

Multi-pathway control of the proliferation versus meiotic development decision in the *Caenorhabditis elegans* germline

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Abstract

An important event in the development of the germline is the initiation of meiotic development. In *Caenorhabditis elegans*, the conserved GLP-1/Notch signaling pathway regulates the proliferative versus meiotic entry decision, at least in part, by spatially inhibiting genes in the *gld-1* and *gld-2* parallel pathways, which are proposed to either inhibit proliferation and/or promote meiotic development. Mutations that cause constitutive activation of the GLP-1 pathway, or inactivation of both the *gld-1* and *gld-2* parallel pathways, result in a tumorous germline in which all cells are thought to be proliferative. Here, to analyze proliferation and meiotic entry in wild-type and mutant tumorous germlines, we use anti-REC-8 and anti-HIM-3 specific antibodies as markers, which under our fixation conditions, stain proliferative and meiotic cells, respectively. Using these markers in wild-type animals, we find that the border of the switch from proliferation to meiotic entry is staggered in late-larval and adult germlines. In wild-type adults, the switch occurs between 19 and 26 cell diameters from the distal end, on average. Our analysis of mutants reveals that tumorous germlines that form when GLP-1 is constitutively active are completely proliferative, while tumors due to inactivation of the *gld-1* and *gld-2* pathways show evidence of meiotic entry. Genetic and time course studies suggest that a third pathway may exist, parallel to the GLD-1 and GLD-2 pathways, that promotes meiotic development.

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Introduction

Germline proliferation, meiosis, and gametogenesis are essential processes for the continued existence of sexually reproducing species. A key step in the development of the germline and the production of gametes is the decision of germ cells to either proliferate or undergo meiotic development. Germline proliferation is essential, first to amplify the germline before the initial onset of meiosis, and then to maintain the germline stem cells so that gametes can be produced continuously in the reproductive adult. Meiosis is essential since it is the means by which a diploid organism makes haploid gametes, which then combine with other haploid gametes to generate diploid offspring. Germ cells that enter meiotic prophase must produce all of the cellular

components necessary for meiosis to occur properly, such as factors that align homologous chromosomes and form the synaptonemal complex, factors involved in reciprocal meiotic recombination, as well as factors that properly segregate the homologous chromosomes to opposite poles (Champion and Hawley, 2002). These cells also must synthesize the components necessary for differentiation into gametes.

Much of our current knowledge of the factors involved in regulating meiotic entry in animals has been obtained through genetic analysis of model organisms such as *Caenorhabditis elegans* and *Drosophila*. In *C. elegans*, the adult hermaphrodite germline is spatially patterned with a proliferative stem cell population in the most distal end of the gonad (Fig. 1). Most cells in the germline are syncytial, but with each nucleus partially enclosed by a plasma membrane. We refer to each nucleus and its surrounding cytoplasm and membranes as a germ cell. As cells move proximally away from the distal tip, they enter meiotic prophase. Genetic and molecular analysis implicates the conserved GLP-1/Notch signaling pathway

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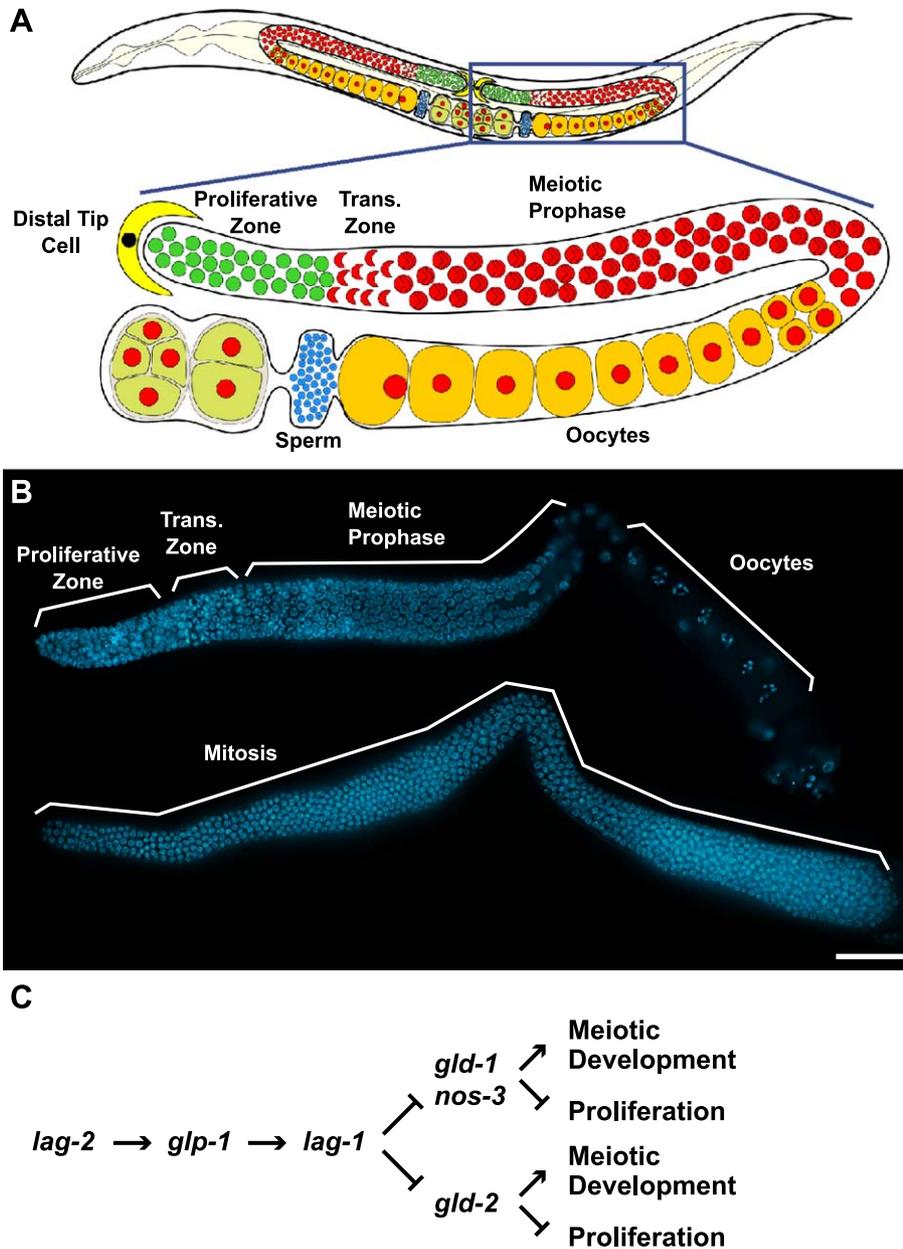


Fig. 1. The proliferation versus meiotic development decision in the *C. elegans* germline. (A) Drawing of a wild-type adult hermaphrodite (top) and a blow-up of one of the gonad arms (bottom). The somatic distal tip cell (DTC) caps the very distal end of the gonad. Proliferative or mitotic germ cells (green) begin meiotic development as they move proximally and enter the transition zone, which corresponds to leptotene/zygotene (Dernburg et al., 1998). Cells continue to progress through meiotic prophase (red) as they move proximally, eventually differentiating as first sperm (blue), then oocytes (yellow). Cells in the gonad are syncytial, sharing a common cytoplasm, but cellularize as sperm and oocytes. For convenience of illustration, the arrangement of the gonad arms relative to the intestine are not depicted accurately. (B) Dissected gonad arms of a wild-type adult hermaphrodite (top) and *glp-1(gf)* mutant (bottom) with a tumorous germline, both stained with DAPI to visualize nuclear morphology. Distal is to the left. Actual genotype of *glp-1(gf)* mutant is *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/dpy-19(e1259) unc-32(e189) glp-1(oz112gf)*; *qDp3* (*qDp3* contains *unc-32(e189)* and wild-type copies of *dpy-19* and *glp-1*; Austin and Kimble, 1987). Scale bar = 20 μ m. (C) Genetic pathway involved in regulating the proliferation versus meiotic entry decision. In this model, GLP-1/Notch signaling, consisting of *lag-2*, *glp-1*, and *lag-1*, inhibits the activities of the *gld-1* and *gld-2* pathways. These two pathways function redundantly to promote meiotic development and/or inhibit proliferation. Adapted from Kadyk and Kimble (1998) and Hansen et al. (2004).

as the major controller of the proliferation versus meiosis decision (Seydoux and Schedl, 2001). Spatially controlled activation of the pathway occurs when membrane-bound LAG-2 ligand (Henderson et al., 1994; Tax et al., 1994), expressed in the somatic distal tip cell (DTC) (Fitzgerald and Greenwald, 1995; Henderson et al., 1994), binds to the

GLP-1 receptor (Austin and Kimble, 1987; Yochem and Greenwald, 1989), which is expressed in the germ cells (Crittenden et al., 1994). Ligand binding is thought to cause the intracellular portion of GLP-1 to translocate to the nucleus of the germ cells and bind the LAG-1 transcription factor, thereby causing the transcription of

target genes (Mumm and Kopan, 2000). If the GLP-1 signaling pathway is rendered inactive, all germ cells enter meiosis, depleting the stem cell population (Austin and Kimble, 1987; Lambie and Kimble, 1991). Conversely, in *glp-1(oz112gf)* gain-of-function mutants, in which the GLP-1 receptor is constitutively active, germ cells fail to enter meiosis, continue to proliferate, and form a germline tumor in which all cells appear to be proliferative, as indicated by nuclear morphology (Berry et al., 1997). In *glp-1(oz112gf)* hemizygotes or in animals carrying the weaker *glp-1(ar202gf)* mutation, the proliferation versus meiotic development decision in the distal germline is initially normal; however, the size of the proliferative zone increases in the adult over time, with germ cells entering meiosis progressively much further from the DTC (Berry et al., 1997; Pepper et al., 2003a). This over-proliferation phenotype is called a ‘late-onset’ tumor.

