Control of the proliferation versus meiotic development decision in the *C. elegans* germline through regulation of GLD-1 protein accumulation

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Summary

Maintenance of the stem cell population in the *C. elegans* germline requires GLP-1/Notch signaling. We show that this signaling inhibits the accumulation of the RNA binding protein GLD-1. In a genetic screen to identify other genes involved in regulating GLD-1 activity, we identified mutations in the *nos-3* gene, the protein product of which is similar to the *Drosophila* translational regulator Nanos. Our data demonstrate that *nos-3* promotes GLD-1 accumulation redundantly with *gld-2*, and that *nos-3* functions genetically downstream or parallel to *fbf*, an inhibitor of GLD-1 translation. We show that the GLD-1 accumulation pattern is important in controlling the proliferation versus meiotic development decision, with low GLD-1 levels allowing proliferation and increased levels promoting meiotic entry.

Supplemental data available online

Key words: Germline development, Stem cells, Proliferation, Tumor, Meiotic entry, Notch signaling, *gld-1*, *nos-3*, *glp-1*

Introduction

Stem cells are of intense interest because of their potential use in regenerative medicine (Daley, 2002; Pfendler and Kawase, 2003), and their possible roles in cancer (Reya et al., 2001). They are also of interest because of their roles in many aspects of development and the continuous turnover of specific tissues. Stem cells have almost limitless proliferation capacity providing a pool of cells available over long periods of time. Their progeny have, in addition, the ability to discontinue proliferation and enter a differentiation pathway. A balance between proliferation and differentiation is therefore required in the normal utilization of stem cells. If too many cells enter the differentiation pathway the stem cell population is depleted and only a small number of the differentiated cells are made. Conversely, if stem cells continue to proliferate and fail to enter a differentiation pathway, tissue homeostasis is not maintained and a tumor may result. The need for controlling proliferation and differentiation is especially important for germline stem cells because the reproductive fitness of many animals relies on the production of large numbers of gametes over long periods of time. A shift in the balance between stem cell proliferation and differentiation can lead to sterility, caused by either a depletion of the stem cells resulting in few gametes being made (Austin and Kimble, 1987), or excess proliferation at the expense of gamete formation (Berry et al., 1997).

The *Caenorhabditis elegans* germline is an excellent system for studying the balance between proliferation and differentiation in a stem cell population because cells can be found in all stages of development in a linear spatial pattern (Schedl, 1997). The most distal end of the adult gonad contains a stem cell population that covers a region of approximately 20 cell diameters in length (Fig. 1A) (Crittenden et al., 1994; Hansen et al., 2004). Cells immediately proximal to the stem cells, in the transition zone, have entered meiotic prophase and continue to progress through meiosis as they move proximally.

The conserved GLP-1/Notch signaling pathway regulates the balance between proliferation and entry into meiotic prophase (Seydoux and Schedl, 2001). LAG-2 is a conserved ligand for the GLP-1/NOTCH receptor (Henderson et al., 1994; Tax et al., 1994) that is expressed in the somatic distal tip cell (DTC), which caps the distal end of the gonad (Fig. 1) (Kimble and White, 1981). GLP-1 is a member of the Notch family of transmembrane receptors (Austin and Kimble, 1987; Priess et al., 1987; Yochem and Greenwald, 1989) that is expressed in the germ cells (Crittenden et al., 1994). It is thought that the interaction of the LAG-2 ligand with the GLP-1 receptor results in a cleavage of the intracellular portion of GLP-1, generating GLP-1(INTRA), followed by its translocation to the nucleus and binding to LAG-1 (Mumm and Kopan, 2000). The GLP-1(INTRA)/LAG-1 complex probably results in transcription of genes that promote proliferation and/or inhibit entry into meiosis. As germ cells move proximally, away from the DTC, signaling decreases and the germ cells enter meiotic prophase. Loss of the activity of *lag-2*, *glp-1* or *lag-1* causes germ cells to enter meiosis prematurely, resulting in a depletion of the stem cell population (Austin and Kimble, 1987; Lambie and Kimble, 1991). Conversely, ligand-independent activation of the GLP-1 receptor resulting from a gain-of-function (gf) mutation results in stem cells failing to enter meiosis (Berry et al., 1997; Pepper et al., 2003). In this case the stem cells continue to proliferate, forming a germline tumor. Together these results support the

model of GLP-1/Notch signaling working as a binary switch in regulating the decision between proliferation and entry into meiotic prophase.

While no direct transcriptional targets of GLP-1 signaling have yet been characterized in the germline, genetic evidence indicates that *gld-1* and *gld-2* function in redundant pathways downstream of GLP-1/Notch signaling to promote meiotic development and/or inhibit proliferation (Fig. 1B) (Francis et al., 1995b; Kadyk and Kimble, 1998). GLD-1 is a KH domain-containing RNA binding protein (Jones and Schedl, 1995), and GLD-2 is the catalytic portion of a poly(A) polymerase (Wang et al., 2002). The gene for either of these is sufficient to promote meiotic entry since in either *gld-1* or *gld-2* single null mutant animals, germ cells enter meiosis normally (Francis et al., 1995a; Kadyk and Kimble, 1998; Hansen et al., 2004). However, in animals that lack both *gld-1* and *gld-2* activity, a germline tumor is formed that is similar to that of *glp-1(gf)* mutants (Kadyk and Kimble, 1998). This tumorous phenotype is epistatic to *glp-1* null indicating that *gld-1* and *gld-2* function downstream of GLP-1/Notch signaling (Kadyk and Kimble, 1998). Therefore GLP-1 signaling promotes proliferation, at least in part, by turning off the activities of *gld-1* and *gld-2*. It is not known how alteration of GLP-1 signaling in the distal germline changes *gld-1* and *gld-2* activities there, or how *gld-1* and *gld-2* become active more proximally. The mechanism appears to involve spatial regulation of GLD-1 protein accumulation. GLD-1 is at the lowest level at the very distal end and increases until reaching maximum levels approximately 20 cell diameters from the distal tip (Jones et al., 1996) (Fig. 1C,D). Since *gld-1* promotes meiotic entry, the low levels of GLD-1 protein in the distal end may be necessary to maintain the stem cell population. Likewise, the high levels of GLD-1 protein achieved at the approximate location of meiotic entry may be important for meiotic entry to occur.

Recently, FBF, a homolog of *Drosophila* Pumilio that is the product of two nearly identical adjacent genes, *fbf-1* and *fbf-2* (Zhang et al., 1997), has been shown to inhibit GLD-1 accumulation in the distal end of the germline (Crittenden et al., 2002). FBF is also necessary for germ cell proliferation in late larvae and adults; loss of FBF activity results in premature entry into meiotic prophase and a depletion of the stem cell population in the late fourth larval stage (Crittenden et al., 2002). FBF is a post-transcriptional repressor of *gld-1* and Crittenden et al. have proposed that FBF promotes proliferation by keeping GLD-1 levels low in the distal most germline (Crittenden et al., 2002).

We show that a major mechanism by which GLP-1/Notch signaling maintains the stem cell population is by inhibiting GLD-1 protein accumulation in the distal end of the germline, thereby restricting its activity to more proximal regions. We further show that not only does low GLD-1 allow proliferation, but that high GLD-1 promotes meiosis. We also show that the position of the rise in GLD-1 levels determines the size of the stem cell population and the location where germ cells begin meiotic development. We find that *nos-3*, whose role we identified in a mutant screen, functions redundantly with *gld-2* to promote the rise in GLD-1 that is necessary for entry into meiosis. Genetic experiments indicate that repression of GLD-1 accumulation by FBF is acting through *nos-3*, while regulation of *gld-2* in this processes is likely by something other than, or in addition to, FBF. Our data suggest a model in which GLP-1 signaling regulates the size of the stem cell population by regulating GLD-1 levels, at least in part, through antagonism between the repressive activity of *fbf* and the positive activities of *nos-3* and *gld-2*.

Materials and methods

Strains

The following mutations were used: *LGI: gld-2(q497), gld-1(q485), gld-1(q361), gld-1(oz10gf), fog-3(q443), LGII: fbf-1(ok91), fbf-2(q704), let-241(mn228), nos-3(oz231), nos-3(q650), unc-4(e120), LGIII: unc-36(e251), dpy-19(e1259), unc-32(e189), glp-1(q175), glp-1(oz112gf), glp-1(bn18).*

Nematode strains and culture

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

Measurement of distal GLD-1 accumulation pattern

Eleven wild-type (N2) gonad arms from animals grown at 20°C and dissected one day past L4 were stained with anti-GLD-1-specific antibodies (see below) and analyzed using a Leica TCS SP2 confocal microscope. Images were collected well below saturation. For the distal end of each arm, images were obtained as 1 μm serial sections and then flattened into one image. Pixel intensity was determined on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In short, the program divided the arm into a grid 20 units in height and 150 units in length, which corresponds to approximately 24 cell diameters. The pixel intensity was measured for each location on the grid and each of the 150 columns was averaged (20 spots per column). These 150 values were then averaged with the 150 values of the remaining 10 gonad arms and plotted on a graph (Fig. 1D).

