate cell fate, the lack of G1 could be an unavoidable consequence of the role of CDK-2-CYE-1 in promoting the proliferative fate. In cell cycle progression, CDK-2-CYE-1 regulates the cell cycle by promoting the transition into S-phase (Hwang and Clurman 2005). Typically, CYE-1 is thought to have periodic expression during the course of cell cycle progression. However, periodic CDK-2-CYE-1 activity during cell cycle progression could cause unstable proliferative fate maintenance in the germline and CYE-1 is expressed continuously throughout the cell cycle in proliferative zone cells (Chapter 2). CYE-1 expression is generally thought to be

rate limiting for progression into S-phase (Hwang and Clurman 2005), suggesting that continuous CDK-2-CYE-1 activity could be a major driver of the abbreviated G1 phase. In doing so, continuous CDK-2-CYE-1 activity is predicted to bypass the early G1 signaling cascade that culminates in CYE-1 induction (Orford and Scadden 2008). In support of this, we found that CDK-4, a G1 CDK essential during larval somatic cell divisions, is not required for cell cycle progression in the germline. If CDK-2-CYE-1 serves a similar role in regulating cell fate in other organisms and tissues, similar cell cycle characteristics, such as a reduced G1, may correlate with this activity based on a need for continuous activity and expression.

Genetic interactions between RNAi depletion of *cdk-2* and *cye-1* with other germline regulatory mutants has helped place CDK-2-CYE-1 into a genetic pathway that explains the regulation of proliferative fate versus meiotic fate. At the core of this genetic pathway, the GLP-1/Notch signaling pathway is upstream of and represses the GLD-1 and GLD-2 pathways (Kadyk and Kimble 1998; Hansen et al. 2004). CDK-2-CYE-1 acts either downstream or in parallel to the GLD-1 and GLD-2 pathways, as RNAi depletion of *cye-1* or *cdk-2* leads to meiotic entry in germlines that lack both GLD-1 and GLD-2 pathway function (Chapter 2). This raises an interesting possibility that CDK-2-CYE-1 acts downstream of and is regulated by the GLD-1 or GLD-2 pathways. This hypothesis is supported by the observations that the GLD-1 and GLD-2 pathways regulate mRNA translation and that CYE-1 is repressed upon entry into meiosis (Hansen and Schedl 2006; Biedermann et al. 2009). However, while GLD-1 is important for maintaining low CYE-1 during meiotic prophase progression (Biedermann et al. 2009; Chapter 2), neither the GLD-1 nor GLD-2 pathway is necessary for the initial repression of CYE-1 upon

entry into meiosis (Chapter 2). Furthermore, this repression is not essential for entry into meiosis to occur, indicating that CYE-1 alone is not sufficient for the proliferative fate (Chapter 2). One explanation for this result is that CDK-2-CYE-1 activity is regulated by a mechanism other than CYE-1 protein abundance. Another possibility is that CDK-2-CYE-1 pathway activity is regulated at a level downstream of CYE-1. If so, it remains possible that the GLD-1 and GLD-2 pathways could be responsible for this downstream regulation. These issues will remain unresolved until CDK-2-CYE-1 activity can be monitored in the germline and the downstream CDK-2-CYE-1 pathway has been identified.

Another important genetic interaction in our epistasis analysis revealed that GLP-1 does not require CDK-2-CYE-1 in order to promote the proliferative fate (Chapter 2). Following RNAi depletion of either *cye-1* or *cdk-2*, GLP-1 signaling still regulates the proliferative fate in the distal germline. RNAi depletion of *cye-1* or *cdk-2* only causes entry into meiosis when GLP-1 signal activity is reduced or not present. Two separate interactions support this conclusion. First, *cye-1* or *cdk-2 RNAi* cause premature meiotic entry in a sensitized background containing a *glp-1* partial loss of function allele but do not cause premature meiotic entry in a wild type germline where GLP-1 activity has not been altered (Chapter 2). Second, *cye-1* or *cdk-2 RNAi* causes complete meiotic entry throughout a *gld-1* pathway *gld-2* pathway tumor double mutant that lacks *glp-1* but fails to cause meiotic entry in the distal region of a *gld-1* pathway *gld-2* pathway tumor double mutant where GLP-1 is thought to be active (Chapter 2). Therefore, if CDK-2-CYE-1 acts downstream of GLP-1 to promote the proliferative fate, GLP-1 must also provide some additional activity that promotes the proliferative fate independent of CDK-2-CYE-