While the tumors described above appear to be due to a defect in meiotic entry, some other tumors are not due to a meiotic entry defect, even though superficially they look similar to *glp-1(oz112gf)* tumors. For example, hermaphrodites mutant for *gld-1*, which encodes a KH domain translational inhibitor (Jan et al., 1999; Jones and Schedl, 1995; Lee and Schedl, 2001), have a germline tumor that is due to a failure of female germ cells to successfully progress through meiotic prophase (Francis et al., 1995a,b). Careful analysis of the germ cells in these animals throughout development has shown that they enter meiosis at the normal stage of development and the correct location in the gonad, but they are unable to complete meiotic prophase. Instead, female pachytene stage germ cells re-enter the mitotic cell cycle and proliferate forming a large germline tumor. Similarly, during spermatogenesis of *Pumilio puf-8* mutants, primary spermatocytes dedifferentiate back into mitotically dividing cells, forming a proximal germline tumor (Subramaniam and Seydoux, 2003). Thus, both *gld-1* and *puf-8* mutants demonstrate that ectopic germline over-proliferation is not always due to a defect in meiotic entry, but that defects in meiotic progression can also cause this general phenotype.

The GLP-1/Notch signaling pathway promotes proliferation and/or inhibits meiotic development. Loss of all of the activities that work in the opposite direction (promote meiotic development and/or inhibit proliferation) is predicted to result in a tumor that is identical to constitutive activation of *glp-1* (*glp-1(oz112gf)*; Berry et al., 1997). GLP-1 signaling has been proposed to inhibit the activities of two genetically downstream genes, *gld-1* and *gld-2*, which each either inhibit proliferation and or promote meiotic entry (Francis et al., 1995b; Kadyk and Kimble, 1998). The role of *gld-1* in the proliferation versus meiotic development decision described here is different from its role in female meiotic prophase progression described above. For meiotic entry, *gld-1* is proposed to function redundantly with *gld-2* (Kadyk and Kimble, 1998), which

encodes a catalytic subunit of a poly(A) RNA polymerase (Wang et al., 2002). In *gld-1* or *gld-2* single null mutants, germ cells enter meiosis normally (Francis et al., 1995a; Kadyk and Kimble, 1998). When the activities of both *gld-1* and *gld-2* are absent, a tumorous germline forms independent of germline sex (Kadyk and Kimble, 1998) as indicated by increased cell numbers, ectopic proliferation [based on phospho-histone (H3) staining, a mitotic M-phase marker], and a lack of meiotic entry based on nuclear morphology as revealed by DAPI staining. Therefore, the tumors in *gld-1* and *gld-2* mutants are thought to result from disruption of the decision to enter meiotic prophase based on nuclear morphology (Kadyk and Kimble, 1998). Because *gld-1* and *gld-2* are redundant in this meiotic entry function, we refer to the tumor formed in *gld-2 gld-1* double null mutant animals as a synthetic tumor. A similar synthetic tumorous phenotype is seen when the activities of both *nos-3* and *gld-2* are eliminated (Hansen et al., 2004). NOS-3 is one of three *C. elegans* genes similar to *Drosophila* Nanos (Kraemer et al., 1999; Subramaniam and Seydoux, 1999), and is proposed to function in the *gld-1* pathway for regulating meiotic entry (Hansen et al., 2004).

DNA morphology, as visualized by DAPI staining, has been the primary means by which meiotic entry has been studied in wild-type and tumorous germlines in *C. elegans*. While certain stages of both mitosis and meiosis are easily recognizable by DAPI-stained DNA morphology, and while this remains a convenient means to assess germline nuclear morphology, the earliest stages of meiotic prophase (leptotene/zygotene) are not always easily distinguishable from neighboring proliferating cells, making it difficult to accurately study meiotic entry. Moreover, these difficulties are magnified in mutants where the positions and borders between these cell populations are abnormal.

Here we use two markers, one proliferation-specific and the other meiotic-specific, to more precisely study the proliferation versus meiotic entry decision in *C. elegans*. In wild-type late-larval and adult animals, we find that meiotic entry is spatially variable with respect to distance from the DTC, resulting in a segment of the germline that contains both proliferating and meiotic cells. We also use these markers to analyze germline tumors thought to result from disruption of the proliferation versus meiotic entry decision. We find that the *glp-1(oz112gf)* tumor is completely proliferative; however, cells within the *gld-2 gld-1* and *gld-2; nos-3* null mutant synthetic tumors show evidence of meiotic entry and thus are not completely proliferative. Therefore, the synthetic tumors are not equivalent to the canonical *glp-1(gf)* tumor. We further show by genetic analysis and time course experiments that GLD-1, GLD-2, and NOS-3 function in regulating meiotic entry. We propose that at least one additional pathway exists, parallel to the *gld-1* and *gld-2* pathways, to regulate the proliferation versus meiotic entry decision.

Materials and methods

Nematode strains and culture

Standard procedures for culture and genetic manipulation of *C. elegans* strains were followed with growth at 20° unless otherwise noted (Sulston and Hodgkin, 1988). Descriptions of genes, alleles, and phenotypes related to this study are in Hodgkin and Martinelli (1999).

The following mutations were used: LGI: *gld-2* (*q497null*) (Kadyk and Kimble, 1998), *gld-1* (*q485null*) (Francis et al., 1995a), *gld-1* (*q361*) (Francis et al., 1995a); LGII: *dpy-10* (*e128*), *nos-2* (*ok230*) (probable null) (generated by the *C. elegans* Gene Knockout Consortium), *nos-1* (*gv5*) (probable null) (Subramaniam and Seydoux, 1999), *nos-3* (*oz231null*) (Hansen et al., 2004), *unc-4* (*e120*), *sqt-1* (*sc13*); LGIII: *unc-36* (*e251*), *dpy-19* (*e1259*), *unc-32* (*e189*), *glp-1* (*q175null*) (Austin and Kimble, 1987), *glp-1* (*oz112gf*) (Berry et al., 1997), *glp-1* (*ar202gf*) (Pepper et al., 2003a), *glp-1* (*q172*) (Austin and Kimble, 1987). The *nos-2* (*ok230*) allele is due to deletion of bases 30999–33076 of cosmid ZK1127 (GenBank accession , U58758) and also deletes a portion of the *him-14* gene (K. Subramaniam and G. Seydoux, personal communication).

The *nos-2* (*ok230*) *nos-3* (*oz231*) *nos-1* (*gv5*) triple mutant chromosome was constructed by first constructing a *nos-3* (*oz231*) *nos-1* (*gv5*) chromosome. This was accomplished by crossing *nos-3* (*oz231*) *sqt-1* (*sc13*)/*unc-4* (*e120*) *nos-1* (*gv5*) heterozygous males with *unc-4* (*e120*) *sqt-1* (*sc13*) hermaphrodites and picking the non-Unc, non-Sqt recombinants. *nos-2* (*ok230*) was recombined onto this chromosome by crossing *dpy-10* (*e128*) *nos-3* (*oz231*) *nos-1* (*gv5*)/*nos-2* (*ok230*) *unc-4* (*e120*) heterozygous males with *dpy-10* (*e128*) *unc-4* (*e120*) hermaphrodites and picking non-Dpy non-Unc hermaphrodites. The presence of the mutations for each of the three *nos* genes on the resulting chromosome was confirmed by PCR analysis.

Antibody staining

Antibody staining of dissected gonads is described (Jones et al., 1996). As determined by immunohistochemical detection, REC-8 protein is found in the nucleoplasm and on the DNA of germ cells in the mitotic zone, in short threads in transition zone cells (corresponding to axial elements of the chromosomes), and long threads in pachytene cells (lateral elements of the meiotic chromosomes; Pasierbek et al., 2001). We see this same pattern using similar fixation conditions (formaldehyde, ethanol, methanol, and acetone) to those previously described (Pasierbek et al., 2001). However, this method leads to a low yield of intact gonads using our batch method (Jones et al., 1996), as well as spurious membrane staining. Instead, our conditions were as follows: dissected gonads were fixed in 3% formaldehyde/0.1 M K₂HPO₄ (pH 7.2) for 1 h at RT followed by 5-min incubation with 100% MeOH at –20°C. Under these conditions, anti-

REC-8 antibody staining was detected in the nucleoplasm and on the DNA of germ cells within the mitotic zone, but was not observed in meiotic prophase chromosomes, presumably because these more interior epitopes were not exposed in the absence of the acetone extraction step. Fluorescent images were captured with a Zeiss Axioskop microscope equipped with Hamamatsu digital CCD camera (Hamamatsu Photonics). Anti-HIM-3 antibodies were kindly provided by Monique Zetka (Zetka et al., 1999) and anti-REC-8 antibodies were generously provided by Pavel Pasierbek and Joseph Loidl (Pasierbek et al., 2001).

Time course analyses

To obtain many synchronized animals, adult hermaphrodites were treated with hypochlorite solution and their eggs were isolated (Sulston and Hodgkin, 1988). The eggs were then suspended in M9 buffer and shaken at 20°C to arrest animals in the L1 stage. Arrested animals were washed with M9 buffer and placed on standard NGM plates (Sulston and Hodgkin, 1988) and grown for specific lengths of time at 20°C (unless otherwise noted), then dissected and stained as described above.

Results

Anti-REC-8 and anti-HIM-3 antibodies serve as mutually exclusive markers for meiotic entry under certain fixation conditions

The *C. elegans* germline is a useful system for studying germline development, partially due to the spatial organization of the gonad (Hubbard and Greenstein, 2000). Simple DAPI staining of dissected gonads to visualize nuclear morphology reveals the general spatial organization (Fig. 1B). At the region where germ cells first show evidence of entering meiotic prophase, roughly 20 cells diameters from the distal end of the adult gonad where the DTC resides (Crittenden et al., 1994), DAPI staining in nuclei of cells in the leptotene/zygotene stages of meiotic prophase takes on a crescent-shaped appearance (we will refer as cells with crescent-shaped nuclei) (MacQueen and Villeneuve, 2001). Although this change in DAPI nuclear morphology is useful for identifying cells that have entered meiotic prophase, it is somewhat subjective. Antibodies that specifically recognize HIM-3, a component of the proteinaceous core that exists between sister chromatids (Zetka et al., 1999), have also been useful for identifying meiotic cells (MacQueen and Villeneuve, 2001; Zetka et al., 1999). To thoroughly study the proliferation versus meiotic entry decision, a marker specific for proliferative germ cells would also be valuable. Antibodies specific to phospho-histone (H3) identify a small subset of proliferative germ cells, those in late prophase and early mitotic M-phase (Ajiro et al., 1996). Therefore, the majority of

proliferative cells (those in late M, interphase, and S phase) is not marked.