Antibody staining and RNA in situ hybridization

Antibody staining of dissected gonads has been described previously (Jones et al., 1996). In short, animals were dissected and fixed with either 3% formaldehyde/0.1 M K_2HPO_4 (pH 7.2) for 1 hour at room temperature (RT) followed by 5 minutes incubation with 100% methanol at –20°C (this fixative was used when not using GLD-1 antibodies), or 3% formaldehyde/0.5× PBS/75% methanol for 5 minutes at –20°C (this fixative used when GLD-1 antibodies were used). The use of nucleoplasmic REC-8 staining to identify proliferative germ cells is described elsewhere (Hansen et al., 2004). Fluorescent images were captured with a Zeiss Axioskop microscope equipped with a Hamamatsu digital CCD camera (Hamamatsu Photonics). For all strains stained with GLD-1, wild-type control animals were dissected in the same dish, co-stained, mounted on the same slide and images were captured with the same camera settings. In many cases, both the N2 and mutant gonads were captured in the same field (Fig. 6C). In order to confirm that the low GLD-1 levels seen in *gld-2(q497); nos-3(oz231)* animals was not due to the germlines being masculinized, we also stained *gld-2(q497) fog-3(q443); nos-3(oz231); unc-32(e189)* animals and found that GLD-1 levels were still low (data not shown).

RNA in situ hybridization has been described previously (Jones et al., 1996). Briefly, dissected gonads were fixed in 0.25% glutaraldehyde/3% formaldehyde, 100 mM K_2HPO_4, pH 7.2. Both sense and anti-sense probes were synthesized using primer extension and digoxigenin-11-dUTP. Protease concentrations and incubation times were roughly doubled from that described (Jones et al., 1996),

which aided in visualizing *gld-1* mRNA in the most distal end of the gonads, presumably because of increased permeablization. Images were captured with a Zeiss Axioplan 2 microscope equipped with a SPOT digital CCD camera (Diagnostic Instruments).

Results

The GLP-1/Notch signaling pathway regulates GLD-1 levels

GLD-1 protein levels are relatively low in the most distal end of the *C. elegans* hermaphrodite gonad, but increase gradually until reaching a high level ~20 cells diameters from the DTC (Jones et al., 1996) (Fig. 1C,D), the approximate region where germ cells first enter meiotic prophase (Crittenden et al., 1994; MacQueen and Villeneuve, 2001; Hensen et al., 2004). GLD-1 has been implicated to act downstream of GLP-1 signaling to repress premeiotic proliferation and/or promote meiotic development (Francis et al., 1995b; Kadyk and Kimble, 1998). Since the level of GLD-1 is spatially controlled in the distal end, where the entry into meiosis decision takes place, we sought to determine if GLP-1 signaling regulates GLD-1 protein accumulation.

We first examined animals that have constitutively active, ligand independent, GLP-1 signaling (Berry et al., 1997), predicting that if GLP-1/Notch signaling inhibits GLD-1 protein accumulation, then constitutively active signaling would result in lower GLD-1 levels. Animals with one copy of the gain-of-function allele *glp-1(oz112)* and one copy of the *glp-1(q175)* null allele have a late onset tumorous phenotype where the distal proliferative zone increases in size over time, reflecting constitutive GLP-1 activity (Berry et al., 1997). In these animals, low GLD-1 levels extend much further proximally than in wild-type (Fig. 2). The maximum level, however, still coincides with the transition of germ cells from proliferation to early meiotic prophase as judged by nuclear non-chromosomal axis REC-8 staining (Pasierbek et al., 2001), which under our fixation conditions stains proliferating germ cells (Hansen et al., 2004). In animals homozygous for *glp-1(oz112gf)*, and carrying an extra copy of *glp-1(+)* on a free duplication, GLD-1 levels do not increase (Fig. 2). These animals have completely tumorous germlines with no evidence of entry into meiosis (Berry et al., 1997; Hansen et al., 2004). Therefore, GLP-1/Notch signaling activity leads to low GLD-1 levels, suggesting that in wild-type animals, GLP-1/Notch signaling inhibits GLD-1 accumulation in the distal end.

An alternative explanation for these results is that proliferation per se, rather than GLP-1 signaling, inhibits GLD-1 accumulation. To distinguish between these possibilities, we looked at GLD-1 protein spatial pattern in germline tumors where GLP-1 signaling is unperturbed; we stained for GLD-1 in animals homozygous for loss-of-function (lf) mutations in *gld-2* and *gld-*

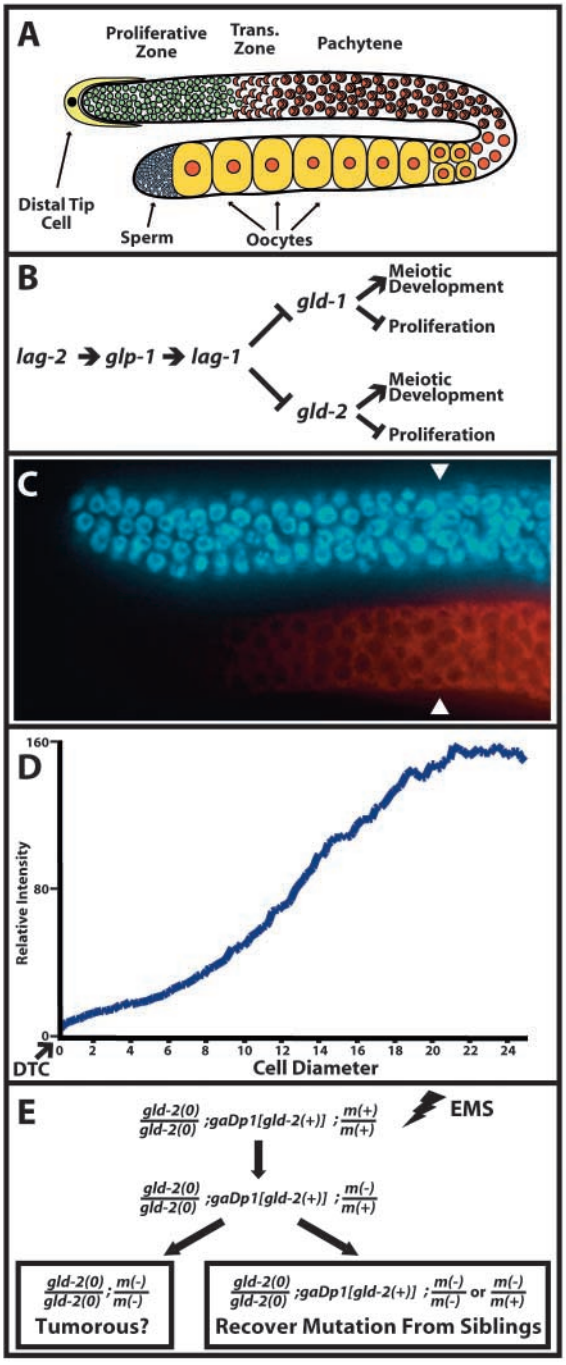

Fig. 1. Polarity of the *C. elegans* germline and the genetic pathway involved in maintaining the stem cell population. (A) Diagram of germline organization of a young adult hermaphrodite gonad arm. Distal germ cells (proliferative zone; green), enter meiotic prophase as they move proximally (red). The somatic distal tip cell (DTC) caps the very distal end. (B) Genetic pathway that regulates the decision to enter meiosis [adapted from Kadyk and Kimble (Kadyk and Kimble, 1998)]. The GLP-1/Notch signaling pathway inhibits the activities of *gld-1* and *gld-2*. (C) Distal end of a wild-type adult gonad arm showing GLD-1 spatial patterning (red; GLD-1-specific antibodies). The same arm stained with DAPI (blue), to reveal nuclear morphology. Arrowheads indicate approximately where transition zone nuclei are first seen. (D) Graph (roughly aligned with C) showing distal GLD-1 accumulation averaged from 11 gonad arms stained with GLD-1 specific antibodies (see Materials and methods). x-axis is the distance in cell diameters from the DTC. y-axis is the relative intensity of antibody staining in arbitrary units. (E) Genetic screen used to identify genes that function with *gld-1* in regulating entry into meiosis. Animals homozygous for a *gld-2(null)*, carrying a free duplication (*gaDp1*) that contains *gld-2(+)*, were mutagenized to generate mutations in genes (*m*). Animals [*m(–)*] are recovered from siblings containing *gaDp1* and are either homozygous or heterozygous for *m(–)*.