1. This finding suggests a model by which GLP-1 and CDK-2-CYE-1 may coordinate meiotic entry in the proliferative zone. The location of GLP-1 ligand in the distal region of the proliferative zone suggests that GLP-1 activity is reduced in the proximal region of the proliferative zone (Hansen and Schedl 2006). Therefore, CDK-2-CYE-1 activity may be a critical determinant of proliferative fate versus meiotic fate in the proximal region of the proliferative zone where GLP-1 activity is reduced. In this role, it is tempting to speculate that CDK-2-CYE-1 activity serves as a critical mediator for coordinating mitotic cell cycle progression with the switch to meiotic entry.

While CDK-2-CYE-1 displays an active role in both cell cycle progression and proliferative cell fate regulation, two lines of evidence suggest that GLP-1 does not directly regulate mitotic cell cycle progression. First, partial loss-of-function *glp-1* mutants have a smaller proliferative zone, but mitotic cell cycle progression appears normal. The M-phase index, S-phase index, and length of G2+M+G1 are equivalent in *glp-1(bn18)* and wild type proliferative zone cells indicating that both cell cycle structure and total generation time are not affected (Chapter 3). Second, *glp-1* is not necessary for cell cycle progression in *gld-1* pathway *gld-2* pathway tumorous double mutants (Kadyk and Kimble 1998; Hansen et al. 2004). Currently, there is no evidence that direct targets of GLP-1 signaling in the germline include cell cycle factors, and CYE-1 expression does not require GLP-1 in the aforementioned *gld-1* pathway *gld-2* pathway tumorous double mutants (Chapter 2). Thus, not only do CDK-2-CYE-1 and GLP-1 act at least partially in parallel to promote the proliferative fate, the mechanism of their respective regulatory activity may be fundamentally different. Whereas CDK-2-CYE-1 links cell cycle progression and cell fate, GLP-1 appears to only regulate the proliferative fate.

Developmental equivalency among germline proliferative cells

An open question in the *C. elegans* germline concerns whether cells throughout the proliferative zone are developmentally equivalent. In this regard it remains unclear whether stem cells give rise to transit amplifying cells as committed precursors to meiosis. This question persists due to the absence of techniques to directly test a germ cell's self-renewal potential. Two models have been proposed for how the proliferative zone may be organized (Hansen and Schedl 2006): 1) The observation that the ligand for GLP-1 is restricted to the distal region of the proliferative zone by expression in the DTC has suggested that only cells in direct contact with the DTC are true stem cells (Crittenden et al. 2006). According to this model, cells displaced from the DTC lose GLP-1 signal activity but temporarily retain the proliferative fate as transit amplifying cells for a set of mitotic cell divisions. 2) All proliferative cells are essentially equivalent with respect to signal input and self renewal potential. This model predicts that GLP-1 is necessary and active in mitotically dividing cells, even if they are displaced from the DTC (Hansen and Schedl 2006).

We tested these models by analyzing the spatial and temporal pattern in which proliferative zone cells complete entry into meiosis following loss of *glp-1*. The hypothesis that proliferative zone cells undergo transit amplifying divisions upon loss of GLP-1 predicted that stem cells and putative transit amplifying cells would respond differently to a loss of GLP-1 signaling; transit amplifying cells would have already lost GLP-1 activity and have initiated a series of requisite intervening cell divisions prior to entry into meiosis. Therefore, stem cells may have taken longer to reach meiotic prophase as they completed a set of intervening mitotic divisions. By and large, we did not observe

transit amplifying divisions after loss of *glp-1*. Rather, we observed that proliferative cells completed mitotic cell cycles that were already underway, followed by immediate entry into meiosis (Chapter 3). This suggests that proliferative cells may be developmentally equivalent. An important exception was that a subpopulation of cells in the proximal-most region did enter meiosis prior to more distally located cells. This proximal subpopulation likely represents cells in a premeiotic stage such as premeiotic S-phase. Still, this analysis provides only indirect support for the model that proliferative zone cells are developmentally equivalent. A direct test for the self-renewal capacity of individual cells awaits the development of more advanced genetic or transplantation techniques.