We found that under certain fixation conditions, REC-8-specific antibodies serve as a useful marker for specifically identifying proliferative germ cells, and in combination with HIM-3 antibodies, can distinguish proliferative from early-meiotic prophase germ cells. REC-8 is part of the sister-chromatid cohesion protein family and is found in the nucleoplasm and on the DNA of cells in the proliferative zone, as well as in the proteinaceous core of sister chromatids in meiotic prophase (Pasierbek et al., 2001). Using relatively mild fixation conditions, we only observed the nucleoplasmic and the non-proteinaceous core chromosomal staining in the proliferative zone (see Materials and methods).

To determine the specificity of anti-REC-8 antibodies for proliferative germ cells, and the usefulness of the combination of anti-REC-8 and anti-HIM-3 antibodies in studying the proliferation versus meiotic entry decision, we analyzed anti-REC-8 and anti-HIM-3 staining patterns relative to (1) each other, (2) transition zone (crescent-shaped DAPI staining) nuclei, and (3) phospho-histone (H3) antibody staining. We found that under our conditions, anti-REC-8 and anti-HIM-3 staining patterns (hereafter called REC-8 and HIM-3 patterns) are mutually exclusive (Figs. 2 and 3): REC-8 marks cells in the proliferative zone, while HIM-3 marks cells within and proximal to the transition zone. All phospho-histone (H3) staining cells (in M-phase) are REC-8-positive and HIM-3-negative, while all cells with crescent-shaped DAPI staining (meiotic-leptotene/zygotene) are REC-8-negative and HIM-3-positive (Figs. 2 and 3). Transition zone cells have crescent-shaped asymmetric DAPI staining due to a spatial reorganization of the chromatin and nucleolus that occurs in early meiotic prophase of many organisms (MacQueen and Villeneuve, 2001; Scherthan, 1997; Zickler and Kleckner, 1999). These results demonstrate that under mild fixation conditions, REC-8 and HIM-3 distinguish proliferative and early meiotic germ cells and thus provide a useful tool in studying the proliferation versus meiotic entry decision.

Meiotic entry in wild-type hermaphrodites

To better characterize meiotic entry during development (from L2 to young adult), we examined REC-8 and HIM-3 staining patterns in synchronous staged populations of wild-type animals (see Materials and methods). We first observed HIM-3-positive/REC-8-negative cells in the proximal end of the early L3 germline, 32 h past L1 arrest, at a time when the germline measures approximately 13 cell diameters from the distal end (Fig. 4). The length of the proliferative zone continues to increase and reaches maximum distance (as measured by cell-diameter-lengths) between late L4 and young adult (44–50 h past L1 arrest). During the L3 and early L4 stages, the length of the proliferative zone increases, and the transition between mitosis and meiosis

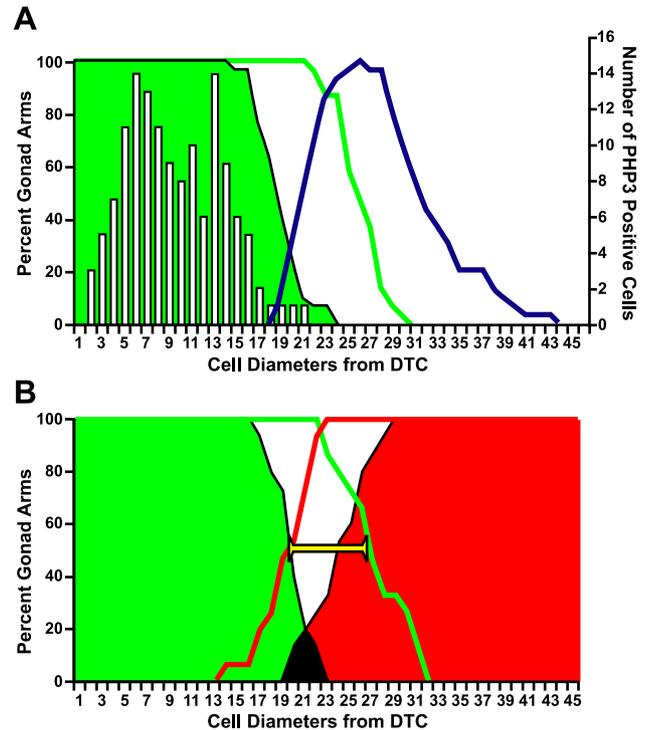


Fig. 2. Proliferative and meiotic prophase cells revealed by staining for REC-8 and HIM-3. (A) Graph showing the percentage of gonad arms (left y axis) that have only REC-8-positive cells (solid green) at specific distances from the DTC as measured by cell diameters (x axis). The most proximal REC-8-positive cells are shown with a green line while the blue line shows the percentage of gonad arms that have transition zone cells (with crescent-shaped DAPI-stained nuclei) at a given position in the gonad arm. Bar graph shows number of phospho-histone (H3) positive cells (right y axis) at specific cell diameters from distal end. Line graphs, left axis; bar graph, right axis. Analysis is based on 30 gonad arms from wild-type hermaphrodites 1 day past L4 grown at 20°C. (B) Comparison of REC-8 and HIM-3 staining patterns. Fifteen dissected wild-type hermaphrodite gonad arms from animals 1 day past L4 at 20°C were stained with anti-REC-8 (green) and anti-HIM-3 (red) specific antibodies. The graph shows the percentage of gonad (y axis) arms with different staining patterns at specific positions along the distal to proximal axis of the gonad arms (x axis). Solid green shows the position where all cells at that distance are REC-8-positive. The green line shows the position of the most proximal REC-8-positive cell. The red line shows the position of the most distal HIM-3-positive cell. Solid red shows the position where all cells at that distance are HIM-3-positive. The yellow bar depicts the region where both REC-8-positive/HIM-3-negative cells and REC-8-negative/HIM-3-positive cells are found, which we call the meiotic entry region. We scored cells with nuclear/chromosomal HIM-3 staining as HIM-3 positive; cells somewhat closer to the DTC can accumulate cytoplasmic HIM-3, but these are scored as HIM-3-negative. Additionally, the cells closest to the DTC that are HIM-3-positive have relatively weaker staining than cells further from the DTC, making the HIM-3 (positive) cells closest to the DTC the most difficult to score.

occurs over a distance of one cell diameter. Thus, during early stages, the switch from proliferation to meiotic entry occurs across a sharp border of REC-8-positive to HIM-3-positive cells at a given distance from the distal tip.

By the late L4 stage, 44 h past L1 arrest, a region becomes evident that contains both proliferative (REC-8-positive/HIM-3-negative) and meiotic (REC-8-negative/