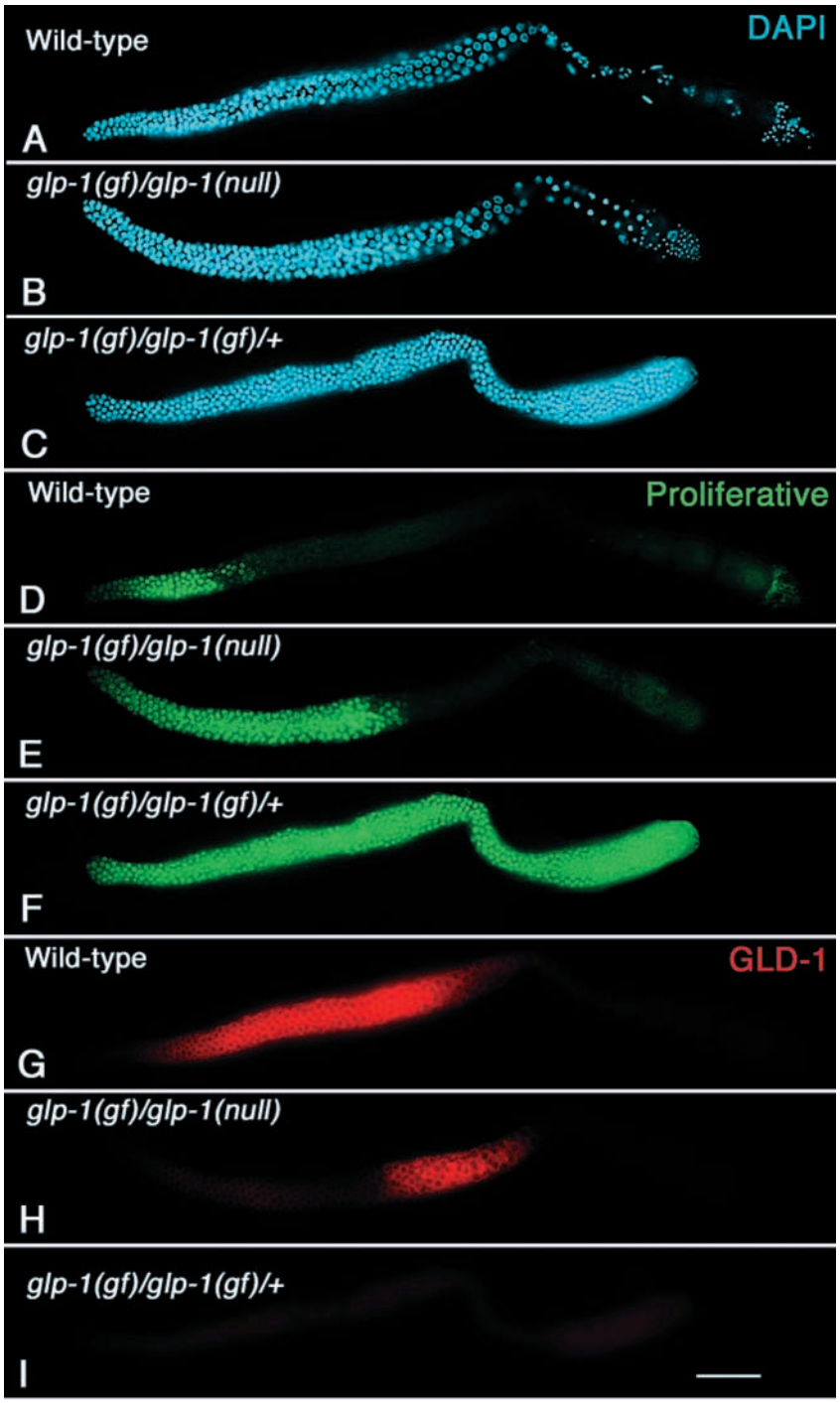

Fig. 2. Increased GLP-1/Notch signaling inhibits GLD-1 accumulation. Hermaphrodite gonad arms, with distal to the left, stained with (A-C) DAPI (blue), (D-F) REC-8 antibodies (proliferative cells; green) and (G-I) GLD-1 antibodies (red). (A,D,G) Wild-type young adult; (B,E,H) *unc-32(e189) glp-1(oz112gf)/ unc-36(e251) glp-1(q175)*; (C,F,I) *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 μm.

1. *gld-2* and *gld-1* function redundantly to inhibit proliferation and/or promote entry into meiosis, and loss of the activities of both genes results in a germline tumor (Kadyk and Kimble, 1998). We used the *q361* allele of *gld-1* that causes a synthetic tumorous phenotype in combination with *gld-2*, but still makes protein that accumulates normally (Francis et al., 1995a; Jones et al., 1996). GLD-1 accumulates in an essentially wild-type pattern, reaching roughly wild-type levels at ~20 cell diameters from the DTC in *gld-2(q497) gld-1(q361)* tumorous germlines (Fig. 3), indicating that GLD-1 accumulation is not inhibited by proliferating germ cells and that GLD-1 does not have an essential function in regulating its own accumulation.

Since high GLP-1/Notch signaling inhibits GLD-1 accumulation, we hypothesized that eliminating *glp-1* activity would increase GLD-1 accumulation in the distal end. However, we could not directly look at GLD-1 levels in animals lacking *glp-1* because in *glp-1(null)* animals all germ cells prematurely enter meiosis during early larval development. Therefore, we removed *glp-1* activity from *gld-2(q497) gld-1(q361)* tumorous animals in which GLD-1 accumulation in the distal end is roughly wild type (Fig. 3B). The loss of GLP-1/Notch signaling results in high GLD-1 levels in the distal end, unlike in *gld-2(q497) gld-1(q361)* or wild-type animals (Fig. 3C). Thus, removal of *glp-1* activity causes an increase of distal GLD-1 accumulation, further supporting the model that GLP-1/Notch signaling inhibits distal GLD-1 accumulation. We further looked at *gld-2(q497) gld-1(q361)* animals that had reduced *lag-1* activity and found that GLD-1 levels were uniform along the distal arm (Fig. S2, http://dev.biologists.org.supplemental/), suggesting that it is not just GLP-1 activity that is needed for repression of GLD-1 accumulation but rather the GLP-1/Notch signaling pathway.

To determine if GLP-1/Notch signaling inhibits GLD-1 accumulation at the level of transcription, we looked at *gld-1* mRNA levels by in situ hybridization in *gld-2(q497) gld-1(q361)* and *gld-2(q497) gld-1(q361); glp-1(q175)* animals. Previous studies suggested that *gld-1* mRNA accumulation is only modestly regulated along the distal proximal axis in wild-type hermaphrodites (Jones et al., 1996). We did not see an increase in *gld-1* mRNA levels in *gld-2(q497) gld-1(q361); glp-1(q175)* animals, but approximately the same spatial patterning as in g*ld-2(q497) gld-1(q361)* animals (Fig. 3). Therefore the lack of GLD-1 accumulation in *glp-1(gf)* tumorous germlines (Fig. 2) and in the distal-most region of wild-type and *gld-2(q497) gld-1(q361)* (Fig. 3A,B), probably reflects the inhibition of GLD-1 accumulation by GLP-1 signaling at a post transcriptional level, possibly through inhibiting translation or promoting protein degradation.

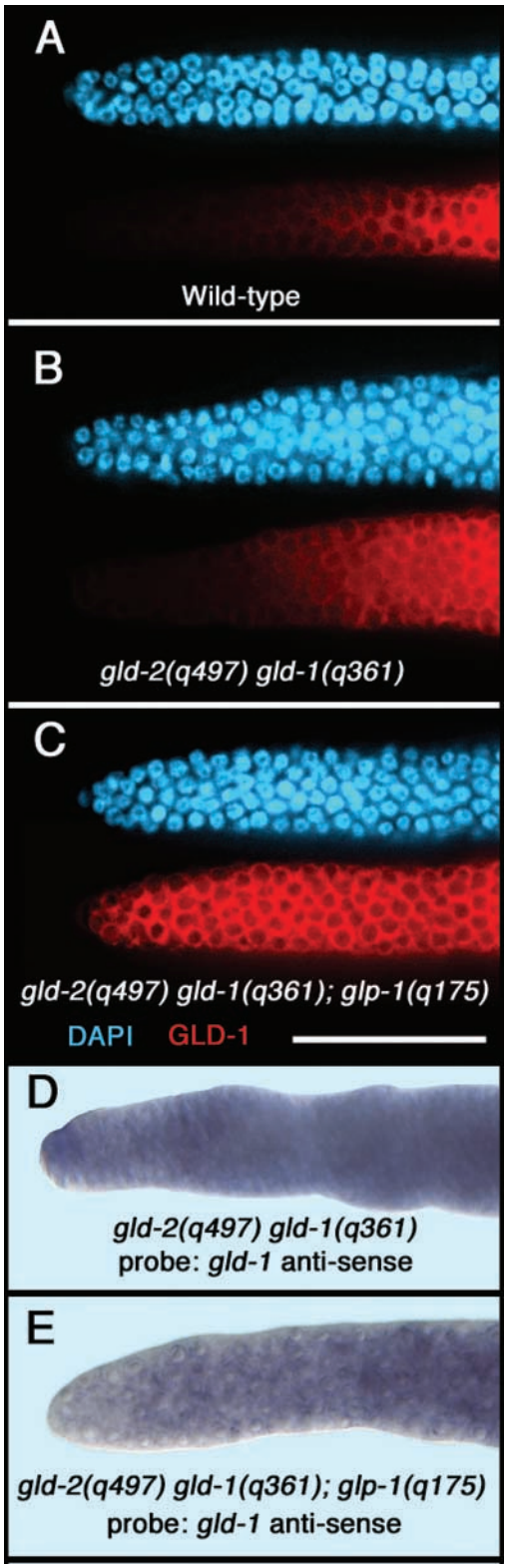

Fig. 3. Loss of GLP-1/Notch signaling causes increased distal GLD-1 accumulation. (A-C) Distal end (left) of dissected hermaphrodite gonad arms stained with DAPI (blue), GLD-1 specific antibodies (red) and REC-8 antibodies (not shown). *gld-2(q497) gld-1(q361); unc-32(e189) glp-1(q175)* animals (C), which lack GLP-1/Notch signaling, have high distal GLD-1 accumulation levels. (D,E) *gld-1* mRNA spatial accumulation is similar in *gld-2(q497) gld-1(q361); unc-32(e189)* (D) and *gld-2(q497) gld-1(q361); unc-32(e189) glp-1(q175)* (E). *gld-1* sense probe shows little or no staining (not shown). Scale bar: 20 μm.

Increased GLD-1 accumulation in the distal most end results in germ cells entering meiosis more distally

We have shown that GLP-1/Notch signaling represses GLD-1 accumulation in the distal end of the gonad. To determine if this repression of GLD-1 is functionally important in maintaining the stem cell population, we sought to determine the effect of ectopically increasing GLD-1 levels in the distal-most end. Since *gld-1* has previously been shown to inhibit proliferation and/or promote meiotic entry, this would imply that *glp-1*-mediated repression of GLD-1 accumulation in the distal end allows for proliferation in this region (see also Crittenden et al., 2002). In order to test this further we utilized *gld-1(oz10gf)* animals, which have increased GLD-1 accumulation in the distal-most end (Jones et al., 1996). *gld-1(oz10gf)* animals display a semi-dominant Mog phenotype (masculinization of the germline), with both heterozygous and homozygous hermaphrodites having increased sperm at the expense of oocytes. This Mog phenotype results from GLD-1's role in regulating germline sex determination, a function that is separate from its function in regulating meiotic entry (Francis et al., 1995a).