Our results analyzing the response of proliferative cells to a loss of *glp-1* also allow us to examine the relationship between commitment to mitotic cell cycle progression and the switch to meiosis. We observed that proliferative cells in mitotic S-phase or G2 appear committed to completing mitosis before they can enter meiosis (Chapter 3). An important caveat for our use of a temperature sensitive mutant for this analysis is the temporal delay in gene inactivation following the temperature shift. The observed commitment to mitosis may have been a passive cause of the delay in *glp-1* inactivation, rather than an active commitment mechanism within the proliferative zone cells. Future advances in monitoring GLP-1 activity will hopefully contribute to this analysis.

We observed a puzzling result when proliferative zone cells were arrested with hydroxyurea treatment. When *glp-1* activity was removed following a shift to the restrictive temperature, these arrested proliferative zone cells went on to initiate meiosis

(Chapter 3). Therefore, despite the observation that proliferative cells in S-phase and G2 will normally complete the mitotic cell cycle before switching to meiosis, proliferative cells can still be forced to enter meiosis when these phases are not completed. This suggests that the decision to enter meiosis can, in part, be uncoupled from mitotic cell cycle progression. However, it is important to note that this experiment relied upon forcing meiotic entry by loss of *glp-1*.

As discussed in this thesis, redundant pathways regulate entry into meiosis. Perhaps certain pathways are responsible for coordinating the switch to meiosis with mitotic cell cycle progression while others act independent of mitotic cell cycle progression. A number of observations suggest that GLD-1 may promote entry into meiosis independently of mitotic cell cycle progression. GLD-1 protein abundance is an important factor in the cell fate decision to either be a proliferative cell or enter meiosis (Hansen et al. 2004b). GLD-1 increases as proliferative zone cells move proximal and near meiotic entry (Jones et al. 1996; Hansen et al. 2004b). In support of GLD-1 acting independent of cell cycle progression, GLD-1 accumulation in proliferative zone cells occurs independent of their position in the mitotic cell cycle (Hansen et al. 2004b). While cells enter meiosis in *gld-1* mutants, the premature meiotic entry that is induced by loss of *glp-1* is partially inhibited in when *gld-1; glp-1(bn18)* double mutants are shifted to the restrictive temperature (Chapter 3). In *gld-1; glp-1(bn18)* double mutants shifted to the restrictive temperature, most proliferative cells enter meiosis however a significant number do not. When HU-treated *gld-1; glp-1(bn18)* double mutants are shifted to the restrictive temperature, entry into meiosis of the cell cycle arrested proliferative zone cells is completely blocked (Chapter 3). While we do not completely understand why cell

cycle arrested proliferative zone cells can be induced to enter meiosis, our results suggest that the GLD-1 pathway is important for this to occur and that GLD-1 may be responsible for promoting entry into meiosis independent of mitotic cell cycle progression. An important future goal will be determining how other meiotic entry pathways compare.

Future Directions

How is germline proliferation regulated?

Cells in the proliferative zone divide mitoticaly and produce daughters that enter meiosis. This rate of entry into meiosis reflects the number of actively cycling cells and their generation time. By monitoring these parameters, we can obtain a fundamental understanding of proliferation in the germline. While we do not have markers for counting the number of actively cycling cells (proliferative zone cells are not necessarily mitotically cycling), this value can be inferred from the cell cycle rate and the output rate. We have measured and compared the output and cell cycle rate of wild type, *glp-1(bn18)* and *eat-2* proliferative zones (Chapters 2 and 3). Both *glp-1(bn18)* and *eat-2* have a smaller proliferative zone size than wild type and therefore presumably have fewer actively cycling cells as well. In all cases, we did not observe a significant difference in cell cycle rate and the output of the proliferative zone was proportional to the number of proliferative zone cells.