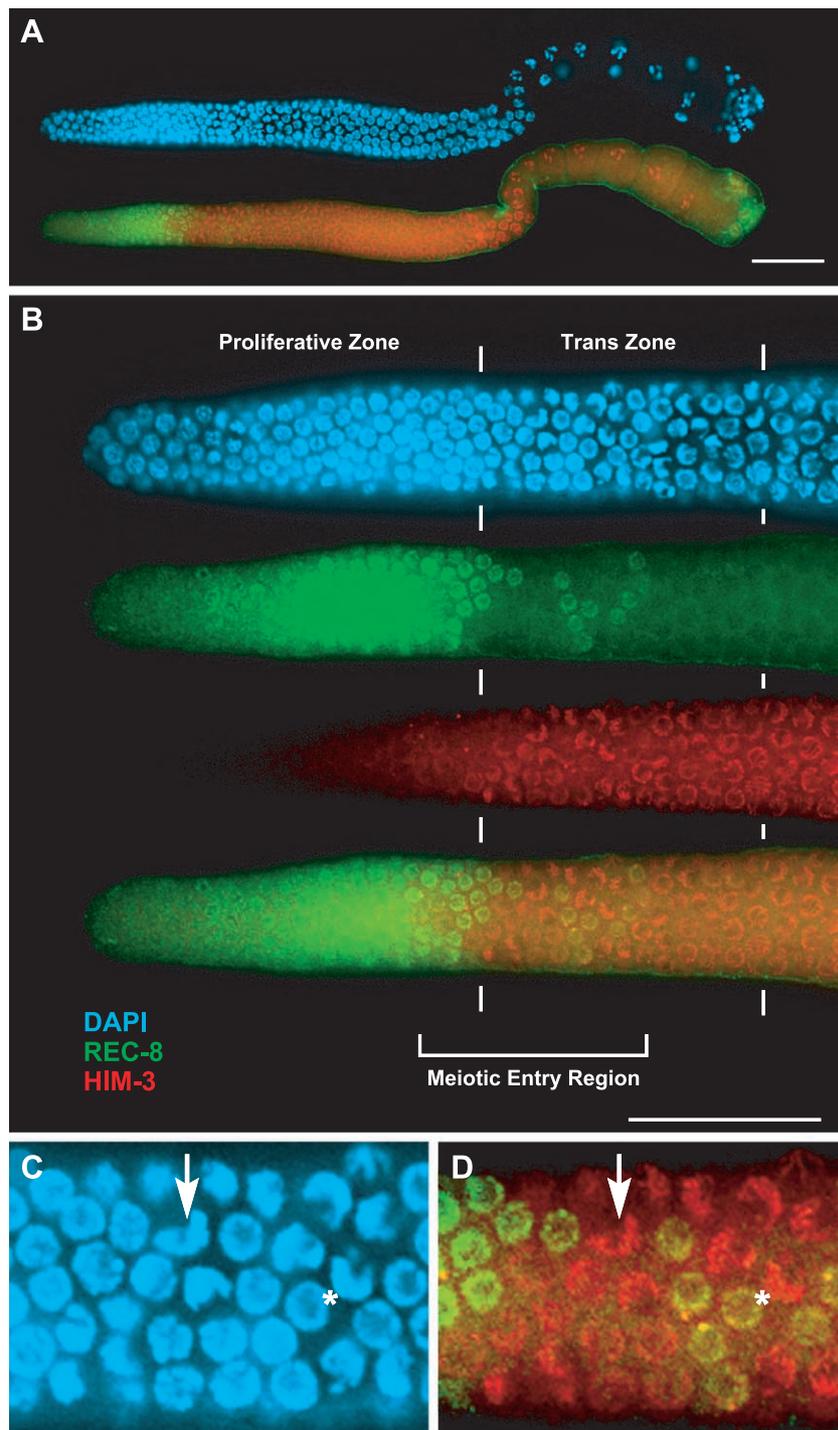


Fig. 3. REC-8 and HIM-3 staining in a wild-type hermaphrodite. Dissected gonad arm from an adult hermaphrodite stained with DAPI (blue), REC-8 antibodies (green), and HIM-3 antibodies (red). (A) Shows the entire gonad arm with distal to the left and proximal to the right. (B) Shows a blow-up of the distal mitotic region and the transition zone of the same gonad arm in (A); however, REC-8 and HIM-3 antibody staining are also shown separately. The boundaries of the mitotic and transition zones are demarcated with vertical lines. The location of the meiotic entry region is within the horizontal bracket. (C) and (D) are further blow-ups of the same gonad arm stained with DAPI (C) and anti-REC-8 and anti-HIM-3 specific antibodies (D). The region shown is part of the meiotic entry region with the arrow pointing to a representative transition zone nucleus with crescent-shaped DAPI staining. The asterisk is beside a REC-8-positive nucleus that has a non-crescent-shaped DNA organization but is further from the DTC than the HIM-3-positive nucleus with crescent-shaped DAPI staining (arrow). This and other REC-8-positive nuclei in the meiotic entry region are more yellow than in the proliferative region, which appears to be due to faint HIM-3 staining and may represent cells that are in the transition from REC-8-positive to HIM-3-positive. Scale bars = 20 μ m.

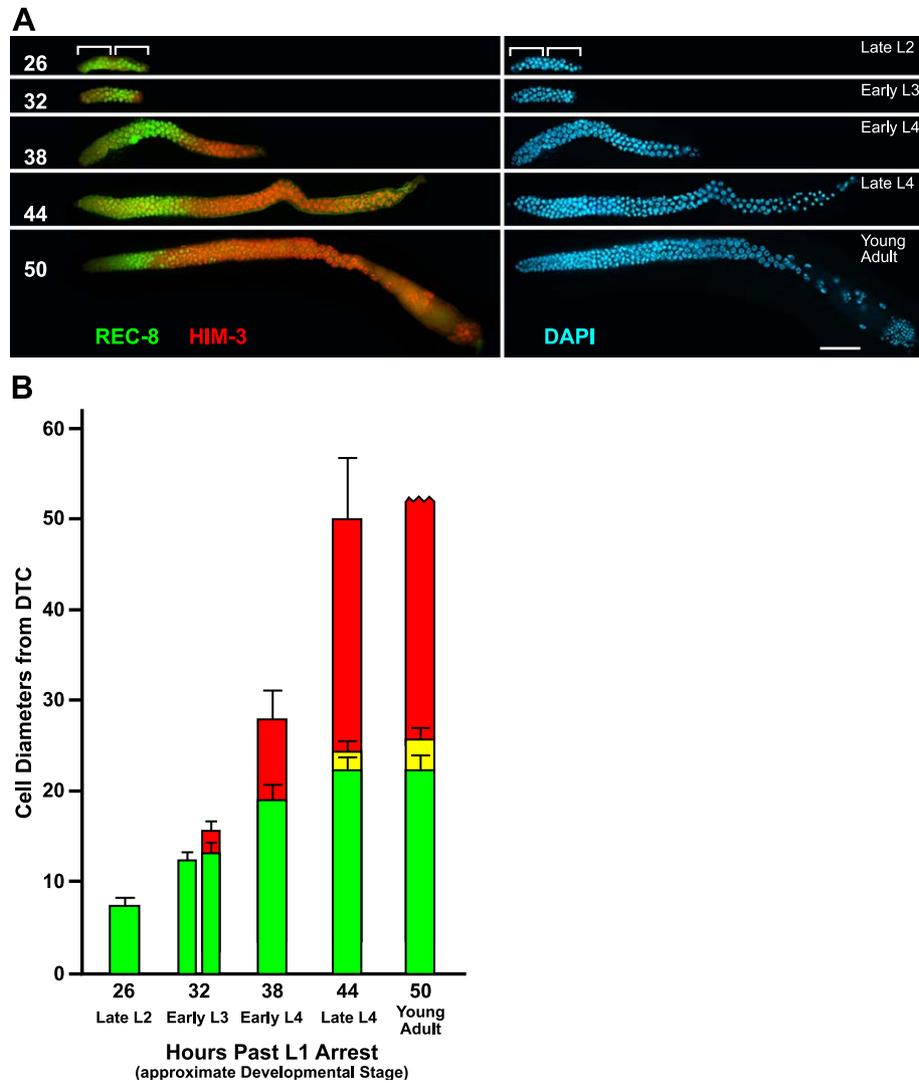


Fig. 4. Proliferation and meiotic entry throughout development in wild-type hermaphrodites. (A) Photographs of dissected gonad arms of wild-type hermaphrodites at various time points in development. Time (left) is the number of hours past L1 arrest. Arms were stained with REC-8 (green) and HIM-3 (red) specific antibodies to show proliferative and meiotic cells, respectively. Gonad arms were also stained with DAPI (blue) so as to show nuclear morphology. At each time point, only one of the gonad arms from a single animal is shown with distal to the left, with the exception of 26 h where the entire gonad is shown with the approximate location of each arm indicated with brackets. For each time point, pictures in the left and right panels are of the same gonad arm. Some arms show proximal green staining that is due to REC-8 staining some somatic structures in the proximal gonad. Scale bar = 20 μ m. (B) Graph showing the positions of REC-8-positive and HIM-3-positive cells in staged wild-type animals as measured as cell diameters from the DTC (y axis). The picture in (A) shows a representative dissected gonad arm of each time point. The x axis shows the number of hours past L1 arrest and the approximate developmental stage (see Materials and methods). Bars in green depict regions of the gonads with REC-8-positive/HIM-3-negative cells. Yellow depicts the meiotic entry region with both proliferative (REC-8-positive/HIM-3-negative) and meiotic (REC-8-negative/HIM-3-positive) cells existing the same distance from the DTC. The regions with only REC-8-negative/HIM-3-positive cells are shown in red. For each time point, a total of 12 gonad arms were analyzed, except for at 32 h. This time point was divided into two based on whether the arms only contained proliferative cells (left, $N = 10$), or both proliferative and meiotic cells (right, $N = 6$). For the 50-h time point, no maximum distance was measured because these animals contained sperm in the proximal end of the gonad making the cell diameter measurement of distance from the distal end inaccurate. Error bars = 1 SD.

HIM-3-positive) cells at the same distance from the distal end (Fig. 4). Adult males show a similar pattern of nonuniform meiotic entry (data not shown). Thus, at this stage, the distal germline can be separated into three distinct regions. The first (closest to the DTC) region consists of only proliferative cells. In adults (1 day past L4) it extends approximately 19 cell diameters from the DTC and all cells are REC-8-positive HIM-3-negative (Fig. 2). It should be

noted that cells in the more distal half of this region often show a lower level of REC-8 expression than the more proximal half, suggesting that the proliferative zone may not be a homogeneous population (Fig. 3). The second region consists of both proliferative and early meiotic prophase cells equidistant from the DTC. We refer to this region as the 'meiotic entry region'. Specifically, we define this region as the segment between the HIM-3-positive cell

closest to the DTC and the REC-8-positive cell furthest from the DTC (Figs. 2B and 3). This meiotic entry region averages seven cell diameters, though it varies from animal to animal (Figs. 2B and 3). For example, among the 15 gonad arms analyzed in Fig. 2B, the closest HIM-3-positive nucleus to the DTC was 14 cell diameters away, while the furthest REC-8-positive nucleus from the DTC was 32 cell diameters away (as measured in different gonad arms). The late larval/adult meiotic entry region should not be confused with the transition zone, which is much larger. Our working definition of the transition zone is that it constitutes the region between the cells closest to the DTC and those furthest from the DTC that have a crescent-shaped DNA organization (this definition is similar though not identical to that described previously; Crittenden et al., 1994). While the distal portion of the transition zone consists of both REC-8-positive and HIM-3-positive cells, the proximal portion contains only HIM-3-positive cells, and therefore would not be considered part of the late larval/adult meiotic entry region (Figs. 2A and 3). The third region consists of only meiotic (REC-8-negative/HIM-3-positive) cells, and encompasses all cells proximal to the REC-8-positive cell furthest from the DTC. Therefore, the late larval/adult gonad consists of both proliferative and meiotic cells spatially separated, with a staggered border between the two populations (Figs. 2 and 3).

Meiotic entry occurs in synthetic tumorous mutants

Using the REC-8 (proliferation) and HIM-3 (meiotic prophase) markers, we analyzed various tumorous mutants

to determine if the tumors consisted solely of proliferative cells by these more stringent criteria. We first analyzed the canonical strong *glp-1(gf)* tumor [*glp-1(oz112gf)/glp-1(oz112gf)/glp-1(+)* at 25°C], in which the GLP-1 receptor is constitutively active (Berry et al., 1997). The germ cells in adult gonads stained positively for REC-8 throughout the germline, and lacked HIM-3 staining (Fig. 5, Table 1), indicating that this germline tumor consists only of proliferating cells with no evidence of meiotic entry. Identical results were observed when larval gonads of this genotype were examined (data not shown).

Surprisingly, synthetic tumorous germlines from *gld-2(q497) gld-1(q485)* double null adult animals contained not only proliferative cells (REC-8-positive and HIM-3-negative), but also meiotic cells (REC-8-negative and HIM-3-positive) (Fig. 5, Table 1). While the number of REC-8-negative/HIM-3-positive cells, and thus the extent of meiotic entry, varied from animal to animal, 92% of gonad arms examined contained meiotic cells (Table 1).