We measured the size of the proliferative zone in *gld-1(oz10gf)* homozygotes following staining for proliferative and meiotic prophase nuclei using anti-REC-8 and HIM-3 antibodies respectively (Pasierbek et al., 2001; Zetka et al., 1999; Hansen et al., 2004). *gld-1(oz10gf)* homozygotes have a proliferative zone 13 cell diameters in length as compared with 19 in wild-type animals of the same age (Fig. 4A).

If low GLD-1 levels are necessary to maintain the distal proliferative zone, then an increase in GLD-1 levels in the distal end should enhance a weak *glp-1(lf)* mutation. Therefore, we tested the ability of the *gld-1(oz10gf)* allele to enhance the temperature sensitive lf *glp-1(bn18)* allele. At 20°C, *glp-1(bn18)* animals are essentially wild type, but at 25°C, the animals display a strong Glp phenotype with all germ cells prematurely entering meiotic prophase, resulting in a loss of the stem cell population. *gld-1(oz10gf)* enhances the Glp phenotype of *glp-1(bn18)* animals at the permissive temperature of 20°C, further suggesting that increased GLD-1 levels, and presumably increased GLD-1 activity, increases inhibition of proliferation and/or promotion of meiotic entry. Therefore the inhibition of GLD-1 accumulation by GLP-1/Notch signaling probably serves to maintain a pool of proliferating cells (see Discussion).

Screen to identify genes that function in the GLD-1 pathway

gld-1 and *gld-2* function redundantly to regulate the switch of germ cells from the mitotic proliferative state to meiotic development (Francis et al., 1995b; Kadyk and Kimble, 1998) (Fig. 1B). In the absence of *gld-1* or *gld-2* activity, cells are able to enter meiosis properly, however, if the activities of both *gld-1* and *gld-2* are absent, cells fail to enter meiosis properly and a germline tumor results (Kadyk and Kimble, 1998). In order to identify genes that function with *gld-1* either to

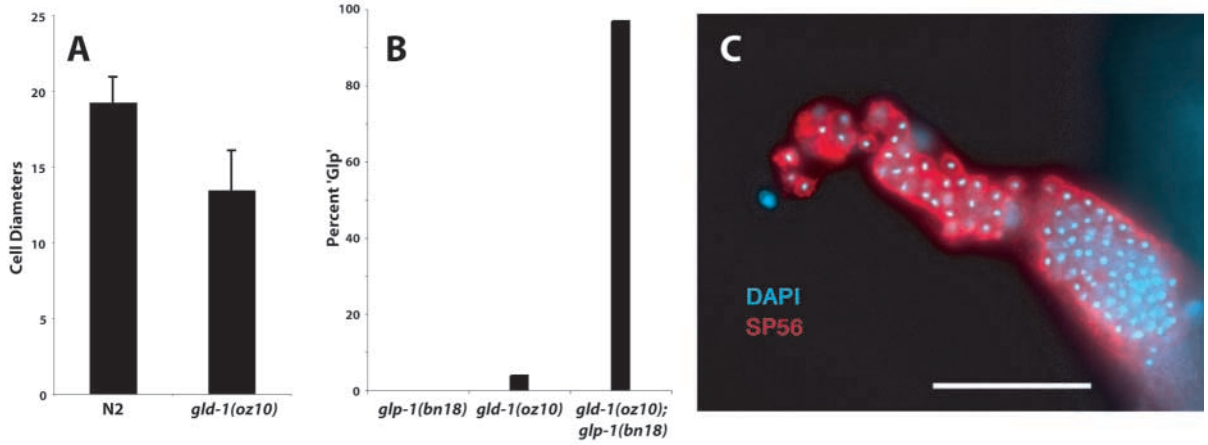

Fig. 4. Excess GLD-1 causes premature meiotic entry. (A) *gld-1(oz10gf)* has a smaller proliferative zone than wild-type animals. Dissected *gld-1(oz10gf)* and wild-type gonad arms from animals grown at 20°C to one day past L4, stained with REC-8- and HIM-3-specific antibodies, and DAPI. Proliferative zone defined as the number of cell diameters from the DTC that are REC-8-positive with all cells at that distance also REC-8-positive. n=15 per genotype. t-test $P<10^{-7}$. The *oz10* allele contains a deletion in the *gld-1* 3′UTR, as well as a missense mutation in an amino acid conserved in some, but not all homologues (Jones and Schedl, 1995). The increased GLD-1 accumulation is probably due to the mutant 3′UTR causing increased translation (Crittenden et al., 2002). However, we cannot rule out the possibility that the missense mutation affects GLD-1 levels or GLD-1 activity. (B) *gld-1(oz10gf)* enhances the 'Glp' phenotype of *glp-1(bn18)* at 20°C. The graph shows the percentage of animals that have lost their distal proliferative zones as measured by Nomarski microscopy. 40/40 *unc-32(e189) glp-1(bn18)* gonad arms had wild-type proliferative zones. For *gld-1(oz10gf); unc-32(e189)*, 52/54 gonad arms had wild-type proliferative zones while 2/54 had smaller gonad arms with enlarged cells in the distal end. In *gld-1(oz10gf); unc-32(e189) glp-1(bn18)* animals, only 3/93 had large proliferative zones while the rest lacked a normal proliferative zone, with either sperm completely filling the distal end (85/93) or sperm with other larger cells (5/93). (C) Dissected *gld-1(oz10gf); unc-32(e189) glp-1(bn18)* adult hermaphrodite gonad arm stained with DAPI (blue) and SP56 monoclonal antibody (red), which is specific to male germ cells (Ward et al., 1986). Scale bar: 20 μm.

promote entry into meiosis and/or inhibit proliferation, we screened for recessive mutations that, when in combination with a *gld-2* null mutant, form a germline tumor (a synthetic tumorous phenotype, Syt). The genetic screen we employed (Fig. 1E) involved mutagenizing animals that were homozygous for *gld-2(q497*) but that carried the *gaDp1* free duplication, which contains a copy of *gld-2(+)*.

The screen yielded new alleles of *gld-1* (three), as well as mutations that define three other loci. We describe the locus initially called *syt-1* in which five alleles were identified. The reference allele, *oz231*, mapped between *let-241* and *unc-4*, although closer to *unc-4* (4/16 Unc non Let recombinants carried the *oz231* allele), approximately 300 kb from *unc-4* on the physical map **(**http://www.wormbase.org, release WS100, May 2003). An examination of genes in the region identified *nos-3*, which encodes a putative translational regulator, as a likely candidate to encode *syt-1*. NOS-3 was previously identified from its similarity to *Drosophila* Nanos (Subramaniam and Seydoux, 1999), as well as for its ability to bind FBF-1 and FBF-2 (Kraemer et al., 1999). FBF-1 and FBF-2 are products of two nearly identical genes, *fbf-1* and *fbf-2* (Zhang et al., 1997), which are members of a larger family of Pumilio-related 'puf' genes (Pumilio and FBF) (Wickens et al., 2002). FBF can bind to the 3′UTR of the mRNA of the sex determining gene *fem-3* (Zhang et al., 1997), and working with NOS-3, is thought to repress FEM-3 translation to allow the switch from spermatogenesis to oogenesis in the L4 hermaphrodite.

Four pieces of evidence confirm that *oz231* and the other four mutations are alleles of the *nos-3* gene. First, reducing the activity of *nos-3* by RNAi in a strain lacking *gld-2* mimics the *gld-2; oz231* double mutant phenotype in that they have tumorous germlines (data not shown). Second, sequencing genomic DNA of all five alleles revealed lesions in the *nos-3* gene with each lesion predicted to result in a truncation of the protein prior to the zinc finger motifs (Fig. 5A). Third, staining of animals carrying one of the alleles *(oz231)* with the NOS-3 antibody (Kraemer et al., 1999) fails to detect a signal, confirming that *oz231* is an allele of *nos-3*, and probably a null (data not shown). Fourth, double mutant animals for *gld-2(q497)* and *nos-3(q650)*, a previously identified allele of *nos-3* (Kraemer et al., 1999), form a germline tumor in hermaphrodites and males similar to that formed in *gld-2(q497) nos-3(oz231)* animals (data not shown). Therefore we conclude that *syt-1* is *nos-3*.

nos-3 functions in the *gld-1* pathway for entry into meiosis

Genetic analysis indicates that *nos-3* functions in the *gld-1* pathway for entry into meiosis. First, animals lacking *nos-3* activity are not tumorous, but rather are essentially wild-type (Kraemer et al., 1999), showing that *nos-3* must function synthetically to regulate meiotic entry. Second, *nos-3 gld-2* double mutants form a tumor (Fig. 5E), while *nos-3 gld-1* double mutants appear to have essentially normal meiotic entry and gametogenesis, as assessed in males, which do not display the oogenesis-specific return to mitosis from pachytene phenotype (Francis et al., 1995a) (although some *gld-1(q485); nos-3(oz231)* males have proliferative cells in the proximal end of the gonad; Fig. S3, http://dev.biologists.org.supplemental/). Third, the *gld-2(q497); nos-3(oz231)* synthetic tumorous phenotype is epistatic to *glp-1* null failure to proliferate (Fig. S4, http://dev.biologists.org.supplemental/), indicating that, like