We focused on *eat-2* because *eat-2* provides a model for caloric restriction. Therefore, these initial observations suggest that lower nutrition causes a decrease in proliferative cell number but does not drastically change cell cycle kinetics (see appendix to Chapter 2). However, in *eat-2*, nutrition is kept constantly low throughout

development. Therefore, the steady state size of the proliferative zone is likely determined during the course of larval development as is true for the reduced proliferative zone size observed in the insulin receptor mutant, *daf-2* (Michaelson et al. 2010). Presuming that proliferative zone size is modulated by changes in nutrition, is the steady state size of the proliferative zone flexible in the adult? To address this question, two independent approaches can be taken to vary nutrition levels in the adult. 1) Culture conditions could be altered to deliver less food to the animals and liquid culture methods have been developed for these purposes. 2) A combination of *eat* mutants and alternative bacterial food sources can be used to modulate food intake (Avery and Shtonda 2003). With the ability to alter nutritional intake during adulthood, one could address whether the size of the proliferative zone is dynamic and whether temporarily changing the proliferative zone size provides a mechanism for coping with unfavorable environmental conditions. In addition, one may be able to determine whether a more substantial decrease in caloric intake would lead to a decrease in cell cycle kinetics and, if so, which part of the cell cycle is affected.

Another important aspect of this topic is which signals are responsible for regulating proliferative zone output. Previous work has demonstrated that the insulin signaling pathway is important for expansion of the proliferative zone during larval development (Michaelson et al. 2010). However, it is unclear whether this signaling pathway also regulates proliferation in the adult. In addition to this, if proliferative zone size is a key variable for regulating output, factors that regulate the proliferative versus meiotic cell fate decision may play a role in regulating the proliferative zone output. This could be tested by investigating possible genetic interactions between *eat* mutants and

mutations in cell fate regulatory factors, an example being *glp-1* partial gain- and loss-of-function mutants.

How is continuous CYE-1 expression achieved in the proliferative zone?

The continuous expression of CYE-1 throughout the cell cycle is likely important for the abbreviated G1 phase in the germline (Chapter 2). This pattern is also observed during embryonic development in a variety of model organisms (White and Dalton 2005). How is CYE-1 regulation altered to switch from periodic to constant expression? Periodic cyclin E expression is largely achieved by the targeted degradation of cyclin E after entry into S-phase (Hwang and Clurman 2005). Perhaps continuous cyclin E accumulation occurs when this step is inhibited. This hypothesis could be tested by comparing CYE-1 regulation in the germline (where CYE-1 is continuous) with CYE-1 regulation in the soma (where CYE-1 is periodic). Making this comparison requires a series of experiments. First, what factors are involved in targeting CYE-1 for degradation in both the germline and the soma? Second, how are these regulatory factors regulated themselves? In regards to the first question, a number of likely candidates for CYE-1 regulation in the germline and soma already exist. In chapter two, we demonstrated that the SCF^{PROM-1} ubiquitin ligase complex is required for CYE-1 repression (Chapter 2). While this strongly suggests that SCF^{PROM-1} targets CYE-1 for degradation, it remains to be established that CYE-1 is indeed a direct target of SCF^{PROM-1}. In the soma, the SCF^{LIN-23} complex is thought to mediate CYE-1 degradation (Kipreos 2005). The identification of distinct regulatory complexes in the germline and soma would support the hypothesis that the regulation of CYE-1 degradation may be a key difference. Once the critical ubiquitin

ligase factors have been established, it would interesting to determine the expression pattern of these factors. Differences in CYE-1 regulation may be explained by differences in expression among SCF ubiquitin ligase factors.

How does CDK-2-CYE-1 contribute to both mitotic cell cycle progression and stem cell fate maintenance?

An additional question for the future regards the mechanism by which CDK-2-CYE-1 regulates cell cycle progression and also stem cell fate. These processes are likely mediated by phosphorylation of specific target genes, and a major priority is identifying these targets. Well developed genetics techniques make the C. elegans germline a powerful model for testing the function of putative kinase targets, for an excellent recent example see (Arur et al. 2009). However, the identification of novel CDK-2-CYE-1 targets is a significant challenge. The analysis of previously identified candidates may be the most practical, albeit limited, approach for the germline. The initial analysis of such candidates should first address two questions: 1) Is the factor involved in germline cell cycle function? 2) Is the factor required for proliferative fate versus meiotic entry regulation? Both of these questions can be addressed by mutant or RNAi analysis using standard cell cycle progression assays and genetic interaction with sensitized genetic backgrounds as have been described in this thesis.

Where is GLP-1 active in the germline?