Synthetic tumorous germlines from *gld-2(q497); nos-3(oz231)* double null adult animals displayed an even greater extent of meiotic entry than in *gld-2(q497) gld-1(q485)* animals. All gonad arms examined contained REC-8-negative/HIM-3-positive cells, and the number of meiotic cells was greater than in *gld-2(q497) gld-1(q485)* animals (Table 1). *nos-3* is one of three related *nos* proteins in the *C. elegans* genome that shows similarity to *Drosophila* Nanos (*nos-1*, *nos-2*, and *nos-3*) (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). To test if the *nos* genes function redundantly in regulating meiotic entry, we examined the extent of meiotic entry in *gld-2; nos-2 nos-3 nos-1* quadru-

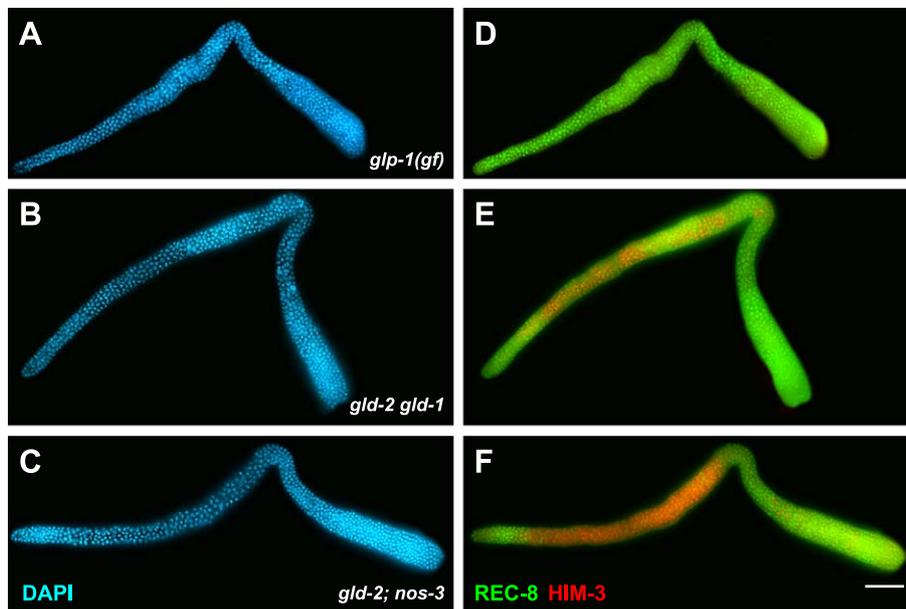


Fig. 5. Proliferating and meiotic cells in germline tumors. Dissected gonad arms stained with DAPI (A, B, and C; blue), REC-8 antibodies (D, E, and F; green), and HIM-3 antibodies (D, E and F; red) with distal to the left. Actual genotypes; (A and D) *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/dpy-19(e1259) unc-32(e189) glp-1(oz112gf)*; *qDp3* (*qDp3* contains *unc-32(e189)* and wild-type copies of *dpy-19* and *glp-1*; Austin and Kimble, 1987) grown at 25°C; (B and E) *gld-2(q497) gld-1(q485); unc-32(e189)*; (C and F) *gld-2(q497); nos-3(oz231); unc-32(e189)*. Scale bar = 20 μ m.

Table 1
Extent of meiotic entry in germline tumors

Genotype	Percent showing meiotic entry (%) ^a	Extent of entry ^b	N ^c
Control ^d	100	NA	hundreds
<i>glp-1(oz112gf)</i> ^e	0	NA	30
<i>gld-2(q497) gld-1(q485)</i> ^f	92	++	61
<i>gld-2(q497); nos-3(oz231)</i> ^g	100	++++	>100
<i>gld-2(q497) gld-1(q485); nos-3(oz231)</i> ^h	46	+	24
<i>gld-2(q497) gld-1(q361)</i> ⁱ	13	+	95
<i>gld-2(q497) gld-1(q485); glp-1(q175)</i> ^j	21	+	72
<i>gld-2(q497); nos-3(oz231); glp-1(q175)</i> ^k	100	+++	77
<i>gld-2(q497); nos-2(ok230) nos-1(gv5) nos-3(oz231)</i>	100	++++	22
<i>gld-2(q497) gld-1(q485); glp-1(ar202)</i> ^l	0	NA	20

^a Entry into meiosis was determined by the presence of HIM-3-positive staining cells coincident with the absence of REC-8 staining in the same cell. All strains were grown at 20°C unless otherwise noted, and were scored 1 day past L4.

^b Extent of entry refers to the approximate number HIM-3-positive cells of a typical gonad arm for those arms that do have HIM-3-positive cells. + ≈ < 5 cells, +++ ≈ greater than 20 cell diameters, which means many more than 20 cells. ++ and +++ reflect relative amounts of entry between + and ++++.

^c N refers to the total number of gonad arms analyzed.

^d *unc-32(e189)*.

^e Actual genotype is *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/dpy-19(e1259) unc-32(e189) glp-1(oz112gf)*; *qDp3* (*qDp3* contains *unc-32(e189)* and wild-type copies of *dpy-19* and *glp-1*) grown at 25°C.

^f Actual genotype; *gld-2(q497) gld-1(q485)*; *unc-32(e189)*.

^g Actual genotype; *gld-2(q497); nos-3(oz231); unc-32(e189)*; however, similar results were obtained with an unmarked strain.

^h Actual genotype; *gld-2(q497) gld-1(q485); nos-3(oz231); unc-32(e189)*.

ⁱ Actual genotype; *gld-2(q497) gld-1(q361); unc-32(e189)*.

^j Actual genotype; *gld-2(q497) gld-1(q485); unc-32(e189) glp-1(q175)*.

^k Actual genotype; *gld-2(q497); nos-3(oz231); unc-32(e189) glp-1(q175)*.

^l Actual genotype; *gld-2(q497) gld-1(q485); unc-32(e189) glp-1(ar202)*. Substantial meiotic entry is normally observed in *unc-32(e189) glp-1(ar202)* double mutants at 20°C (Pepper et al., 2003a).

ple mutants and found that the extent of meiotic entry was essentially the same as that seen in *gld-2; nos-3* double mutants (Table 1). We conclude that *nos-1* and *nos-2* likely do not function redundantly with *nos-3* in regulating meiotic entry.

Since the *glp-1(oz112gf)* tumor shows no evidence of meiotic entry while both *gld-2 gld-1* and *gld-2; nos-3* synthetic tumors contain meiotic cells, the synthetic tumorous mutants are not equivalent to the *glp-1(gf)* tumor. Therefore, the elimination of *gld-1* and *gld-2* pathway genes is not equivalent to constitutive activation of the GLP-1/Notch pathway. Since gametes are not formed in the synthetic tumorous mutants, although cells have entered meiosis, meiotic prophase progression must also be disrupted. *gld-1* single mutants are known to have a meiotic progression defect in female germ cells (Francis et al.,

1995a), and *gld-2* single mutants have meiotic progression defects in both male and female germ cells (Kadyk and Kimble, 1998).

The synthetic tumorous phenotype derives from a defect in meiotic entry, rather than meiotic progression

Since some cells enter meiosis in *gld-2 gld-1* and *gld-2; nos-3* double mutant adults, this raises the possibility that these synthetic tumors derive from a defect in meiotic prophase progression rather than meiotic entry, analogous to the etiology of the female germ cell tumorous phenotype in the *gld-1* single mutant. As mentioned previously, female germ cells in *gld-1* null single mutants enter meiotic prophase; however, the cells fail to progress beyond the pachytene stage, but rather exit meiotic prophase and return to mitosis, resulting in a large germline tumor (Francis et al., 1995a,b). To determine if the synthetic germline tumors in *gld-2(q497) gld-1(q485)* and *gld-2(q497); nos-3(oz231)* animals are due to a defect in the entry into meiosis decision, as in *glp-1(gf)* mutants, or due to an inability to progress through meiotic prophase, as in the *gld-1* single mutant female germ cells, we asked if each single synthetic tumorous mutant could enhance a weak *glp-1(gf)* allele. We reasoned that null mutations in genes that promote meiotic entry should enhance a weak *glp-1(gf)* allele, whereas those that act solely in meiotic progression should not enhance. *glp-1(ar202gf)* is a temperature sensitive allele that at the restrictive temperature, displays a Pro phenotype and a late-onset-tumorous phenotype (Pepper et al., 2003a,b) where the size of the distal proliferative zone increases over time. At the permissive temperature (15°C), however, neither of these mutant phenotypes is observed. We found that each of three double mutant combinations of *glp-1(ar202gf)* with *nos-3*, *gld-1*, or *gld-2* enhances the late-onset tumorous phenotype of *glp-1(ar202gf)* at the permissive temperature (Fig. 6), suggesting that *nos-3*, *gld-1*, and *gld-2* are involved in the proliferation versus meiotic entry decision. Therefore, the meiotic cells that are observed in these synthetic tumorous mutants are likely due to an incomplete inhibition of meiotic entry rather than normal meiotic entry followed by reversion to mitosis.