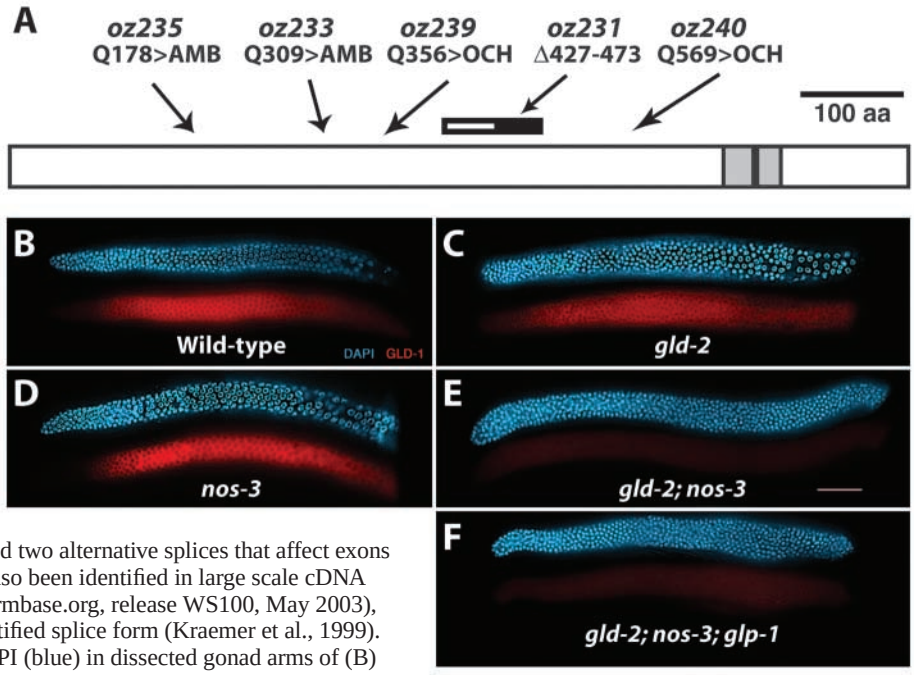

Fig. 5. *gld-2* and *nos-3* function to promote GLD-1 protein accumulation. (A) Diagram of NOS-3 protein drawn to scale showing the location of the lesions associated with the *nos-3* alleles obtained in the genetic screen described (Fig. 1). Shaded boxes represent the two putative zinc fingers that are similar to *Drosophila* Nanos. The *oz233*, *oz235*, *oz239* and *oz240* alleles are associated with nonsense mutations predicted to result in truncated proteins 308, 177, 355 and 568 amino acids in length respectively, as compared to 871 amino acids of full-length NOS-3 (Kraemer et al., 1999). The *oz231* allele is associated with a 139 base pair deletion (open box), deleting amino acids 427-473, as well as changing the reading frame, therefore adding 39 amino acids (filled box) before encountering a stop codon. All lesion locations refer to the previously published splice form of *nos-3* (Kraemer et al., 1999), however we have identified two alternative splices that affect exons five and seven. The alternative splice sites have also been identified in large scale cDNA sequencing efforts and are noted (http://www.wormbase.org, release WS100, May 2003), with *nos-3b* corresponding to the previously identified splice form (Kraemer et al., 1999). (B-F) GLD-1 protein accumulation (red) and DAPI (blue) in dissected gonad arms of (B) wild-type, (C) *gld-2(q497)*, (D) *nos-3(oz231)*, (E) *gld-2(q497); nos-3(oz231)* and (F) *gld-2(q497); nos-3(oz231); unc-32(e189) glp-1(q175)* animals one day past L4 at 20°C. The distal end is to the left and the proximal portion of each arm is not shown. Wild-type (B) and mutant animals (C-F) were dissected, fixed and stained together and pictures taken with the same settings and processed identically (see Materials and methods). Scale bar: 20 μm.

gld-1, *nos-3* functions redundantly with *gld-2*, downstream of GLP-1/Notch signaling.

NOS-3 and GLD-2 function redundantly to promote GLD-1 accumulation

Since *gld-1* and *nos-3* function in the same pathway for entry into meiosis (see above), we next wanted to determine their regulatory relationship. As both proteins are thought to be translational regulators, we looked at the level of protein accumulation. GLD-1 accumulation in *nos-3* mutants was very similar to the accumulation in wild type (Fig. 5D), as was NOS-3 accumulation in *gld-1* mutants (data not shown). This suggests that neither GLD-1 nor NOS-3 is solely responsible for promoting the expression or stability of the other. However, we already knew through genetic analysis that *nos-3* functions redundantly with *gld-2* in regulating entry into meiosis, therefore we looked at protein accumulation in *gld-2; nos-3* double mutants and found that GLD-1 accumulation is greatly reduced or absent (Fig. 5E). Since GLD-1 accumulates at wild-type levels in *gld-2* single mutant (Fig. 5C), we infer that *nos-3* and *gld-2* function redundantly to promote GLD-1 accumulation.

To determine the relationship between GLP-1/Notch signaling and the redundant activities of *gld-2* and *nos-3* in regulating GLD-1 accumulation, we assayed GLD-1 levels in *gld-2*; *nos-3*; *glp-1* triple mutants and found that GLD-1 levels were low (Fig. 5F). This suggests that the high level of GLD-1 found in the absence of GLP-1/Notch signaling requires *nos-3* and *gld-2* activity, and that *nos-3* and *gld-2* function downstream of GLP-1/Notch signaling in regulating GLD-1 accumulation. Furthermore, RNA in situ hybridization of *gld-2(q497); nos-3(oz231)* animals (data not shown) shows *gld-1* mRNA levels similar to *gld-2(q497) gld-1(q361)* animals, which express GLD-1 protein at near wild-type levels. This suggests that *gld-2* and *nos-3* are promoting GLD-1 accumulation at the level of translation or protein stability.

fbf-1 fbf-2 proliferation/meiosis phenotype depends on *nos-3* activity

Animals lacking FBF activity have germ cells entering meiotic prophase prematurely resulting in a depletion of the proliferative germ cells (Crittenden et al., 2002; Zhang et al., 1997). This depletion is suggested to be due to high levels of GLD-1 in the distal end (Crittenden et al., 2002). FBF is a negative regulator of GLD-1 accumulation and binds to the 3′UTR of *gld-1* mRNA in the region deleted by the *oz10gf* allele (Crittenden et al., 2002). FBF and NOS-3 physically interact in vitro and in a yeast 2-hybrid assay (Kraemer et al., 1999), and are thought to function together in repressing *fem-3* translation relating to germline sex determination. This is apparently analogous to the canonical Puf/Nanos interaction where *Drosophila* Pumilio and Nanos form a ternary complex with *hunchback* RNA to prevent its translation (Sonoda and Wharton, 1999). It is, therefore, interesting that FBF and NOS-3 function in *opposite* directions to regulate meiotic entry. FBF promotes proliferation and/or inhibits meiotic entry (Crittenden et al., 2002), while NOS-3 inhibits proliferation and/or promotes meiotic entry (this work), both accomplishing these functions, at least in part, by regulating GLD-1 accumulation.

To determine the epistatic relationship between *nos-3* and *fbf* for entry into meiosis, we compared the size of the proliferative zone and pachytene region of *fbf-1(ok91) fbf-2(q704)* double null mutants with *fbf-1 fbf-2 nos-3(oz231)* triple null mutants, in young adults (Fig. 6A). While all *fbf-1 fbf-2* germlines lacked a proliferative zone, and all but one lacked any pachytene cells, all *fbf-1 fbf-2 nos-3* germlines have extensive

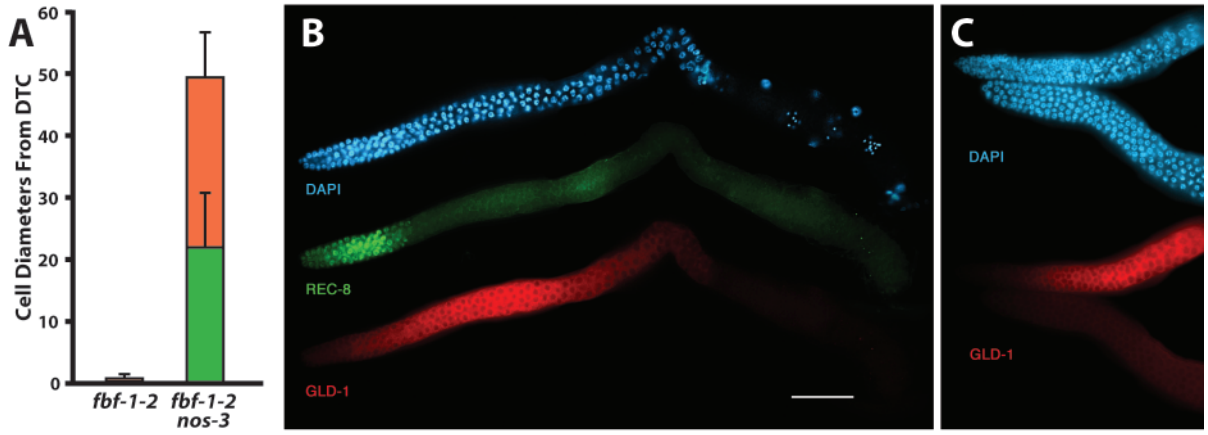

Fig. 6. NOS-3 is required for *fbf-1 fbf-2* double mutant Glp phenotype. (A) *fbf-1(ok91) fbf-2(q704)* and *fbf-1(ok91) fbf-2(q704) nos-3(oz231)* animals one day past L4 were dissected and stained with REC-8 (proliferative) and HIM-3 (meiotic) antibodies (Hansen et al., 2004). The graph shows the average number of cell diameters along the length of the gonad arm that cells are proliferative (REC-8, green) or meiotic (HIM-3, red). The proliferative zones of 8/10 *fbf-1(ok91) fbf-2(q704) nos-3(oz231)* arms were smaller or of similar size to those of wild-type, while 2/10 were much larger (33 and 40 cell diameters). The phenotype is independent of germline sex as *fog-3(q443); fbf-1(ok91) fbf-2(q704); unc-32(e189)* and *fog-3(q443); fbf-1(ok91) fbf-2(q704) nos-3(oz231); unc-32(e189)* animals were similar to the unfeminized animals (data not shown). Error bars = 1 s.d. (B) Dissected gonad arm of *fog-3(q443); fbf-1(ok91) fbf-2(q704) nos-3(oz231); unc-32(e189)* young adult animal stained with DAPI (blue), REC-8 (green) and GLD-1 (red). Distal is to the left. Scale bar: 20 μm. (C) Dissected gonad arms of wild-type (top) and *gld-2(q497) fog-3(q443); fbf-1(ok91) fbf-2(q704) nos-3(oz231); unc-32(e189)* (bottom) stained with DAPI (blue) and GLD-1 (red). Only a portion of the distal arms are shown with distal to the left.

proliferative zones and pachytene regions, although somewhat smaller than those of wild type (Fig. 6A). Therefore the lack of *nos-3* activity suppresses the *fbf-1 fbf-2* null late-onset Glp lf phenotype, suggesting that *nos-3* functions downstream or parallel to *fbf* in regulating meiotic entry.