The response of proliferative cells to a loss of GLP-1 likely depends on their position in the cell cycle. Transit amplifying divisions are not observed after loss of GLP-

1. Rather, proliferative cells complete the mitotic division that is underway and then enter meiosis. This result supports the hypothesis that cells throughout the proliferative zone are equivalent in terms of developmental potential because it implies that all mitotically proliferating cells have and are affected by active GLP-1 signaling. However, reporters for GLP-1 activity are unavailable and the location of GLP-1 activity remains unclear. An important future goal is testing whether GLP-1 is active throughout the proliferative zone. One strategy for investigating GLP-1 activity is by determining GLP-1(INTRA) abundance. Developing reagents for identifying GLP-1(INTRA) in the germline is an area of active research.

How do the GLD-1 and GLD-2 pathways contribute to meiotic entry of arrested proliferative cells?

Surprisingly, when proliferative cells become arrested in the cell cycle due to drug inhibition of DNA replication or loss of critical cell cycle factors, they can still be induced to enter meiosis. Two redundant genetic pathways are required for cells to enter meiosis: the GLD-1 pathway and the GLD-2 pathway. However, it remains unclear why redundant pathways are present and whether they involve distinct regulatory mechanisms. We discovered that *gld-1* is required for cell cycle arrested cells to enter meiosis. This observation raises the question of whether the other known components of the GLD-1 and GLD-2 pathways are also required for entry into meiosis in this situation. This can be tested by analyzing double mutants with *glp-1(bn18)* and putative null alleles of members of the GLD-1 and GLD-2 pathways. The assays presented in Chapter 3 of this thesis can then be used to determine whether these factors are also important for the

timing of meiotic entry following loss of GLP-1 signaling. One interesting possibility is that, while the GLD-1 and GLD-2 pathways act redundantly to promote entry into meiosis, their activities act during different parts of the cell cycle or respond differently to cell cycle arrest.

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Curriculum vitae

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Education

Washington University, Saint Louis, Missouri, 2004-present

- Ph.D. in Developmental Biology, (expected completion date: December 2010)

University of Wisconsin, Madison, Wisconsin, 2000-2004

- B.S. with honors in Molecular Biology

Research Experience

Doctoral Research: Department of Genetics, Washington University, 2005-present (advisor: Dr. Tim Schedl)

- Analysis of the mitotic cell cycle in the *C. elegans* germline.
- Characterization of CYE-1-CDK-2 as a critical regulator of stem cell fate in addition to cell cycle regulation.
- Analysis of the decision of germ cells to enter meiosis relative to their progression through the mitotic cell cycle.

Undergraduate honors thesis: Department of Molecular Biology, University of Wisconsin-Madison, 2003-2004 (advisor: Dr. Ching Kung)

- Set up and performed a screen in *S. cerevisiae* to identify mutants sensitive to low calcium with the goal of identifying an experimentally predicted low affinity calcium ion channel.

Research Interests

- Cell cycle regulation during development
- Regulation of the proliferation and differentiation of stem cells
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Honors and Awards

- 2010 Best talk award, Developmental Biology retreat, Washington University
- 2009 Best talk award, Genetics retreat, Washington University
- 2007 Poster Session Honorable Mention, DNA Day symposium, Washington University
- 2006 Best poster award, Developmental Biology Retreat, Washington University
- 2003 Undergraduate research award, University of Wisconsin-Madison

Publications

- **Fox PM**, Vought V, Hanazawa M, Lee MH, Maine E, Schedl T. "Cyclin E-CDK-2 regulates proliferative cell fate and cell cycle progression in the *C.elegans* germline" (submitted to Development)

- Hadwiger G, Dour S, Arur S, **Fox P**, Nonet ML. "A monoclonal antibody toolkit for *C. elegans*" (2010) PLoS One. Apr 13;5(4):e10161.

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Talks

2010 Model Organism to Human Biology Conference, Stem cell session platform talk, Genetics Society of America, Boston

- "Cyclin E/CDK-2 regulates proliferative cell fate and cell cycle progression in the *C.elegans* germline"

2009 International C. elegans Conference, Germline parallel session talk, UCLA

- "CYE-1 and CDK-2 may link the proliferation versus meiotic entry decision with the mitotic cell cycle"

Teaching Experience

- 2006 Teaching Assistant: Principles of Human Physiology
- 2007-2009 I mentored three undergraduate students, one during each summer as part of the school's BioMedRep and Opportunities in Genomics Research minority outreach proprams. The projects focused on using next generation sequencing technologies to identify molecular lesions in the *C. elegans* genome and also using genetic mosaic animals to analyze the role of specific cell cycle factors in the *C.elegans* germline.