A third pathway promotes initiation of meiotic development in late larvae/adults and is negatively regulated by GLP-1-mediated signaling

To further understand the processes controlling meiotic entry, we examined the temporal and spatial pattern of meiotic entry during development in the synthetic tumorous mutants. For this analysis, we examined the pattern of REC-8 and HIM-3 staining in synchronous populations of staged animals at 6-h intervals throughout development (Fig. 7). In synthetic tumorous animals, germ cells entered meiosis later in development as compared to control animals (Fig. 7). Indeed, all wild-type control animals display REC-8-nega-

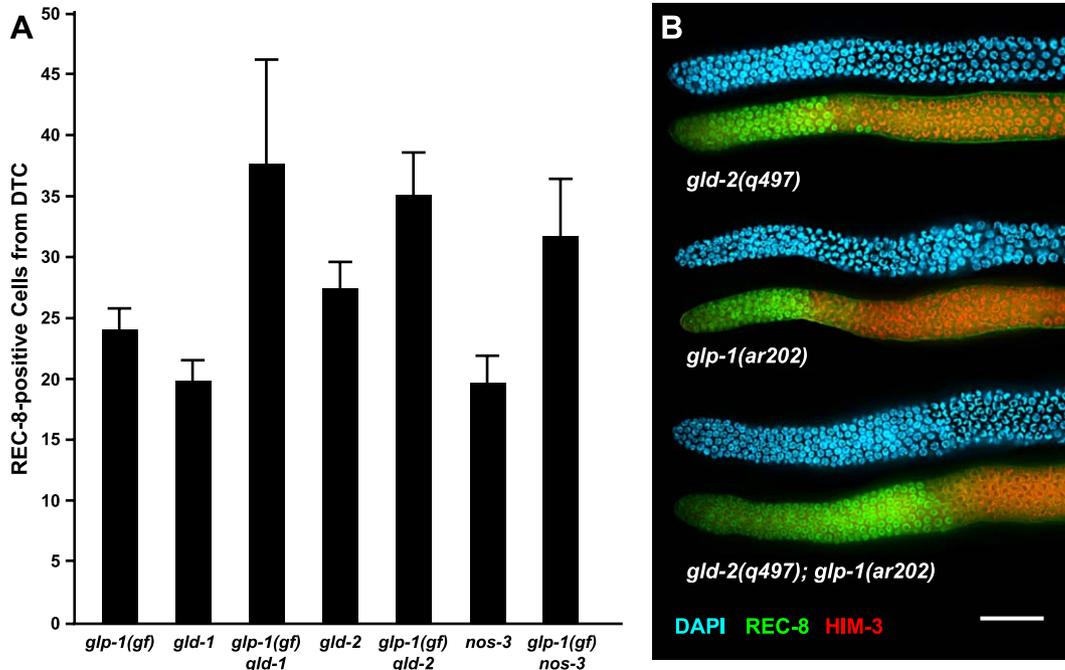


Fig. 6. *gld-1*, *gld-2*, and *nos-3* mutants each enhance a weak *glp-1(gf)* allele. (A) Graph showing the average size of the distal proliferative zones (*y* axis) of various mutant animals as measured by the number of cell diameters where all cells are REC-8-positive and HIM-3-negative. The *x* axis shows the genotypes of the mutant animals. Actual genotypes are *glp-1(ar202gf)*, *gld-1(q485)*, *gld-2(q497)*, and *nos-3(oz231)*. All strains were also marked with *unc-32(e189)* and grown at 15°C. For each strain, the mitotic zones of at least 15 gonad arms were measured. For each of *gld-1*, *gld-2*, and *nos-3*, when in combination with *glp-1(ar202gf)*, the region containing REC-8-positive/HIM-3-negative cells (proliferative zone) was larger and the meiotic entry region displaced proximally as compared to the single mutants. For single mutants versus relative double mutants, $P < 6.5 \times 10^{-6}$ *t* test. Error bars = 1 SD. (B) A distal portion of typical dissected gonad arms of three mutant animals from part (A) showing the enhancement of the size of the mitotic zone in *glp-1(ar202gf)* animals by *gld-2(q497)*. Each arm is stained with DAPI (blue), anti-REC-8 antibodies (green), and anti-HIM-3 antibodies (red). Each strain is marked with *unc-32(e189)*. Scale bar = 20 μm.

tive/HIM-3-positive cells by 38 h past L1 arrest, whereas REC-8-negative/HIM-3-positive cells are not visible until 56 h in *gld-2(q497) gld-1(q485)* gonads and until 44 h in *gld-2(q497); nos-3(oz231)* animals. Furthermore, when meiotic entry occurs in the tumorous mutants, it occurs at a greater distance from the distal end than in wild type (Figs. 7A, B, and C). Therefore, early in development removal of the activities of GLD-1 and GLD-2 (or NOS-3 and GLD-2) appears equivalent to constitutive activation of the GLP-1 receptor (*glp-1(oz112gf)/glp-1(oz112gf)/glp-1(+)*), in that germ cells fail to enter meiosis. However, later in development, unlike the *glp-1(gf)* tumor, the synthetic tumors show meiotic entry. This suggests that later in development, *glp-1* negatively regulates at least one additional pathway that promotes meiotic entry, in addition to the *gld-1* and *gld-2* pathways.

If a putative third pathway functions downstream of GLP-1/Notch signaling, then elevating the activity of the *glp-1* pathway in *gld-2 gld-1* animals should reduce the extent of meiotic entry. Conversely, if only the *gld-1* and *gld-2* pathways, and not a third pathway, are regulated by GLP-1/Notch signaling, then elevating the activity of GLP-1 should not affect the number of cells entering meiosis in a *gld-2 gld-1* tumor. We examined the extent of meiotic entry in *gld-2 gld-1; glp-1(ar202gf)* triple mutant animals and

found no evidence of meiotic entry (Table 1). These results suggest that a third pathway acts in opposition to and downstream of the GLP-1/Notch signaling pathway to allow meiotic entry to occur.

We also examined the extent of meiotic entry in the synthetic tumorous mutants in the absence of *glp-1*. We reasoned that although the *gld-2 gld-1* synthetic tumorous phenotype is epistatic to the *glp-1(lf)* phenotype, the removal of *glp-1* might result in a qualitatively less tumorous phenotype (that is, more REC-8-negative/HIM-3-positive cells or more cells in meiosis) than the synthetic tumorous phenotype in an otherwise *glp-1(+)* background. Surprisingly, removal of GLP-1 activity from synthetic tumorous mutants in the triple mutants *gld-2(q497) gld-1(q485); glp-1(q175)* and *gld-2(q497); nos-3(oz231); glp-1(q175)* results in gonads with fewer cells exhibiting meiotic entry than when the GLP-1/Notch pathway is active (Fig. 7, Table 1). Furthermore, the meiotic entry that does occur in these triple mutants occurs later in development than when the GLP-1 activity is present (Fig. 7, Table 1). One explanation for these counterintuitive results is that when GLP-1/Notch signaling is removed, germ cells enter meiosis much earlier in development and return to proliferation before the time of our analysis. To examine this possibility, we analyzed dissected *gld-2(q497) gld-1(q485); glp-1(q175)* gonads at

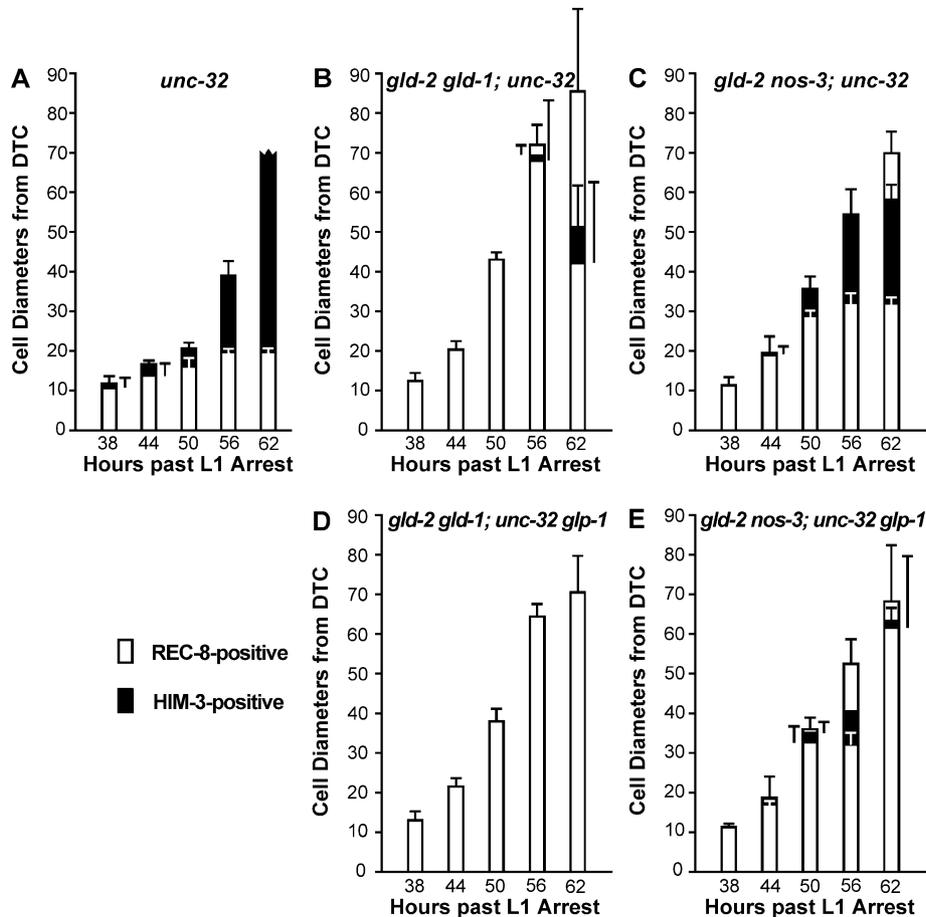


Fig. 7. Synthetic tumorous mutants enter meiosis later in development. Graphs showing the extent of meiotic entry at various points in development in synthetic tumorous mutants with the numbers of cell diameters from the DTC on the y axes and number of hours past L1 arrest on the x axes. Meiotic entry was measured by determining the position from the DTC of REC-8 and HIM-3 staining cells. Actual genotypes (A) *unc-32(e189)* (B) *gld-2(q497) gld-1(q485); unc-32(e189)* (C) *gld-2(q497); nos-3(oz231); unc-32(e189)* (D) *gld-2(q497) gld-1(q485); unc-32(e189) glp-1(q175)* (E) *gld-2(q497); nos-3(oz231); unc-32(e189) glp-1(q175)*. For all time points, the region containing meiotic cells [HIM-3-positive] is defined as the region from the most distal REC-8-negative HIM-3-positive cell to the most proximal REC-8-negative HIM-3-positive cell even though some cells between these two boundaries could be REC-8-positive HIM-3-negative. For most time points, 10 gonad arms were analyzed with 5 gonad arms for one time point (*gld-2; nos-3; unc-32 glp-1* at 38 h). For the 62-h time point in *unc-32* animals, no maximum distance was measured because these animals contained sperm in the proximal end of the gonad making the cell diameter measurement of distance from the distal end inaccurate. Error bars = 1 SD.

two earlier time points, 26 and 32 h past L1 arrest (late L2/early L3), and all germ cells were proliferative (REC-8-positive HIM-3-negative). The average number of germ cells per animal (not arm) at 26 h was 12.6 ($n = 15$, range 10–16). Meiotic entry likely would not have occurred before this time because both *gld-1; glp-1* and *gld-2; glp-1* double mutant animals produce a total of approximately 32 cells per animal (Francis et al., 1995b; Kadyk and Kimble, 1998). Therefore, the reduced amount of meiotic entry in synthetic tumorous mutants when GLP-1 activity is removed is not likely due to germ cells entering meiosis earlier in development, but rather could reflect a role for GLP-1 in inhibiting proliferation and/or promoting meiotic entry (see Discussion).