We next analyzed GLD-1 levels in *fbf-1 fbf-2 nos*-3 animals. The rise in GLD-1 protein accumulation in the distal germline is similar in wild-type males and hermaphrodites (female), but the magnitude of the rise is much lower in the male germline (Jones et al., 1996). Since *fbf-1 fbf-2 nos*-3 animals have a masculinized germline, and to allow a comparison of the GLD-1 accumulation pattern with other strains in this study, we feminized *fbf-1 fbf-2 nos-3* animals with *fog-3(q443)*, which did not affect the suppression of the *fbf-1 fbf-2* mutant Glp phenotype by *nos-3* null. In these animals the pattern of GLD-1 accumulation is very similar to that of wild type, with low levels at the very distal end and increasing to a high level as germ cells enter meiosis, although overall levels appear to be slightly lower (Fig. 6B). Thus, NOS-3 activity is required for the higher distal GLD-1 levels thought to occur in *fbf* mutants.

We next examined the relationship of *gld-2* to *fbf* to test whether the *fbf-1 fbf-2* Glp phenotype requires *gld-2* activity. We examined the germlines of *gld-2(q497); fbf-1 fbf-2* triple null adult hermaphrodites and found that they lacked a distal proliferative region (n=24), although the total number of germ cells appears to be slightly higher (data not shown). Thus, in contrast to *nos-3*, the activity of *gld-2* is not required for the *fbf-1 fbf-2* double mutant Glp phenotype.

GLD-1 levels rise as germ cells enter meiosis in the feminized *fbf-1 fbf-2 nos-3* triple null mutants (Fig. 6B). Removal of *gld-2* activity (in feminized *gld-2(q497); fbf-1(ok91) fbf-2(q704) nos-3(oz231)* quadruple mutants), results in GLD-1 levels that are very low or absent (Fig. 6C). This result supports the view that GLD-2 is sufficient to promote high levels of GLD-1. However, since there is a proliferative region and low levels of GLD-1 in the very distal end of feminized *fbf-1 fbf-2 nos-3* triple mutants (Fig. 6B), GLD-2 must be inactive in the very distal end, even in the absence of *fbf*. Taken together, these results suggest that GLD-2 is sufficient to promote high levels of GLD-1 and that its activity in the most distal end of a wild-type germline is inhibited by something other than, or in addition to, FBF.

Discussion

Our studies demonstrate that a number of factors regulate the spatial patterning of GLD-1 accumulation in the *C. elegans* germline and that this pattern sets the border between proliferating and differentiating germ cells. We have shown that the GLP-1/Notch signaling pathway inhibits GLD-1 accumulation in the distal end, probably indirectly through translational regulation or protein stability. We also have shown that *gld-2* and *nos-3* function redundantly in promoting GLD-1 accumulation. Interestingly, NOS-3 functions in opposition to FBF, a protein that inhibits GLD-1 accumulation (Crittenden et al., 2002). Furthermore, we have shown that the spatial distribution of GLD-1 is important for regulating the balance between stem cell proliferation and differentiation in the *C. elegans* germline.

GLP-1/Notch signaling controls spatial accumulation of GLD-1

The spatial pattern of GLD-1 accumulation is important for regulating the balance between proliferation and meiotic entry (Crittenden et al., 2002). The extended low GLD-1 levels in the larger than normal proliferative zone of *glp-1(oz112gf)/glp-1(null)* hemizygotes, as well as the low or absent GLD-1 levels in *glp-1(oz112gf)/glp-1(oz112gf)/glp-1(+)* animals (Fig. 2), supports the hypothesis that GLD-1 levels in the most distal end of wild-type animals must be low in order to enable the

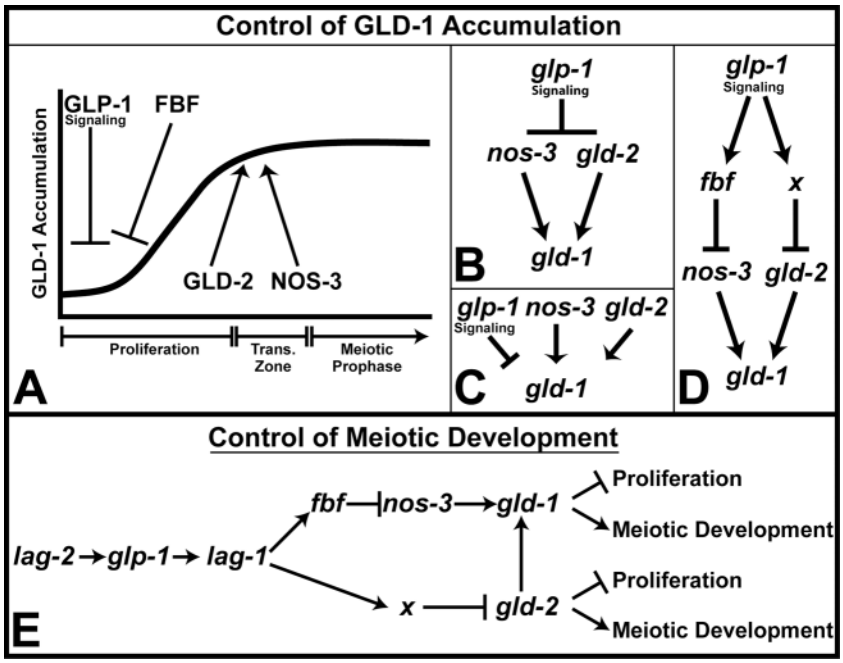

Fig. 7. Models of factors regulating GLD-1 accumulation levels. (A) Schematic representation of GLD-1 accumulation in the distal germline with factors inhibiting accumulation (barred lines) and factors promoting accumulation (arrows). GLP-1/Notch signaling and FBF sequentially inhibit GLD-1 accumulation at the distal-most end of the germline, while GLD-2 and NOS-3 redundantly promote GLD-1 accumulation. (B,C) Alternative models describing the genetic relationships between *glp-1* signaling and *nos-3* and *gld-2* relative to GLD-1 accumulation. (B) *glp-1* signaling inhibits GLD-1 accumulation by inhibiting the redundant activities of *nos-3* and *gld-2*. Alternatively (C), *glp-1* signaling works in parallel with *nos-3* and *gld-2*, and GLD-1 accumulation reflects the net influence of these factors. (D) Genetic pathway regulating GLD-1 accumulation. In the distal end *fbf* and gene *x* inhibit *nos-3* and *gld-2*, respectively. More proximally, where *glp-1* signaling is low, *nos-3* and *gld-2* promote GLD-1 accumulation. (E) Genetic model of genes functioning in the proliferation versus meiotic entry decision. *glp-1* signaling inhibits the *gld-1* and *gld-2* pathways in the most distal end. For *gld-1*, this inhibition involves *fbf-1/-2* inhibiting the promotion of *gld-1* by *nos-3*. *gld-2* is inhibited by something (*x*) other than, or in addition to, *fbf-1/-2*. As *glp-1* signaling is reduced in more proximal cells, *nos-3* and *gld-2* promote GLD-1 protein accumulation, and both *gld-1* and *gld-2* promote meiotic development and/or inhibit proliferation (see text).

stem cell population to be maintained. Conversely, the correlation of increased GLD-1 levels with meiotic entry in *glp-1(oz112gf)/glp-1(null)* hemizygotes (Fig. 2) and of increased GLD-1 levels in the distal end resulting in more distal meiotic entry (Fig. 4), indicates that the wild-type rise in GLD-1 levels causes germ cells to enter meiotic prophase. It is currently unknown, however, what level of GLD-1 is necessary to promote meiotic entry. Cells may commit to enter meiotic prophase when GLD-1 levels are near their highest, or it is possible that cells commit to enter meiotic prophase more distally, where GLD-1 levels are still increasing.

GLP-1/Notch signaling, activated by a ligand produced by the DTC, is the initial spatial polarizing cue in regulating the proliferation versus entry into meiosis decision (Seydoux and Schedl, 2001). The rise in GLD-1 accumulation as cells move proximally is probably due to a lowering of GLP-1/Notch signaling. Inhibition of distal GLD-1 accumulation is probably achieved post-transcriptionally because when GLP-1/Notch signaling is absent, *gld-1* mRNA levels do not increase (Fig. 3E), even though there is a dramatic increase in protein levels (Fig. 3C). However, since the culminating third component of the core Notch signaling pathway is a CSL transcription factor [LAG-1 bound to GLP-1(INTRA)], *gld-1* is unlikely to be directly regulated by this complex. Instead a factor(s), whose transcription is regulated by LAG-1/GLP-1(INTRA), may control GLD-1 protein levels. None of the genes known to regulate GLD-1 levels, and that have known expression patterns (NOS-3, GLD-2 and FBF-1), have significant changes in accumulation in the region where GLD-1 protein levels increase (Crittenden et al., 2002; Kraemer et al., 1999; Wang et al., 2002), therefore they probably are not transcriptional targets of LAG-1/GLP-1(INTRA).

Even though GLP-1/Notch signaling inhibits GLD-1 accumulation, it is interesting that GLP-1 protein levels are still high at the same location where GLD-1 levels are high (~20 cell diameters from the DTC) (Crittenden et al., 1994; Jones et al., 1996). This suggests that the level of GLP-1 visible on the membrane does not, necessarily, reflect the level of signaling that is occurring.

GLD-2 and NOS-3 promote GLD-1 accumulation

We have shown that GLP-1/Notch signaling inhibits GLD-1 accumulation, while NOS-3 and GLD-2 function redundantly to promote GLD-1 accumulation (Fig. 7A). Therefore both positive and negative influences shape the pattern of GLD-1 accumulation, allowing a spatially controlled balance between proliferation and differentiation to be maintained. One possible model for how these opposing factors regulate GLD-1 accumulation is that GLP-1/Notch signaling could inhibit the activities of GLD-2 and NOS-3 in the most distal end of the germline (Fig. 7B). As germ cells move proximally, away from the DTC-bound LAG-2 ligand, GLP-1 signaling is reduced, allowing for NOS-3 and GLD-2 to promote the accumulation of GLD-1. Supporting this model are the low GLD-1 accumulation and tumorous germline phenotypes in *gld-2; nos-3; glp-1* triple mutants, indicating that *gld-2* and *nos-3* are epistatic to *glp-1* with respect to GLD-1 accumulation. As mentioned above, however, NOS-3 and GLD-2 are unlikely to be direct targets of GLP-1/Notch signaling. Current data do not rule out an alternate model where GLP-1/Notch signaling, *nos-3* and *gld-2* each function independently on GLD-1 accumulation and that the sum of their positive and negative regulation determines GLD-1 levels (Fig. 7C). In this model NOS-3 and GLD-2 may continually promote GLD-1 accumulation, but only when the inhibiting influence of GLP-1 signaling is reduced by distance from the DTC, are high GLD-1 levels achieved.

GLD-2 is the catalytic portion of a cytoplasmic poly(A)

polymerase thought to translationally activate or stabilize mRNAs through lengthening their poly(A) tails (Wang et al., 2002). It is currently unknown if GLD-2 directly promotes GLD-1 accumulation through lengthening its poly(A) tail, or if there are one or more intermediates between these genes. (i.e. *gld-2* could regulate another gene, which then in turn regulates *gld-1*). Interestingly, GLD-2 lacks an RNA binding domain but binds another protein, GLD-3, which contains KH RNA binding domains and presumably recruits GLD-2 to specific mRNAs (Eckmann et al., 2002; Wang et al., 2002). We have identified lf alleles of *gld-3* in a screen for mutants that are synthetic tumorous with *nos-3*. Furthermore, *nos-3 gld-3* double mutants have low GLD-1 germline accumulation, and genetic experiments indicate that *gld-3* acts with *gld-2* to promote entry into meiosis (D.H. and T.S., unpublished), suggesting that GLD-2 and GLD-3 probably function together to promote GLD-1 accumulation, possibly by GLD-2 and GLD-3 increasing *gld-1* mRNA poly(A) tail length and increasing its translation.

NOS-3 is an RNA binding protein similar to *Drosophila* Nanos (Kraemer et al., 1999). It is currently unclear how *nos-3* functions redundantly with *gld-2* in promoting GLD-1 accumulation. One possibility is that *gld-2* and *nos-3* (or genes that they regulate) accomplish similar biochemical functions that are mutually compensatory. Alternatively, each may be involved in promoting the translation of GLD-1 through independent means and only when both activities are reduced is a threshold crossed where a dramatic decrease in GLD-1 levels is realized. Since *nos-3* and *gld-2* activity are each sufficient to achieve the normal pattern of GLD-1 accumulation, both genes must be negatively regulated in the distal-most germline to keep GLD-1 levels low and allow proliferation.

Antagonistic relationship between FBF and NOS-3

FBF probably functions downstream of GLP-1/Notch signaling in inhibiting GLD-1 accumulation (Fig. 7D), because loss of FBF and GLP-1/Notch signaling have similar germline phenotypes, and because FBF appears to directly inhibit GLD-1 translation. FBF binds the *gld-1* 3′UTR, and there are putative binding sites in the UTR that are removed in the *gld-1(oz10gf)* deletion (Crittenden et al., 2002). In *gld-1(oz10gf)* mutants, distal GLD-1 levels are increased (Jones et al., 1996) and meiotic entry occurs more distally than normal (see Results). Therefore, FBF probably functions directly to translationally inhibit GLD-1 accumulation. Furthermore, since GLP-1 signaling also inhibits GLD-1 accumulation, GLP-1/Notch signaling probably positively regulates FBF. It should be noted that GLD-1 accumulation reaches a high level at ~20 cell diameters from the DTC (Jones et al., 1996), where FBF-1 levels are high (Crittenden et al., 2002), therefore the spatial patterning of FBF-1 does not explain the distribution of GLD-1 in the distal arm.

Since FBF inhibits GLD-1 accumulation, it functions in opposition to NOS-3, which promotes GLD-1 accumulation. We have shown that *nos-3* mutants suppress the Glp lf phenotype of *fbf-1 fbf-2* mutants, and that *fbf-1fbf-2 nos-3* triple mutants display near wild-type distal GLD-1 patterning. This suggests that *nos-3* functions genetically downstream of *fbf* (Fig. 7D), or parallel to it. The antagonistic relationship between FBF and NOS-3 contrasts with their relationship in hermaphrodite germline sex determination where they are thought to work together to inhibit *fem-3* translation (Kraemer et al., 1999) and is at odds with their *Drosophila* homologues, Nanos and Pumilio, which function together to repress translation (Sonoda and Wharton, 1999).

There are a number of possibilities to explain this unique antagonistic relationship between Nanos and Pumilio homologues. First, although both FBF and NOS-3 regulate entry into meiosis, they may not partner in this process. Instead, FBF may partner with one of the other two NOS homologues (Kraemer et al., 1999; Subramaniam and Seydoux, 1999), and NOS-3 may partner with one of the other ten PUF proteins (Wickens et al., 2002). The genetic epistasis of *fbf* and *nos-3* suggests that the FBF/NOS-X complex could function upstream and inhibit the PUF-X/NOS-3 complex. However, this model is unlikely to be correct since FBF directly binds to the *gld-1* 3′UTR in vitro (Crittenden et al., 2002). Also, *nos-3* cannot be a direct target of translational inhibition because NOS-3 protein accumulation is uniform throughout the gonad (Kraemer et al., 1999), although its partner PUF protein could be a target. Furthermore, FBF can bind NOS-3, but not NOS-1 or NOS-2 in a two-hybrid assay or as GST-fusion proteins in vitro (Kraemer et al., 1999). The possibility still remains, however, that binding between FBF and NOS-1 or NOS-2 is dependent upon the presence of the target RNA, as is the case with *Drosophila* Pumilio and Nanos (Sonoda and Wharton, 1999).

A second possible reason why FBF and NOS-3 have an antagonistic relationship, unlike Nanos and Pumilio, could have to do the divergence of the Nanos and NOS-3 proteins. Nanos is 401 amino acids in length while NOS-3 is over twice that size at 871. Most similarity between the proteins exists in the putative zinc finger domains, and even there they are only 26% identical over 57 amino acids (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). Furthermore, while Nanos and Pumilio are unable to interact, except in the presence of target RNA (Sonoda and Wharton, 1999), interaction of NOS-3 and FBF-1 is not RNA dependent (Kraemer et al., 1999). Nanos appears to require its zinc finger motifs to complex with Pumilio and the *hunchback* RNA (Sonoda and Wharton, 1999), while the NOS-3 zinc fingers are dispensable for binding to FBF-1 (Kraemer et al., 1999). Perhaps the extensive differences between Nanos and NOS-3 reflect different molecular functions, and the relationship between NOS-3 and FBF may not be completely analogous to Nanos and Pumilio, allowing an inhibitory relationship to exist between NOS-3 and FBF.

Repression of GLD-2 activity in the proliferative zone

gld-2 and *nos-3* are each sufficient to promote high levels of GLD-1 since only in the double mutant are levels of GLD-1 dramatically reduced (Fig. 5, Fig. 6C). Therefore, in the most distal end of a wild-type germline, where GLD-1 levels are low, the activities of GLD-2 and NOS-3 must each be repressed (Fig. 7D). FBF probably represses NOS-3 activity since *nos-3* lf mutants suppress the premature entry into meiosis phenotype of *fbf-1 fbf-2*, and since *fbf-1 fbf-2 nos-3* triple mutants have low GLD-1 levels in the distal end (see above), and higher GLD-1 levels at ~20 cell diameters away, probably as a result of GLD-2 activity (Fig. 6C). However, if repression of GLD-

2 was solely accomplished through FBF activity, then in *fbf-1 fbf-2 nos-3* triple mutant animals, the repression of *gld-2* would be relieved and wild-type *gld-2* would be sufficient to promote not just proximal (~20 cell diameters), but also distal GLD-1 accumulation. Since distal GLD-1 accumulation is low in *fbf-1 fbf-2 nos-3* triple mutants, GLD-2 activity must be repressed in the most distal end by something (X) other than (or in addition to) FBF (Fig. 7D).