In the course of conducting these experiments, we found that the *gld-2 gld-1* double mutant with *gld-1(q361)* displayed fewer cells undergoing meiotic entry (that is, the tumorous phenotype was more proliferative)

than when the *gld-1* null allele was used (Table 1). The *gld-1(q361)* missense mutation prevents the GLD-1 protein product from binding target mRNAs, but allows production of the nonfunctional protein at essentially wild-type levels (Jan et al., 1999; Jones and Schedl, 1995; Jones et al., 1996; Lee and Schedl, 2001). *gld-1(q361)* behaves as a genetic null for GLD-1's essential function in female germ cell meiotic prophase progression and has some dominant negative properties relative to male sex determination (more heterozygous feminized animals are seen than with the null Francis et al., 1995a). This enhanced tumorous phenotype suggests that the GLD-1(*q361*) protein could bind to and interfere with the normal activity of proteins that would otherwise promote meiotic entry and/or inhibit proliferation. The factor(s) that the GLD-1(*q361*) protein is poisoning could normally function in the putative third pathway regulating meiotic entry.

We also found that *gld-2(q497) gld-1(q485); nos-3(oz231)* triple mutants have fewer germ cells enter meiosis than in either *gld-2(q497) gld-1(q485)* or *gld-2(q497); nos-3(oz231)* double mutants (Table 1). Previous data indicate that NOS-3 functions to promote GLD-1 accumulation in the GLD-1 pathway (Hansen et al., 2004). These current results further suggest that NOS-3 may regulate a meiosis-promoting factor that is distinct from the GLD-1 and GLD-2 pathways.

Discussion

REC-8 and HIM-3 antibodies as tools for investigating meiotic entry

The initiation of meiosis is a key step in germline development and must be tightly regulated to ensure reproductive fitness. We have characterized the temporal and spatial properties of meiotic entry in the *C. elegans* germline using antibodies that specifically distinguish proliferative and meiotic germline cells. The current model of meiotic entry proposes that germ cells close to the DTC have elevated GLP-1 signaling, which inhibits the GLD-1 and GLD-2 pathways, while germ cells further away from the DTC have low levels of GLP-1 signaling, allowing for activation of the GLD-1 and GLD-2 pathways and meiotic entry ensues. We have used antibodies that recognize REC-8 (Pasierbek et al., 2001) and HIM-3 (Zetka et al., 1999) to study the initiation of meiotic development. REC-8 is in the nucleoplasm and on the chromosomes during proliferation and then localizes to the proteinaceous core of sister chromatids in meiotic prophase. Under our fixation conditions, only the nucleoplasmic and non-proteinaceous core chromosomal REC-8 localization is observed. The appearance of HIM-3 on chromosomes, during meiotic prophase, corresponds to the loading of HIM-3 onto chromosomal axes (Zetka et al., 1999). The first overt event in meiotic development is DNA replication (Forsburg, 2002). It appears to be distinct from mitotic S phase and is important for inter-homolog recombination. It is also important for chromosome segregation in the MI and MII divisions, which is, at least in part, a consequence of loading the meiotic cohesion REC-8 onto chromosomes during meiotic S phase (Forsburg, 2002). It is likely that conversion from REC-8 staining to HIM-3 staining is a consequence of meiotic S phase or its completion.

Spatial control of meiotic entry

Our analysis of the border of proliferation and meiotic prophase using REC-8 and HIM-3 antibodies reveals a developmental change in the proliferation versus meiotic prophase decision. In early larval germlines, in which meiotic prophase has begun but the distal proliferative zone is still growing, cells switch from proliferation to meiosis as

they cross a distance of one germ cell diameter. In contrast, the switch from proliferation to meiotic prophase in late L4/adulthood occurs, on average, over a region of seven cell diameters in length (from 19 to 26 cell diameters from the distal tip; the meiotic entry region, Figs. 2 and 3). The temporal difference in the sharpness of the proliferation versus meiotic entry boundary may be a reflection of differences in the regulation of meiotic entry during development. Alternatively (or additionally), this temporal difference may have to do with there being significantly fewer germ cells in early larval gonads and that these cells are larger than in later stages.

Although the distal proliferation zone in the adult *C. elegans* germline is a stem cell population in the sense that it is both self-renewing and produces cells that differentiate, it is currently unknown whether all cells in this zone are stem cells. While cells undergoing mitotic divisions are observed throughout this zone, it is formally possible that only the cells closest to the DTC are stem cells and that they undergo transit amplification before entering pre-meiotic S phase. This situation would be analogous to gonialblast proliferation in the *Drosophila* germline (Kiger and Fuller, 2001; Xie and Spradling, 2001). Most of the surface area of the DTC spreads over several cell diameters of the germline, and on average, DTC processes extend only as far as the distal-half of the proliferative zone (Hall et al., 1999), though processes can extend to the edge of the proliferative zone (Fitzgerald and Greenwald, 1995). Therefore, cells in the proximal portion of the proliferative zone may come in contact with a significantly lower level of LAG-2 ligand than those in the distal portion, and cells could commit to enter meiotic prophase more distally than anti-REC-8 and anti-HIM-3 antibodies reveal. Alternatively, the region between the majority of DTC-bound LAG-2 ligand and meiotic entry could reflect a slow decay rate of GLP-1/Notch signaling [e.g., the half life of nuclear GLP-1(INTRA)]. A developmental analysis of the dynamics of cell division compared to signaling levels in the distal proliferative zone will be of significant interest, although this analysis is currently not feasible since there is no available method to monitor nuclear GLP-1(INTRA).

It is also important to note that GLD-1, which promotes meiotic entry, reaches a high level of accumulation distal to some cells that appear not to have entered meiosis. GLD-1 levels are low in the distal end, then increase until reaching a high level approximately 20 cell diameters from the distal end (Hansen et al., 2004; Jones et al., 1996). It is currently unknown what level of GLD-1 is necessary to promote meiotic entry, although the fact that not all cells have entered meiosis when GLD-1 levels are high would suggest that either cells commit to enter meiosis while still REC-8-positive/HIM-3-negative (distal to the meiotic entry region), or that cells differ in their competence to respond to meiotic entry signals.

What could account for the nonuniform entry into meiotic prophase in the late larval/adult germline? One

possible contributing factor could be that the mitotic cell cycle within the proliferation zone is asynchronous. Previous studies of the wild-type proliferative zone showed that cells in mitotic M-phase are infrequent and relatively randomly scattered. Cells at a given distance from the distal tip appear to be at various stages of the cell cycle (Crittenden et al., 1994; Francis et al., 1995a; Kadyk and Kimble, 1998; Kuwabara et al., 2000). This result is not expected given the syncytial nature of the germline. For example, in the *Drosophila* syncytial blastoderm embryo, there is a very high degree of cell cycle synchrony (Foe and Alberts, 1983). This difference has led to the proposal that while the distal *C. elegans* germline is syncytial, each germline nucleus, cytoplasm, and surrounding membranes (called a germ cell) acts as an autonomous unit with respect to mitotic cell cycle progression, and that diffusion of cell cycle control factors is limited. If, for example, the switch from proliferation to meiotic development can only be made in G1, then the nonuniform or staggered initiation of meiotic development may be a consequence of cells, at various stages of the mitotic cell cycle, entering a critical region of the gonad where signaling is sufficient to promote entry into meiotic prophase. Therefore, some cells must progress to G1 (moving more proximally as they do so) before entering meiotic prophase, while other cells that are in G1 as they enter the critical region can immediately enter meiotic prophase. It is also possible that if there are transit amplifying cycles in the proliferative germline (see above), the number or length of the cell cycles could differ between cells, thereby causing cells to enter meiotic prophase at different positions in the gonad.

Another related mechanism that may account for the staggered border between proliferation and meiotic prophase in late L4/adults could be cell autonomous differences in the levels of GLP-1 signaling—either stochastic differences in the half-life of nuclear GLP-1 (INTRA), differences in contact with LAG-2 due to the nonuniform shape of the DTC (Hall et al., 1999), or some other differences in signaling. A caveat to this model is that GLD-1 accumulation, our only available molecular read-out of GLP-1 signaling in the germline (Hansen et al., 2004), appears to be uniform between cells at a given distance from the distal end in the adult (Jones et al., 1996). However, since GLD-1 accumulation is cytoplasmic, and germ cells are not completely separate, some diffusion of GLD-1 may occur between germ cells. Indeed, this diffusion may be a means of leveling differences in GLP-1 signaling between adjacent cells, thereby reducing the potential amount of nonuniform meiotic entry.

Evidence for additional pathway/pathways regulating the adult proliferation versus meiotic entry decision

We have shown that constitutive activation of GLP-1 results in a homogeneously proliferative tumor of REC-8-positive/HIM-3-negative cells, likely due to a failure of cells

to enter meiotic prophase. However, we cannot completely rule out the possibility that some cells in these tumors briefly enter meiosis but do not progress far enough into meiotic prophase to be detected by these markers. Nevertheless, these markers do show that the tumors resulting from inactivation of the downstream GLD-1 and GLD-2 pathways contain significant numbers of cells in the early stages of meiosis (Fig. 4, Table 1). If prevention of meiotic entry via GLP-1/Notch signaling were acting through the *gld-1* and *gld-2* pathways alone, then eliminating these two pathways should be phenotypically equivalent to constitutive activation of *glp-1*. The presence of meiotic cells in *gld-2 gld-1* and *gld-2; nos-3* tumors are either due to a defect in meiotic progression (analogous to the *gld-1* single mutant; Francis et al., 1995a), or that there is a third (at least) pathway downstream of GLP-1/Notch signaling regulating meiotic entry. Our genetic results demonstrating enhancement of a weak *glp-1(gf)* allele by mutations in *gld-1*, *gld-2*, or *nos-3*, and results of our time course analysis, support a role for GLD-1, GLD-2, and NOS-3 in the meiotic entry decision. Taken together, these results implicate a third pathway promoting meiotic entry and/or inhibiting proliferation, acting downstream of GLP-1/Notch signaling and parallel with the GLD-1 and GLD-2 pathways.

The relative strength of the putative third pathway is apparently lower than that of the *gld-1* or *gld-2* pathways. Even though some meiotic entry occurs in a *gld-2 gld-1* tumor, most of the germ cells are proliferative and the tumor is epistatic to the *glp-1(null)* premature meiotic entry phenotype. Additionally, the reliance on this third pathway appears to be lower or absent in hermaphrodite larvae relative to adults because meiotic entry in the synthetic tumorous mutants is delayed relative to meiotic entry in wild type.

While no components of the putative third pathway have yet been identified, our data provide some clues that may assist in their identification. It is possible that a component of the third pathway binds to the GLD-1 protein since fewer cells enter meiosis in *gld-2(q497) gld-1(q361)* than in *gld-2(q497) gld-1(q485null)* gonads. The phenotype of *gld-1(q361)* single mutant is very similar to that of the *gld-1* null; however, it produces protein at equivalent levels to wild-type that is unable to bind at least some of its target mRNAs (Jan et al., 1999; Jones et al., 1996; Lee and Schedl, 2001). Thus, the nonfunctional GLD-1(*q361*) protein may bind a component of the third pathway and prevent it from performing its normal function. Additionally, NOS-3 may be involved in regulating the activity of a component of the third pathway. First, *gld-2 gld-1; nos-3* hermaphrodite germlines contain fewer meiotic cells than either *gld-2 gld-1* or *gld-2; nos-3* double mutants indicating a function in meiotic entry independent of the *gld-1* or *gld-2* pathways. Second, many *gld-1(q485);nos-3(oz231)* males have a synthetic proximal proliferation phenotype (Hansen et al., 2004), which is presumably due to a failure of some proximal germ cells to enter meiosis. At a minimum, this

demonstrates that NOS-3 is performing a function in regulating meiotic entry in addition to its role in promoting GLD-1 accumulation (Hansen et al., 2004). Further, we propose that the third pathway is unable to compensate for the loss of GLD-1 and GLD-2 in younger hermaphrodites. It is not until *gld-2(q497) gld-1(q485)* hermaphrodites are older that meiotic cells are seen. This could mean that the third pathway only functions in late larvae/adult hermaphrodites (Fig. 8), or that a single pathway is not sufficient to cause meiotic entry in younger hermaphrodites, but is sufficient in adults. These data are consistent with previous results (Berry et al., 1997; Pepper et al., 2003b) suggesting that some differences exist between the control of meiotic entry during different stages of development.

The effect of GLP-1(+) on synthetic tumorous mutants

Our analysis uncovered a paradox in the role of *glp-1* in the proliferation versus meiotic development decision. A wealth of data support the conclusion that the role of *glp-1*(+) is to promote proliferation and/or inhibit meiotic entry

(Austin and Kimble, 1987; Berry et al., 1997; Lambie and Kimble, 1991; Pepper et al., 2003a). Our observation that *gld-2 gld-1; glp-1(ar202gf)* animals are completely tumorous (that is, contain only proliferating cells; Table 1) also supports this role for *glp-1*. Our unexpected result is that genetic removal of *glp-1*, using the null allele *glp-1(q175)*, reduced the number of meiotic cells in *gld-2 gld-1* and *gld-2; nos-3* synthetic tumors. A possible model explaining this result is that *glp-1* could function in both promoting and inhibiting proliferation (and/or inhibiting and promoting meiotic development) (Fig. 8). For example, the proposed third pathway could require GLP-1 activity in order for it to promote meiotic entry and/or inhibit proliferation.

Another possible, perhaps more likely, explanation for this apparent paradox can best be understood in the context of a proposed positive feedback mechanism between proliferation or active GLP-1 signaling and GLP-1 protein expression and/or activity (Berry et al., 1997; Kadyk and Kimble, 1998; Kodoyianni et al., 1992). *gld-2 gld-1* synthetic tumorous mutants (in the presence of wild-type GLP-1) have excess membrane-associated GLP-1 (Kadyk and Kimble, 1998). This excess GLP-1, which is distant from the LAG-2 ligand and therefore is unlikely to produce GLP-1(INTRA) and activate signaling, could bind and titrate proliferation-promoting or meiosis-inhibiting factors. In tumorous animals that do not make GLP-1 protein (e.g., *gld-2 gld-1; glp-1*), the titration of these factors would not occur and fewer cells would enter meiosis. *glp-1(oz112gf)* animals, which also have GLP-1 on membranes throughout the germline, do not show titration-associated meiotic entry, possibly because the ectopically expressed GLP-1 is constitutively active, presumably continually generating GLP-1(INTRA), which would inhibit downstream pathways throughout the germline.

Even though the positive feedback regulation of GLP-1 could be involved in the extent of meiotic entry in synthetic tumorous mutants, it likely does not play a role in regulating meiotic entry in wild-type adults. Recent work indicates that a GLP-1 positive feedback mechanism could be accomplished through GLD-1 (Marin and Evans, 2003). GLD-1 binds the GLP-1 3'UTR and represses its translation. In the part of the germline closest to the DTC, GLP-1 signaling is high and represses GLD-1 accumulation (Hansen et al., 2004). More proximally, further away from the DTC-bound LAG-2 ligand, GLP-1 signaling is reduced, allowing for increased expression of GLD-1, which then can bind to the 3'UTR of GLP-1, inhibiting its translation. This inhibition of GLP-1 translation is not necessary for the proliferation versus meiotic entry decision because in *gld-1(null)* animals, where no GLD-1-mediated translational inhibition of *glp-1* can occur, germ cells enter meiosis at approximately the normal position (Fig. 6; Francis et al., 1995a), even though excess GLP-1 is produced (Marin and Evans, 2003). Also, high levels of GLP-1 and GLD-1 coexist at approximately 20 cell diameters from the DTC, and little LAG-2 ligand is likely present at this distance from the DTC (Hall

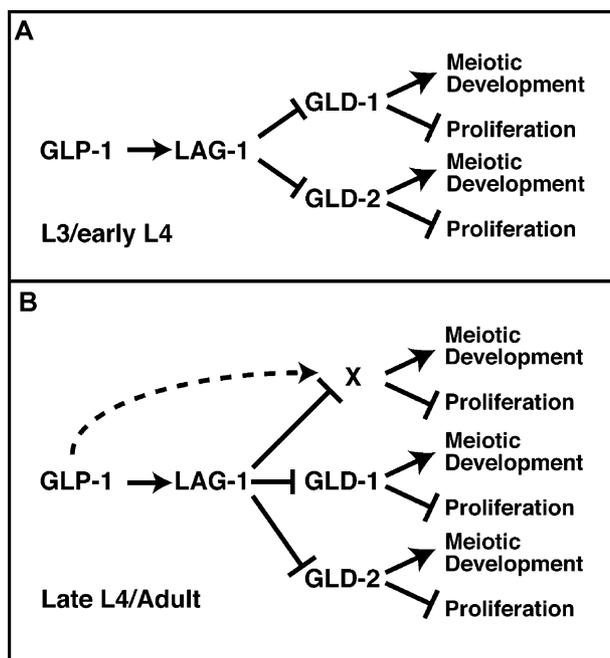


Fig. 8. Model depicting genetic interaction regulating meiotic entry in L3/L4 versus adult hermaphrodites. (A) In L3/L4 hermaphrodites, GLP-1/Notch signaling inhibits the GLD-1 and GLD-2 pathways in the distal end of the gonad, but as cells move proximally, these pathways become active causing meiotic entry to occur. When the activities of these pathways are eliminated through genetic mutation, germ cells fail to enter meiotic prophase forming a homogenous tumor of proliferating cells. (B) In adult hermaphrodites, elimination of GLD-1 and GLD-2 pathways is not sufficient to completely prevent meiotic entry, suggesting that a third pathway (X) may still be promoting meiotic development and/or inhibiting proliferation. Therefore, in the wild-type adult hermaphrodite germline, all three pathways may be regulating the proliferation versus meiotic entry decision. The relative importance of each pathway may vary depending on age, sex, and environmental conditions. It is further possible that the third pathway requires GLP-1 activity to function properly.

et al., 1999; Henderson et al., 1994), making it unlikely that GLD-1 repression of GLP-1 translation is important for meiotic entry in the distal germline. Rather, this feedback loop is more likely part of the mechanism to prevent GLP-1 from being expressed in the proximal germline and interfering with GLP-1 function in the early embryo, as previously suggested (Kadyk and Kimble, 1998).

Why do multiple redundant pathways control meiotic entry?

One proposed purpose of redundancy is to increase the fidelity of the regulated process (Thomas, 1993). The proliferation versus meiotic entry decision requires a tight balance: too much proliferation (too little meiotic entry) results in a germline tumor, and too little proliferation (too much meiotic entry) causes a depletion of the stem cell population. Either result causes sterility or a reduction in reproductive fitness. The reproductive fitness of a species is central to its survival and would likely have enormous evolutionary pressure pushing toward maximizing efficiency. GLD-1 is a KH domain translational inhibitor (Jan et al., 1999; Jones and Schedl, 1995; Lee and Schedl, 2001), while GLD-2 is the catalytic portion of a poly(A) polymerase (Wang et al., 2002); therefore, redundancy between these two pathways cannot simply be explained by the two proteins performing similar compensatory biochemical functions. Perhaps in this case, the GLD-1, GLD-2, and putative third pathways allow several different control points (and the importance of each point may differ depending on age, sex, or environmental condition), resulting in an overall more robust system and the maintenance of a perfect balance.

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