Furthermore, since meiotic entry is normal in both *gld-1* and *gld-2* single mutants (Francis et al., 1995a; Kadyk and Kimble, 1998), the activities of either *gld-1* or *gld-2* are sufficient for the switch from proliferation to meiotic prophase to occur. The premature meiotic entry phenotype of *fbf-1 fbf-2* double mutants is probably primarily due to increased *gld-1* activity, and not *gld-2* activity, because reducing the amount of *gld-1* by half (*gld-1/+; fbf-1 fbf-2)*, suppresses the *fbf-1 fbf-2* premature meiotic entry phenotype (Crittenden et al., 2002). Since *gld-2* activity is sufficient to cause the switch from proliferation to meiotic prophase, if *fbf-1 fbf-2* inhibits *gld-2* activity in the most distal end then in *gld-1/+; fbf-1 fbf-2* mutants, the *gld-2* suppression would be relieved and cause premature meiotic entry. However, this is not seen, and therefore we suggest that something other than *fbf*, or in addition to *fbf*, inhibits *gld-2* activity in the most distal end of the germline (Fig. 7E). We note that the lack of *gld-2* activity does seem to weakly repress the *fbf-1 fbf-2* Glp phenotype, with *gld-2 fbf-1 fbf-2* having slightly larger germlines than *fbf-1 fbf-2* double mutants. This repression is minimal compared to that observed in *fbf-1 fbf-2 nos-3* mutants, and could be caused by the lack of *gld-2* activity slightly reducing GLD-1 levels, since GLD-2 is a positive regulator of GLD-1 accumulation. Alternatively, FBF may function redundantly with the activity of another factor(s) in repressing GLD-2 activity, therefore only minimal repression of the *fbf-1 fbf-2* Glp phenotype is observed when *gld-2* activity is removed.

gld-2 must have another role(s) in regulating entry into meiosis in addition to promoting GLD-1 accumulation (Fig. 7E). If *gld-2* only promoted GLD-1 accumulation, then a *gld-2 gld-1* double mutant would have a similar phenotype to a *gld-1* single mutant, however, this is not the case. *gld-2 gld-1* double mutants have a germline tumor because of a defect in entry into meiosis (Kadyk and Kimble, 1998), while germ cells in a *gld-1* single mutant enter meiosis normally (Francis et al., 1995a). In addition, *gld-2 gld-1* double mutant males have tumorous germlines (Kadyk and Kimble, 1998), while *gld-1* single mutant males have wild-type germlines (Francis et al., 1995b). Therefore, there must be another downstream target(s) of *gld-2* activity in regulating entry into meiosis. GLD-2, in part, may be a positive regulator of meiosis-specific genes since it is thought to lengthen poly(A) tails of target mRNAs (Wang et al., 2002), thereby promoting translation. Conversely, GLD-1 functions as an inhibitor of translation (Clifford et al., 2000; Jan et al., 1999; Lee and Schedl, 2001), and therefore may, in part, represses proliferation-specific gene products.

Maintenance of a stem cell population

The balance between proliferation and differentiation must be tightly controlled in order for a stem cell population to be maintained and for required tissues to be generated. In order to understand the behavior of stem cells, and thereby harness their therapeutic potential, it is important that we understand the mechanisms involved in regulating the proliferation versus differentiation decision. In the *C. elegans* germline, we have shown that this decision relies on the spatial pattern of GLD-1 levels. The genetic hierarchy controlling this spatial pattern, beginning with the restriction of GLP-1/Notch signaling to the most distal end of the gonad and culminating in the promoting influence of *gld-2* and *nos-3*, provides an excellent example of how tight control of protein levels can set the boundary for a niche, within which stem cell proliferation can occur.

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References

Austin, J. and Kimble, J. (1987). *glp-1* is required in the germline for regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell* **51**, 589-599.

Berry, L., Westlund, B. and Schedl, T. (1997). Germline tumor formation caused by activation of *glp-1*, a member of the *Notch* family of receptors. *Development* **124**, 925-936.

Clifford, R., Lee, M. H., Nayak, S., Ohmachi, M., Giorgini, F. and Schedl, T. (2000). FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. *Development* **127**, 5265-5276.

Crittenden, S. L., Troemel, E. R., Evans, T. C. and Kimble, J. (1994). GLP-1 is localized to the mitotic region of the *C. elegans* germline. *Development* **120**, 2901-2911.

Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M. and Kimble, J. (2002). A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. *Nature* **417**, 660-663.

Daley, G. Q. (2002). Prospects for stem cell therapeutics: myths and medicines. *Curr Opin Genet Dev* **12**, 607-613.

Eckmann, C. R., Kraemer, B., Wickens, M. and Kimble, J. (2002). GLD-3, a bicaudal-C homolog that inhibits FBF to control germline sex determination in *C. elegans*. *Dev Cell* **3**, 697-710.

Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995a). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* **139**, 607-630.

Francis, R., Maine, E. and Schedl, T. (1995b). Analysis of multiple roles of *gld-1* in germline development: interactions with the sex determination cascade and the *glp-1* signaling pathway. *Genetics* **139**, 607-630.

Hansen, D., Hubbard, E. J. and Schedl, T. (2004). Multi-pathway control of the proliferation versus meiotic development decision in the *C. elegans* germ line. *Dev. Biol.* (in press).

Henderson, S. T., Gao, D., Lambie, E. J. and Kimble, J. (1994). *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development* **120**, 2913-2924.

Hodgkin, J. and Martinelli, S. (1999). 1999 genetic map of *Caenorhabditis elegans*. St Paul, MN: Caenorhabditis Genetics Center.

Jan, E., Motzny, C. K., Graves, L. E. and Goodwin, E. B. (1999). The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. *EMBO J.* **18**, 258-269.

Jones, A. R. and Schedl, T. (1995). Mutations in *gld-1*, a female germ cell-specific tumor suppressor gene in *Caenorhabditis elegans*, affect a conserved domain also found in Src-associated protein Sam68. *Genes Dev.* **9**, 1491-1504.

Jones, A. R., Francis, R. and Schedl, T. (1996). GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. *Dev. Biol.* **180**, 165-183.

Kadyk, L. C. and Kimble, J. (1998). Genetic regulation of entry into meiosis in *C. elegans*. *Development* **125**, 1803-1813.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-219.

Kraemer, B., Crittenden, S., Gallegos, M., Moulder, G., Barstead, R., Kimble, J. and Wickens, M. (1999). NANOS-3 and FBF proteins physically interact to control the sperm-oocyte switch in *Caenorhabditis elegans*. *Curr. Biol.* **9**, 1009-1018.

Lambie, E. J. and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-240.

Lee, M. H. and Schedl, T. (2001). Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for *C. elegans* germ cell development. *Genes Dev.* **15**, 2408-2420.

MacQueen, A. J. and Villeneuve, A. M. (2001). Nuclear reorganization and homologous chromosome pairing during meiotic prophase require C. elegans *chk-2*. *Genes Dev.* **15**, 1674-1687.

Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* **228**, 151-165.

Pasierbek, P., Jantsch, M., Melcher, M., Schleiffer, A., Schweizer, D. and Loidl, J. (2001). A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes Dev.* **15**, 1349-1360.

Pepper, A. S., Killian, D. J. and Hubbard, E. J. (2003). Genetic analysis of *Caenorhabditis elegans glp-1* mutants suggests receptor interaction or competition. *Genetics* **163**, 115-132.

Pfendler, K. C. and Kawase, E. (2003). The potential of stem cells. *Obstet. Gynecol. Surv.* **58**, 197-208.

Priess, J. R., Schnabel, H. and Schnabel, R. (1987). The *glp-1* locus and cellular interactions in early *C. elegans* embryos. *Cell* **51**, 601-611.

Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111.

Schedl, T. (1997). Developmental Genetics of the Germline. In *C. elegans II*, vol. 2 (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 241-269. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Seydoux, G. and Schedl, T. (2001). The germline in *C. elegans*: origins, proliferation, and silencing. *Int. Rev. Cytol.* **203**, 139-185.

Sonoda, J. and Wharton, R. P. (1999). Recruitment of Nanos to *hunchback* mRNA by Pumilio. *Genes Dev.* **13**, 2704-2712.

Subramaniam, K. and Seydoux, G. (1999). *nos-1* and *nos-2*, two genes related to *Drosophila nanos*, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. *Development* **126**, 4861-4871.

Sulston, J. and Hodgkin, J. (1988). Methods. In *The Nematode Caenorhabditis elegans* (ed. W. B. Wood), pp. 587-606. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tax, F. E., Yeargers, J. J. and Thomas, J. H. (1994). Sequence of *C. elegans lag-2* reveals a cell-signaling domain shared with *Delta* and *Serrate* of Drosophila. *Nature* **368**, 150-154.

Wang, L., Eckmann, C. R., Kadyk, L. C., Wickens, M. and Kimble, J. (2002). A regulatory cytoplasmic poly(A) polymerase in *Caenorhabditis elegans*. *Nature* **419**, 312-316.

Ward, S., Roberts, T. M., Strome, S., Pavalko, F. M. and Hogan, E. (1986). Monoclonal antibodies that recognize a polypeptide antigenic determinant shared by multiple *Caenorhabditis elegans* sperm-specific proteins. *J. Cell Biol.* **102**, 1778-1786.

Wickens, M., Bernstein, D. S., Kimble, J. and Parker, R. (2002). A PUF family portrait: 3′UTR regulation as a way of life. *Trends Genet* **18**, 150-157.

Yochem, J. and Greenwald, I. (1989). *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in *Caenorhabditis elegans*, encode similar transmembrane proteins. *Cell* **58**, 553-563.

Zetka, M. C., Kawasaki, I., Strome, S. and Muller, F. (1999). Synapsis and chiasma formation in *Caenorhabditis elegans* require HIM-3, a meiotic chromosome core component that functions in chromosome segregation. *Genes Dev.* **13**, 2258-2270.

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J. and Wickens, M. P. (1997). A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germline. *Nature* **390**, 477-484.